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Inhibition of Apoplastic and Symplastic Peroxidase Activity from Norway Spruce by the Photooxidant Hydroxymethyl Hydroperoxide[1]

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Young, clonal Norway spruce trees (*Picea abies* L.) were exposed for 2 years at high altitudes to ambient atmospheric concentrations of photooxidants containing hydroxymethyl hydroperoxide (HMHP) as an important constituent. In spruce needles from a site with higher concentrations of organic peroxides in air, the apoplastic peroxidase activities were significantly lower than in needles exposed to lower organic peroxide concentrations. Guaiacol peroxidase activities in total needle extracts were not affected. In vitro HMHP at a concentration of 35 μM inhibited apoplastic and total needle guaiacol peroxidase activities by 50% at pH 5.25. At the same pH, ascorbate-specific peroxidase activity required about 100 μM HMHP for 50% inhibition. At pH 7, 1.46 mM HMHP caused a 50% reduction in guaiacol peroxidase and a 13% reduction in ascorbate peroxidase activity. The present results suggest that HMHP in ambient air may affect peroxidase activity in spruce needles. Peroxidases located in the relatively acidic aqueous phase of the cell walls appear to be more susceptible to HMHP inhibition than those present in neutral or slightly alkaline symplastic compartments of cells such as the cytosol or chloroplasts.

Gaseous peroxides are trace constituents in ambient air (0.1–5 nL L^{-1}; Sakugawa et al., 1990). Their production involves photochemical reactions of ozone with volatile hydrocarbons of anthropogenic or biogenic origin such as formaldehyde, isoprene, terpenes, etc. (Gäb et al., 1985; Becker et al., 1990; Hewitt and Kok, 1991; Simonaitis et al., 1991). The organic peroxide HMHP is a major product of ozone and isoprene gas-phase reactions (Hewitt and Kok, 1991; Simonaitis et al., 1991). It has also been detected in leaves of isoprene-emitting plants after ozone fumigation (Hewitt et al., 1990). Therefore, it was suggested that this compound might be involved in mediating air pollution damage to plants (Hewitt et al., 1990).

Peroxidases are possible targets of HMHP. Marklund (1971) found that an acidic guaiacol peroxidase isolated from horseradish is rapidly and irreversibly inhibited by HMHP. The susceptibility of peroxidases to HMHP in plant species such as Norway spruce (*Picea abies* L.) that are suffering greatly from environmental pollution has not yet been studied. In spruce needles about 2 to 5% of total "nonspecific" guaiacol peroxidase activity is found in the apoplastic space (Polle et al., 1990). Ascorbate peroxidase activity is localized in the cytosol and in chloroplasts of plant cells (Asada, 1992) but not in apoplastic washing fluids of spruce needles (Polle et al., 1990). It is unknown whether the ascorbate-specific peroxidases, whose enzymic characteristics are different from the family of "nonspecific" guaiacol peroxidases (Asada, 1992), are inactivated by HMHP.

In the present study we investigated peroxidase activities in needles of clonal spruce trees exposed for 2 years at high altitudes to ambient atmospheric concentrations of photooxidants including HMHP. Since airborne hydrophilic pollutants enter the leaves predominantly via the stomata and have to cross the aqueous apoplastic space before they reach intracellular targets (Polle and Rennenberg, 1993), apoplastic peroxidase activities were also investigated. The effect of HMHP on ascorbate and guaiacol peroxidase activities was analyzed in vitro. The significance of ambient atmospheric HMHP concentrations for damage to plants is discussed on the basis of the present results.

MATERIALS AND METHODS

Preparation of HMHP Stock Solutions

In aqueous solution HMHP is a product of H_2O_2 and formaldehyde according to the following equilibrium reaction:

$$H_2O_2 + H_2CO \rightleftarrows HOCH_2O_2H$$

To produce stock solutions of HMHP, concentrated solutions of H_2O_2 (30%) and formaldehyde (37%) were mixed stoichiometrically and stirred for 12 h at room temperature. The concentrations of HMHP, H_2O_2, and formaldehyde in the resulting "HMHP stock solution" were slightly variable from batch to batch. Therefore, the composition of each freshly prepared HMHP solution was fluorimetrically analyzed according to Lazrus et al. (1986). A typical HMHP stock solution contained 1.46 M HMHP, 1.04 M H_2O_2, and 0.92 M HCHO, pH 2.2. The HMHP stock solution was stored in the dark at

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Abbreviation: HMHP, hydroxymethyl hydroperoxide.

