PHYSIOLOGIA PLANTARUM 106: 378–385. 1999
*Printed in Ireland—all rights reserved*

*ISSN 0031-9317*

# Effects of chronic ozone and elevated atmospheric $CO_2$ concentrations on ribulose-1,5-bisphosphate in soybean (*Glycine max*)

**Chantal D. Reid[a,*], Edwin L. Fiscus[a,b] and Kent O. Burkey[a,b]**

[a]*Department of Crop Science, Agricultural Research Service, North Carolina State University, 3908 Inwood Road, Raleigh, NC 27603, USA*
[b]*USDA-ARS, Air Quality Plant Growth and Development Research Unit, North Carolina State University, 3908 Inwood Road, Raleigh, NC 27603, USA*
**Corresponding author, e-mail: chantal_reid@ncsu.edu*

Received 10 November 1998; revised 23 April 1999

**Ribulose-1,5-bisphosphate (RuBP) pool size was determined at regular intervals during the growing season to understand the effects of tropospheric ozone concentrations, elevated atmospheric carbon dioxide concentrations and their interactions on the photosynthetic limitation by RuBP regeneration. Soybean (*Glycine max* [L.] Merr. cv. Essex) was grown from seed to maturity in open-top field chambers in charcoal-filtered air (CF) either without (22 nmol $O_3$ $mol^{-1}$) or with added $O_3$ (83 nmol $mol^{-1}$) at ambient (AA, 369 μmol $CO_2$ $mol^{-1}$) or elevated $CO_2$ (710 μmol $mol^{-1}$). The RuBP pool size generally declined with plant age in all treatments when expressed on a unit leaf area and in all treatments but CF-AA when expressed per unit ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) binding site. Although $O_3$ in ambient $CO_2$ generally reduced the RuBP pool per unit leaf area, it did not change the RuBP pool per unit Rubisco binding site. Elevated $CO_2$, in CF or $O_3$-fumigated air, generally had no significant effect on RuBP pool size, thus mitigating the negative $O_3$ effect. The RuBP pools were below 2 mol $mol^{-1}$ binding site in all treatments for most of the season, indicating limiting RuBP regeneration capacity. These low RuBP pools resulted in increased RuBP regeneration via faster RuBP turnover, but only in CF air and during vegetative and flowering stages at elevated $CO_2$. Also, the low RuBP pool sizes did not always reflect RuBP consumption rates or the RuBP regeneration limitation relative to potential carboxylation (%RuBP). Rather, %RuBP increased linearly with decrease in the RuBP pool turnover time. These data suggest that amelioration of damage from $O_3$ by elevated atmospheric $CO_2$ to the RuBP regeneration may be in response to changes in the Rubisco carboxylation.**

## Introduction

Atmospheric carbon dioxide concentrations ($[CO_2]$) and tropospheric ozone concentrations ($[O_3]$) are increasing on a global scale, mainly as a result of increased anthropogenic emissions (IPCC 1996). At the current rate of $CO_2$ emissions, a doubling of pre-industrial $[CO_2]$ by the end of the 21st century is forecast (IPCC 1996). In contrast, high $[O_3]$ occurs mainly around urban regions (Thompson et al. 1990), although the global $[O_3]$ is increasing (Ashmore and Bell 1991). Individually, both trace gases affect photosynthesis (see Drake et al. 1997 for elevated $CO_2$ and Pell et al. 1994 for $O_3$), but in divergent ways. Elevated $CO_2$ usually increases photosynthesis because of increased carboxylation, while $O_3$ decreases photosynthesis because of reduced ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity. Therefore, understanding the combined effects of these trace gases on biochemical controls of photosynthesis is necessary to predict photosynthesis in future atmospheres.

Studies of the combined effects of elevated $CO_2$ and $O_3$ on photosynthesis show that elevated $CO_2$ reduces most of the adverse effects of $O_3$ (McKee et al. 1995, Booker et al. 1997, Kellomäki and Wang 1997, Reid and Fiscus 1998). The amelioration of $O_3$ damage is presumed to be due

*Abbreviations* – AA, ambient $CO_2$ air; CABP, carboxyarabinitol bisphosphate; CF, charcoal-filtered air; CO2, elevated $CO_2$ air; DAP, days after planting; OZ, $O_3$-fumigated air; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; %RuBP, RuBP regeneration limitation relative to potential Rubisco capacity.

largely to a reduction in leaf conductance with elevated $CO_2$ and, thus, of ozone flux into the leaf (McKee et al. 1995, Fiscus et al. 1997, McKee et al. 1997). In addition, McKee et al. (1995) suggested that the protection from $O_3$ with elevated [$CO_2$] might involve a shift of the biochemical limitation of photosynthesis away from Rubisco capacity towards ribulose-1,5-bisphosphate (RuBP) regeneration. However, such a shift can lead to limitations by RuBP regeneration. Indeed, we reported a higher limitation by RuBP regeneration in elevated $CO_2$ both in charcoal-filtered and $O_3$-fumigated air during vegetative growth of soybean (Reid and Fiscus 1998). This response to elevated $CO_2$ was without a change in carboxylation efficiency (Reid and Fiscus 1998), but with a decrease in initial Rubisco activity through a reduced activation state (Reid et al. 1998). However, during reproductive growth, photosynthetic limitations by RuBP regeneration have decreased for plants grown with elevated $CO_2$ in charcoal-filtered or $O_3$-fumigated air. Changes in the RuBP pool size were not characterized in those studies. Characterization of the RuBP pool size and time for pool turnover may help in understanding how elevated $CO_2$ reduces $O_3$ damage to the biochemical processes of photosynthesis.

