Plant, Cell and Environment (2001) **24**, 327–336

Stomatal and non-stomatal limitation to photosynthesis in two trembling aspen (*Populus tremuloides* Michx.) clones exposed to elevated CO_2 and/or O_3

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ABSTRACT

Leaf gas exchange parameters and the content of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the leaves of two 2-year-old aspen (*Populus tremuloides* Michx.) clones (no. 216, ozone tolerant and no. 259, ozone sensitive) were determined to estimate the relative stomatal and mesophyll limitations to photosynthesis and to determine how these limitations were altered by exposure to elevated CO_2 and/or O_3. The plants were exposed either to ambient air (control), elevated CO_2 (560 p.p.m.) elevated O_3 (55 p.p.b.) or a mixture of elevated CO_2 and O_3 in a free air CO_2 enrichment (FACE) facility located near Rhinelander, Wisconsin, USA. Light-saturated photosynthesis and stomatal conductance were measured in all leaves of the current terminal and of two lateral branches (one from the upper and one from the lower canopy) to detect possible age-related variation in relative stomatal limitation (leaf age is described as a function of leaf plastochron index). Photosynthesis was increased by elevated CO_2 and decreased by O_3 at both control and elevated CO_2. The relative stomatal limitation to photosynthesis (l_s) was in both clones about 10% under control and elevated O_3. Exposure to elevated CO_2 + O_3 in both clones and to elevated CO_2 in clone 259, decreased l_s even further – to about 5%. The corresponding changes in Rubisco content and the stability of C_i/C_a ratio suggest that the changes in photosynthesis in response to elevated CO_2 and O_3 were primarily triggered by altered mesophyll processes in the two aspen clones of contrasting O_3 tolerance. The changes in stomatal conductance seem to be a secondary response, maintaining stable C_i under the given treatment, that indicates close coupling between stomatal and mesophyll processes.

Key-words: elevated CO_2; elevated O_3; light-saturated photosynthesis; Rubisco; stomatal conductance; stomatal limitation.

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Abbreviations: A, light-saturated photosynthesis at ambient $[CO_2]$; C_a, ambient CO_2 concentration; C_i, intercellular CO_2 concentration; g_s, stomatal conductance; l_s, relative stomatal limitation; LPI, leaf plastochron index; r^*, cotangent of A–C_i curve at the operating $[CO_2]$; r_{bl}, boundary layer resistance; r_s, stomatal resistance ($1/g_s$); Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

INTRODUCTION

The concentration of atmospheric carbon dioxide (CO_2) and ozone (O_3) are increasing (Bazzaz 1990; Chameides *et al*. 1995) due to increasing consumption of fossil fuels, and these increases have been implicated in changes in terrestrial ecosystems (Miller 1973; Ciais *et al*. 1995; Keeling, Chine & Whorf 1996). Elevated atmospheric CO_2 is associated with increased photosynthetic rates, increased plant growth and higher yields (Bowes 1993; Drake, Gonzalez-Meler & Long 1997; Will & Ceulemans 1997; Pan, Wang & Quebedeaux 1998). The CO_2-induced stimulation of photosynthesis has often been found to decrease over time (Rey & Jarvis 1998; Tissue, Griffin & Ball 1999; Jach & Ceulemans 1999), but it may also last over a longer time if strong carbon sinks persist (Stitt 1991).

Ozone, on the other hand, inhibits the growth of plants (Heath 1994; Pell, Schlagnhaufer & Arteca 1997) by decreasing stomatal conductance and photosynthesis, decreasing the content and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), decreasing the content of chlorophyll and inducing accelerated senescence (Darrall 1989; Pell, Eckardt & Enyedi 1992; Landry & Pell 1993; Pell, Eckardt & Glick 1994; Karnosky *et al*. 1996; Nali *et al*. 1998). How these two greenhouse gases together affect plant growth and metabolism has been studied by some laboratories but the results are often contradictory (Polle *et al*. 1993; Rao, Hale & Ormrod 1995; Karnosky *et al*. 1996, 1998; Kull *et al*. 1996; McKee, Eiblmeier & Polle 1997; Dickson *et al*. 1998; Reid, Fiscus & Burkey 1998). For *Gramineae* family members elevated CO_2 often ameliorates the harmful effects of O_3 (Rao *et al*. 1995; McKee *et al*. 1997), whereas for other plants the data are less conclusive.

We have found that the inhibitory effect of O_3 on height, diameter and biomass growth of aspen was not ameliorated by elevated CO_2 (Karnosky *et al*. 1998) and that photosynthetic rates were, in fact, more inhibited under simultaneous exposure to CO_2 and O_3 than under elevated O_3 alone (Kull *et al*. 1996). It is still unclear, however, what causes the changes in photosynthetic performance. The correlation between changing photosynthesis and stomatal conductance has sometimes been interpreted as evidence for stomatal control over photosynthesis (McKee *et al*. 1997; Ishida, Toma & Marjenah 1999), whereas the calculated values of relative stomatal limitation are often low (Jones 1985, 1998; Assmann 1988). In fact, the decreased stomatal conductance may be the result rather than the cause of decreased photosynthesis (Fiscus *et al*. 1997).

