Plant Physiol. (1991) 95, 529–535
0032-0889/91/95/0529/07/$01.00/0

Received for publication July 23, 1990
Accepted October 15, 1990

The Sequence of Change within the Photosynthetic Apparatus of Wheat following Short-Term Exposure to Ozone[1]

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ABSTRACT

The basis of inhibition of photosynthesis by single acute O_3 exposures was investigated *in vivo* using analyses based on leaf gas exchange measurements. The fully expanded second leaves of wheat plants (*Triticum aestivum* L. cv Avalon) were fumigated with either 200 or 400 nanomoles per mole O_3 for between 4 and 16 hours. This reduced significantly the light-saturated rate of CO_2 uptake and was accompanied by a parallel decrease in stomatal conductance. However, the stomatal limitation, estimated from the relationship between CO_2 uptake and the internal CO_2 concentration, only increased significantly during the first 8 hours of exposure to 400 nanomoles per mole O_3; no significant increase occurred for any of the other treatments. Analysis of the response of CO_2 uptake to the internal CO_2 concentration implied that the predominant factor responsible for the reduction in light-saturated CO_2 uptake was a decrease in the efficiency of carboxylation. This was 58 and 21% of the control value after 16 hours at 200 and 400 nanomoles per mole O_3, respectively. At saturating concentrations of CO_2, photosynthesis was inhibited by no more than 22% after 16 hours, indicating that the capacity for regeneration of ribulose bisphosphate was less susceptible to O_3. Ozone fumigations also had a less pronounced effect on light-limited photosynthesis. The maximum quantum yield of CO_2 uptake and the quantum yield of oxygen evolution showed no significant decline after 16 hours with 200 nanomoles per mole O_3, requiring 8 hours at 400 nanomoles per mole O_3 before a significant reduction occurred. The photochemical efficiency of photosystem II estimated from the ratio of variable to maximum chlorophyll fluorescence and the atrazine-binding capacity of isolated thylakoids demonstrated that photochemical reactions were not responsible for the initial inhibition of CO_2 uptake. The results suggest that the apparent carboxylation efficiency appears to be the initial cause of decline in photosynthesis *in vivo* following acute O_3 fumigation.

Ozone is widely recognized as a major air pollutant affecting crop yields (12). Photosynthesis has been shown to be particularly sensitive, with O_3 producing a variety of effects ranging from altered chloroplast ultrastructure to loss of electron transport capacity and enzyme activity (reviewed in ref. 9). In particular, many studies have detailed changes in stomatal conductance that accompany exposure of leaves to O_3 (for review see ref. 3). However, it is uncertain which of these changes in the individual steps of the photosynthetic process may cause reduced CO_2 uptake and which are secondary effects responding to decreased photosynthesis. Subcellular investigations have shown differences in the susceptibility of different parts of the photosynthetic apparatus to O_3. Whether O_3 actually reaches the chloroplast is contentious; recently, Laisk *et al.* (14) have calculated the intercellular O_3 concentration of leaves to be near zero. Instead, photosynthesis may be affected indirectly through the generation of oxidizing radicals or through alteration of the plasmalemma (9). Resultant changes in the cytoplasmic ion and solute concentrations have the potential to affect all stages in the photosynthetic process.

Decrease in the A_{sat}[2] may result from three underlying causes: (a) decreased carboxylation efficiency; (b) a decrease in the rate of regeneration of RuBP for carboxylation; or (c) an increase in stomatal limitation. Changes in all three factors have been implicated in previous studies of the effects of O_3 inhibition of photosynthesis (3, 9). Analysis of the response of A_{sat} to variation in the intercellular CO_2 concentration, the A/c_i curve (6, 7), provides an *in vivo* method of quantitatively assessing the importance of each of these three factors in decreasing A_{sat}.

The majority of previous investigations have considered the effects of O_3 on light-saturated or near light-saturated photosynthesis, but under field conditions most leaves in plant canopies do not experience light-saturation or do so for only part of the day. Under light-limiting conditions a different set of factors may lower the rate of CO_2 uptake. A decrease in the initial slope of the light response or maximum ϕ_c may result from three types of change: (a) a decreased absorptance of light energy, which would result from a change in pigment concentration; (b) a decrease in the efficiency of energy transduction of the photosynthetic membrane, which could result

[1] Funding for this work was provided by the Natural Environment Research Council grant No. GST/02/235.