Changes in RuBP pool size may signal the need for an adjustment between the Rubisco capacity to consume RuBP and the RuBP regeneration capacity. In a review on the regulation of photosynthetic carbon assimilation, Geiger and Servaites (1994) suggested that the activity of Rubisco was regulated by the concentrations of its substrate RuBP and its product 3-phosphoglycerate (PGA). With an increase in internal $CO_2$ concentration ($C_i$), the increased carboxylation by Rubisco reduces the RuBP pool size that, in turn, signals the need for an increase in the rate of RuBP regeneration. In short-term atmospheric $CO_2$ studies, the RuBP pool size was found to be inversely proportional to $C_i$ until a minimum low RuBP pool was reached (Badger et al. 1984, Mott et al. 1984), and the RuBP pool size becomes limiting to photosynthesis when the quantity of RuBP relative to Rubisco binding sites is below 1.7 (Seemann and Sharkey 1986, von Caemmerer and Edmondson 1986). Also, the photosynthetic model of von Caemmerer and Farquhar (1981) suggests that RuBP regeneration becomes limiting to photosynthesis at higher $C_i$. Long-term exposure to elevated $CO_2$ and $O_3$ may alter the RuBP pools because of changes in photosynthetic limitations.

Photosynthesis can be reduced because of altered RuBP regeneration capacity. The RuBP regeneration may be limited by electron transport in thylakoid-related processes or by a lack of inorganic phosphate ($P_i$) regeneration due to reduced activity of enzymes in the Calvin cycle (reviewed by Sage and Reid 1994). Using a tobacco mutant for the reversible conversion of PGA to triose phosphate, which reduced RuBP regeneration, Price et al. (1995) showed a decrease in the RuBP pool size that was a main factor for the decrease in photosynthesis. With long-term exposure to elevated [$CO_2$], an increase in leaf starch and sucrose accumulation can limit photosynthesis by reducing RuBP regeneration via reduced $P_i$ regeneration (Stitt 1991). With [$O_3$] exposure, damage to the electron transport capacity has been reported (Guidi et al. 1997, Reichenauer et al. 1997). Long-term exposure to elevated $CO_2$ and $O_3$ may alter the balance among the regulated processes between RuBP utilization and regeneration and, hence, the RuBP pool size and its role as a regulatory signal.

Limited data are available on the effect of long-term elevated atmospheric $CO_2$ or $O_3$ singly on the RuBP pool size. When grown over a range of 160–990 μmol $CO_2$ $mol^{-1}$, the RuBP pool decreased 64% for soybean in the vegetative stage, but was little affected by elevated $CO_2$ at the onset of reproduction (Campbell et al. 1987). On the other hand, when exposed to long-term $O_3$ fumigation, the RuBP pool per activated Rubisco binding site was decreased for $O_3$-fumigated wheat during anthesis, suggesting the possibility of a RuBP regeneration limitation (Lehnherr et al. 1988). Long-term exposure to combined $CO_2$ and $O_3$ may have a positive interactive effect on the RuBP pool size.

The objective of our study was to elucidate the main effects of elevated $CO_2$ and $O_3$, singly and in combination, on RuBP regeneration by determining the RuBP pool size and the RuBP turnover time in soybean grown in charcoal-filtered or $O_3$-fumigated air at either ambient or elevated $CO_2$. Because we previously measured differential photosynthetic limitations by RuBP regeneration with $CO_2$ in charcoal- and $O_3$-fumigated air for soybean during vegetative and reproductive phases, the RuBP pool was characterized at different stages of growth.

## Materials and methods

### Plant material and experimental design

The experimental design was the same as described by Booker et al. (1997) and Fiscus et al. (1997). On 31 May 1995, seeds of soybean (*Glycine max* [L.] Merr. cv. Essex) were planted in 21-l pots that contained a 2:1:1 (by volume) mixture of clay-loam topsoil:sand:vermiculite-sphagnum-perlite horticultural mix (MetroMix 220; W. R. Grace Co., Cambridge, MA, USA). Eight plants, one per pot, were grown in each of eight open-top field chambers equipped with charcoal filters at the Air Quality Plant Growth and Development Laboratory, USDA, in Raleigh, NC, USA. $CO_2$ and $O_3$ were dispensed from seedling emergence (6 days after planting [DAP]) until the end of the experiment (106 DAP). The two-way factorial design consisted of two atmospheric $CO_2$ concentrations (24 h $day^{-1}$) and two $O_3$ treatments (12 h $day^{-1}$) replicated twice. The two $CO_2$ treatments were current ambient (average $369 \pm 2$ μmol $mol^{-1}$, AA) and elevated $CO_2$ (average $710 \pm 6$ μmol $mol^{-1}$, CO2) and the two $O_3$ treatments were charcoal-filtered air (average $22 \pm 0.8$ nmol $O_3$ $mol^{-1}$, CF) and charcoal-filtered + $1.5 \times$ ambient $O_3$ (average $83 \pm 1.2$ nmol $mol^{-1}$, OZ). Dispensing and monitoring of $CO_2$ and $O_3$ are described by Booker et al. (1997).

### Sampling procedure

Leaves were harvested periodically during the growing season for measurement of Rubisco and RuBP content. Sampling was done between 11:00 and 13:00 h EST. The center leaflet of the first fully expanded mature leaf below the apex

on the main stem was sampled through pod formation (77 DAP). Afterwards, the main stem apical leaf was used because vegetative growth had stopped. Thus, leaves had the same physiological age through 77 DAP, leaves increased in age afterwards, and all were canopy leaves in full sun. At each sampling period, four plants were sampled per chamber. For sampling of Rubisco, a leaf disk was cut on the center leaflet and immediately immersed in liquid nitrogen. For sampling of RuBP, a rapid-freeze clamp (described below) cooled in liquid nitrogen was used to cut a leaf disk of 2.7 $cm^2$ area in less than 150 ms without shading the sample. The frozen leaf disk was immediately transferred to liquid nitrogen. The sample for Rubisco determination was always collected before the one for RuBP, each sample collected from opposite sides of the midvein. The samples were transferred to an ultra-freezer at $-80°C$ until the time of assay. All samples were taken in full sunlight. Photosynthetically active radiation averaged $1415 \pm 28$ μmol $m^{-2}$ $s^{-1}$ during sampling.

