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Effects of low ozone exposure of spring wheat on net CO_2 uptake, Rubisco, leaf senescence and grain filling

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SUMMARY

Effects of ozone on spring wheat (*Triticum aestivum* L. cv. Satu) were studied in an open-top chamber experiment during two growing seasons (1992–1993) at Jokioinen in south-west Finland. The wheat was exposed to filtered air (CF), non-filtered air (NF), non-filtered air + 35 nl l^{-1} ozone for 8 h d^{-1} (NF^+) and ambient air (AA). Each treatment was replicated five times. Two wk after anthesis, after 4 wk of ozone treatment (NF^+, 45 nl l^{-1} 1000–1800 hours, seasonal mean) the net CO_2 uptake of wheat flag leaves was decreased by *c.* 40 % relative to CF and NF treatments, both initial and total activity of Rubisco and the quantity of protein-bound SH groups were decreased significantly. Added ozone also significantly accelerated flag leaf senescence recorded as a decrease in chloroplast size. The effect was significant 2 wk after anthesis, and senescence was complete after 4 wk. In the CF and NF treatments senescence was complete 5 wk after anthesis. The significant effect of ozone on the chloroplasts and net CO_2 uptake 2 wk after anthesis did not affect the grain filling rate. However, since the grain filling period was shorter for ozone fumigated plants, kernels were smaller. The decrease in 1000-grain weight explained most of the yield reduction in the plants under NF^+ treatment. The results indicate that wheat plants are well buffered against substantial decrease in source activity, and that shortened flag leaf duration is the major factor causing ozone-induced yield loss.

Key words: *Triticum aestivum* L., open-top chambers, chloroplasts, sulphydryl groups.

INTRODUCTION

It is well established that ambient ozone concentrations in the US and Europe are sufficiently high to decrease yields of agricultural crops including wheat (*Triticum aestivum* L.) (Heck, Taylor & Tingey, 1988; Skärby *et al.*, 1993). Although it is well documented that ozone causes yield loss, the physiological mechanisms that induce these losses remain largely unexplained. Numerous studies have provided evidence of ozone induced changes in plants that could reduce yield, including accelerated senescence and leaf drop and decrease in net photosynthesis (Grandjean & Fuhrer, 1989; Darrall, 1989; Pell, Eckardt & Glick, 1994*a*, Ojanperä *et al.*, 1992). However, few studies have been conducted in an attempt to link those data with responses in yield.

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Ozone is known to inhibit foliar photosynthesis (Lehnherr *et al.*, 1987; 1988; Dann & Pell, 1989; Grandjean, Grimm & Fuhrer, 1992), with the degree of inhibition depending on ozone concentration, duration of exposure and the plant species and cultivar (Darrall, 1989; Pell, Eckardt & Enyedi, 1992). Although evidence exists that ozone can alter normal patterns of stomatal opening and closing (Darrall, 1989), thereby affecting CO_2 uptake, it has been demonstrated that O_3-induced reduction in net photosynthesis occurs soon after initial exposure and is independent of stomatal closure (Atkinson, Robe & Winner, 1988; Pell *et al.*, 1992). It has been proposed that the stomatal closure might result from the build-up of internal CO_2 in leaves which occurs when photosynthesis is impaired (Atkinson *et al.*, 1988; McKee, Farage & Long, 1995). In studies with wheat, data suggest that light capture/electron transport mechanisms are not involved (Farage *et*

al., 1991; Grandjean *et al.*, 1992; Nie, Tomasevic & Baker, 1993). Reduction in carboxylation capacity due to ozone is probably the most satisfactory explanation for decreases in net photosynthesis (Lehnherr *et al.*, 1988; Dann & Pell, 1989; Farage *et al.*, 1991).

The object of most investigations concerning air pollution effects on photosynthetic carbon assimilation is ribulose bisphosphate carboxylase/oxygenase (Rubisco), which is the first of the 13 enzymes regulating the Calvin cycle in the chloroplast stroma. The enzyme has two functions: (1) the carboxylation of ribulose bisphosphate (RuBP) to yield two molecules of 3-phosphoglycerate (PGA) and (2) the oxygenation of RuBP to phosphoglycolate and PGA (Knight, 1989). Ozone has been reported to decrease the activity and quantity of Rubisco in several plant species, including wheat. Evidently it affects both the synthesis and the degradation of Rubisco. The ozone induced degradation of Rubisco is probably triggered by oxidation rather than by a change in protease activity; ozone might induce oxidative modification of Rubisco leading to increased susceptibility to proteolysis (Pell *et al.*, 1994*a*).

Loss of Rubisco from mature leaves has been identified as the key feature of senescence (Dalling, 1987). Although there is evidence that ozone induces accelerated senescence (Ojanperä *et al.*, 1992), it is not known whether the loss in Rubisco is caused by ozone-induced senescence, or if the ozone-induced loss of Rubisco promotes accelerated senescence (Pell *et al.*, 1994*a*). McKee *et al.* (1995) have reported decrease in the maximum quantum efficiency of carboxylation in ozone-treated wheat flag leaves appearing ahead of the general senescent trend, indicating that the latter scenario is more likely.

