Plant Physiol. (1989) 91, 427–432
0032-0889/89/91/0427/06/$01.00/0

Received for publication January 24, 1989
and in revised form May 17, 1989

Decline of Activity and Quantity of Ribulose Bisphosphate Carboxylase/Oxygenase and Net Photosynthesis in Ozone-Treated Potato Foliage[1]

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ABSTRACT

The effect of ozone (O_3) on ribulose bisphosphate carboxylase/oxygenase (Rubisco) activity and quantity and net photosynthesis in greenhouse-grown *Solanum tuberosum* L. cv 'Norland' foliage was studied in relation to oxidant-induced premature senescence. Plants, 26 days old, were exposed to 0.06 to 0.08 microliters per liter O_3 from 1000 to 1600 hours for 4 days in a controlled environment chamber. On day 5, plants were exposed to a 6-hour simulated inversion in which O_3 peaked at 0.12 microliters per liter. Net photosynthesis declined in response to O_3 but recovered to near control levels 3 days after the exposure ended. Rubisco activity and quantity in control potato foliage increased and then decreased during the 12-day interval of the study. In some experiments foliage studied was physiologically mature and Rubisco activity had peaked when O_3 exposure commenced. In those cases, O_3 accelerated the decline in Rubisco activity. When less mature foliage was treated with O_3, the leaves never achieved the maximal level of Rubisco activity observed in control foliage and also exhibited more rapid decline in initial and total activity. Percent activation of Rubisco (initial/total activity) was not affected significantly by treatment. Quantity of Rubisco decreased in concert with activity. The decrease in activities is most likely due to a decrease in available protein rather than a decrease in the percentage of Rubisco activated *in vivo*. The reduction in the quantity of Rubisco, an important foliar storage protein, could contribute to premature senescence associated with toxicity of this air pollutant.

Ozone (O_3) is a major phytotoxic air pollutant responsible for crop loss in the United States (5). An early event observed in plants exposed to O_3 is a reduction in net photosynthesis (18). Later in development, plants stressed by O_3 often exhibit premature senescence (15). Both reduced photosynthetic rate and premature senescence may lead to reduced plant productivity.

A key protein involved in both photosynthesis and, indirectly, in senescence is Rubisco[3] (2). This protein performs a dual role, serving as the enzyme for net CO_2-fixation during photosynthesis (13) and as the major storage protein in the leaf (7). Changes in synthesis and degradation of Rubisco may be divided into three phases corresponding to leaf age (2, 11). The first phase is a period of rapid synthesis and almost undetectable degradation, coinciding with the period of leaf expansion. At full leaf expansion, rubisco quantity reaches a maximum and may constitute up to 70% of the total soluble leaf protein (12). The second phase is a steady state period in which synthesis and degradation rates are similar. The final phase is characterized by a period in which degradation is the main process. After full leaf expansion, less Rubisco is needed for photosynthesis, and the enzyme begins to degrade to export nitrogen and carbon to growing regions of the plant (7).

Several studies have been conducted to investigate the effects of O_3 on Rubisco. Nakamura and Saka (14) reported a 50 to 80% decline in activity of Rubisco in rice leaves after a 2-h exposure to 0.12 $\mu L\ L^{-1}$ O_3. Pell and Pearson (16) demonstrated a decrease in Rubisco quantity in alfalfa after a similar acute O_3 exposure of 0.25 $\mu L\ L^{-1}$ for 2 h. The results of the latter study were especially intriguing, showing a 36 to 80% decrease in enzyme quantity even in those leaves exposed to O_3 but without foliar symptoms. More recently, Lehnherr *et al.* (9) exposed field-grown wheat to 0.1 $\mu L\ L^{-1}$ O_3 for 8 h/d for 2 months and reported a decrease in Rubisco activity in flag leaves.

