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Original Research

Changes in Antioxidant Enzyme Activities of European Mistletoe (*Viscum album* L. subsp. *Album*) Leaves as a Response to Environmental Stress Caused by Pollution of the Atmosphere by Nitrogen Dioxide

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Abstract

In the present study we investigated the seasonal pattern of activity of antioxidant enzymes such as superoxide dismutase (SOD) catalase (CAT) ascorbate peroxidase (APX), guaiacol peroxidase (POD) and syringaldazine peroxidase (SPOD) as well as the total protein concentration in the European mistletoe (*Viscum album* L. subsp. album). We studied mistletoe leaves that grew on the selected tree species in different parts the city of Lodz, exposed to a greater or lesser extent to the nitrogen dioxide. Sampling campaigns were conducted during the growing season 2013 in early May (at the beginning of the growing season) and in November (at the end of the growing season). We showed considerable seasonal variations of antioxidant enzymatic activity and total protein concentrations for all the samples studied. The test parameters varied depending on the host plants exposition to pollution with nitrogen dioxide. The changes in enzymatic activity did not depend on the host plants. In mistletoe leaves greater changes of SOD activity and total protein concentration were observed in autumn. There is correlation between the level of nitrogen dioxide in atmosphere and activities the enzymes. SOD activity was significantly higher in autumn when the host plants were defoliated. Increased CAT activity was observed in late spring. We demonstrated the positive correlation between changes in enzyme activities and the progress of growing season. Increased activities of POD, CAT and APX to a limited extent depended on the place of growing and exposition to air pollution. In late spring activity of the enzymes did not significant grow because of the protective umbrella from host plant leaves preventing the access of nitrogen dioxide to mistletoe. The higher SOD activity in mistletoe is a consequence of oxidative stress causes by nitrogen dioxides evidently observed in the city center. The test parameters, mainly the activity of SOD, can be used in the future as markers of the

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environment purity, especially in the autumn and winter when the temperatures are above freezing, and there are no leaves on the trees.

Keywords: stress, Peroxidase, European mistletoe

Introduction

Plants are exposed to many harmful factors during growth and development. Biotic factors such as bacterial or fungal pathogens, abiotic pollutants of soil and atmosphere, changes of temperature or humidity as well as solar exposure can cause disorders of cell metabolism called oxidative stress [1-7]. These factors disturb respiration processes, which results in generation of excess amounts of reactive oxygen species (ROS) in the form of singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^\bullet$) but mainly of superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) [8-10].

Plants growing in great urban agglomerations are especially vulnerable to the oxidative stress mostly caused by air pollution with sulfur dioxides or nitrogen dioxide. An excess of reactive oxygens can damage structural and enzymatic proteins which is harmful for cellular organels, e.g., DNA [11]. Long-term (24 h) heat shock (42ºC) causes damage and oxidative stress in *Triticum aestivum* seedlings and results in overproduction of $O_2^{\bullet-}$ [12]. Reactive oxygen species (ROS) can induce leaf senescence, which is executed through the programmed cell death and which plays an important role in plant survival. Persistent oxidative stress leads to death of cells, tissues and finally of whole plants [13].

All plants in order to survive must deal with the oxidative stress and they employ different defensive strategies to reduce harmful effects of ROS overproductions. Antioxidative enzymes such as catalase (CAT), ascorbate peroxidases (APX) and peroxidase measured with guaiacol (GPOD) and superoxide dismutase (SOD) scavenge ROS resulting in oxidative stress.

SOD plays a crucial role because it transforms more reactive and very harmful $O_2^{\bullet-}$ to less reactive H_2O_2 which is involved in many important biochemical reactions, e.g., process of lignification of a cell wall.

The European mistletoe (*Viscum album* L. subsp. *album*) is an evergreen, hemi-parasitic plant, normally found growing on a variety of trees. Mistletoe plants are able to accumulate NO_2 at a larger concentration than host species [14, 15]. These findings may indicate that *V. album* subsp. *album* is a higher resistance to the harmful effects of city environment and attacks hosts with lowered viability caused by environmental pollution [16].

