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Combined effects of chronic ozone and elevated CO_2 on Rubisco activity and leaf components in soybean (*Glycine max*)

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Abstract

Content and activity of Rubisco and concentrations of leaf nitrogen, chlorophyll and total non-structural carbohydrates (*TNC*) were determined at regular intervals during the 1993 and 1994 growing seasons to understand the effects and interactions of [O_3] and elevated [CO_2] on biochemical limitations to photosynthesis during ontogeny. Soybean (*Glycine max* var. Essex) was grown in open-top field chambers in either charcoal-filtered air (CF, 20 nmol mol^{-1}) or non-filtered air supplemented with 1.5 × ambient [O_3] (c. 80 nmol mol^{-1}) at ambient (AA, 360 μmol mol^{-1}) or elevated [CO_2] (700 μmol mol^{-1}). Sampling period significantly affected all the variables examined. Changes included a decrease in the activity and content of Rubisco during seed maturation, and increased nitrogen (*N*), leaf mass per unit area (*LMA*) and total non-structural carbohydrates (*TNC*, including starch and sucrose) through the reproductive phases. Ontogenetic changes were most rapid in O_3-treated plants. At ambient [CO_2], O_3 decreased initial activity (14–64% per unit leaf area and 14–29% per unit Rubisco) and content of Rubisco (9–53%), and *N* content per unit leaf area. Ozone decreased *LMA* by 17–28% of plants in CF-AA at the end of the growing season because of a 24–41% decrease in starch and a 59–80% decrease in sucrose. In general, elevated [CO_2], in CF or O_3-fumigated air, reduced the initial activity of Rubisco and activation state while having little effect on Rubisco content, *N* and chlorophyll content, per unit leaf area. Elevated CO_2 decreased Rubisco activity by 14–34% per unit leaf area and 15–25% per unit Rubisco content of plants in grown CF-AA, and increased *LMA* by 27–74% of the leaf mass per unit area in CF-AA because of a 23–148% increase in starch. However, the data suggest that, at elevated [CO_2], increases in starch and sucrose are not directly responsible for the deactivation of Rubisco. Also, there was little evidence of an adjustment of Rubisco activity in response to starch and sucrose metabolism. Significant interactions between elevated [CO_2] and [O_3] on all variables examined generally resulted in alleviation or amelioration of the O_3 effects at elevated CO_2. These data provide further support to the idea that elevated atmospheric CO_2 will reduce or prevent damage from pollutant O_3.

Key words: $O_3 \times CO_2$ interaction, carbohydrate metabolism, Rubisco, sugars, non-structural carbohydrates.

Introduction

Global atmospheric CO_2 concentration is increasing steadily at a rate of 1.5 μmol mol^{-1} per year while the tropospheric O_3 concentration has about doubled since the pre-industrial period (IPCC, 1996). In plants, elevated [CO_2] is thought to increase photosynthesis and resource-use-efficiency (Drake *et al.*, 1997), and thus productivity, while fumigation by O_3 causes serious injury to plants, reduces photosynthesis (Heath, 1994) and decreases productivity (Heck, 1989). Concurrent increases in atmospheric [CO_2] and [O_3] suggest that plants must adjust

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simultaneously to both trace gases, although few studies have addressed their interactive effects.

Studies of biochemical limitations to photosynthesis have focused on ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) regulation (Portis, 1992; Salvucci, 1989; Woodrow and Berry, 1988). Woodrow and Berry (1988) speculate that Rubisco is the primary rate-limiting component of photosynthesis and possibly the most limiting enzyme for plant productivity. Carboxylation by Rubisco depends on its activity and on the availability of the substrate ribulose-1,5-bisphosphate (RuBP), which partly depends on carbohydrate metabolism. Since Rubisco is the largest nitrogen investment in the leaf, its activity should be optimized by maintaining a balance between the Rubisco capacity to consume RuBP and the regeneration of RuBP (Sage *et al*., 1989).

Both elevated CO_2 and O_3 affect photosynthesis but in different ways. In elevated CO_2, the short-term enhancement of photosynthesis may shift the limitation from Rubisco capacity to RuBP regeneration, either through thylakoid processes or starch and sucrose metabolism (Stitt, 1991). In the long-term, elevated CO_2 may cause adjustments of the photosynthetic machinery that include the re-establishment of a new balance among these limitations. A potential adjustment involves the reduction of Rubisco content and, thus, reduction of Rubisco capacity to consume RuBP. For field-grown soybean in elevated [CO_2], a reduction in Rubisco carboxylation efficiency was reported during reproduction and was accompanied by a reduction in limitation by RuBP regeneration (Reid and Fiscus, 1998), indicating such an adjustment. A potential signal for the adjustment of Rubisco activity may be the increased content of leaf carbohydrates with elevated CO_2. In plants grown at elevated CO_2, increased carbohydrate content has been associated with decreased Rubisco activity (McKee and Woodward, 1994; Jacob *et al*., 1995) and decreased transcription of Rubisco genes (Van Oosten and Besford, 1995; Nie *et al*., 1995). In addition, high starch content in the leaf has been associated with reduced RuBP regeneration (Stitt, 1991). Ozone, on the other hand, appears mainly to inhibit photosynthesis by a loss of Rubisco carboxylation. The decline in Rubisco activity with O_3 is caused by a decline in Rubisco content rather than a decline in activation state (Dann and Pell, 1989; Pell *et al*., 1992). However, plants exposed to O_3-fumigation may also show a reduction in RuBP regeneration capacity, suggesting that the decline in photosynthesis with increasing O_3 is both enzyme and substrate limited (Atkinson *et al*., 1988). With O_3-fumigation, the capacity of RuBP regeneration may be caused by the reduced translocation of carbohydrate assimilates (McLaughlin and McConathy, 1983) and increased accumulation of leaf carbohydrates (Rennenberg *et al*., 1996). A better understanding of the combined effect of the two trace gases on the biochemical limitations to photosynthesis is required to predict photosynthesis and productivity in future environments.

Reports of the combined effects of elevated [CO_2] and [O_3] on photosynthesis suggest an amelioration of O_3 damage by elevated [CO_2] (McKee *et al*., 1995; Kellomäki and Wang, 1997; Reid and Fiscus, 1998). A reduction of O_3 flux into the leaf because of reduced stomatal conductance was suggested for this amelioration (McKee *et al*., 1995; Fiscus *et al*., 1997). In addition, McKee *et al*. (1995) suggested that a shift from Rubisco capacity to other limitations might also be involved in the moderation of O_3 damage by elevated CO_2. Changes in photosynthetic rates under elevated CO_2 may be attended by reduced Rubisco activity as a consequence of increased leaf carbohydrates during long-term exposure. On the other hand, reduced Rubisco content with O_3-fumigation have reduced photosynthesis and thus potentially decreased carbon metabolism. It is possible that changes in photosynthetic rates with elevated CO_2 might offset some of the limitations imposed by O_3 on Rubisco activity. The objective of this study was to test this possibility by examining the effects of O_3 fumigation and elevated atmospheric [CO_2] on Rubisco activity and leaf components associated with carbohydrate metabolism throughout plant development.

Materials and methods

Plant materials and experimental design

Soybean (*Glycine max* [L.] Merr. cv. Essex) was grown in open-top field chambers (Heagle *et al*., 1973) at Raleigh, NC, USA. At the end of May in 1993 and 1994, seeds of soybean were inoculated with *Bradyrhizobium japonicum* and planted in 0.021 m^3 pots containing a 2:1:1 (by vol.) mixture of clay-loam topsoil:sand:vermiculite-sphagnum-perlite horticultural mix (MetroMix 220[4] WR Grace Co., Cambridge, MA, USA). Cultural conditions and practices are described in Fiscus *et al*. (1997).