4°C and used for 4 to 5 h after its preparation. Dilutions were prepared immediately before use.

Sampling and Extraction of Spruce Needles

Three-year-old Norway spruce seedlings (*Picea abies* L., clone 252, Laufen) were exposed to ambient climate and pollution near three meteorological stations, namely Garmisch-Partenkirchen at the foot of Wank mountain (735 m above sea level), Wank basis (1175 m above sea level), and Wank summit (1780 m above sea level). The stations are located in the Calcareous Alps (Bavaria, Germany) close to spruce forests for which data on climate and pollution have been reported previously (Polle et al., 1992b). The seedlings were kept in brown forest earth in pots that had been sunk in the ground. After 2 years, in September 1990, 1-year-old needles were harvested from six trees at each site. The fresh material was transported to the laboratory and used for the extraction of apoplastic and total needle peroxidase activities as described previously (Polle et al., 1990).

For the in vitro studies, apoplastic and total needle extracts were prepared from the current year's needles of approximately 100-year-old, healthy Norway spruce trees. The material was collected in fall and winter 1992 at the field site Katzenstein mountain (Garmisch-Partenkirchen, Bavaria), which has been described in previous reports (Polle et al., 1990, 1992a).

Before the analysis of peroxidase activities, apoplastic washing fluids and needle extracts were desalted on Sephadex G-25 (NAP-5 column, Pharmacia, Freiburg, Germany). The contamination of the apoplastic fraction with symplastic components amounted to 0.01% as determined by measurement of catalase activity (Aebi, 1983). The protein content of desalted extracts was determined with the bicinchoninic acid reagent kit from Pierce (München, Germany).

Analyses of Peroxidase Activities

Enzymic activities were determined in a final volume of 1 mL at 25°C using methods tested for spruce needles (Polle et al., 1990). All measurements were performed in triplicate. The assay for guaiacol peroxidase (EC 1.11.1.7) activity contained 50 mM K_2HPO_4/KH_2PO_4 (pH 5.25 or 7.0), 40 mM guaiacol, 50 μL of enzyme extract, and 10 mM H_2O_2. The assay for ascorbate peroxidase (EC 1.11.1.11) activity contained 50 mM K_2HPO_4/KH_2PO_4 (pH 5.25 or 7.0), 800 μM sodium ascorbate, 100 μL of enzyme extract, and 1 mM H_2O_2. Each reaction was started by addition of H_2O_2. Control rates recorded in the absence of substrate or extract were subtracted. HMHP at concentrations ranging from 0.1 to 1400 μM was added to guaiacol or ascorbate peroxidase assays before the addition of H_2O_2. The mixture was incubated for 2 min before starting the reaction. Because the HMHP solutions contained residual amounts of H_2O_2 and formaldehyde, controls were run by replacing HMHP solutions by the appropriate concentrations of these two compounds. Formaldehyde affected peroxidase activity at a concentration above 60 mM and, therefore, was kept below this concentration.

Apoplastic peroxidase isozymes were separated under nondenaturing conditions on a Phastsystem (Pharmacia, Freiburg, Germany) by IEF in a pH gradient from 3 to 9 or by native PAGE on 20% homogeneous polyacrylamide gels (precast minigels, Pharmacia). To visualize guaiacol peroxidase activity in the presence or absence of HMHP, the gels were preincubated for 10 min in HMHP solutions or the appropriate solutions of H_2O_2 and formaldehyde before the transfer into the staining solution (50 mM K_2HPO_4/KH_2PO_4, pH 5.25, 10 mM guaiacol, and 5 mM H_2O_2).

Nonenzymic Oxidation of Ascorbate by H_2O_2 and HMHP

Nonenzymic oxidation of ascorbate by peroxides was determined at 290 nm in a total volume of 1 mL at 25°C. The reaction mixture contained 20 mM K_2HPO_4/KH_2HPO_4 at different pH values (indicated in Fig. 3), 1 mM ascorbate, and peroxide concentrations from 0.5 to 2 mM. In the controls, H_2O_2 was replaced by water or, where appropriate, by formaldehyde. After subtraction of the control rate the nonenzymic oxidation rate of ascorbate (v) was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate at 290 nm (Nakano and Asada, 1987). The apparent reaction constants for the oxidation of ascorbate by H_2O_2 (k_1) or HMHP (k_2) were calculated according to the following equations:

$$k_1 = v/[\text{ascorbate}]\,[H_2O_2]$$

$$k_2 = (v/[\text{ascorbate}] - k_1\,[H_2O_2])/[\text{HMHP}]$$

Statistical Analysis

Data were analyzed by one-way analysis of variance (Statgraphics, St. Louis, MO). Significant differences at $P \leq 0.01$ are indicated by different letters.

