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ORIGINAL ARTICLE

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Foliar physiology of yellow-poplar (*Liriodendron tulipifera* L.) exposed to O_3 and elevated CO_2 over five seasons.

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Abstract The chronic effects of ozone (O_3) alone or combined with elevated carbon dioxide (CO_2) on the foliar physiology of unfertilized field-grown yellow-poplar (*Liriodendron tulipifera* L.) seedlings were studied from 1992 to 1996. Within open-top chambers, juvenile trees were exposed to the following: charcoal-filtered air (CF); 1× ambient ozone ($1XO_3$); 1.5× ambient ozone ($1.5XO_3$); 1.5× ambient ozone plus 700 ppm carbon dioxide ($1.5XO_3+CO_2$); or chamberless open-air (OA). Seasonal 24-h mean ambient O_3 concentrations ranged from 32 to 46 ppm over the five seasons. Averaged over 5 years, midseason net photosynthesis at saturating light (A_{sat}) was reduced by 14% (P=0.029) and stomatal conductance (g_s) was reduced, albeit non-significant, by 13% (P =0.096) in upper canopy foliage exposed to $1.5XO_3$-air relative to CF controls. There were no significant differences over the 5 years in A_{sat} and g_s between trees grown in $1XO_3$- and $1.5XO_3$-air. Our results support the hypothesis that the magnitude of O_3 effects on A_{sat} and g_s decreases as saplings age. When averaged over the five seasons of exposure, total chlorophyll concentration (*chl*) was not significantly affected by exposure to elevated O_3; however, in $1.5XO_3+CO_2$-air, foliar *chl* was reduced by 33% relative to all others (P<0.001). In $1.5XO_3+CO_2$-air, A_{sat} was 1.4–1.9 times higher (P<0.001) and g_s was 0.7 times lower (P=0.022) than all others. O_3 uptake in juvenile trees exposed to elevated O_3 plus elevated CO_2 ($1.5XO_3+CO_2$-air) was most comparable to trees exposed to ambient air ($1XO_3$) throughout the study. These findings suggest that elevated CO_2 may minimize the negative effects of O_3 by reducing O_3 uptake through decreased stomatal conductance.

Keywords Elevated ozone · Elevated carbon dioxide · Hardwoods · Open-top chambers · Photosynthesis

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Introduction

Trees in eastern USA forests are being exposed to potentially damaging levels of tropospheric ozone (O_3) and future predictions suggest these levels are expected to continue to increase (Chameides et al. 1994). Levels of ambient O_3 in rural areas often exceed 50 ppb and can exceed 100 ppb (Lefohn et al. 1994). Oxidant levels in the Ohio River Valley and the Piedmont/Mountain/Ridge and Valley physiographic regions have been cited as some of the highest in eastern USA (Chappelka and Samuelson 1998). O_3 has long been known to be phytotoxic (Middleton 1956). Typically, carbon fixation is reduced by elevated O_3, resulting in diminished shoot and root growth. Concurrent with the increase of tropospheric O_3, has been the increase of atmospheric CO_2 levels (1.5 ppm $year^{-1}$ over the past 20 years) (IPCC 2001). Numerous reports indicate that elevated CO_2 stimulates carbon fixation and reduces water loss from plants by reducing stomatal conductance (increased water use efficiency), but the impact of these stimulatory-type responses in the presence of harmful pollutants such as O_3 is not well understood. A common hypothesis is that increasing CO_2 will reduce O_3 uptake and subsequent damage by decreasing stomatal conductance (Olszyk et al. 2000). Some studies support this hypothesis (Volin et al. 1998), while others have demonstrated that the deleterious effects of O_3 can be enhanced in O_3-sensitive clones or genotypes in the presence of elevated CO_2 (Kull et al. 1996). Noormets et al. (2001) suggested that the photosynthetic response to the combined effects of elevated O_3 and CO_2 is due to altered metabolic mesophyll processes and that changes in stomatal conductance appear to be a secondary response.

Our knowledge of the effects of simultaneous exposure to elevated O_3 and elevated CO_2 on long-lived perennial species is limited (Olszyk et al. 2000; Volin et al. 1998; Grams et al. 1999; Lütz et al. 2000). Researchers have characterized the short-term growth effects of elevated O_3 and CO_2, but few have reported results for more than two to three seasons of exposures (Broadmeadow and Jackson 2000). Also few investigators have studied the effects of exposing the same population of trees to gaseous pollutants during their development from seedlings to saplings or mature trees (Lee and Jarvis 1995; Isebrands et al. 2001).

Over five seasons, we examined the growth and physiological responses of the combined effects of elevated O_3 and CO_2 on field-planted yellow-poplar. We found that O_3 effects on growth were slow to manifest and limited. Enriched CO_2 appeared to ameliorate the negative effects of elevated O_3 on shoot and root growth (Rebbeck and Scherzer 2002). We monitored the physiological response of yellow-poplar to O_3 with and without elevated CO_2 by intensively measuring leaf-level gas exchange, chlorophyll concentration, and xylem water potential over the five exposure seasons. The primary focus of this paper was to determine whether the magnitude of O_3 effects lessens over time; and whether elevated CO_2 ameliorates any negative O_3 effects on foliar physiology.

Materials and methods

Study site, seedling culture, and fumigation

From 1992 (year 1) to 1996 (year 5), yellow-poplar seedlings were exposed continuously to O_3 and CO_2 treatments within open-top chambers (OTC) in a field plantation at the Northeastern Research Station's Forestry Sciences Laboratory at Delaware, Ohio, USA (40°21′N 83°04′W) from mid-May through mid-October, 24 h per day (Rebbeck and Scherzer 2002). The soil in the field plantation was a rich clay-loam glacial till under conditions of ambient water and nutrient availability. The experiment was a randomized block design with three replicates of the following targeted treatments: (1) charcoal-filtered air (CF); (2) 1× ambient O_3 ($1XO_3$); (3) 1.5× ambient O_3 ($1.5XO_3$); (4) 1.5× ambient O_3 plus 350 ppm CO_2 above ambient with a target CO_2 concentration of 700 ppm ($1.5XO_3$+CO_2); and (5) open-air chamberless plot (OA). O_3 was added to all $1XO_3$-, $1.5XO_3$-, and $1.5XO_3$+CO_2-chambers. Beginning in year 1, 12 seedlings were exposed within each of the 12 standard 3-m diameter OTCs (Heagle et al. 1973) and three open-air plots. Six seedlings within each chamber were harvested in September 1993 (year 2) and three additional saplings were removed in September 1994. The three remaining saplings were exposed to treatments through September 1996. As the trees increased in size, chamber heights were first doubled from 2.5 to 5 m in year 3; in year 4, these chambers were replaced with larger OTCs (4.6 m diameter and 9.2 m tall) similar to those developed by Heagle et al. (1989). The latter were twice as tall and equipped with two blowers. Gas exchange, chlorophyll, leaf mass per area, and leaf-water potential were measured throughout the study. The tree culture, experimental setup, and pollutant exposure system are discussed in detail in Rebbeck and Scherzer (2002).