[2] Abbreviations: A_{sat}, net CO_2 uptake rate per unit leaf area at light saturation; A, net CO_2 uptake rate per unit leaf area; A_{max}, net CO_2 uptake rate per unit leaf area at CO_2 and light saturation; ϕ_c, quantum yield of CO_2 uptake for absorbed light; ϕ_o, quantum yield of oxygen evolution for incident light; c_a, mole fraction of CO_2 in air surrounding the leaf; g_s, stomatal conductance to CO_2; c_i, mole fraction of CO_2 in the intercellular air space; F_v, variable Chl fluorescence; F_m, maximum Chl fluorescence; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate.

from changes in the organization of the photosynthetic membrane (for review see ref. 22), of particular importance could be loss of active photosystem II; (c) a decrease in quantum yield of CO_2 uptake could occur even in the absence in changes in (a) and (b). First, $\emptyset_c$ would decrease if the proportion of energy utilized in reductive processes other than CO_2 uptake, *e.g.* nitrogen metabolism and protein synthesis, was increased. Second, a range of environmental stresses have been shown to cause stomatal closure in patches over the leaf such that CO_2 diffusion will limit photosynthesis even at the low light levels used in the determination of $\emptyset_c$ (27). These causes would result in a decline in $\emptyset_c$ but not in $\emptyset_o$ when measured in an atmosphere saturated with CO_2. This is because noncyclic electron transport will result in O_2 evolution regardless of the electron acceptor, whereas the use of saturating CO_2 concentrations should overcome stomatal limitations if closure had occurred in patches (27).

The objectives of this study were to examine the kinetics of change in light-saturated and light-limited photosynthesis following acute exposures to O_3. The sequence of change in carboxylation efficiency, CO_2-saturated photosynthesis, and stomatal limitation were monitored to assess their relative significance as contributors to change in light-saturated photosynthesis. Similarly, to assess the causes of change in light-limited photosynthesis, the kinetics of change in the quantum yields of CO_2 uptake and O_2 evolution together with Chl fluorescence, were monitored following O_3 exposure. To avoid the complication of indirect effects that O_3 may produce by modifying growth and development during long-term fumigation, this study was limited to brief single fumigations of high concentrations of O_3, at the extreme of concentrations recorded in the field (1).

MATERIALS AND METHODS

Plant Material

Seeds of wheat, *Triticum aestivum* L. (cv Avalon; Kings Seeds Ltd., Coggsehall, UK), were sown in a soil-free compost (Levingtons, F2; Fisons Ltd., Ipswich, UK) and grown in a controlled-environment cabinet (Fi-totron 600H; Fisons Ltd., Loughborough, UK). The air temperature was maintained at 20°C during the 16-h photoperiod and at 15°C during the dark period. Water vapor pressure deficit was maintained at 0.8 kPa and the PPFD at the level of the leaves was about 300 μmol m^{-2} s^{-1}. All experiments were performed on the fully expanded second leaf at 17 to 24 d after sowing. Preliminary studies showed that during this period both the quantum yield and light-saturated rate of photosynthesis remained constant. The background O_3 concentration in the cabinet was negligible, at <3 nmol $mole^{-1}$.

Ozone Fumigation

To ensure a uniform microclimate, leaves were fumigated individually inside a stirred and water-jacketed glass chamber connected to a heater/circulator (C400; Techne Ltd., Duxford, UK) and refrigeration unit (EN-350; Neslab, Portsmouth, NH). Leaf temperature was maintained at 22° ± 1°C. The leaf was held horizontal by a nylon cradle and the opening of the chamber sealed with a flexible silicone rubber (Terostat VII/IX; Teroson GmbH, Heidelberg, FRG). A high-pressure sodium vapor lamp (SON/T 1000W; Thorn, Middlesex, UK) above and parallel to the chamber produced even illumination over the length of the leaf of 300 ± 15 μmol m^{-2} s^{-1}. Photon flux was determined with a quantum sensor (SKP 215; Skye Instruments Ltd., Llandrindod Wells, UK) placed in the position normally occupied by the leaf. A small fan (V363M; Radiatron Ltd., London, UK) incorporated inside the chamber provided rapid air recirculation and produced a high boundary layer conductance of 2.1 moles m^{-2} s^{-1}, as calculated from a filter paper leaf replica by the method of Long and Hällgren (18). Ozonated air was supplied to the leaf chamber via polytetrafluoroethylene tubing (Visijar Plastics Ltd., Croydon, UK) and connectors (Fluorocarbon Inc. Anaheim, CA). Ozone was generated from an UV source by a combined generator and analyzer unit (1008-PC; Dasibi Environmental Corp., Glendale, CA). This monitored and maintained the air within the leaf chamber to an O_3 content within 8 nmol $mole^{-1}$ of the required target concentration, by operating the analyzer in feedback mode with the generator. Ozone uptake by the leaves was determined from the increase in O_3 required to maintain the correct chamber concentration with leaves enclosed relative to the empty chamber.

Treatments

Attached second leaves that had just completed extension growth were sealed into the leaf cuvette. Illumination was provided for 15 min before ozonation. Ninety percent of the target O_3 concentration was attained within 5 min. Leaves were fumigated for 4, 8, or 16 h, while control leaves were treated identically without O_3.