Non-denaturated Rubisco has a large number of free sulphydryl residues (Garcia-Ferris & Moreno, 1993). The SH groups are responsible for maintaining the correct structural conformation of Rubisco (Takabe & Akazawa, 1975). Ozone-induced oxidation of SH groups in Rubisco could alter the structural integrity of this enzyme, resulting in reduced catalytic activity and increased vulnerability to proteolysis (Pell *et al.*, 1994*b*).

Ojanperä *et al.* (1992) have reported that ozone accelerates the senescence of flag leaves of spring wheat. Decrease in grain yield reflects the effects on flag leaf ultrastructure (Pleijel *et al.*, 1991, 1996) and it has been proposed that accelerated senescence of the flag leaves shortens the grain filling period, thus decreasing yield. The present study was conducted to measure the effect of ozone in concentrations known to occur in the environment, on flag leaf area duration, net CO_2 uptake, Rubisco activity and the quantity of protein-bound SH groups in flag leaves of spring wheat over their entire life span. The aim was to establish the relationship between ozone exposure and changes in these processes affecting yield as well as the effect on ultimate yield.

MATERIALS AND METHODS

Experimental details

The experiment was carried out during two summers (1992–1993) at Jokioinen in south-west Finland. The experimental site was located at 60° 49′ N, 23° 29′ E in 1992 and at 60° 47′ N, 23° 28′ E in 1993. The elevation was *c.* 100 m above sea level. No major air pollutant sources were located in the vicinity. The soil in both sites was classified as heavy clay. In 1992, 550 kg ha^{-1} of NPK compound fertilizer (N-P-K, 20-6-6) and in 1993 500 kg ha^{-1} (23-5-4) was applied. MCPA and mecoprop-p herbicides were used during both years, powdery mildew and aphids were controlled with propiconazole and pirimicarb sprayings.

Open-top chambers (OTCs) were placed on a field of spring wheat when the crop was at the three leaf stage, and experiments were run 18 June–3 September in 1992, and 19 June–8 September in 1993. Chambers were placed on the field in a completely randomized design. Four treatments were applied: filtered air (CF), unfiltered air (NF), and unfiltered air + 35 nl l^{-1} of ozone added between 1000 and 1800 hours local time (NF^{+}), and open-field plots (AA). Each treatment was replicated five times.

The OTCs were of Raleigh design (Heagle, Body & Heck,1973) with added frustum (Kohut, Krupa & Russo, 1978). The aluminium frames (3 m in diameter, 2·8 m in height) of the chambers were covered with clear PVC plastic film panels. The flow rate in the chambers corresponded to approximately three air changes of the full chamber volume (18 m^3) per minute. Envopleat® 40SC prefilters (Environmental Filter Corp., USA) were used in all treatments. In CF treatments filter units consisting of 20 trays were fitted in the stainless-steel fan-boxes. Charcoal and Purafil® filters were used in 1992, and solely charcoal filters in 1993. The activated carbon for the filters was made from crushed coconut shells that had been carbonized by heating at 400–500 °C and activated by exposure to steam at 800–1000 °C (RSE manufacturer pers. comm.). This is the raw material most commonly used in charcoal filters (Wellburn, 1990). Purafil filters (Purafil Inc., Atlanta, GA) consist of alumina pellets impregnated with $KMnO_4$ and are commonly used for additional filtration because activated charcoal filter has little capacity to adsorb NO. Purafil filters oxidize any incoming NO to NO_2, which can then be trapped by the activated charcoal (Wellburn, 1990). The filters and OTCs were purchased from Program Resources, Inc., USA.

Ozone was monitored with Dasibi 1008-PC and Thermo Environmental Instruments Model 49 ozone monitors at *c.* 10 cm above the crop. The ozone monitors were cross-calibrated with the EMEP monitor (European Monitoring and Evaluation Programme) at the Finnish Meteorological Institute. SO_2, NO_2 and NO were monitored by Thermo Environmental Instruments Models 43S and 42S. T.E.I. 43S and 42S monitors were calibrated with the VE 3M calibrator (Environnemet, S.A., France). Ozone was generated by electrical discharge in pure oxygen with a Fischer 502 ozone generator and was bubbled through ultrapure water before it was added to the air in the NF^+ chambers. Water traps were used in order to remove harmful compounds other than ozone possibly generated from oxygen (Wellburn, 1990).

Measurement of net CO_2 uptake

Net CO_2 uptake was measured from the wheat flag leaves during summer 1993, starting at anthesis. A portable IRGA (LAC-3) (ADC Company, UK) was used with PLC-3 as the measuring cuvette. Measurements were always performed at a minumum photon fluence rate of 800 μmol m^{-2} s^{-1}, which was determined to be saturating for the wheat. Owing to the very rainy summer of 1993, with 43 rainy days during the grain-filling period (July and August), the measurements were only carried out on three occasions.