Most of gas exchange research is performed on leaves of one particular developmental stage (Field, Jackson & Mooney 1995) and the age-related variation is not addressed. Although this approach is adequate for studying the mechanisms of regulation in response to changes in environmental conditions, it does not always adequately characterize the response at whole plant level. Wait *et al*. (1999) showed that expanding and expanded leaves in *Populus deltoides* respond differently to elevated CO_2 treatment and that the ratio of expanding to expanded leaves determines the plant level response. In fact, the age-related changes are more dynamic and the division into expanding and expanded leaves may not be sufficient. It is known that photosynthetic capacity increases as leaves develop, peaking at full expansion and remains the same or decreases in maturity (Kozlowski, Kramer & Pallardy 1991). Moreover, O_3 can lead to premature leaf senescence (Pell *et al*. 1999). In order to account for the possible age-related differences we measured light-saturated photosynthesis (A) and partitioned relative stomatal limitation for all leaves on branches from three different crown positions.

The goal of this work was to elucidate whether the interactive effect of elevated CO_2 and O_3 on photosynthesis under steady-state conditions is primarily mediated by stomatal or mesophyll processes. For that we estimated the stomatal limitation based on instantaneous light-saturated photosynthesis (A), complemented with parameters from A–C_i curves. Since the plants were young and the canopy unclosed, most of the leaves were exposed to near full sunlight. Therefore, light-saturated photosynthesis (A) was chosen to describe CO_2 assimilation rates. We also recorded stomatal conductance and estimated the content of Rubisco enzyme in leaves. The work was carried out on two aspen clones previously shown to have differential O_3 tolerance (Karnosky *et al*. 1996).

MATERIALS AND METHODS

Experimental site and plant material

Two aspen (*Populus tremuloides* Michx.) clones (no. 216, O_3 tolerant and no. 259, O_3 sensitive), were grown in a free air carbon dioxide enrichment (FACE) facility (Karnosky *et al*. 1999) near Rhinelander, WI, USA. The experimental site is located at 45°30′ N, 89°30′ W, on sandy loam soil. The differential O_3 tolerance of these two clones has been characterized on the basis of the visual foliar symptoms and growth parameters (Karnosky *et al*. 1996, 1998). The plant material was propagated from greenhouse-grown stock plants. The rooted cuttings were 6-months-old by the time of planting in July 1997 and about 1·5 m tall by the time of measurement in 1998.

The treatments: control, elevated CO_2, elevated O_3 and elevated $CO_2 + O_3$ were triplicated and arranged in a randomized complete block design. Each ring was 30 m in diameter and the trees were planted at a density of one tree per square metre. The detailed description of the experimental set-up and conditions can be found elsewhere (Dickson *et al*. 2000).

Fumigation

Control plants were exposed to ambient air (daytime [CO_2] was 360 p.p.m., night-time [CO_2] varied from 360 to 500 p.p.m. and daytime [O_3] averaged 36 p.p.b.). Elevated CO_2 was applied from 1 May (bud break) to 15 October (leaf drop) and elevated O_3 from 15 May to 15 October 1998. Elevated CO_2-treated plants (alone and in combination with O_3) were exposed to 560 p.p.m. CO_2 from sunrise to sunset. The 1 min integrated CO_2 concentration was within 10% of the target concentration 81% of the time and within 20% of the target 93% of the time. Elevated O_3-treated plants (alone and in combination with CO_2) received 97·8 p.p.m. × h of O_3 seasonally, with average daytime (0700 to 1900 h) exposure concentration of 55 p.p.b. compared with the ambient seasonal O_3 dose of 65·3 p.p.m. × h averaging at 36 p.p.b. from 0700 to 19·00 h. Ozone fumigation followed a typical diurnal curve (with peak concentrations in early afternoon) and generally lasted from 0700 to 1900 h; there were no O_3 fumigations during rain, fog, mist or dew conditions.

Measurements

Gas exchange

Gas exchange of the aspen clones was measured from 9 to 30 July with a LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Two plants per clone were sampled from each ring, totalling six plants per treatment. Light-saturated photosynthesis (A) measurements were taken at the CO_2 concentration at which the plants were grown ('operating [CO_2]' was 360 p.p.m. for control and elevated O_3 and 560 p.p.m. for elevated CO_2 and $CO_2 + O_3$) under saturating light of 1000 μmol m^{-2} s^{-1} at ambient temperature (28–34 °C) and air humidity (40–60%). A was measured in all leaves of the current terminal and of two lateral branches (one from the upper and one from the lower crown) to account for differences by leaf age and the hierarchical order of a branch. Leaf plastochron index (LPI) (Larson & Isebrands 1971) was used as a measure of

physiological age of the leaf. The LPI system provides an easy way to estimate relative leaf age on trees with indeterminate growth habit based on its position on the shoot. According to Larson & Isebrands (1971), the youngest leaf on a branch longer than 2·5 cm was assigned LPI = 1, leaves older than that were assigned successively higher LPI values. The LPI numeration was applied independently to each branch. Based on changes in morphological and physiological characteristics (Larson & Isebrands 1971; Coleman *et al*. 1995), the leaves were divided into age classes (young, LPI = 1–8; recently mature, LPI 9–14; mature, LPI = 15–23; old, LPI > 23). Since the leaf maturation process may vary depending on environmental conditions, the assignment into different age classes may slightly vary from study to study.