The experiment was a two-way factorial design that consisted of two atmospheric CO_2 concentrations and two O_3 treatments. The two atmospheric [CO_2] were current ambient (average 371 μmol mol^{-1}, AA) and double ambient (average 708 μmol mol^{-1}, CO_2) and the two O_3 treatments were charcoal-filtered (average 24 nmol mol^{-1}, CF) and non-filtered + 1.5 × ambient O_3 (average 81 nmol mol^{-1}, O3). Seasonal averages for 1993 and 1994 are described in Fiscus *et al*. (1997). Dispensing of CO_2 (24 h d^{-1}) and O_3 (12 h d^{-1}), and monitoring are as described in Booker *et al*. (1997). Carbon dioxide and O_3 were dispensed from planting until the end of the experiment (108 days after planting [DAP] and 105 DAP in 1993 and 1994, respectively). In each year, treatments were replicated twice and were assigned randomly to chambers within each of two blocks.

[4] The use of trade names in this publication does not imply endorsement by the US Department of Agriculture, the North Carolina Agricultural Research Service, the North Carolina State University nor criticism of similar ones not mentioned.

Sampling procedure

Leaf samples for biochemical assays were harvested at 1- to 2-week intervals during the growing season. Between 11.00 and 13.00 h EST, one leaf disc of 2.84 cm^2 area was cut from a centre leaflet that was in full sunlight and immediately frozen in liquid nitrogen. The frozen leaf samples were kept in vials in liquid nitrogen for the determination of Rubisco activity and content, and chlorophyll content. Between 16.00 and 17.00 h EST, one leaf disc of 2.94 cm^2 was cut and immediately dipped in liquid nitrogen for the determination of total non-structural carbohydrates. Following rapid freezing, the sample was placed in a 7 ml plastic vial containing 3 ml of 80% ethanol. At the same time, three leaf discs of 2.24 cm^2 were harvested on a lateral leaflet for the determination of leaf mass per unit area and nitrogen content. The samples for Rubisco and carbohydrate analysis were transferred to an ultrafreezer at $-80\,^{\circ}C$ until the time of assay while the samples for mass and nitrogen analysis were transferred to a forced-air dying oven at $60\,^{\circ}C$ until they reached a constant mass.

Different leaf positions were harvested during reproduction in 1993 and 1994. Samples were collected from the centre leaflet of the most recent fully expanded leaf on the main stem through pod formation (71 DAP in 1993 and 62 DAP in 1994). After the pod formation stage in 1993, lateral branches, which remain in the vegetative stage longer than the main stem, were sampled. Thus in 1993, leaves that were in the same phenological stage were sampled until late seed maturation. After the pod formation stage in 1994, the main stem apical leaf was still used. Thus, leaves at the same physiological age through 79 DAP and leaves increasing in age afterwards were sampled.

Biochemical analyses

Rubisco activity and content: Procedures follow Sage *et al.* (1993) and are described in Reid *et al.* (1997). Rubisco activity was assayed immediately after extraction (initial activity) and following a 12–15 min incubation in 10 mol m^{-3} $NaHCO_3$ buffer (final activity) via $^{14}CO_2$ incorporation into acid-stable compounds. Rubisco content was determined by binding of ^{14}CABP (Collatz *et al.*, 1979), and radioactivity was determined using liquid scintillation counting. For correlation with assimilation and productivity, Rubisco activity and content were expressed per unit leaf area. In addition, Rubisco activity was expressed per unit Rubisco to assess the catalytic competency of the enzyme, and Rubisco content was expressed per unit nitrogen to assess the nitrogen investment in Rubisco. Chlorophyll content was determined using a spectrophotometric assay after extraction of a 0.2 cm^3 aliquot of the leaf crude extract in 80% acetone (Lichtenthaler and Wellburn, 1983).

Nitrogen content: All leaf discs were ground to fine powder in a glass vial with a glass rod. The nitrogen content was determined by mass spectrometry at the Soil Science Analytical Laboratory, North Carolina State University.

Carbohydrate determination: After thawing the leaf disc in ethanol, the tissue was homogenized in a Ten-Broeck glass homogenizer. The tissue was then extracted in hot ethanol following Kerr *et al.* (1984) to separate soluble sugars from starch. After centrifugation, the pellet was used for starch analysis, and the supernatant was stored at $-80\,^{\circ}C$ for the determination of soluble sugars. The supernatant was analysed for total soluble sugars and sucrose following the procedures of Jones *et al.* (1977) as modified by Kerr *et al.* (1985). The ethanol-soluble fraction was evaporated to dryness, resuspended and enzymatically analysed for sucrose and hexoses. As suggested by Hendrix (1993), the resuspended extract was mixed with activated charcoal to remove ethanol-soluble materials which might interfere with subsequent enzyme-coupled assays. Similar to Kerr *et al.* (1984), starch content was determined by amyloglucosidase (EC 3.2.1.3) digestion using prepared enzymes from starch test kits (Boehringer Mannheim Corp., Indianapolis, IN). In 1993, the final supernatant from the digested starch solution was enzymatically assayed for free glucose using hexokinase and glucose-6-phosphate dehydrogenase and no soluble sugars could be detected. In 1994, the free glucose was not determined. Glucose was used as the standard for starch and sugars.

Statistical analysis

For each parameter, a two-way analysis of variance (ANOVA, SAS Institute, 1986) was used to assess treatment effects using $[CO_2]$ and $[O_3]$ as main effects, and period of sampling as a split-plot effect. Because no significant block effect was found, a complete random factorial design was used. Plants were used as replicates within a block because the mean squares of chambers within a treatment and plants within chambers within treatments were similar. In all cases, period of sampling was the most highly significant effect and further analyses were done for each sampling period to determine interactions. Pair-wise comparisons were carried out on each parameter.

Results

Effect of sampling period

A similar temporal pattern was generally observed among treatments for Rubisco activity, and content of Rubisco, nitrogen (*N*), chlorophyll (*Chl*), and leaf carbohydrates; and, for clarity, only plants grown in charcoal-filtered air and ambient CO_2 are presented (Figs 1, 2). The effects of sampling period, expressed as DAP, on Rubisco initial activity and content differed in 1993 and 1994 (Fig. 1). In 1993, Rubisco initial activity was significantly affected by DAP ($P<0.001$ per unit leaf area, Fig. 1A; $P<0.0001$ per unit Rubisco, Fig. 1B) mostly because of a decline during reproduction. Likewise in 1993, the Rubisco content declined significantly with DAP during reproduction on a unit leaf area ($P<0.05$, Fig. 1C). However, on a unit leaf *N*, the decline in Rubisco content was during the vegetative to flowering stage ($P<0.0001$ per unit *N*, Fig. 1D). In 1994, the Rubisco initial activity generally showed no pattern through the growing season even though DAP had a significant effect per unit Rubisco ($P<0.05$, Fig. 1B). Likewise in 1994, Rubisco content showed no significant trend with DAP on a unit leaf area (Fig. 1C). On a unit leaf *N*, however, the pattern of decline was similar to the one observed in 1993: Rubisco content decreased significant with DAP until the beginning of pod formation ($P<0.0001$, Fig. 1D). Furthermore, sampling period had no significant effect on activation state, which averaged from 76–90% in 1993 and 81–97% in 1994 (data not shown).