Previous studies demonstrated that nitrogen pollution is generally considered an important factor responsible for the decline of vascular plant species in large cities [17]. In the previous report, we documented that nitrogen accelerated mistletoe post dispersal distribution [18].

This work examines the activities of enzymes involved in ROS scavenging. The activities of CAT, APX, and two forms of peroxidases (one measured with guanaco, the other with syringaldazine) were measured. The latter substance is involved in cell wall lignification. CAT triggered disproprotionation of H_2O_2 while APX, POD and SPOD used H_2O_2 to oxidise bonds as well as ascorbate and phenolic compounds and SOD converts $O_2^{\bullet-}$ to H_2O_2. The enhanced level of total protein content seems to indicate the increase in the above enzymes.

The objective of this study was to investigate the influence of seasons (May and November) on antioxidant activity of *V. album* samples originating from different host trees, located in the city of Lodz as well as to find whether there is correlation between the antioxidant activity *V. album* and NO_2 contamination of the air which would allow to use mistletoe as a bio-indicator.

Material and Methods

Site Description

Lodz is the third largest city in Poland located at 19º20' N/19º38' E at an altitude of 170 to 284.1 m a.s.l. in the centre of the country. It covers an area of 293 km^2 and in 2014 had about 706.000 inhabitants. The average annual precipitation in the period 1931-1998 ranged between 530 and 580 mm. The average annual temperature (1931-1998) ranged between 7.5 and 8.4ºC [19].

Acer saccharinum L. is the most common tree (24.1%) in the city of Lodz, followed by *T.* × *euchlora, Sorbus aucuparia, Salix alba,* and *P.* ×*canadensis* mainly occurring at the border of the urban area. Frequent forestry newcomers include *Robinia pseudoacacia* and *Acer negundo* which are often planted as street trees because they are tolerant towards polluted city air [20]. *Acer saccharinum* L., *P.* ×*canadensis* Moench (in the varieties of 'Serotina'. 'Robusta') and *R. psedoacacia* L. are frequently infested with mistletoe [18].

Air pollution in Lodz is a well known phenomenon. Nitrogen oxides (NO_x, and especially nitrogen dioxide, or NO_2) are one of the most common air pollutants. For example, from the city centre (nearer the pollutant source) to the outskirts of the city (farther from the source) in the Lodz city NO_2 concentrations were high at 52 Drewnowska St. (28.6 µg/m^3, over 24 h, from January to December in 2013; Regional Inspectorate for Environmental Protection in Lodz) and low (16.2 µg/m^3) 6 km further west near the 144 Aleksandrowska St. (Fig. 1).

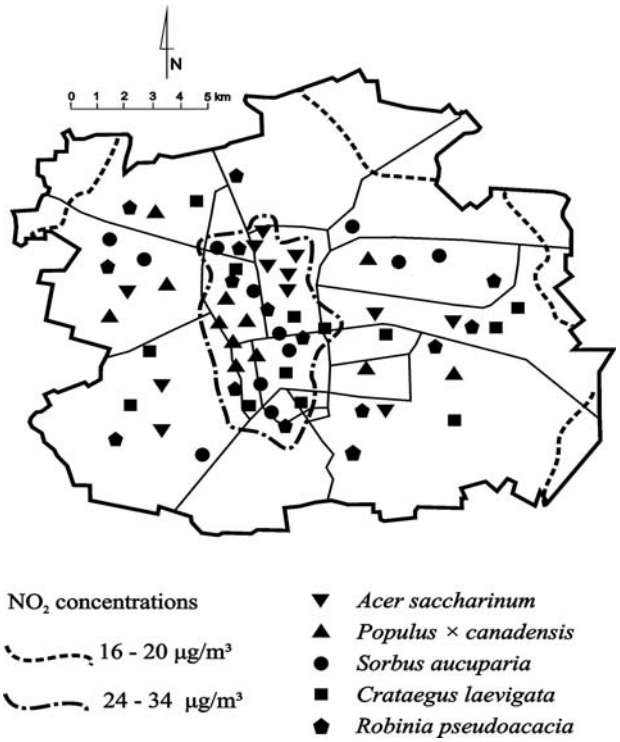

Fig. 1. Regression coefficient for SOD and SPOD activities in the leaves of mistletoe from different host trees.

