Effects of elevated ozone on growth and foliar traits of European and hybrid aspen

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We studied growth and foliar responses of two clones of both European aspen (*Populus tremula*) and hybrid aspen (*P. tremula × P. tremuloides*) to elevated O_3 (45 ppb, 14-h mean) over one growing season using a free-air fumigation system in central Finland. All clones exhibited O_3-specific foliar injury and accelerated leaf senescence under elevated O_3. Yet, exposure to $1.5 \times$ ambient O_3 had only minor effects on the growth and biomass production of clones grown under optimal nutrient and water supply, and no O_3 effects on leaf morphology were observed. Slower-growing European aspen was more sensitive to elevated O_3 than hybrid aspen. Exposure to O_3 decreased the root/stem ratio (–11%) and leaf N concentration (–9%) of European aspen. Inter- or intraspecific differences in the O_3 sensitivity of the trees could not be explained by stomatal conductance, but some xeromorphic leaf traits were related to increased susceptibility to O_3. Intraspecific differences in the O_3 sensitivity have implications e.g. for nurseries producing commercial tree material.

Introduction

Tropospheric ozone (O_3) is considered one of the most widespread and phytotoxic air pollutants, and is generally detrimental to forest growth (Percy *et al*. 2003). The tropospheric concentration of O_3 has more than doubled during the last century and is currently increasing at an annual rate of 0.5%–2%, mostly due to human activity (Vingarzan 2004, IPCC 2007). Fowler *et al*. (1999) estimated that by 2100, 50% of the world's temperate/subpolar forests will be exposed to O_3 levels that may damage vegetation. Indeed, simulations suggest that in future, warmer climate conditions, summer O_3 may pose a much more serious threat to agriculture and natural ecosystems, particularly in western and central Europe (Meleux *et al*. 2007). In Finland, the annual mean O_3 concentration is around 30 ppb (Laurila 1999), which is in the mid-range of the background levels of 20–45 ppb, usually present in the northern hemisphere (Vingarzan 2004). Regional emission scenarios also propose considerable increases in background O_3 con-

centrations in Finland up to about 2050 (Laurila *et al.* 2004). Although O_3 concentrations in Fennoscandia are lower than in central and southern Europe, the environmental conditions in Nordic countries, meaning long summer days, and the cooler and more humid climate compared to southern Europe, tend to promote stomatal conductance and subsequent O_3 uptake (Pleijel *et al.* 1999).

Tree species and genotypes differ in their sensitivity to O_3 and, in general, broadleaved trees are considered more susceptible to O_3 than conifers are (Reich 1987, Wittig *et al.* 2007). Species of the genus *Populus* are regarded as one of the most O_3 sensitive ones, based on the considerable amount of research conducted on North American trembling aspen (*P. tremuloides*) (Isebrands *et al.* 2001, Karnosky *et al.* 2005, King *et al.* 2005, Kubiske *et al.* 2007, Wittig *et al.* 2007) and various poplar species (Dickson *et al.* 1998, Pell *et al.* 1999, Bortier *et al.* 2000, Bussotti *et al.* 2007). A recent study (Percy *et al.* 2007) has shown that growth loss from ambient O_3 levels in large areas of the natural aspen range in North America could be in the range of 5%–30%. In contrast, few studies have addressed the sensitivity of European aspen (*P. tremula*) to O_3 (Matyssek *et al.* 1993, Häikiö *et al.* 2007).

European aspen, with its wide distribution range covering the whole of northern Eurasia, is a keystone species maintaining biodiversity and ecosystem function in boreal forest ecosystems. Various animal and fungus species are entirely dependent on aspen, and its calcium-rich leaf litter raises the pH of otherwise acidic soils of boreal forests, thus affecting soil-related biota and processes (Siitonen 1999, Suominen *et al.* 2003). Moreover, although previously overlooked in forestry in northern Europe, aspen has increased in economic value since the 1990s, especially through the interest of the paper industry in its production. Recently, species of *Populus* have also been considered as candidate species in the production of bioenergy (Karačić and Weih 2006).

In commercial tree plantations, hybrid aspen (*P. tremula* × *P. tremuloides*), a cross between European aspen and North American trembling aspen, is mainly utilized because of its faster growth rate and better fibre quality compared with native European aspen (Yu *et al.* 2001). Suvanto *et al.* (2004) demonstrated that gene flow between native European aspen and hybrid aspen is possible in nature as well. This may have implications for the success of native aspen and thereby affect the other species dependent on it, especially if hybrid aspen additionally shows a better tolerance to environmental stress. Since considerable genetic variation in the response to O_3 exists within trembling aspen (Karnosky *et al.* 2005, Kubiske *et al.* 2007), knowledge on the intraspecific differences in the sensitivity of hybrid aspen would be beneficial in choosing suitable genotypes for forest tree breeding programmes.

Exposure to elevated O_3 levels can cause visible leaf injuries and accelerated senescence in *Populus* species (Pell *et al.* 1999, Novak *et al.* 2007), as well as reductions in photosynthetic capacity and growth (Bortier *et al.* 2000, Isebrands *et al.* 2001, Noormets *et al.* 2001, King *et al.* 2005, Bussotti *et al.* 2007). O_3 can also affect the morphological and chemical traits of trees, such as specific leaf area, epicuticular waxes, nutrient concentrations and defensive metabolites (Karnosky *et al.* 2002, Percy *et al.* 2002, Holton *et al.* 2003, Bussotti *et al.* 2005), and this can lead to increased susceptibility to insect damage and disease. Changes in foliar morphology may be associated with acclimatisation to O_3-related stress (Bussotti *et al.* 2005).

So far, it is still unclear which factors determine the O_3 sensitivity of a species or of populations and individuals of a certain species. Trees with high growth rate, within and between species, appear in general to be more sensitive to O_3 than slow-growing ones (Skärby *et al.* 1998). This is hypothesized to result from the higher stomatal conductances and thus greater O_3 uptake of the faster-growing individuals (Reich 1987). However, various other physiological, anatomical, biochemical and environmental factors have been proposed to explain sensitivity differences (Pääkkönen *et al.* 1998, Chen and Gallie 2005).

In this paper, we report the results on growth and foliar responses of European and hybrid aspen clones exposed to elevated (1.5 × ambient) O_3 using free-air fumigation technology.

Our main hypothesis was that the faster-growing hybrid aspen would be more responsive to O_3 than native European aspen. In addition, we wanted to study if some foliar or growth traits could be associated with the possible inter- and intraspecific differences in the O_3 sensitivity of the two species and their clones.

Materials and methods

Plant material

In the experiment, we used two clones of both European aspen and hybrid aspen. The European aspen clones, R1 and R6, are of southern Finnish origin. Hybrid aspen clone 14 is a cross between *P. tremuloides* female, originally from Canada but grown in southern Sweden, and *P. tremula* male from southern Finland. Hybrid aspen clone 34 is a cross between *P. tremula* female of southern Finnish origin and *P. tremuloides* male of Canadian origin. The hybrid aspen clones have been in commercial use since 1999, but none of the clones had previously been tested for their O_3 sensitivity. The clones were propagated from root cuttings in late April 2006 at the Haapastensyrjä Breeding Station of the Finnish Forest Research Institute. The root cuttings of the European aspen clones were obtained from two-year old trees, while the age of the mother trees of the hybrid aspen clones 14 and 34 were 36 and 20 years, respectively, at the time of cloning. The cuttings were grown in a bottom-heated greenhouse (mean daily temperature 18.5 °C and relative humidity 90%) until the end of June 2006, when they were transplanted into small peat-filled pots and allowed to grow in a cooler greenhouse for a week. The cuttings were planted into 15-l pots (diam. 29 cm) containing a 2:1 mixture of fertilized white *Sphagnum* peat (N:P:K 16:10:20, Kekkilä horticultural peat, Finland) and sand, and transferred to the experimental field on 4–5 July 2006. At the beginning of the experiment, we measured five individuals of each clone for height, basal diameter, and number of leaves, and subsequently harvested them destructively to obtain the initial dry weight of stem, leaves, and roots.

Experimental design and ozone fumigation

The study was conducted using the free-air exposure facility located at the experimental field of the University of Kuopio (62°13´N, 27°13´E), central Finland, at 80 m above sea level. Complete details on experimental design and O_3 exposure have been published elsewhere (Karnosky *et al*. 2007). The site contained eight ring-shaped plots (diam. 10 m), with four plots each allocated to the ambient air treatment (control) and elevated O_3 treatment (referred to as elevated O_3). The potted trees were placed randomly into the plots to form a circular pattern in the centre, with the distance from the gas inlets being the same for each tree. The number of individuals per clone per treatment at the beginning of the experiment was: clone R1 20, clone R6 19, clone 14 18, and clone 34 16.

O_3 was generated from pure oxygen (Fisher OZ500 generator) and released into the elevated O_3 plots through vertical perforated tubes (Karnosky *et al*. 2007). Fumigation occurred 14 h per day (08:00 to 22:00), seven days a week, except during rain or at very high or low wind velocities. O_3 concentrations were continuously monitored at 2 m height in the centre of each plot with Dasibi 1008-RS ozone analyzers. The target O_3 exposure in the elevated O_3 treatment was twice that in the ambient air. The O_3 exposures were run from 6 July until 19 September 2006 (76 days) in this experiment. Ambient temperature, precipitation, relative humidity and photosynthetically active radiation (PAR) were measured continuously during the experiment at one location in the middle of the experimental area.

To avoid potential water stress and its interaction with O_3, we watered the trees with lake water daily when it was not raining. The trees were fertilized once a week with 0.5 l of 0.2% Taimi-Superex (N:P:K 19:4:20, Kekkilä, Finland) between 19 July and 16 August 2006. This resulted in a total dose of 139 kg N ha^{-1}, which corresponds to N fertilization used in Finnish forest nurseries for one-year old seedlings (Juntunen and Rikala 2001). To prevent potential block effects, we randomized the positions of the trees within plots each week throughout the experiment.