Photosynthesis versus intercellular [CO_2] (A–C_i) response curves were measured on intact leaves under saturating light intensity of 1000 μmol m^{-2} s^{-1} at 25 ± 1 °C and 40–55% air humidity. A typical A–C_i curve is shown on Fig. 1 to exemplify the parameters estimated from the curves. Five A–C_i curves per clone per leaf class [young (LPI = 5–8), recently mature (LPI = 10–13) and mature leaves (LPI = 15–22)] were measured during the course of study. Because of obvious time constraints it was not possible to measure A–C_i curves on all leaves where an A reading was taken. The parameters estimated from the A–C_i curves were assumed to be constant for a given leaf class and the same values were used for the whole leaf class in stomatal limitation calculations.

The relative stomatal limitation to photosynthesis was calculated with sensitivity analysis method according to Jones (1998):

$$l_s = 100 * \frac{r_s}{r_s + r^* + r_{bl}} \quad (1)$$

where l_s is relative stomatal limitation, r_s is stomatal resistance, r^* is the cotangent to the A–C_i curve at the operating point (cot α in Fig. 1) and r_{bl} is boundary layer resistance (r_{bl} = 0·352, provided by the software for the LI-6400).

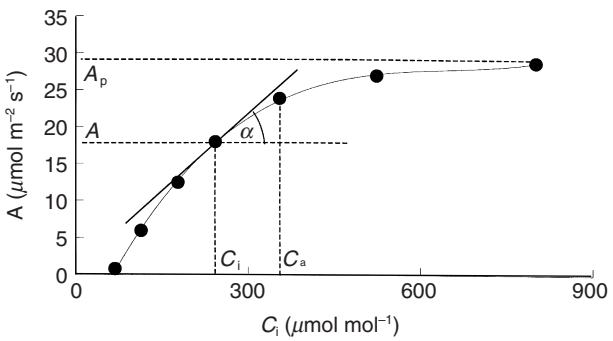

Figure 1. A typical A–C_i curve measured in clone 259 grown under elevated O_3 treatment (ambient [CO_2] = 360 p.p.m.). A, assimilation rate at growth C_i; A_p, photosynthetic capacity (CO_2- and light-saturated); C_a, ambient CO_2 concentration; C_i, intercellular CO_2 concentration; α, the angle of ascent of the A–C_i curve at the operating point. cot α is used for calculating relative stomatal limitation as described in Materials and Methods.

The weather conditions were stable and favourable during the course of measurements and no drought had occurred prior to or during the measurement period. Plants of at least two treatments were sampled each day to eliminate the possibility of variations in weather conditions confounding treatment effects. Plants from different treatments were sampled in random order, which should eliminate the possibility of diurnal patterns confounding treatment effects.

Protein extraction and Rubisco content

Leaf samples (LPI = 9–13) for Rubisco analysis were collected from the current terminal of trees on 4 and 5 August between 1500 and 1800 h, packed in aluminium foil, fast-frozen in liquid nitrogen and stored at –80 °C until further analysis. Leaf punches (14 mm diameter) were collected and weighed immediately for calculating leaf weight per area. The leaf punches were then dried at 80 °C for 24 h for calculating the dry : wet weight ratio.

Total soluble protein extraction, quantification and separation by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) were performed as described by Brendley & Pell (1998). The images of protein gels were digitized by using the Eagle Eye II still video system (Stratagene, La Jolla, CA, USA). Purified Rubisco from spinach (Sigma, St. Louis, MO, USA) was run alongside the samples on each gel to verify the identity of the bands and to adjust for differences in background intensity. The intensity of bands representing Rubisco subunits and the cumulative intensity of all bands was estimated with a Line Profile tool in ImageTool 2·0 software package (The University of Texas Health Science Center, San Antonio, TX, USA). The amount of Rubisco in each sample was calculated from total soluble protein content and Rubisco percentage of total protein.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using treatment, LPI and branch as class variables (SAS, 1996; SAS Institute, Cary, NC, USA). Significant differences at a given leaf position were calculated with two-tailed t-test at $P < 0·05$. For three-way analysis a general linear models procedure was used and statistically significant effects were calculated with Duncan's multiple range test at $P < 0·05$ level. Data on the figures is given as mean ± SE, $n = 3$.