Evidence exists that O_3 reduces net photosynthesis in many species (18) and changes in the status of Rubisco have been offered as a partial explanation. The implications of O_3-induced decreases in Rubisco content could extend beyond the role of this enzyme in photosynthesis. As described above, Rubisco goes through a process of synthesis and degradation which characterizes, in part, the maturation and aging processes of the leaf. The O_3-induced decrease in Rubisco could reflect an acceleration of this process and offer a partial explanation for O_3-induced premature senescence. In the present study, we sought to more thoroughly evaluate the effect of O_3 on the status of Rubsico throughout foliar maturation and senescence in potato.

MATERIALS AND METHODS

Plant Culture

Solanum tuberosum L. cv 'Norland' plants were used for these experiments. This C_3 species has been shown to exhibit

[1] Supported in part by U.S. Department of Agriculture Grant No. 84-CSRS-22394. Contribution No. 1723, Department of Plant Pathology, The Pennsylvania State Agricultural Experiment Station. Authorized for publication as Journal Series Paper No. 8086.

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[3] Abbreviations: Rubisco, ribulose bisphosphate carboxylase/oxygenase; PPFD, photosynthetic photon flux density.

premature senescence after exposure to O_3 (17). The Norland cultivar has been shown to be O_3-sensitive (3).

Norland potato tubers (W. Bauman, Coudersport, PA) were cut into 2.5 cm diameter pieces and dusted with 8% potato fungicide (zinc ion maneb complex; Agway, Inc., Syracuse, NY) and allowed to suberize at 16°C and 90% RH for 4 to 6 d. Tuber pieces were planted in 10-cm plastic pots containing a 1:1 mixture of peat:vermiculite with a controlled release fertilizer, 14:14:14 N:P:K, at a rate of 1.6 kg m^{-3}. Plants were grown in the greenhouse and supplemented with artificial lighting from 600 to 2200 h for 22 d. The supplemental lighting ensured that plants in all experiments, conducted from July through November, received the same 16-h photoperiod. While natural light intensity fluctuated daily, supplemental lighting also ensured that intensity at the leaf surface did not fall below 450 μE m^{-2} s^{-1}. Greenhouse temperature was maintained from 22 to 28°C. On d 23, plants were transplanted into 15-cm plastic pots in the same soil mix in preparation for O_3 exposure.

Ozone Exposure

The plants were 26 d old on the first day of the O_3 treatment. Approximately 18 h before O_3 exposure, plants were transferred to charcoal-filtered environmental growth chambers maintained at 21°C and 70% RH and averaging 450 μE m^{-2} s^{-1} PPFD at plant height with the same photoperiod as the greenhouse.

Plants were treated with charcoal-filtered air with or without O_3 from 1000 to 1600 h for 5 consecutive days. After the 6-h treatment each day, plants were returned to the greenhouse overnight. The O_3 regimen was designed to simulate a 5-d exposure that plants might experience in the ambient environment. On d 1 through 4, plants were exposed consecutively to 2 h of O_3 at 0.06 μL L^{-1}, 3 h at 0.08 μL L^{-1}, and 1 h at 0.06 μL L^{-1}. The exposure schedule on d 5 was designed to simulate an inversion. Plants were exposed consecutively to 1 h of O_3 at 0.06 μL L^{-1}, 1 h at 0.09 μL L^{-1}, 3 h at 0.12 μL L^{-1}, and 1 h at 0.06 μL L^{-1}. Ozone was generated by passing O_2 through an OREC O3V1 ozonator (Ozone Research and Equipment Corp, Phoenix, AZ) and was monitored continuously with a Dasibi 1003AH O_3 monitor (Dasibi Environmental Corp., Glendale, CA). Five point calibrations of the O_3 monitor were performed with a dedicated Dasibi 1008-PC analyzer.

Rubisco Extraction and Activity Assays

Rubisco activity was assayed by a modification of the procedure from Seemann *et al.* (20). The sixth oldest leaves from each of two plants were used for all analyses. Leaves were excised, midribs removed, and 0.04 g used for extraction. The remaining tissue was weighed and dried to a constant weight at 70°C.

