Age effects on Norway spruce (Picea abies) susceptibility to ozone uptake: a novel approach relating stress avoidance to defense

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Summary Cumulative ozone (O₃) uptake and O₃ flux were related to physiological, morphological and biochemical characteristics of Norway spruce (Picea abies (L.) Karst.) trees of different ages. Under ambient CO₂ conditions, photosynthetic capacity (Aₘₐₓ) declined in mature trees when cumulative O₃ uptake into needles, which provides a measure of effective O₃ dose, exceeded 21 mmol m⁻² of total needle surface area. A comparable decline in Aₘₐₓ of seedlings occurred when cumulative O₃ uptake was only 4.5 mmol m⁻². The threshold O₃ flux causing a significant decline in Aₘₐₓ ranged between 2.14 and 2.45 mmol m⁻² s⁻¹ in mature trees and seedlings subjected to exposure periods of ≥ 70 and ≥ 23 days, respectively. The greater O₃ sensitivity of young trees compared with mature trees was associated with needle morphology. Biomass of a 100-needle sample increased significantly with tree age, whereas a negative correlation was found for specific leaf area, these changes parallel those observed during differentiation from shade-type to sun-type needles with tree ontogeny. Age-dependent changes in leaf morphology were related to changes in detoxification capacity, with area-based concentrations of ascorbate increasing during tree ontogeny. These findings indicate that the extent of O₃-induced injury is related to the ratio of potentially available antioxidants to O₃ influx. Because this ratio, when calculated for ascorbate, increased with tree age, we conclude that the ratio may serve as an empirical basis for characterizing age-related differences in tree responses to O₃.

Keywords: antioxidants, ascorbate, cumulative ozone uptake, detoxification, needle morphology, ontogeny, ozone flux, scaling, tree age.

Introduction Tropospheric ozone (O₃) is one of the most detrimental air pollutants affecting forest trees (Lefohn 1991, Sandermann et al. 1997). Trees respond to O₃-induced stress by mechanisms of avoidance and defense (i.e., stress tolerance; Hogsett and Andersen 1998) such as restriction of O₃ uptake by stomatal closure or detoxification through biochemical reactions in the tissue. Much of the information on O₃ effects on trees is based on chamber studies conducted with seedlings (Reich 1987, Pye 1988, Sandermann et al. 1997, Matyssek and Innes 1999). Seedlings, however, are uncertain surrogates for mature trees (Kelly et al. 1995, Kolb et al. 1997, Kolb and Matyssek 2001), because mature trees and seedlings differ in morphological and physiological characteristics that strongly influence plant responses to O₃ (Ryan et al. 1997, Bond 2000). Understanding the influence of age on the response of trees to O₃ is, therefore, essential to a better interpretation of the available experimental data.

The effect of age on tree responses to O₃ is poorly understood (Samuelson and Kelly 2001), because of the difficulty of exposing large trees to O₃ experimentally (Samuelson and Edwards 1993, Grulke and Miller 1994, Fredericksen et al. 1995, 1996, Werner and Fabian 2001). Limited data for Norway spruce (Picea abies (L.) Karst.) trees indicate the potential for large differences in physiological response to O₃ among trees of different ages and sizes (Wieser 1997). The magnitude of tree responses to O₃ depends on several factors, including exposure regime (Wieser et al. 1998), site conditions that influence stomatal aperture (Maurer et al. 1997, Kronfuß et al. 1998) and hence O₃ influx (i.e., O₃ uptake rate, defined as external O₃ concentration times stomatal conductance) and cumulative O₃ uptake (i.e., integrated flux during the exposure period; Wieser et al. 2000), biochemical defense mechanisms (Polle 1998) that vary with tree age, size (Kolb et al. 1997) and genotype (Taylor 1994).