*Rapid-freeze clamp*

A portable freeze clamp that was easy to use in multiple sampling was made for field sampling in hot weather. The main body of the clamp was made of 2.5-cm thick brass (1.5 kg $block^{-1}$) because of its thermal conductivity (Fig. 1). The clamp has a fixed lower portion and a spring-loaded movable upper portion that is triggered by a lever on a wooden handle. In its center, the upper portion has a circular cutting area made of stainless steel that was milled out to avoid crushing the leaf. The upper portion moves down to the lower portion to simultaneously freeze-kill and cut the leaf in less than 150 ms. For a clean cut, the movement is guided by two stainless steel rods. The maximum area of contact with the leaf is 2.5 cm × 5.3 cm. Care was taken during sampling not to shade the leaf with the upper portion of the clamp by holding it at an angle. This clamp allowed for sampling of the leaf in its environment with no prior disturbance. Although the leaf area in contact with the clamp was killed, the remainder of the leaf was still functional.

## Biochemical analyses

*Rubisco extraction and assay*

Procedures followed Sage et al. (1993). After extraction, the crude Rubisco extract was incubated 12–15 min in 10 m*M* sodium bicarbonate ($NaHCO_3$) buffer to achieve full activation. The Rubisco content was then determined by liquid scintillation counting of $^{14}$CABP (carboxyarabinitol bisphosphate) bound to Rubisco (Collatz et al. 1979). Because all samples were taken at midday in full sunlight, all Rubisco binding sites, 8 per mole of Rubisco, were assumed to be free of inhibitors.

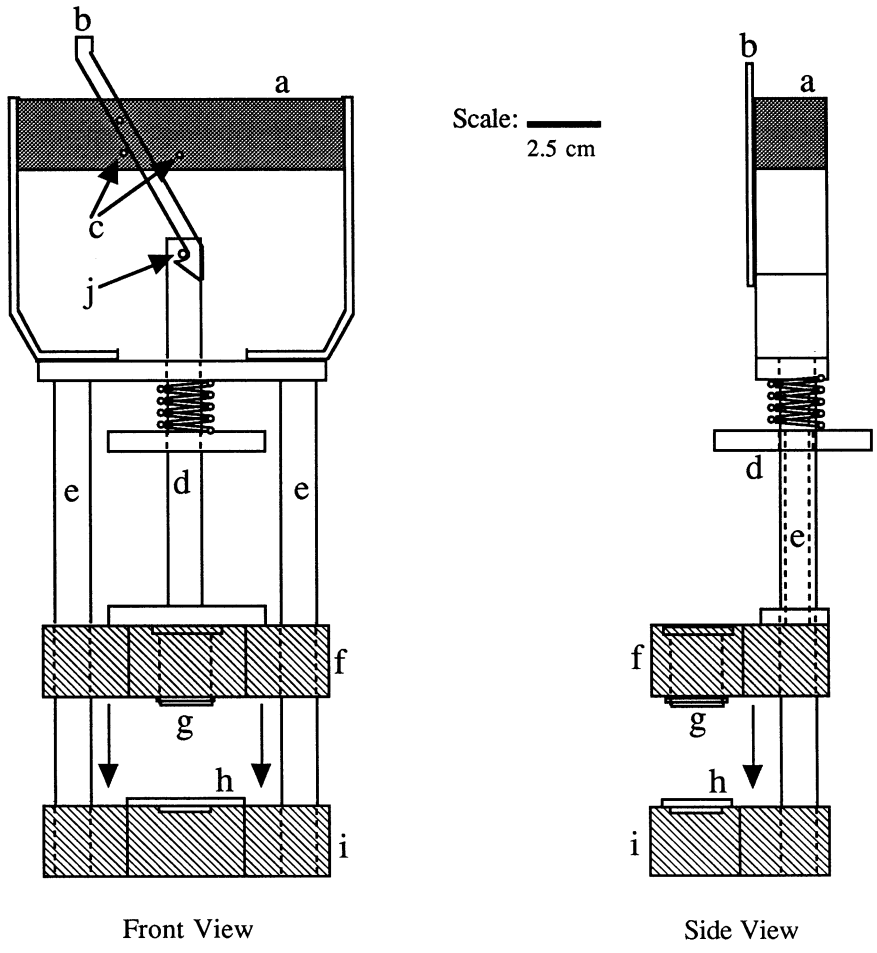


Fig. 1. Schematic of a rapid-freeze portable clamp. The clamp was made of brass (shaded area) and stainless steel (clear) except for the wood handle (a). Other components are as follows: (b) quick-release lever; (c) guides for lever; (d) spring-loaded movable piston; (e) guiding rods; (f) brass block on movable piston; (g) circular cutter on piston; (h) cutting plate; (i) fixed brass block; and (j) piston release pin.

