Free radical scavengers and photosynthetic pigments in *Pinus cembra* L. needles as affected by ozone exposure

G. WIESER*, M. TAUSZ**+, A. WONISCH** and W.M. HAVRANEK*

**Forstliche Bundesversuchsanstalt, Institut für Immissionsforschung und Forstchemie, Abteilung für Forstpflanzenphysiologie, Rennweg 1, A-6020 Innsbruck, Austria*
***Institut für Pflanzenphysiologie, Karl-Franzens-Universität Graz, Schubertstraße 51, A-8010 Graz, Austria*

Abstract

The goal of this study was the characterization of the antioxidative protection system of current and 1-year-old needles of a cembran pine (*Pinus cembra* L.) and its possible responses to elevated concentrations of atmospheric O_3. Twigs of a mature cembran pine at the alpine timberline (1950 m a.s.l.) were exposed in climate-controlled twig chambers for 91 d to charcoal-filtered air (CF), ambient air O_3 concentration (A), and two-fold ambient air O_3 concentration (2A). Additionally, a chamberless control group (AA) was used to examine chamber effects. At the end of the fumigation period the contents of free radical scavengers and photosynthetic pigments were measured in the needles. Independent from O_3 exposure, total ascorbate and α-tocopherol contents were higher in 1-year-old needles compared to the current flush while the opposite was found for glutathione. The amounts of pigments and antioxidants in *P. cembra* needles were comparable to those in other conifers growing at high-elevation sites. The only hint toward O_3 induced changes in the composition of antioxidants was an increase in the glutathione redox state toward more oxidation in 1-year-old needles upon exposure to A or AA conditions, but not upon 2A exposure. Chlorophyll and carotenoid contents were not affected by O_3 neither in current- nor in previous-year needles. The de-epoxidation state of the xanthophyll cycle pigments, however, was significantly increased in 1-year-old needles under A and AA compared to the CF control, but not under 2A. Hence, *Pinus cembra*, which is well adapted to the extreme environment of the timberline ecotone, exhibited only marginal biochemical changes in response to elevated O_3.

Additional key words: ascorbic acid, cembran pine, glutathione, oxidative stress, stress, xanthophyll cycle

Introduction

During the last century the atmosphere over Europe has changed in the composition of trace gas components and recent analyses indicate that tropospheric O_3 concentrations have at least doubled during the last 100 years (Volz and Kley 1988, Marenco *et al.* 1994). Nowadays alpine forests in central Europe can experience O_3 episodes above 120 $mm^3\ m^{-3}$ (Schneider *et al.* 1996) and mean annual values of 50 $mm^3\ m^{-3}$ coincide with the elevation of montane and subalpine forests in Central Europe (Smidt and Gabler 1994). Such concentrations have negative effects on the physiological performance of conifer seedlings in chamber experiments (Sandermann *et al.* 1997). Under field conditions, impaired photosynthetic capacity and reductions in stomatal conductance has been reported for mature spruce and larch trees after more than 12 weeks of exposure to mean O_3 concentrations higher than 100 $mm^3\ m^{-3}$ (Wieser 1999a).

The negative impacts of ozone on forest trees (Reich 1987, Matyssek *et al.* 1995, Sandermann *et al.* 1997) depend on the amount of O_3 entering the needles (Guderian *et al.* 1985, Heath 1994) and on the presence

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Abbreviations: A - chambers with ambient air ozone concentration; AA - chamberless control group; 2A - chambers with two-fold ambient air ozone concentration; An - antheraxanthin; CF - chambers with charcoal-filtered air; CU - cumulative O_3 uptake; F_{O_3} - O_3 uptake rate; g_{O_3} - mean stomatal conductance for O_3; GSH - glutathione; GSSG - oxidized glutathione; SUMO - total external O_3 dose; V - violaxanthin; Z - zeaxanthin.
+ E-mail: michael.tausz@kfunigraz.ac.at

