Seasonal Changes in the Activities of Apoplastic, Cytoplasmic, Ionically and Covalently Bound Isoperoxidases from Norway Spruce (*Picea abies* (L.) Karst.) Needles: A Comparison between Three Collection Sites with Different Ambient Ozone Concentrations

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Apoplastic, cytoplasmic, ionically and covalently bound isoperoxidases were investigated from needles of Norway spruce (*Picea abies* (L.) Karst.) and a pronounced genetic variability of the isoenzyme pattern was found. The enzymatic activity of each group of isoperoxidases was determined between November 1989 to November 1990 from needles of three locations (Velmerstot, Glindfeld, Haltern) with different ozone concentrations. Apoplastic peroxidases of needles from trees grown in Velmerstot and Glindfeld showed up to two-fold higher activities than the same group of peroxidases from Haltern. Based on the recorded increased ozone concentrations in Velmerstot and Glindfeld a positive correlation between the activity of the apoplastic peroxidases and ambient ozone concentrations at the different locations could be demonstrated. A putative role of the apoplastic peroxidases as a bioindicator for air pollutants is discussed.

Introduction

Since about twenty years novel forest decline has become an increasingly important problem in Central Europe. The typical damage symptoms of this phenomenon were first reported for fir *(Abies alba)* and spruce *(Picea abies)*, but later also for deciduous trees. Substantial efforts have been made to reveal the cause of this decline and a variety of hypotheses have been published. In most cases single factors such as air pollutants, acidic rain, drought, frost, nutrient status, soil acidification, climate or pathogens have been favoured. However, novel forest decline appears to be caused by multifactor stress [1–6].

Air pollutants like ozone, sulphur dioxide, nitrogen monoxide and nitrogen dioxide are considered to play a key role within such a multifactor stress system. Especially the annual ozone maximum during summer represents an important oxidative stress acting on all living organisms. The effect of ozone upon plants and the subsequent alterations of physiological processes are well known [7–9].

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Plant tissues possess powerful defense mechanisms against oxidative stress [10]. Peroxidases, which are involved in an antioxidative defense system, play an important role in the detoxification of highly reactive oxygen species like hydrogen peroxide [11]. Peroxidases are also known to be stress – related enzymes: because changes in temperature, pathogen attack, wounding and heavy metalions [12–14] may lead to alterations of peroxidase activities or the induction of new isoenzymes. Furthermore, many reports deal with the influence of air pollutants on peroxidase activities. The resulting effects are discussed controversely and it is still unclear, whether peroxidase activity represents an useful bioindicator for the impact of air pollutants on plants [15, 16] or not [17, 18].

In general, many efforts have been made to discover biochemical or physiological bioindicators for air pollutants as an additional possibility for the classification of damaged forests [19–21].

Alterations of peroxidase activities are normally obtained by measuring crude protein extracts of total plant tissues. Since peroxidases occur in different plant tissues and cell compartments [22–25], the effects of stress should be investigated more specifically by using the isolated different groups of isoperoxidases.

Our current work is part of a research project to characterize the physiological and biochemical

status of field grown Norway spruce (*Picea abies* (L.) Karst.) from three sites of the state of North-rhine-Westfalia/Germany. Two sites (Velmerstot and Glindfeld) are located in remote mountain regions and they are characterized by increased ozone values. At the third site (Haltern), which is located close to an industrial area, the ozone is half the concentrations normally found. Our interest was focussed on the determination of the enzymatic activities of apoplastic, cytoplasmic, ionically and covalently bound isoperoxidases from needles obtained from several trees at each location. Attempts were made to obtain a correlation between enzyme activities and the ambient concentrations of air pollutants. Furthermore, we proved whether different groups of peroxidase isoenzymes could be taken as bioindicators for air pollutants.