Sampling period had a highly significant effect on each

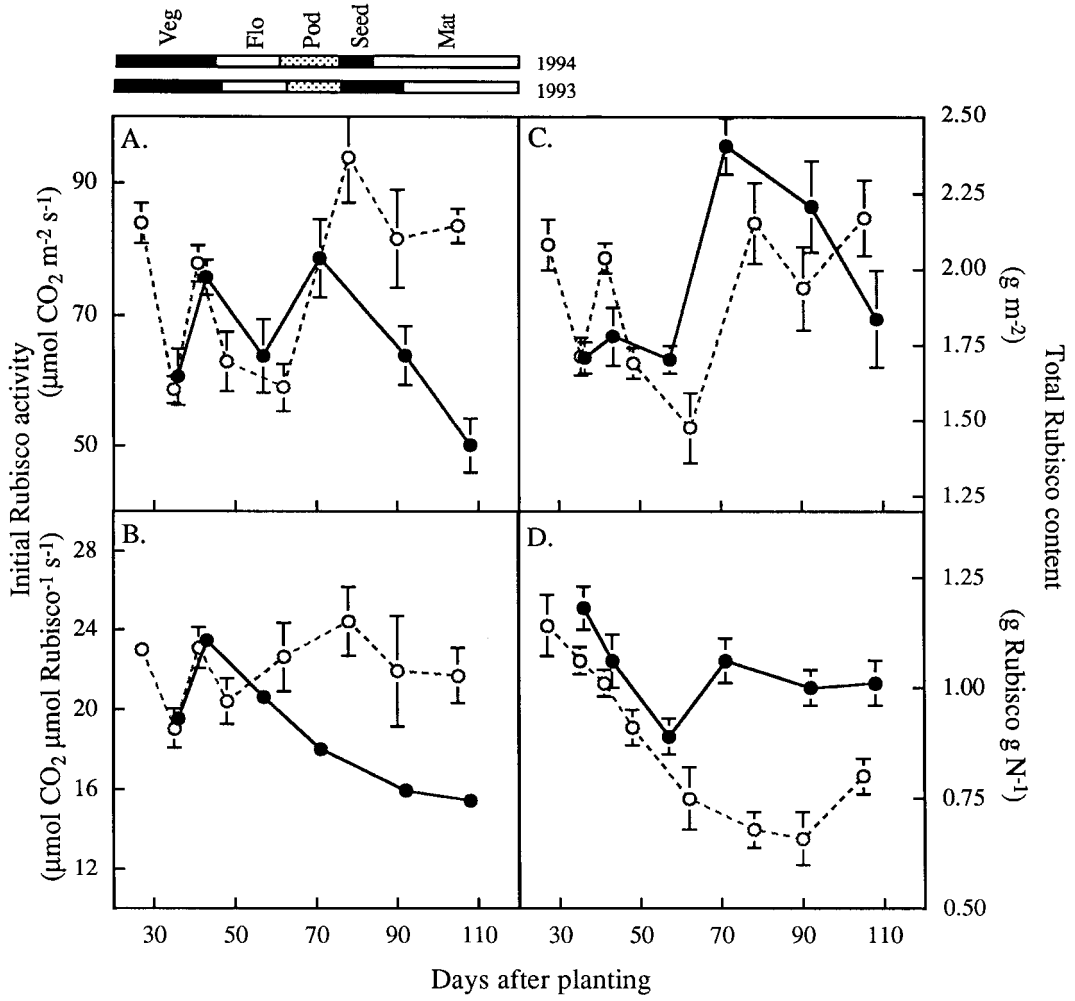

Fig. 1. Activity and content of Rubisco for soybean grown in charcoal-filtered air at ambient [CO_2] through the 1993 (—●—) and 1994 (- -○- -) seasons. Rubisco initial activity (A, B), and total Rubisco content (C, D) represent the mean ±s.e. ($n=6$) for each day sampled. Above (A), the length of each developmental stage is shown for each year: Veg is vegetative growth, Flo is flowering, Pod is pod formation, Seed is seed development, and Mat is seed maturation.

Table 1. *Analysis of variance for the effects of [CO_2], [O_3], and ontogeny on leaf constituents of soybean*

The soybeans were treated with ambient [CO_2] (350 μmol mol^{-1}, AA) or elevated [CO_2] (700 μmol mol^{-1}, CO_2), combined with either charcoal-filtered (CF) or O_3-fumigated air (OZ) in open-top field chambers. Nitrogen content (N), total chlorophyll content (Chl), leaf mass per unit area (LMA), total non-structural carbohydrates (TNC), starch, and sucrose were determined at five and six sampling periods (DAP) in 1993 and 1994, respectively. Values represent probability for significant treatment effect (ns is not significant).

Treatment	N		Chl		LMA		TNC		Starch		Sucrose	
	1993	1994	1993	1994	1993	1994	1993	1994	1993	1994	1993	1994
O_3	0.02	0.01	—	ns	ns	ns	ns	ns	ns	ns	ns	ns
CO_2	ns	ns	—	ns	0.02	0.05	ns	0.02	0.04	0.03	ns	ns
$O_3 \times CO_2$	ns	ns	—	ns	ns	ns	ns	ns	ns	ns	ns	ns
DAP	0.0001	0.0001	—	0.0002	0.0001	0.0001	0.0001	0.0001	0.04	0.04	0.0002	0.0001
DAP × O_3	0.0004	0.0001	—	0.0001	0.01	0.0008	ns	0.01	0.002	ns	ns	0.03
DAP × CO_2	0.008	ns	—	ns	0.02	ns	ns	ns	ns	ns	ns	0.0007
DAP × CO_2 × O_3	ns	ns	—	ns	ns	ns	ns	ns	0.001	ns	ns	ns

of the leaf constituents (Table 1). Both leaf nitrogen (*N*) and total chlorophyll (*Chl*) content increased until the end of pod formation to decrease afterwards (Fig. 2A, B), and these temporal patterns observed in CF-AA were generally similar among treatments. Likewise, leaf mass per unit area (*LMA*) increased from the vegetative to the pod formation stage in 1993 and from flowering to the seed maturation stage in 1994 (Fig. 2C), and the patterns