Materials

Plant Material

Leaves of *V. album* were harvested in May and November 2013, from different host trees located either in the city centre (very high pollution) or at the outskirts of the city (low pollution). Due to the temperature above zero in both months, mistletoe plants were in a vegetative state. Locations were chosen on the basis of air pollution data from Regional Inspectorate for Environmental Protection in Lodz and terrestrial mapping of mistletoes conducted by Kołodziejek et al. [18].

The following host plant species were selected: *Acer saccharinum, Sorbus aucuparia* L. emend. Hedl., *P. ×canadensis* cv. 'Robusta', *Robinia pseudoacacia* and *Crataegus laevigata* (Poir.) DC. Five isolated or dominating trees per species were chosen in the city centre and in the outskirts. Fully expanded leaves (3-5 leaves) were cut from the south side of the canopy at a height of 1.70-2.50 m above the ground. A total of 60 plant samples were collected they were kept in a cool box during transport to the laboratory and until the moment of analysis.

Methods

Enzyme Assays

Half a gram of the tissue located up to 1 mm beneath the peel was homogenized in a mortar with ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM ascorbate, 1 M NaCl, 1 mM EDTA and 1% polvinylpyrrolidone. After centrifugation (20 000 × g, 15 min) the supernatant was used to determine the activities of peroxidase, superoxide dismutase and protein contents.

Assay of Peroxidase (EC 1.11.1.7) Activity with Guaiacol and Syringaldazine

Peroxidase activity with guaiacol (GPOD) was determined by increase in absorbance at 470 nm (= 26.6 mM^{-1} cm^{-1}) [21]. The reaction mixture contained 25 mM acetate buffer pH 5.6, 5mM guaiacol, 15 mM H_2O_2 and enzyme extracts. The addition of H_2O_2 started the reaction. Peroxidase activity with syringaldazine (SPOD) was determined spectrophotometrically by an increase in absorbance at 530 nm (= 27 $mM^{-1}cm^{-1}$). The reaction mixture contained 25 mM phosphate buffer pH 6.0, 41.6 µM syringaldazine (0.050 cm^3) solution (3.1 mg in 4 cm^3 methanol), 0.11 mM H_2O_2 and enzyme extracts. The addition of syringaldazine started the reaction.

Assay of Ascorbate Peroxidase (EC 1.11.1.11) Activity

The APX activity was assayed in extracts from fresh leaves by the Nakano and Asada method [22]. Two cm^3 of the reaction mixture contained: 50 mM potassium phosphate buffer of pH 7.0, 0.05 cm^3 of enzymatic extract, 0.05 cm^3 of 5 mM the ascorbate. The reaction was initiated by adding 0.10 cm^3 of 0.5 mM H_2O_2. Activities of enzymes were measured by the reduction of the optical density at wavelength 265 nm for 5 minutes. To assess activity of APX the following coefficient for ascorbate was used ε = 13.7 mM^{-1} cm^{-1}. The amount of enzyme oxidizing 1 mM of ascorbate during 1 minute at 30ºC was accepted as one unit (U) of the enzyme activity.

Assay of Catalase (EC 1.11.1.6) Activity

The CAT activity was assayed spectrophotometric according to Dhindsa et al. [23]. 2 cm^3 of the incubation mixture contained 50 mM potassium phosphate buffer pH 7.0 15 mM H_2O_2 and 0.03-0.05 cm^3 of the enzymatic extract. The decrease in the H_2O_2 concentration was monitored spectrophotometrically at 240 nm wavelength. To assess activity of CAT the following coefficient for H_2O_2 was used ε = 45.2 mM^{-1} cm^{-1}.