Materials and Methods

Description of the collections sites

Eggegebirge

The spruce forest is located near Velmerstot on a western slope in the southern part of the Teutoburger Wald called Eggegebirge 410 m above sea level. The trees are about 57 years old and belong to a damage class (1990) 1 to 2. In 1989 the air pollutants were measured with an annual average of 65.7 $\mu g/m^3$ O_3, 20.2 $\mu g/m^3$ SO_2 and 17.6 $\mu g/m^3$ NO_2.

Rothaargebirge

The 50 year old spruce grow near Glindfeld on a southern slope in the eastern part of the Sauerland called Rothaargebirge 630 m above sea level and belong to a damage class (1990) from 0 to 1. Air pollutants were measured as 64.6 $\mu g/m^3$ O_3, 11.9 $\mu g/m^3$ SO_2 and 14.6 $\mu g/m^3$ NO_2 as the annual average of 1989.

Haard

The 80 year old spruce (damage class 1990: 0 to 2) grow near Haltern close to the northern part of the Ruhrgebiet 80 m above sea level. In 1989 the air pollutants were determined to be 33.1 $\mu g/m^3$ O_3, 34.8 $\mu g/m^3$ SO_2 and 40.2 $\mu g/m^3$ NO_2 as the annual average.

All immission data were determined by the Landesanstalt für Immissionsschutz Essen, Germany. The damage classes are defined as class 0 (no visible needle bleaching) and damage class 1, 2, 3 and 4, with up to 10%, 25%, 60% and 100% needle bleaching, respectively.

Plant material

During a one year period, twigs of Norway spruce *(Picea abies)* were harvested in November 1989, April 1990, June 1990, August 1990 and November 1990. At each harvest twigs of the same six trees from each site were collected. The twigs were placed in a mineral salt solution according to [26] and stored several hours at 6 °C until protein extraction. For the extraction of peroxidases material from the three youngest needle generations (needles of the current year, one year old and two year old needles) was taken. The needles of each tree were collected and extracted separately. All needles were taken from the upper part of the canopy and revealed no visible necrosis or bleaching.

Extraction of isoperoxidases

Fresh needles (2 g per extraction) were infiltrated with a buffer solution containing 2 mM Ca^{2+}-lactate, 4 mM KCl and 100 mM sorbitol under vacuum in a plastic syringe (Pfanz *et al.*, 1990). The infiltrated needles were placed in test tubes and centrifuged at 800 × *g* for 10 min. The recovered fluid was considered as the crude extract containing the **apoplastic peroxidases.** Investigations [27] reveal a contamination of the apoplastic fluids by cytoplasmic components of less than 0.02%. Furthermore, the extracted needles were then homogenized in a solution of 25 ml cold (−20 °C) acetone/water (3:1) and 2 g Polyclar AT (Sigma) using an Ultra-Turrax [28]. The resulting mixture was taken to dryness under vacuum and the obtained greyish powder was extracted by shaking for 1 h in 30 ml potassium phosphate buffer (50 mM, pH 7.0). The extract was centrifuged at 500 × *g*. 10 min and the supernatant was considered as the crude extract containing the **cytoplasmic peroxidases.** The remaining pellet was washed with 70 ml 1% Triton X-100 solution and subsequently with 50 ml potassium phosphate buffer (50 mM, pH 7.0). The following extraction procedure with 20 ml 1 M NaCl solution (3 h) liberates the **ionically bound peroxidases** [29, 30].

After centrifugation at 2000 × *g* for 10 min the

pellet was washed again with 50 ml 1 M NaCl solution and 50 ml potassium phosphate buffer. A final digestion of the remaining cell-wall residues with a solution of 0.5% cellulase and 0.5% pectinase in 20 ml 50 mM sodium acetate buffer (pH 5.0) led to a supernatant (after centrifugation at 2000 × *g* for 10 min) containing the **covalently bound peroxidases** [29, 30]. All extracts were stored at −20 °C.

