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Oxidative stress generation in *Taxus baccata* leaves affected by *Pestalotiopsis funerea* under sunny and shaded conditions

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Abstract: In the last few years on old yew trees growing in the parks and gardens extensive disease symptoms were observed on leaves, indicating affection with pathogenic microorganisms. The causal factor was the presence of a fungal pathogen, *Pestalotiopsis funerea*. Physiological responses involving the oxidative stress, i. e. superoxide anion and hydrogen peroxide levels, and the superoxide dismutase were analyzed as an element of a plant defense mechanism. Diseased leaves from plants growing under sunny and shaded conditions, from May to July, were investigated.
The increased generation of superoxide anion-radicals and hydrogen peroxide were observed in the leaves with disease symptoms simultaneously with the activation of superoxide dismutase, which may indicate the induction of host defense response to the *P. funerea*.

Additional key words: *Taxus*, superoxide anion, hydrogen peroxide, superoxide dismutase

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Introduction

A common feature of abiotic and biotic stress factors is the generation of reactive oxygen species (ROS). i.e. superoxide anion-radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) in plant cells (Low and Merida 1996, Mehdy et al. 1996, Lamb and Dixon 1997, Wojtaszek 1997a, b, Scandalios 2002). ROS formation also accompanies normal metabolic processes, in particular photosynthesis and respiration. The enhanced production of ROS is a common phenomenon of oxidative stress (Langebartels et al. 2002, Mittler 2002) and was considered to be an integral part of the host defence responses (De Gara et al. 2003).

ROS due to their reactivity have antimicrobial properties directly inhibiting pathogenic organisms and are involved in the formation of structural barriers, or like e.g. hydrogen peroxide have signalling functions in plant-pathogen interaction (Mittler 2002, Neil et al. 1999, 2002, Veljavic-Jovanovic et al. 2002). Excessive amounts of reactive oxygen species may be harmful for plants, thus they are eliminated by the antioxidant system involving superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and non-enzymatic compounds: ascorbic acid (AA), cysteine (Cys), carotenoids and polyamides (PAs) (Baker and Orlandi 1995, De Gara et al. 2003, Scandalios 2002).

On old common yew plants the symptoms of shoot blight are observed, a disease which may be caused by both abiotic factors and pathogenic microorganisms. According to Łobanowski et al. (2002) *Pestalotiopsis funerea*, *Fusarium* sp., *Rhizoctonia* sp., and *Phomopsis* sp.

fungi could be responsible for it. The first disease symptom is yellowing of needles proceeding from the tip to their base, next needle browning and dropping are observed. Disease symptoms on shoots are manifested as necroses, on which black acervuli, containing fungal spores are found (Łabanowski and Orlikowski 1997, Łabanowski et al. 2002).

The aim of the study was to investigate oxidative stress intensity and SOD activity in yew needles, on which disease symptoms were observed. Moreover, the locality of plant growth, i.e. sunny and shaded conditions were considered.

Material and methods

Plant material. Shoots, on which disease symptoms were observed, were cut off from common yew (*Taxus baccata* L) trees growing in the natural localities in the Dendrological Garden of the Agricultural University in Poznań (Fig. 1). Perennial individual yews of 5–6 high were grown on two localities, sunny and shaded by other plant formation. Shoots with no visible disease symptoms were selected as controls; one-year-old and growing on perennial shoots (2–3 year old). The plant material was sampled four times during May till July. Immediately after carrying to laboratory 200 mg samples of leaves were weighted and frozen in liquid nitrogen. In the winter season needles were collected from the experimental trees to identify the causal factor.

Fig. 1. A branch of *Taxus baccata* showing the leaves with disease symptoms

Identification of pathogen. After surface sterilization with 96% of ethanol (3 min) and subsequently 0,01% $HgCL_2$ (15 min), needles were washed few times in sterilized water and were transferred onto Petri dishes on PDA (Difco) medium and were left under the light at room temperature for 4 weeks. The identification of *P. funerea* were based on sporulation process.