Ozone uptake is commonly estimated from assessments of gas exchange. Differences in O₃ sensitivity of trees differing in age have been attributed to ontogenetic changes in stomatal conductance (Kolb et al. 1997). However, differences in stomatal conductance can only partially explain age-related differences in O₃ susceptibility. There is evidence linking O₃ sensitivity to ontogenetic trends in leaf morphology (Pääkkönen et al. 1995, 1997, Ferdinand et al. 2000) and biochemistry (Taylor and Hanson 1992, Wellburn and Wellburn 1996).
To improve our ability to scale data from O₃ sensitivity studies on seedlings to mature trees, we need information on O₃ dose–response relationships in forest trees of different ages and sizes. With the exception of studies by Baumgarten et al. (2000) on beech (Fagus sylvatica L.) trees, no O₃ uptake thresholds for performance effects on either young or old trees have been determined. Moreover, little information on age-related differences in needle morphology and antioxidative defense capacity; and link stress avoidance (exclusion of O₃ uptake) and tolerance (detoxification) to O₃ sensitivity in a conceptual framework.

Materials and methods

Experimental design and ozone treatment

To characterize effects of long-term exposure to O₃ on photosynthetic performance of current-year needles of Norway spruce trees, we compared experimental data obtained from seven O₃ fumigation experiments with mature trees and seedlings (Table 1). Two exposure facilities were used, twig chambers and growth chambers. In field experiments, twigs from the upper crown of 60- to 65-year-old trees were sealed in transparent fumigation cuvettes (Havranek and Wieser 1990, 1994) throughout one or two growing seasons between 1986 and 1990. The O₃ exposure regimes were charcoal-filtered air, ambient air, and up to threefold ambient O₃ concentrations (Table 1). The system provided control of ambient climatic conditions and O₃ concentrations, and tracked their diurnal and seasonal fluctuations inside the cuvettes, in addition to controlling several replicates per treatment in parallel. In 1994 and 1995, 4- and 5-year-old seedlings were placed in growth-chambers (Kronfuß et al. 1998) and exposed continuously (day and night) to either charcoal-filtered air (control), or a constant concentration of O₃ (100 nl l⁻¹) (1994), or O₃ concentrations that increased weekly in steps of 25 nl l⁻¹ from 0 to 100 nl l⁻¹ (1995) (Table 1).

<table>
<thead>
<tr>
<th>Year</th>
<th>Tree type</th>
<th>Experiment</th>
<th>Mean O₃ concentration (nl l⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1986</td>
<td>Mature tree</td>
<td>Twig chamber</td>
<td>0⁻¹, 65², 120³</td>
<td>Wieser and Havranek 1996</td>
</tr>
<tr>
<td>1987</td>
<td>Mature tree</td>
<td>Twig chamber</td>
<td>0⁻¹, 64², 100³</td>
<td>Wieser and Havranek 1996</td>
</tr>
<tr>
<td>1988</td>
<td>Mature tree</td>
<td>Twig chamber</td>
<td>0⁻¹, 47², 77³, 107³, 137³</td>
<td>Wieser and Havranek 1996</td>
</tr>
<tr>
<td>1989</td>
<td>Mature tree</td>
<td>Twig chamber</td>
<td>0⁻¹, 37², 67³, 97³, 127³</td>
<td>Wieser and Havranek 1996</td>
</tr>
<tr>
<td>1990</td>
<td>Mature tree</td>
<td>Twig chamber</td>
<td>0⁻¹, 30², 55³, 90³, 115³</td>
<td>Wieser and Havranek 1996</td>
</tr>
<tr>
<td>1994</td>
<td>Seedling</td>
<td>Growth chamber</td>
<td>0⁻¹, 100⁴</td>
<td>Kronfuß et al. 1998</td>
</tr>
<tr>
<td>1995</td>
<td>Seedling</td>
<td>Growth chamber</td>
<td>0⁻¹, 34⁴, 75⁴, 100⁴</td>
<td>Wieser et al. 1998</td>
</tr>
</tbody>
</table>

¹ Charcoal-filtered air.
² Ambient air.
³ Tracking ambient O₃ concentration.
⁴ Constant (day and night) O₃ concentration.