Determination of the peroxidase activity

The enzyme assay (1 ml) contained 2 mM guaiacol, 12 mM hydrogen peroxide, 50 mM potassium phosphate buffer, pH 7.0 and 10−100 µl enzyme extract. The increase in the absorption was continuously measured at 436 nm for 5 min. The specific peroxidase enzyme activities were calculated for each fraction of the 18 trees. Based on the values of the six trees per harvest site the median was evaluated. These medians are calculated for the current year needles, the one year old needles (apoplastic, cytoplasmic, ionically and covalently bound peroxidases) and additionally for the two year old needles of the apoplastic peroxidases.

Native gel electrophoresis

Electrophoresis was performed as horizontal gel electrophoresis with the Mulitiphor II-System of LKB, Pharmacia. The gel consists of 7.5%/0.2% acrylamide/bis-acrylamide and 0.28 M Tris/HCl pH 8.0. 40 µl protein extracts were placed on 1 cm^2 filter discs in the middle of the gel on a 2 cm broad stacking gel (with 5%/0.15% acrylamide(bis-acrylamide and 0.1 M Tris/HCl pH 6.8). Tris/glycine (10 g/48 g per liter) served as the electrode buffer. This method allows the simultaneous separation of acidic and basic peroxidases of the protein extracts. Prior to separation the protein extracts of the ionically and covalently bound peroxidases were concentrated 10-fold by Centricon 10 tubes.

The electrophoresis was carried out at 250 V/15 mA for 0.5 h and 500 V/30 mA for 3.5 h with a cooling temperature of 4 °C. Xylene cyanole FF was used as the acidic front marker and cytochrome c as the basic marker.

After electrophoretic separation the gels were immediately stained by using 100 ml sodium acetate buffer pH 5.0 containing 20 mg 3-amino-9-ethylcarbazole dissolved in 500 µl N-N-dimethylformamide and 100 µl 30% hydrogen peroxide.

Results

For the separate determination of the different groups of peroxidases a fractionation protocol was developed in which the apoplastic isoforms were first extracted from the needles by vacuum infiltration. The cytoplasmic, ionically bound and covalently bound isoenzymes were subsequently obtained by a stepwise procedure using buffer extraction, detergent application and enzymatic digestion of cell debris for further fractionation (for details see Materials and Methods). Simultaneous separation of acidic and basic peroxidases by native gel electrophoresis indicates a great genetic variability of these isoenzymes among the eighteen trees used in our experiments (Fig. 1). The number of isoperoxidases for each spruce differs from one to six. There are no or only trace amounts of cationic peroxidases which were barely perceptible as reddish spots in the lower part of the gel (Fig. 1). The bulk of the separated peroxidases were anionic isoforms and these proteins were the dominant, most active isoperoxidases from all trees. These proteins were routinely found in all enzyme extracts of the four fractions (apoplastic, cytoplasmic, ionically bound and covalently bound peroxidases). Furthermore the peroxidase extracts from each tree can be differentiated by the occurrence of minor, less active isoperoxidases (data not shown).

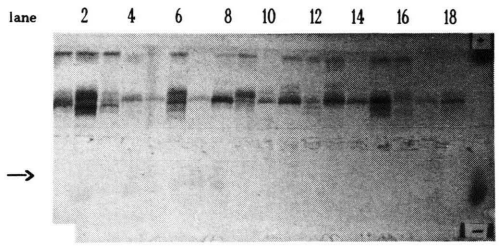

Fig. 1. Gel electrophoretic separation of cytoplasmic peroxidases from eighteen different spruces of three locations in Northrhine-Westfalia (**lane 1−6:** Haltern, **lane 7−12:** Glindfeld, **lane 13−18:** Velmerstot). The samples were placed in the middle of the gel and acidic and basic (weak spots indicated by the arrow) isoperoxidases were separated simultaneously and stained with 3-amino-9-ethylcarbazole and H_2O_2 as substrates. Xylene cyanole FF and cytochrome *c* were used as the acidic and basic front marker, respectively.