Superoxide anion contents were measured based on its ability to reduce nitroblue tetrazolium (NBT) (Doke 1983). Samples were immersed in 3 ml 0.01 M K phosphate buffer (pH 7) containing 0.05% NBT and 10 mM NaN_3 for 1 h. After that the reaction solution was heated at 85°C for 15 min and cooled. O_2^- was measured spectrophotometrically at 560 nm and calculated as absorbance per 10 g of fresh weight.

Hydrogen peroxide concentration was assayed using the method by Messner and Boll (1994). Leaf samples were grounded in 3 ml cold K phosphate buffer (pH 7.0) containing 0.02 g Polyclar AT, a substance binding low-molecular compounds. The homogenate was centrifuged for 25 min. at 15 000 g. The reaction mixture contained 1.5 ml of extract, 0.15 ml K phosphate buffer (pH 7.0), 50 ml horseradish

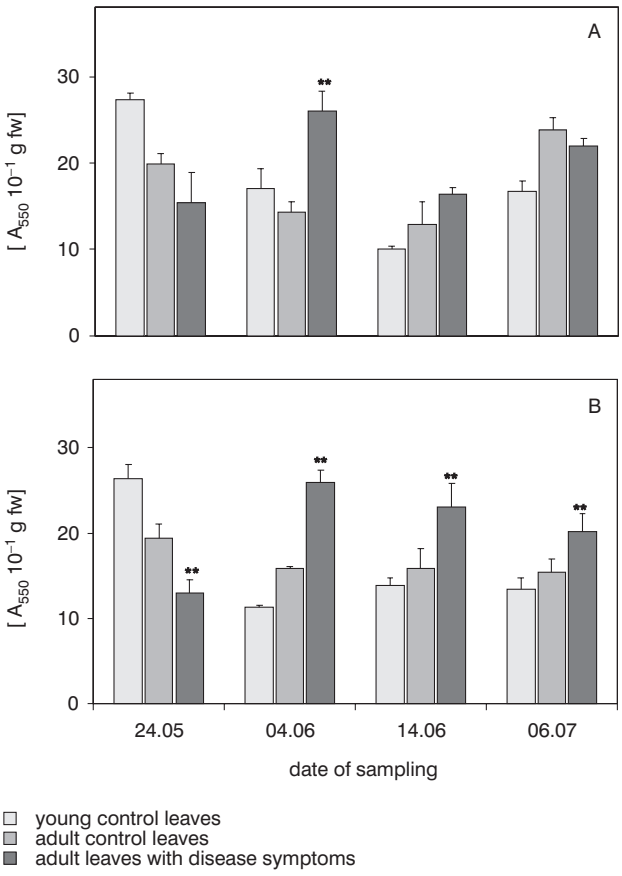

Fig. 2. Superoxide anion level in *T. baccata* leaves: A – sunny conditions, B – shaded conditions (mean ± SD). Data significantly different from adult control: *P< 0.05, **P< 0,01

peroxidase (1 mg ml^{-1}, dissolved in medium) and 0.05 ml ABTS (solution (0.05 M). Absorbance at 415 nm was measured after 3 min. Results were given in nmoles H_2O_2 per g of fresh weight.

Superoxide dismutase (SOD) activity was assayed using the method of Beauchamp and Fridovich (1971), which measures inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm. Samples were prepared by homogenizing frozen leaves in 3 ml cold solution containing: 0.05 M Na phosphate buffer (pH 7.0), 1% PVP, 1 mM EDTA and 0.5 M NaCl. The homogenate was centrifuged for 25 min. at 15 000 g. The 3 ml reaction mixture contained: 0.05 M Na phosphate buffer (pH 7.8), 0.1 mM EDTA, 97 mM L-Methionine, 120 mM Ribiflavin, 2 mM NBT and 30 μl of enzyme extract. The reaction was carried out for 10 min under a fluorescent lamp. One unit of activity was estimated as the enzyme quantity reducing the absorbance to 50% in comparison to that of tubes lacking the enzyme. Total enzyme activity was expressed in units × mg^{-1} protein.