Disproportionating 1µmol H_2O_2 during 1 minute at 30ºC was accepted as one unit (U) of the enzyme activity.

Assay of Superoxide Dismutase (EC 1.15.1.1) Activity

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Beauchamp & Fridovich modified by Patykowski and Kołodziejek [19]. Three cm^3 of the

reaction mixture contained 50 mm phosphate buffer at pH 7.8, 13 mM methionine (Sigma), 75 μM NBT, 2 μM riboflavin (Sigma), 0.1 mM EDTA (Sigma) and 0.02 cm^3 enzyme extract. Riboflavin was added last and the tubes were placed 30 cm below two 15 W fluorescent lamps. The reaction was initiated by switching on the light and for 10 min. Switching off the light stopped the reaction and the tubes were covered with a black cloth. The unilluminated tubes were the control. The absorbancies at 560 nm were read. The volume of extract corresponding to 50% inhibition of the reaction was considered one enzyme unit.

Assay of Protein Contents

Proteins were determined by the method of Bradford [24] using bovine serum albumin (Sigma) as a standard.

Statistical Analysis

Prior to data analysis all data were arcsine square root transformed to correct sample heterogenity. Two-way statistically significant differences between 'seasons' (two levels: spring and autumn) and 'sites' (two levels: the outskirts of a city and the city centre) were calculated under two-way ANOVA. Tukey's multiple comparison post hoc test (HSD-test) was carried out to show significant ($P < 0.05$) differences between individual treatments. Statistical analysis was carried out using Statistica v. 10 (Statsoft. Inc., 2011).

Results

The mean values of antioxidant enzyme activities and protein contents in the leaves of mistletoe from different host trees are shown in Table 1.

The specific activities of CAT in spring were almost twice higher than those in autumn. However, the activities of APX, GPOD, SPOD, and mainly of SOD were in general significantly lower in spring than in autumn. It is worth showing that SOD activity increased significantly in the material from all five host plants, while that of APX only in the material from three host species. In autumn, V. album collected from Crataegus had the lowest protein content. Protein content in the leaves of mistletoe hosted by Acer, Populous, and Robinia did not change significantly in different seasons (Table 1). In general, antioxidant activity was higher in autumn than in spring.

The most statistical changes depended on the season and site (Table 2). Correlation between total protein content and enzymatic activities may be of major importance.

Two-way ANOVA indicates that activity of these antioxidant enzymes and total content of protein were significantly affected by seasons ($P < 0.05$) (Table 2).

It was shown that the regression coefficients for SOD and SPOD were consistent, which indicates an important role of these enzymes for mistletoe functioning (Fig. 2).

The map (Fig. 1) shows the levels of nitrogen dioxide pollution of the atmosphere of where the spaces were collected from the host trees.

Discussion

Many articles concerning the problem of pollution have been written. Environmental pollution factors include heavy metals [25], dusts, and gases that cause acid rains [26] and other problems. They damage chloroplasts [27], which leads to disturbances of plant growth and development.

Table 1. ANOVA results of antioxidant enzyme activities and protein contents in the leaves of mistletoe from different host trees based on post-hoc mean comparison (Tukey's multiple comparison post hoc test). Data are means ± SD of 3 replicates (n = 3).