Extraction and assay of Rubisco activity

Flag leaves were frozen in liquid nitrogen in the field immediately after measurement of the net CO_2 uptake. The frozen flag leaves were transported from the field in liquid nitrogen and stored in the ultrafreezer at −80 °C until the assays were performed. While still frozen the flag leaves were homogenized with mortar and pestle in liquid nitrogen with the extraction buffer (100 mM Tricine-NaOH, pH 8·2; 5 mM $MgCl_2$; 5 mM DTT; 1 mM Na_2EDTA). The extraction buffer was CO_2-free and was freshly prepared before use. The homogenates were centrifuged in microfuge tubes at 9000 ***g*** for 1 min. Supernatants were collected and kept on ice until enzyme activities were measured. The procedure from extraction to initial assay was always performed within 5 min. The spectrophotometric assay was based on the measurement of 3-phosphoglycerate-dependent NADH oxidation as described by Lilley & Walker (1974). The reaction was started by adding 20 μl of enzyme extract and 0·66 mM RuBP to a cuvette that contained 88 mM Tricine, pH 8·2; 8·8 mM $NaHCO_2$; 17·6 mM $MgCl_2$; 5 mM phosphocreatine; 5 mM ATP; 0·2 mM NADH; 3 units of GAPDH-PGP (glyceraldehyde-3-phosphate dehydrogenase/phosphoglyceratekinase) and 3 units of creatine phosphokinase. The total volume of solution was 500 μl. The 'initial activity' of Rubisco was followed for 3 min at 340 nm. This initial activity reflects the *in vivo* activity. After this measurement Rubisco was fully activated at 20–25 °C for 10 min with a solution containing 10 mM $NaHCO_3$ and 20 mM $MgCl_2$. Total activity was measured after activation.

SH groups

Frozen flag leaves (0·5 g) were homogenized with mortar and pestle in liquid nitrogen and transferred to centrifuge tubes, after which 5 ml of 0·15 % sodium ascorbate solution was added to each sample (Grill, Esterbauer & Klösch, 1979). The homogenates were thoroughly vortexed before centrifugation at 19000 ***g*** for 23 min (+3 °C). Protein SH content was determined by the method of deKok & Kuiper (1986) except that DTNB (Ellman, 1959) was dissolved in 0·1 M sodium phosphate, pH 8·0. Soluble protein content was assayed according to Lowry *et al.* (1951) with bovine serum albumin used as standard.

Microscopy

Sampling of the leaves for microscopy began each year 2 wk before anthesis, when the flag leaves were fully developed, and was continued weekly until the leaves had completely senesced. Four flag leaves from each chamber were sampled randomly and processed for microscopy. The 1-cm leaf sections were excised 5 cm from the leaf base and put directly in a solution containing 1·5 % glutaraldehyde and 1·5 % paraformaldehyde, 0·015 M sucrose and 2 mM $CaCl_2$ in 0·05 M sodium cacodylate buffer at pH 7·0. Samples, 1 × 2 mm, were cut adjacent to the central midrib of the leaves and kept overnight at 4 °C in the fixative described above. The samples were then rinsed with the sodium cacodylate buffer, post-fixed with a 1 % buffered solution of osmium tetroxide for 4 h, rinsed again with the buffer and dehydrated in the graded series of ethanol (50–99·5 %) followed by propylene oxide. The samples were then embedded in Ladds epoxy medium according to Luft (1961), and sectioned and stained for electron microscopy as described by Sutinen (1987). Chloroplast size was measured with an Olympus® CUE-2 image analyser connected to an Olympus BH-2 light microscope as described by Ojanperä *et al.* (1992).

Grain filling

Grain filling was followed by determining the d. wt of the developing grains twice per week from anthesis to maturity. Three ears were sampled from plants in each chamber. Kernels from the eight spikelet from the base of each ear were threshed by hand and dried to constant weight at 70 °C, after which mean d. wt of the kernels was determined.

Statistics

Descriptive statistics (mean, SD) were used to characterize the data. Statistical analyses for microscopy and plant physiological data were performed using SAS (SAS System for Linear Models, SAS Institute Inc., Cary, NC, USA) computer routines, ANOVA General Linear Model Procedure. Significance was accepted at the $P < 0{\cdot}05$ level (Sokal & Rohlf, 1981).

RESULTS

Air pollution data

Cumulative doses and 8-h (1000–1800 hours) seasonal means of ozone during 1992 and 1993 in different treatments are presented in Table 1, together with the cumulative doses of SO_2, NO, and NO_2. As can be seen from the data, charcoal filters removed up to two thirds of the ozone and most of the sulphur dioxide and nitrogen dioxide. However, the cumulative dose of nitric oxide was almost twice as high in the CF treatment as in the other treatments. This should be borne in mind in the interpretation of the plant physiological data presented below.

Flag leaf duration

Visible injury. The visible symptoms attributable to ozone were similar to those associated with normal senescence. When leaves from the different treatments were compared, however, the visible injury due to the relatively low ozone concentration in the NF^+ treatments could be distinguished. The ozone fumigation caused mottled chlorosis that progressed basipetally, starting from the tips of the leaves, and the leaves were chlorotic 2–3 wk earlier in ozone-fumigated chambers than in the other chambers.