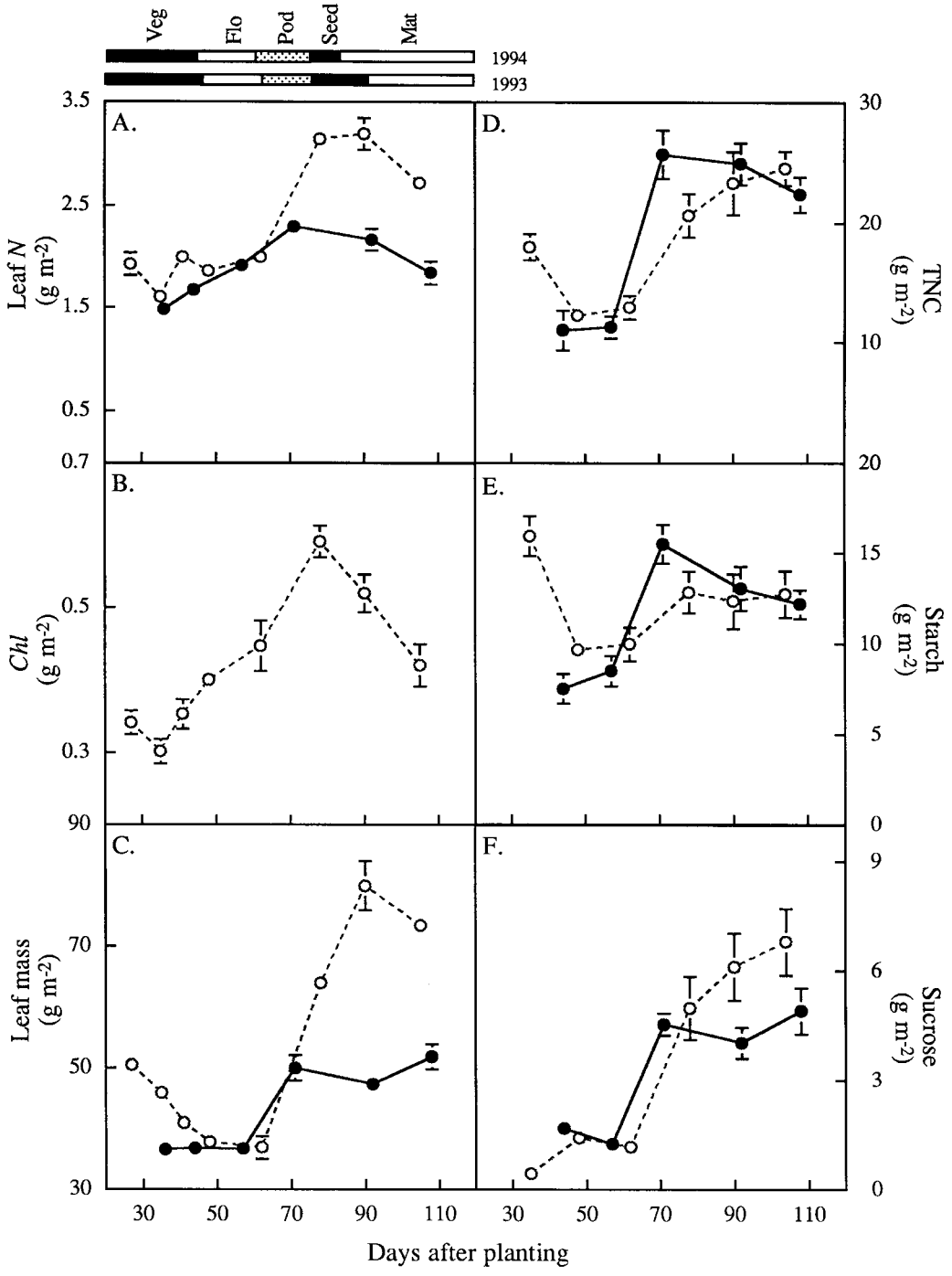

Fig. 2. Leaf constituents of soybean grown in open-top field chambers in charcoal-filtered air at ambient [CO_2] through the 1993 (—●—) and 1994 (- -○- -) seasons. Nitrogen (*N*, panel A), total chlorophyll content (*Chl*, B), leaf mass per unit area (C), and total carbohydrate content (*TNC*, D), starch (E) and sucrose (F) content represent the mean ±s.e. ($n=6$) for each point. Above (A), the length of each developmental stage is shown for each year, as described in Fig. 1.

of change with DAP were generally similar among treatments. In addition, total non-structural carbohydrates (*TNC*), starch and sucrose generally changed in a pattern similar to *LMA* (Fig. 2D, E, F) although, in 1994, the starch content remained constant from the beginning of seed development onwards.

Treatment effects

Ozone and elevated [CO_2] generally decreased the initial Rubisco activity and content (Figs 3, 4). Yet, no significant O_3 or CO_2 effects were usually found in a general ANOVA model because significant effects of DAP pre-

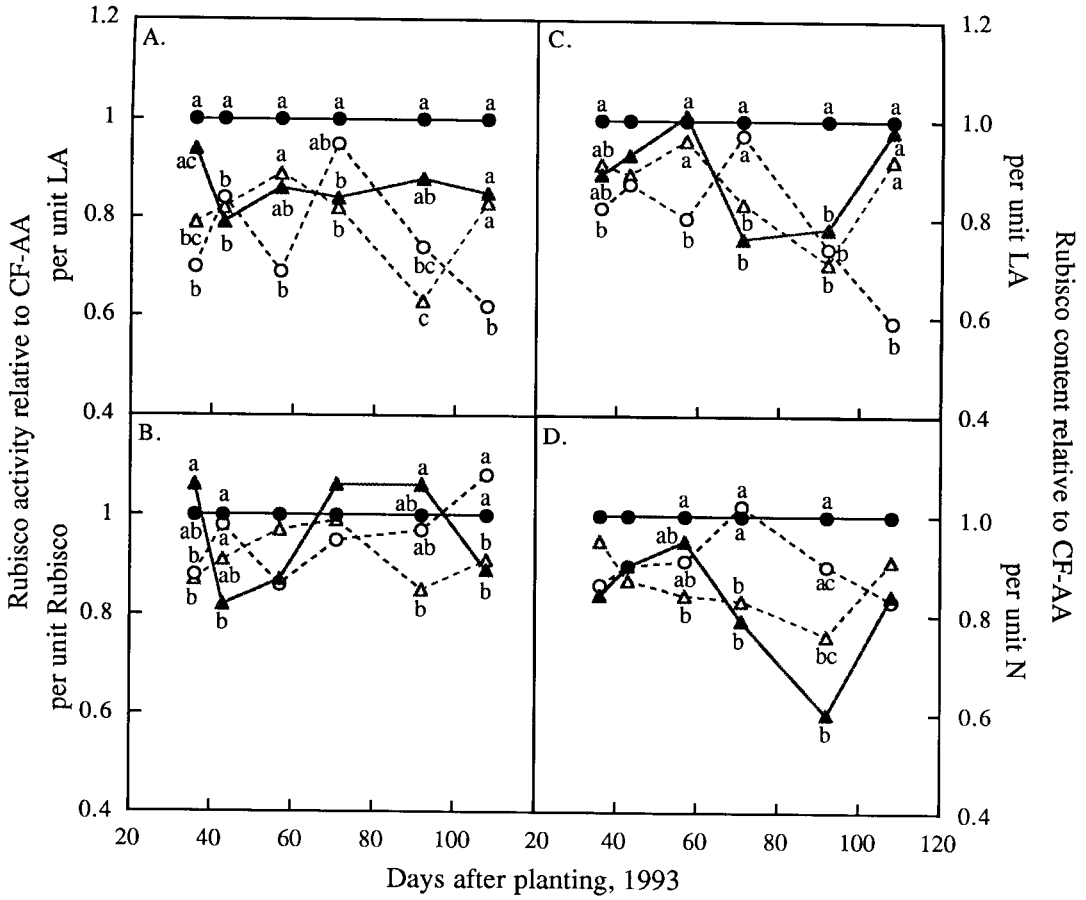

Fig. 3. Changes in activity and content of Rubisco for soybean grown in different [CO_2] and [O_3] treatments through the 1993 growing season. Treatments were charcoal-filtered air (CF, full symbols) or O_3-fumigated air (open symbols) at ambient (AA, circles) or elevated [CO_2] (triangles). Rubisco activity (A, per unit leaf area [LA]; B) and Rubisco content (C, D) were expressed relative to activity and content of plants grown in CF-AA. Within a day, significant differences between treatments at $P<0.05$ are indicated by different letters. ANOVA per sampling period showed significant $CO_2 \times O_3$ effect at 44, 57, and 108 DAP in (A) ($P<0.05$); and at 36 DAP ($P<0.05$) in (C). Each point represents the mean ($n=6$).