Leaf gas exchange

Treatment and seasonal effects

To determine the impacts of O_3 alone and combined with elevated CO_2 on gas exchange of yellow-poplar, net photosynthesis at saturating light (A_{sat}) and stomatal conductance (g_s) were measured during the 5-year study. Data were collected with a LI-COR 6200 portable photosynthesis system (LI-COR, Lincoln, Neb.) and a 0.25 l cuvette. Throughout the study, three to six seedlings/saplings were measured within each chamber/plot. Initially, measurements were made on attached primary leaves of the main terminal. By year 3, tree architecture had increased in complexity such that leaves were not easily accessible and canopy light was too variable for consistent in situ measurements. Therefore, detached leaves were used for all measurements in years 3–5. Immediately following leaf removal, the cut end of a leaf petiole was immersed and stored in a sealed 5-ml tube of tap water. Measurements of detached leaves did not differ from equivalent attached leaves when readings were completed within 4 min.

Monthly gas exchange measurements were collected from June to August each year in all treatments. Gas exchange measurements were made between 1000–1400 hours on relatively sunny days to minimize diurnal variability. Measurements were taken over 3 days with one treatment replicate completed per day. Since yellow-poplar photosynthetic rates were determined to be saturated at 800 μmol $m^{-2}s^{-1}$ PAR (photosynthetically active radiation) (Loats and Rebbeck 1999; Rebbeck and Loats 1997), leaves were illuminated with an artificial light (GE Cool-Beam PAR Lamp Model 300PAR 56/2WFL) when ambient light dropped below saturating conditions. Leaves were measured at the CO_2 concentration at which they were grown. CO_2 averaged 343 ppm for ambient-grown plants and 709 ppm for those grown in $1.5XO_3$+CO_2-air, 24-h day^{-1}. Midday dark respiration of attached leaves was measured on terminal mature leaves (node 9–10) on 12 August, year 1. During these measurements, ambient light was excluded by covering the cuvette with aluminum foil for approximately 2 min to achieve steady-state measurements prior to a reading (Rebbeck and Loats 1997).

Leaf age. The interactive effects of pollutant treatment and leaf age on gas exchange rates were examined in late July and August, year 2. One recently mature leaf (node 4–6) and one older leaf (node 10–11) attached to the main terminal on each seedling were measured.

Canopy position. To determine whether treatment responses varied with canopy position, each tree was first divided visually into three equal portions: lower, mid, and upper canopy; one branch from each position was selected randomly and flagged in year 3. A_{sat} and g_s of a detached mature leaf (node 11–13 from apex) from each of the flagged branches were measured monthly from mid-June through mid-August. The same branches were measured again in years 4 and 5.

Diurnal responses. To assess how treatments affected A_{sat} and g_s diurnally, a mature leaf (node 9) from the primary stem was measured every 3 h from 0600–1800 hours) on 5 August, year 1. Three seedlings per chamber were sampled (n=9 seedlings/treatment).

O3 uptake. Stomatal uptake of O_3 at each sampling time was calculated from mid-day conductance (g_s) rates and O_3 exposure (ppm × hours) using the following equation:

$$\text{total stomatal } O_3 \text{ uptake} = (g_s/1.68)\ (\text{Cumulative } O_3 \text{ exposure}) \quad (1)$$

where 1.68 is a factor accounting for the molecular weight and critical volume differences between O_3 and water (Wang et al. 1995). Cumulative O_3 exposure (ppb × hours) was estimated as the sum of all hourly average concentrations up to the time of a given gas exchange measurement.

Leaf-water potential

Midday (1000–1400 hours) leaf-water potentials (Ψ_{leaf}) were measured with a pressure chamber (PMS Instruments, Corvallis Ore.) concurrently with A_{sat} and g_s measurements, initially with upper canopy foliage in August, year 3. The same protocols used to select branches and leaves for gas exchange measurements to determine canopy effects were followed except that only mid-canopy leaves were measured in June and July, year 4.

Chlorophyll and leaf mass per area

Frequency of chlorophyll extractions varied each year due to amount of foliage available for sampling. In year 1, a mature (node 9–11) and a new (node 4–5) leaf were collected from each of two seedlings within a chamber treated with CF-, $1.5XO_3$- and $1.5X+CO_2$-air in August to test leaf age effects. In year 2, late July and August, new (node 6) and mature (node 10–11) leaves were sampled in all treatments. In years 3–5, saplings were sampled monthly from June to August. Excluding year 1, leaves measured for A_{sat} and g_s were sampled for total chlorophyll (*chl*) and leaf mass per area. Immediately following gas exchange measurements, two 15-mm-diam discs were punched from a leaf with care taken to avoid primary and secondary veins. The leaf discs were incubated at 65°C for 3 h with dimethyl sulfoxide (DMSO) (Hiscox and Israelstam 1979). Extinction coefficients previously reported were used (Rebbeck et al. 1993). Leaf mass per area (M_a), expressed as total leaf dry mass per fresh leaf area, was determined by measuring leaf area on a LI-COR 3100 leaf area meter, then drying leaf material at 70°C for 48 h. M_a was calculated on leaves with petioles except in 1992.

Statistical analyses

Treatment effects were tested using only the chambered plots (CF, $1XO_3$, $1.5XO_3$, and $1.5XO_3+CO_2$) while comparisons were made between OA- and $1XO_3$-exposed trees to assess the effect of chambers on foliar physiology.

Treatment effects on dark respiration were tested with SAS General Linear Model analysis (SAS 1999) and single degree of freedom contrasts tested the statistical significance of treatment means.

To assess treatment effects on diurnal gas exchange of yellow-poplar leaves, A_{sat} and g_s integrals (area under diurnal curves) were estimated using SAS PROC MIXED and then tested for statistical significance using single degree of freedom contrasts.

Canopy position and treatment responses of A_{sat}, g_s, *chl*, and M_a, from mature foliage of low, mid, and upper branches, measured midseason (July) in years 3–5, were analyzed by repeated measures analysis of variance (RMA) with a randomized block design using SAS System for Mixed Models (Littell et al. 1996). Canopy position and treatment effects on Ψ_{leaf} also were tested in year 5 using SAS General Linear Model analysis. Three chamber replicates per treatment were used in these analyses.