Chloroplast size. Microscopy showed that the size of the chloroplasts decreased during senescence both in ozone-treated plants and control plants. The duration of flag leaves could thus be quantified by measuring the choloroplast size with an image analyser. Ozone fumigation significantly shortened the flag leaf duration. The effect of ozone fumigation was observable after 1 wk from anthesis (Fig. 1 *a*, *b*; $P < 0{\cdot}0035$ in 1992, $P < 0{\cdot}022$ in 1993). No significant difference was recorded for chloroplast size between CF and NF treatments in 1993, but in 1992, chloroplast size decreased faster in the CF treatment than the NF treatment ($P < 0{\cdot}005$). In plants in NF^+

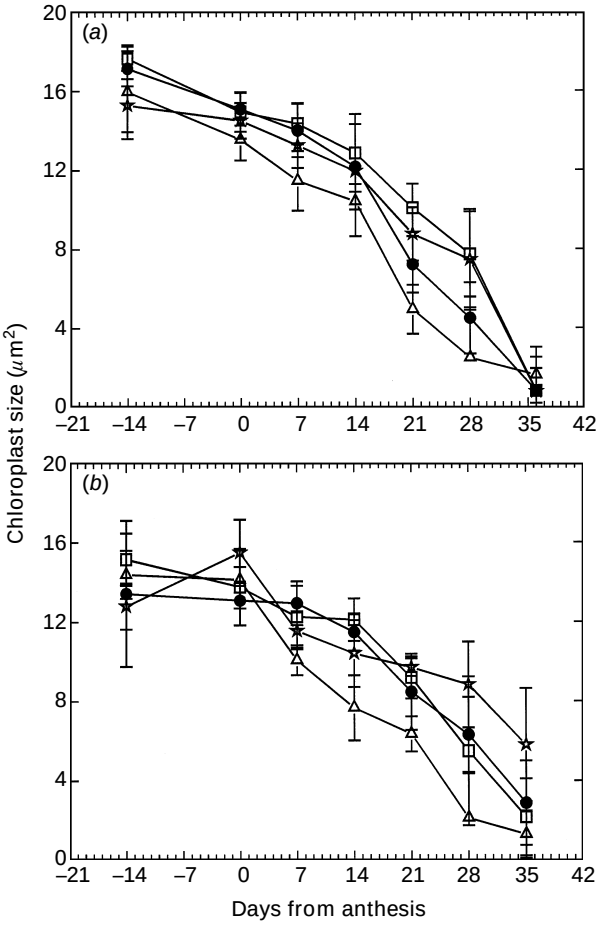

Figure 1. Chloroplast size (μm^2) in different treatments in relation to days from anthesis. (*a*) 1992. (*b*) 1993. ●, CF; □, NF; △, NF^+; ☆, AA. Bars, SD ($n-1$).

Table 1. *AOT0, AOT40 and 8-h (1000–1800 hours) seasonal means of ozone and AOT0 of SO_2, NO_2 and NO ($nl\ l^{-1}$) in 1992–1993*

Treatment	AOT0 O_3	AOT40 O_3	8-h mean O_3	AOT0 SO_2	AOT0 NO_2	AOT0 NO
1992						
CF	16277	0	14	337	1182	1331
NF	34780	671	30	567	2376	671
NF^+	54598	13744	61	630	2460	588
AA	37266	1050	32	685	2398	643
1993						
CF	10189	0	9	93	2069	858
NF	24840	0	21	160	2983	603
NF^+	40608	4609	45	157	3144	479
AA	28843	13	24	233	3090	520

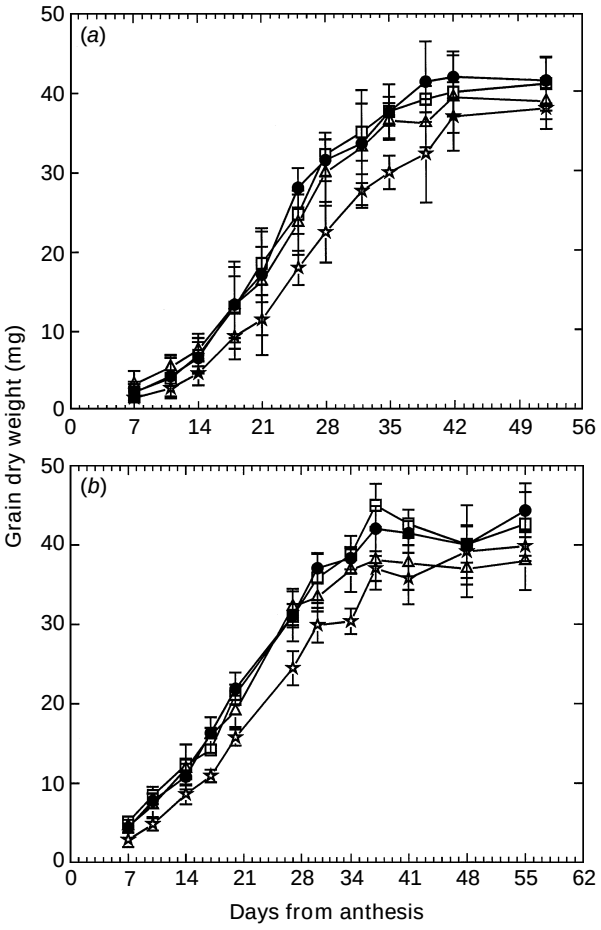

Figure 2. Grain d. wt (mg) during the grain-filling period in different treatments in relation to days from anthesis. (*a*) 1992. (*b*) 1993. ●, CF; □, NF; △, NF$^+$; ☆, AA. Bars, SD ($n-1$).