*RuBP extraction and assay*

Extraction procedures followed Badger et al. (1984) as modified by Seemann and Sharkey (1986). After grinding the frozen leaf disk in a large mortar pre-cooled with liquid nitrogen using a small pre-cooled funnel, the frozen leaf powder was transferred to a microcentrifuge tube containing 0.5 ml of 3% (v/v) perchloric acid. The microcentifuge tube was shaken by hand while the leaf powder thawed. The sample was then centrifuged for 3 min at 16 000 *g*. An aliquot of the supernatant was incubated for 10 min on ice with a neutralizing agent and charcoal and centrifuged again for 3 min. The RuBP content was assayed via $^{14}CO_2$ incorporation into acid-stable compounds using purified Rubisco (EC 4.1.1.39; all chemicals from Sigma Chemicals Co., St Louis, MO, USA) that was activated in 10 m*M* $NaHCO_3$ buffer. Prior to assay, the 400 µl RuBP extract was incubated for 10 min with 10 µl of 100 m*M* hexokinase (EC 2.7.1.1) and 10 µl of 1 m*M* glucose to avoid contamination by other sugar phosphates (Seemann and Sharkey 1986). Three assays were averaged per sample. The RuBP pool was expressed per unit leaf area and per unit Rubisco binding site.

### RuBP turnover time

The time for RuBP pool turnover was calculated as the ratio of RuBP pool size to RuBP consumption rate. The rate of RuBP consumption was calculated as described in Sharkey (1988) using data from assimilation responses to internal $[CO_2]$ ($A/C_i$ curves) done on soybean in a similar experimental design in 1994 (Reid and Fiscus 1998). The in situ assimilation rates and Rubisco initial activities were similar in 1994 and 1995 (unpublished data; Reid et al. 1998) and, thus, we assumed that the rate of RuBP consumption was comparable between the 2 years. The rate of RuBP consumption and the RuBP pool size were paired according to DAP for each treatment.

In their previous study, Reid and Fiscus (1998) used an elimination technique to assess limitations to photosynthesis. Using $A/C_i$ curves, they assumed that the potential Rubisco capacity for carboxylation was the upper limit of photosynthesis, and stomatal and RuBP regeneration limitations were relative to photosynthesis at Rubisco capacity. The RuBP regeneration limitation relative to potential Rubisco capacity (%RuBP) was the difference between photosynthesis at the potential Rubisco capacity and photosynthesis at $C_i$ equal to ambient $CO_2$. The %RuBP that corresponded to the rate of RuBP consumption was combined with the pool turnover time to examine the relationship between RuBP turnover and RuBP limitation.

### Statistical analysis

The effects of $CO_2$ and $O_3$ on Rubisco content, RuBP content and rate of RuBP consumption were analyzed using a two-way analysis of variance (ANOVA; SAS Institute, Inc. 1986). The statistical model had $CO_2$ and $O_3$ as the main effects and period of sampling as a split-plot effect in a completely randomized design because no block effect was found. The mean squares of chambers within treatments and plants within chambers within treatments were tested and found to be similar, so plants were used as replicates. Because period of sampling was the most significant effect and could hide potential interactions, pair-wise comparisons were also carried out for each parameter at each sampling period. A linear regression model of turnover time against %RuBP was determined for all treatments pooled.

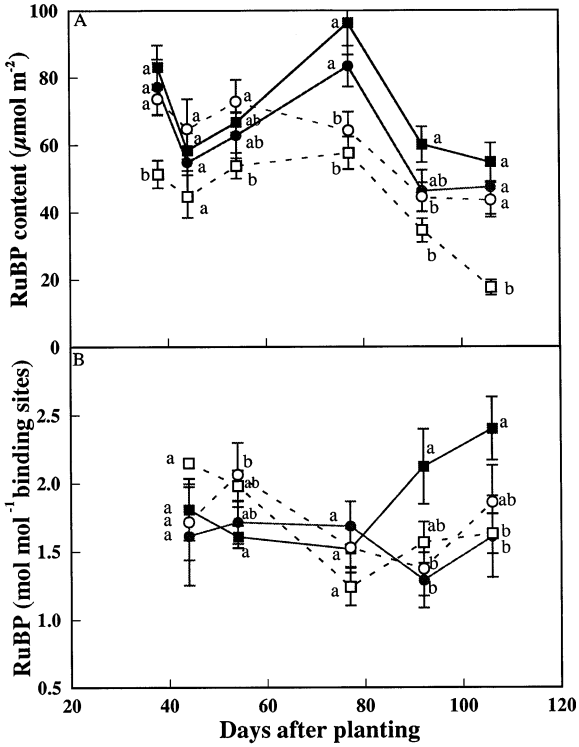


Fig. 2. Changes in the RuBP pool size of soybean grown at different $[O_3]$ and $[CO_2]$ during one growing season. The RuBP pool was expressed per unit leaf area (A) and per unit Rubisco binding site (B). Soybean was grown in charcoal-filtered air at ambient $CO_2$ (■) or at elevated $CO_2$ (●) and in $O_3$-fumigated air at ambient $CO_2$ (□) or at elevated $CO_2$ (○). Different letters within a sampling period indicate significant differences at $P \leq 0.05$.

## Results

### Sampling periods

Sampling period had a significant effect on the RuBP pool size and rate of RuBP consumption. When expressed per unit leaf area, the significant effects of sampling period ($P \leq 0.0001$) were because the RuBP pool decreased from mid-reproduction (77 DAP) onward (Fig. 2A). However, when expressed per unit Rubisco binding site, the change with sampling period was significant because of a period $\times$ $O_3$ interaction ($P < 0.04$). The interaction was because the RuBP pool size decreased from vegetative (44–54 DAP) to reproductive stages in $O_3$-treated plants, but increased during seed maturation (92–106 DAP) in plants treated with CF-AA (Fig. 2B). On the other hand, the significant effect of sampling period on RuBP consumption rate ($P < 0.0001$) was due to a decline from 58 DAP in all treatments but