and efficiency of defence mechanisms available inside the tissue (Runeckles and Vaartnou 1997). Since O_3 is a strong oxidant and will give rise to several reactive oxygen species, antioxidative systems may provide an important protection against O_3 injury (Polle 1998). Biochemical damages to the photosynthetic apparatus may lead to an overreduction of the electron transport system in the thylakoids, a situation that promotes the formation of toxic oxygen species due to an excess of absorbed light energy (Elstner and Osswald 1994). Photoprotective pigments quench excess light energy and avoid overreduction of the photosystems (Demming-Adams and Adams 1994). The antioxidative defense systems protect the plant cells through removal of toxic oxygen species. Important low molecular mass antioxidants are ascorbate, glutathione, and α-tocopherol. Antioxidants and protective pigments are important markers of stress to plants. The contents of apoplastic ascorbate (Polle *et al.* 1995) as well as ascorbate and glutathione in the symplast of conifer needles increased after O_3 exposure under controlled conditions (Barnes 1972, Mehlhorn *et al.* 1986, Manderscheid *et al.* 1991, Kronfuss *et al.* 1998, Wieser *et al.* 1998). On the other hand, the capacity of protective systems is generally higher in plants growing at higher elevations (Rennenberg 1988, Wildi and Lütz 1996), where plants are chronically exposed to higher oxidative stress including an increased ambient ozone concentration as compared with lower elevations. Needle concentrations of ascorbate and glutathione increase with increasing altitude in the foliage of Norway spruce (Polle and Rennenberg 1992, Polle *et al.* 1995, Tausz *et al.* 1996) and of pine species (Tausz *et al.* 1998a, 1999c).

Ecosystems at the distribution limits of species, such as timberlines, may be of particular interest with respect to environmental changes. *Pinus cembra* L. is a subalpine conifer species ecologically well adapted to high natural stress at high altitude plots. In contrast to spruce which has a broad ecological amplitude, the natural ecological optimum of *P. cembra* is restricted to the timberline ecotone. Hence, the performance of this species under elevated ozone conditions may give important insight into responses of potentially sensitive subalpine forest species to rising ambient ozone concentrations.

Although *P. cembra* is of major importance in the treeline ecosystems in the Alps, there is a lack of specific knowledge of the antioxidative protection systems of this subalpine species. Furthermore, the possible effect of ozone on the biochemical defense system of this species is unknown. The present study characterizes the antioxidative system of cembran pine needles under subalpine field conditions. Possible responses of the antioxidant and photoprotective systems to a 91-d exposure in twig chambers to ozone-free air, ambient, and twofold ambient O_3 concentrations were investigated. These values are important for biochemical stress indication systems possibly applicable to this species at field stands (Tausz *et al.* 1998b).

Materials and methods

Plants and site: Measurements were carried out on a mature 12-m high *P. cembra* tree growing in a podsol on a south west slope at 1950 m a.s.l. near the Klimahaus Research Station on Mt. Patscherkofel (47° N, 12° E) close to Innsbruck, Austria. The field site is characterized by a cool subalpine climate with frequent precipitation in summer and the possibility of frost in every month. O_3 was the dominant air pollutant, whereas the concentrations of SO_2, NO_2, and NO were negligible.

Experimental design and ozone fumigation: Aluminum scaffolding provided access to branches in the upper half of the canopy. Twigs of similar size on the south-west facing part of the tree were enclosed in double-walled climatized twig chambers. The experimental design consisted of four treatments with three replicate twigs per treatment: *1*) chambers with charcoal-filtered air (CF), *2*) chambers with ambient air O_3 concentration (A), and *3*) chambers with two-fold ambient air O_3 concentration (2A). An additional *4*) chamberless control group (AA) was included to check chamber effects. The 2A treatment was selected to achieve O_3 concentrations close to 100 mm^3 m^{-3}, a concentration known to impair gas exchange in mature field grown Norway spruce (*Picea abies*) trees after long term exposure (Havranek *et al.* 1989, Wieser and Havranek 1994, 1996).