Protein content was measured by the dye-binding method of Bradford (1976).

Statistical analysis: Experimental data were subjected to a one-way analysis of variance (Anova) and significant differences between among means were determined by Tukey multiple range test.

Results

The level of superoxide anion in young control leaves throughout the experimental period was similar, irrespective of the fact whether plants were growing in the sunny or shaded locality (Fig. 2). In the late spring, i.e. the time of intensive shoot growth, it was the highest and higher than in the 2–3 year old leaves. Later on the radical level was lower and showed higher stability both in the young and 2–3 year old leaves. In leaves with disease symptoms, except for the first assay date, increase of superoxide anion generation was found, and irrespectively of the growth conditions; 82 and 27% for the sunny and 64, 45 and 31%, respectively for the shaded conditions.

The level of hydrogen peroxide in young control leaves was rather low, though it was approx 1.5 times higher from the sunny than from the shaded position (Fig. 3). In 2–3 year old control leaves the level of that

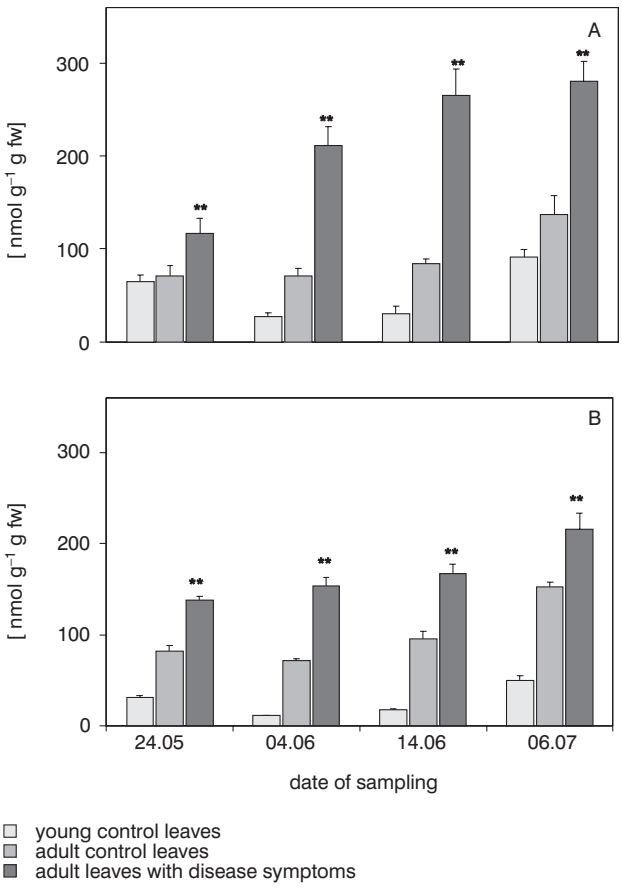

Fig. 3. Hydrogen peroxide level in *T. baccata* leaves: A – sunny conditions, B – shaded conditions (mean ± SD). Data significantly different from adult control: *P< 0.05, **P< 0,01