Leaf tissue was frozen with liquid nitrogen and ground to a fine powder with a cold mortar and pestle. The powder was transferred to a Dounce glass homogenizer and ground for about 30 s under nitrogen with 4.0 mL of extraction buffer consisting of 0.1 M Tris-HCl (pH 7.8), 0.1 mM EDTA, 1.5% polyvinyl-pyrrolidone-40T, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride (PMSF), and 10 μM leupeptin sulfate (Sigma Chemical Co., St. Louis, MO). The extraction buffer was prepared 'CO_2-free' by gently boiling the distilled H_2O for 15 min prior to addition of reagents. The homogenate was filtered through two layers of cheesecloth. A 1.0-mL sample of the homogenate was centrifuged at 15,850*g* for 15 s in a microfuge. Aliquots were then removed to measure initial and total Rubisco activity. Initial activity was defined as that activity assayed without a preincubation in the presence of the enzyme activators CO_2 and Mg. To initiate the assay of initial activity, a 50-μL sample of supernatant was added to 450 μL of assay buffer consisting of 0.1 M Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 1 mM EDTA, 13 mM $NaH^{14}CO_3$ (2.6 μCi per assay vial, equal to 48.1 kBq), and 0.5 mM ribulose bisphosphate. The reaction was terminated after 30 s with 100 μL 2 N HCl.

Total activity was measured by adding 900 μL of the extract supernatant to 100 μL of a preincubation mixture consisting of 100 mM $NaHCO_3$ and 200 mM $MgCl_2$ to bring to a final concentration of 10 mM $NaHCO_3$ and 20 mM $MgCl_2$. The extract was allowed to incubate for 10 min and served to activate any unactivated Rubisco. A 50-μL aliquot of the diluted extract was used to assay activity as described for initial activity. Acid-stable ^{14}C-radioactivity was determined in a Beckman LS7000 scintillation counter.

Rubisco Quantity Determination

Rubisco quantity was determined for the 6th oldest leaves. Rubisco was extracted as described for activity assays except that the buffer contained 20 mM $MgCl_2$ and 10 mM $NaHCO_3$. The supernatant was used for all protein determinations. Total soluble protein concentration was determined with the Coomassie blue reagent (1) and BSA as standard.

To concentrate the protein, 0.1 mL of 50% TCA was added to a 0.5-mL sample of the extract supernatant in a 1.5-mL microfuge tube (10). After 30 min at 0°C, the sample was centrifuged at 15,850*g* for 5 min, excess TCA was decanted, and 1.0 mL of cold acetone was added. The sample was incubated at 4°C for 1 h. The sample was centrifuged at 15,850*g* for 5 min, the supernatant was decanted, and the acetone wash was repeated. After the second wash, the supernatant was decanted and the pellet allowed to dry at 4°C.

To each pellet was added 100 μL of electrophoresis buffer (0.062 M Tris-HCl (pH 6.8), 2% SDS, 0.286 mM 2-mercaptoethanol, 0.05% bromophenol blue, and 10% glycerol) and 400 μL of extraction buffer. Each vial was heated for 4 min at 95°C and allowed to cool before electrophoresis.

Rubisco subunits were separated electrophoretically in SDS-PAGE slab gels with the Mini-Protean II system (Bio-Rad Laboratories, Rockville Centre, NY). A discontinuous buffer system based on that of Laemmli (8) was used, consisting of a 0.375 M Tris (pH 8.8), 12% polyacrylamide resolving gel (5.5 cm), and a 0.125 M Tris (pH 6.8), 4% polyacrylamide stacking gel (2.5 cm). The gels were 0.75 mm thick. The running buffer consisted of 0.025 M Tris, 0.192 M glycine (pH 8.3), with 0.1% SDS.

