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Combined effects of CO_2 and O_3 on antioxidative and photoprotective defense systems in needles of ponderosa pine

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Abstract

Ponderosa pine (*Pinus ponderosa* Dougl. *ex* Laws.) seedlings were exposed to near ambient or elevated CO_2 (average concentrations during the last growing season 446 *versus* 699 μmol mol^{-1}), combined with low or elevated O_3 for three seasons. Ozone exposure during the last growing season (accumulated dose above threshold 0.06 μmol mol^{-1}) was 0.05 *versus* 26.13 μmol mol^{-1} h. Needles of the youngest age class were harvested after the dormancy period. Ozone exposure decreased needle contents of chlorophyll *a*, chlorophyll *b*, and ascorbate, and resulted in a more oxidized total ascorbate and a more de-epoxidized xanthophyll cycle pool irrespective of the CO_2 level. Trees under elevated CO_2 had a more oxidized glutathione pool and lower chlorophyll *a* content. Contents of glutathione, tocopherol, and carotenoids were not affected by the CO_2 or O_3 treatments. There were no interactive effects between elevated CO_2 and elevated O_3 on any of the parameters measured. The results suggest that elevated atmospheric CO_2 concentration does not compensate for ozone stress by increasing antioxidative capacity in ponderosa pine.

Additional key words: air pollution, ascorbate, glutathione, oxidative stress, pigments, *Pinus ponderosa*, xanthophyll.

Introduction

Responses of antioxidative defense systems are widely accepted and investigated biochemical markers of plant stress (Tausz *et al*. 2003). As a result, measurements of defense system components may provide a method for assessment of pollution effects (Heath 1999). In particular, antioxidative responses induced by O_3 have been extensively studied and reviewed (Polle 1998, see also *e.g.* Wieser *et al.* 2001, Bernardi *et al.* 2004).

An increased CO_2/O_2 ratio in the photosynthetic apparatus by elevated CO_2 may reduce the rate of active oxygen formation. On the other hand, down-regulation of protective pathways such as photorespiration or, in the long term, lower antioxidant concentrations, may make plants more susceptible to oxidative stress (Karnosky 2003). Both CO_2-induced activation (Schwanz *et al.* 1996) and down-regulation (Polle *et al.* 1993) of antioxidant systems have been reported. Whereas in some studies chlorophyll (Tausz *et al*. 1996) and foliar N (Karnosky 2003) concentrations were lower with elevated CO_2, chlorophyll and carotenoid concentrations were higher in oak species exposed to long-term elevated CO_2 near natural CO_2-springs, than in similar trees growing further away from these springs (Schwanz and Polle 1998). Contradictory effects on foliar N concentrations of different Mediterranean species growing near such springs were also reported (Tognetti and Peñuelas 2003).

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Abbreviations: A - antheraxanthin; DEPS - de-epoxidation state; V - violaxanthin; Z - zeaxanthin.
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Elevated CO_2 near the springs also increased ascorbic acid concentrations and reduced the redox state compared with control trees (Marabottini *et al.* 2001), while in another year and season of the year, lower ascorbate and higher glutathione concentrations were reported with elevated CO_2 for the same sites (Schwanz and Polle 1998).

Investigations on the interactive effects of combinations of O_3 and elevated CO_2 on plant antioxidative defense systems have been conducted since the early 1990s (Polle *et al.* 1993); but the picture emerging from these studies is still highly contradictory. While some authors concluded a protective effect of elevated CO_2 against ozone impact (Schwanz *et al.* 1996, McKee *et al.* 1997, Gaucher *et al.* 2003), others did not (Polle *et al.* 1993). When a protective effect was postulated, it might have been due to CO_2-induced activation of antioxidant systems, or to decreased stomatal conductance leading to decreased pollutant uptake (McKee *et al.* 1997). If elevated CO_2 leads to down-regulated defense systems, it will exacerbate ozone effects (Karnosky 2003). Published results show the difficulties in comparing effects among different experimental conditions, across woody and herbaceous plants, and across evergreen or deciduous species. An understanding of interactive effects of CO_2 and O_3 on antioxidative defense capacity of plants is still a major knowledge gap (Karnosky 2003).