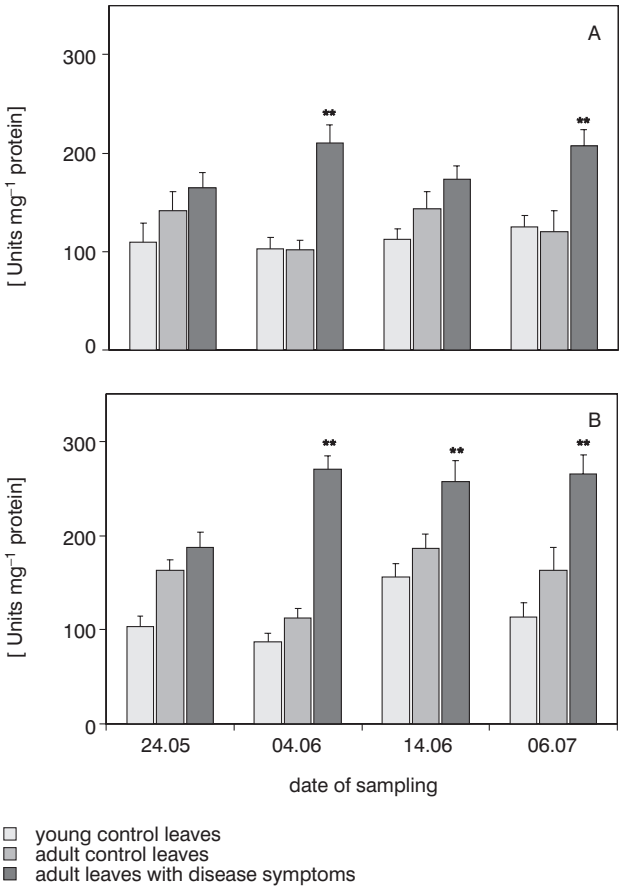

Fig. 4. Superoxide dismutase activity in *T. baccata* leaves: A – sunny conditions, B – shaded conditions (mean ± SD). Data significantly different from adult control: *P< 0.05, **P< 0,01

metabolite was stable, being slightly higher on the beginning of July. In the fungal affected leaves hydrogen peroxide level was 2–3 fold higher than in the adult control leaves, whereas it was generated in higher amounts on the sunny stand. This increase was 65, 200, 216 and 105% on the sunny stand and 68, 113, 76 and 42% on the shaded one, respectively.

Superoxide dismutase activity in young control leaves showed high stability, irrespective of the plants growth conditions and it was slightly higher in older shoots (Fig. 4). In leaves with disease symptoms an increase in the activity was found, reaching 16, 104, 21 and 73% on the sunny position and 15, 141, 38 and 57% on the shaded, respectively.

Discussion

On old yew trees commonly grown in parks and gardens extensive disease symptoms were observed, indicating disorders caused by adverse environmental factors or affection with pathogenic microorganisms (Kozłowska et al. 2002). Phytopathological testing confirmed a fungal pathogen, *Pestalotiopsis funerea* Desm., responsible for shoot blight of trees and shrubs of numerous coniferous species (Łobanowski et. al. 1997, 2002). Yew, as compare to other conifers, belongs to stress-tolerant species, however, shoot blight, especially in older specimens, results in they deteriorated decorative value. Exposure to high irradiation of shade bearing plant may also influence on the tolerance to other stresses.

Generation of reactive oxygen species in the amounts exceeding physiological levels of these metabolites is a common plant response to stress factors. In yew leaves, except for the first assay date, enhanced generation of $O_2^{\cdot-}$ was found in tissues with disease symptoms when compared to controls. This result confirms numerous studies on the mechanism of diseases induce by biotic and abiotic stresses (Mehdy et al. 1996, Low and Merida 1996, Dat et al. 2000). High level of that radical found in control leaves at the beginning of experiment (end of May) may be on effect of level irradiation in relation to CO_2 fixation. The insolation have no effect on the superoxide anion accumulation.