Growth measurements

We recorded the number of leaves (live and dead) and branches, and the height of trees (± 0.5 cm) weekly, and stem basal diameter (± 0.1 mm) every three weeks throughout the experiment. At the end of the experiment, we chose three individuals per clone per plot for a destructive harvest on 20–21 September 2006. The trees not included in the final harvest had been damaged by hares. We separated the trees into stems, leaves and roots, which were then dried (60 °C for at least 48 h) and weighed. Relative growth rate (RGR) was calculated for each clone using initial (W_1) and final (W_2) total dry weights as follows:

$$RGR = (\ln W_2 - \ln W_1)/\text{number of experimental days} \quad (1)$$

The trees not included in the final harvest were left to overwinter with their pots buried in the ground. In early June 2007, we observed the degree of freezing damage on these trees.

Light-saturated photosynthesis and stomatal conductance

We measured stomatal conductance (g_s) on two randomly chosen individuals per clone per plot using a portable infrared gas analyzer (LCA-3, ADC Ltd., Hoddesdon, UK). Measurements were carried out every time from the same leaf (leaf plastochron index, LPI 4 at the time of the first measurement). Each plot was measured four times a day (08:00–09:30, 11:30–13:00, 15:00–16:30 and 18:30–20:00) between 9 and 12 August and again on 15 August. In addition, we determined light-saturated photosynthesis (P_s) and g_s from the same leaves on 23 August between 10:00 and 15:00 h using a portable photosynthesis system (Li-6400, Li-Cor, Lincoln, NE, USA) using the irradiance of 1200 μmol m^{-2} s^{-1}.

Leaf injuries, morphology and chemistry

Visible foliar injuries were recorded weekly throughout the study. O_3-related injuries were manifested as necrotic spots and earlier senescence, appearing as yellow leaves (Brace *et al.* 1999). In addition, we monitored the occurrence of foliar *Melampsora* rust fungus infection. The proportion of injured leaves per tree was scored for each injury type separately.

On 1 September, we collected three green leaves (LPI 7, 8, 10) from four individuals per clone per plot for the determination of specific leaf area (SLA). The leaves were scanned for the determination of leaf area with ASSESS (Image Analysis Software for Plant Disease Quantification), after which they were dried (60 °C for 48 h) and weighed. On 18 September, we collected one leaf (LPI 9) from the same four individuals per clone per plot to form a pooled sample for analyses of foliar epicuticular wax characteristics. The leaves were stored at –20 °C until the analyses. Epicuticular waxes were extracted by rinsing the leaves with CHCl$_3$ using a glass syringe. Solvent/wax solution was filtered, solvent evaporated, and epicuticular waxes weighed to ± 10 μg and expressed as μg cm^{-2} leaf area. We determined the quantitative wax chemical composition (± 0.001%) using a high-temperature capillary Varian 3410 gas chromatograph equipped with an FID (Percy *et al.* 1994). Varian Workstar software programming was used to integrate peak areas and calculate homologue percentages. We completed the final confirmation of homologue assignments using a Hewlett-Packard 5989 GC-MS.

We determined the carbon (C) and nitrogen (N) concentrations of green leaves collected on 1 September, and of leaves and roots from the final harvest on 20–21 September. From 12 September on until the end of the experiment, we collected leaf litter twice a week from the pots of all trees. The litter material from different dates was pooled, dried at 60 °C for 48 h, and analyzed for C and N concentrations. For all C and N measurements we used pooled samples from each clone per plot. All analyses were made from dried and milled samples using high-temperature combustion (Vario MAX CN analyzer, Elementar Analysesysteme GmbH, Germany). Finally, we determined the nitrogen resorption efficiency (NRE) of each clone using N concentrations of green (collected on 1 September, N_g) and senescent (litter, N_s) leaves as follows:

$$\text{NRE} = (N_g - N_s)/N_g \qquad (2)$$

Soil analyses

In addition to plant measurements, we collected soil samples at the onset of the experiment from the initial planting soil and again after the harvest in late September 2006 from the pots of the harvested trees. We pooled the latter subsamples to form a composite soil sample from each clone per plot. The samples were stored at –20 °C until chemical analyses. We extracted the soils samples with deionised water (v:v, 1:2.5) and measured the pH and conductivity with an electrochemical analyzer (C933, Consort, Belgium). The rest of the soil sample was dried (72 h at 60 °C), sieved (diam. 2 mm), milled, and analyzed for total C and N concentration by combusting (Vario MAX CN analyzer).

Statistical analyses

We used two-way analysis of variance (ANOVA) to test the main effects and interactions of O_3, species and clone on measured variables. To test the effects of elevated O_3 treatment on individual clones, we used the *t*-test, or when needed, the non-parametric Mann-Whitney *U*-test. In the case of time-repeated measurements, we used repeated measures ANOVA to evaluate differences between treatments. Pearson's or Spearman's correlation test was used to examine the relationships between various variables. We considered the results statistically significant at $p < 0.05$. All statistical analyses were performed with SPSS 15.0 for Windows.

Results

Climate, O_3 exposure data and soil chemistry

July and August 2006 were warmer (0.4 °C and 3.1 °C, respectively) and exceptionally dry (about half of the normal average precipitation) compared with the long-term average (years 1971–2000) measured in Kuopio (Finnish Meteorological Institute 2002) (Table 1). September was also slightly warmer than the long-term average but with normal precipitation. The actual O_3 enrichment averaged 1.5 × the ambient O_3, with the 14-h mean O_3 concentrations being 45 ppb (elevated O_3) and 29 ppb (control) (Table 2). The highest O_3 concentrations (121 ppb) were experienced at the beginning of the experiment. Higher O_3 concentrations (70–90 ppb) occurred also in early and late August and in mid-September (Fig. 1). The AOT40 value in the elevated O_3 treatment (10.2 ppm h) was almost ten-fold compared with the control treatment (1.1 ppm h) (Table 2).

At the end of the experiment, the soil total N concentration (averaged per clone per plot) ranged between 0.05% and 0.09% and the C concentration between 3.03% and 4.15%. The pH ranged from 5.49 to 5.61 and the electrical conductivity from 20.3 to 23.9 μS cm^{-1}. No statistically significant differences between the treatments were detected in the measured soil variables (data not shown).

Growth and biomass production

Elevated O_3 did not have an effect on height or diameter growth, biomass production or RGR

Table 1. Mean temperature, precipitation, and mean relative humidity at the experimental field from 6 July to 19 September 2006. Temperature and relative humidity values are calculated from 24 h daily mean values.

	July	August	September
Temperature (°C)	17.1	17.1	12.4
Precipitation (mm)	35.8	39.2	43.2
Relative humidity (%)	68.0	76.5	87.8

Table 2. Ozone exposure in the control and elevated O_3 treatments between 6 July and 19 September 2006.

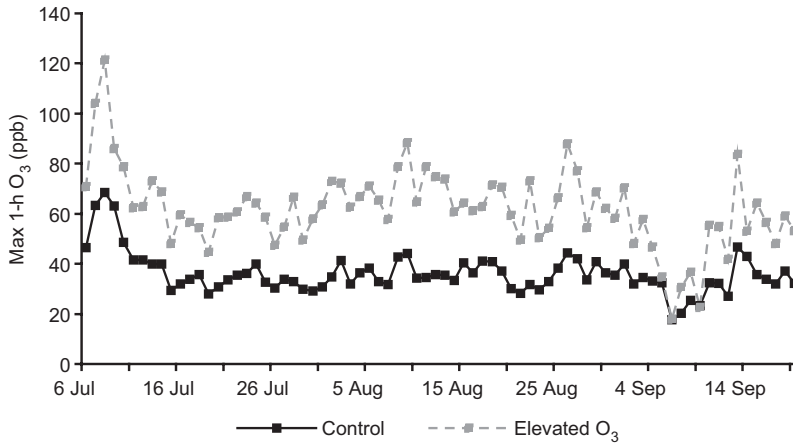|	Control	Elevated O_3
14-h mean (ppb)	29	45
24-h mean (ppb)	27	36
1-h maximum (ppb)	68	121
AOT0 (ppm h)	48.8	65.0
AOT40 (ppm h)	1.1	10.2

Fig. 1. Daily maximum 1-hour O_3 concentrations during the experiment.

in either species (Table 3). At the clone level, however, elevated O_3 tended to enhance slightly (+10%) the height growth of European aspen clone R1 (Table 4).

Although the biomass production of different parts of trees was not affected by O_3 exposure, elevated O_3 decreased the root/stem biomass ratio of the trees by 2% to 14% (Tables 3 and 4). However, at the species level elevated O_3 tended ($p = 0.076$) to decrease the root/stem ratio only in European aspen. When clones were tested separately, no effect of O_3 was noted.

The two species differed from each other in the measured growth variables, except in the case of root biomass. Hybrid aspen was superior in height and diameter growth as well as in total dry weight production and RGR compared with native European aspen in both treatments. Compared with hybrid aspen, these attributes were 12% to 29% lower in European aspen. On the other hand, hybrid aspen had considerably lower (–11%) root/shoot and root/stem ratios than European aspen. Within hybrid aspen, clone 14 produced more biomass than clone 34, while within European aspen clone R6 was faster-growing than clone R1 in terms of diameter growth and RGR.

Light-saturated photosynthesis and stomatal conductance

Based on the gas exchange measurements conducted with photosynthesis system (Li-6400)

Table 3. Multivariate ANOVA results (*p* values) for the main effects and interactions of O_3, species and clone on growth variables.

	Height growth	Diameter growth	Leaf biomass	Stem biomass	Root biomass	Total biomass	RGR	Root/ shoot ratio	Root/ stem ratio
All clones									
O_3	0.448	0.593	0.750	0.559	0.715	0.855	0.866	0.053	0.014
Species	< 0.001	< 0.001	0.001	0.002	0.423	0.012	< 0.001	< 0.001	< 0.001
$O_3 \times$ species	0.856	0.812	0.945	0.805	0.723	0.795	0.994	0.652	0.386
European aspen									
O_3	0.370	0.704	0.655	0.458	0.993	0.683	0.820	0.118	0.076
Clone	0.339	< 0.001	0.382	0.918	0.757	0.770	< 0.001	0.944	0.606
$O_3 \times$ clone	0.494	0.298	0.513	0.653	0.420	0.494	0.449	0.455	0.539
Hybrid aspen									
O_3	0.729	0.909	0.889	0.821	0.609	0.960	0.851	0.317	0.116
Clone	0.080	0.244	0.200	0.025	0.017	0.032	0.092	0.252	0.524
$O_3 \times$ clone	0.855	0.569	0.378	0.631	0.387	0.434	0.474	0.439	0.267

and when all clones were pooled, elevated O_3 tended to increase both P_s ($p = 0.062$) and g_s ($p = 0.054$) (Tables 5 and 6). The increases within clones ranged between 4% and 15% for P_s and 6% and 21% for g_s. When species were analysed separately, a significant stimulatory effect ($p = 0.048$) of O_3 on g_s, and to lesser extent on P_s ($p = 0.090$), was detected only in European aspen. Within European aspen, elevated O_3 tended to increase P_s in clone R1 and g_s in clone R6.