The twig chamber system, allowing a tracking of ambient climatic conditions and the control of O_3 concentrations inside the chambers, was operated from July 20 to October 19, 1996. The chambers were 12 cm in diameter and 32 cm long and made of thin perspex. Gas-tight nylon bags were used for twig enclosure allowing flexibility in order to prevent breakage of the twigs through high wind movement. Each chamber was provided with 139 cm^3 s^{-1} of forced air, corresponding to two exchanges of the chamber volume per minute. Four inlet ports and fans inside the chambers prevented concentration gradients, allowed a throughout mixing of the air and minimised the needle boundary layer resistance.

The fumigation system was supplied with ambient air drawn through charcoal-filters, which completely removed O_3. In order to remove short-term fluctuations in humidity and CO_2-concentration the air passed a 250 dm^3

buffer vessel. For each of the O_3 treatments a manifold supplied air to the three twig exposure chambers set to the same O_3 concentration.

Supplemental O_3 was generated from CF ambient air using an *Osram, HNS-UOZ 10* ultraviolet lamp and diluted in two steps to the demand of A and 2A O_3 concentrations, respectively, before continuously (day and night) entering the manifolds of the A and 2A treatment. Previous tests had shown that this method did not produce amounts of nitrogen oxides which were above the detection limit of 1 mm^3 m^{-3} of a nitrogen oxide analyser (model *8840, Monitor Labs*, San Diego, USA).

Ambient air O_3 concentration was sampled above the scaffold and measured continuously with an O_3 analyser (model *8810, Monitor Labs*, San Diego, USA). O_3 concentrations of the A and 2A treatment were sampled through teflon tubing at the outlet of two control chambers. The sampling was automatically switched to a different chamber every 10 min according to a prescribed schedule. Chamber O_3 concentration was analysed by a second O_3 analyser (model *8410, Monitor Labs*, San Diego, USA). Signals from both the ambient air and twig chamber O_3 monitors were connected to a data acquisition system (*Campbell Scientific*, Shepshed, UK) and values were processed and used to adjust the O_3 concentrations in the A and 2A treatment by means of motor operated mixing valves (*Walz*, Effeltrich, Germany), while the CF air chambers were always O_3 free. O_3 concentrations were imposed continuously (day and night). The A and 2A control chambers were changed in a sequential mode every second to third day. The CO_2 concentration inside the chambers was checked with a *LI 6200* (*Li-Cor*, Lincoln, USA) gas analyser and adjusted by hand to about 350 ± 30 µmol mol^{-1} during the day and was somewhat above ambient CO_2 during the night due to respiration processes of the twigs.

Air temperature and humidity of the air entering and leaving the chambers were measured with *EE20* air temperature/humidity sensors (*E&E Electronics*, Engerwitzdorf, Austria). A sensor was also mounted externally to provide ambient temperature and humidity. The values were recorded using a *Campbell* data acquisition system which also controlled temperature and humidity. Chamber temperature was controlled by water cooling, and humidity inside the chambers by adjusting the humidity of the air supplying the chambers in 1-min intervals. For a detailed description of the twig chamber performance see Havranek and Wieser (1994).

Prior to the fumigation treatments, the empty chambers and tubing systems were "saturated" with O_3. Tests indicated that after two days of fumigation with 250 to 400 mm^3 m^{-3} of O_3 the system was saturated with O_3 and a further uptake by the system was within the error of measurement.

Ozone uptake: Ozone uptake rate or flux into the needles (F_{O_3}) was calculated independently for the six enclosed A and 2A branches. Ozone concentrations of the A and 2A control chamber, respectively, were multiplied with stomatal conductance values for water vapour (determined from temperature and humidity measurements of the air entering and leaving the individual chambers) and divided by 1.68 to adjust for the diffusion rate of O_3. Ozone concentration inside the needles was assumed to be zero (Laisk *et al.* 1989). An uptake through the cuticle was neglected because the cuticle is highly impermeable to O_3 (Kerstiens and Lendzian 1989), and because measurements in spruce (Wieser and Havranek 1993, Polle *et al.* 1995) and larch (Wieser 2001) indicated that calculated O_3 uptake rates were in good agreement with uptake rates measured as the difference between the O_3 concentrations entering and leaving the chamber. Cumulative O_3 uptake (CU) for each A and 2A twig was calculated as the flux or uptake rate (F_{O_3}) integrated over time.