RESULTS

Gas exchange and stomatal conductance

The instantaneous light-saturated photosynthesis (A) versus LPI profiles were similar in both clones for current terminals and lateral branches. Elevated CO_2 increased values of A in both clones at all leaf positions. Elevated O_3 decreased A in the leaves with LPI > 15 in the O_3-sensitive

clone (259) at both ambient and elevated CO_2, whereas in the O_3-tolerant clone (216) the decrease was observed only in a few of the oldest leaves. The maximum values of A were reached by leaves of LPI = 10–14, whereas the rates in older leaves declined slightly (Fig. 2). The average change of A in this leaf class of clone 216 was +33% under elevated CO_2 and +1% under elevated O_3, in comparison with control (Fig. 2). The corresponding values for clone 259 were +38% under elevated CO_2 and –11% under elevated O_3. The response of clone 216 to the combination of elevated CO_2 and O_3 was greatest of all, +49%, and in clone 259 the increase was +38%.

The stomatal conductance (g_s) increased in young and recently mature leaves with increasing leaf age (LPI = 1–15) and stayed at that level or decreased in the mature and old leaves (LPI = 15–30). Elevated CO_2, alone and in combination with O_3, decreased g_s in both clones (Fig. 3). Elevated O_3 decreased g_s in the mature and old leaves of clone 259 at both ambient and elevated CO_2, whereas it did not affect g_s in clone 216. The ratio of intercellular to ambient CO_2 concentration was constant ($C_i/C_a = 0{\cdot}70 \pm 0{\cdot}035$; mean ± SD; data not shown) across different LPI values and was not altered by treatment conditions.

Relative stomatal limitation

Relative stomatal limitation (l_s) to A under control conditions was 9–12% in the recently mature and mature leaf zone (LPI = 10–23) of both clones. The l_s was not affected by elevated CO_2 in clone 216, but it decreased by about half in clone 259 (Fig. 4). Elevated O_3 did not significantly alter l_s in either clone. Elevated CO_2 + O_3 decreased the l_s in both clones by about one-third.

Rubisco content

Little clonal and developmental variability was observed in the Rubisco content of leaves under control conditions