vailed. In the two cases where DAP was not significant, significant treatment effects were found: (1) O_3 significantly changed the Rubisco content per unit leaf area in 1994 ($P<0.03$, Fig. 4C), and significant $CO_2 \times O_3$ interactions were found ($P<0.04$); and (2) elevated CO_2 significantly reduced Rubisco activation state to 89–94% of CF-AA in 1993 ($P<0.01$) and 88–95% of CF-AA in 1994 ($P<0.02$). Because significant DAP effects may have masked the main treatment effects, each period was analysed separately. Ozone in ambient CO_2 significantly decreased initial Rubisco activity and content, per unit leaf area, through the season in 1993 (Fig. 3A, C), and in 1994, excluding the period of 48–78 DAP (Fig. 4A, C). Also in 1994, O_3 in ambient CO_2 decreased the Rubisco content, per unit leaf *N*, although significant increases were observed from 78 to 92 DAP (Fig. 4D). However, O_3 generally had no significant effect on Rubisco activity per unit Rubisco (Figs 3B, 4B) and on activation state, which ranged from 102 to 106% of CF-AA in 1993 and 97% to 104% of CF-AA in 1994. On the other hand, elevated [CO_2] in charcoal-filtered air decreased initial Rubisco activity per unit leaf area, but significantly so only at 44 and 71 DAP in 1993 (Fig. 3A) and through 1994 (Fig. 4A). Elevated CO_2 decreased Rubisco activity per unit Rubisco only in 1994 (Fig. 3B, 4B). Furthermore, elevated CO_2 significantly decreased the Rubisco content from 71 DAP through 92 DAP in 1993 (Fig. 3C, D) whereas it had little effect on Rubisco content in 1994 (Fig. 4C, D).

The effects of O_3 and elevated CO_2 on leaf constituents were dependent on sampling periods. First, significant interactions between DAP and O_3 were observed for leaf *N* and *Chl* (Table 1) because the direction of the O_3 effect on *N* and *Chl* contents changed depending on DAP (Fig. 5). In addition, a significant DAP $\times$ CO_2 was observed for leaf *N* in 1993 because elevated [CO_2] increased leaf *N* significantly only at 57 DAP and 92 DAP (Fig. 5A). Thus, because the significant effect of DAP prevailed, the treatment effects were examined for each sampling period. Ozone significantly reduced leaf *N* through most of the season in 1993 (Fig. 5A) and reduced leaf *N* and *Chl* from 92 DAP on in 1994 (Fig. 5B, C).

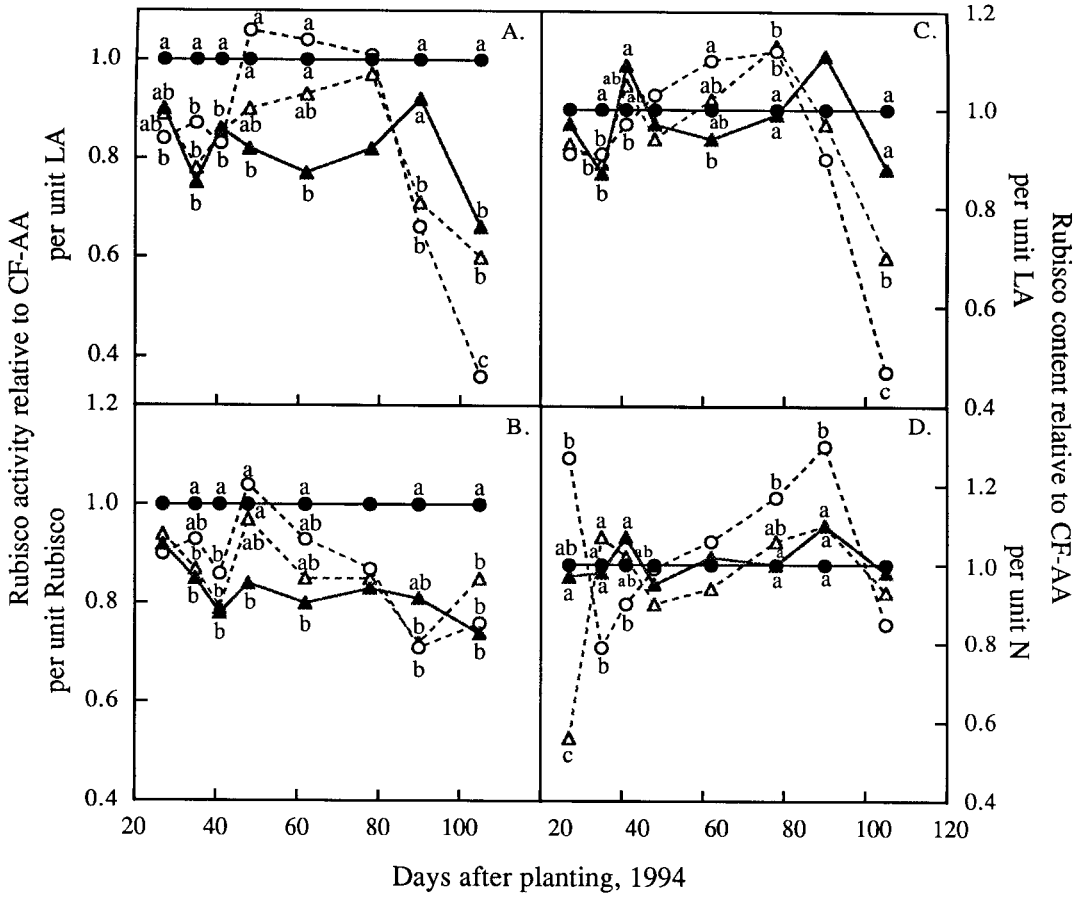

Fig. 4. Changes in activity and content of Rubisco for soybean grown in different [CO_2] and [O_3] treatments through the 1994 growing season. Parameters, treatments and significance of pairwise comparisons are described in Fig. 3. ANOVA per sampling period showed significant $CO_2 \times O_3$ effect at 105 DAP in (A) and (C) ($P<0.0001$ and $P<0.002$, respectively); and at 27, 35, and 90 DAP ($P<0.002$, $P<0.02$, and $P<0.02$, respectively) in (D). Each point represents the mean ($n=6$).

Furthermore in 1994, O_3 in ambient air significantly reduced the *Chl* content at 27 DAP (Fig. 5C). The *Chl* data were not available in 1993. In contrast, elevated CO_2 in charcoal-filtered air had little effect on leaf *N* (Fig. 5A, B) and *Chl* for most of the growing season (Fig. 5C).

Ozone generally decreased while CO_2 significantly increased *LMA* and leaf carbohydrates (Figs 6, 7; Table 1). With a general ANOVA model, however, the significant effect of O_3 was usually by interactions between DAP $\times$ O_3 (Table 1). The DAP by O_3 interactions were generally because of an effect of O_3 at the end of the growing season (Figs 6, 7). Because the effect of O_3 on *LMA* and leaf carbohydrates was dependent on DAP, each period was analysed separately. Compared to plants grown in CF-AA, O_3 at ambient [CO_2] significantly reduced *LMA* (17% and 28% in 1993 and 1994) and sucrose content (20% and 29% in 1993 and 1994) only at 108 DAP in 1993 and from 92 DAP on in 1994 (Figs 6A, 7A). In addition, O_3 significantly reduced *TNC* (18% to 46%) and starch (20% to 67%) from 71 DAP on in 1993 and during all periods but 62 DAP in 1994. In contrast, elevated CO_2 in charcoal-filtered or O_3-fumigated air significantly increased the *LMA* by 27% to 74% over the leaf mass of plants grown in CF-AA, and the *TNC* by 26% to more than double, mostly because of an increase in starch content but also in sucrose (Figs 6, 7).