Determination of the enzymatic activities of the four different groups of isoperoxidases from each tree during a one year period was expected to provide data for a general comparison of the three collection sites Velmerstot, Glindfeld and Haltern and the expression of these enzymes. According to our expectation the cytoplasmic peroxidases showed the highest total enzyme activities (>90%), whereas the ionically bound (3–5%) and the covalently bound peroxidases (5–7%) are only expressed in low activities. The soluble peroxidases of the apoplastic space contribute less than one percent to the total peroxidase activity of the needles (data not shown, but compare Fig. 2).

The seasonal changes of the four groups of peroxidase activities revealed a typical time course for each isoperoxidase group (Fig. 2 and 3). Distinct differences among the three collection sites are especially obvious in case of the apoplastic peroxidases (Fig. 2). Although we found a pronounced increase in the enzyme activity of this group for each site between April to June 1990 this rise is up to three times higher in needles from Velmerstot and Glindfeld than in the plant material from Haltern. At the two former sites ozone values significantly rose and averaged at 80–100 $\mu g/m^3$ during the April to June period whereas 40–60 $\mu g\ O_3/m^3$ were only recorded as the monthly average for Haltern. Other air pollutants (*i.e.* NO, NO_2 and SO_2) remained practically unchanged at all three sites during the 1990 summer period (data not shown). After a steep increase the peroxidase enzyme activities of the apoplastic space again decreased between June to August. The different extent of apoplastic peroxidase induction in relation to increasing ozone concentrations at the three sites is clearly seen by the correlation shown in Fig. 4. In contrast to the seasonal changes of the enzyme activities of the apoplastic space the cytoplasmic peroxidase activities were practically identical for each site (Fig. 2). In addition, the ionically bound and covalently bound peroxidases also revealed no significant differences among the investigated collection sites (Fig. 3). Furthermore, the activities of cytoplasmic, ionically bound and covalently bound peroxidases even seemed to be slightly higher during the winter period. In essence a comparable increase of enzyme activities as it

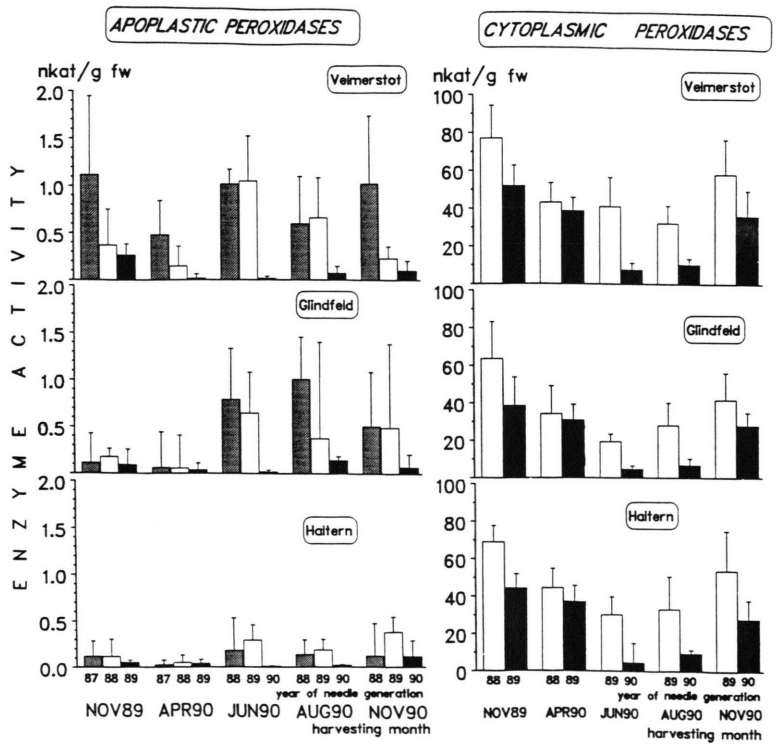

Fig. 2. Seasonal changes of **apoplastic** and **cytoplasmic** isoperoxidase activities of spruce needles from two ozone-dominant sites (Velmerstot and Glindfeld) and the control site Haltern. Needles were harvested during November 1989 to November 1990 and the medians (each calculated from the data obtained from six different trees) are represented. For the extraction of peroxidases the three youngest (for apoplastic peroxidases) and the two youngest (for cytoplasmic peroxidases) needle generations were taken. fw = freshweight; kat = katal.