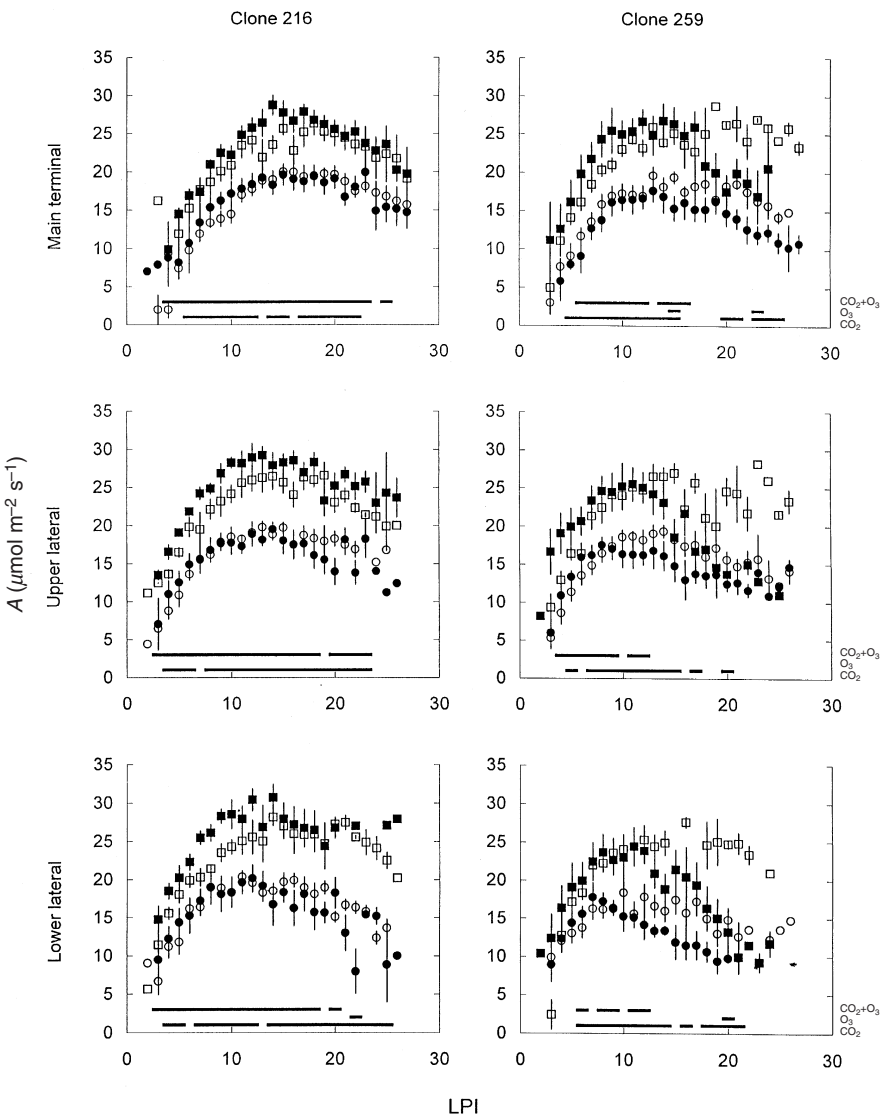

Figure 2. Light saturated photosynthesis (A) versus leaf plastochron index (LPI) relationships for three different shoot classes (main terminal, lateral branch in the upper one-third of the crown and lateral branch in the lower one-third of the crown) of two aspen clones exposed to different CO_2 and O_3 regimes, plotted against LPI. Control (○), elevated CO_2 (□), elevated O_3 (●) and elevated CO_2 + O_3 (■). Data are given as mean ± SE (n = 3). Statistically significant difference of treatments compared to control at a given leaf position (calculated with two-tail t-test, $P < 0{\cdot}05$) is shown with solid bars at the bottom of the graph.

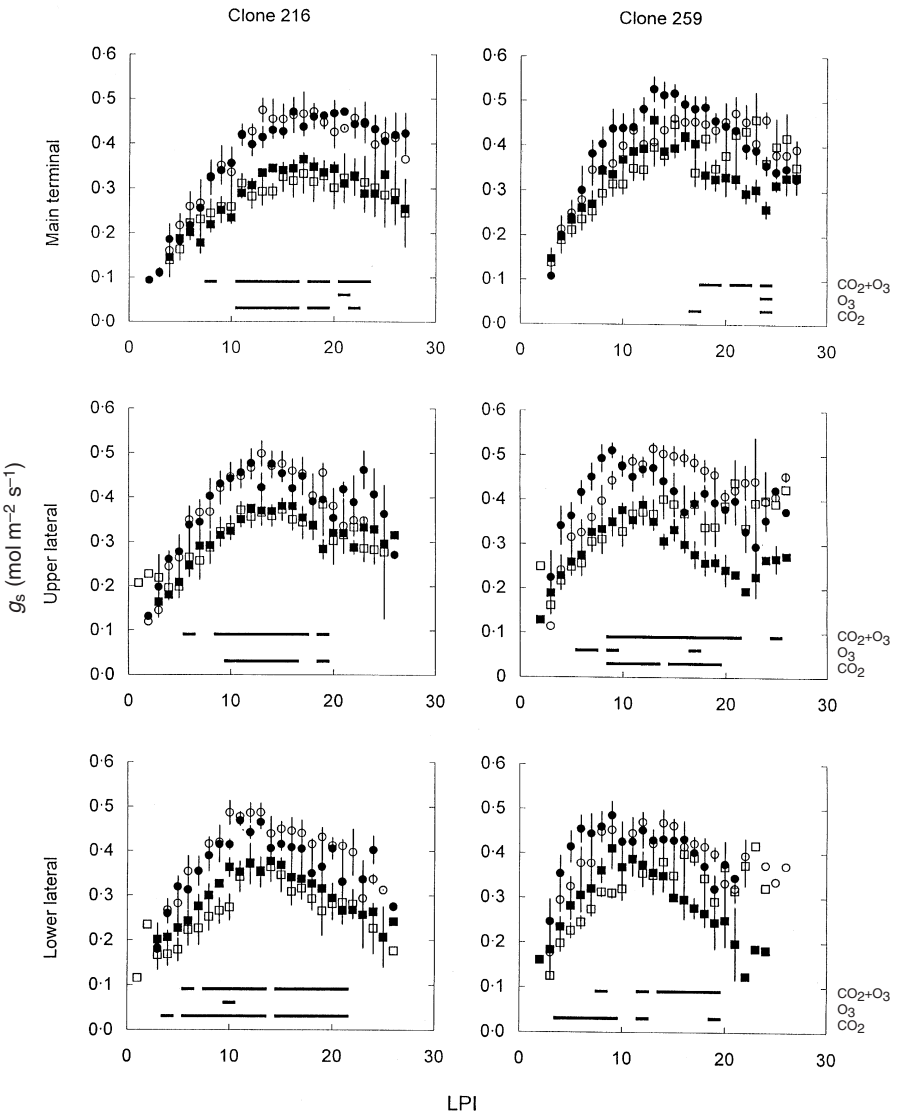

Figure 3. Stomatal conductance (g_s) versus leaf plastochron index (LPI) relationships for three different shoot classes (main terminal, lateral branch in the upper one-third of the crown and lateral branch in the lower one-third of the crown) of two aspen clones exposed to different CO_2 and O_3 regimes, plotted against LPI. Symbols are the same as in Fig. 2.

(Fig. 5). Exposure to elevated CO_2 resulted in a slight increase in Rubisco content in clone 216 and in a slight decrease in the old leaves (LPI > 23) in clone 259. Exposure to elevated O_3 decreased Rubisco content in the young leaves and increased it in the old leaves of both clones. Exposure to elevated $CO_2 + O_3$ decreased Rubisco content in all leaves of both clones (Fig. 5). The significance of treatment differences at $P < 0{\cdot}05$ level could be shown in mature and old leaves but not in young and recently mature leaves. The changes reported were due to specific decrease in Rubisco enzyme and not due to changes in total soluble protein content (data not shown).