The g_s measurements carried out at different periods during a day (with LCA-3) revealed that the O_3-related increase in g_s only occurred around the noon ($p = 0.020$, repeated measures ANOVA, all clones pooled). Again, when examined at the species level, elevated O_3 tended to enhance g_s only in European aspen ($p = 0.098$). Species and clones did not differ in g_s rates at any times of day.

Specific leaf area and epicuticular waxes

Elevated O_3 did not affect SLA or the amount of leaf epicuticular waxes (Table 5), but some inter- and intraspecific differences were recorded in these morphological traits. Hybrid aspen had a higher (+18%) SLA than European aspen

(Table 6). Although the species did not differ in the amount of epicuticular waxes, both species exhibited intraspecific variation, with clone R1 having more waxes (+45%) than clone R6, and clone 14 more (+27%) than clone 34.

Of the three major epicuticular wax classes (alkyl esters, fatty acids and alkanes) recovered from leaves, alkyl esters in all clones comprised 52%–72% of the epicuticular wax deposit (Table 6). The proportion of fatty acids was similar between species (10%–19%), but the proportion of alkanes as well as alkane:fatty acid ratio ($p = 0.007$) were significantly higher in European aspen than hybrid aspen (Table 5). Elevated O_3 had only a marginal effect on the chemical composition of epicuticular waxes. When all clones were pooled, exposure to elevated O_3 tended to ($p = 0.079$) decrease the proportion of alkanes. At the species level, elevated O_3 tended to increase the proportion of alkyl esters and decrease the proportion of alkanes in European aspen, while the wax chemical composition of hybrid aspen remained unaffected under elevated O_3. However, European aspen exhibited considerable intraspecific variation in wax composition, and when clones were analysed separately, no effect of O_3 on wax chemical composition was noted.

Table 4. Growth variables measured in late September 2006 (mean ± SD, $n = 4$). Statistically significant differences ($p < 0.05$) among all the clones irrespective of treatment are indicated with different letters (ANOVA followed by Tukey's test). Asterisk (*) indicates differences between the treatments within each clone at $p < 0.1$ (*t*-test).

Variable	Treatment	European aspen		Hybrid aspen	
		Clone R1	Clone R6	Clone 14	Clone 34
Height growth (cm)	Control	81.2 ± 4.4a	89.9 ± 14.8a	126.7 ± 16.9b	109.7 ± 6.2b
	Elevated O3	89.5 ± 6.3*	91.0 ± 11.5	128.1 ± 17.9	114.1 ± 20.2
Diameter growth (mm)	Control	6.9 ± 0.7a	9.1 ± 0.2b	11.4 ± 1.6c	11.1 ± 0.8c
	Elevated O_3	7.4 ± 0.7	8.9 ± 0.8	11.7 ± 0.9	10.7 ± 0.7
Leaf biomass (g)	Control	14.1 ± 2.0a	13.8 ± 1.9a	22.0 ± 3.0b	16.6 ± 3.9ab
	Elevated O_3	15.1 ± 0.9	13.5 ± 3.6	20.2 ± 2.3	19.1 ± 7.9
Stem biomass (g)	Control	20.6 ± 4.5a	22.0 ± 4.2a	34.1 ± 7.1b	24.5 ± 6.6a
	Elevated O_3	23.5 ± 3.1	22.7 ± 6.4	33.3 ± 3.9	26.8 ± 7.3
Root biomass (g)	Control	24.6 ± 4.5ab	25.9 ± 3.3ab	33.4 ± 6.8b	22.3 ± 6.4a
	Elevated O_3	26.7 ± 3.4	23.8 ± 7.6	29.1 ± 3.9	23.4 ± 6.5
Total biomass (g)	Control	59.3 ± 10.7a	61.7 ± 8.7a	89.5 ± 16.7b	63.4 ± 16.7a
	Elevated O_3	65.8 ± 7.3	60.0 ± 17.1	82.6 ± 9.0	69.5 ± 20.2
RGR (% day^{-1})	Control	0.058 ± 0.002a	0.071 ± 0.002b	0.072 ± 0.003bc	0.074 ± 0.003c
	Elevated O_3	0.060 ± 0.001	0.070 ± 0.005	0.072 ± 0.002	0.076 ± 0.004
Root/stem ratio	Control	1.22 ± 0.08a	1.23 ± 0.18a	0.98 ± 0.03b	0.91 ± 0.05b
	Elevated O_3	1.13 ± 0.11	1.06 ± 0.14	0.88 ± 0.10	0.89 ± 0.09

Leaf C and N

The average leaf N concentration values ranged between 2.9% and 3.7% at the end of the experiment. The leaf N concentration was lower (2%–12%) and C:N ratio higher in trees in the elevated O_3 treatment compared with the ones in the control (Table 5, Fig. 2a and b), whereas leaf C concentrations did not differ between treatments (data not shown). At the species level, the O_3 effect on leaf N concentration and C:N ratio was statistically significant ($p = 0.010$ and $p = 0.007$, respectively) in European aspen, but not significant ($p = 0.073$ and $p = 0.063$, respectively) in hybrid aspen. Although the species did not differ from each other in the foliar N and C traits, both species exhibited intraspecific variation in these characteristics. When clones were analysed

Table 5. Multivariate ANOVA results (p values) for the main effects and interactions of O_3, species and clone on light-saturated photosynthesis (P_s), stomatal conductance (g_s), leaf morphology, wax chemistry, leaf N and C concentrations and/or ratios, and nitrogen resorption efficiency (NRE).

	P_s	g_s	SLA	Wax amount/ leaf area	Wax alkyl esters	Wax fatty acids	Wax alkanes	Leaf N	Leaf C:N	Litter C:N	NRE
All clones											
O_3	0.062	0.054	0.894	0.641	0.111	0.589	0.079	0.023	0.012	0.372	0.324
Species	0.795	0.058	< 0.001	0.162	0.562	0.709	0.018	0.813	0.126	0.003	0.020
$O_3 \times$ species	0.648	0.648	0.355	0.470	0.216	0.287	0.494	0.882	0.791	0.280	0.239
European aspen											
O_3	0.090	0.048	0.516	0.405	0.067	0.274	0.063	0.010	0.007	0.078	0.117
Clone	0.088	0.025	0.927	0.026	0.040	0.021	0.061	0.009	0.016	0.717	0.273
$O_3 \times$ clone	0.388	0.347	0.452	0.311	0.497	0.844	0.210	0.626	0.813	0.517	0.355
Hybrid aspen											
O_3	0.349	0.361	0.623	0.893	0.766	0.642	0.527	0.073	0.063	0.875	0.886
Clone	0.717	0.747	0.052	0.009	0.854	0.342	0.105	0.002	0.002	0.023	0.058
$O_3 \times$ clone	0.956	0.795	0.411	0.496	0.996	0.402	0.866	0.162	0.192	0.649	0.383

Table 6. Light-saturated photosynthesis (P_s), stomatal conductance (g_s), leaf morphology, wax chemistry, and nitrogen resorption efficiency (NRE) of the clones (mean ± SD, $n = 4$). Statistically significant differences ($p < 0.05$) among all the clones irrespective of treatment are indicated with different letters (ANOVA followed by Tukey's test). Asterisk indicates differences between the treatments within each clone at $p < 0.1$ (t-test).

Variable	Treatment	European aspen		Hybrid aspen	
		Clone R1	Clone R6	Clone 14	Clone 34
P_s (µmol m^{-2} g^{-1})	Control	18.3 ± 2.7a	21.1 ± 1.5a	20.0 ± 4.1a	19.7 ± 1.6a
	Elevated O_3	21.1 ± 0.7*	22.0 ± 2.5	21.2 ± 1.2	20.7 ± 1.5
g_s (mol m^{-2} g^{-1})	Control	0.63 ± 0.11a	0.71 ± 0.10a	0.59 ± 0.21a	0.63 ± 0.10a
	Elevated O_3	0.69 ± 0.10	0.86 ± 0.08*	0.67 ± 0.15	0.67 ± 0.05
SLA (cm^2 g^{-1})	Control	181.9 ± 17.5a	173.8 ± 5.4a	222.5 ± 12.6b	210.4 ± 20.7ab
	Elevated O_3	180.9 ± 18.6	187.3 ± 22.9	225.7 ± 14.8	197.9 ± 21.6
Wax amount (µg leaf cm^{-2})	Control	19.9 ± 4.8a	16.2 ± 7.9b	21.3 ± 1.9a	17.7 ± 3.5ab
	Elevated O_3	20.3 ± 1.2	11.4 ± 3.1	22.6 ± 3.9	16.9 ± 0.5
Wax alkyl esters (%)	Control	66.2 ± 7.5a	52.4 ± 11.5a	65.6 ± 6.0a	64.8 ± 6.8a
	Elevated O_3	72.2 ± 6.9	64.9 ± 10.0	66.7 ± 6.4	66.0 ± 11.4
Wax fatty acids (%)	Control	13.7 ± 4.9a	19.4 ± 3.3b	15.5 ± 4.4ab	15.3 ± 1.5ab
	Elevated O_3	10.5 ± 2.8	17.2 ± 6.8	18.0 ± 4.2	14.5 ± 4.1
Wax alkanes (%)	Control	11.9 ± 1.0ab	19.2 ± 7.3b	8.5 ± 1.9a	11.7 ± 5.0ab
	Elevated O_3	10.3 ± 3.3	11.9 ± 3.1	6.8 ± 3.8	10.7 ± 5.0
NRE (%)	Control	36.2 ± 21.6a	50.2 ± 4.1ab	66.0 ± 17.2b	57.8 ± 10.8ab
	Elevated O_3	53.8 ± 11.9	55.1 ± 8.9	71.2 ± 6.0	50.5 ± 17.7

separately, the effects of elevated O_3 were discovered as significant ($p = 0.042$ and $p = 0.030$) in clone R1, but not significant ($p = 0.089$ and $p = 0.083$) in clone R6. No O_3-related changes in the C and N concentrations of roots were noted (data not shown).