Constituent		CAT U/g fr.w.	APX U/g fr.w.	GPOD U/g fr.w.	SPOD U/g fr.w.	SOD U/g fr.w.	Protein mg/1 g fr.w.
Spring	Viscum/Acer	0.0589±0.0242b	0.059 ± 0.0241a	18.16 ± 7.73ab	20.06 ± 4.19a	23.57 ± 14.54a	6.41 ± 0.94a
	Viscum/Crataegus	0.0509±0.0198a	0.051±0.0198b	23.68±9.09b	22.44±5.56a	24.88±11.61a	6.28±0.66a
	Viscum/Populus	0.0461±0.0004b	0.046±0.0001a	22.81±9.35ab	18.22±9.35a	22.52±14.64a	6.31±1.13a
	Viscum/Robinia	0.0389±0.0008a	0.039±0.0008b	14.48±0.96a	17.43±2.09a	29.09±5.37a	6.28±0.02a
	Viscum/Sorbus	0.0494±0.0174a	0.049±0.0174a	19.51±5.99ab	17.97±5.87a	33.32±12.64a	7.37±1.24b
Autumn	Viscum/Acer	0.0066±0.0023a	0.579±0.126a	24.14±7.54a	23.33±5.06a	50.19±37.33a	7.33±0.95a
	Viscum/Crataegus	0.0066±0.0030a	0.465±0.069a	26.03±8.88a	23.29±2.46a	39.50±31.48a	6.70±1.15b
	Viscum/Populus	0.0063±0.0019a	0.489±0.109a	26.81±9.64a	18.68±5.45a	26.96±28.74a	7.53±1.09a
	Viscum/Robinia	0.0057±0.0026a	0.502±0.037a	17.26±3.82a	22.34±4.85a	21.01±9.93a	7.85±1.20a
	Viscum Sorbus	0.0055±0.0015a	0.518±0.089a	24.29±5.41a	19.74±5.68a	51.95±28.26a	7.37±1.24a

Different letters in each column for the same season indicate significant differences ($P < 0.05$) across all host trees (N = 12 mistletoes per group).

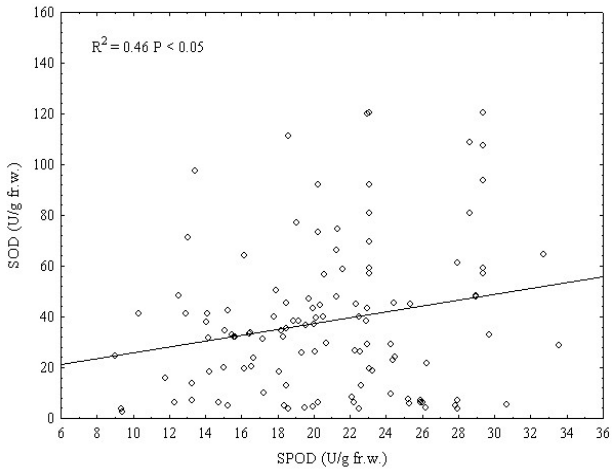

Fig. 2. Sampling sites in Lodz: very high and low pollution areas.

Similarly a lot of research work focused on plant defence mechanisms against pathogens (bacteria and fungi) as well dehydration, low temperature and salinity [28]. All these factors lead to molecular changes.

To the best of our knowledge there is little information concerning enzymatic changes in parasitic and hemiparasitic plants. European mistletoe is a semi-parasitic evergreen shrub, its growth and development are closely adapted to the host [15, 29] and seasons of the year [21, 30]. The seasonal changes of physiological activity of *V. album* are similar to those of woody evergreen species and depend on the development stages of the parasite [31].

It has been shown that mistletoe's influence on hosts was similar to the effect of long-lasting drought stress [32]. It is known that the mistletoe parasite has a huge impact on the transport of primary metabolites in a host plant and has a decisive influence on its growth [33]. During plant-pathogen interactions the deposition of β-1,3-glucan, triggered by reactive oxygen species [34], leads to callose accumulation in the form of a structural scaffold for toxic molecules [35] and reflects its participation in host self-defence mechanisms. Callose localization in root cells may lead to oxidative burst and host cell death.

Activities of many plant enzyme involved in defence mechanisms were examined after the oxidative stress. We compared the parameters in the material collected in autumn and late spring because in autumn host plants are already deprived of leaves and when temperatures are above zero the mistletoe vegetation is most intensive due to access to light. While in spring host plant leaves deprive mistletoe of sun light.