CO_2 and H_2O Gas Exchange Measurements

After fumigation, CO_2 uptake and transpiration were measured in an open gas exchange system using a leaf section chamber (LSC; ADC Ltd., Hoddesdon, UK), which was modified by the addition of a polytetrafluoroethylene coating and black base to prevent back reflection of light onto the leaf. This small chamber (total volume 6.6 cm^3) enabled the microclimate to be controlled precisely and provided a very rapid response time to changes in c_a or PPFD. The passage of coolant through each half of the chamber enabled leaf temperature to be held at 20° ± 0.5°C. Leaf temperature was measured using a copper-constanton thermocouple with 0.2-mm-diameter silver-soldered junction appressed against the abaxial leaf surface. The thermocouple was connected to an electronic thermometer display (1625 Cu/Con; Comark Ltd., Littlehampton, UK). The leaf was illuminated by a 250-W quartz iodide source (Schölly Fiberoptick, GmbH, FRG) used in conjunction with a heat-reflecting filter (OCLI Ltd., High Wycombe, UK) and water filter. The PPFD at the leaf surface was attenuated by using thin sheets of glass fiber as neutral density filters mounted in front of the chamber window. Photon flux density (400–700 nm) was determined by placing a quantum sensor (SKP 215; Skye Instruments Ltd.) below the window at the position normally occupied by the leaf.

Gas mixtures were supplied to the chamber from three cylinders, N_2, O_2, and 10% CO_2 in N_2 (BOC Special Gases,

London, UK). A gas blender (853 VI-5; Signal Instrument Co. Ltd., Camberley, UK) was used to supply the leaf chamber with 80% N_2, 20% O_2, and a range of CO_2 concentrations from 40 to 1600 μmol mole^{-1}. Before reaching the chamber, the dry air was passed through a temperature-controlled column of ferrous sulfate crystals that rehumidified the air to a water vapor pressure of 1.66 kPa, which ensured that the leaf-air water vapor pressure deficit was <1 kPa. Absolute and differential water vapor pressure were measured using an infrared gas analyzer (Series 225/2; ADC Ltd.), which was calibrated with a water vapor generator (WD600; ADC Ltd.). Absolute and differential CO_2 concentrations (mole fraction) were measured using a two-channel infrared CO_2 analyzer (Binos 2; Leybold-Heraeus, FRG) calibrated against a standard CO_2 concentration in air supplied in an aluminum cylinder (Cryo Services Ltd., Worcester, UK). Gas flow through the chamber was measured using precision-variable area flow meters (Fisher Controls Ltd., Croydon, UK) calibrated against a bubble flow meter (18). Projected leaf area was measured with an area meter (AM1; Delta T Devices Ltd., Burwell, UK).

Transpiration, A, g_s, and c_i were calculated using the equations of von Caemmerer and Farquhar (29). The response of A to c_i was analyzed according to the biochemical model of Farquhar *et al.* (6), and the stomatal limitation to photosynthesis was estimated by the method of Farquhar and Sharkey (7).

Measurement of the Quantum Yield of CO_2 Uptake and O_2 Evolution

$\emptyset_c$ was determined in an Ulbricht integrating sphere leaf chamber as described by Ireland *et al.* (13). Oxygen evolution at 20°C was measured with a leaf-disc electrode (LD2; Hansatech Ltd., Kings Lynn, UK) using the procedure of Walker (30). A bicarbonate buffer (1 M) solution was used to provide a CO_2 concentration of 5% by volume. Illumination in both systems was produced from quartz iodide sources (Schölly Fiberoptick, GmbH). The PPFD was varied with neutral density filters and measured in the oxygen electrode with a quantum sensor at the position occupied by the leaf. Quantum yields of CO_2 uptake and O_2 evolution were calculated as the slope of the best fitting line, determined by least-squares linear regression analysis, to measured rates of CO_2 uptake and O_2 evolution at photon fluxes between 50 to 150 and 40 to 135 μmol m^{-2} s^{-1}, respectively, for each replicate.

Measurement of Chl Fluorescence (F_v/F_m)

Chl fluorescence was measured using a modulated fluorimeter (MFMS, Hansatech Ltd.) as described previously (21). A tungsten light source provided saturating actinic light (PPFD, 2500 μmol m^{-2} s^{-1}). The ratio of F_v to F_m was measured after leaves had been in complete darkness for 30 min.

Measurement of the Capacity of Thylakoids to Bind Atrazine

^{14}C-Atrazine was used to determine the number of plastoquinone binding sites associated with PSII (28) in isolated thylakoids as described previously by Habash and Baker (10).

RESULTS

Light Response of Photosynthesis

Following exposure of *T. aestivum* leaves to O_3, there was a marked decease in leaf gas exchange. The light-saturated rate of CO_2 uptake declined markedly after 8-h fumigation with O_3 at 400 nmol mole^{-1} ($P < 0.001$, Fig. 1B). The light-limited rate of CO_2 uptake (*i.e.* the initial slope of the response to light) also began to show a significant decrease after this treatment ($P < 0.05$, Fig. 1A) and decreased further after 16-h exposure to 400 nmol mole^{-1} O_3 ($P < 0.01$, Fig. 1A). Subsequent sections analyze the possible causes of these two changes to the response of photosynthesis.