European aspen also tended ($p = 0.078$) to produce leaf litter with higher (+8%) C:N ratio under elevated O_3 than in the ambient air (Table 5 and Fig. 2c). However, NREs of neither of the species were affected by elevated O_3 (Tables 5 and 6). Averaged over treatments, hybrid aspen exhibited higher litter C:N ratio (+50%) and NRE compared with European aspen. Within hybrid aspen, clone 14 produced litter with a considerably higher (+65%) C:N ratio and tended to have higher NRE than clone 34, whereas European aspen clones were similar to each other in litter quality and NRE. When clones were studied separately, no significant effect of elevated O_3 treatment on litter C:N ratio was detected.

Foliar senescence, visible injuries and rust infection

Both species exhibited accelerated foliar yellowing under elevated O_3 (Table 7, significant $O_3 \times$ time interaction). Elevated O_3 increased the proportion of yellow leaves in both clones of European aspen, but markedly more so in clone R6 (significant $O_3 \times$ clone interaction) (Fig. 3a). In the case of hybrid aspen, the proportion of yellow leaves increased significantly in clone 34, but only tended ($p = 0.065$) to increase in clone 14 under elevated O_3 (Fig. 3b).

Visible leaf injuries specific to O_3 (bifacial necrotic lesions) occurred in all clones in the elevated O_3 treatment, but to a minor extent also in the control treatment in clones R1 and 14 (Figs 4 and 5). The severity of injury was in general low, the proportion of injured leaves ranging from 2% to 11% and from 0% to 5% in the elevated O_3 and control treatment, respectively. From all the trees, about half of the individuals of clone R1, about 25% of clones R6 and 14, and 9% of clone 34 exhibited foliar injury. At the species level, a significant O_3 effect on the occurrence of visible injury was detected only in European aspen, which had more visible O_3 injury than
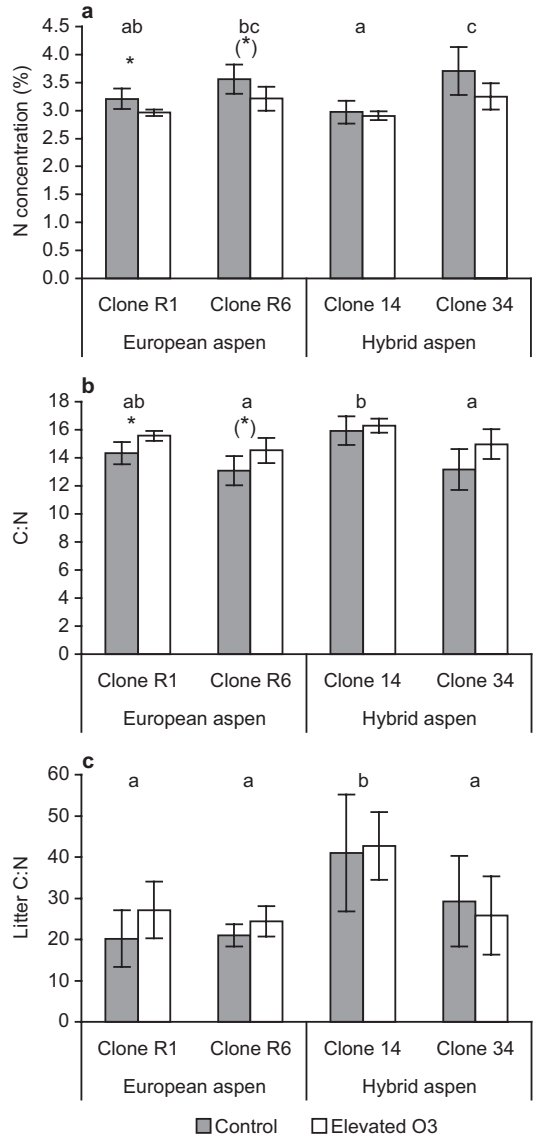

Fig. 2. Leaf (**a**) N concentration and (**b**) C:N ratio of harvested trees in late September 2006, and (**c**) leaf litter C:N ratio of the clones in the two treatments (mean ± SD, $n = 4$). Significant differences ($p < 0.05$) among all the clones irrespective of treatment are indicated by different letters (ANOVA followed by Tukey's test). Differences between the treatments within each clone are indicated as (*) = $p < 0.1$, * = $p < 0.05$ (*t*-test).

hybrid aspen (Table 7). Within European aspen, the clones differed from each other, and significant differences in the occurrence of visible injuries between the treatments were found only in clone R6. However, the overall proportion of

injured leaves was largest in clone R1. No significant intraspecific variation occurred between the hybrid aspen clones.

In late July 2006, a rust fungus infection (*Melampsora* sp.) was detected on the trees (Fig. 6). By mid-September, all trees except five individuals were infected, and a significant ($p < 0.001$) difference in rust occurrence between the treatments was apparent at the species level (Table 7). The rust was twice as abundant in the control treatment as in the elevated O_3 treatment (Fig. 7). At the clone level, significant differences in the rust occurrence between the treatments were detected only in both clones of European aspen, which were more susceptible to the rust infection than hybrid aspen clones.

The trees manifested very little overwintering damage, and no difference in the amount of freezing injury was detected between treatments, species or clones (data not shown).

Relationships among studied variables

At the treatment level, the extent of visible foliar O_3 injury correlated positively with the proportion of yellow leaves ($p = 0.001$) and negatively with RGR ($p = 0.013$), whereas only positive and negative trends were noted in the case of g_s ($p = 0.082$) and leaf N concentration ($p = 0.064$), respectively. Within European aspen, foliar O_3 injury correlated positively with the biomass of the above-ground parts and total biomass of the trees, as well as with the proportion of yellow leaves, whereas negative correlation was found between O_3 injury and leaf N concentration (Table 8). However, within hybrid aspen no correlations between O_3 injury and other variables were observed.

The severity of *Melampsora* rust infection was strongly correlated with biomass measures, being negatively correlated with P_s, RGR, SLA and the biomass of the above-ground parts and total biomass of the trees (Table 8). The abundance of rust infection tended to increase with increasing proportion of alkanes and alkane:fatty acid ratio in epicuticular waxes ($p = 0.061$ and $p = 0.063$, respectively).

Stomatal conductance was strongly and positively coupled with P_s ($p = 0.002$), but neither

Table 7. Repeated measures ANOVA results (*p* values) for the main effects and interactions of O_3, species, clone and time on the proportion of yellow leaves, and multivariate ANOVA results for the main effects and interactions of O_3, species and clone on visible O_3 injury and *Melampsora* rust occurrence in mid-September.

|		Yellow leaves	O_3 injury	Rust occurrence
All clones	O_3	< 0.001	0.029	< 0.001
	Species	0.122	0.030	< 0.001
	$O_3 \times$ species	0.793	0.270	0.712
	Time	< 0.001		
	Time $\times O_3$	< 0.001		
	Time $\times$ species	0.003		
	Time $\times O_3 \times$ species	0.828		
European aspen	O_3	< 0.001	0.049	0.001
	Clone	0.933	0.032	0.322
	$O_3 \times$ clone	0.007	0.767	0.958
	Time	< 0.001		
	Time $\times O_3$	< 0.001		
	Time $\times$ clone	0.110		
	Time $\times O_3 \times$ clone	0.010		
Hybrid aspen	O_3	< 0.001	0.249	0.034
	Clone	0.039	0.387	0.806
	$O_3 \times$ clone	0.242	0.898	0.997
	Time	< 0.001		
	Time $\times O_3$	0.006		
	Time $\times$ clone	0.867		
	Time $\times O_3 \times$ clone	0.235		
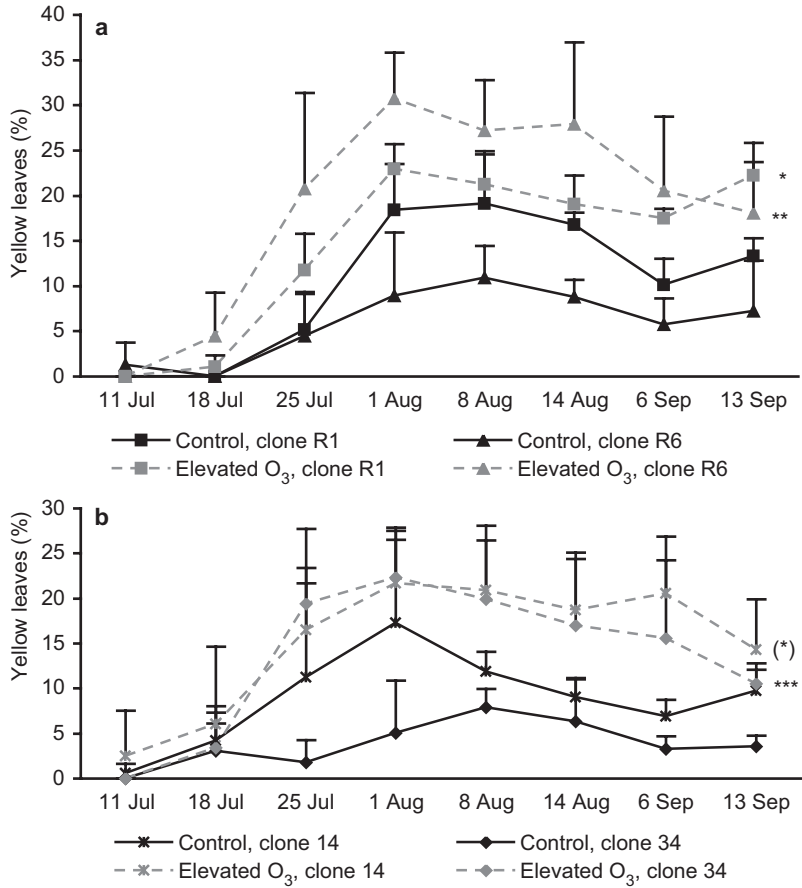
Fig. 3. Proportion of yellow leaves in (**a**) European and (**b**) hybrid aspen clones in the two treatments during the experiment (mean + SD, n = 4). Differences between the treatments within each clone are indicated as (*) = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (repeated measures ANOVA).