Chronic treatment effects on gas exchange (A_{sat} and g_s), *chl*, and M_a measurements each July (years 1–5) were analyzed using RMA. This mid-season measurement period was used since it represented the most uniform and constant gas exchange rates within a growing season. Typically A_{sat} rates peaked in late June to early July and did not decline substantially until late August. Only measurements of mature leaves from upper canopy branches or the main terminal were used in those analyses as they were most comparable physiologically over the five seasons and all represented sun leaves.

The MIXED procedure for RMA within SAS was used to determine the most appropriate covariance structure for each of the repeated measures models used. A likelihood-based approach was used for assessing model adequacy. In most cases, a compound symmetry covariance structure was considered the most appropriate. Least square means ± 1 SE are presented. A priori single degree of freedom contrasts were used to determine differences among treatments and included: (1) the effects of elevated O_3 relative to clean-air controls—CF versus $1.5XO_3$; (2) the effects of ambient and elevated O_3 relative to controls—CF versus $1XO_3$ and $1.5XO_3$; (3) the effects of elevated CO_2 relative to ambient CO_2—CF, $1XO_3$ and $1.5XO_3$ versus $1.5XO_3+CO_2$; and (4) the effects of elevated CO_2 in the presence of elevated O_3—$1.5XO_3$ versus $1.5XO_3+CO_2$ (SAS 1999). Effects were considered significant if $P \leq 0.05$.

Results

Pollutant levels and environmental conditions

During the study, seasonal ambient 24-h mean O_3 concentrations ranged from 32 to 46 ppb (Table 1). In years 1–5, (1992–96), 51, 46, 7, 84 and 89%, respectively, of the exposure days had daily hourly maximum ambient O_3 concentrations that exceeded 50 ppb. Seasonal cumulative O_3 exposure (SUM00), a sum of every hourly O_3 mean during an active growing season, is an index used to assess O_3 impacts on woody and herbaceous vegetation. The cumulative exposures for years 1–5 are summarized in Table 1. During gas exchange measurements, seasonal air temperature ranged from 27.7 (±2.6) to 34.3 (±0.1)°C, relative humidity ranged from 51.5 (±2.1) to 54.4 (±5.0)%, and PAR ranged from 1,191 (±137) to 1,580 (±69) μmol m^{-2} s^{-1} within the 0.25 l LI-COR leaf cuvette.

Table 1 Seasonal 24-h O_3 concentration and seasonal cumulative O_3 exposure (SUM00), Delaware, Ohio, USA, 1992–96. (*OA* open-ambient air chamberless treatment; *CF* charcoal-filtered air; *1XO₃* 1 times ambient O_3; *1.5XO₃* 1.5 times ambient O_3; *1.5XO₃ +CO₂* 1.5 times ambient O_3 plus 2 times ambient carbon dioxide (target CO_2 concentration was 700 ppm). O_3 was added to all $1XO_3$-, $1.5XO_3$-, and $1.5XO_3+CO_2$-chambers)

Seasonal 24-h Treatment	O_3 concentration					Seasonal cumulative O_3 exposure (SUM00) [a]					
	1992	1993	1994	1995	1996	1992	1993	1994	1995	1996	Total (92–96)
	(ppb)					(ppm·h)					
OA	35	32	35	42	46	103	105	128	120	126	582
CF	10	5	11	12	11	27	16	39	36	27	145
$1XO_3$	35	32	35	42	48	104	107	125	117	130	583
$1.5XO_3$	46	51	49	59	78	136	171	176	166	212	861
$1.5XO_3+CO_2$	42	50	47	61	73	124	166	170	172	198	830

[a] Seasonal cumulative O_3 exposure: SUM00 represents sum of each daily 24-h total exposure for entire exposure season

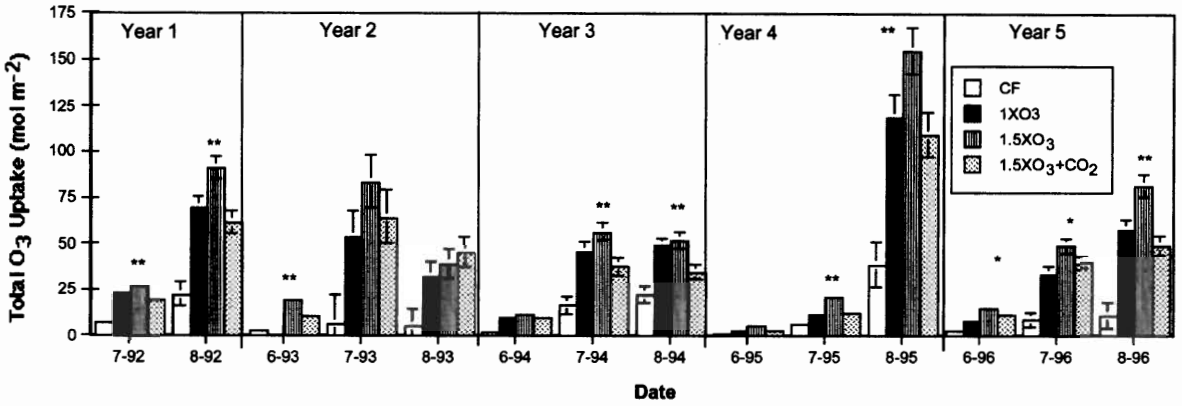

Fig. 1 Mean total O_3 uptake of yellow-poplar foliage in 1992–96 (years 1–5), at each physiological sampling date. Uptake was calculated based on cumulative O_3 exposures and stomatal conductance readings. The following treatments are represented: *CF* (charcoal-filtered air, control), *1XO3* (one times ambient ozone), *1.5XO3* (1.5 times ambient ozone), and *1.5XO3+CO2* (1.5 times ambient ozone plus two times ambient carbon dioxide). O_3 additions were made in $1XO_3$, $1.5XO_3$, and $1.5XO_3+CO_2$ chambers. These values represent the O_3 exposure only during a given growing season, not an accumulation from previous seasons. Least square means ±1SE are represented. Note: * denotes that $1.5XO_3+CO_2$ uptake rates for a given date were significantly lower that $1.5XO_3$ alone at $P<0.10$; ** denotes that $1.5XO_3+CO_2$ uptake rates for a given date were significantly lower that $1.5XO_3$ alone at $P<0.001$. $1XO_3$ leaves were not measured in June 1993

See Rebbeck and Scherzer (2002) for a discussion of rainfall, temperature, PAR, and detailed O_3 exposure data collected during the 5-year study.