Light-Saturated Photosynthesis

The effect on the light-saturated rate of photosynthesis after exposing *T. aestivum* leaves to 200 or 400 nmol mole^{-1} O_3 for 4 to 16 h is shown in Figure 2. When fumigated with 200 nmol mole^{-1} O_3, A_{sat} declined during the first 4 h and was significantly reduced ($P < 0.05$) after 8 h, but showed little further decrease after 16 h. When fumigated at 400 nmol mole^{-1} O_3, A_{sat} declined more rapidly and was still declining after 16-h exposure, by which time it was 34% of the rate in control leaves ($P < 0.001$). The reductions in A_{sat} were not, therefore, directly correlated with O_3 dose (*i.e.* O_3 concentration × exposure). However, inhibition of A_{sat} paralleled the rate of O_3 taken up by the leaf. Consequently, at 200 nmol mole^{-1} O_3, the rate of O_3 uptake began to stabilize after 4 h (Fig. 3) and was followed by A_{sat} showing no further decrease after 8 h. With fumigation of 400 nmol mole^{-1} O_3, the rate of O_3 uptake was not double the rate measured at 200 nmol mole^{-1} O_3 and most probably reflects the marked decrease in stomatal conductance (Fig. 4). However, the additional O_3 absorbed by the leaves was particularly significant because of the disproportionately greater inhibition of A_{sat} that occurred.

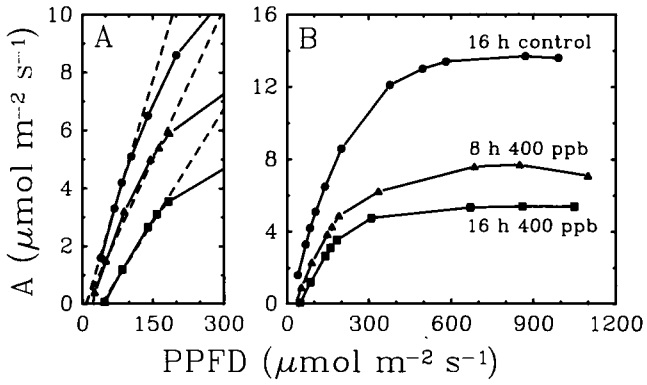

Figure 1. Effect of O_3 fumigations on (A) light-limited CO_2 uptake and (B) the complete light response curve for individual *T. aestivum* leaves. Measurements were made at 20°C in an external CO_2 concentration of 340 μmol mole^{-1}. Control leaves (●) were illuminated at a PPFD of 300 μmol m^{-2} s^{-1} for 16 h; ▲, leaves previously illuminated for 8 h with 400 nmol mole^{-1} O_3; ■, leaves illuminated for 16 h with 400 nmol mole^{-1} O_3. For clarity, control leaves illuminated for 8 h are omitted; rates were not significantly different from those obtained from 16-h control leaves.

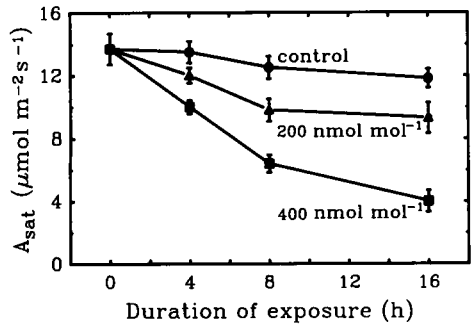

Figure 2. Change in the light-saturated rate of CO_2 uptake (A_{sat}) of *T. aestivum* leaves resulting from increasing lengths of exposure to O_3 concentrations of 200 (▲) and 400 (■) nmol mole^{-1}. Control leaves (●) were illuminated for corresponding time periods without O_3 before measurement of A_{sat}. Measurements were made at 20°C with a PPFD of 1500 μmol m^{-2} s^{-1} in an external CO_2 concentration of 340 μmol mole^{-1}. Points are the mean of four to six replicates; vertical bars represent SEM.

This suggests that for these experimental conditions a threshold exists above 200 nmol mole^{-1} O_3, for which the capacity of leaves to counter the toxic effects of O_3 becomes outstripped. The reductions in A_{sat} could result from a decrease in the conductance to CO_2 of the stomata, the mesophyll, or both. Decrease in A_{sat} was closely correlated with reduction in g_s, which was significantly reduced after 4 h at 400 nmol mole^{-1} O_3 ($P < 0.05$, Fig. 4). However, the intercellular CO_2 concentration, as a proportion of the external CO_2 concentration (c_i/c_a) did not change ($P > 0.05$, Fig. 4). This suggests that decrease in g_s results from loss of photosynthetic capacity rather than the converse. However, such a conclusion assumes that the shape of the A/c_i response is unchanged by the treatment.