Table 8. Correlation coefficients (Pearson's or Spearman's correlation test) for the relationships between the proportion of leaves with O_3 injury or *Melampsora* rust infection and other growth and foliar variables at the end of the experiment, unless stated otherwise. Significant correlations are indicated as * = $p < 0.05$, ** = $p < 0.01$.

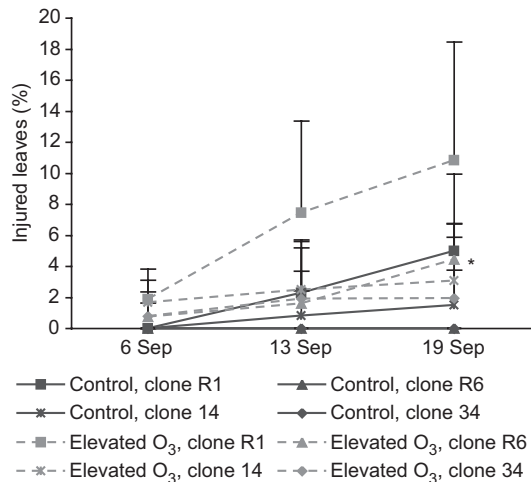|	O_3 injury		*Melampsora*
	European aspen	Hybrid aspen	All clones pooled
Yellow leaves	0.504*	0.365	–0.142
P_s (22 Aug)	0.267	0.255	–0.360*
g_s (22 Aug)	0.075	0.237	–0.094
RGR	–0.176	–0.315	–0.379*
Total biomass	0.588*	0.025	–0.386*
Leaf biomass	0.556*	0.139	–0.402*
Stem biomass	0.542*	0.045	–0.468**
Root biomass	0.458	0.043	–0.201
SLA (1 Sep)	0.070	0.323	–0.561**
Leaf N (1 Sep)	–0.572*	–0.236	0.011
Wax amount	0.165	0.259	–0.022

Fig. 4. Development of visible foliar O_3 injuries in the two treatments during the experimental period (mean + SD, n = 4). Significant differences between the treatments within each clone are indicated as * = $p < 0.05$ (repeated measures ANOVA).
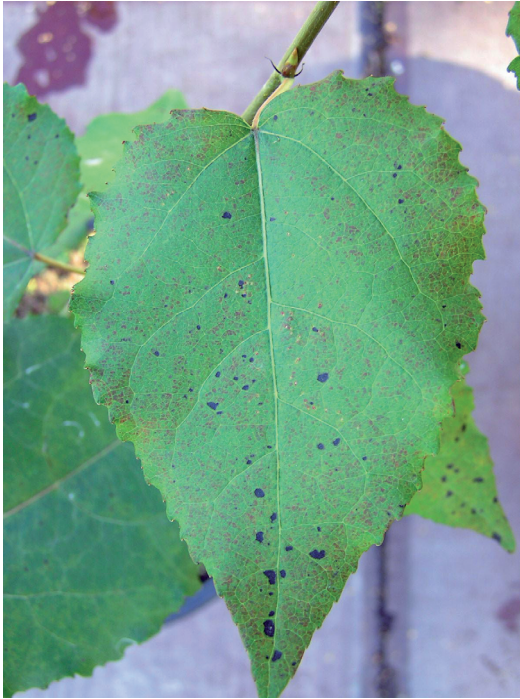
Fig. 5. O_3-induced bifacial necrotic lesions on the leaf of European aspen clone R1 in the elevated O_3 treatment.
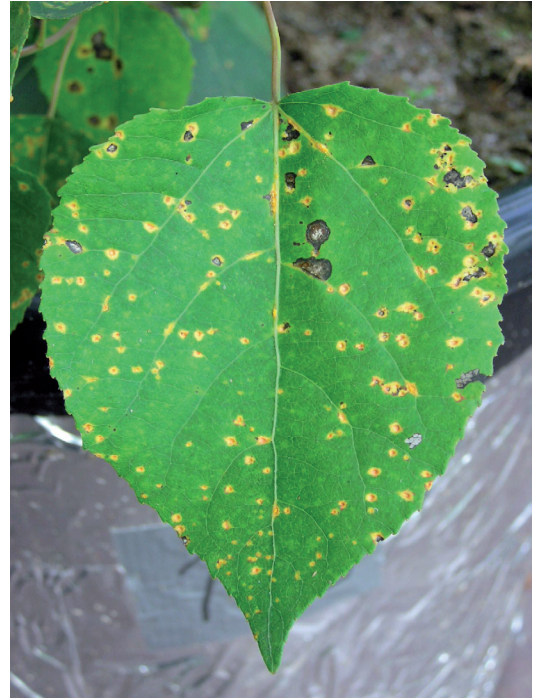

Fig. 6. *Melampsora* rust fungus infection on the leaf of hybrid aspen clone 34 in the control treatment.

of them correlated with RGR, total biomass or SLA ($p > 0.1$). The latter three variables were positively correlated with each other ($p < 0.05$), while total biomass correlated negatively with

Fig. 7. Proportion of leaves infected by *Melampsora* rust fungus in the two treatments in mid-September (mean ± SD, $n = 4$). Significant differences ($p < 0.05$) among all the clones irrespective of treatment are indicated by different letters (ANOVA followed by Tukey's test). Significant differences between the treatments within each clone are indicated as * = $p < 0.05$ (*t*-test).

leaf N concentration ($p = 0.001$). In addition, wax amount showed negative correlations with g_s ($p = 0.001$) and leaf N concentration ($p = 0.031$), and RGR with the proportion of yellow leaves ($p = 0.003$).

Discussion

Visible O_3 injuries and leaf senescence

By the end of the experiment, all clones exhibited O_3-specific foliar injury in the form of bifacial necrotic lesions, a typical symptom of O_3 injury on aspen leaves (Brace *et al*. 1999). Although the severity of injury was low, it is noteworthy that European aspen clone R1 and hybrid aspen clone 14 developed visible injuries also in the control treatment, indicating sensitivity even to ambient O_3 concentrations (24-h mean concentration of the whole experimental season 27 ppb). O_3-specific leaf injuries on vegetation are rare in the ambient O_3 concentrations prevailing in Fennoscandia (Timonen *et al*. 2004). The ambient O_3

levels in central Europe are higher, however, and black poplar (*Populus nigra*), a close relative of aspen, is one of the most sensitive tree species to ambient O_3 concentrations with regard to visible injuries (Novak *et al*. 2003). Visible foliar O_3 injuries are often acute responses that result from exposure to short-term, high O_3 peaks, whereas effects on growth are mediated through chronic exposure to lower O_3 concentrations (Krupa and Manning 1988). In the present experiment, high O_3 hourly concentrations (Fig. 1) likely contributed to the onset of visible injuries. In addition, the occurrence of visible injuries was likely promoted by higher than average ambient O_3 exposure. The AOT40 values measured in the summer 2006 ranged from 5.8 to 8.5 ppm h in southern and central Finland, and were 55%–85% higher than the long-term average (years 2002–2006) (Finnish Meteorological Institute unpubl. data). Due to regular irrigation, the growing conditions of our trees favoured stomatal opening, and thus O_3 flux, more than natural conditions would have done, which may have promoted the emergence of visible injuries (cf. Schaub *et al*. 2003).

Accelerated leaf senescence is a widely reported phenomenon under exposure to elevated O_3 (e.g. Pell *et al*. 1999, Ribas *et al*. 2005, Novak *et al*. 2007), and was also previously observed in trembling aspen (Karnosky *et al*. 2005) and European aspen (Matyssek *et al*. 1993). In our experiment, accelerated leaf senescence was detected in both species, manifested as O_3-related enhanced foliar yellowing. The senescence process involves degradation of chlorophyll and Rubisco, and can be characterized by reductions in foliar N levels (Yamaji *et al*. 2003, Ribas *et al*. 2005), as we also found. A high proportion of yellow leaves was associated with increased leaf O_3 injury. Within both species, the effect of elevated O_3 on senescence was more pronounced on the clones that showed no visible O_3 injuries at ambient O_3 levels (clones R6 and 34), compared with that on the other two clones. On the other hand, the clones that exhibited visible O_3 injuries at ambient O_3 concentrations (clones R1 and 14) produced significantly more yellow leaves in the ambient air control treatment than the other two clones, suggesting that ambient O_3 concentrations may also have affected the level of yellowing in the control trees of these clones.

Photosynthesis, growth and biomass allocation

The g_s rates of the clones in this experiment (ca. 0.64 mol m^{-2} s^{-1}) were similar to those measured in hybrid aspen of the same age (Oksanen *et al*. 2001), but relatively high as compared with those for somewhat older trees of different *Populus* species (g_s values range between 0.14 and 0.50 mol m^{-2} s^{-1}) (Matyssek *et al*. 1993, Noormets *et al*. 2001, Yu 2001, Orendovici-Best *et al*. 2008). The rates of P_s were also slightly higher than usually recorded in European or hybrid aspen (8–20 μmol m^{-2} s^{-1}) (Matyssek *et al*. 1993, Oksanen *et al*. 2001, Yu 2001, Hermle *et al*. 2007), which may be attributed to the regular N fertilization and a good water status.

In contrast to a recent review (Wittig *et al*. 2007), we detected a slight increase in the P_s and g_s of European aspen under O_3 exposure. This stimulatory effect of O_3 may indicate compensatory reactions for stress, as suggested by some previous studies. Greitner *et al*. (1994) demonstrated that the photosynthetic capacity of young leaves of trembling aspen increases under O_3 exposure as a response to the decrease in the photosynthetic capacity of old leaves, and Brendley and Pell (1998) found a similar compensatory mechanism with the quantity and synthesis of Rubisco in hybrid poplar (*Populus maximowizii × trichocarpa*) leaves when exposed to O_3. With silver birch (*Betula pendula*), Oksanen and Saleem (1999) reported that an O_3-tolerant genotype compensated for stress caused by low O_3 concentrations by enhanced g_s.