O_3 uptake

Peak total O_3 uptake, a function of O_3 exposure and g_s (Reich 1987), varied from season to season, but were observed most often in July or August (Fig. 1). The highest total uptake, averaging 153 mol m^{-2}, was observed in $1.5XO_3$-exposed trees in August, year 4. In 73% of the measurement dates, O_3 uptake rates in saplings exposed to $1.5XO_3+CO_2$-air were significantly lower than $1.5XO_3$-seedlings (P <0.09) [60% of the dates were highly significant (P <0.001)]. O_3 uptake rates of $1.5XO_3+CO_2$-exposed saplings were most similar to those grown in $1XO_3$-air.

Chamber effects

Over the 5-year study, July measurements of A_{sat} (P=0.79), g_s (P=0.89), *chl* (P=0.63), and M_s (P=0.28) of upper canopy sun leaves exposed to ambient O_3 within OTCs were not significantly different than those grown in open-air chamberless plots (data not shown). No differences in diameter growth or biomass were detected between the chambered and open-air chamberless-grown trees throughout the study (Rebbeck and Scherzer 2002).

Leaf gas exchange

Dark respiration

In August, year 1, midday dark respiration of mature leaves in CF-air averaged −2.11 (±0.16) μmol m^{-2} s^{-1}. Dark respiration increased by 35.5% in $1.5XO_3$- and by 20% in $1.5XO_3+CO_2$-exposed seedlings relative to CF-air controls (P =0.001).

Treatment effects

Midseason (late July) A_{sat} and g_s measurements (years 1–5) of mature upper canopy sun leaves were significantly affected by treatment, year, and treatment × year (Table 2). Consistently, throughout the study, the highest A_{sat} was observed in trees exposed to $1.5XO_3+CO_2$-air (Fig. 2a). When averaged over the five seasons of exposure, $1.5XO_3$-air reduced A_{sat} by 14% (P=0.029) and g_s by 13% (P=0.096) relative to trees grown in CF-air. A_{sat} increased by 64% in foliage exposed to $1.5XO_3+CO_2$-air compared with $1.5XO_3$-air alone. The lowest g_s were observed most frequently in foliage of trees grown in $1.5XO_3+CO_2$-air (Fig. 2b).

Diurnal responses

Treatments impacted diurnal A_{sat} and g_s after 11 weeks of exposure in year 1 (Fig. 3). Significantly smaller integrals of diurnal A_{sat} were estimated for seedlings grown in $1XO_3$- (16% reduction) or $1.5XO_3$-air (43%) compared with those in CF-air (P=0.001). Plants grown in $1.5XO_3+CO_2$-air had integrals which were almost equal to those calculated for CF-air. Significantly

Table 2 Summary of repeated measures analyses to test the effect of five seasons of exposure to elevated O_3 alone and/or combined with elevated CO_2 on gas exchange, chlorophyll concentration, and leaf mass per area of mature upper canopy yellow-poplar leaves using July measurements from years 1–5 (1992–96). Mean values presented in Figs. 2 and 4

Source	Numerator *df*	Denominator *df*	A_{sat}[a]	g_s	*chl*	M_a
			P-value			
Treatment (Trt)	3	6	**<0.001**	0.057	**0.016**	**0.001**
Year	4 (3)[b]	32 (24)	**<0.001**	**<0.001**	**0.013**	**<0.001**
Trt × Year	12 (9)	32 (24)	**0.003**	0.083	0.737	0.705
Treatment contrasts[c]						
CF vs $1.5XO_3$	1	6	**0.029**	0.096	0.623	0.584
CF vs $1XO_3$ and $1.5XO_3$	1	6	**0.029**	0.114	0.059	**0.038**
All vs $1.5XO_3$+CO_2	1	6	**<0.001**	**0.022**	**0.003**	**<0.001**
$1.5XO_3$ vs $1.5XO_3$+CO_2	1	6	**<0.001**	0.160	**0.008**	**0.001**

[a] A_{sat} = μmol CO_2 $m^{-2}s^{-1}$; g_s = mol H_2O m^{-2} s^{-1}; *chl* = mg g^{-1} fresh weight and M_a = g m^{-2}
[b] Since data from years 2–5 used for repeated measures analyses for *chl* and M_a, the degrees of freedom differ in parenthesis
[c] Single *df* contrasts represent treatment effects over the five seasons of exposure for A_{sat} and g_s and four seasons for *chl* and M_a.

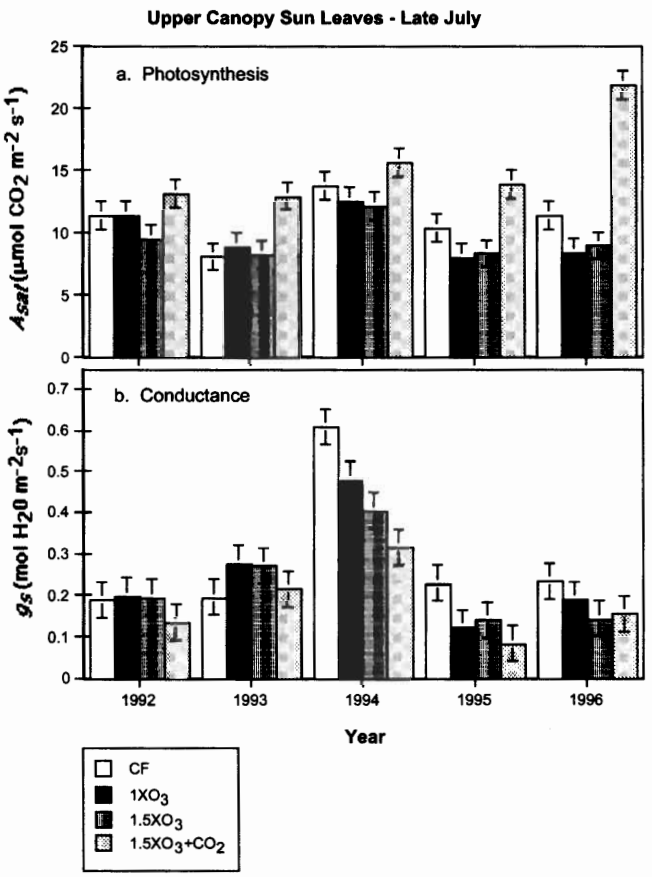

Fig. 2 Late July gas exchange measurements of upper canopy yellow-poplar foliage exposed to O_3 treatments over five seasons (1992–96); **a** net photosynthesis (A_{sat}), and **b** stomatal conductance (g_s) (measured under growth conditions). Least square means ± 1SE are represented. Repeated measures analysis (RMA) was run on July measurements each season. Probability values and details of RMA are presented in Table 2