Analysis of the Response of A to c_i

The response of A to changing c_i was radically altered by O_3 treatment. Comparison of A at $c_a = 340$ μmol mole^{-1} with A at infinite stomatal conductance (*i.e.* $c_i = 340$ μmol mole^{-1}) indicated that the stomatal limitation initially increased, even though c_i/c_a was unchanged. However, the increase in the stomatal limitation was only observed for the first 8 h at 400 nmol mole^{-1} O_3. For all other treatments there was no significant change ($P > 0.05$) in the stomatal limitation (Fig. 5, A and B), suggesting that there was no increased restriction in the supply of CO_2 at the mesophyll. There was, however, an increase in the variability of the stomatal limitation after 16 h at both O_3 concentrations, as shown by the larger SE (Fig. 5, A and B; the variance of g_s increased from 7×10^{-4} to 2×10^{-2} during 16-h exposure to 400 nmol mole^{-1} O_3). This could reflect the development of a heterogeneity in stomatal opening.

Ozone fumigation significantly decreased the initial slope of the A/c_i curve, which is dependent on the efficiency of the primary carboxylase (Rubisco; see ref. 6). However, the CO_2-saturated rate of photosynthesis (A_{max}) was inhibited less. The effects on these two parameters are shown for all the treatments in Fig. 5, A and B. A statistically significant decrease in A_{max} ($P < 0.05$) occurred only with fumigations of 400 nmol mole^{-1} O_3. Conversely, the carboxylation efficiency was significantly decreased after 8 h at 200 nmol mole^{-1} O_3 ($P < 0.01$) and by just 4 h at 400 nmol mole^{-1} O_3 ($P < 0.01$). Thereafter, further inhibition continued at both O_3 concentrations resulting in a decline to 21% of the control value after 16 h at 400 nmol mole^{-1} O_3. The A/c_i response suggests that the primary carboxylase is the first phase of light-saturated photosynthesis to be affected in *T. aestivum*. The effect on A_{max}, which relates to the maximum rate of RubP regeneration, is much smaller. The maximum rate of regeneration of RubP has been related to the maximum rate of coupled electron transport *in vivo* (6), but may also be restricted by the balance of chloroplast sugar phosphate export and inorganic phosphate import. The A/c_i data therefore imply that electron transport capability is less susceptible to O_3 than carboxylation.

Light-Limited Photosynthesis

Leaves of control plants gave values of quantum yield ($\emptyset_c = 0.052 \pm 0.001$; $\emptyset_o = 0.091 \pm 0.002$; Fig. 6, A and B) within

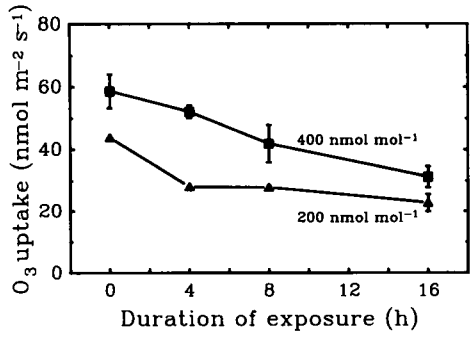

Figure 3. Rates of O_3 uptake for *T. aestivum* leaves exposed to external O_3 concentrations of 200 (▲) and 400 (■) nmol mole^{-1}. Ozone uptake was calculated from the additional O_3 required to maintain the correct chamber concentration when leaves were enclosed relative to the empty chamber. Measurements were made at 22°C, with a PPFD of 300 μmol m^{-2} s^{-1} in an external CO_2 concentration of 340 μmol mole^{-1}. Points are the mean of three replicates; vertical bars represent SEM.

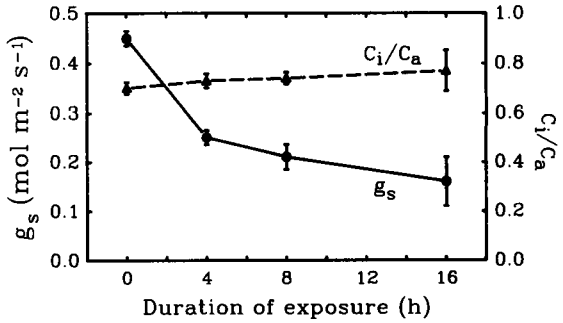

Figure 4. Effect on g_s (●) and the ratio of c_i to c_a CO_2 concentration (▲) of *T. aestivum* leaves exposed to an O_3 concentration of 400 nmol mole^{-1} for 4 to 16 h. Measurements were made at 20°C with a PPFD of 1500 μmol m^{-2} s^{-1} in an external CO_2 concentration of 340 μmol mole^{-1}. Points are the mean of four to six replicates; vertical bars represent SEM.