In the present work we determine the effects of elevated CO_2 and O_3 on antioxidative defense capacities in seedlings of ponderosa pine (*Pinus ponderosa* Dougl. *ex* Laws.). Several European conifers investigated in this respect earlier, such as Norway spruce [*Picea abies* (L.) Karst], European larch (*Larix decidua* L.), or stone pine (*Pinus cembra* L.) seem to be relatively resistant to ozone (Wieser and Havranek 2001), which leads to vague evaluations of ozone effects in European forests (Matyssek and Sandermann 2003). Ponderosa pine, in contrast, is among the most susceptible forest species to ozone, based on foliar injury (Miller 1983), growth reductions (Temple and Miller 1994), decreased photosynthetic capacities (Grulke 1999), or needle loss.

Materials and methods

The experiment was conducted in sun-lit chambers that track and control air and soil factors while maintaining natural environmental variability (Olszyk *et al.* 2001). Each chamber was 2 m wide, 1 m front-to-back, 1.3 to 1.5 m tall, on a 0.2 m high wooden base. The chamber surface area was 2 m^2 and enclosed volume was 3.2 m^3. The mineral soil was an ashy, glassy Xeric Vitricry, probably of the Steiger series from the east side of the Oregon Cascade Mountains. Chamber air temperature and vapor pressure deficit levels were set at current ambient levels. More details on the chambers in Olszyk *et al.* (2001). There were 12 chambers, three for each CO_2 by O_3 combination treatment.

During the year prior to plant harvest and sampling of needles for this study (4 April 2000 - 3 April 2001) the average hourly CO_2 concentration in "ambient" CO_2 chambers was 446 ± 3 (mean ± SD) μmol mol^{-1}, and in elevated CO_2 chambers was 699 ± 6 μmol mol^{-1}. These averages were for 5 ambient and 6 elevated CO_2 chambers across all daily average values. They were essentially the same as the average concentrations of 443 and 717 μmol mol^{-1} CO_2 for the ambient and elevated CO_2 chambers across the entire three years of the experiment (Olszyk *et al.* 2002). The CO_2 in ambient chambers was slightly (by about 20 μmol mol^{-1}) higher than in outside air to facilitate concentration control (Olszyk *et al.* 2001). Ozone was added to the chambers from mid-May though early-October when the trees were actively growing. Ozone concentrations in ambient air were low (Olszyk *et al.* 2001, 2002). Target O_3 levels were based on hourly O_3 data from the Midwestern United States, with O_3 added continuously (24 h d^{-1} and 7 d $week^{-1}$) following a pattern of varying high and low concentrations. Growing-season-long O_3 exposure was expressed as a single value, SUM06, which was the accumulation over the growing season of all hourly average values > 0.06 μmol mol^{-1} (Lee *et al.* 1988). During the 2000 growing season (18 May - 7 October), prior to needle sampling, the SUM06 for elevated O_3 chambers was 26.13 ± 0.39 μmol mol^{-1}·h across six replicate chambers, and for low O_3 chambers was 0.05 ± 0.05 μmol mol^{-1}·h across five replicate chambers. From mid-October through mid-Spring, chamber O_3 concentrations simulated the low natural O_3 conditions. Because no O_3 was added and some ambient O_3 was removed from the air by chamber surfaces, this resulted in the same, low O_3 concentrations (nearly always < 0.06 μmol mol^{-1} on an hourly basis) in all chambers.