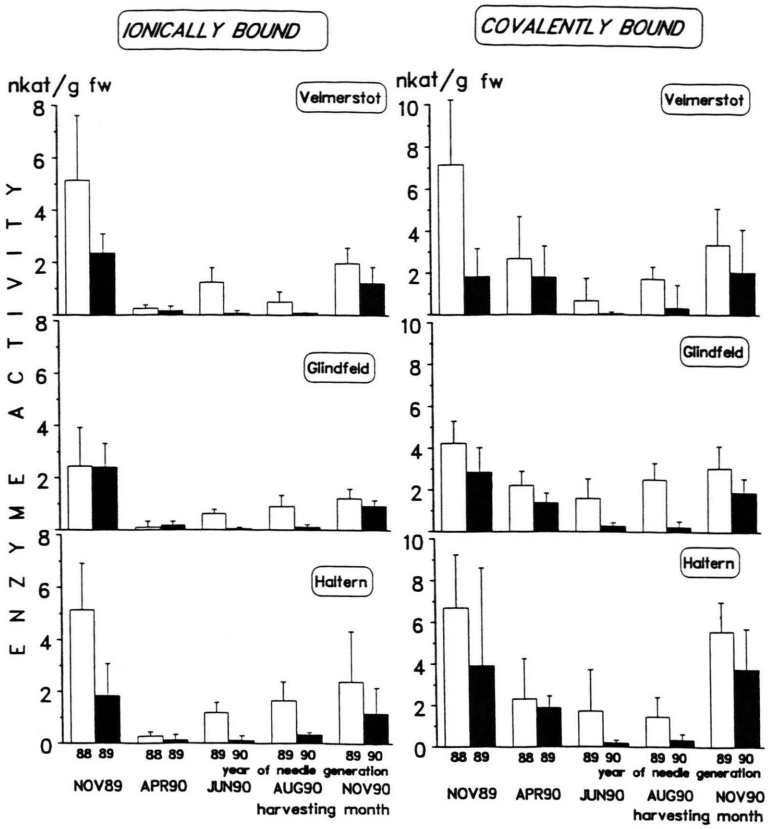

Fig. 3. Seasonal changes of **ionically** and **covalently** bound isoperoxidase activities of spruce needles from two ozone-dominant sites (Velmerstot and Glindfeld) and the control site Haltern. Needles were harvested during November 1989 to November 1990 and the medians (each calculated from the data obtained from six different trees) are represented. For the extraction of peroxidases the two youngest needle generations were taken. fw = freshweight; kal = katal.

was found for the apoplastic peroxidases could not be observed for the other three groups of isoenzymes.

Discussion

Electrophoretic separation and gel-staining of total isoperoxidases of eighteen Norway spruce *(Picea abies)* revealed the pronounced genetic variability of this group of enzymes. According to [28] and [31] each individual spruce possesses its own characteristic isoenzyme pattern. However, the previously claimed high number of up to 12 acidic isoperoxidases in Norway spruce [31] could not be confirmed in our studies. It is unknown whether experimental or genetic differences are responsible for this discrepancy. Because of the high variability of the isoperoxidase pattern, these isoenzymes are discussed as useful markers to distinguish between clones, which were classified as "relative resistance to air pollution" and those with the quality "resistance to air pollution" [32]. Genetic variability among isoperoxidases has also been reported for other *Pinaceae* such as *Larix decidua* and *Abies alba* [28] as well as different *Pinus*-species [33, 34].