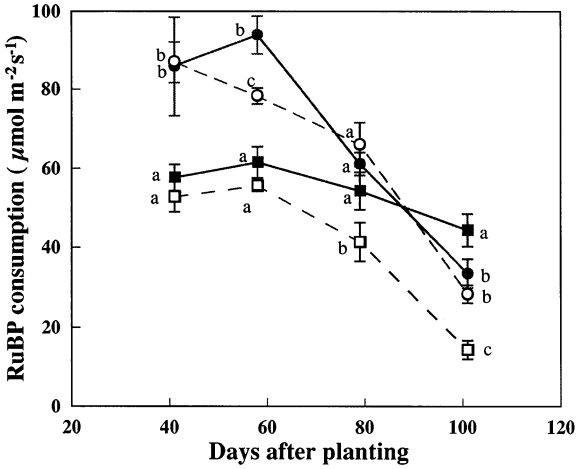


Fig. 3. Changes in the RuBP consumption rate for soybean grown at different [$O_3$] and [$CO_2$] during one growing season. Data are derived from 1994 measurements of $A/C_i$ (Reid and Fiscus 1998), as both years showed similar in situ photosynthesis and Rubisco activities. Soybean was grown in charcoal-filtered air at ambient $CO_2$ (■) or at elevated $CO_2$ (●) and in $O_3$-fumigated air at ambient $CO_2$ (□) or at elevated $CO_2$ (○). Different letters within a sampling period indicate significant differences at $P \leq 0.05$.

OZ-CO2, which decreased from the beginning (Fig. 3). Furthermore, although sampling period had no significant effect on the time for RuBP turnover, faster turnover times were calculated during the vegetative than reproductive stages (Fig. 4).

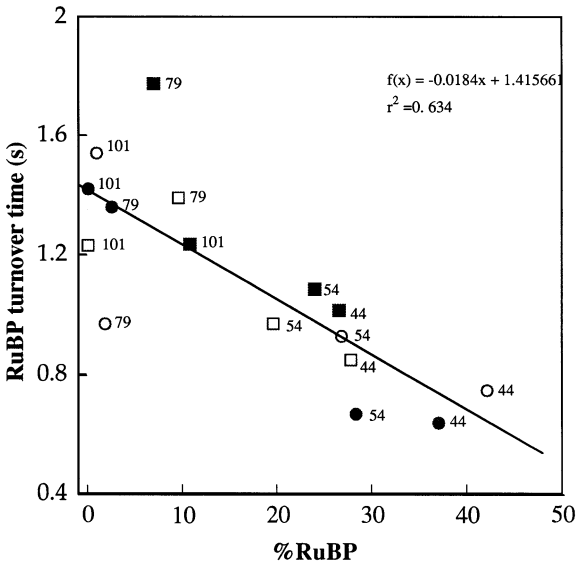


Fig. 4. Time for RuBP pool turnover as a function of the RuBP regeneration limitation relative to the potential Rubisco carboxylation (%RuBP). Soybean was grown in charcoal-filtered air at ambient $CO_2$ (■) or at elevated $CO_2$ (●) and in $O_3$-fumigated air at ambient $CO_2$ (□) or at elevated $CO_2$ (○). Time for turnover was estimated from the RuBP pool size per unit leaf area and from RuBP consumption rate. The %RuBP is as described in Materials and methods. The numbers next to each point are DAP. Data from all treatments are pooled for the regression model.

### Treatment effects

Ozone and elevated $CO_2$ concentrations had different effects on the RuBP pool size and the rate of RuBP consumption. Ozone had the most significant effect on the RuBP pool per unit leaf area as, at ambient $CO_2$, the pool size of plants in $O_3$-fumigated air was reduced 20–40% below plants in charcoal-filtered air (Fig. 2A). However, the effect of $O_3$ on the RuBP pool size depended upon what basis the pool was expressed, as $O_3$ had little effect on the pool size per unit Rubisco binding site until 92 DAP (Fig. 2B). In addition, $O_3$ significantly decreased the RuBP consumption rate from 58 DAP onward. In contrast, elevated $CO_2$ generally had no statistically significant effect on the RuBP pool per unit leaf area (Fig. 2A) although, relative to plants in CF-AA, it significantly decreased the RuBP pool size per unit binding site from 92 DAP onward (Fig. 2B). Furthermore, relative to CF-AA, elevated $CO_2$ increased the RuBP consumption rate in the first part of the season, but decreased it at the end of the growing season (Fig. 3).

Significant $CO_2 \times O_3$ interactions were observed on the RuBP pool size and RuBP consumption rate. For the RuBP pool size per unit leaf area, the significant $CO_2 \times O_3$ interactions ($P < 0.03$, 0.04, 0.02 and 0.006, at 38, 54, 92 and 106 DAP, respectively) occurred because the RuBP pool in the combined $CO_2 \times O_3$ treatment was similar to pools in elevated $CO_2$ in charcoal-filtered air, i.e., $O_3$ had no effect in the presence of elevated $CO_2$ (Fig. 2A). For the RuBP pool per unit binding site, the significant interactions ($P < 0.04$) occurred at the end of the season (106 DAP) when the pool for plants grown in the $CO_2 \times O_3$ treatment was intermediate between the pool in CF-AA and in either treatment alone (Fig. 2B). For RuBP consumption rate, the significant $CO_2 \times O_3$ interactions ($P < 0.05$ and 0.002, at 79 and 101 DAP, respectively) were observed during reproduction when elevated $CO_2$ alleviated the effect of $O_3$.

RuBP turnover time was not significantly affected by either the $CO_2$ or $O_3$ treatment. Rather, a linear regression model showed that the time for RuBP turnover was negatively proportional to the %RuBP, regardless of treatments (Fig. 4). In all treatments, the highest %RuBP and shortest RuBP turnover times were observed in the vegetative and flowering stages, whereas the lowest %RuBP and longest turnover time were observed at the end of the growing season.