One-year-old half-sib ponderosa pine (*Pinus ponderosa* Dougl. *ex* Laws.) seedlings were obtained from the US Forest Service nursery near Bend, Oregon, USA. The seed source was a tree exposed to low ambient O_3 on the east side of the Cascade Mountains. Seedlings were planted in the chambers (11 trees per chamber) on 8 April 1998 (full details in Olszyk *et al.* 2001). Watering was controlled throughout the year to provide a typical seasonal soil moisture cycle (wet winter and dry summer) as found in Pacific Northwest ponderosa pine forests. No fertilizer was added to the soil during this experiment, thus the seedlings relied on soil processes for nutrients. No chemical pesticides were used in the chambers.

Needles were harvested for analysis at the end of the

study on 4 April 2001, after three growing and three dormant seasons (Olszyk *et al.* 2002). We harvested needles after the dormant season to evaluate cumulative long-term effects of O_3 over the previous growing season, and to exclude potential transitory short-term effects of O_3 during the growing season itself. Needles were taken from the current age class, which developed during the summer of 2000. For each chamber, one fascicle (three needles) was taken from nearly every tree and all needles pooled to obtain one sample per chambers. The fascicles were in full sun, on the south-facing sides of the trees midway up the terminal shoot of the seedling.

Needle preparation and biochemical analyses were conducted as described in detail in Tausz *et al.* (2003). In brief, upon removal from the trees, needles were immediately frozen in liquid nitrogen, then freeze-dried and sealed in plastic bags with silica gel. Needle tissue was ground in a dismembrator (*Braun*, Melsungen, Germany), and stored frozen in humidity-proof plastic vials prior to analysis. The chlorophylls, carotenoids and xanthophylls were determined on acetone extracts of the needle dry powder with a HPLC gradient method. The violaxanthin, antheraxanthin, and zeaxanthin concentrations were summed and represented as V+A+Z. Tocopherol was measured on the same acetone extracts with an isocratic HPLC method using fluorometric detection. Determination of ascorbate and dehydroascorbate was done by an isocratic HPLC method with an ion-pairing reagent and photometric detection, and glutathione in its oxidized and reduced state was measured after derivatization with the thiol specific fluorescent dye monobromobimane with a gradient HPLC method. The HPLC system consisted of automatic injector (*Midas 830*, *Spark Holland*, Emmen, The Netherlands), 2 analytical pumps (*SunFlow 100*, *SunChrom*, Friedrichsdorf, Germany) or 1 pump with gradient former (*GF 46*, *SunChrom*) and degasser (*K5002*, *Knauer*, Berlin, Germany), UV-vis (*SpectraFlow 550*, *SunChrom*) and fluorescence detector (*F1300*, *Hitachi*, Tokyo, Japan), and integration software (*CromStar*, *SCPA*, Stuhr, Germany). All separations were carried out on *Spherisorb S5 ODS2* 250 × 4.6 mm columns with precolumns *S5 ODS2* 50 × 4.6 mm; details on solvents, gradients, and detection settings are given in Tausz *et al.* (2003).

There were four treatments: ambient or elevated CO_2 with low or elevated O_3. Since needles from within individual chambers were pooled to one sample, replicate chambers were the experimental units for each treatment. Because of the small replication number [3 chambers, except for 2 for ambient CO_2 and low O_3 treatment due to loss of one chamber (Olszyk *et al*. 2001)] statistical evaluation of O_3, CO_2, and interactive $O_3 \times CO_2$ effects was done by an enhanced Kruskal-Wallis test using data alignments [non-parametric analogon to a two-way *ANOVA*, according to the Hildebrandt method reported in Bortz *et al.* (1990)]. For the same reason data are presented as medians and median deviations, which are best suited for small replication numbers (Sachs 1992).

Results and discussion

Both chlorophyll *a* and *b* concentrations were depressed significantly by O_3 exposure (Table 1). Chlorophyll *a* concentration also was significantly decreased upon elevated CO_2, whereas all interaction effects $O_3 \times CO_2$ were not significant. Concentrations of carotenoids and α-tocopherol were not affected by the treatments (Table 1). Neither elevated CO_2 nor elevated O_3 altered the concentration of xanthophylls. However, the de-epoxidation status [DEPS = (Z + 0.5 A)/(V + A + Z)] of the xanthophylls was significantly reduced for trees in elevated O_3.