treatments, in both years, chloroplast size decreased from 15 μm^2 to *c.* 4 μm^2, and the flag leaves were senesced completely 1 wk earlier than in CF and NF treatments (Fig. 1 *a*, *b*).

Grain filling. In both 1992 and 1993 (Fig. 2 *a*, *b*) the d. wt of the wheat kernels increased linearly during the first 4 wk after anthesis, at which point the grains had reached their final weight and grain filling ceased. The grains in the open field plots (AA) gained less weight per day throughout the entire grain-filling period each year, indicating that the climate had a continuous effect on the grain-filling rate. In chambers, there was no difference in the grain-filling rate attributable to the treatments. However, the grain-filling period was a few days shorter in plants from the NF$^+$ treatments than in plants from the other two treatments, and the final grain weight was somewhat smaller (Fig. 5 *a*, *b*; $P < 0{\cdot}07$ in 1992, $P < 0{\cdot}008$ in 1993). This was also reflected in 1000-grain weight in both years (Table 2). There was no significant difference in either year between the grain filling periods or the final grain weights for plants in the CF and NF treatments.

Yield. Grain yield, straw yield, 1000-grain weight and harvest index in the different treatments are summarized in Table 2. Relative to plants in NF and CF treatments, grain yield was decreased for plants in NF$^+$ treatment, where 35 ppb ozone was added to non-filtered air. In 1992, the decrease was 18 % relative to NF and in 1993 6 %. In 1992 the yield was lower in the CF treatment than the NF treatment. Probably this was due in part to the extremely dry spring in 1992 (1 mm rainfall during May), which caused poor germination of seeds in two of the five CF plots. In spite of the irrigation applied later in the spring when the experiment began, the stands in these two plots remained sparse and both the grain and the straw yields were *c.* 20 % lower in the corresponding two chambers than in the other CF chambers. When data for these two chambers were excluded from analyses, the yield reduction in the

Table 2. *Grain and straw yields in different treatments years 1992–1993 in Jokioinen, Finland*

	Treatment			
	CF	NF	NF$^+$	AA
1992				
Straw yield (t ha^{-1})	5·4 ab	5·9 a	5·4 b*	6·0
Grain yield (t ha^{-1})	3·5 a	3·7 a	3·05 b*	3·5
Difference from NF(%)	−5·4		−17·6	−5·4
1000 grain weight	33·8 a	34·4 a	30·8 b***	29·5
Harvest index (HI)	39	38	36	36
1993				
Straw yield (t ha^{-1})	7·3 a	6·2 a	6·6 a	7·4
Grain yield (t ha^{-1})	4·8 a	4·5 a	4·2 b*	4·4
Difference from NF(%)	6·6		−6·6	−2·3
1000 grain weight	36·4 a	36·7 a	32·1 b***	34·1
Harvest index (HI)	40	42	41	37

Values marked with different letters are significantly different (ANOVA, * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$).

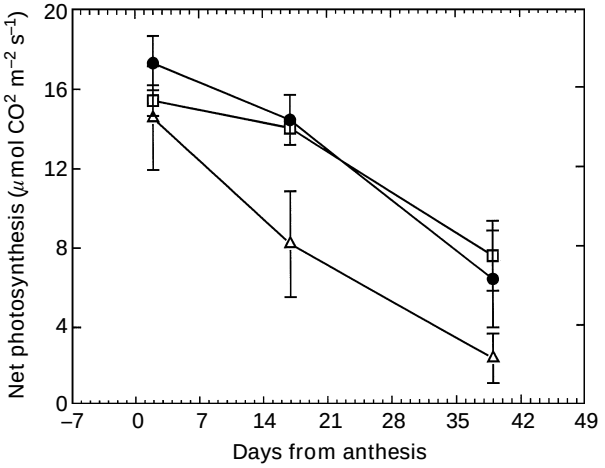

Figure 3. Net photosynthesis (μmol CO_2 m^{-2} s^{-1}) in open-top chambers in 1993 in relation to days from anthesis. ●, CF; □, NF; △ NF^+. Bars, SD ($n-1$).

CF chambers relative to NF chambers was only *c.* 5 %, which corresponds to the plant physiological data measured in plants from those chambers.