Important role in plant defense response is played by hydrogen peroxide, realatively stable metabolite and the $O_2^{\cdot-}$ dismutation by SOD may be the source H_2O_2 (Ogawa et al. 1997). As well as other alternative pathways of hydrogen peroxide generation might be considered in plants (Blokhina et al. 2003). It was directly involved in the pathogen inhibition and served as a signal, contributing to the initiation of defense mechanisms (Pietras et al. 1997, Kuźniak and Urbanek 2000). Małolepsza and Urbanek (2000), while conducting *in vitro* studies on tomato inoculated with *Botrytis cinerea*, showed an inhibitory effect of hydrogen peroxide on the mycelium growth. Lu and Higgins (1999) found a similar effect on the germination and growth of *Cladosporium fulvum*. Endogenous level of hydrogen peroxide in yew leaves increased along with age. Moreover, as a result of *P. funerea* affection significant increase of H_2O_2 generation was noted too and correlated with the SOD activity. But, it is hard to say whether it can be responsible for inhibition of pathogen spread or whether it may play a signaling function in a rather undefined defense mechanism.

One of the essential factors determining the metabolic activity in plants are the light conditions. It was revealed that in the yew plants under the sunny conditions H_2O_2 levels, was higher and correlated with peroxidase activity (Kozłowska et al. 2002). It might be a physiological effect of high photosynthetic activity, or more likely this metabolite was generated in a shade bearing plant as a result of high insolation.

Changes in the SOD activity as effect environmental stresses is well documented. Babitha et al. (2002) observed that the activity of this enzyme depends on the pathogen resistance. They showed 2–3-fold higher activity in the resistant Indian millet genotype than in susceptible genotype infected with *Sclerospora graminicola*. Floryszak-Wieczorek (2000) confirmed this hypothesis on potato cultivars differing in the susceptibility to *Phytophthora infestans*. Pukacka i Pukacki (2000), under the investigations of Scot pine growing in polluted area observed increase of SOD depended on growing season, population provenance and age of needles. According Madamanchi at al. (1991) SOD activity in spruce was related to ozone concentration, growth locality and other environmental factors. Under shaded conditions as compare to sunny one, SOD activity of yew was significantly higher and not correlated with the level of hydrogen peroxide. It additionally confirms generation of hydrogen peroxide in a spontaneous reaction, especially under sunny conditions.

Summing up it may be stated that *P. funerea*, similarly as many stress factors, induces an oxidative burst in yew leaves, which has led to a considerable accumulation of H_2O_2. Excessive accumulation of this metabolite may be an effect of SOD activity and possibly carring to the restriction of pathogen.

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References

Babitha M.P., Bhat S.G., Prakash H.S., Shetty H.S. 2002. Differential induction of superoxide dismutase in downy mildew-resistant and -suscepti-

ble genotypes of pearl millet. Plant Pathology 51: 480–486.

Baker C.J., Orlandi E.W. 1995. Active oxygen in plant pathogenesis. Annual Review of Phytopathology 33: 299–321.

Beuchamp CH., Fridovich I. 1971. Superoxide dismutase: improved assays and an assays applicable to acrylamide gels. Analytical Biochemistry 44: 276–287.

Blokhina O., Virolainen E., Fagerstedt K. V. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals of Botany 91: 179–194.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254.

Doke N. 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiological Plant Pathology 23: 345–357.

Dat J., Vandenabeele S., Vranova E., Van Montagu M., Inze D., VanBreusegem F. 2000. Dual action of the active oxygen species during plant stress responses. CMLS. Cell Molecular Life Science 57; 779–795.

De Gara L., de Pino M.C., Tommasi F. 2003. The antioxidant systems vis-s-vis reactive oxygen species during plant-pathogen interaction. Plant Physiology and Biochemistry 41: 863–870.

Floryszak-Wieczorek J. 2000. Effect of *Phytophthora infestans* on the activity of oxygen scavenging enzymes in leaves of different potato genotypes. Phytopathologia Polonica 19: 147–155.

Kozłowska M., Rybus–Zając M., Gniazdowska–Skoczek H. 2002. Reakcja metaboliczna *Taxus baccata* L. na porażenie grzybem *Pestalotiopsis funerea* Desm. w zależności od warunków siedliskowych. Acta Agrobotanica 55(1): 149–155.

Kuźniak E., Urbanek H. 2000. The involvement of hydrogen peroxide in plant responses to stresses. Acta Physiologiae Plantarum 22(2): 195–203.