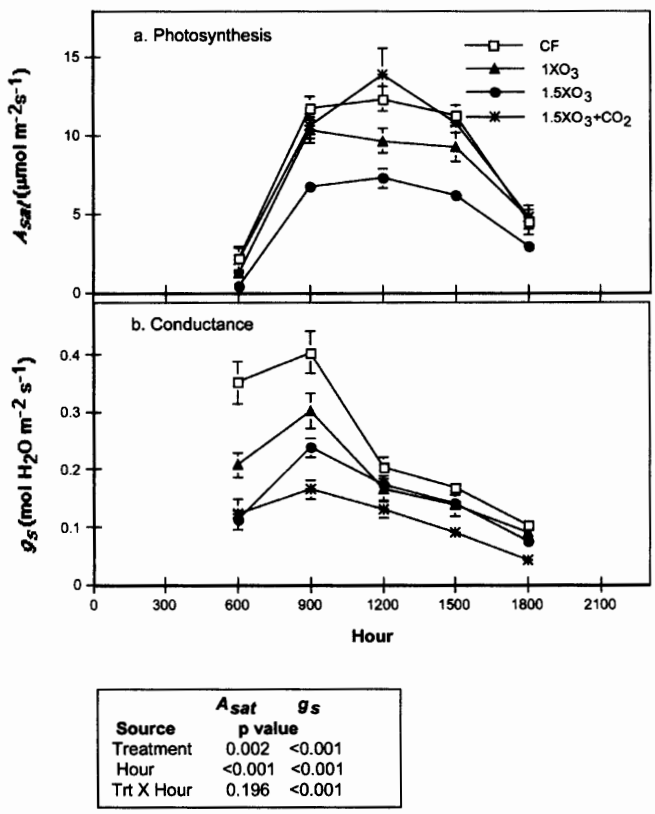

Fig. 3 Net photosynthesis of mature (node 9 from terminal apex) sun leaves of yellow-poplar measured every 3 h from 0600 to 1800 hours after 11 weeks of exposure in year 1. Diurnal A_{sat} integrals (a summation of the area under the diurnal curve of each treatment) were estimated as follows: CF = 417.9; $1XO_3$ = 349.0; $1.5XO_3$ = 237.5; and $1.5XO_3$+CO_2 = 419.1 mol m^{-2}day^{-1}. Least square means ±1SE are represented (n =9 trees per treatment)

smaller g_s integrals were estimated for foliage of seedlings grown in $1XO_3$- (25% reduction), $1.5XO_3$- (37%), and $1.5XO_3$+ CO_2-air (53%) compared with those in CF-air (P=0.001).

Leaf age

Overall, younger leaves (node 4–6 from apex) had significantly higher A_{sat} (48%) and g_s (152%) than older leaves (node 10–11) (P=0.001) (data not shown). How-

Table 3 Summary of repeated measures analyses to test canopy position (low, mid, upper) and treatment effects on gas exchange, chlorophyll and mass per area of mature upper canopy yellow-poplar leaves using July measurements from years 3–5 (1994–96). Mean values presented in Table 4

Source	Numerator *df*	Denominator *df*	A_{sat}[a]	g_s	*chl*	M_a
			P-value			
Treatment (Trt)	3	6	**0.001**	**0.040**	0.072	**0.001**
Canopy position (Canopy)	2	48	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Year (Yr)	2	16	**<0.001**	**<0.001**	**0.021**	**<0.001**
Trt × Canopy	6	48	**0.002**	**0.004**	**0.024**	**0.018**
Trt × Yr	6	16	**0.010**	0.051	0.120	0.504
Canopy × Yr	4	48	**0.001**	**<0.001**	**<0.001**	**<0.001**
Trt × Canopy × Yr	12	48	**0.033**	0.863	0.068	0.355
Contrasts[b]						
CF vs $1.5XO_3$	1	6	0.296	0.097	0.989	0.848
CF vs $1XO_3$ and $1.5XO_3$	1	6	0.186	0.117	0.862	0.591
All vs $1.5XO_3$+CO_2	1	6	**<0.001**	**0.014**	**0.014**	**<0.001**
$1.5XO_3$ vs $1.5XO_3$+CO_2	1	6	**<0.001**	0.105	**0.028**	**<0.001**

[a] A_{sat} = μmol CO_2 $m^{-2}s^{-1}$; g_s = mol H_2O m^{-2} s^{-1}; *chl* = mg g^{-1}; and M_a = g m^{-2}
[b] Single *df* contrasts represent treatment effects over final three seasons of exposure; all contrasts of canopy position effects significant at P <0.001

Table 4 Canopy position and treatment means* (±1 SE) of net photosynthesis (A_{sat}), conductance (g_s), mass per area (M_a), and chlorophyll concentration (*chl*) of mature foliage of yellow-poplar exposed to O_3 alone or combined with twice ambient CO_2 using July measurements from years 3–5 (1994–96). Probability values for treatment, canopy position and year effects are shown in Table 3

Canopy position and treatment	A_{sat} (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	*chl* (mg g^{-1})	M_a (g m^{-2})
Canopy				
Lower	7.23±0.35	0.12±0.01	3.43±0.14	126.2±2.0
Mid	9.93±0.35	0.17±0.01	3.16±0.14	158.0±2.0
Upper	12.12±0.35	0.26±0.01	2.93±0.14	175.7±2.0
Treatment × Canopy[a]				
CF				
Lower	6.82±0.69	0.15±0.02	3.60±0.24	124.6±4.0
Mid	8.89±0.69	0.19±0.02	3.40±0.24	155.5±4.0
Upper	11.87±0.69	0.36±0.02	3.25±0.24	166.0±4.0
$1XO_3$				
Lower	6.50±0.69	0.15±0.02	3.53±0.24	122.0±4.0
Mid	8.08±0.69	0.18±0.02	3.36±0.24	148.7±4.0
Upper	9.65±0.69	0.26±0.02	3.08±0.24	163.1±4.0
$1.5XO_3$				
Lower	6.08±0.69	0.11±0.02	3.55±0.24	124.1±4.0
Mid	9.03±0.69	0.19±0.02	3.45±0.24	153.1±4.0
Upper	9.81±0.69	0.23±0.02	3.24±0.24	171.1±4.0
$1.5XO_3$+CO_2				
Lower	9.55±0.69	0.07±0.02	3.04±0.24	134.1±4.0
Mid	13.70±0.69	0.12±0.02	2.42±0.24	174.9±4.0
Upper	17.16±0.69	0.18±0.02	2.13±0.24	202.7±4.0

[a] Significant treatment × canopy position effects were detected for all physiological parameters

ever, treatment responses of new and older leaves did not vary significantly when measured in year 2.