In the present study, the stimulation of gas exchange in the elevated O_3 treatment translated into growth enhancement only in the European aspen clone R1. The slight increase in the height growth of clone R1 under elevated O_3 may be linked to the subtle O_3-related increase in the P_s of this clone. Similar stimulatory effects of relatively low concentrations of O_3 on growth were previously reported on some Finnish hybrid aspen (Oksanen *et al*. 2001) and trembling aspen (Karnosky *et al*. 1996) clones, as well as on silver birch clones (Yamaji *et al*. 2003). However, under prolonged O_3 exposure, the initial response of growth enhancement may disappear (Pääkkönen *et al*. 1993), and be followed by

cumulatively impaired growth, as reported in silver birch (Oksanen 2003).

The growth of none of the clones, except European aspen clone R1, was affected by elevated O_3, even though the AOT40 value was two-fold as compared with the critical level of O_3 for forest trees (AOT40 5 ppm h) (ICP 2004). Several studies reported previously on reductions in growth under elevated O_3 (Dickson *et al.* 1998, Bortier *et al.* 2000, Isebrands *et al.* 2001, King *et al.* 2005). The lack of growth reductions under elevated O_3 in our study may partly be explained by the relatively short exposure time of only one growing season. In multi-year free-air O_3 fumigation studies with trembling aspen and birch, O_3-induced growth reductions often became apparent only after some exposure seasons (Oksanen 2003, Karnosky *et al.* 2005). In the study of Matyssek *et al.* (1993) on European aspen, branch length and weight and leaf size were reduced only when the trees were exposed to an O_3 concentration of 100 ppb, roughly twice the concentration used in our experiment. In addition, the trees in the present study were grown in otherwise optimal conditions with adequate N fertilization and irrigation in order to minimize other stresses. Pääkkönen and Holopainen (1995) reported previously that ample N supply can improve the trees' resistance to O_3, while high soil moisture, on the other hand, promotes the uptake of O_3 by encouraging stomatal opening (Schaub *et al.* 2003). Thus, the present results point to the significance of soil N supply as a factor ameliorating or modifying the negative effects of O_3 on vegetation even under conditions that promote high stomatal O_3 flux.

The relatively short O_3 exposure period was, however, sufficient to induce changes in the biomass partitioning of the trees at the expense of the roots, as demonstrated by the decrease in the root/shoot and root/stem biomass ratios, especially in European aspen. Similar O_3-induced shifts in resource allocation towards the shoot are particularly common in tree species with an indeterminate growth pattern (Dickson *et al.* 1998, Oksanen *et al.* 2001, Yamaji *et al.* 2003), such as aspen. The mechanism underlying this shift is likely to be induced by an increased carbon demand in the O_3-exposed above-ground part for detoxification, repair or compensation reactions

(Pearson and Mansfield 1994). The observed change in biomass allocation suggests that in an environment with elevated O_3 concentration, trees may need to support growth with relatively smaller root systems, which weakens their competitive ability below ground (cf. Matyssek and Innes 1999). Impaired root/shoot balance may also predispose trees to drought stress, winter damage and parasites, and limit growth in the long term through changed resource acquisition (cf. Oksanen *et al.* 2001). At the species level, hybrid aspen exhibited lower root/shoot and root/stem ratios than European aspen, which can make it more vulnerable under competition. However, in natural forests root/shoot ratios may be higher due to low nutrient supply.

Although elevated O_3 may affect soil components indirectly through alterations in plant resource allocation (Andersen 2003), we did not observe changes in the C and N concentrations of roots or in the soil chemistry. Neither did we detect any carry-over effects of O_3 exposure in the form of increased winter damage to trees, which fits in with the inconsistent results reported on O_3 effects on the frost sensitivity of trees (Skärby *et al.* 1998).

Leaf morphology, chemistry and diseases

Even though the leaf N levels of our trees (ca. 3.3% of leaf dry weight in the control treatment) were somewhat higher than previously measured on European aspen (ca. 2.6%) (Matyssek *et al.* 1993), they fall within the range observed in various other *Populus* species (Tjoelker *et al.* 1999, Marinari *et al.* 2007). The negative correlation found between the total biomass and leaf N concentration probably results from growth dilution suggesting that the trees were not over-fertilized (Salifu and Jacobs 2006).

The decrease in leaf N concentration under O_3 exposure, as observed here, has also been found in other studies (Lindroth *et al.* 2001, Yamaji *et al.* 2003, Kopper and Lindroth 2003, Ribas *et al.* 2005), and is linked to O_3-induced activation of senescence-related processes (Bielenberg *et al.* 2002). The decline in leaf N level resulted in a higher leaf C:N ratio, since leaf C concentrations

remained unaffected under elevated O_3. Similar to the findings of Lindroth *et al*. (2001) on trembling aspen, we did not find any influence of O_3 on the N resorption of the trees. Thus the N levels in leaf litter reflected that of green leaves, which contributed to the trend towards a higher C:N ratio in the European aspen litter that was produced under elevated O_3. Nitrogen concentration and the C:N ratio of green leaves and litter have important roles in herbivory and decomposition (Kopper and Lindroth 2003, Cortez *et al*. 2007). Since litter with a high C:N ratio decomposes slower than that with a low C:N, an increase in litter C:N ratio may retard decomposition rates and consequently affect nutrient cycling in forests (Parsons *et al*. 2004). O_3 had no statistically significant effect on the C:N of the litter produced in our experiment. The interspecies difference in C:N ratio was found far more significant and it may have implications on ecosystem functioning if hybrid aspen becomes more common in ecosystems outside commercial plantations.

Although some studies suggest that leaves produced under O_3 stress may exhibit more xeromorphic traits (Bussotti *et al*. 2005, Ribas *et al*. 2005), we discovered no O_3-induced changes in the foliar morphological characteristics that we examined (SLA, the amount of epicuticular waxes) in the trees. The few studies that have previously been carried out on the effects of O_3 exposure on the amount of epicuticular waxes of deciduous trees are contradictory: an increase in the amount of waxes has been reported in trembling aspen (Karnosky *et al*. 2002) and no effect in beech (*Fagus sylvatica*) (Paoletti *et al*. 2007). Leaf cuticle properties are important in determining plant sensitivity to pathogen infections (Kerstiens 1996), yet the amount of wax does not explain the increased tolerance of trees to *Melampsora* rust infection under elevated O_3. Previously, the tolerance of O_3-exposed *Populus* species to *Melampsora* rust increased in a short-term (5 h) study (Coleman *et al*. 1987), but decreased in longer-term studies (Beare *et al*. 1999, Karnosky *et al*. 2002). Karnosky *et al*. (2002) showed that the increased susceptibility of trembling aspen to the rust was related to O_3-induced changes in the chemical composition of the wax, as the ensuing alterations in leaf surface characteristics made the leaves more wettable.

Leaf wetting depends on the ratio of the most hydrophobic cuticular wax class (alkanes) to the least hydrophobic one (fatty acids) (Percy *et al*. 2002). We found O_3-related tendencies that were similar to the changes in wax composition observed by Karnosky *et al*. (2002), i.e. an increase in alkyl esters and a decrease in alkanes in European aspen. Despite this, the rust tolerance increased under elevated O_3.

In the present study, a higher proportion of alkanes and a higher alkane:fatty acid ratio in cuticular wax seemed to be related to increased rather than decreased susceptibility to rust infection. Previously, Hegde and Kolattukudy (1997) reported that appressorium formation of fungi can indeed occur on both hydrophilic and hydrophobic surfaces. It is likely that the increased rust tolerance of the O_3-exposed trees in our study was linked to O_3-induced general defence against oxidative stress through increased antioxidative enzymes, phenolics and the ascorbate-glutathione cycle. As a harmful substance to living organisms, O_3 might also have affected the rust fungus directly. At the species level, hybrid aspen exhibited more epicuticular waxes than European aspen, which may have contributed to its better tolerance to the rust infection in both treatments.

Differences in O_3 sensitivity between the species and their clones

According to our results, the slower-growing European aspen was more sensitive to elevated O_3 than hybrid aspen, in terms of both visible injuries and other O_3-induced alterations. This is in contrast with the hypothesis of species with higher growth rates being more sensitive to O_3 (Skärby *et al*. 1998). This contrast is also indicated by the negative correlation between RGR and the severity of visible O_3 injuries and foliar yellowing. The hypothesis is mainly based on the idea of faster-growing species exhibiting higher g_s and thus greater O_3 uptake (Reich 1987). In the present study, however, we detected no correlation between RGR and g_s, but neither did we find interspecific differences in g_s. Our results are thus more in accordance with the notions of, for example, Pell *et al*. (1999) and Orendovici-Best

et al. (2008), who stated that species sensitivity to O_3 cannot be explained by O_3 flux alone.

At the species level, we found no correlation between growth rate and O_3 injury. Instead, in European aspen the abundance of visible O_3 injury was associated with a high dry weight of the above-ground part of the tree in particular, suggesting that compensatory investment in the above-ground part took place in response to the necrotic injuries in leaves (cf. Yamaji *et al.* 2003). This also led to a reduced root/shoot ratio, as discussed earlier. In hybrid aspen, however, we found no such correlation. In general, the detected effects of O_3 on hybrid aspen were minor, suggesting little need for compensatory mechanisms.

Differences in the O_3 sensitivity of individual clones also became apparent during the experiment. European aspen clone R1 was the most O_3-responsive clone, while hybrid aspen clone 34 appeared to be the most tolerant to O_3. Within both species, a more O_3-sensitive clone exhibited a greater amount of epicuticular waxes than a more O_3-tolerant one, and European aspen clones had smaller SLAs compared with those of hybrid aspen clones. Greater amounts of epicuticular waxes and smaller SLAs are considered as xeromorphic adaptations (Bussotti *et al.* 2005, Shepherd and Griffiths 2006). Indeed, Paoletti *et al.* (2007) observed that xeromorphism may increase the O_3 sensitivity of species not adapted to drought, such as aspen, when experiments are conducted with full irrigation, as in our study. Yamaji *et al.* (2003) reported that an unaffected root/shoot ratio under O_3 exposure was typical for the most O_3-tolerant silver birch clones, as observed also in the present experiment. In their study, the O_3 tolerance was related to a high constitutive amount of total phenolics, indicating antioxidants as important in O_3 response. The ability to detoxify O_3 is stated as one of the cornerstones in determining species sensitivity to O_3 (De Temmerman *et al.* 2002), and it might also better explain the differential O_3 responses of the clones in this study.