Lamb C., Dixon R.A. 1997. The oxidative burst in plant disease resistance. Annual Review Plant Physiology and Plant Molecular Biology 48: 251–275.

Langebartels Ch., Wohlgemmuth H., Kschieschan S., Grun S., Sandermann H. 2002. Oxidative burst and cell death in ozone-exposed plants. Plant Physiology and Biochemistry 40: 567–575.

Low P.S., Merida J.R. 1996. The oxidative burst in plant defense: function and signal transduction. Physiologia Plantarum 96: 533–542.

Lu H., Higgins V.J. 1999. The effect of hydrogen peroxide on the viability of tomato cells and of the fungal pathogen *Cladosporium fulvum*. Physiological and Molecular Plant Pathology 54: 131–143.

Łabanowski G., Orlikowski L. 1997. Ochrona roślin iglastych i wrzosowatych. Wyd. Plantpress, Kraków.

Łabanowski G., Orlikowski L., Soika G., Wojdyła A. 2002. Ochrona drzew i krzewów iglastych. Wyd. Plantpress. Kraków.

Madamanchi-Nageswara R., Hausladen A., Alscher R. G., Amundson R. G., Fellows S. 1999. Seasonal changes in antioxidants in red spruce (*Picea rubens* Sarg.) from three field sites in the northeastern United States. New Phytologist 118: 331–338.

Małolepsza U., Urbanek H. 2000. The oxidants and antioxidant enzymes in tomato leaves treated with o-hydroxyethylorutin and infected with *Botrytis cinerea*. European Journal of Plant Pathology. 106: 657–665.

Mehdy M.C., Sharma Y.K., Sathasivan K., Bays N.W. 1996. The role of activated oxygen species in plant disease resistance. Physiologia Plantarum 98: 365–374.

Messner B., Boll M. 1994. Cell suspension cultures of spruce (*P. abies*): inactivation of extracellular enzymes by fungal elicitor-induced transiet release of hydrogen peroxide (oxidative burst). Plant Cell, Tissue and Organ Culture 39: 69–78.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7: 405–410.

Neil S., Desikan R., Clarke A., Hancock J. 1999. H_2O_2 signalling in plant cells. In: „Plant Responses to Environmental Strees. Oxford 8: 59–64.

Neil S., Desikan R., Hancock J. 2002. Hydrogen peroxide signalling. Current Opinion in Plant Biology 5: 388–395.

Pietras T., Małolepsza U., Witusik A. 1997. Udział nadtlenku wodoru i reaktywnych postaci tlenu wytwarzanych przez oksydazę NADPH w odporności roślin przeciwko patogenom. Wiadomości Botaniczne 41: 43–50.

Ogawa K, Kanematsu S., Asada K. 1997. Generation of superoxide anion and localization of CuZn-superoxide dismutase in the vascular tissue of spinach hypocotyls: their association with lignification. Plant Cell Physiology 38: 1119–1126.

Pukacka S., Pukacki P. M. 2000. Seasonal changes in antioxidant level of Scot pine (*Pinus sylvestris* L). needles exposed to industrial pollution. II. Enzymatic scavengers activities. Acta Physiologiae Plantarum 4: 457–464.

Scandalios J.G. 2002. The rise of ROS. Trends in Biochemical Sciences. 27: 483–486.

Veljovic–Jovanovic S., Noctor G., Foyer C.H. 2002. Are leaf hydrogen peroxide concentrations commonly overestimated? The potential influence of

artefactual interference by tissue phenolics and ascorbate. Plant Physiology and Biochemistry 40: 501–507.

Wojtaszek P. 1997a. Mechanisms for the generation of reactive oxygen species in plant defence response. Acta Physiologiae Plantarum 19: 581–589.

Wojtaszek P. 1997b. Oxidative burst: an early plant response to pathogen infection. Biochemistry Journal 322: 681–692.