Net CO_2 uptake and Rubisco. At anthesis, the difference in net CO_2 uptake in the three treatments was not significant. Two wk after anthesis the net CO_2-uptake was 40 % lower in NF^+ chambers than in NF and CF treatments ($P < 0{\cdot}002$). At the end of the study, 5 wk after anthesis, net CO_2 uptake had declined in all the treatments compared with 2 wk after anthesis. The NF^+ value was close to zero whereas the CF and NF were higher and not significantly different (Fig. 3).

When Rubisco activity was determined per unit f. wt of flag leaves, a combination of two effects was observed: decrease in the quantity of Rubisco and decrease in its activity. The pattern of the Rubisco activity per unit f. wt (Fig. 4*a*, *b*) was similar to the pattern of the changes in net CO_2 uptake in the different ozone exposures during the growing season.

At all times of sampling the initial Rubisco activity was between 40 and 60 % of the total activity. The % activity of Rubisco in the different ozone exposures varied with the growth stage of the plants. At early senescence, initial Rubisco activity seemed to be less affected by ozone than total activity, and % activity increased in the ozone treatments. In the late senescence stage the % activity was lower in the NF^+ plants than in the NF plants.

SH groups. The concentration of SH groups decreased in all treatments, from *c.* 40 nmol mg^{-1} soluble protein to near zero during the duration of the flag leaves (Fig. 5*a*, *b*). Compared with other treatments, however, ozone fumigation accelerated the degradation of SH groups. Differences were statistically significant in 1992 starting at anthesis ($P < 0{\cdot}02$) to *c.* 3 wk after anthesis ($P < 0{\cdot}0001$). In 1993 the difference between the NF treatment and

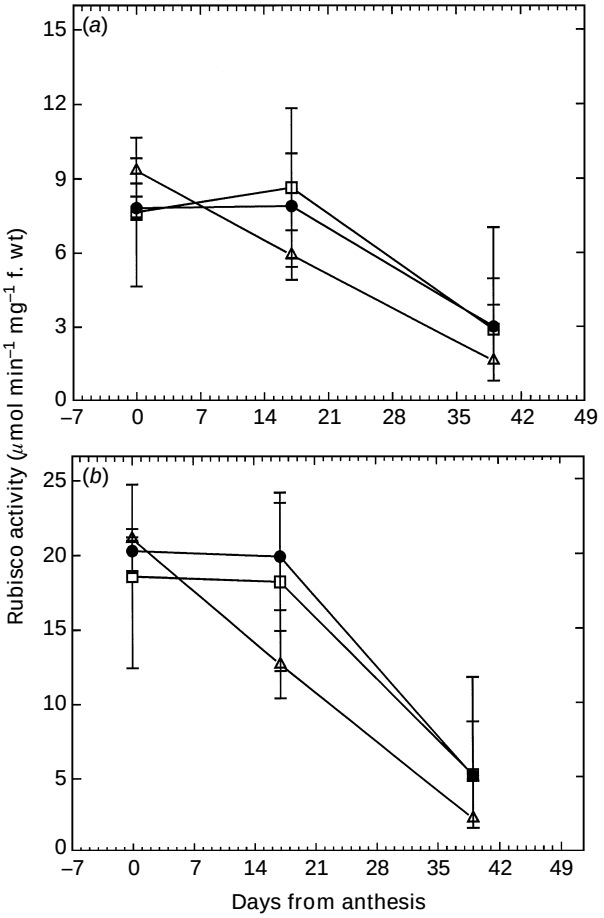

Figure 4. Activity of Rubisco (μmol min^{-1} mg^{-1} f. wt) in different treatments in 1993 in relation to days from anthesis. (*a*) Initial activity. (*b*) Total activity. ●, CF; □, NF; △, NF^+. Bars, SD ($n-1$).

NF^+ treatment was statistically significant from 3 wk after anthesis ($P < 0{\cdot}03$), corresponding to the decrease in the net CO_2 uptake and Rubisco activity, and continued to be significant for 3 wk ($P < 0{\cdot}002$, $P < 0{\cdot}04$). The concentration of protein-bound SH groups was lower in charcoal-filtered air than in non-filtered air in both years, but the difference was statistically significant only in 1992 ($P < 0{\cdot}002$).

DISCUSSION

Schnyder (1993) has detailed three processes limiting grain yield: source activity (net photosynthesis in the green organs linked to grain filling), translocation of photosynthate from source to sink and sink activity in the growing grain (quantitatively dominated by starch synthesis and storage in the endosperm).