Interactions between O_3 and elevated CO_2

Interactions between elevated [CO_2] and [O_3] that were observed in the vegetative stages and at the end of reproduction ranged from amelioration to elimination of O_3 damage. In the vegetative stages, significant $CO_2 \times O_3$ interactions were observed for Rubisco activity, Rubisco content, and *LMA* in 1993 (Figs 3A, C, 6A); and for *TNC* and starch in 1994 (Fig. 7A, C) because O_3 had no effect in elevated CO_2 atmosphere. Also in the vegetative stages, a synergistic effect between elevated CO_2 and O_3 was observed for *N*, *Chl* (Fig. 5), and Rubisco content per unit leaf *N* (Fig. 3D) because these contents were higher in the $CO_2 \times O_3$ treatment than in either treatment alone. At the end of the growing season, some of the CO_2 by O_3 interactions were because elevated CO_2 partly

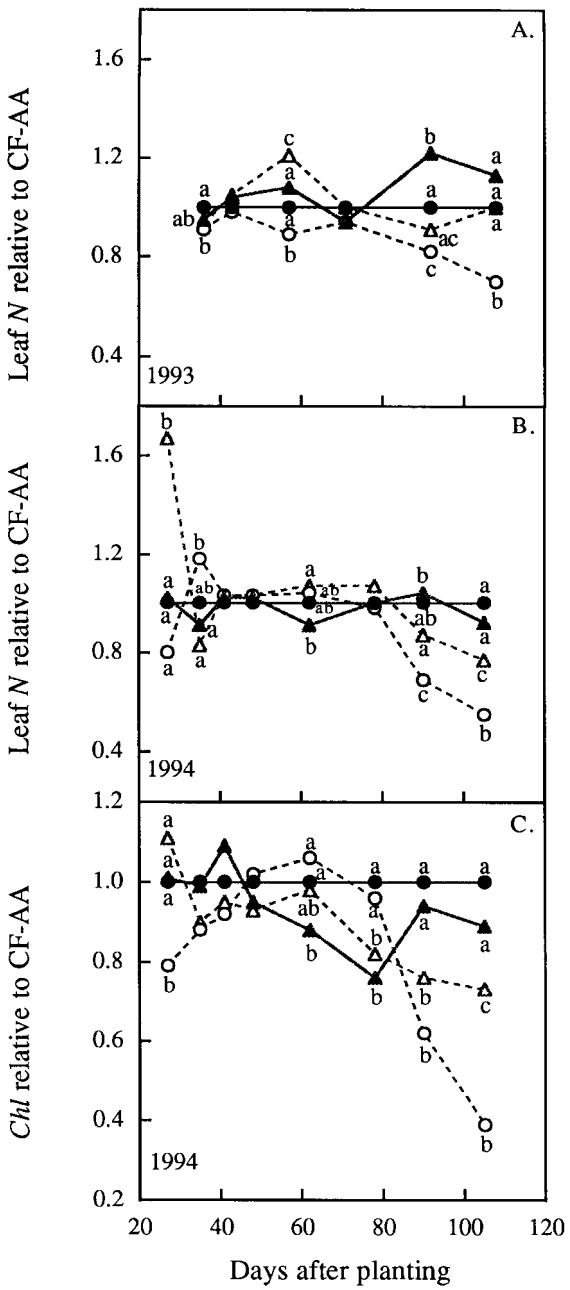

Fig. 5. Changes in nitrogen (N) and total chlorophyll content (*Chl*) for soybean grown in different [CO_2] and [O_3] treatments. Nitrogen content in 1993 (A) and 1994 (B) and *Chl* (C) were expressed as a proportion of *N* and *Chl* per unit leaf area, of plants grown in charcoal-filtered air (CF) at ambient CO_2 (AA). Treatments and significance of pairwise comparisons are described in Fig. 3. ANOVA per sampling period showed significant $CO_2 \times O_3$ effects at 57 DAP ($P < 0.0009$) in (A); at 27 and 105 DAP ($P < 0.0001$ and 0.006, respectively) in (B); and at 27, 90, and 105 DAP ($P < 0.02$, 0.05, and 0.0007, respectively) in (C). Each point represents the mean ($n = 6$).

alleviated the negative effect of O_3. This amelioration was observed for contents of Rubisco, *N*, *Chl* and sucrose in 1994 (Figs 4C, 5B, C, 7D). Other significant $CO_2 \times O_3$ interactions were observed for Rubisco activity (Figs 3A, 4A), *TNC* and starch in 1993 (Fig. 6B, C) because elevated CO_2 eliminated the negative effect of O_3. In addition, one synergistic interaction was observed for sucrose in 1993 (Fig. 6D).

Discussion

Effects of ontogeny

Different effects of sampling periods on Rubisco activity, Rubisco content, and on leaf biochemistry were observed in 1993 compared to 1994. Year-to-year variations were most obvious during reproduction and were presumably caused by sampling different leaf positions during the reproductive stages (Figs 1, 2). In 1993, leaves of the same phenological stage were used in vegetative and reproductive growth by moving to lateral branches later during reproduction, as indicated by the maintenance of constant *LMA*, *N* and carbohydrates during reproduction. Thus, the decline in Rubisco activity and content through reproduction was because of plant ontogeny rather than leaf ageing. In contrast, in 1994, leaf ontogeny was examined by consistently sampling the same leaf position, and the increase in *LMA*, and in contents of *N*, *Chl* and carbohydrates until late maturation are consistent with such leaf ageing. Thus, for these field-grown soybean plants, leaf ageing appears to have little effect on Rubisco activity and content during reproduction. Yet, in both years, variations in starch content with DAP were decoupled from variations in Rubisco or other carbohydrates with DAP. The patterns reported here appear to be related to developmental stages rather than to adjustment of Rubisco and leaf biochemistry to optimize the photosynthetic machinery.

Ozone and elevated CO_2 appear to accelerate ontogeny. In both years, faster ontogenetic declines of Rubisco activity and content were observed during reproduction in elevated CO_2 and/or O_3 treatments relative to CF-AA (Figs 3, 4), and these patterns are consistent with other studies on [O_3] fumigation (Dann and Pell, 1989; Pell *et al.*, 1994*a*) and elevated [CO_2] (Besford *et al.*, 1990; Nie *et al.*, 1995). Also, the changes in Rubisco and leaf biochemistry reported here are consistent with the accelerated ontogeny suggested by photosynthesis (Reid and Fiscus, 1998) and leaf conductance (Fiscus *et al.*, 1997) under elevated CO_2 and/or O_3.