Table 1. Summary of the ozone response studies.
Halo Star KLR 51, Osram, Germany)). Measurements were made repeatedly throughout and at the end of each fumigation period. All gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981) and expressed on the basis of total needle surface area, which was determined by the glass-bead technique (Thompson and Leyton 1971).

At each measurement date, differences in $A_{\text{max}}$ between O$_3$-treated plants and controls in charcoal-filtered air were examined by the Student’s $t$-test at $P < 0.05$. To account for the effects of age (Yoder et al. 1994), season (Kronfuß et al. 1998) and year-to-year variation (Wieser and Havranek 1996) on $A_{\text{max}}$ and O$_3$ exposure, photosynthetic rates obtained at each date were expressed as percentages relative to the respective rates of control twigs grown in charcoal-filtered air.

Data from the seedling and tree experiments were pooled to determine dose–response functions for seedlings and mature trees. The two data sets were then combined to determine incipient effects of cumulative O$_3$ uptake on $A_{\text{max}}$ of mature trees and seedlings by boundary-line analysis. Because cumulative O$_3$ uptake is an integrated measure expressing the quantity but not the time span of O$_3$ exposure, we also determined O$_3$ uptake rate on a short timescale.

Biochemical analysis and morphological characteristics

We determined antioxidant concentrations in needles from 8- to 60-year-old field-grown Norway spruce trees. All study trees grew in a gap in a forest near the Austrian Timberline Research Station Klimahaus on Mt. Patscherkofel (1950 m a.s.l., 47° N, 11° E), Innsbruck, Austria. Samples were taken from exposed, southwest-facing branches in similar light conditions (G. Wieser, unpublished data). In 1998, to account for microclimatic influences within a stand on needle antioxidant concentrations, we planted 4-year-old Norway spruce seedlings in 70-l containers (20 seedlings per container) filled with natural forest soil. In spring 1999, five containers were transferred to the upper sun crown (20 m high) of a 50-year-old Norway spruce forest stand at the free-air ozone fumigation plot at Kranzberg (485 m a.s.l., 48° N, 11° E) near Munich, Germany (cf. Pretsch et al. 1998, Häberle et al. 1999). The potted seedlings were irrigated regularly throughout the growing season.

In September 1999, current-year needles from both mature trees and seedlings were sampled under uniform light conditions (1200 to 1430 h, mean PPFD of 1500 µmol m$^{-2}$ s$^{-1}$) at both study sites from five trees per age class (one seeding per container). Needles were removed from the twigs, immediately frozen in liquid nitrogen and stored at −80°C. The material was lyophilized, ground in a microdisembrator (Braun, Melsungen, Germany) and stored in plastic vials at −80°C until analyzed.

Reduced ascorbate and dehydroascorbate were quantified in meta-phosphoric acid extracts after derivatization with o-phenylenediamine. An isocratic reversed-phase chromatographic method using an ion-pairing reagent was employed (Tausz et al. 1996). Ascorbate concentrations were expressed on a needle dry mass basis, determined after 72 h at 80°C and on a total needle surface area, estimated as described by Thompson and Leyton (1971). Determination of area-based concentrations of ascorbate allowed us to define O$_3$ sensitivity as the equilibrium between needle O$_3$ uptake and detoxification processes.

Finally, dry mass of 100-needle samples and specific leaf area (SLA; cm$^2$ leaf area per g dry weight) were determined from twigs adjacent to twigs used for the biochemical analyses. Measurements of needle water potential, net photosynthesis and stomatal conductance in trees of different ages performed immediately before harvest indicated that there were no significant age-related differences at either the Mt. Patscherkofel or the Kranzberg site. Means were $–1.3 ± 0.15$ MPa for needle water potential, $1.2 ± 0.5$ µmol CO$_2$ m$^{-2}$ s$^{-1}$ for net photosynthesis and $22.9 ± 3.1$ mmol H$_2$O m$^{-2}$ s$^{-1}$ for stomatal conductance for trees on Mt. Patscherkofel. Corresponding values for trees at the Kranzberg site were $–1.6 ± 0.10$ MPa, $3.2 ± 0.2$ µmol CO$_2$ m$^{-2}$ s$^{-1}$ and $57.7 ± 7.5$ mmol H$_2$O m$^{-2}$ s$^{-1}$, respectively. Mean predawn needle water potentials assessed in trees one day after harvest (0400–0500 h) were $–0.7 ± 0.15$ MPa at Mt. Patscherkofel and $–1.2 ± 0.21$ MPa at the Kranzberg site.