## Discussion

We showed that sampling period, $O_3$ and elevated $CO_2$ had significant effects on the RuBP pool size and the rate of RuBP consumption. Sampling period, which had the largest overall effect, reflected plant and leaf ontogeny as the parameters generally decreased during reproduction. Ozone in ambient air reduced only the RuBP pool size per unit leaf area and the rate of RuBP consumption. In contrast, elevated $CO_2$, in charcoal-filtered or $O_3$-fumigated air, generally had little effect on the RuBP pool size but increased the rate of RuBP consumption, which resulted in a faster turnover of the RuBP pool. These data suggest an ameliora-

tion of the negative effect of $O_3$ on the RuBP regeneration by elevated $CO_2$. Also, the RuBP pool sizes were limiting to photosynthesis in most cases and acted as signals for increased RuBP regeneration, but only in CF air. Nevertheless, the time for RuBP turnover was shorter when the %RuBP was highest, regardless of treatments.

### Ontogenetic changes

The change in RuBP pool size with period of sampling initially reflected plant ontogeny and later reflected plant and leaf ontogeny. On a unit leaf area basis, the highest RuBP pool size in the CF-AA treatment was at the beginning of seed development (77 DAP), when maximum carboxylation capacity (Reid and Fiscus 1998) and Rubisco initial activity (Reid et al. 1998) were highest. RuBP was thus most abundant concomitantly with other components of the photosynthetic machinery, suggesting coordination among the limiting photosynthetic components. Such coordination was also found in $O_3$-fumigated plants. Makino et al. (1985) reported similar correlations among changes in photosynthesis, Rubisco content and RuBP pool size during ontogeny of developing rice leaves. However, these patterns were different from the ones observed for the rate of RuBP consumption suggesting that, although at its maximum, the RuBP pool size was still limiting to photosynthesis.

The RuBP pool size per unit Rubisco binding site is a more photosynthetically relevant variable for understanding the balance between RuBP regeneration and Rubisco carboxylation. Previous short-term studies on *Phaseolus vulgaris* have shown that photosynthesis becomes limited by RuBP when the RuBP pool size ranges from 1.6 to 2 mol $mol^{-1}$ binding site (Seemann and Sharkey 1986, Seemann et al. 1987). In our long-term study, the RuBP pool size remained below 2 mol $mol^{-1}$ binding site for most of the season in all treatments (Fig. 2B), suggesting a limitation by RuBP regeneration. These low RuBP pool sizes agree with limiting RuBP regeneration, but only during the vegetative stages for plants in CF air, and they were associated with a shorter RuBP turnover time that indicated higher RuBP regeneration. These low pools were likely signals for increased regeneration, as suggested by Geiger and Servaites (1994). However, this signaling from RuBP pools was not effective in $O_3$-fumigated tissue or during reproduction at elevated $CO_2$. During reproduction, carboxylation efficiency was decreased (Reid and Fiscus 1998) and time for turnover of RuBP was increased, possibly indicating alteration in the regulation of RuBP regeneration. Thus, the relationship between RuBP pool size per Rubisco binding site and the RuBP regeneration limitation was dependent on ontogeny.

### Treatment effects

The reduced RuBP pool sizes per unit leaf area we observed with $O_3$ in ambient air indicate a reduced RuBP regeneration capacity. The decrease in RuBP pool size with $O_3$ is consistent with a study on wheat grown in chronic $O_3$ exposure up to 100 nmol $mol^{-1}$ (Lehnherr et al. 1988) and a report of the substantial inhibition of the capacity for RuBP regeneration in *Quercus robur* with short-term $O_3$ exposure (Farage and Long 1995). In our study, the reduced RuBP pool size was probably not related to a decrease in RuBP regeneration via reduced electron transport capacity because $O_3$ had little effect on chlorophyll variable fluorescence ratio until late reproduction (E. L. Fiscus and K. O. Burkey, unpublished data). Likewise, the leaf $P_i$ of soybean was not limiting (F. L. Booker, unpublished data) and was at concentrations greater than the ones reported to reduce the RuBP pool size (Rao et al. 1989, Jacob and Lawlor 1992). However, $O_3$ effects on the RuBP pool size per unit leaf area may be altered by changes in the cell ultrastructure, such as changes in functional cell volume (Miyake et al. 1989), and changes in RuBP pools per unit binding site may be a better representation of $O_3$ damage to the biochemical machinery.

The lack of change in RuBP pool size per Rubisco binding site during vegetative and early reproduction suggested that the RuBP regeneration was balanced with the Rubisco capacity, which is reduced in $O_3$-stressed plants (Pell et al. 1997, Reid et al. 1998). Such a concomitant decrease in the Rubisco and RuBP content per unit leaf area may result from the accelerated senescence observed in $O_3$-fumigated soybean (e.g., Reid et al. 1998). In addition, these data are consistent with a primary effect of $O_3$ on biochemical rather than stomatal limitations of photosynthesis (McKee et al. 1995, Fiscus et al. 1997) because stomatal limitation (Fiscus et al. 1997, Reid and Fiscus 1998) and the ratio of internal to ambient $CO_2$ (C. D. Reid and E. L. Fiscus, unpublished data) were not affected by $O_3$. However, there was little correlation between the low RuBP pool size per binding site and %RuBP, suggesting that other components of the Calvin cycle were affected. Such disturbances in components of the Calvin cycle have been proposed for *Populus nigra* exposed to $O_3$ fumigation (Reichenauer et al. 1997).