European mistletoe is highly resistant to the harmful effects of city environment and attacks trees with lowered viability caused by environmental pollution [16]. In the classical research model plants exposed to anthropogenic air pollution, e.g., nitrogen dioxides. The plant defence reaction consists in activation of the enzymes responsible for ROS scavenging. An enhanced level of ROS can cause damage to biomolecules such as lipids, proteins and DNA. These reactions can alter membrane properties like fluidity, ion transport, enzyme activity, protein cross-linking, inhibition of protein synthesis, DNA damage, and so forth ultimately resulting in cell death [6]. The global climates changes brings about novel combinations of severity and timing of different stresses, the effects of which on tree performance are hard to predict. Photosynthesis and growth rates decline with increasing tree age and size, while support biomass in roots, stem and branches accumulates and the concentrations of non-structural carbohydrates increase. It was suggested that combined stresses can influence survival of large trees even more than chronic exposure to a single predictable stress such as drought [36].

ROS are a hallmark of successful recognition of infection and activation of plant defences [3]. Rai and Agrawal [37] reported that in rice seedlings exposed to high concentrations of nitrogen dioxide, mainly SOD activity (but also those of POD and APX) increased. Hussain and Reigos [38] showed that after treatment of Arabidopsis thaliana seedlings with rutin, a secondary metabolite increases POD activity, which reflected to stress. During oxidative stress have problems homeostasis and normal functioning of the activity of chloroplasts [39]. Kim et al. [40] observed modulation of 20 different peroxidases during rice interaction with the plant-pathogenic fungus *Magnaporthe oryzae*, which suggests intracellular ROS homeostasis. SOD isoenzymes are essential for scavenging excess reactive oxygen species in living organisms. High SOD activity have been corelated with the generation of $O_2^{\bullet-}$ radical as a consequence of air pollution with nitrogen dioxides. This enzyme is involved in the metabolism of reactive oxygen species by dismutation of $O_2^{\bullet-}$ to molecular oxygen and hydrogen peroxide. Radwan et al. [41] showed that particular isoforms of SOD interacted with other enzymes protecting cells from the oxidative damage. A recent study showed that the expression of genes encoding SOD isoforms may in the future explain the mechanism of stress induction [42].

In plants exposed to pathogens as well as to abiotic stress factors, ROS generation is triggered leading to H_2O_2 overproduction [6]. During the host-pathogen interaction the ability to synthesise H_2O_2 degrading enzymes such as catalase and peroxidase depends on plant resistance [10, 43]. This proves, that in plants exposed to abiotic stress CAT activity increases most significantly because it is responsible for disproportionation of H_2O_2 excess under stress [44]. It can be indirectly involved in the process of deactivation of increased H_2O_2 concentrations by different isoforms of peroxidases [43]. Overproduced reactive oxygens such as $O_2^{\bullet-}$ and H_2O_2 are often deactivated already in the apoplastic fraction and this is closly connected with cell wall reconstrution [45]. Peroxidases participate in the process of the cell wall remodeling during oxidative stress [45, 46]. Activities of special peroxidases responsible for lignification process and for cell wall strengthening are often higher under stress [47]. The activity of peroxidase involved lignification measured with a specific substrate of syringaldazine (SPOD) increased [48]. Especially upon

Table 2. Two-way of ANOVA effects of season (spring vs. autumn), site (the outskirts of a city vs. the city centre), and their correlation on antioxidant enzyme activity and protein contents in leaves of mistletoe from different host tree.