Our findings of seasonal changes of the cytoplasmic, ionically and covalently bound peroxidases with their maxima of activities during the winter season together with the increase in peroxidase activities in current year needles between June to November (Fig. 2 and 3) confirm previous reports [18, 31]. Although comprehensive knowledge on isoperoxidases from whole needles exists comparatively little is known about individual isoperoxidases separated from different tissues and cell compartments. Therefore, our work aimed at measuring the course of seasonal activities of apoplastic, cytoplasmic, ionically and covalently bound peroxidases in spruce needles. Prior to the subsequent extraction of cytoplasmic peroxidases, ionically bound and covalently bound peroxidases

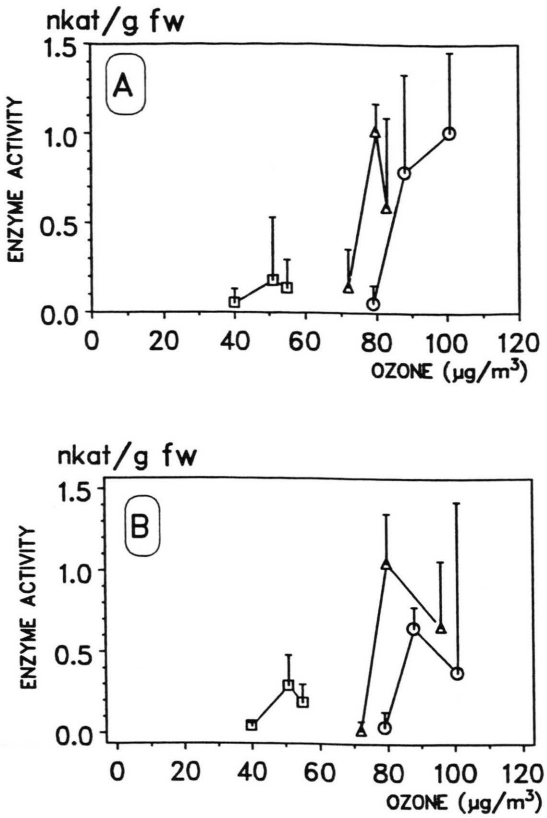

Fig. 4. Changes of apoplastic peroxidase activities of spruce needles (**A:** needles grown in 1988; **B:** needles grown in 1989) in comparison to the ambient ozone concentrations measured at the three different collection sites (Velmerstot, Haltern, Glindfeld). Each line represents the time course of enzyme activities from April 1990 through June to August 1990. The ozone data were provided by the Landesanstalt für Immissionsschutz Essen, Germany. fw = freshweight; kat = katal.

with low molar extraction buffer, high molar extraction buffer and enzymatic digestion of cell wall residues, respectively, apoplastic peroxidases were obtained by vacuum infiltration of fresh needles.

Influences of air pollutants on peroxidase activities were normally investigated in chamber experiments by single gas or gas mixture (mostly O_3, NO_X and SO_2) applications, whereas little is known concerning the impact of ambient air pollutants on isoperoxidases in needles of field grown spruce.

Many reports deal with determinations of peroxidase activities from chamber grown plants mostly fumigated with gas concentrations which are much higher than naturally occurring concentrations of air pollutants (*i.e.* [18, 35]). On the other hand it is difficult to reach a better understanding of forest decline caused by the impact of air pollutants on older trees by fumigation results obtained from chamber experiments with saplings, young trees and herbaceous plants. An increase of total peroxidase activities was often demonstrated by chamber experiments. After a fumigation of fir *(Abies alba)* and beach *(Fagus sylvestirs)* with 30–180 µg ozone/m³ (7–21 h per day) an increase of total peroxidase activities of these tree species was recorded, whereas the total peroxidase activity of Norway spruce remained unchanged [36]. Additionally, after several days of fumigation an experiment using 310 ppb ozone (= 620 µg/m³) and seven year old spruce no alteration of total peroxidase activities was observed [18]. In contrast to these findings of chamber experiments without an alteration of peroxidase activities after ozone application, a single ozone fumigation with 300 µg/m³ provokes an 1.3–2.5 times enhanced total peroxidase activity in four year old saplings of Norway spruce [35]. Furthermore, gas mixture experiments (*i.e.* O_3/SO_2 and $O_3/SO_2/NO_2$ combinations) often led to a significant induction of total peroxidase activities in spruce and fir needles [37, 38]. Chamber fumigation experiments, performed by the Landesanstalt für Immissionsschutz (Essen, Germany), with different applications of $O_3/SO_2/NO_2$ gas mixture revealed the significant increase of apoplastic, cytoplasmic, ionically bound and covalently bound peroxidase activites in spruce needles, respectively (publication in preparation).