RESULTS

Exposure of Spruce Seedlings to Photooxidants at High Altitudes

Guaiacol peroxidase activities in extracts of needles from young spruce trees grown for 2 years at three altitudes, i.e. 735, 1175, and 1780 m above sea level, were not affected by differences in climate or pollution at Wank mountain (Table I). However, apoplastic peroxidase activities were significantly lower in needles from middle and high altitude than in needles from valley level (Table I). The reduction in peroxidase activity could not be attributed to soil-borne factors or genetic differences, since potted, clonal plants were used. The lowest apoplastic peroxidase activities were found at middle altitude (Table I). It was unlikely that climatic factors caused a specific reduction in apoplastic peroxidase activity at the middle altitude, since mean temperatures decreased (0.5°C/100 m) and radiation increased with increasing altitude. Air pollution by sulfur and nitrogen oxides was low at Wank mountain (Polle et al., 1992b). Ozone increased gradually from valley to summit (Table I). However, the concentration of organic peroxides was 1.85-fold higher at Wank basis (1175 m above sea level) than at Wank summit (1780 m above sea level), whereas the atmospheric H_2O_2 concentrations were similar at both sites (Table I). Since HMHP constituted a major fraction of about 70% of the organic peroxides at Wank mountain in summer (Fels and

Table I. *Mean concentrations of photooxidants in air and peroxidase activities in spruce needles at three altitudes at Wank mountain (735, 1175, and 1780 m above sea level)*

Guaiacol peroxidase activity was determined in apoplastic washing fluids and total needle extracts of clonal spruce trees exposed for 2 years near meteorological stations located at three altitudes at Wank mountain. The figures indicate means (±SD, $n = 6$). Different letters in the columns indicate significant differences at $P \leq 0.01$. The concentrations of photooxidants are means recorded at daytime during the growth period (May to September). ROOH, Organic peroxides; ND, not determined.

Altitude	Photooxidant			Peroxidase Activity	
	O_3	H_2O_2	ROOH	Needles	Apoplastic space
m		*nL L*$^{-1}$		*nkat mg*$^{-1}$ *protein*	
735	45.8	ND	ND	266 ± 32a	6501 ± 2706c
1175	50.3	0.86	0.33	316 ± 75a	1764 ± 853a
1780	58.2	0.73	0.18	331 ± 76a	3083 ± 505b

Junkermann, 1993), we investigated whether apoplastic and symplastic peroxidase activities of spruce needles were affected by HMHP.

Effect of HMHP on Peroxidase Activities in Vitro

Separation of peroxidases in apoplastic washing fluids by anodic, native PAGE resolved tree-specific patterns with three to four acidic isozymes (not shown) similar to previous studies with spruce needles from other sites (Polle et al., 1991). IEF of apoplastic washing fluids showed only two activity bands of guaiacol peroxidases, which were located at the acidic and alkaline ends of the pH gradient in the gel (Fig. 1). Apparently, the isoelectric points of the individual peroxidase isozymes in the apoplastic washing fluid from spruce needles were strongly alkaline (≥pH 9) and strongly acidic (≤pH 3). After preincubation of the gels with 0.1 mM HMHP, both the acidic and alkaline peroxidases retained residual activity, but they were completely inhibited by 10 mM HMHP (Fig. 1).

For quantitative investigations, HMHP was added to spectrophotometric assays. When the HMHP concentration was increased from 10 to 100 μM, apoplastic guaiacol peroxidase activity was almost completely inhibited (Fig. 2A). Apparently, the inhibition of peroxidase activity was greater in soluble assays than in the gel. A 50% inhibition was observed at an HMHP concentration of 35 μM (Fig. 2A). The inhibition was specific for HMHP and not caused by residual amounts of formaldehyde, as shown by the appropriate controls (cf. "Materials and Methods").