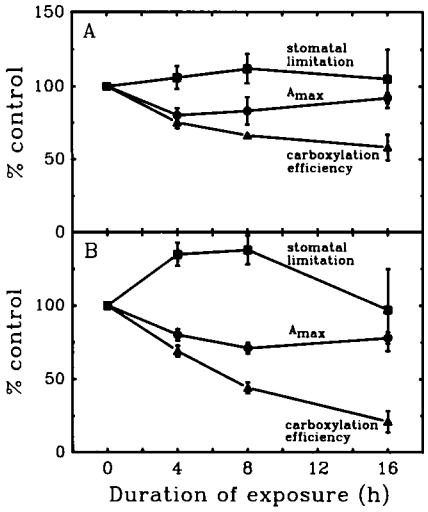

Figure 5. Changes in the CO_2-saturated rate of CO_2 uptake (●) (4 h control = 30.6, 8 h control = 29.9, 16 h control = 29.4 μmol m^{-2} s^{-1}); the apparent carboxylation efficiency (▲) (4 h control = 0.08, 8 h control = 0.09, 16 h control = 0.08 mole m^{-2} s^{-1}); and stomatal limitation (■) (4 h control = 0.11, 8 h control = 0.14, 16 h control = 0.16) of *T. aestivum* leaves fumigated with (A) 200 nmol $mole^{-1}$ O_3 and (B) 400 nmol $mole^{-1}$ O_3 relative to controls treated without ozone. Measurements were made at 20°C with a PPFD of 1500 μmol m^{-2} s^{-1}. Points are the mean of three to six replicates; vertical bars represent SEM.

the range typical for healthy C_3 photosynthetic organs (17, 30). Fumigation with O_3 at 200 nmol $mole^{-1}$ had no significant ($P > 0.05$) effect on light-limited photosynthesis (Fig. 6, A and B). However, with increasing duration of exposure at 400 nmol $mole^{-1}$ O_3, the quantum yield of CO_2 uptake fell significantly by 27% after 8 h ($P < 0.05$) and was decreased by 46% ($P < 0.01$) after 16-h exposure (Fig. 6A). In an elevated CO_2 environment, the quantum yield of O_2 evolution showed a decrease proportionate to that reported for CO_2 uptake (Fig. 6B), *i.e.* a 23% decline after 8 h ($P < 0.05$) increasing to 51% after 16 h at 400 nmol $mole^{-1}$ O_3. The very close similarity in the inhibition kinetics for $\emptyset_c$ and $\emptyset_o$ suggests that the decrease in $\emptyset_c$ was not due to stomatal patchiness. The high CO_2 concentration used with the oxygen electrode (50 mmol mol^{-1}) would predominantly overcome any decreased stomatal conductance. Although the measurements of $\emptyset_c$ were for absorbed light, whereas $\emptyset_o$ were determined for incident light, no significant differences ($P > 0.05$) in leaf absorptance were observed following O_3 fumigation.

Chl Fluorescence—F_v/F_m

Reductions in quantum yield have most frequently been associated with photoinhibition, which occurs when excess light is absorbed by the reaction centers. Environmental stress significantly increases susceptibility to photoinhibition, which is manifest as damage to PSII and characterized by a decline in the fluorescence ratio F_v/F_m. However, no significant reduction in F_v/F_m ($P > 0.05$) was recorded after any of the 200 nmol $mole^{-1}$ O_3 treatments (Fig. 6C). There was a small reduction after 8 h at 400 nmol $mole^{-1}$, increasing further after 16 h to a significantly lower level, 0.68 ($P < 0.05$). The pattern of change of F_v/F_m was qualitatively similar to the $\emptyset_c$ measurements, but the decrease, relative to controls after 16 h 400 nmol $mole^{-1}$ O_3, was only 11% compared with 46% for $\emptyset_c$. This suggests that photoinhibition of PSII, inferred by the decrease in F_v/F_m, could only account for a small part of the observed decrease in $\emptyset_c$ and $\emptyset_o$. However, no significant change was observed in the ability of thylakoids isolated from leaves to bind atrazine after 16-h exposure to O_3 at 400 nmol $mole^{-1}$ (2.04 ± 0.15 nmol atrazine mg^{-1} Chl) compared with