Biochemical analyses: For the determination of the concentrations of antioxidants and chloroplast pigments current year's and previous year's needles were sampled at the end of the fumigation period under uniform light conditions. The needles were removed from the twigs, frozen in liquid nitrogen immediately, and stored at -80 °C. The material was lyophilised and ground in a microdismembrator (*Braun*, Melsungen, Germany). Subsequently, the powder was stored in humidity proof plastic vials at -80 °C until further biochemical analysis.

Pigment contents were determined in acetone extracts of the needle dry powder using the HPLC gradient method by Pfeifhofer (1989). α-Tocopherol was determined in acetone extracts of the needle powder using an isocratic HPLC method with fluorometric detection (Wildi and Lütz 1996). Reduced ascorbate and dehydroascorbate were quantified in *m*-phosphoric acid extracts after derivatisation with *o*-phenylene-diamine. An isocratic reversed-phase chromatography method using an ion-pairing reagent was employed (Tausz *et al.* 1996). Glutathione was determined in HCl extracts after labelling of thiol groups with the specific fluorescent dye monobromobimane. For the determination of oxidised glutathione free -SH groups were blocked by N-ethylmaleimide. Separation was performed on a gradient HPLC with fluorometric detection (Kranner and Grill 1993).

Statistics: Statistical analyses were carried out with the help of *Statistica* (*StatSoft*, Tulsa, USA). Three replicate branches per experimental variant and control, respectively, were used. The restriction to this small sample size was necessary due to the high technical requirements for the twig-chamber fumigation system.

Median values and the total range of the data are recommended for small samples sizes (Bortz *et al.* 1992) and used in tables and figures. Differences between ozone variants and the charcoal filtered air treatment (CF) were evaluated by Mann-Whitney U-test using the exact probabilities for small sample sizes reported in Bortz *et al.* (1992). $P < 0.1$ was regarded significant according to recommendations for small sample sizes (Bortz *et al.* 1992). Differences between needles of the current flush and previous year's needles were evaluated separately by Mann-Whitney U-test after pooling the samples with regard to the experimental variant.

Results

Ozone exposure and uptake: During the study mean ambient air O_3 concentration was 44 $mm^3\ m^{-3}$ (24 h-mean for 91 d) with half hour maxima up to 68 $mm^3\ m^{-3}$ (Table 1). O_3-fumigated needles showed slightly higher stomatal conductances for O_3 than foliage exposed to charcoal-filtered air (Table 1). However, this was within the range of the natural variation from 15.6 to 29 mmol $m^{-2}\ s^{-1}$. Consequently, O_3 flux into the needles was mainly dependent on the external O_3 concentration. The observed variations in cumulative O_3 uptake (CU) (Table 1) within the different O_3 treatments may be attributed to differences in needle biomass which can cause slight differences in the vapour pressure deficit of the air within the individual twig chambers (Havranek and Wieser 1994). There is strong evidence that vapour pressure deficit has a significant influence on stomatal aperture in cembran pine (Wieser 1999, 2001).

Antioxidants: In all the treatments the contents of total ascorbate and α-tocopherol were higher in the 1-year-old needles than in current season needles of *P. cembra*. The opposite trend was found for the glutathione pool (Table 2).

At the end of the experiment the different O_3 treatments did not affect total ascorbate concentrations, neither in the current flush nor in previous-year needles (Table 2). In the needles of the current flush the contents of dehydroascorbate showed no differences between the treatments. Previous-year needles exposed to ambient air O_3 concentration, both in A and outside the chambers AA, contained more dehydroascorbate than CF controls (Table 2). However, there was no trend toward a further increase of dehydroascorbate content with increasing O_3 exposure, because the 2A variant did not exhibit higher values than the control (Table 2).

Table 1. Exposure indices for the different O_3 treatments as well as mean stomatal conductance for O_3 (g_{O3}) during the experimental period July 20 to October 19, 1996. (Conductance values after Havranek, unpublished.) Values are medians ± half total range of 3 branches; nd = not determined; SUMO = total external O_3 dose; CU = cumulative O_3 uptake.