The ozone dependent induction of apoplastic peroxidase activities was previously reported for the leaves of *Sedum album* as well as for spruce needles. In the case of *Sedum album* a two hour ozone treatment led to a differentiated increase of cationic and anionic apoplastic peroxidase activities, whereas the synthesis of new apoplastic isoperoxidases could not be demonstrated [39]. Additionally, the apoplastic peroxidase activities of spruce needles were enhanced up to 3.4-fold by short time (2 days) and long time (30 days) fumigation experiments with 300 µg ozone/m³ [35].

In spite of the good knowledge of fumigation induced total peroxidase activities in chamber grown plants, little efforts have been taken to investigate the influence of ambient air pollutants on field

grown older trees. According to our current results concerning the seasonal changes of the cytoplasmic peroxidase activities (representing about 99% of the soluble total peroxidase activity) the seasonal total peroxidase activities of spruce needles from a collection site near Hamburg, Germany, remained unchanged in spite of maximum ozone values up to 260 $\mu g/m^3$ during the summer period [18]. In addition, no differences of total peroxidase activities obtained from needles of damaged spruce and obviously undamaged spruce, grown at a collection site located in the Northern Black Forest, were found [40], so that the group of cytoplasmic isoperoxidases cannot be considered as a useful bioindicator for oxidative air pollutants or forest decline.

In conclusion our current results lead to the assumption that neither cytoplasmic peroxidase activities nor ionically and covalently bound peroxidase activities of spruce needles from the collection sites Velmerstot, Glindfeld and Haltern are influenced by ambient air pollutants. In contrast to these groups of isoperoxidases the induction of apoplastic isoperoxidase activities during the early summer period at each collection site is considered to be caused by increasing ozone concentrations. A comparable study concerning apoplastic peroxidase activities was performed in the Calcareuos Alps [41]. Their results show a general decrease of apoplastic peroxidase activities in spruce needles by increasing sea level, but a transient maximum of apoplastic peroxidase activities at a sea level (about 1270 m) with highest ambient ozone concentrations.

The signal transduction of the ozone impact to compartments of peroxidase activation or the synthesis of new isoperoxidases remains unclear. An ozone dependent redistribution of Ca^{2+}-ions as a second messenger, an additional involvement of ethylene and the following increase of apoplastic peroxidase activities in *Sedum album* are discussed to be part of a putative signal transduction of oxidative stress [16]. In general, increase of the antioxidative system within the apoplastic space of needles should be a powerful defense mechanism against oxidative stress. Especially hydrogen peroxide, which increased concomitantly with increasing ozone concentrations [42], as well as organic peroxides as products of ozonolysis of alkenes and terpenoids [43] could be detoxified by apoplastic peroxidases [27]. On the other hand ascorbate [27, 35] or phenolic compounds [44] within the apoplastic space of spruce needles could serve as electron donators for peroxidase reactions. Furthermore, the capability of sulfite oxidation by apoplastic as well as cell wall bound peroxidases from barley *(Hordeum vulgare)* and spruce needles was demonstrated and the role of apoplastic peroxidases as a defense mechanism against cytoplasmic acidification was discussed [45].

In essence, our current work clearly indicates, that future investigations concerning stress impact and possible responses of peroxidase activity in plants should focus on specific isoperoxidases of different plant tissues and compartments. Furthermore, investigations should be extended to other collection sites to confirm whether the apoplastic peroxidase activities of spruce needles are useful bioindicators for the prediction of effects caused by oxidative air pollutants.

Acknowledgement

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