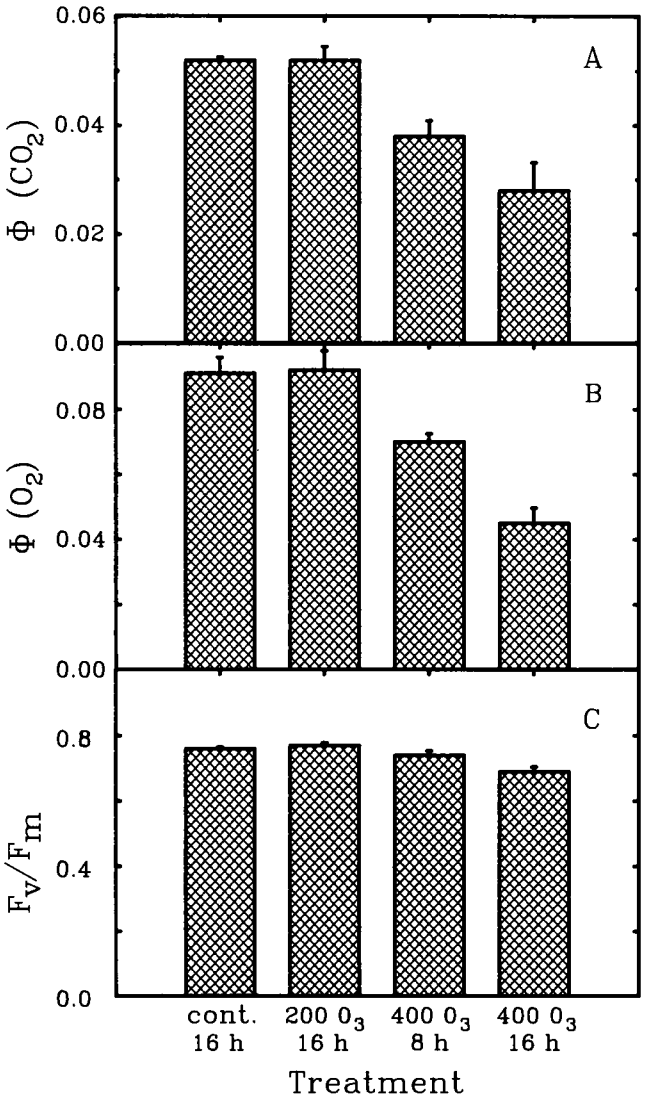

Figure 6. Effect on *T. aestivum* leaves of O_3 fumigations of 200 or 400 nmol $mole^{-1}$ on: (A) $\emptyset_c$; measurements were made at 20°C in an external CO_2 concentration of 340 μmol $mole^{-1}$ using photon fluxes of 50 to 150 μmol m^{-2} s^{-1}. (B) The quantum yield of O_2 evolution measured at 20°C using a bicarbonate buffer (1 M) solution to provide a CO_2 concentration of 5% by volume. The quantum yield was determined from the initial slope of the light response curve for incident photon fluxes of 40 to 135 μmol m^2 s^{-1}. (C) The ratio of F_v to F_m. Leaves were dark-adapted for 30 min after O_3 fumigation. Points are the mean of five to eight replicates; vertical bars represent SEM.

control leaves (2.17 ± 0.15 nmol atrazine mg^{-1} Chl). Western blotting of polypeptide profiles of thylakoids with antibodies specific to the D1 protein also showed no difference in the amounts of the protein between control and treated plants (data not shown). This demonstrates that damage to the D1 reaction center of PSII does not occur and that the decreases in F_v/F_m, $\emptyset_c$, and $\emptyset_o$ cannot be attributed to photoinhibitory damage to PSII reaction centers. The observed small decreases in F_v/F_m, which indicate a decrease in the quantum yield of PSII in the absence of damage to the D1 protein, are probably indicative of an increase in deactivation of excitation of PSII by nonphotochemical processes associated with a regulated decrease in photochemistry and photosynthetic rate (8).

DISCUSSION

Exposing *T. aestivum* leaves to elevated levels of O_3 produced a subsequent decrease in CO_2 uptake and transpiration. *T. aestivum* has been classified as an O_3-sensitive species (9). The reductions in A_{sat} ranging from 11 to 60% of control values found in the present investigation are comparable with those reported for other agricultural crops susceptible to short-term O_3 fumigations (3, 25). However, as noted by Darrall (3), there is little information on the inhibitory effects of short-term O_3 exposure on photosynthetic rate.

The results from the present investigation suggest that the first inhibitory effect to develop in photosynthesis during short-term O_3 fumigation is a loss of carboxylation efficiency, inferring a decrease in amount of active carboxylase. Effects on the thylakoid membrane, inferred from changes in quantum yield and Chl fluorescence, occur only after a marked reduction (>50%) in carboxylation efficiency. The longer term significance of these effects will depend on the capacity for recovery. Although it is not possible to tell whether repeated low-level O_3 exposure will effect plants in a similar way, the results obtained here suggest that light-saturated photosynthesis would be vulnerable through the effect on carboxylation. The efficiency of light-limited photosynthesis was less susceptible, and decreased only after the longest fumigation, but may become important if repeated O_3 exposures were to occur. The effects of repeated low-level O_3 exposures on photosynthesis form the basis of a separate study.

The marked effect of O_3 on carboxylation efficiency found in the present investigation could result from a decrease in either the amount or the activity of Rubisco. A reduction in Rubisco content, which more than offset an increase in activation state, has been reported in spring wheat subjected to prolonged O_3 exposure (100 nmol $mole^{-1}$, 8-h d^{-1} for 2 months; ref. 15). However, Pell and Pearson (24) found a decrease in the quantity of Rubisco after just 2-h exposure of alfalfa cvs to 250 to 300 nmol $mole^{-1}$ O_3. Their assay was not performed until 48 h after fumigation and so it is not possible to establish whether this could account for the immediate reductions in photosynthesis following O_3 treatment that were observed here. A decrease in Rubisco activity has been found to occur in rice plants fumigated with O_3 (120–200 nmol $mole^{-1}$) for 2 to 3 h (20). Although their first measurement was not made until 12 h after fumigation, Rubisco activity could well decrease sooner. Chloroplast ultrastructural changes are observed soon after the onset of O_3 fumigation, even before ultrastructural changes of the plasmalemma become apparent (9). It is likely that the visible alterations of the chloroplast stroma (9) are accompanied by changes in ion and solute concentrations that could alter the activation state of Rubisco. Furthermore, Rubisco has many sulfhydryl groups, and these are especially susceptible to oxidation (19). However, even if the stomata are not limiting the supply of CO_2 into the leaf, an apparent decrease in carboxylation efficiency, indicated by the A/c_i analysis, could result from a decreased liquid-phase conductance to CO_2 diffusion in the mesophyll. Possible causes of this could be change in the plasmalemma or an inactivation of the carbonic anhydrase, which would reduce the supply of CO_2 from bicarbonate ions in the chloroplast stroma (17).