Conclusions

The O_3 exposure in our experiment was two-fold compared with the critical level of O_3 and induced visible foliar O_3 injury on the aspen clones, but had minor effects on growth in otherwise optimal conditions with adequate N and water supply. At any rate, exposure to O_3 tended to cause a shift in the biomass allocation of European aspen towards the above-ground part as well as accelerated leaf senescence in both species. Both of these effects may negatively affect growth in the long term, if the trees were exposed to elevated O_3 for several growing seasons. The occurrence of visible O_3 injuries under ambient air suggests that aspen may be adversely affected by current O_3 concentrations under conditions that promote O_3 flux into the leaves. European aspen was more sensitive to elevated O_3 than hybrid aspen, regardless of its slower growth rate. Stomatal conductance did not explain inter- or intraspecific differences in the sensitivity to O_3, but some xeromorphic leaf traits appeared to be related to increased O_3 sensitivity of the trees under regular irrigation. Response to O_3 stress differs with tree age (Samuelson and Kelly 2001), which complicates the extrapolation of these results to mature trees. Furthermore, results from monoculture studies on O_3 effects may not be entirely applicable to plants growing under inter- and intraspecific competition, which typically occurs in natural forests (Kozovits *et al.* 2005). Yet these results do indicate that young trees may experience relatively high O_3 fluxes in optimal conditions, as suggested by the occurrence of visible O_3 injuries, and this has implications e.g. for nurseries producing commercial tree material.

References

Andersen C.P. 2003. Source-sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytol.* 157: 213–228.

Beare J.A., Archer S.A. & Bell J.N.B. 1999. Effects of *Melampsora* leaf rust disease and chronic ozone exposure on poplar. *Environ. Pollut.* 105: 419–426.

Bielenberg D.G., Lynch J.P. & Pell E.J. 2002. Nitrogen dynamics during O_3-induced accelerated senescence in hybrid poplar. *Plant Cell Environ.* 25: 501–512.

Bortier K., De Temmerman L. & Ceulemans R. 2000. Effects of ozone exposure in open-top chambers on poplar (*Populus nigra*) and beech (*Fagus sylvatica*): a comparison. *Environ. Pollut.* 109: 509–516.

Brace S., Peterson D.L. & Bowers D. 1999. *A guide to ozone injury in vascular plants of the Pacific Northwest*. U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station.

Brendley B.W. & Pell E.J. 1998. Ozone-induced changes in biosynthesis of Rubisco and associated compensation to stress in foliage of hybrid poplar. *Tree Physiol.* 18: 81–90.

Bussotti F., Pancrazi M., Matteucci G. & Gerosa G. 2005. Leaf morphology and chemistry in *Fagus sylvatica* (beech) trees as affected by site factors and ozone: results from CONECOFOR permanent monitoring plots in Italy. *Tree Physiol.* 25: 211–219.

Bussotti F., Desotgiu R., Cascio C., Strasser R.J., Gerosa G. & Marzuoli R. 2007. Photosynthesis responses to ozone in young trees of three species with different sensitivities, in a 2-year open-top chamber experiment (Curno, Italy). *Physiol. Plantarum* 130: 122–135.

Chen Z. & Gallie D.R. 2005. Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. *Plant Physiol.* 138: 1673–1689.

Coleman J.S., Jones C.G. & Smith W.H. 1987. The effect of ozone on cottonwood-leaf rust interactions: independence of abiotic stress, genotype and ontogeny. *Can. J. Bot.* 65: 949–953.

Cortez J., Garnier E., Pérez-Harguindeguy N., Debussche M. & Gillon D. 2007. Plant traits, litter quality and decomposition in a Mediterranean old-field succession. *Plant Soil* 296: 19–34.

De Temmerman L., Vandermeiren K., D'Haese D., Bortier K., Asard H. & Ceulemans R. 2002. Ozone effects on trees, where uptake and detoxification meet. *Dendrobiology* 47: 9–19.

Dickson R.E., Coleman M.D., Riemenschneider D.E., Isebrands J.G., Hogan G.D. & Karnosky D.F. 1998. Growth of five hybrid poplar genotypes exposed to interacting elevated CO_2 and O_3. *Can. J. For. Res.* 28: 1706–1716.

Finnish Meteorological Institute 2002. *Climatological statistics of Finland 1971–2000*. Climatic statistics of Finland 2002/1. [In Finnish and English].

Fowler D., Cape J.N., Coyle M., Flechard C., Kuylenstierna J., Hicks K., Derwent D., Johnson C. & Stevenson D. 1999. The global exposure of forest ecosystems to air

pollutants. *Water Air Soil Pollut.* 116: 5–32.

Greitner C.S., Pell E.J. & Winner W.E. 1994. Analysis of aspen foliage exposed to multiple stresses: ozone, nitrogen deficiency and drought. *New Phytol.* 127: 579–589.

Hegde Y. & Kolattukudy P.E. 1997. Cuticular waxes relieve self-inhibition of germination and appressorium formation by the conidia of *Magnaporthe grisea*. *Physiol. Mol. Plant. Pathol.* 51: 75–84.

Hermle S., Vollenweider P., Günthardt-Goerg M.S., McQuattie C.J. & Matyssek R. 2007. Leaf responsiveness of *Populus tremula* and *Salix viminalis* to soil contaminated with heavy metals and acidic rainwater. *Tree Physiol.* 27: 1517–1531.

Holton M.K., Lindroth R.L. & Nordheim E.V. 2003. Foliar quality influences tree-herbivore-parasitoid interactions: effects of elevated CO_2, O_3, and plant genotype. *Oecologia* 137: 233–244.

Häikiö E., Freiwald V., Pasanen T., Beuker E., Holopainen T. & Oksanen E. 2007. Impacts of elevated ozone and nitrogen on growth and photosynthesis of European aspen (*Populus tremula*) and hybrid aspen (*P. tremula* × *P. tremuloides*). *Can. J. For. Res.* 37: 232–2336.

ICP 2004. *Manual on methodologies and criteria for modelling and mapping critical loads & levels and air pollution effects, risks and trends*. ICP Mapping and Modelling, UNECE CLRTAP.

IPCC 2007. *Climate Change 2007: The physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge, United Kingdom and New York, USA.

Isebrands J.G., McDonald E.P., Kruger E., Hendrey G., Percy K., Pregitzer K., Sober J. & Karnosky D.F. 2001. Growth responses of *Populus tremuloides* clones to interacting elevated carbon dioxide and tropospheric ozone. *Environ. Pollut.* 115: 359–371.

Juntunen M.-L. & Rikala R. 2001. Fertilization practice in Finnish forest nurseries from the standpoint of environmental impact. *New Forest.* 21: 141–158.

Karačić A. & Weih M. 2006. Variation in growth and resource utilisation among eight poplar clones grown under different irrigation and fertilisation regimes in Sweden. *Biomass Bioenerg.* 30: 115–124.

Karnosky D.F., Gagnon Z.E., Dickson R.E., Coleman M.D., Lee E.H. & Isebrands J.G. 1996. Changes in growth, leaf abscission, and biomass associated with seasonal tropospheric ozone exposures of *Populus tremuloides* clones and seedlings. *Can. J. For. Res.* 26: 23–37.

Karnosky D.F., Pregitzer K.S., Zak D.R., Kubiske M.E., Hendrey G.R., Weinstein D., Nosal M. & Percy K.E. 2005. Scaling ozone responses of forest trees to the ecosystem level in a changing climate. *Plant Cell Environ.* 28: 965–981.

Karnosky D.F., Percy K.E., Xiang B., Callan B., Noormets A., Mankovska B., Hopkin A., Sober J., Jones W., Dickson R.E. & Isebrands J.G. 2002. Interacting elevated CO_2 and tropospheric O_3 predisposes aspen (*Populus tremuloides* Michx.) to infection by rust (*Melampsora medusae* f. sp. *tremuloidae*). *Glob. Change Biol.* 8: 329–338.

Karnosky D.F., Werner H., Holopainen T., Percy K., Oksanen T., Oksanen E., Heerdt C., Fabian P., Nagy J., Heilman W., Cox R., Nelson N. & Matyssek R. 2007. Free-air exposure systems to scale up ozone research to mature trees. Review Article. *Plant Biology* 9: 181–190.

Kerstiens G. 1996. Signalling across the divide: A wider perspective of cuticular structure-function relationships. *Trends Plant Sci.* 1: 125–129.

King J.S., Kubiske M.E., Pregitzer K.S., Hendrey G.R., McDonald E.P., Giardina C.P., Quinn V.S. & Karnosky D.F. 2005. Tropospheric O₃ compromises net primary production in young stands of trembling aspen, paper birch and sugar maple in response to elevated atmospheric CO₂. *New Phytol.* 168: 623–636.

Kopper B.J. & Lindroth R.L. 2003. Responses of trembling aspen (*Populus tremuloides*) phytochemistry and aspen blotch leafminer (*Phyllonorycter tremuloidiella*) performance to elevated levels of atmospheric CO₂ and O₃. *Agr. Forest Entomol.* 5: 17–26.

Kozovits A.R., Matyssek R., Blaschke H., Göttlein A. & Grams T.E.E. 2005. Competition increasingly dominates the responsiveness of juvenile beech and spruce to elevated CO₂ and/or O₃ concentrations throughout two subsequent growing seasons. *Glob. Change Biol.* 11: 1387–1401.

Krupa S.V. & Manning W.J. 1988. Atmospheric ozone: formation and effects on vegetation. *Environ. Pollut.* 50: 101–137.

Kubiske M.E., Quinn V.S., Marquardt P.E. & Karnosky D.F. 2007. Effects of elevated atmospheric CO₂ and/or O₃ on intra- and interspecific competitive ability of aspen. *Plant Biology* 9: 342–355.