Canopy position

In years 3–5, significant differences in A_{sat} and g_s were associated with treatment, canopy position, year, and treatment × position for July measurements (Table 3). A_{sat} and g_s increased linearly from lower to upper canopy foliage (Table 4). Upper canopy A_{sat} was 68% greater (P<0.001) while g_s was 117% greater (P<0.001) than lower canopy foliage. When separated by treatment, the highest A_{sat} was observed in upper canopy foliage of $1.5XO_3$+CO_2-treated trees while the lowest rates were observed in lower canopy foliage of $1.5XO_3$-treated trees. The highest g_s were observed in the upper canopy foliage of CF- treated trees, while the lower canopy foliage of $1.5XO_3$+CO_2-treated trees had the lowest g_s. To better assess canopy x treatment interactions, slopes (b) were estimated for the change in A_{sat} within the canopy for each treatment. The greatest slope was detected in $1.5XO_3$+CO_2 trees (b=3.81), followed by CF (b=2.53), $1.5XO_3$ (b=1.86), and $1XO_3$ (b=1.58) -trees.

Leaf-water potential

Leaf-water potential (Ψ_{leaf}) was less negative in $1.5XO_3$- and $1.5XO_3$+CO_2-air. In year 4, Ψ_{leaf}, was 8% less negative (−1.13 MPa) in $1.5XO_3$- and 23% less negative (−0.95 MPa) in $1.5XO_3$+CO_2-air, relative to CF-controls (−1.23 MPa) (P<0.001). In year 5, significant treatment (P=0.009), canopy position (P<0.001), month (P<0.001), treatment × canopy position (P=0.009), and canopy position × month (P=0.019) effects were detected. During the fifth season, upper canopy sun leaves (−1.25 MPa) were the most water stressed as indicated by the most negative Ψ_{leaf} readings. Ψ_{leaf} of lower (−0.88 MPa) and mid-canopy leaves (−1.01 MPa) from $1.5XO_3$+CO_2-grown trees were the least negative, while other treated foliage did not differ (−1.13 MPa). Upper canopy Ψ_{leaf} of trees exposed to $1.5XO_3$- (−1.27 MPa) and $1.5XO_3$+CO_2-air (−1.06 MPa) were similar but were less negative than leaves of CF- (−1.36 MPa) or $1XO_3$-grown trees (−1.3 MPa).

Chlorophyll and leaf mass per area

Leaf age

During the first season, younger leaves had 114% higher *chl* than mature leaves (P<0.001). Leaf age influenced the magnitude of treatment impacts on *chl* (P=0.019). In mature leaves of $1.5XO_3$-grown seedlings, *chl* was 1.06 (±0.21) mg g^{-1} compared with 2.13 (±0.21) and 1.43 (±0.21) mg g^{-1} in CF- and $1.5XO_3$+CO_2-grown seedlings, respectively. In young leaves exposed to CF-, $1.5XO_3$-, $1.5XO_3$+CO_2-air, *chl* was 3.67 (±0.21), 3.57 (±0.21), and 2.66 (±0.21) mg g^{-1}, respectively. M_a averaged 132 (±3) g m^{-2} and did not vary with leaf age or treatment. Contrary to the first season, no differences were associated with leaf age or treatment for *chl* or M_a in the second season.

Treatment effects

Significant treatment and year effects on *chl* and M_a of mature upper canopy sun leaves (measured in late July, years 2–5) were identified (Table 2, Fig. 4). The responses were consistent from year to year and no interactions were detected. In years 2–5, elevated O_3 alone had no significant effect on *chl* or M_a. In foliage exposed to $1.5XO_3$+CO_2-air, *chl* was 33% lower (P=0.003) and M_a was 20% higher (P=<0.001) relative to all others throughout the study.

Canopy position

Significant canopy position, treatment, and treatment × canopy position effects were detected in years 3–5 (Tables 3, 4). Mature foliage from lower canopy branches had 15% higher *chl* and 39% lower M_a than upper canopy leaves. The lowest *chl* was detected in upper canopy foliage of $1.5XO_3$+CO_2-trees and the highest *chl* in lower canopy foliage of CF-trees. Upper canopy foliage of $1.5XO_3$+CO_2-trees had the highest M_a while the lowest M_a was found in lower canopy branches irrespective of treatment.

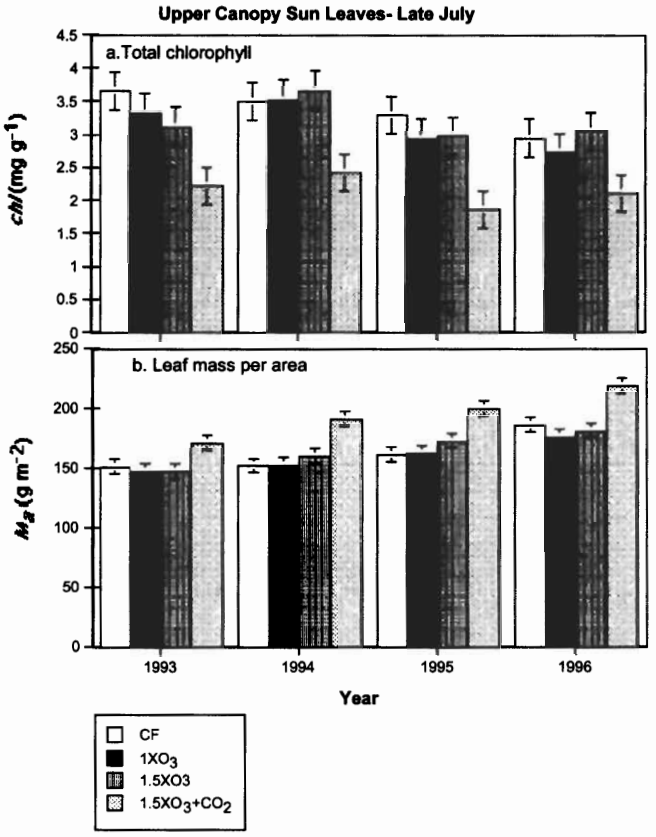

Fig. 4 Late July measurements of upper canopy yellow-poplar foliage exposed to O_3 treatments over five seasons (1992–96). **a** total chlorophyll concentration (*chl*), and **b** leaf mass per area (M_a). Least square means ±1SE are represented. Repeated measures analysis (RMA) was run on July measurements each season. Probability values and details of RMA are presented in Table 2

Discussion

Elevated O_3 effects

Most earlier studies report no significant O_3 effects on photosynthesis of yellow-poplar seedlings in short-term (one growing season or less), controlled environment exposures (Cannon et al. 1993; Loats and Rebbeck 1999; Roberts 1990; Tjoelker and Luxmoore 1992). In our current study, intensive gas exchange measurements of juvenile yellow-poplar exposed to O_3 over five growing seasons revealed that midseason A_{sat} of upper canopy foliage declined 10% when grown in 1× ambient O_3- and 14% in 1.5× ambient O_3-air relative to the CF-air controls. These trees received no supplemental watering

beyond the first season and were not fertilized (Rebbeck and Scherzer 2002). We anticipated that they would be less sensitive to O_3 since they were not grown under optimal conditions. However, the observed O_3-induced seasonal reductions in photosynthesis were comparable to A_{sat} declines of 12–42% we reported for potted yellow-poplar seedlings exposed to 1.7× ambient O_3 (cumulative exposure of 107–197 ppm·h^{-1}) over two growing seasons in a previous OTC study (Rebbeck and Loats 1997).