The long-term effect of elevated $CO_2$ on the RuBP pool size has received little attention compared with short-term studies (e.g., Badger et al. 1984, von Caemmerer and Edmondson 1986, Sage et al. 1988). For example, greenhouse-grown *Phaseolus vulgaris* exposed to elevated $CO_2$ for 30 min had similar non-limiting RuBP pool size per unit Rubisco binding site at ambient and elevated $CO_2$ (Sage et al. 1988). Likewise, in our long-term study, elevated $CO_2$ had no significant effect on the RuBP pool size until after the beginning of seed filling. Yet, our RuBP pool sizes were limiting to carboxylation, both at ambient and elevated $CO_2$, and the maintenance of the RuBP pool sizes in the $CO_2$ treatments was accomplished by a shorter RuBP turnover time. These data indicate a faster RuBP regeneration in elevated $CO_2$.

Reduction in $O_3$ damage by elevated $CO_2$ may be by reduction of damage to the biochemistry. Although $O_3$ flux into the leaf was reduced because of reduced leaf conductance with elevated $CO_2$, the midday $O_3$ fluxes of soybean were higher in $CO_2 \times O_3$ than in CF-AA air (Fiscus et al. 1997), suggesting that biochemical limitations were also ameliorated with elevated $CO_2$. In our study, $CO_2$ generally alleviated the detrimental $O_3$ effect on the RuBP pool sizes per unit leaf area and RuBP consumption rates. However, the alteration may result from changes in the leaf ultrastruc-

ture and this possibility remains to be investigated. Furthermore, the alleviation of $O_3$ damage by elevated $CO_2$ is consistent with amelioration of the relative RuBP regeneration limitation for soybean (Reid and Fiscus 1998). Nevertheless, these data contrast with a study on sensitive and resistant clones of aspen that reported a similar effect of $O_3$ in ambient or elevated $CO_2$ on regeneration of RuBP (Kull et al. 1996). In addition, in the $CO_2 \times O_3$ treatment, the decrease in the RuBP consumption rate from the vegetative growth onwards is consistent with the accelerated senescence proposed by the earlier decrease in carboxylation efficiency for this treatment (Reid and Fiscus 1998). The RuBP regeneration may be to balance the Rubisco activity.

The RuBP pool sizes reported in our study are not always consistent with the %RuBP for the different treatments. The RuBP pool sizes do not appear to act as a signal for increased RuBP regeneration in $O_3$-fumigated environments and during reproduction in elevated $CO_2$. The amelioration of the negative $O_3$ effect with elevated $CO_2$ may be because of a RuBP regeneration response to Rubisco carboxylation when the carboxylation rate is high.

*Acknowledgements* – We are grateful to Robert Philbeck and Walt Pursley for field operations, Adam Dewitt and Evan Brady for plant maintenance and field sampling, and Mary-Catherine Kuralt-Smith for laboratory assistance. We also thank Drs F. L. Booker and M. A. Gonzalez-Meler for comments on an earlier version of the manuscript. The research was supported by USDA Agricultural Research Service, CRIS # 6645-11000-003-00D. The use of trade names in this publication does not imply endorsement of the products named by North Carolina Agriculture Research Service, US Department of Agriculture, or North Carolina State University, nor criticism of similar ones not mentioned.

## References

Ashmore MR, Bell JNB (1991) The role of ozone in global change. Ann Bot 67: 39–48

Badger MR, Sharkey TD, von Caemmerer S (1984) The relationship between steady-state gas exchange of bean leaves and the levels of carbon-reduction-cycle intermediates. Planta 160: 305–313

Booker FL, Reid CD, Brunschön-Harti S, Fiscus EL (1997) Photosynthesis and photorespiration in soybean [*Glycine max* (L.) Merr.] chronically exposed to elevated carbon dioxide and ozone. J Exp Bot 48: 1843–1852

Campbell WJ, Allen LH Jr, Bowes G (1987) Effects of short-term and long-term exposures to varying $CO_2$ concentrations on soybean photosynthesis. In: Biggens J (ed) Progress in Photosynthesis Research IV. Martinus Nijhoff, Dordrecht, pp 253–256. ISBN 90-247-3449-5

Collatz GJ, Badger MR, Smith C, Berry JA (1979) A radioimmune assay for $RuP_2$ carboxylase protein. Carnegie Inst Washington Yearb 78: 171–175

Drake BG, Gonzàlez-Meler MA, Long SP (1997) More efficient plants: A consequence of rising atmospheric $CO_2$? Annu Rev Plant Physiol Plant Mol Biol 48: 609–639

Farage PK, Long SP (1995) An in vivo analysis of photosynthesis during short-term $O_3$ exposure in three contrasting species. Photosynth Res 43: 11–18

Fiscus EL, Reid CD, Miller JE, Heagle AS (1997) Elevated $CO_2$ reduces $O_3$ flux and $O_3$-induced yield losses in soybeans: Possible implications for elevated $CO_2$ studies. J Exp Bot 48: 307–313

Geiger DR, Servaites JC (1994) Diurnal regulation of photosynthetic carbon metabolism in $C_3$ plants. Annu Rev Plant Physiol Plant Mol Biol 45: 235–256

Guidi L, Nali C, Ciompi S, Lorenzini G, Soldatini GF (1997) The use of chlorophyll fluorescence and leaf gas exchange as methods for studying the different responses to ozone of two bean cultivars. J Exp Bot 48: 173–179

Intergovernmental Panel on Climate Change (IPCC) (1996) Climate change 1995. In: Houghton JT, Meira Filho LG, Callander BA, Harris N, Kattenberg A, Maskell K (eds) The Science of Climate Change. Cambridge University Press, Cambridge, pp 572. ISBN 0-521-56436-0