Two subsamples of protein extract, 5 and 10 μL each, were added to adjacent wells. Low mol wt standards (range: 94,000–14,400 D; Pharmacia, Piscataway, NJ) were used to

calibrate each gel. Electrophoresis was performed at 150 V (10–20 mA per gel) for 55 to 65 min or until the dye front migrated to about 1 cm from the bottom of the gel. Gels were stained for 30 min in Coomassie blue stain (45.4% methanol, 9.2% glacial acetic acid, 0.25% Coomassie brilliant blue R, Sigma Chemical Co.). The gels were destained overnight in a solution of 24% ethanol and 8% glacial acetic acid.

To quantitate Rubisco, gels were scanned with a Quick-Scan, Jr. densitometer (Helena Laboratories, Beaumont, TX). Rubisco quantity, expressed on a dry weight basis, was calculated from the proportion of total soluble protein that consisted of the large and small subunits of Rubsico, the concentration of total soluble protein, and the extract volume.

Photosynthesis Measurements

Net photosynthesis by potato foliage was determined with an LI-6000 photosynthesis system (Li-Cor, Lincoln, NE). Leaf (average area 18 cm^2) photosynthetic rates were determined in growth chambers. All measurements were made at 21°C, 70% RH, 350 to 380 $\mu L\ L^{-1}$ CO_2, and a PPFD averaging 450 $\mu E\ m^{-2}\ s^{-1}$ at the leaf surface. Measurements were made on the same leaves as Rubisco activity assays and within an hour before excision and subsequent assay.

Experimental Design

Two experiments were conducted. The objective of experiment 1 was to measure changes in Rubisco activity with O_3 exposure over time. Initial and total activity were measured in order to determine whether O_3 altered the percent activation of the protein, where

$$\% \text{ activation} = \frac{\text{initial activity}}{\text{total activity}} \times 100$$

Rubisco activities were determined before the O_3 treatments began (26 d after planting), before the simulated inversion on the fifth day of the O_3 exposure (30 d after planting), after the inversion (31 d after planting), and 4 and 7 d after the exposure ended (34 and 37 d after planting). In a preliminary experiment (data not shown), an older leaf (third or fourth oldest) and a younger leaf (6th oldest) were sampled to determine which leaf was more responsive to O_3 treatment. Because there was little difference in Rubisco activities between the two age groups, the younger leaf was chosen; at this age, Rubisco quantity in other species was close to maximum (11). The experiment was repeated four times between August and November 1986.

The objective of experiment 2 was to measure changes in Rubisco quantity as well as activity in response to O_3 exposure over time. Plant age during sampling and O_3 regimen were the same as in experiment 1 except a sampling day was added at 28 d after planting to better characterize the effects of the O_3 treatment. Total Ribusco activity was measured on these leaves as described before. The experiment was repeated three times between September and November 1987.

Data Analysis

Data were analyzed by analysis of variance using general linear regression models (19).

RESULTS

The response of Rubisco activities and quantity and net photosynthesis to O_3 exposure varied with the time of the year in which the plants were grown. Analyses of variance performed on the trials conducted within an experiment revealed interactions which indicated that it was inappropriate to average the results from each experiment. The first three trials from experiment 1, conducted in August, September, and October 1986, respectively, showed a linear response to O_3 exposure as exemplified by the August trial (Fig. 1, A and B). Initial and total activities declined in both O_3-exposed and control plants. Ozone accelerated this decline to a significant