Results and discussion

Quantification of tree response to cumulative O$_3$ uptake and O$_3$ flux

In the field experiments with mature trees, O$_3$ treatments tracked ambient fluctuations, whereas seedlings in the chambers were exposed to a constant concentration of O$_3$. Although trees and seedlings were exposed to different O$_3$ regimes, only mean O$_3$ concentrations above 100 nl l$^{-1}$ induced a statistically significant decline in $A_{\text{max}}$, by 20–40% in current-year needles of mature trees (Wieser and Havranek 1996, Matyssek et al. 1997) and 20–30% in seedlings (Kronfuß et al.1998, Wieser et al. 1998). The basic assumption underlying the use of branch chambers on trees is that branches export but do not import carbon during the growing season (Sprugel et al. 1991). It is unclear, however, whether detoxification in individual branches is supported by substrates translocated from other tree parts (Matyssek et al. 1997). Nevertheless, dose–response functions demonstrated seedlings were more sensitive to O$_3$ than mature trees (Figure 1).

Thresholds based on cumulative O$_3$ uptake can be derived from the plot shown in Figure 2A for $A_{\text{max}}$. The boundary line represents the threshold below which the combination of exposure period and cumulative O$_3$ uptake did not cause statistically significant reductions in $A_{\text{max}}$ relative to controls in O$_3$-free air. In mature trees, there was a significant reduction in $A_{\text{max}}$ when cumulative O$_3$ uptake exceeded 21 mmol m$^{-2}$ per unit of total needle surface area (Figure 2A), which occurred after 70 days of exposure to mean O$_3$ concentrations persistently higher than 100 nl l$^{-1}$. The derived threshold O$_3$ uptake was about 45% higher than that determined over one growing season for evergreen conifers growing in ambient O$_3$ in the central European Alps (11.4 ± 1.7 to 14 mol m$^{-2}$; Wieser et al. 2000). There was a comparable decline in $A_{\text{max}}$ of seedlings when cumulative O$_3$ uptake was only 4.5 mmol m$^{-2}$ (Fig-
cause of the shortcomings in scaling O₃ effects from seedlings. The finding of a similar threshold O₃ flux in mature Norway spruce seedlings (22.5 ± 2.1 and 23.1 ± 1.65 mmol O₃ m⁻², respectively). Therefore, we determined O₃ flux, a measure of O₃ exposure over a short timescale. Although the study plants differed by O₃ exposure, genotype and environmental conditions, the threshold flux for inducing a significant reduction in $A_{max}$ ranged between 2.14 to 2.45 mmol m⁻² s⁻¹ for both mature trees and seedlings (Figure 2B). Again, the threshold O₃ flux exceeded the values determined in the field for mature conifers (0.5 to 0.85 mmol m⁻² s⁻¹; Wieser et al. 2000). The finding of a similar threshold O₃ flux in mature Norway spruce trees and seedlings in chambers (Figure 2B) is consistent with studies on beech (Baumgarten et al. 2000). Based on a modeling approach, Emerson et al. (1998, 2000) determined that a cumulative O₃ uptake of 3.1 and 3.0 mmol m⁻² was required to induce visible leaf injury in mature beech trees and seedlings in chambers, respectively. In both studies, the mature forest trees exhibited a significant delay in O₃ uptake (Figure 2).