Jacob J, Lawlor DW (1992) Dependence of photosynthesis of sunflower and maize leaves on phosphate supply, ribulose-1,5-bisphosphate carboxylase-oxygenase activity, and ribulose-1,5-bisphosphate pool size. Plant Physiol 98: 801–807

Kellomäki S, Wang K-Y (1997) Effects of elevated $O_3$ and $CO_2$ concentrations on photosynthesis and stomatal conductance in Scots pine. Plant Cell Environ 20: 995–1006

Kull O, Sober A, Coleman MD, Dickson RE, Isebrands JG, Gagnon Z, Karnosky DF (1996) Photosynthetic responses of aspen clones to simultaneous exposures of ozone and $CO_2$. Can J For Res 26: 636–648

Lehnherr B, Mächler F, Grandjean A, Fuhrer J (1988) The regulation of photosynthesis in leaves of field-grown spring wheat (*Triticum aestivum* L. cv. Albis) at different levels of ozone in ambient air. Plant Physiol 88: 1115–1119

Makino A, Mae T, Ohira K (1985) Photosynthesis and ribulose-1,5-bisphosphate carboxylase/oxygenase in rice leaves from emergence through senescence. Quantitative analysis by carboxylation/oxygenation and regeneration of ribulose 1,5-bisphosphate. Planta 166: 414–420

McKee IF, Farage PK, Long SP (1995) The interactive effects of elevated $CO_2$ and $O_3$ concentration on photosynthesis in spring wheat. Photosynth Res 45: 111–119

McKee IF, Eiblmeier M, Polle A (1997) Enhanced ozone-tolerance in wheat grown at an elevated $CO_2$ concentration: ozone exclusion and detoxification. New Phytol 137: 275–284

Mott KA, Jensen RG, O'Leary JW, Berry JA (1984) Photosynthesis and ribulose-1,5-bisphosphate concentration in intact leaves of *Xanthium strumarium* L. Plant Physiol 76: 968–971

Miyake H, Matsumura H, Fujinuma Y, Totsuka T (1989) Effects of low concentrations of ozone on the fine structure of radish leaves. New Phytol 111: 187–195

Pell EJ, Eckardt N, Glick RE (1994) Biochemical and molecular basis for impairment of photosynthetic potential. Photosynth Res 39: 453–462

Pell EJ, Schlagnhaufer CD, Arteca RN (1997) Ozone-induced oxidative stress: Mechanisms of action and reaction. Physiol Plant 100: 264–273

Price GD, Evans JR, von Caemmerer S, Yu J-W, Badger MR (1995) Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces $CO_2$ assimilation via a reduction in ribulose bisphophate regeneration in transgenic tobacco plants. Planta 195: 369–378

Rao M, Arulanatham AR, Terry N (1989) Leaf phosphate status, photosynthesis and carbon partitioning. II. Diurnal changes in sugar phosphates, adenylates and nicotinamide nucleotides. Plant Physiol 90: 820–826

Reichenauer T, Bolhàr-Nordenkampf HR, Erlich U, Soja G, Postl WF, Halbwachs F (1997) The influence of ambient and elevated ozone concentrations on photosynthesis in *Populus nigra*. Plant Cell Environ 20: 1061–1069

Reid CD, Fiscus EL (1998) Effects of elevated [$CO_2$] and/or ozone on limitations to $CO_2$ assimilation in soybean (*Glycine max*). J Exp Bot 49: 885–895

Reid CD, Fiscus EL, Burkey KO (1998) Combined effects of chronic ozone and elevated $CO_2$ on Rubisco activity and leaf components in soybean (*Glycine max*). J Exp Bot 49: 1999–2011

Sage RF, Reid CD (1994) Photosynthetic response mechanisms to environmental change in $C_3$ plants. In: Wilkinson RE (ed) Plant-Environment Interaction. Marcel Dekker, New York, NY, pp 413–499. ISBN 0-8247-8940-7

Sage RF, Sharkey TD, Seemann JR (1988) The in vivo response of the ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated $CO_2$ in *Phaseolus vulgaris* L. Planta 174: 407–416

Sage RF, Reid CD, Moore Bd, Seemann JR (1993) Long-term kinetics of the light-dependent regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in plants with and without 2-carboxyarabinitol 1-phosphate. Planta 191: 222–230

SAS Institute, Inc. (1986) SAS/STAT™ User's Guide, Release 6.03 Edition. SAS Institute, Cary, NC, pp 549–640. ISBN 1-55544-088-6
Seemann JR, Sharkey TD (1986) Salinity and nitrogen effects on photosynthesis, ribulose-1,5-bisphosphate carboxylase and metabolite pool sizes in *Phaseolus vulgaris* L. Plant Physiol 82: 555–560
Seemann JR, Sharkey TD, Wang JL, Osmond CB (1987) Environmental effects on photosynthesis, nitrogen-use efficiency, and metabolite pools in leaves of sun and shade plants. Plant Physiol 84: 796–802
Sharkey TD (1988) Estimating the rate of photorespiration in leaves. Physiol Plant 73: 147–152
Stitt M (1991) Rising $CO_2$ levels and their potential significance for carbon flow in photosynthetic cells. Plant Cell Environ 14: 741–762
Thompson AM, Huntley MA, Stewart RW (1990) Perturbations to tropospheric oxidants, 1985–2035. 1. Calculations of ozone and OH in chemically coherent regions. J Geophys Res 95: 9829–9844
von Caemmerer S, Edmondson DL (1986) Relationship between steady-state gas exchange, in vivo ribulose bisphosphate carboxylase activity and some carbon reduction cycle intermediates in *Raphanus sativus*. Aust J Plant Physiol 13: 669–688
von Caemmerer S, Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153: 376–387

Edited by C. H. Foyer