Dependent variable	Source	d.f.	SS	MS	F-value	P-value
CAT	Season	1	0.003	0.003	9.704	<0.001
	Site	1	0.006	0.006	0.168	0.682
	Season × site	1	0.006	0.006	0.015	0.902
APX	Season	1	0.365	0.365	17.771	<0.001
	Site	1	0.007	0.007	0.003	0.954
	Season × site	1	0.010	0.010	0.533	0.466
GPOD	Season	1	479.17	479.17	9.219	<0.001
	Site	1	83.48	83.48	0.708	<0.05
	Season × site	1	36.80	36.80	0.709	0.401
SPOD	Season	1	259.27	259.27	10.191	<0.01
	Site	1	116.50	116.50	4.579	<0.05
	Season × site	1	111.45	111.45	4.381	<0.05
SOD	Season	1	9881.9	9881.9	15.08	<0.001
	Site	1	7992.2	7992.2	12.20	<0.001
	Season × site	1	4087.8	4087.8	6.246	<0.05
Protein	Season	1	6.605	6.605	4.629	<0.05
	Site	1	5.668	5.668	3.972	<0.05
	Season × site	1	4.271	4.271	2.994	<0.05

direct pathogen attack peroxidases (POD) or oxalate oxidases participate in plant defense through reinforcement of plant cell wall [49].

It was shown in this study that SOD activity was generally higer in autumn period and that increased nitrogen dioxide concentration in the atmosphere intensified this process (Tables 1 and 2). We put forward of hypothesis that in mistletoe the high cosntitutive activities of CAT and APX indicated its high adaptability thus it seems that these two enzymes are sufficient for H_2O_2 scavenging. That is why no correlation between CAT and APX activities and the level of air pollution was observed. It was shown that the regression coefficients for SOD and SPOD indicated their important role for mistletoe functioning (Fig. 1).

Mistletoe is a hemiparasitic plant able to biosynthesise necessary compounds but it also takes some nutrients from host trees. Vicas et al. [50] showed that aqueous extracts obtained from the leaves of mistletoe had higher antioxidant potentials in autumn than in spring, but this activity depended not only on the harvesting time, but on host trees as well. Low molecular compounds such as phenolic acids and flavonoids were found to be mainly responsible for antioxidant properties of the mistletoe leaves [30]. These authors demonstrated that the differences in antioxidant activity between leaves and stems of mistletoe speciments harvested from different trees could be attributed to environmental factors such as season, climate and temperature which can significantly affect the accumulation of antioxidant components in the plant tissue [30]. Inhibition of oxidative stress generating free radicals and lipid peroxidation was noted in vitro cultured cells after treatment with extracts of mistletoe lectin (KML) [51-52]. These results suggest that KML is a promising antioxidant due to its ability to scavenge free radicals, thus protecting cell against oxidative damage induced by free radicals.

The present study revealed significant influence ($P < 0.05$) of the season on the antioxidative activities of enzymes (CAT, APX, GPOD, SPOD, SOD) in mistletoe samples originating from different species of trees located in the city of Lodz. We observed a significant decrease in CAT and APX activities in November in comparison with May. The habitat type was significant ($P < 0.05$) for SPOD and SOD enzyme activities (Table 2) in both periods (May and November). The enzyme activities were higher in the city centre in comparison with the outskirts (Fig. 2). The season also influenced the protein contents in the leaves of mistletoe from different host trees.

We observed the correlation between GPOD, SPOD, SOD and protein contents and sites of growth and season, but no such relationship was observed for CAT and APX (Fig. 1).

The mistletoe reaction to stress is not typical because it may affect host plants similarly to pathogens. However, it

seems that due to high SOD induction and to the fact that host plants are deprived of leaves, mistletoe functions as a non-parasitic plant. We showed that host plants did not influence SOD activity. The mistletoe grew well in spite of high nitrogen dioxide pollution of the atmosphere.

It still needs elucidation whether mistletoe can be regarded as a biological marker of the environmental pollution with gasses.

Conclusions

Our study shows that seasonal changes in the activity of antioxidant enzymes in mistletoe occurred independently of the host plant species, and they were higher in the autumn. We believe that especially noticeable higher SOD activity in the leaves of mistletoe growing in the city center the nitrogen dioxide concentration in the atmosphere is high, proving its adaptation to pollution. Mistletoe can perfectly cope in spite of the conditions for increased pollution. We suggest that mistletoe can be used as a biological marker of environmental pollution, toxic gases and the general health of trees.

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