Elevated O_3 caused a significant reduction in the total ascorbate concentration and increased the concentration of its oxidized form (Table 1). Glutathione concentrations were not changed by the CO_2 or O_3 treatments. However, glutathione was more oxidized in needles from CO_2-treated trees but not in the O_3-treated trees (Table 1).

Our seedlings responded similarly to mature field-grown ponderosa pine trees; concentrations of pigments, lipophilic and hydrophilic antioxidants were in the range of data from ponderosa pine needles collected in forests (Tausz *et al.* 1999a,b,c, 2001, 2002).

In previous studies, long-term O_3 exposures increased antioxidant pools and activities of antioxidant enzymes (Polle *et al.* 1993, Elvira *et al.* 1998, Alonso *et al.* 2001). In those studies, samples were taken directly after exposure, which does not distinguish between acute and possible long-term effects. The lack of an ozone effect on glutathione, tocopherol, or carotenoid pools in our study is not surprising as the ozone exposures were stopped about 6 months before the tissue was harvested for analysis, which allowed sufficient time for homeostatic re-adjustment processes to occur. However, the persistent ozone effects on ascorbate and dehydroascorbate concentrations in our study were unexpected and likely are examples of ozone carry-over effects (Andersen *et al.* 1997, Langebartels *et al.* 1998, Oksanen and Saleem 1999). Langebartels *et al.* (1998) proposed that ozone carry-over effects are caused by soluble and/or wall-bound compounds such as catechin, stilbenes and tyramine. These ozone-induced compounds contribute to endogenous stress leading to ozone symptoms.

In contrast to other antioxidative compounds studied in this paper (glutathione, tocopherol, carotenoids,

Table 1. Effects of CO_2 and O_3 on pigments and antioxidants in *Pinus ponderosa* needles. ACLO - ambient CO_2 + low O_3; ACEO - ambient CO_2 + elevated O_3; ECLO - elevated CO_2 + low O_3; ECEO - elevated CO_2 + elevated O_3; d.m. needle dry mass; V + A + Z - the sum of the violaxanthin, antheraxanthin and zeaxanthin pigments; DEPS (de-epoxidation state) is calculated as (Z+0.5 A)/(V+A+Z) × 100; DHA - dehydroascorbate; total GSH - total glutathione (reduced + oxidized); GSSG - oxidized glutathione. Medians ± median deviations for 3 replicate chambers per treatment except for 2 replicate chambers for ACLO.

Parameter	Treatment				*P*-values of effects		
	ACLO	ACEO	ECLO	ECEO	CO_2	O_3	$CO_2 \times O_3$
Chlorophyll *a* [μmol g^{-1}(d.m.)]	2.42 ± 0.51	1.24 ± 0.28	1.53 ± 0.11	0.76 ± 0.13	0.018	0.006	0.935
Chlorophyll *b* [μmol g^{-1}(d.m.)]	0.75 ± 0.09	0.49 ± 0.08	0.66 ± 0.02	0.51 ± 0.04	0.584	0.006	0.923
Lutein [nmol g^{-1}(d.m.)]	360 ± 81	296 ± 29	340 ± 2	358 ± 38	0.201	0.361	0.909
Neoxanthin [nmol g^{-1}(d.m.)]	90 ± 46	80 ± 11	89 ± 23	92 ± 12	0.855	0.465	0.948
V+A+Z [nmol g^{-1}(d.m.)]	86 ± 10	90 ± 33	93 ± 15	96 ± 3	0.855	0.715	0.971
DEPS [%]	10 ± 1	2 ± 2	11 ± 2	7 ± 3	0.272	0.018	0.808
α-Carotene [nmol g^{-1}(d.m.)]	18 ± 5	13 ± 6	19 ± 2	15 ± 1	0.855	0.361	0.995
β-Carotene [nmol g^{-1}(d.m.)]	204 ± 32	189 ± 20	179 ± 14	194 ± 6	0.855	0.584	0.892
α-Tocopherol [nmol g^{-1}(d.m.)]	588 ± 85	592 ± 1	584 ± 29	614 ± 61	0.855	0.715	0.981
Total ascorbate [μmol g^{-1}(d.m.)]	28 ± 1	13 ± 2	20 ± 1	13 ± 3	0.068	0.006	0.066
DHA [% of total]	16 ± 2	33 ± 1	23 ± 1	35 ± 5	0.201	0.006	0.394
Total GSH [nmol g^{-1}(d.m.)]	311 ± 52	225 ± 14	222 ± 3	236 ± 74	0.201	0.361	0.624
GSSG [% of total]	10 ± 2	9 ± 2	14 ± 1	17 ± 2	0.018	0.736	0.273