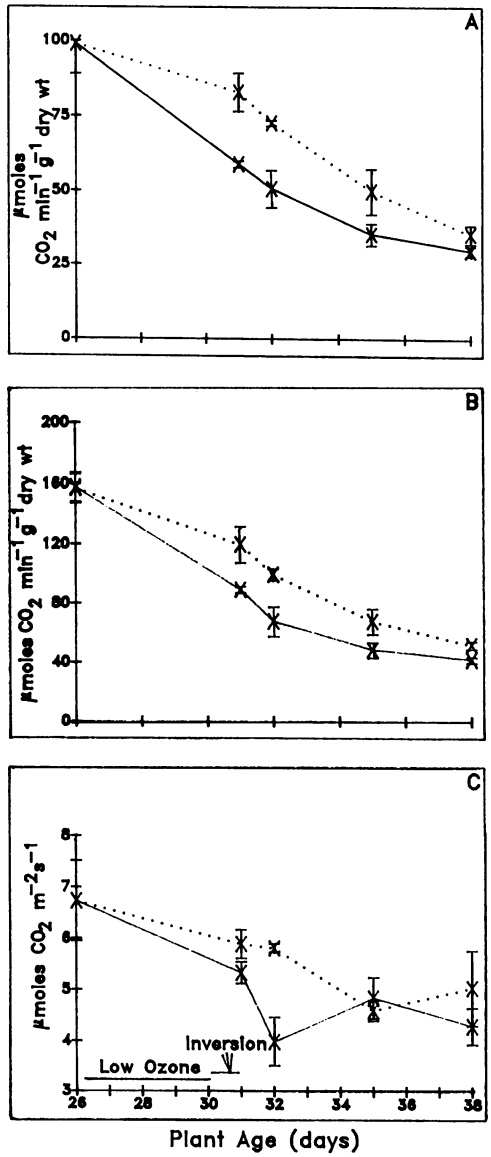

Figure 1. Effects of a 5-d O_3 exposure on (A) initial Rubisco activity, (B) total Rubisco activity, (C) net photosynthesis of foliage from Norland potato plants in August beginning at 26 d after planting. Plants were exposed to low O_3 concentrations for 4 d and a simulated inversion on d 30. The sixth oldest leaf was used for analysis. Values represent means ± SE of three trials. (——), O_3; (· · ·), control.

degree by the fifth day of O_3 exposure. Net photosynthesis showed a similar response to O_3 exposure (Fig. 1C).

The fourth trial of experiment 1, conducted in November 1986, showed a cubic response to O_3 exposure (Fig. 2, A and B). Rubisco activities increased initially before peaking at d 34. After this time, activities decreased, with the O_3-treated plants declining more rapidly than those of the control group. Net photosynthesis remained elevated until 31 d after planting when decline began (Fig. 2C); in the earlier trials net photosynthesis declined throughout the study period (Fig. 1C). Percent activation of Rubisco varied between 36 and 81% independent of sampling time and did not differ significantly between treatments.

In experiment 2, conducted from September through November 1987, total Rubisco activity was similar to that observed in the fourth trial of experiment 1 (Figs. 3A and 2B). Activity increased initially before a peak was observed and then declined. The decline was more rapid following exposure to O_3. When the quantity of Rubisco was determined, variation inherent in the technique resulted in interactions between trials which also made pooling of data inappropriate. When trials were analyzed separately, we were able to detect O_3-induced reductions in Rubisco quantity in two of three cases as exemplified by the trial conducted in October (Fig. 3B).

DISCUSSION

The activity and quantity curves correspond to the Rubisco synthesis and degradation curves observed in rice (11). Rubisco is synthesized with little degradation until full leaf expansion, at which time degradation takes on a more prominent role (11). While O_3 exposure in all trials was begun at the same chronological age of the plant (26 d after planting), those plants grown in November 1986 and in all trials in 1987 may have been physiologically younger than those grown between August and October 1986. Since plants were grown in the greenhouse during a 16-month period, it is not surprising that