Laurila T. 1999. Observational study of transport and photochemical formation of ozone over northern Europe. *Geophys. Res.* 104: 26235–26243.

Laurila T., Tuovinen J.-P., Tarvainen V. & Simpson D. 2004. Trends and scenarios of ground-level ozone concentrations in Finland. *Boreal Env. Res.* 9: 167–184.

Lindroth R.L., Kopper B.J., Parsons W.F.J., Bockheim J.G., Karnosky D.F., Hendrey G.R., Pregitzer K.S., Isebrands J.G. & Sober J. 2001. Consequences of elevated carbon dioxide and ozone for foliar chemical composition and dynamics in trembling aspen (*Populus tremuloides*) and paper birch (*Betula papyrifera*). *Environ. Pollut.* 115: 395–404.

Marinari S., Calfapietra C., De Angelis P., Mugnozza G.S. & Grego S. 2007. Impact of elevated CO₂ and nitrogen fertilization on foliar elemental composition in a short rotation poplar plantation. *Environ. Pollut.* 147: 507–515.

Matyssek R. & Innes J.L. 1999. Ozone — a risk factor for trees and forests in Europe? *Water Air Soil Pollut.* 116: 199–226.

Matyssek R., Keller T. & Koike T. 1993. Branch growth and leaf gas-exchange of *Populus tremula* exposed to low ozone concentrations throughout 2 growing seasons. *Environ. Pollut.* 79: 1–7.

Meleux F., Solmon F. & Giorgi F. 2007. Increase in summer European ozone amounts due to climate change. *Atmos. Environ.* 41: 7577–7587.

Noormets A., Sôber A., Pell E.J., Dickson R.E., Podila G.K., Sôber J., Isebrands J.G. & Karnosky D.F. 2001. Stomatal and non-stomatal limitation to photosynthesis in two trembling aspen (*Populus tremuloides* Michx.) clones exposed to elevated CO₂ and/or O₃. *Plant Cell Environ.* 24: 327–336.

Novak K., Skelly J.M., Schaub M., Kräuchi N., Hug C., Landolt W. & Bleuler P. 2003. Ozone air pollution and foliar injury development on native plants of Switzerland. *Environ. Pollut.* 125: 41–52.

Novak K., Cherubini P., Saurer M., Fuhrer J., Skelly J.M., Kräuchi N. & Schaub M. 2007. Ozone air pollution effects on tree-ring growth, delta ¹³C, visible foliar injury and leaf gas exchange in three ozone-sensitive woody plant species. *Tree Physiol.* 27: 941–949.

Oksanen E. 2003. Responses of selected birch (*Betula pendula* Roth) clones to ozone change over time. *Plant Cell Environ.* 26: 875–886.

Oksanen E. & Saleem A. 1999. Ozone exposure results in various carry-over effects and prolonged reduction in biomass in birch (*Betula pendula* Roth). *Plant Cell Environ.* 22: 1401–1411.

Oksanen E., Amores G., Kokko H., Santamaria J.M. & Kärenlampi L. 2001. Genotypic variation in growth and physiological responses of Finnish hybrid aspen (*Populus tremuloides × P. tremula*) to elevated tropospheric ozone concentration. *Tree Physiol.* 21: 1171–1181.

Orendovici-Best T., Skelly J.M., Davis D.D., Ferdinand J.A., Savage J.E. & Stevenson R.E. 2008. Ozone uptake (flux) as it relates to ozone-induced foliar symptoms of *Prunus serotina* and *Populus maximowizii × trichocarpa*. *Environ. Pollut.* 151: 79–92.

Paoletti E., Nali C. & Lorenzini G. 2007. Early responses to acute ozone exposure in two *Fagus sylvatica* clones differing in xeromorphic adaptations: Photosynthetic and stomatal processes, membrane and epicuticular characteristics. *Environ. Monit. Assess.* 128: 93–108.

Parsons W.F.J., Lindroth R.L. & Bockheim J.G. 2004. Decomposition of *Betula papyrifera* leaf litter under the independent and interactive effects of elevated CO₂ and O₃. *Glob. Change Biol.* 10: 1666–1677.

Pearson M. & Mansfield T.A. 1994. Effects of exposure to ozone and water stress on the following season's growth of beech (*Fagus sylvatica*). *New Phytol.* 126: 511–515.

Pell E.J., Sinn J.P., Brendley B.W., Samuelson L., Vinten-Johansen C., Tien M. & Skillman J. 1999. Differential response of four tree species to ozone-induced acceleration of foliar senescence. *Plant Cell Environ.* 22: 779–790.

Percy K.E., McQuattie C.J. & Rebbeck J.A. 1994. Effects of air pollutants on epicuticular wax chemical composition. In: Percy K.E., Cape J.N., Jagels R. & Simpson C.M. (eds.), *Air pollutants and the leaf cuticle*, NATO ASI Series, vol. G 36, Springer-Verlag, Heidelberg, pp. 67–79.

Percy K.E., Legge A.H. & Krupa S.V. 2003. Tropospheric ozone: a continuing threat to global forests? In: Karnosky D.F., Percy K.E., Chappelka A.H., Simpson C. & Pikkarainen J. (eds.), *Air pollution, global change and forests in the new millennium*, Elsevier, Amsterdam, pp. 85–118.

Percy K.E., Nosal M., Heilman W., Dann T., Sober J., Legge A.H. & Karnosky D.F. 2007. New exposure-based metric approach for evaluating O₃ risk to North American aspen forests. *Environ. Pollut.* 147: 554–566.

Percy K.E., Awmack C.S., Lindroth R.L., Kubiske M.E., Kopper B.J., Isebrands J.G., Pregitzer K.S., Hendrey G.R., Dickson R.E., Zak D.R., Oksanen E., Sober J., Harrington R. & Karnosky D. 2002. Altered performance of forest pests under atmospheres enriched by CO₂ and O₃. *Nature* 420: 403–407.

Pleijel H., Grennfelt P. & Skärby L. 1999. Synthesis and evaluation. In: Pleijel H. (ed.), *Ground-level ozone — a threat to vegetation*, Swedish Environmental Protection Agency, Report 4970, pp. 91–105.

Pääkkönen E. & Holopainen T. 1995. Influence of nitrogen supply on the response of clones of birch (*Betula pendula* Roth.) to ozone. *New Phytol.* 129: 595–604.

Pääkkönen E., Vahala J., Pohjola M., Holopainen T. & Kärenlampi L. 1998. Physiological, stomatal and ultrastructural ozone responses in birch (*Betula pendula*) are modified by water stress. *Plant Cell Env.* 21: 671–684.

Pääkkönen E., Paasisalo S., Holopainen T. & Kärenlampi L. 1993. Growth and stomatal responses of birch (*Betula pendula* Roth.) clones to ozone in open-air and chamber fumigations. *New Phytol.* 125: 615–623.

Reich P.B. 1987. Quantifying plant response to ozone: a unifying theory. *Tree Physiol.* 3: 63–91.

Ribas A., Peñuelas J., Elvira S. & Gimeno B.S. 2005. Ozone exposure induces the activation of leaf senescence-related processes and morphological and growth changes in seedlings of Mediterranean tree species. *Environ. Pollut.* 134: 291–300.

Salifu K.F. & Jacobs D.F. 2006. Characterizing fertility targets and multi-element interactions in nursery culture of *Quercus rubra* seedlings. *Ann. For. Sci.* 63: 231–237.

Samuelson L. & Kelly J.M. 2001. Scaling ozone effects from seedlings to forest trees. *New Phytol.* 149: 21–41.

Schaub M., Skelly J.M., Steiner K.C., Davis D.D., Pennypacker S.P., Zhang J., Ferdinand J.A., Savage J.E. & Steveson R.E. 2003. Physiological and foliar injury responses of *Prunus serotonina*, *Fraxinus americana*,

and *Acer rubrum* seedlings to varying soil moisture and ozone. *Environ. Pollut.* 124: 307–320.

Shepherd T. & Griffiths D.W. 2006. The effects of stress on plant cuticular waxes. *New Phytol.* 171: 469–499.

Siitonen J. 1999. Haavan merkitys metsäluonnon monimuotoisuudelle. *Metsäntutkimuslaitoksen tiedonantoja* 725: 71–80.

Skärby L., Ro-Poulsen H., Wellburn F.A.M. & Sheppard L.J. 1998. Impacts of ozone on forests: a European perspective. *New Phytol.* 139: 109–122.

Suominen O., Edenius L., Ericsson G. & de Dios V.R. 2003. Gastropod diversity in aspen stands in coastal northern Sweden. *Forest Ecol. Manage.* 175: 403–412.

Suvanto L., Stenvall N., Vares A. & Pulkkinen P. 2004. Hybridihaavan geenivirta. *Metsätieteen aikakauskirja* 1/2004: 89–97.

Timonen U., Huttunen S. & Manninen S. 2004. Ozone sensitivity of wild field layer plant species of northern Europe. A review. *Plant Ecol.* 172: 27–39.

Tjoelker M.G., Reich P.B. & Oleksyn J. 1999. Changes in leaf nitrogen and carbohydrates underlie temperature and CO₂ acclimation of dark respiration in five boreal tree species. *Plant Cell Environ.* 22: 767–778.

Vingarzan R. 2004. A review of surface ozone background levels and trends. *Atmos. Environ.* 38: 3431–3442.

Wittig V.E., Ainsworth E.A. & Long S.P. 2007. To what extent do current and projected increases in surface ozone affect photosynthesis and stomatal conductance of trees? A meta-analytic review of the last 3 decades of experiments. *Plant Cell Environ.* 30: 1150–1162.

Yamaji K., Julkunen-Tiitto R., Rousi M., Freiwald V. & Oksanen E. 2003. Ozone exposure over two growing seasons alters root-to-shoot ratio and chemical composition of birch (*Betula pendula* Roth). *Glob. Change Biol.* 9: 1363–1377.

Yu Q.B. 2001. Can physiological and anatomical characters be used for selecting high yielding hybrid aspen clones? *Silva Fenn.* 35: 137–146.

Yu Q., Tigerstedt P.M.A. & Haapanen M. 2001. Growth and phenology of hybrid aspen clones (*Populus tremula* L. × *Populus tremuloides* Michx.). *Silva Fenn.* 35: 15–25.