xanthophylls), ascorbate is present in the apoplast of leaf tissues, constituting a "first line of defense" detoxifying ozone in the cell walls; its apoplastic levels change in response to ozone exposure (Polle 1998, Smirnoff 2000, Bichele *et al.* 2000, Burkey *et al.* 2003). Ozone exposure leads to an increased oxidation of the ascorbate pool indicating that ascorbate regeneration can not keep up with ascorbate oxidation. Oxidation without subsequent regeneration to the reduced form will lead to degradation and loss of ascorbate. Previous studies found O_3-related changes in the glutathione system, which is the main regeneration pathway for ascorbate (reviewed in DeKok and Tausz 2001). There are more possible explanations for the persistent ozone effects on ascorbate and dehydroascorbate concentrations; *1*) the ozone treatment caused the formation of chemicals that are continuing to oxidize ascorbate or *2*) the ozone treatment has altered membrane transport limiting the movement of ascorbate into the apoplast and the movement of dehydroascrobate to its site of reduction**,** or *3*) ozone induced premature senescence resulted in the observed changes. However, leaf senescence is marked by increased tocopherol concentrations (Polle and Rennenberg 1994), which were not observed in the present study.

Furthermore, chlorophyll contents were clearly depressed upon O_3 exposure (Table 1) which is commonly regarded a biochemical damage symptom in connection with O_3. An O_3-induced reduction in chlorophyll has been seen in a number of other studies (*e.g.*, Polle *et al.* 1993, Elvira *et al.* 1998), but not for current year needles as we observed. In our study there were no visible needle symptoms of O_3 injury.

As in previous studies (Ormrod *et al.* 1999), CO_2 alone also depressed chlorophyll concentrations in ponderosa pine needles. This effect may be regarded an adaptation to higher internal leaf CO_2 concentrations (c_i) which decrease the need for more effective photosynthesis. Decreased chlorophyll concentrations may also be related to decreased foliar N contents commonly observed under elevated CO_2 (Karnosky 2003).

As summarized in the introduction, reports as to whether or whether not elevated CO_2 increases antioxidative defense capacity, are contradictory. In our experiment there were no hints towards of an increased antioxidative defense under elevated CO_2. In contrast, the glutathione pool tended to be more oxidized, indicating a less effective regeneration of glutathione as observed under various stress conditions (Tausz 2001).

Consistent with the effects of the single CO_2 and O_3 stresses, the combination of CO_2 and O_3 did not reveal a mediation of O_3 effects, since no significant interactive effects of $O_3 \times CO_2$ on the antioxidant systems were observed. The O_3 effects that were observed (depressed ascorbate levels) occurred irrespective of CO_2 treatment. Since there was no effect on stomatal conductance from elevated CO_2 during the 2000 growing season when the leaves evaluated were exposed to O_3 (Olszyk *et al.* 2002), the antioxidant defense system results in this study reflected differences in leaf metabolism and not differences in internal O_3 exposure with different CO_2 treatments. Thus, based on the results of our study, a protective effect of elevated CO_2 towards O_3 due to an increased antioxidative defense capacity is unlikely in this sensitive forest species.

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