During the first season of our current study, diurnal (predawn to post sunset) photosynthesis of seedlings was reduced by 16% and 43% by exposure to ambient and elevated O_3, respectively. Mean diurnal g_s was reduced by 25% in ambient O_3 and by 43% in elevated O_3 compared to CF-air controls. Peak g_s occurred between 0600 and 0900 hours across treatments, preceding typically observed ambient daily O_3 peaks between 1200–1600 hours. This indicates that stomates may be open only for a short period during the early morning when O_3 levels are typically low, suggesting that O_3 uptake may have been limited. Stomatal closure appeared to be proportionally greater in ambient and sub-ambient O_3 air than elevated O_3 air indicating some factor other than O_3 may have caused this reduction in g_s. Information is scarce on the diurnal gas exchange response of woody plants exposed to O_3; however, we previously observed similar impacts on diurnal photosynthesis and conductance with potted seedling yellow-poplar grown out of doors (Rebbeck and Loats 1997). A more thorough characterization of diurnal stomatal conductance and carbon assimilation of the major forest tree species is needed to better predict the potential impacts of changing climate conditions on forested ecosystems.

In addition to stomatal behavior, O_3 uptake is also dependent on the concentration, frequency and duration of ambient O_3. Climatic, environmental and edaphic factors mediate O_3 uptake and subsequent physiological processes. Seasonal ambient O_3 patterns varied considerably during the five-season study. In the third season, only 7% of the treatment exposure days had daily hourly maximum ambient O_3 concentrations that exceeded 50 ppb compared with 51 and 46% of exposure days in years 1 and 2, respectively, and 84 and 89% of days in years 4 and 5, respectively. The amount and frequency of rainfall also varied throughout the study (Rebbeck and Scherzer 2002). For example, in August 1995 (year 4), saplings received 112 mm of rainfall during a 12-day period that coincided with 3 days of gas exchange measurements. The "normal" (50-year average) total monthly precipitation for the site in August is 85 mm. During those August 1995 foliar gas measurements, we observed some of the highest conductance values across all treatments and highest O_3 uptake rates throughout the study. These observed temporal variations in O_3 response emphasize the need for longer-term field pollutant-exposure experiments conducted under realistic growing conditions when studying long-lived species such as trees.

Some studies have reported O_3-induced reductions in foliar chlorophyll in tree species (Bortier et al. 2000; Broadmeadow and Jackson 2000; Kull et al. 1996; Reich 1983), while others have shown little effect (Amundson et al. 1991; Grams et al. 1999; Jensen and Noble 1984; Rebbeck et al. 1993). In our study, O_3 effects on chlorophyll were observed only in the first season. We speculate that because these seedlings were just being established, they may have been more sensitive to elevated O_3. Chlorophyll formation is affected by many environmental factors such as light, temperature, water stress, and mineral nutrition that can affect metabolic processes (Darrall and Jager 1984). It is likely that mineral nutrition was not a contributing factor in the reduction in chlorophyll. Concentrations of foliar nitrogen (N) (26.3 mg g^{-1} mean across treatments and seasons) in these same leaves were not significantly altered by O_3 exposures (Scherzer et al. 1998). Foliar N was within the adequate range for this species (Leaf 1973).

As these juvenile trees increased in stature (heights averaged 7.2 m after five seasons), A_{sat}, g_s, and M_a increased and *chl* decreased in mature leaves from lower to upper canopy branches. M_a was not affected by the O_3 treatments. The change in A_{sat} from lower to upper canopy foliage was greater in CF-trees (slope of 2.5) than trees exposed to ambient and elevated O_3 (slopes of 1.6–1.9). Tjoelker et al. (1995) reported increased O_3 sensitivity of shade leaves of mature sugar maple trees with greater reductions in A_{sat} and lower chlorophyll concentrations. Kolb and Matyssek (2001) hypothesized that shaded portions of older tree canopies may have increased O_3 sensitivity because photosynthate available for repair and defense may be limited in shaded leaves. We found no evidence to support that hypothesis with our young yellow-poplar saplings. Foliage of yellow-poplar, a shade-intolerant species, displayed typical sun- and shade-leaf morphology.

During the final season, O_3-induced changes in leaf-water potential Ψ_{leaf} were greater in upper canopy leaves than in mid or lower canopy leaves. Elevated O_3 may have reduced water stress by triggering stomatal closure, as evidenced by increased Ψ_{leaf} relative to controls. In a 3-month shaded greenhouse study, Roberts (1990) reported that drought and elevated O_3 together had a greater impact on Ψ_{leaf} of seedling yellow-poplar than either stress alone when grown under low light (200 mE m^{-2}s^{-1} photosynthetically active radiation). In our field study, Ψ_{leaf} ranged from −0.25 to −2.00 MPa with an overall average of −1.08 MPa, suggesting there were periods when soil moisture was limited. However, no expression of drought-induced changes in morphology or growth was observed. O_3 uptake most likely was reduced during these episodes, thereby minimizing O_3 effects.

Our current study represents a comprehensive and unique data set with five seasons of intensive A_{sat}, g_s, Ψ_{leaf}, *chl* , and M_a (presented here), and foliar N, growth, shoot and root mass, and environmental (O_3, CO_2, relative humidity, air and soil temperature, rainfall, and solar radiation) measurements (Rebbeck and Scherzer 2002; Scherzer et al. 1998). These data are valuable for refining physiologically based models such as TREGRO (Lau-

rence et al. 2001; Constance and Retzlaff 1997) and P_{net} (Aber et al. 1996; Ollinger et al. 2002), and for enhancing predictions of the long-term effects of O_3, CO_2, and other changing climate variables on the health and sustainability of USA forests. In simulations for southern Appalachia, USA, TREGRO predicted that mature yellow-poplar tree growth would decrease by 28% at ambient O_3 and 42.5% at 1.5× ambient O_3 (180 ppm·h^{-1} cumulative exposure) (Weinstein et al. 2001). TREGRO simulations predicted little O_3 effect on leaf growth or fine root mass per unit leaf. Over five seasons, we observed no significant O_3 impacts on basal-area growth, leaf area, or mass (Rebbeck and Scherzer 2002). In the TREGRO simulations, the maximum net carbon assimilation rate of 7.67 μmol m^{-2}s^{-1} used was based on reported A_{sat} of juvenile yellow-poplar foliage. That value is approximately 24% lower than the photosynthetic rates we observed during midseason measurements of upper canopy sun leaves. Clearly, our data set, which characterizes carbon assimilation rates within a developing canopy over five seasons, would augment predictions of the impacts of future O_3 and climate scenarios on eastern U.S. forests.