The role of ontogeny in tree responses to O₃ is unclear because of the shortcomings in scaling O₃ effects from seedlings in chambers to stands of mature trees in the field (Kelly et al. 1995, Hogsett and Andersen 1998, Kolb and Matyssek 2001). Although the threshold values were similar in mature trees and seedlings, the temporal variation in O₃ flux differed. At night, O₃ flux into needles was reduced to 75 and 25% of daytime values in seedlings (Kronfuß et al. 1998) and mature Norway spruce trees (Wieser and Havranek 1993), respectively, as a result of high nighttime concentrations of O₃ in mountainous regions (Wieser and Havranek 1995). Nocturnal O₃ uptake may promote susceptibility (cf. Musselman and Minnick 2000), resulting in leaf injury in Betula pendula Roth., Populus × euramericana Dode. and Alnus glutinosa L. (Günthardt-Goerg et al. 1997). Carbon allocation and plant productivity data also indicate that birch is more sensitive to O₃ at night than during the day (Matyssek et al. 1995). This is probably because the detoxification system requires photosynthesis to maintain antioxidants in a reduced (active) state (Esterbauer et al. 1980, Schupp and Rennenberg 1988, Wieser et al. 1996, Wildi and Lütz 1996). In addition, ontogenetic differences in O₃ sensitivity might relate to contrasting allocation patterns. Young trees...
typically have a high proportion of total biomass in foliage and thus a large surface area vulnerable to O₃ attack. On the other hand, the high proportion of non-green biomass in mature trees may result in enhanced maintenance respiration. Such age-dependent carbon demands may conflict with increases in respiration induced by O₃ defense (Matyssek and Innes 1999). Net photosynthesis declined with increasing age in our trees (data not shown), as has been observed in *Pinus contorta* var. *latifolia* Engelm. and *Pinus ponderosa* Laws. (Yoder et al. 1994), indicating that, compared with mature trees, young trees are more susceptible to O₃ stress because of their high metabolic activity (cf. Laurence et al. 1994). In the long term, chronic O₃ exposure may weaken stress tolerance because of the persistently enhanced energy costs for defense (Langebartels et al. 1997).

**Morphological, physiological and biochemical differences among trees of different age**

Tree responses to cumulative O₃ uptake and O₃ flux are linked with morphological (Fredericksen et al. 1995, Ferdinand et al. 2000) as well as physiological and biochemical traits of leaves (Taylor and Hanson 1992, Wellburn and Wellburn 1996). We found a significant effect of tree age on morphology of current-year needles. Seedlings had thinner needles than mature trees. In trees at the timberline, dry mass of 100-needle samples increased significantly with increasing tree age, whereas specific leaf area (SLA) declined with tree age (Figure 3). This trend was confirmed at low elevation (Kranzberg), where container-grown seedlings were placed in the sun crowns of mature trees. In the same light environment, needles of seedlings had lower mass, but higher SLA, than needles of mature trees (Figure 3). Needle differentiation during tree ontogeny resembled a change from shade-type toward a sun-type foliage, being consistent with findings by Perterer and Körner (1990) on sun and shade needles in Norway spruce, and may account for age-related differences in O₃ sensitivity. Thin leaves, but not thick leaves, of *Betula pendula* (Pääkkönen et al. 1995), *Betula pubescens* J. H. Ehrh. (Pääkkönen et al. 1997), *Fraxinus pennsylvanica* Marsh. and *Prunus serotina* J. F. Ehrh. (Bennet et al. 1992; but see Ferdinand et al. 2000) are O₃-sensitive, probably because of differences in the intercellular air space (Evans and Miller 1972, Pääkkönen et al. 1995, 1997) and in the diffusion pathway (Chappelka and Samuelson 1998) between thick and thin leaves. Data linking needle anatomy to gas diffusion for Norway spruce trees of different ages or light exposures are unavailable; however, we found no evidence that differences in morphology between sun and shade needles influenced detoxification capacity. There were no significant differences in mass-based concentrations of needle ascorbate among Norway spruce trees of different age (Figure 4A), and the redox state of the ascorbate pool (reduced ascorbate expressed as % of reduced ascorbate + dehydroascorbate) was about 70 ± 10% irrespective of tree age.