In modern wheat cvs the three processes are likely to be in balance and an increase in one process does not necessarily lead to an increase in yield since one of the other processes might be limiting. However, if all three processes could proceed for a longer time they would lead to an increase in yield. This explains why in a highly bred crop like wheat the green leaf area duration is a more important predictor of grain

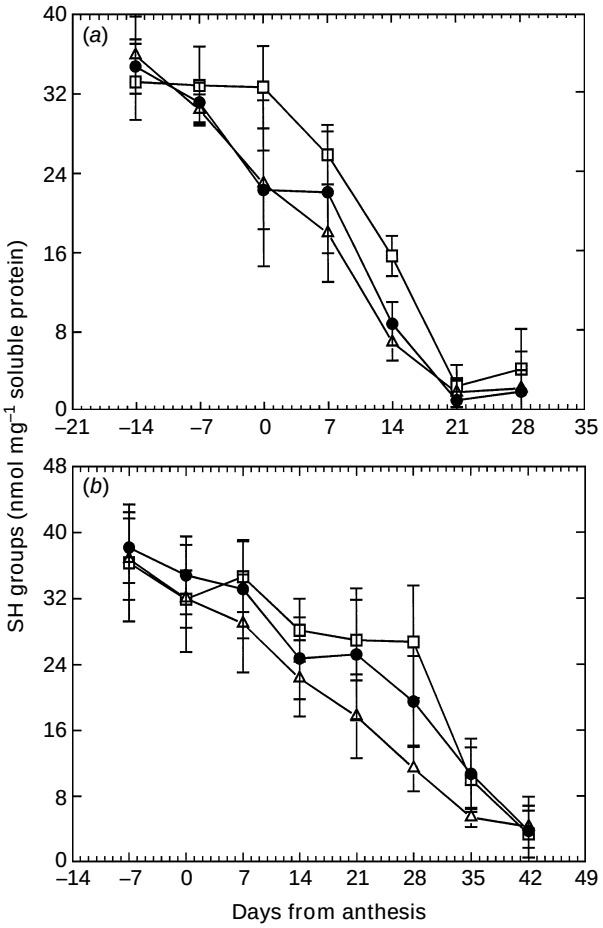

Figure 5. Protein-bound SH groups (nmol mg^{-1} soluble protein) in different treatments in relation to days from anthesis. (*a*) 1992. (*b*) 1993. ●, CF; □, NF; △, NF^{+}; Bars, SD ($n-1$).

yield than the photosynthetic capacity (Evans *et al.*, 1993).

In the present study, the effect of ozone on flag leaf duration, measured as decrease in chloroplast size, was clear and significant in both years of the experiment. The decrease in chloroplast size also corresponded with physiological changes. Thus the 40 % decrease in chloroplast size in flag leaves during the 2 wk after anthesis in NF^{+} treatments in 1993 corresponded with a 40 % decrease in net CO_2 uptake and Rubisco activity during the same period. A similar correspondence was found in both years between the decrease in chloroplast size and the decrease in protein-bound sulphydryl groups. In the case of both parameters, the effects of ozone fumigation began to appear after anthesis and were statistically significant within 2 wk after anthesis. In 1992 chloroplast size decreased faster in plants in the CF treatment than in those in the NF treatment, corresponding to the changes that year in the protein-bound SH group concentrations that year.

The protein-bound sulphydryl groups have long been considered one of the prime targets for ozone induced oxidation (e.g. Mudd (1973)). Tingey & Taylor (1982) reported that SH concentration of crude foliar extracts of spinach (*Spinacia oleracea*), bean (*Phaseolus vulgaris*) and tobacco (*Nicotiana tabacum*) became smaller after exposure to O_3 by the formation of disulphide bonds. In a sensitive variety of common bean, Dominy & Heath (1985) observed severe ozone-induced inhibition of plasmalemma ATPase activity, which could be reversed by treatment with sulphydryl compounds. They concluded that the inactivation was caused by the oxidation of a protein sulphydryl to a disulphide bridge and that sulphydryls can repair ozone-damaged plasmalemma proteins.

Eckhardt & Pell (1995) found that the number of available SH groups declined when they treated purified Rubisco with ozone *in vitro*. However, no sulphydryl oxidation was detected when they exposed whole plants to ozone and they purified Rubisco after the treatment. It is not yet established whether sulphydryl oxidation in Rubisco does not occur *in planta* or whether the purification of Rubisco after ozone exposure influences the results (Pell *et al.*, 1994*b*). The standard purification methods for Rubisco involve using buffers containing chemicals such as DTE (Eckardt & Pell, 1995), which is known to reduce disulphides quantitatively (Cleland, 1964); so sulphydryl oxidation could therefore have been reversed during the purification process.

An additional factor, that could cause confusion when interpreting the ozone effects on SH groups, is that according to the results of the present study SH groups seem to react with charcoal-filtered air, which is the most commonly used control treatment in ozone experiments. In comparing the effects of filtered air (CF) with those of non-filtered air (NF), a decrease in the concentration of SH groups in CF was observed during both years, but the difference was statistically significant only in 1992. Although the difference in the concentration of SH groups was not statistically significant in 1993, it probably was not accidental either, since the pattern persisted throughout the duration of the flag leaves, even though different plants were harvested each time. The corresponding results with the chloroplast data strengthen this interpretation.