The statistical significance of main and combined effects of treatment, branch and LPI on A, g_s, l_s and Rubisco content is given in Table 1.

DISCUSSION

The instantaneous rates of photosynthesis were increased under elevated CO_2, yet the photosynthetic capacity (light- and CO_2-saturated photosynthesis; A_p in Fig. 1) of leaves can decrease under both elevated CO_2 and O_3 (Lippert *et al.* 1997; Grams *et al.* 1999). This is known as photosynthetic acclimation and it is most often observed if plant growth volume becomes restricted (Drake *et al.* 1997). However, in the present study no such acclimation of photosynthetic capacity to elevated CO_2 was observed (Sôber *et al.* unpublished).

We did not observe that clone 216 was more sensitive to O_3 at elevated CO_2 than clone 259, as has been suggested earlier (Kull *et al.* 1996). The contradiction could arise from different soil fertility and different nitrogen content of leaves between the two studies (Sôber *et al.* unpublished) as inadequate nitrogen supply can render plants more susceptible to O_3 (Pääkkönen & Holopainen 1995). However, similar to Kull *et al.* (1996) but contrary to others (Dickson *et al.* 1998; Volin, Reich & Givnish 1998), we found that elevated CO_2 does not always ameliorate the negative effects of O_3, as it did not prevent the O_3-induced drop in A in mature and old leaves of the O_3-sensitive clone 259 (Fig. 2).

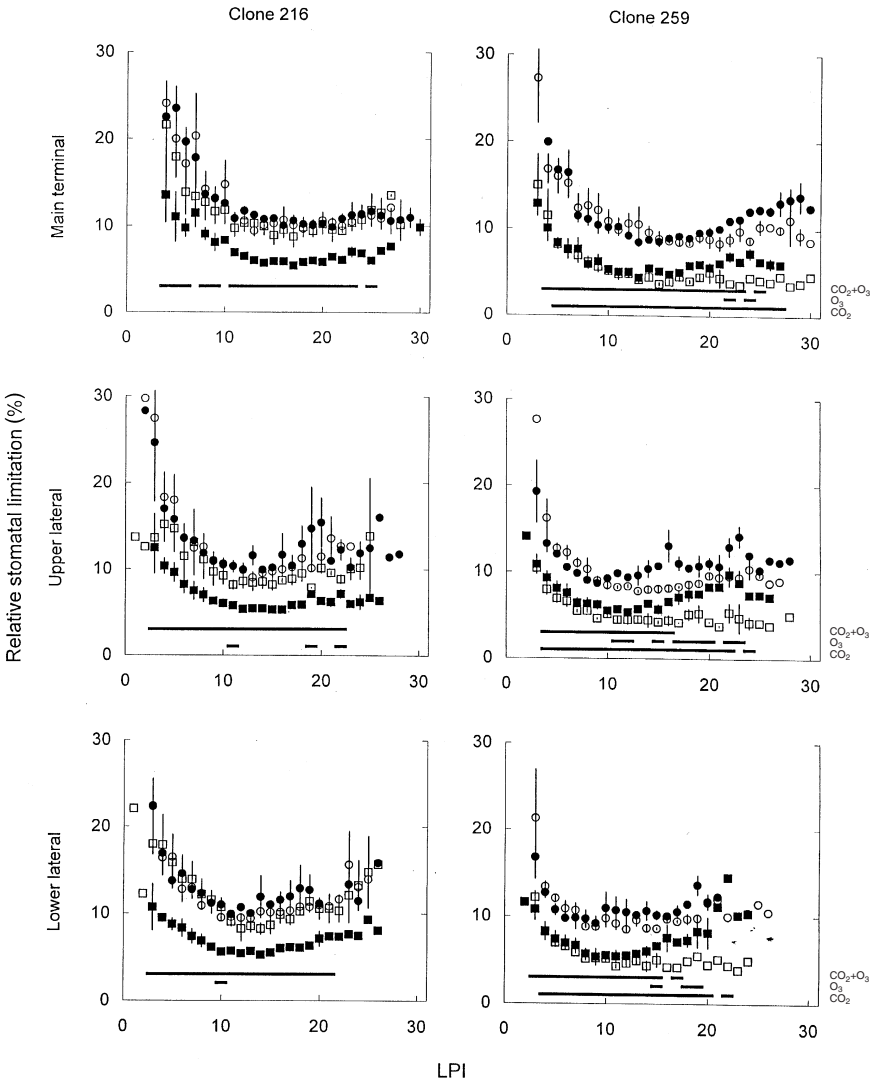

Figure 4. Relative stomatal limitation (l_s) to light-saturated photosynthesis calculated with sensitivity analysis method (Jones 1985) for three different shoot classes (main terminal, lateral branch in the upper one-third of the crown and lateral branch in the lower one-third of the crown) of two aspen clones exposed to different CO_2 and O_3 regimes, plotted against leaf plastochron index (LPI). Symbols are the same as in Fig. 2.