Treatment	Mean O_3 [$mm^3\ m^{-3}$]	SUMO [$\mu mol\ mol^{-1}\ h^{-1}$]	CU [$mmol\ m^{-2}$]	g_{O3} [$mmol\ m^{-2}\ s^{-1}$]
AA	43.6	94.2	nd	nd
CF	0	0	0	17.7 ± 1.5
A	44.0	95.0	7.15 ± 1.13	20.9 ± 3.3
2A	88.7	192.0	14.42 ± 3.10	20.9 ± 4.5

Table 2. Antioxidants in *Pinus cembra* needles exposed to different ozone concentrations. Values are medians ± half total range of 3 branches; nd = not determined. Asterisks indicate differences to the CF variant at $P < 0.10$ (two tailed Mann-Whitney U-test using exact probabilities for small sample sizes). d.m. = needle dry mass.

Antioxidants	Needle flush	CF	A	2A	AA
Total ascorbate [$mg\ g^{-1}$(d.m.)]	current	1.85 ± 0.36	1.74 ± 0.13	1.84 ± 0.11	1.83 ± 0.12
	previous	2.01 ± 0.16	2.00 ± 0.21	2.08 ± 0.16	2.26 ± 0.27
Dehydroascorbate [% of total ascorbate]	current	17 ± 2	13 ± 3	16 ± 2	12 ± 6
	previous	6 ± 1	15 ± 5*	7 ± 2	18 ± 7
Total glutathione [$nmol\ g^{-1}$(d.m.)]	current	349 ± 39	nd	348 ± 76	423 ± 53
	previous	322 ± 49	166 ± 16*	330 ± 10	231 ± 27*
Oxidized glutathione [% of total glutathione]	current	22 ± 1	nd	22 ± 12	23 ± 2
	previous	16 ± 0	26 ± 3*	19 ± 1	22 ± 2*
α-Tocopherol [$\mu g\ g^{-1}$(d.m.)]	current	84 ± 35	63 ± 3*	102 ± 4	77 ± 20
	previous	185 ± 30	186 ± 19	159 ± 12	131 ± 15

Table 3. Photosynthetic pigments of *Pinus cembra* needles exposed to different ozone concentrations. Values are medians ± half total range of 3 branches. Asterisks indicate differences to the CF variant at $P < 0.10$ (two-tailed Mann-Whitney U-test using exact probabilities for small sample sizes). Deepoxidation state ((Z+0.5*A)/(V+A+Z)*100; Z = zeaxanthin, A = antheraxanthin, V = violaxanthin; d.m. = needle dry mass.

Pigments	Needle flush	CF	A	2A	AA
Total chlorophyll [mg g^{-1}(d. m.)]	current	1.90 ± 0.11	1.74 ± 0.19	1.74 ± 0.59	1.98 ± 0.04
	previous	2.24 ± 0.60	2.45 ± 0.07	2.28 ± 0.19	2.51 ± 0.32
Chlorophyll *a* [mg g^{-1}(total chlorophyll)]	current	610 ± 11	598 ± 40	580 ± 26	593 ± 8
	previous	658 ± 10	601 ± 12	631 ± 5	603 ± 39
Chlorophyll *b*	current	390 ± 11	402 ± 40	420 ± 26	407 ± 8
	previous	342 ± 10	399 ± 12	369 ± 35	397 ± 39
Neoxanthin	current	58 ± 5	53 ± 9	57 ± 5	56 ± 14
	previous	73 ± 6	70 ± 8	60 ± 8	71 ± 4
Lutein	current	201 ± 16	199 ± 35	206 ± 7	186 ± 51
	previous	222 ± 27	227 ± 28	185 ± 32	234 ± 13
V+A+Z	current	32 ± 8	30 ± 7	38 ± 5	38 ± 11
	previous	59 ± 10	61 ± 5	48 ± 9	62 ± 7
α-Carotene	current	13 ± 5	18 ± 3	17 ± 2	16 ± 5
	previous	22 ± 1	22 ± 5	20 ± 1	26 ± 4
β-Carotene	current	75 ± 14	65 ± 26	73 ± 12	77 ± 28
	previous	107 ± 11	108 ± 12	91 ± 14	110 ± 4
De-epoxidation state [%]	current	78 ± 8	82 ± 2	75 ± 2	74 ± 2
	previous	75 ± 3	86 ± 4*	77 ± 4	85 ± 1*