Several research groups have reported a decrease in crop yield (Skärby *et al.*, 1993; Colls *et al.*, 1993) and in photosynthesis (Wallin, Skärby & Selldén, 1990) in filtered air relative to air containing low levels of ozone. Some authors explain these results in terms of an earlier adaptation of the plant species to ambient levels of ozone, which is of course possible, given the efficient breeding strategies currently employed for modern cvs of agricultural crops. The results of Barnes *et al.*(1990) in a study of the ozone sensitivity of old and modern wheat cvs did not, however, support this hypothesis since the modern cvs of wheat showed higher foliar sensitivity to ozone than the old ones. Perhaps low concentrations of ozone *per se* stimulate photosynthesis. Nevertheless,

it is difficult to think of a mechanism that would allow ozone to stimulate the concentration of protein-bound SH groups in plants, when the opposite is the case when ozone is added to the air.

The negative effect of CF air vs. NF air was probably observable in our study, because the concentration of ozone in the NF treatment was relatively low, especially in 1993 when the seasonal 8-h mean of 21 nl l^{-1} corresponded to that reported in some of the CF treatments in studies of the European Open-Top Chamber Programme (Skärby *et al.*, 1993). If the ozone concentration in an NF treatment is high enough to cause significant plant response, this might mask the slightly negative filtering effect. This appears to have been the case for our CF and NF^+ treatments, where the seasonal 8-h mean of 45 nl l^{-1} in the NF^+ treatment was similar to that of the NF treatments in some European studies (Skärby *et al.*,1993). If the plants in our experiment really had suffered from excessively low ozone concentrations to which they had not adapted, the significant negative filtering effect would probably have occurred in 1993, when the mean ozone concentration in the CF treatment was lowest (9 nl l^{-1}).

Wellburn (1990) has stated that charcoal filters can actually desorb, as well as absorb, chemical constituents. Olszyk, Bytnerowicz & Takemoto (1989) studied the effect of $CF + O_3$ treatment vs. NF when the amount of ozone was similar in both treatments, and found slightly higher concentrations of nitric oxide (NO) in $CF + O_3$ treatment than in NF chambers. They also measured a statistically significant, higher chlorophyll content of alfalfa (*Medicago sativa* L.) leaves in NF chambers. In addition, there was a significant reduction in d. wt across four harvests for $CF + O_3$ vs. CF plants but not for NF vs. CF plants, although the concentration of ozone was similar in the $CF + O_3$ and NF treatments. They concluded that the results indicate the presence or absence of other oxidants besides ozone in charcoal-filtered air.

In our open-top chambers the cumulative dose of NO was twice as high in the filtered air as in the other treatments. The concentrations of NO were nevertheless low in all cases, and it seems surprising that such levels could be phytotoxic. The negative effects on plants that were observed in the CF treatment vs. NF treatment, however, correspond to the different NO levels in the two years, the effects being smaller in 1993 when the amount of NO was lower.

Relatively low concentrations (8 nl l^{-1}) of NO have been reported to have negative effects on yield and physiological parameters of wheat similar to those of increased ozone concentrations (Nussbaum *et al.*, 1995). Nussbaum *et al.* (1995) concluded that the plant physiological effects of NO were not due to additional ethylene production, and the actual reaction mechanisms remained unclear. Elevated concentrations of NO can cause formation of nitrite in plant cells (Wellburn, 1990), and nitrite can react with thiol-containing proteins (Hewitt, 1975). It is possible therefore that the negative effects of low NO are due to the effects on SH groups. However, since very little evidence exists as to how low concentrations of NO actually affect plants, it is simply suggested that charcoal-filtered air is probably not the best environment for plants and caution should be exercised when using charcoal-filtered air as a control treatment in ozone experiments.

In conclusion, the results of the present study show that exposure of wheat in field conditions to the seasonal mean concentrations of ozone occurring in the larger part of Europe (Beck & Grennfelt, 1994), causes many physiological changes in flag leaves. Changes include decrease in net CO_2 uptake, decrease in Rubisco activity and decrease in concentration of SH groups.

However, neither the ozone effects on chloroplast size nor the effects on net photosynthesis were expressed as a difference in the grain-filling rate. This was constant in all chamber treatments, during 4 wk after anthesis, in spite of the 40 % decrease in the net photosynthesis in NF^+ treated plants 2 wk after anthesis.

Grain filling proceeded each year until the chloroplast size neared zero. The senescence of the flag leaves, reflected as decrease in chloroplast size occurred *c.* 1 wk earlier in plants in the NF^+ treatments in both years that ozone fumigation was carried out. The grain-filling period was correspondingly a few days shorter in the NF^+ treatment, leaving the grains somewhat smaller. This effect on the grain-filling period was reflected in the 1000-grain weight, showing that the grains of plants in NF^+ treatments were smaller, on average, than CF and NF treatments. Smaller 1000-grain weight explained most of the reduction in grain yield of plants in ozone-fumigated chambers.

Evidently, the shortened flag leaf duration caused by relatively low concentrations of ozone shortens the grain-filling period of wheat. This in turn decreases the grain weight and consequently grain yield. According to the results of this study, therefore, the 40 % decrease in net photosynthesis was not directly connected to the reduction in grain yield. The wheat plants, apparently, were well buffered against a substantial decrease in source capacity, and the shortened flag leaf duration was the most important factor causing ozone-induced loss of grain yield.

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