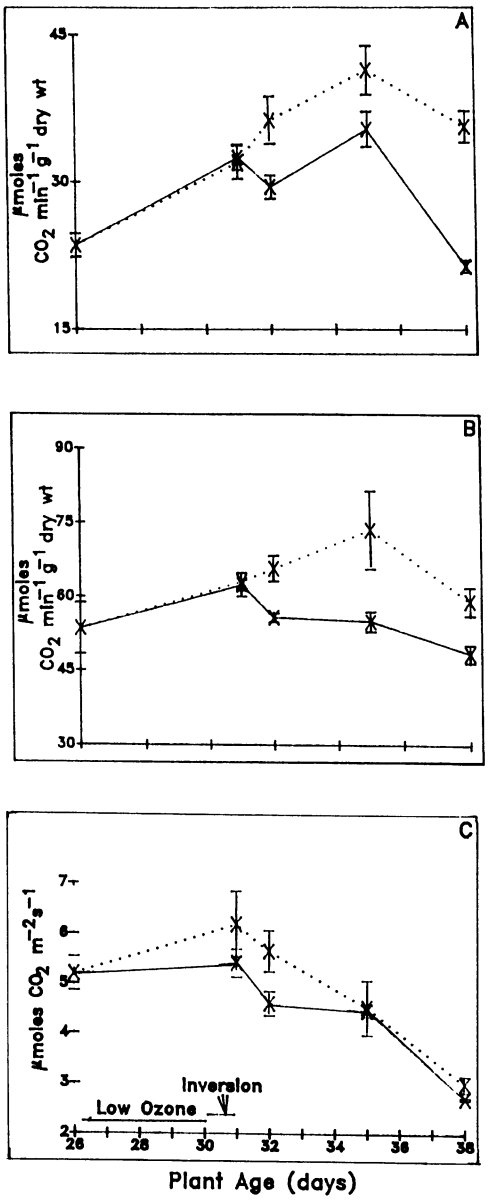

Figure 2. Effects of a 5-d O_3 exposure on (A) initial Rubisco activity, (B) total Rubisco activity, (C) net photosynthesis of foliage from Norland potato plants in November beginning at 26 d after planting. Plants were exposed to low O_3 concentrations for 4 d and a simulated inversion on d 30. The sixth oldest leaf was used for analysis. Values represent means ± SE of three trials. (——), O_3; (· · ·), control.

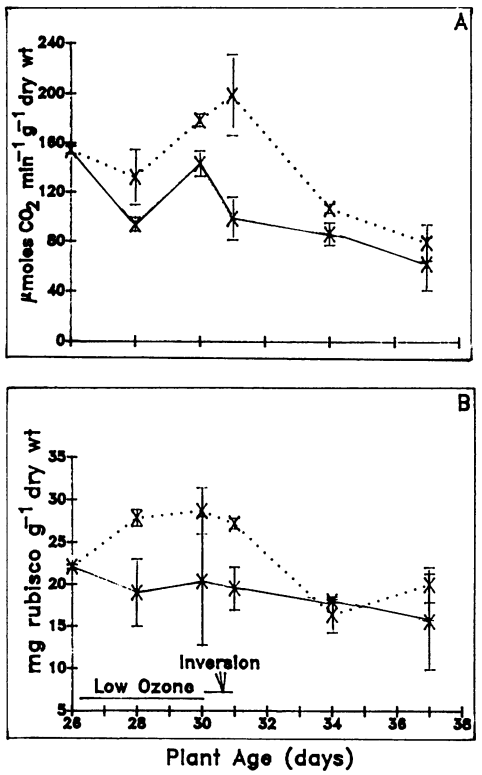

Figure 3. Effects of a 5-d O_3 exposure on (A) total Rubisco activity, (B) quantity of Rubisco large and small subunits as determined spectrophotometrically by scanning stained SDS-PAGE gels. These experiments were performed on a different set of plants but using the same O_3 exposure conditions as in Figures 1 and 2. Values represent means ± SE of two trials. (——), O_3; (· · ·), control.

there would be developmental variation among trials and experiments. Thus, the response seen in trials conducted in August to October 1986 (Fig. 1, A and B), reflects plants which were exposed to O_3 after most Rubisco synthesis had ceased and the process of degradation had become important. In these trials, a decrease in Rubisco activities was observed from the beginning of the treatment period in both O_3-exposed and control plants, with O_3 accelerating the decline.

In contrast, in those trials performed with plants grown in November 1986 (Fig. 2, A and B) and in September to November 1987 (Fig. 3, A and B) O_3 exposure probably occurred prior to, as well as after, cessation of Rubisco synthesis. Ozone appeared to prevent Rubisco levels from ever reaching those of control plants. Since degradation of Rubisco in rice begins before synthesis stops (11), any effect which O_3 might have on degradation could be detected when examining net quantity of the enzyme.