The different conclusion reached by Dickson *et al.* (1998) and Volin *et al.* (1998) in comparison with our current work may derive from the fact that the conclusion by Dickson *et al.* (1998) and Volin *et al.* (1998) was based on the changes observed in whole-plant biomass accumulation instead of the photosynthetic parameters of single leaves as measured in our study. It has been shown that O_3 exposure may in some cases stimulate leaf growth (Pääkkönen *et al.* 1996), that could compensate at the whole plant level for the accelerated senescence of individual leaves.

The higher l_s values in the leaves with LPI = 1–6 in comparison with the older leaves are probably the result of our experimental methods. As mentioned earlier, A–C_i curves were measured in only a few leaves per leaf age category and not in each leaf where an A reading was taken. The developmental changes from one leaf position to the next are greatest in the young leaf zone and using the A–C_i curves from leaves with LPI = 5–8 probably resulted in extrapolation errors in leaves younger than that. Another source of variation could be the non-functional developing stomata that are characteristic to expanding leaves (Choinski & Wise 1999).

The decrease in l_s, observed under elevated CO_2 in clone 259 and under elevated CO_2 + O_3 in both clones, implies increased mesophyll limitation, which we have characterized with the cotangent of the A–C_i curve at the operating [CO_2] and that more specifically refers to ribulose-1,5-bisphosphate (RuBP) regeneration limitation (Sage 1994). Under elevated CO_2 A_a is of course closer to A_p (Fig. 1) than under ambient CO_2 and the slope of the curve is smaller, because A_p did not change in our study (Sôber *et al.* unpublished). Therefore, the change in l_s is expected. What is curious about l_s is that in clone 216 the drop only occurs under CO_2 + O_3 but not under elevated CO_2 alone. Since l_s is calculated from three component parameters, of which one (r_{bl}) is constant, the change in stomatal limitation means a change in opposite direction in mesophyll limitation. If l_s decreases under CO_2 + O_3 in clone 216, but not under CO_2, we can say that there must be a mesophyll component compensating for the increase in C_i that occurs

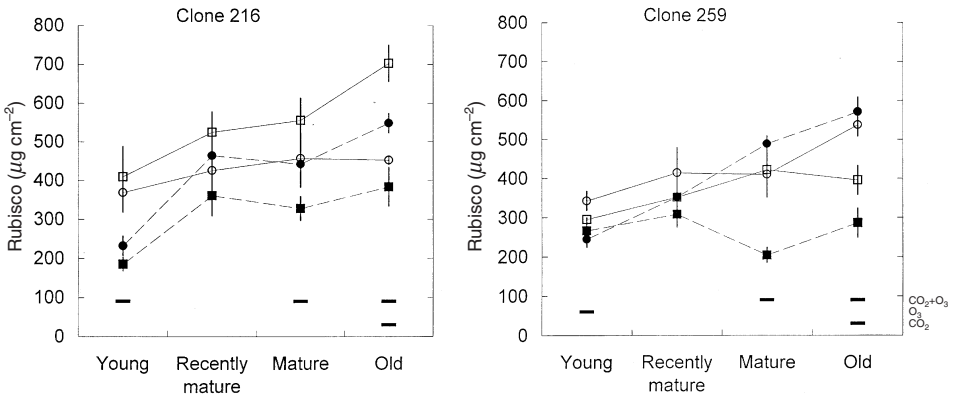

Figure 5. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content in the leaves of current terminal of two aspen clones exposed to different CO_2 and O_3 regimes. Symbols are the same as in Fig. 2. Leaf age classes are as follows: young, LPI = 5–8; recently mature, LPI = 10–13; mature, LPI = 15–21; old, LPI = 24–30.

under elevated CO_2. Mesophyll conductance can be characterized as a product of two main components: the CO_2 binding capacity and the electron transport capacity. The factor most often found to determine mesophyll conductance is Rubisco activity (Pell *et al*. 1992; Eichelmann & Laisk 1999), which is more responsive to CO_2 and O_3 than the light harvesting/electron transport component (Farage *et al*. 1991; Soja, Pfeifer & Soja 1998; Li *et al*. 1999). Our data on Rubisco content suggest, that the differential response in this enzyme between elevated CO_2 and $CO_2 + O_3$ treatments may indeed provide this compensatory link. The elevated CO_2-exposed plants of clone 216 had significantly higher Rubisco content than plants exposed to $CO_2 + O_3$ and this may have been sufficient to match the increased CO_2 availability. No such increase was observed in CO_2-exposed plants of clone 259 and the Rubisco content decreased in $CO_2 + O_3$ exposed plants of both clones, thus potentially increasing mesophyll resistance. Our report of older leaves having higher Rubisco content than younger leaves contrasts with commonly observed patterns (e.g.