Effects of O_3

The decline in Rubisco and other leaf components suggests accelerated senescence in O_3-fumigated plants (Nie *et al.*, 1993; Pell *et al.*, 1994*a*, *b*). Accelerated senescence may result in several adjustments: (1) the lack of an O_3 effect on Rubisco content in early reproductive phases of growth at ambient CO_2 in younger leaves may be triggered by O_3-induced compensatory mechanisms in older tissue,

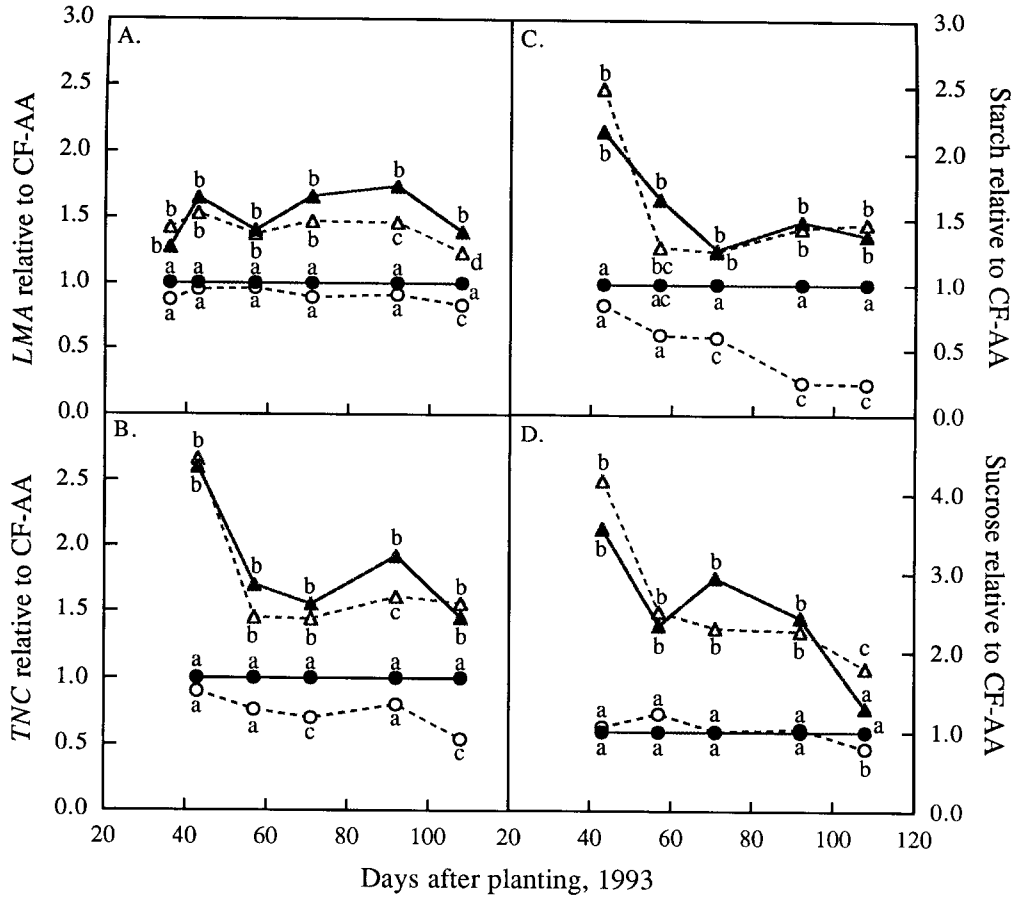

Fig. 6. Changes in leaf carbohydrates for soybean grown in different [CO_2] and [O_3] treatments through the 1993 growing season. Leaf mass (*LMA*, A), total nonstructural carbohydrates (B), starch (C), and sucrose (D) were expressed as a proportion of their respective content, per unit leaf area, in plants grown at CF-AA. Treatments and significance of pairwise comparisons are described in Fig. 3. ANOVA per sampling period showed significant $CO_2 \times O_3$ effects at 36 and 57 DAP ($P<0.05$ and $P<0.005$, respectively) in (A) at 92 and 108 DAP ($P<0.001$ and <0.002, respectively) in (B); at 71, 92, and 108 DAP ($P<0.0003$) in (C); and at 108 DAP ($P<0.03$) in (D). Each point represents the mean ($n=6$).

as proposed by Held *et al.* (1991) and Pell *et al.* (1994*b*); and (2) the similar Rubisco activity and content per unit leaf area in CF and O_3-fumigated air in early reproductive stages may suggest that Rubisco reached its maximum earlier in leaves exposed to O_3 fumigation. Such a pattern of faster leaf development has been suggested by changes in leaf conductance with O_3 (Fiscus *et al.*, 1997). Furthermore, the decline in the transcription of the small subunit of Rubisco has been involved in the O_3-accelerated senescence (Glick *et al.*, 1995) although signalling of O_3 damage at the molecular level is not well understood.

Rennenberg *et al.* (1996) hypothesized that sucrose accumulation in the leaf was partly responsible for the decrease in photosynthesis of O_3-damaged tissue and carbohydrate accumulation has been postulated as a possible signal for reduction of Rubisco gene expression (Krapp *et al.*, 1993). In this study, starch and *TNC* were decreased in O_3-fumigated leaves at ambient CO_2, but sucrose content did not change with O_3 exposure relative to CF air. Thus, these data give little support to the hypothesis of sucrose accumulation mediating O_3 damage. On the other hand, the decrease of starch in soybean during reproduction may result from the cumulative effect of O_3. The reduction in starch partitioning under O_3-fumigation may be caused by increased carbon invested into seeds when carbon assimilation is already reduced. The changes in carbohydrates reported in this study are consistent with previous reports on cotton and soybean (Miller *et al.*, 1989, 1995), and *Trifolium repens* (Rebbeck *et al.*, 1988). However, they differ from Balaguer *et al.* (1995) who reported a significant increase in dark respiration and starch content with a decrease in sucrose for *Triticum aestivum* grown in O_3-fumigated air. Changes in carbon metabolism, indicated by lower starch and sucrose content in O_3-fumigated than in CF air, do not appear to be associated with the reduction in Rubisco activity. Thus, these data give little support to the concept that O_3 damage causes a readjustment of the Rubisco capacity and RuBP regeneration through carbon metabolism.