It appears that O_3 effects may have lessened over time because of suboptimal growing conditions, as statistically significant O_3 effects within a given growing season were not detected after the first season. However, reductions in net photosynthesis of juvenile trees grown in 1.5× ambient O_3 were observed throughout the study. Also significant O_3 effects on A_{sat} were detected when the data were analyzed over the five seasons. O_3-induced shoot and root growth reductions (albeit nonsignificant) were not observed until the fifth season, suggesting that yellow-poplar may compensate for the direct effects of O_3 on carbon fixation for a number of seasons before the onset of any detectable growth impairment.

Combined effects of elevated O_3 and CO_2

Throughout the five seasons of exposure, seasonal net photosynthesis of yellow-poplar foliage grown in the combination of elevated O_3 and elevated CO_2 (700 ppm) was 57–80% higher than trees exposed to elevated O_3 alone. This strongly suggests that elevated CO_2 counteracted the negative effects of O_3 on photosynthesis. This amelioration likely resulted from reduced leaf conductance that was consistently observed throughout the study. Seasonal conductance of foliage exposed to the combination of elevated O_3 and elevated CO_2 was 25–57% lower than that of all other trees. Repeatedly, estimates of O_3 uptake of trees exposed to 1.5× ambient O_3 plus 2× ambient CO_2 were comparable to trees grown in 1× ambient O_3. This study supports the hypothesis that elevated CO_2 protects trees from O_3 by lowering uptake by reducing stomatal conductance. Findings on other deciduous tree species including ash, beech, birch, oak, and trembling aspen also support this hypothesis (Broadmeadow and Jackson 2000; Lűtz et al. 2000; Volin et al. 1998; Volin and Reich 1996), but our study is one of the first to demonstrate long-term sustained reductions in stomatal conductance.

Although O_3 effects on chlorophyll and leaf mass per area were not seen, we did detect differences in foliage grown in elevated O_3 plus elevated CO_2. Leaf mass per area increased by about 20% in foliage exposed to both elevated O_3 and CO_2. Grams et al. (1999) observed a 17% increase in leaf mass per area of beech exposed to elevated CO_2 alone or combined with elevated O_3. This type of change in leaf anatomy often is significant in foliage exposed to elevated CO_2 alone (Saxe et al. 1998), with leaf thickness and starch content often increasing. Ultrastructural examination of yellow-poplar foliage exposed to elevated O_3 plus CO_2 indicated starch accumulation and thicker cuticular membranes (McQuattie and Rebbeck 1994).

Seasonal chlorophyll levels throughout the study were 16–30% lower in foliage exposed to the combination of elevated O_3 and elevated CO_2 relative to all others. Reductions in chlorophyll content and leaf proteins such as Rubisco often are observed in foliage exposed to elevated CO_2 (Lütz et al. 2000; Van Oosten et al. 1993). It has been suggested that less N is invested into light harvesting proteins, and if nutrient supply is high, increased rates of photosynthesis and growth can be sustained under elevated CO_2 (Saxe et al. 1998). Some have proposed that reduced leaf chlorophyll in high CO_2-grown leaves may reflect a dilution of leaf N (Mousseau and Saugier 1992; Lütz et al. 2000). Reduced N concentrations in mature leaves may be related to the reallocation of leaf N to newly expanding foliage or other sinks (Norby et al. 1996). In our study, foliar N content decreased by 18–40% in the combined O_3+CO_2 treatment and leaf litter decomposition was significantly lower (Scherzer et al. 1998). Boerner and Rebbeck (1995) reported that both mass loss and N release of litter from yellow-poplar leaves grown in elevated O_3 plus elevated CO_2 were significantly reduced. That foliage had lower N content and higher lignin content. Although lignin and other secondary plant compounds were not measured in our study, a vacuolar accumulation of dense polyphenolic compounds within leaves of saplings exposed to 1.5XO_3+CO_2-air typically were found with ultrastructural examination (McQuattie, personal communication). This suggests that levels of secondary plant compounds such as tannins, alkaloids, phenols, and glycosides may have been increased in yellow-poplar in response to the combination of elevated CO_2 and O_3.

We recognize that inferences regarding the effects of elevated CO_2 alone cannot be made. From the inception of this study, we anticipated that pollutant exposures would continue for 8–10 years; therefore, the cost of three additional CO_2 open-top chambers was prohibitive. However, the physiological and growth responses observed in these trees are comparable to other deciduous tree species exposed to both elevated O_3 and CO_2 (Broadmeadow and Jackson 2000; Isebrands et al. 2001; Lütz et al. 2000), as well as to yellow-poplar exposed to

elevated CO_2 alone (Norby et al. 1992). In investigating the effects of elevated CO_2 on the growth and physiology of yellow-poplar in a series of controlled and field experiments, Norby and his colleagues observed increased rates of photosynthesis but did not always detect increases in above-ground mass (Gunderson et al. 1993; Norby et al. 1996; Norby and O'Neill 1991). We speculate that since these current trees were field grown, rooting volume would not be restricted and downward acclimation of photosynthesis would not occur. There is no evidence of down-regulation of photosynthesis in these saplings exposed to the combination of elevated O_3 and CO_2-air. Photosynthetic rates remained elevated throughout the study, with some of the highest rates observed during the final season. On average, the root mass was 1.7 times greater in trees grown in elevated O_3 plus elevated CO_2 than that of those grown in elevated O_3 at ambient CO_2 (Rebbeck and Scherzer 2002). This suggests a shift in the allocation of surplus carbon below ground. N nutrition can be an important mediator of the impacts of elevated CO_2 and O_3 (Bortier and Vandermeiren 2001). When N is limiting, trees can be less responsive to elevated CO_2 (Isebrands et al. 2001). However, in our study, foliar and soil N levels did not appear to be limiting (Leaf 1973; Rebbeck and Scherzer 2002; Scherzer et al. 1998).

The results of this five-season field study suggest that elevated CO_2 may ameliorate the negative effects of increased tropospheric O_3 on yellow-poplar. However, long-term studies are needed to better understand how other abiotic and biotic stressors interact with elevated O_3 and CO_2 to affect major deciduous forest species.

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