Table 1. Analysis of variance for light-saturated photosynthesis (A), stomatal conductance (g_s), relative stomatal limitation (l_s) and Rubisco content showing the significance of main and combined effects of treatment (TRT), leaf plastochron index (LPI) and branch (not applicable for Rubisco)

		Clone 216			Clone 259		
Parameter	Source of variation	Mean square	d.f.	P	Mean square	d.f.	P
A	TRT	2611	3	0·0001	2128	3	0·0001
	LPI	270	33	0·0001	191	33	0·0001
	Branch	63·0	2	0·0012	41·8	2	0·0519
	TRT*LPI	8·78	84	0·6143	23·1	75	0·0014
	Branch*LPI	20·9	52	0·0001	19·3	52	0·0494
	Branch*TRT	24·6	6	0·0155	33·8	6	0·0269
	TRT*LPI*Branch	5·02	137	1·0000	5·82	120	1·0000
g_s	TRT	0·370	3	0·0001	0·278	3	0·0001
	LPI	0·109	29	0·0001	0·0756	33	0·0001
	Branch	0·014	2	0·0256	0·0134	2	0·0178
	TRT*LPI	0·00293	75	0·8963	0·00917	82	0·0001
	Branch*LPI	0·0098	51	0·0001	0·00934	48	0·0001
	Branch*TRT	0·00576	6	0·1601	0·0164	6	0·0001
	TRT*LPI*Branch	0·0021	128	0·9999	0·00262	114	0·9263
l_s	TRT	1265	3	0·0001	1169	3	0·0001
	LPI	194	32	0·0001	99·4	33	0·0001
	Branch	60	2	0·0042	5·7	2	0·1307
	TRT*LPI	14·6	78	0·0336	9·3	83	0·0001
	Branch*LPI	25·7	52	0·0001	11	49	0·0001
	Branch*TRT	24·8	6	0·0343	14·8	6	0·0001
	TRT*LPI*Branch	10·9	131	0·4662	2·4	130	0·8469
Rubisco	TRT	94445	3	0·0001	66797	3	0·0001
	LPI	77031	3	0·0001	42081	3	0·0002
	TRT*LPI	6518	9	0·3293	15536	9	0·0055

d.f., degrees of freedom; P, probability.

Brendley & Pell 1998). We hypothesize that our results reflect seasonal changes in nutrient availability, that relatively more Rubisco protein was synthesized early in the season than later, when the upper leaves developed. This would be consistent with the notion that Rubisco may be produced in excess of actual photosynthetic needs and used as a storage compound for nitrogen (Miller & Huffaker 1982).

The high g_s values indicate that the plants had adequate water supply and that the changes in stomatal limitation represent treatment effects and are not obscured by water deficit. Although the mature and old leaves of clone 259 showed decreasing g_s values under elevated O_3 and $CO_2 + O_3$, the stability of the C_i/C_a ratio at 0·7 suggests that this is a secondary response after changes in mesophyll processes. This conclusion is supported by similar findings by Fiscus *et al*. (1997), who showed that decreased stomatal conductance could be the result and not the cause of decreased photosynthesis.

The changes in the above parameters indicate that there is tight coordination between mesophyll and stomatal processes. A high degree of co-regulation between these two processes was also observed by Allen & Pearcy (2000), who looked at the transient effects under changing light conditions. An elegant way to coordinate the mesophyll processes with stomatal conductance was characterized by Mott & Woodrow (1993), who showed that the Rubisco activation state is responsive to C_i. Therefore, we could expect higher Rubisco activity under elevated CO_2 and $CO_2 + O_3$ treatments if the concentration of the enzyme was equal to that of controls. In the absence of data on the Rubisco activation state, however, we assume that total Rubisco activity is proportional to its content under given CO_2 concentration.

In conclusion we can say that elevated CO_2 did not prevent the O_3-induced decrease of A in mature and old leaves of the O_3-sensitive clone, 259, whereas it did prevent it in the O_3-tolerant clone, 216. The treatment differences in A are likely to accrue over time and show up as differential biomass accumulation. Our results suggest that the treatment differences in photosynthesis are primarily caused by non-stomatal factors, but the mesophyll and stomatal processes are closely coordinated. Changes in Rubisco content may have contributed to specific changes in mesophyll conductance under elevated CO_2 and $CO_2 + O_3$. Despite the significant age-related changes in A and g_s profiles in both clones, the l_s showed little dependence on leaf age suggesting the universality of the underlying regulative mechanisms.

ACKNOWLEDGMENTS

This work was in part supported by the USDA Forest Service Northern Global Change Program, The US Department of Energy (DE-FG02–95ER62125), National Science Foundation (DBI-9601942; IBN-9652675), the National Council of the Paper Industry for Air and Stream Improvement (NCASI) and Michigan Technological University. We gratefully acknowledge J. Sinn, Dr G. Torsethaugen, and Dr S. M. Lukyanova for help and advice on performing Rubisco analysis and Dr Olevi Kull, Dr David Ellsworth and two anonymous reviewers for constructive comments on the earlier versions of the manuscript.

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Received 25 August 2000; received in revised form 25 October 2000; accepted for publication 25 October 2000