Generally, the responses of photosynthesis and Rubisco activity to O_3 exposure were similar. However, initial Rubisco activity did not correlate strongly with net photosynthesis (r=0.65, P=0.0001). Measurement of photosynthesis with the LiCor system was influenced by other parameters, such as stomatal aperture, which were not directly related to Rubisco activity. Initial Rubisco activity (measured outside the cell) is only indirectly related to stomatal status in the attached leaf. The poor correlation may be also attributed to the basis for expression of the two parameters, i.e. leaf area (photosynthesis) *versus* dry weight (Rubisco activity). The differences may also be a reflection of the dual role of Rubisco for CO_2 fixation and for nitrogen storage. Quantity of protein, as a means of nitrogen storage, may be more important than photosynthetic capacity at this point in the life cycle of the leaf.

In nonstressed, normally senescing plants, Rubisco quantity attains a maximum value and maintains this steady concentration briefly until it is required for remobilization at which time it is quickly degraded (21). Barring environmental stress, Rubisco begins to degrade just after full leaf expansion when less enzyme is needed for photosynthesis (2). If nitrogen is needed for production of new tissue, degradation of Rubisco in mature leaves is one mechanism to provide amino acids for growth elsewhere in the plant. As such, Rubisco concentration in the leaf is responsive to nutrient demand brought on by stress or source/sink changes during senescence (7). Proteolysis of Rubisco occurs, furnishing the nitrogen requirements for the sink.

Rubisco quantity decreases after exposure to O_3. This loss could occur because O_3 directly oxidizes the enzyme. It is unlikely, however, that O_3 or its byproducts would penetrate to the stroma in a high enough concentration to chemically degrade detectable quantities of protein. Alternatively, Rubisco may degrade because of enhanced enzymatic proteolysis after O_3 exposure. Dalling (2) has proposed that proteolysis occurs when protein, Rubisco in particular, undergoes chemical modification and becomes more 'acceptable' to proteases. Free radicals may be responsible for producing changes in Rubisco in nonstressed plants (2). The proposal that O_3 might produce changes in Rubisco similar to those which occur normally, but at an earlier time, seems quite plausible. Elsewhere, we provide data which demonstrate that O_3 has the potential to enhance proteolysis of Rubsico (MS Dann, EJ Pell, unpublished data).

Lehnherr *et al.* (9) reported an increase in activation percentage of Rubisco in flag leaves of wheat exposed to environmental levels of O_3 in open-top chambers. We did not find an increase in activation state with O_3 exposure in our experiments. They reported decreased Rubisco quantity and net photosynthesis which is consistent with results reported here.

On the last day of the exposure, O_3 concentration was increased to simulate an inversion. This O_3 peak did not elicit any major response beyond that already observed. Elkiey and Ormrod (4) noted a decreased uptake of O_3 during a 3-d study with petunia as the experiment progressed. They suggested that subtle internal injury in the leaves may have caused the decrease. If potato plants sustained similar injury, the apparent lack of additional plant response to the simulated inversion could be explained by reduced O_3 flux.

The recovery of net photosynthesis after the O_3 exposure ended (Fig. 1C) is consistent with the observations of other investigators. Hill and Littlefield (6) noted that net photosynthesis returned to control levels within 24 h after plants were removed from O_3. The recovery was reportedly related to stomatal reopening after the stress was removed.

The results of this series of experiments demonstrate an O_3-induced decrease in Rubisco activities and quantity and net photosynthesis similar to that seen in other species (9, 14, 16). Net photosynthesis recovered to near control levels 4 d after plants were removed from O_3 stress while the decline in activity and quantity of Rubisco could still be detected. Initial activity declined in tandem with total activity. The decrease in initial activity is likely accounted for by a loss of total Rubisco protein rather than a decrease in the proportion of the protein in an activated form. The loss of this major storage protein may have important consequences in O_3-induced premature senescence.

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