**Relating avoidance to tolerance of O₃ stress**

Injury results from an imbalance between O₃ uptake and detoxification (cf. Massman et al. 2000), the latter being determined by resource availability in the tree (Matyssek and Innes 1999). Although we did not measure detoxification rates directly in Norway spruce, we propose a measure that relates O₃ flux to the antioxidant pool in the needles. As a preliminary approach, we considered the area-based concentration of reduced needle ascorbate (*ASC*leaf) a central component in detoxification:

\[
ASC_{\text{leaf}} = \frac{ASC_{\text{dw}} \cdot SLA_{\text{leaf}}}{F_{\text{O}_3}}.
\]  

\[\text{(2)}\]

(ASC<sub>dw</sub> = mass-based concentration of ascorbate), because ASC<sub>leaf</sub> (Figure 4B), but not ASC<sub>dw</sub> (Figure 4A), increased with tree age. Use of needle surface area as the basis of expression of ascorbate concentration is appropriate because detoxification has to cope with the area-related flux density of O₃ uptake. Hence, the potentially available amount of reduced ascorbate in needles (AAASC, nmol ASC<sub>leaf</sub> nmol O₃<sup>–1</sup> s<sup>–1</sup>) per unit of O₃ flux into needles can be expressed as:

\[
AA_{\text{ASC}} = \frac{ASC_{\text{leaf}}}{F_{\text{O}_3}}.
\]  

\[\text{(3)}\]

This ratio may form an empirical basis for comparing age-related differences in tree response to O₃. Because significant effects on photosynthesis occurred above an O₃ flux threshold
of 2.45 nmol m\(^{-2}\) s\(^{-1}\) in both mature trees and seedlings (Figure 2B), the amount of reduced ascorbate in needles available under conditions of critical O\(_3\) uptake tended to increase with tree age (Figure 5). A similar trend was observed for glutathione (data not shown). These trends are consistent with higher O\(_3\) susceptibility in young trees than in mature trees. It is important to emphasize, however, that AA ASC cannot reflect O\(_3\) effects on the antioxidant pool. There is evidence that, in Norway spruce seedlings, ascorbate concentrations increase during continued O\(_3\) exposure and uptake (Kronfuß et al. 1998, Wieser et al. 1998). We determined that the apoplastic ascorbate pool, which is depleted rapidly in response to O\(_3\) (Polle 1998, Polle et al. 2000), was no better as an indicator of antioxidative capacity than the total ascorbate pool for two reasons. First, only 5–10% of apoplastic ascorbate interacted with the O\(_3\) taken up in Norway spruce needles (Polle et al. 1995). Second, it is known that ascorbate regeneration cannot occur in the apoplast, but requires reentry of dehydroascorbate into the cytoplasm where it is reduced through the ascorbate–glutathione cycle (Noctor and Foyer 1998). These studies together with the observation that O\(_3\) in plant cells promotes reactive oxygen species production, possibly depleting the detoxification systems in chloroplasts (Sakaki 1998), led us to conclude that use of the total leaf ascorbate pool to estimate overall detoxification capacity is justified. If Figure 5 reflects age-related control of tree responses to O\(_3\) through both stomata-dependent regulation of O\(_3\) influx (stress avoidance) and antioxidant pools (stress tolerance), then determination of area-based antioxidative capacity is as important as flux-related assessments of O\(_3\) doses in developing air quality standards.

In conclusion, antioxidative capacity may provide a convenient means of assessing age-related differences in O\(_3\) susceptibility. This age-dependent relationship now needs to be examined in mechanistic terms by direct experimental comparisons of antioxidative concentrations, leaf morphology, O\(_3\) uptake and physiological leaf responses across several ontogenetic stages. One approach may be to place seedlings in the canopy of older trees of the same genotype (Kolb and Matyssek 2001, Samuelson and Kelly 2001), thereby ensuring similar exposure. The “free air” O\(_3\) canopy exposure system (Karñosky et al. 2001) that has recently begun operation at the Kranzberg site can be used to validate our proposed approach (Häberle et al. 1999, Werner and Fabian 2001).

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