Ozone fumigation did not affect the contents of total and oxidized glutayhione in the current flush. In 1-year-old needles total glutathione content decreased in the presence of ambient air O_3 concentration, while the proportion of oxidized glutathione increased at ambient air O_3 concentration, both in A and outside AA the fumigation chambers (Table 2). α-Tocopherol contents were slightly lower after the application of ambient air O_3 concentration in the current flush and were not affected by O_3 in previous-year needles (Table 2).

Photosynthetic pigments: Chlorophyll and carotenoid contents as well as the xanthophyll composition were not affected by O_3 neither in current- nor in previous-year needles (Table 3). In general, chlorophyll contents were higher in 1-year-old needles compared to the current flush (Table 3). Furthermore, independent from O_3 exposure, there was also a trend towards higher ratios of carotenoids over total chlorophyll with increasing needle age (Table 3).

The de-epoxidation state of the xanthophyll-cycle was not affected by O_3 in the current flush, but significantly increased in one-year-old needles under the presence of ambient air O_3 concentration in the A and in the AA variant compared to O_3 free controls (Table 3).

Discussion

Responses of the antioxidative and photoprotective defence systems are widely used markers of oxidative stress to plants (Polle and Rennenberg 1994). The range of antioxidant and pigment concentrations of *P. cembra* needles measured in our study is comparable to those reported for other spruce and pine needles growing at high altitudes (Tausz *et al.* 1998a, 1999b). Previous year's needles of *P. cembra* contained more pigments, tocopherols and ascorbate and less glutathione than younger foliage. These results are in coincidence with previous reports on evergreen conifers (Polle and Rennenberg 1994, Tausz *et al.* 1999b). However, since oxidative stress may increase on many factors increasing with altitude (including lower temperature, higher irradiance and higher ambient O_3 concentrations) it is impossible to trace high contents of antioxidants back to O_3 as a single factor in field studies (Matyssek *et al.* 1997). Spruce trees from different sites with similar exposure to oxidative pollutants exhibited different concentrations of foliar ascorbate (Tausz and Grill 1996) and pine needles from a clean-air high-altitude site contained more antioxidants than those from a highly polluted low-elevation site (Tausz *et al.* 1999c).

In the present study no consistent effects of increasing

O_3 concentration and dose on the levels of ascorbate, glutathione, and α-tocopherol in cembran pine needles exposed to ambient climatic conditions in branch chambers were observed. Ozone effects on ascorbate and water soluble thiol contents were also absent in a field fumigation experiment with 60 to 65 year-old mature Norway spruce trees at 1000 m a.s.l. (Wieser and Havranek 1996) in which twigs in the shade and the sun crown were exposed to mean O_3 concentrations between zero and 137 $mm^3\ m^{-3}$ for one growing season (Matyssek *et al.* 1997). Furthermore, after short-term exposure of field grown Norway spruce trees to high O_3 doses, Polle *et al.* (1995) also did not find changes in total foliar ascorbate and glutathione contents but only an increase in apoplastic ascorbate. These findings from mature field grown trees are in contrast to what has been reported for young conifers where an increase in foliar ascorbate and glutathione concentrations has been reported after exposure to O_3 in controlled environmental chambers (Barnes 1972, Kunert and Hofer 1987, Mehlhorn *et al.* 1986, Wellburn and Wellburn 1996, Kronfuss *et al.* 1998, Wieser *et al.* 1998).