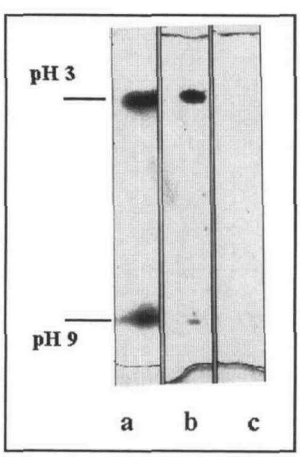

Figure 1. IEF of apoplastic peroxidases from spruce needles. Peroxidase activity corresponding to 0.9 nkat was applied to each lane and stained for activity with guaiacol and H_2O_2. a, Control; b, 0.1 mM HMHP; c, 10 mM HMHP.

The effect of HMHP on guaiacol- and ascorbate-specific peroxidase activities in total needle extracts was tested under the same assay conditions as were used for the apoplastic peroxidases. Ascorbate peroxidase activity was less sensitive to HMHP than guaiacol peroxidase activity (Fig. 2B). A 50% reduction in ascorbate peroxidase activity was found in the presence of about 100 μM HMHP. The lower susceptibility of ascorbate peroxidase activity for HMHP was not caused by a nonenzymic removal of HMHP by ascorbate, as shown in Figure 3. At pH 5.3, the presence of HMHP had almost no effect on the oxidation of ascorbate, whereas H_2O_2 caused a slow nonenzymic oxidation rate that corresponded to a reaction constant of about 6 M^{-1} s^{-1} (Fig. 3).

Since the pH of most symplastic compartments is neutral or slightly alkaline (except for the vacuole), the effect of HMHP on ascorbate and guaiacol peroxidase from needles was also investigated at pH 7 (Table II). At pH 7, the HMHP concentration that caused 50% inhibition of guaiacol peroxidase activity was approximately 40-fold higher than at pH 5.25 (1.46 mM versus 35 μM, Table II, Fig. 2). Ascorbate peroxidase activity was reduced by only 13% at this HMHP concentration (Table II).

DISCUSSION

Both guaiacol and ascorbate peroxidase activities were more susceptible to HMHP at acidic than at neutral pH values, possibly because of the greater stability of HMHP at low pH (Zhou and Lee, 1992). This observation is important because the pH value of the apoplastic compartment, into which air pollutants are initially taken up when entering the leaves, is acidic (pH 5–6.5; Pfanz and Dietz, 1987). Apoplastic guaiacol peroxidase from spruce needles was 50% inhibited

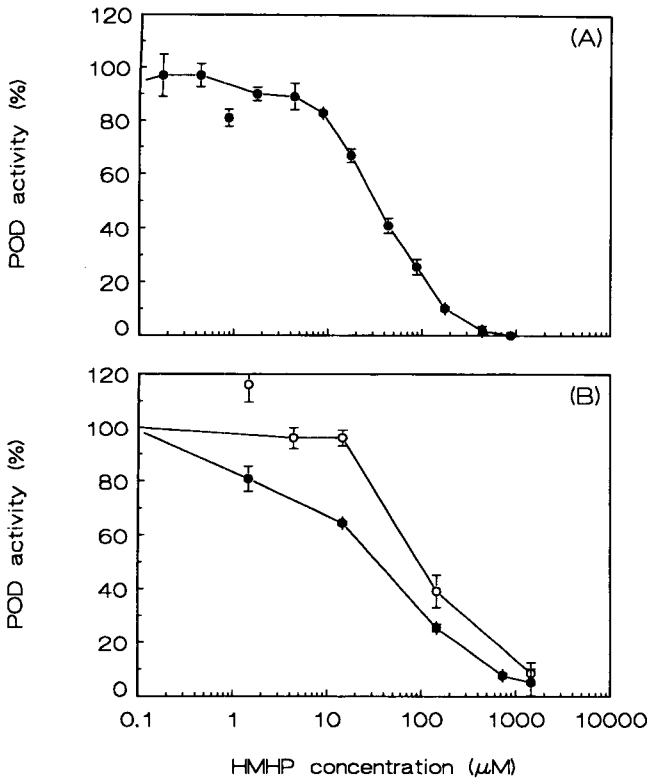

Figure 2. The inhibition of apoplastic (A) and symplastic peroxidases (B) by HMHP at pH 5.25. Guaiacol and ascorbate peroxidase activities were determined in the presence of HMHP at pH 5.25 as indicated in the figure. The data are means (±SD) from three independent experiments. One hundred percent corresponded to the following peroxidase activities (g^{-1} fresh weight): 369 nkat and 13.7 μkat for guaiacol peroxidases (●) from the apoplastic space and total needle extracts, respectively, and 195 nkat for ascorbate peroxidase (O).