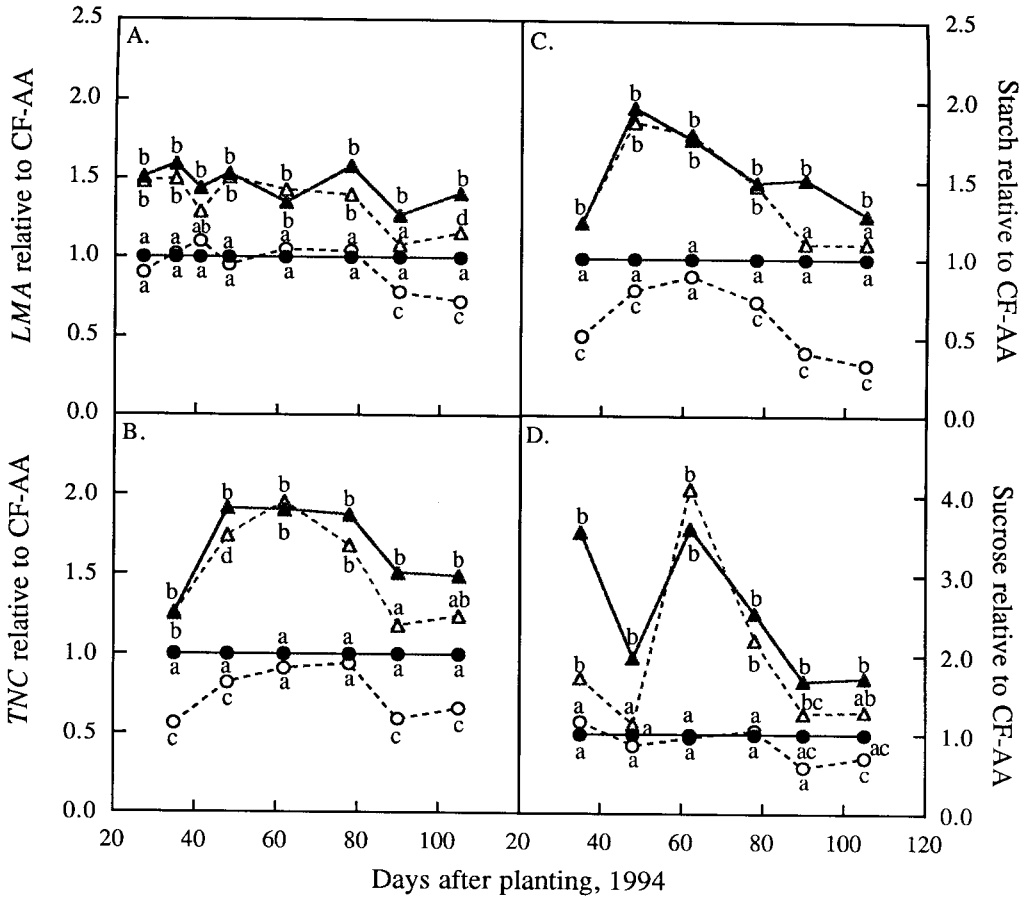

Fig. 7. Changes in leaf carbohydrates of soybean grown in different [CO_2] and [O_3] through the 1994 growing season. Parameters, treatments and significance of pairwise comparisons are the same as in Fig. 6. ANOVA per sampling period showed significant $CO_2 \times O_3$ effects at 35 DAP ($P < 0.0001$) in (B), at 35 DAP ($P < 0.0007$) in (C); and at 35, 78, and 105 DAP ($P < 0.0001$, 0.03, 0.02, respectively) in (D).

Effects of elevated CO_2

Early reports on feedback inhibition of photosynthesis by starch accumulation in the leaf (e.g. diurnal photosynthetic midday depression; Azcòn-Bieto, 1983) were extended to explain down-regulation of photosynthesis in long-term CO_2 enrichment studies (Stitt, 1991). For soybean in this study, decreased Rubisco activity with elevated CO_2 was correlated with a near doubling of starch and soluble sugars. This increase in non-structural carbohydrates may indicate a decrease in carbohydrate translocation to sinks and, consequently, feedback inhibition of Rubisco activity by limiting regeneration of inorganic phosphate (P_i) and, thus, RuBP regeneration. However, the relative increase in starch concentration with elevated CO_2 was not correlated with the decrease in initial Rubisco activity because activity was not significantly different from controls at the end of the season when the starch concentration was highest. For starch-storing species with high invertase activity, such as soybean and cotton, Goldschmidt and Huber (1992) suggested that, rather than starch, the accumulation of transient hexose sugars is responsible for the inhibition of photosynthesis.

In elevated [CO_2], the deactivation of Rubisco was concomitant with an increase in sucrose and other hexoses (*TNC* minus starch and sucrose). When the sucrose hydrolysis exceeds the capacity for rephosphorylation of free hexoses, a build-up of these hexoses could occur and could initiate down-regulation of the Calvin cycle (Goldschmidt and Huber, 1992). The proposed regulation of Rubisco activity by carbohydrate metabolism is consistent with data from this study because the increase in sucrose concentrations with elevated CO_2 observed here was correlated with decreased Rubisco activity. This control mechanism is also consistent with the proposal of Krapp *et al.* (1993) that Rubisco gene expression was inhibited by metabolic factors associated with high carbohydrate content rather than by the high Rubisco content *per se*. In addition, Van Oosten and Besford (1995) have reported a decrease in gene expression for the Rubisco small subunits for plants grown in elevated CO_2.

In elevated [CO_2], the decrease in Rubisco activity was often caused by decreased activation state rather than

content of Rubisco. Van Oosten *et al.* (1994) reported a decrease in the expression of the gene coding for Rubisco activase with tomato exposure to elevated CO_2, and Rubisco activase has been shown to decrease in P_i-limited conditions (Salvucci, 1989). Furthermore, the decrease in Rubisco activity was observed whether it was expressed on a unit leaf area or on a Rubisco content basis. The lack of a [CO_2] effect on Rubisco content per leaf area observed at different stages in this study was consistent with previous findings on soybean (Campbell *et al.*, 1988). Also, although the increase in *LMA* suggested a reduction in leaf *N* per unit dry mass, the *N* content per unit leaf area was not affected by elevated CO_2. Thus, in 1993, when leaves of the same phenological stage were measured, the Rubisco content per unit *N* was reduced during reproduction, supporting the hypothesis of *N* reallocation from Rubisco to other limiting processes (Sage *et al.*, 1989).

Effects of elevated $CO_2 \times O_3$ interactions

The effect of combined O_3 and elevated CO_2 on Rubisco activity and carbohydrate content was generally similar to the effect of elevated CO_2 in CF air, indicating a total alleviation or prevention of O_3 damage by CO_2 enrichment until seed maturation. These findings are consistent with results on Rubisco activity (McKee *et al.*, 1995) and Rubisco content (Rao *et al.*, 1995) of wheat. However, they are not consistent with the work of Barnes *et al.* (1995*a*, *b*) on photosynthesis and carbohydrate accumulation of spruce and wheat, and of Balaguer *et al.* (1995) also on wheat. These authors argued that the relative effect of O_3 remained the same whether in charcoal-filtered or CO_2-fumigated air even though the stomatal conductance, and thus O_3 flux into the leaf, was decreased. Fiscus *et al.* (1997) reported on the O_3 flux estimated from leaf conductance on the same plants used in this study and found a 35% reduction in O_3 flux in the $CO_2 \times O_3$ treatment. Yet, these O_3 fluxes, which averaged 24.4 nmol m^{-2} s^{-1} (average midday), still represented twice the level received in the CF-AA treatment. Thus the reduced O_3 fluxes in the combined CO_2 by O_3 may not account for all the amelioration observed here because O_3 fluxes were still significant. Rao *et al.* (1995) reported that, in wheat, the amelioration of O_3 damage in elevated CO_2 was because of increased antioxidant enzymes and, hence, increased detoxification of O_3. Such an increase in some antioxidants has been reported for soybean grown in natural CO_2 springs (Badiani *et al.*, 1993) and could partially explain the amelioration of O_3 damage observed in this study.

The possibility that the reduced level of O_3 damage in the elevated CO_2 treatment was partly due to the amelioration of biochemical limitations of photosynthesis was suggested for greenhouse-grown wheat (McKee *et al.*, 1995). The data from this study showed that elevated CO_2 reduced the negative effect of O_3 on the Rubisco content and carbohydrate accumulation. Also, the regulation of Rubisco capacity by increased carbohydrate metabolism appear similar at elevated CO_2, whether in CF or O_3- fumigated air. These data on field-grown soybean thus support the notion of amelioration of biochemical limitation in $O_3 \times CO_2$ treatments.

Conclusions

In this study, the effects of [O_3] fumigation and elevated atmospheric [CO_2] on Rubisco activity and leaf components associated with carbohydrate metabolism were examined throughout the growing season. Accelerated development affected the response of Rubisco and associated biochemical components to [O_3] and elevated atmospheric [CO_2], O_3 having the strongest effect. Generally, O_3 reduced Rubisco activity and other biochemical components, although this effect was not consistent in 1994. Elevated CO_2 usually decreased Rubisco activity while increasing carbohydrates including starch and sucrose. However, starch and sucrose data showed little support for the down-regulation of Rubisco via feedback inhibition, but other soluble sugars may be involved. At elevated [CO_2], plants receiving CF air and O_3-fumigated air did not differ. Thus, elevated CO_2 appears to protect soybean against the adverse effect of O_3 for most of the season.

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