The lack of a significant response to increasing O_3 exposure in mature trees may be attributed to differences in stomatal conductance and, hence, also in different O_3 uptake between seedlings in chambers and field grown mature trees shown for Norway spruce (Wieser 1997). Growth conditions in controlled environmental chambers are often non-limiting except for the impact of O_3. On the other hand, field sites are characterized by a broad natural variation in environmental factors possibly limiting O_3 uptake (Wieser 2000). High ambient-air O_3 concentrations generally occur during periods of high irradiance and temperature and O_3 concentration increase with increasing leaf-to-air water vapour pressure difference. In cembran pine stomatal aperture decreased with increasing leaf/air water vapour pressure difference (Wieser 1999a,b). Therefore, high O_3 uptake rates at peak O_3 concentrations are avoided (Wieser *et al.* 2000). Well-watered seedlings in growth chambers are rarely forced to restrict their water loss through stomatal closure (Kronfuss *et al.* 1998) and allow higher O_3 uptake rates.

The only sign of O_3 induced changes in the antioxidant composition of *P. cembra* needle tissues was a decrease of total glutathione content in one-year-old needles together with a shift to a more oxidized redox state. Changes in the redox state of glutathione are supposed to be an indicator for early stages of hidden ozone injury as recently shown for the ozone sensitive species *Pinus ponderosa* (Tausz *et al.* 1999a). Glutathione is essential for the recycling of ascorbate from dehydroascorbate (Foyer 1997). Ascorbate is the prevailing water soluble antioxidant, it is furthermore required for the regeneration of α-tocopherol and the conversions in the photoprotective xanthophyll cycle (Polle and Rennenberg 1994). Ascorbate must be present in its reduced stage to fulfil these tasks. Results on *P. ponderosa* strongly suggest that the GSH/GSSG redox ratio is the most sensitive part in this chain of protective reactions against active oxygen species (Tausz *et al.* 1999a). Upon a shift in the thiol/disulfide status, subsequent consequences in cell metabolism such as changes in enzyme activities or even changes in gene transcription might follow, because all these processes may be regulated by the GSH redox state (Noctor *et al.* 1998). The redox state of ascorbate, by contrast, seems to be more stable than that of glutathione (Tausz *et al.* 1999a,c). However, since the higher ozone concentration applied in the present study (2-fold ambient) had no effect on the GSH/GSSG ratio in *P. cembra* needles compared to clean-air controls, the responses must be regarded marginal and a causal connection with ozone fumigation can hardly be established.

In addition to antioxidants, the photosynthetic apparatus is also protected by xanthophyll cycle pigments violaxanthin, antheraxanthin, and zeaxanthin (Demmig-Adams and Adams 1994). In the present study no clear O_3 effects were found on the contents of xanthophylls and carotenes. Only 1-year-old cembran pine needles exposed to ambient air O_3 concentrations exhibited a higher de-epoxidation state of the xanthophyll cycle pool compared to ozone free controls. Similar results were also reported for Norway spruce seedlings exposed to $mm^3\ m^{-3}$ of O_3 in growth chambers (Kronfuss *et al.* 1998). However, needles exposed to 2A O_3 concentration exhibited no difference in the de-epoxidation state compared to controls. Since the chlorophyll concentrations remained unaffected by both A and 2A O_3 concentration, oxidative degradation of photosynthetic pigments was prevented.

In conclusion, changes in the antioxidative systems of cembran pine needles following O_3 exposure were only observed at the ambient air ozone level (A and AA), but not at the double ambient (2A) compared to charcoal filtered air. Regardless of the small sample size in the present experiment, the ambient ozone variant in the chamber and the outside variant, both experiencing the same O_3 dose, exhibited similar changes in the glutathione system and the xanthophyll cycle state. However, a clear ozone effect on the antioxidative system of *P. cembra* could not be established, since responses were absent under double ambient O_3 concentrations. *P. cembra*, a species particularly well-adapted to natural high altitude stress conditions, seems to be only marginally affected by ambient ozone concentrations at the investigated field stand. To predict possible responses to an increase of ozone concentrations in high altitude ecosystems of the Alps, further investigations on this tree which can be related to the basic antioxidant and photoprotection data reported in the present study, are desirable.

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