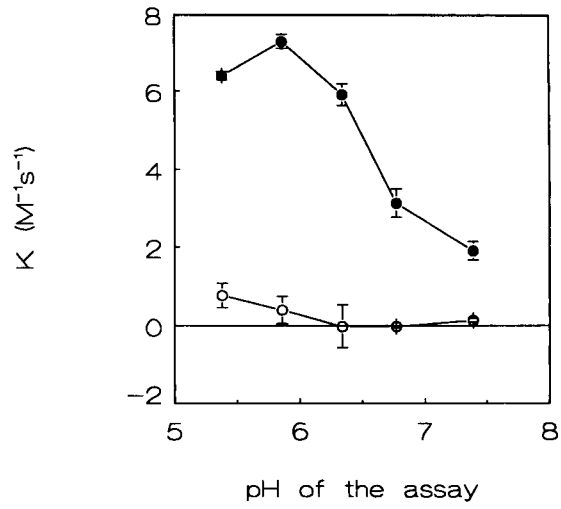

Figure 3. pH dependency of the apparent reaction constant of ascorbate with H_2O_2 (●) and HMHP (O). Nonenzymic oxidation rates of ascorbate were determined at 290 nm in the presence of H_2O_2 or HMHP and used to calculate rate constants as described in "Materials and Methods."

at 35 μM HMHP (Fig. 2). A similar sensitivity was found for acidic horseradish peroxidase (Marklund, 1971).

Because atmospheric HMHP concentrations are generally low (0.2–5 nL L^{-1}; Hewitt and Kok, 1991; Fels and Junkermann, 1993; Lee et al., 1993), the question was addressed whether ambient HMHP concentrations may be a potential threat to plants. HMHP is highly soluble in water (Henry constant of 5×10^5 M^{-1} atm^{-1}; Zhou and Lee, 1992). Thus, the maximum gas-phase concentrations of HMHP observed at Wank mountain of 0.8 nL L^{-1} (W. Junkermann, personal communication) corresponds to an aqueous equilibrium concentration of 400 μM. In vitro, this concentration caused 80 to 100% inhibition of peroxidases at acidic pH values, whereas millimolar concentrations of HMHP would be required for similar reductions in peroxidase activity at neutral pH (Fig. 2, Table II). In vivo, equilibration of pollutants between the external gas phase and the aqueous intrinsic compartments of plants is prevented by a series of resistances (Hicks et al., 1987). The uptake rates of ambient HMHP might still be sufficiently high to affect peroxidases in the cell wall, since we found a reduction in apoplastic peroxidase activity in spruce needles grown in air polluted with organic peroxides as compared with needles grown in air containing about 2-fold lower organic peroxide concentrations (Table I). Total guaiacol peroxidase activity was not reduced in the field experiment (Table I). Ascorbate peroxidase activity in needles of mature spruce trees grown close to the site where enhanced organic peroxide levels were recorded was also not affected as compared with higher or lower altitude (Polle and Rennenberg, 1992). Therefore, it seems possible that ascorbate and guaiacol peroxidases localized in the cytosol or in chloroplasts, i.e. in neutral or alkaline cellular compartments, are better protected from HMHP-mediated inactivation than apoplastic peroxidases.

Hewitt and co-workers (1990) observed that ozone caused considerable formation of HMHP in isoprene-emitting leaves (30 μmol HMHP kg^{-1} dry weight of leaves). The present results attribute a role to HMHP in mediating injury. Whether HMHP present in ambient air can cause damage to plants alone or in combination with other environmental or biogenic factors needs to be addressed in controlled fumigation experiments.

Table II. *Inhibition of guaiacol and ascorbate peroxidase activities from needle extracts by HMHP at pH 7*

HMHP (1.46 mM) was added to the reaction mixtures at pH 7. The figures indicate the mean of three independent experiments (±SD).

Peroxidase Substrate	Peroxidase Activity	
	Control	HMHP
	g^{-1} fresh weight	
Guaiacol (μkat)	8.79 ± 1.32	4.29 ± 0.34
Ascorbate (nkat)	267 ± 22	233 ± 7

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