Stomatal closure has often been considered to be the cause of decreased photosynthesis following O_3 exposure (11), but more recently causes within the mesophyll have been recognized as contributory factors (3, 25). This present *in vivo* analysis now suggests that stomatal closure is predominantly secondary to a loss of carboxylation efficiency. As observed elsewhere, O_3 concentrations of ≥200 nmol $mole^{-1}$ decrease stomatal conductance (3). However, analysis of the A/c_i response suggests that only 38% of the decline in A_{sat} over the first 8 h at 400 nmol $mole^{-1}$ O_3 could be attributed to stomatal closure: by 16 h the stomatal limitation was no longer significantly different from the control value and no significant increase in the stomatal limitation occurred at any time in the 200 nmol $mole^{-1}$ O_3 treatments. This assumes that stomatal patchiness is not confounding the A/c_i analysis and hence the estimation of the stomatal limitation. The consequence of this stomatal patchiness is that some areas of the leaf become CO_2 limited, and this will restrict A. Such an occurrence will go undetected in the estimation of c_i, which is based on gas exchange measurements averaged over the whole leaf area being measured, whereas, in reality the leaf area for gas exchange is effectively reduced by stomatal heterogeneity (5). Induction of stomatal patchiness by ABA-treated *Helianthus annuus* leaves reduced the quantum yield of CO_2 uptake. However, no decrease was observed in the quantum yield of O_2 evolution measured in a disc oxygen electrode, in which the high CO_2 concentration used is likely to overcome stomatal limitation (27). Since the proportionate changes in $\emptyset_c$ and $\emptyset_o$ reported in the present investigation were similar (Fig. 5, A and B), the results suggest that the reduction of $\emptyset_c$ was not a consequence of stomatal limitation but resided in the mesophyll, and suggests that closure of stomata in patches had not been induced by these O_3 treatments, lending support to the interpretation applied to the A/c_i analysis here. It was noted that the stomatal limitation became more variable as the length of fumigation increased, and this could reflect increasingly uneven stomatal aperture across the leaf. A loss of stomatal control might well be expected to occur following O_3 treatment, *e.g.* O_3 markedly increases membrane permeability to potassium ions (4).

The results of the current investigation (A_{max}, $\emptyset$, F_v/F_m, and the atrazine-binding capacity of the thylakoids) did not suggest that short-term O_3 exposure induced any major perturbations of electron transport or PSII functions. This is in contrast to the findings of Shreiber *et al.* (26), who fumigated

Phaseolus vulgaris with 300 or 500 nmol mole^{-1} O_3 for 6 h and deduced that the initial effects of O_3 were on water-splitting activity. Electron transport in PSI PSII was found to be inhibited (2), but this inconsistency may be explained by the fact that Coulson and Heath (2) worked with isolated chloroplasts. The small reduction of A_{max} (10–29%) found here shows that the production of ATP and NADPH, required for the regeneration of RubP, was not severely affected. Other investigators working with O_3 have found that the ATP/ADP ratio increases. For instance, 40-d exposure to 70 or 100 nmol mole^{-1} O_3 for 8 h/d in open-top chambers produced an increase in the ATP/ADP ratio in *T. aestivum* leaves (16). Similar findings were made from short (3 h) exposures of *P. vulgaris* to 250 to 300 nmol mole^{-1} O_3 (23). Electron transport disturbances *per se* therefore do not appear to be responsible for the eventual decline in $\emptyset_c$. The fact that the measurements were made on an absorbed light basis precludes a reduction in light capture efficiency, nor can the effect be a consequence of electron transfer to acceptors not involved in CO_2 reduction (*e.g.* increased photorespiration) because the $\emptyset_o$ was similarly affected.

In conclusion, *in vivo* analysis of the effects of short-term O_3 fumigation on photosynthesis in wheat shows apparent carboxylation efficiency to be the first point of damage and the major initial cause of decline in the light-saturated rate of CO_2 uptake. Changes in stomatal conductance, the rate of regeneration of RubP, and the quantum yield of PSII photochemistry have only a very small quantitative effect on the rate of CO_2 uptake or occur later.

ACKNOWLEDGEMENT

We thank J. Cook for technical assistance with the atrazine-binding experiments.

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