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Ozone inhibits phloem loading from a transport pool: compartmental efflux analysis in Pima cotton

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Abstract. The rate of export of recent photoassimilate from source leaves of Pima cotton (*Gossypium barbadense* L.) is inhibited by ozone (O_3). To characterize these effects on export, source leaves of Pima cotton were exposed to pulses (0.75 h) of O_3 (0.0, 0.2, 0.5 and 0.8 μL L^{-1}) followed by pulses of $^{14}CO_2$. Leaves were monitored by gas exchange and with a Geiger–Muller tube, for a sufficient period to characterize carbon assimilation (A) and a rapid and a slower phase of export. Double exponential decay functions (two-compartment model) were fitted and a compartmental analysis conducted. O_3 reduced by half the fast rate constant describing export from a transport pool, without affecting the rate constants for transport from or to a storage compartment. Measured soluble sugar contents increased slightly from control concentrations (1.2 g C m^{-2}) by about 5–10% at all O_3 concentrations. The calculated soluble sugar content in the transport pool increased from about 200 to 300 mg C m^{-2} with increasing exposure to O_3. The calculated storage pool did not respond to O_3 but exceeded measured contents. This discrepancy is attributed to starch deposition and mobilization, which are not considered in the two-compartment model, uncertainties in slower decay parameters, and non-steady-state A induced by O_3 exposure. Specific inhibition of rapid efflux suggests oxidant damage at the plasmalemma or plasmodesmata of mesophyll or phloem companion cells, and little effect on the tonoplast. A was affected less than export. Future research should target oxidation of components involved in phloem loading.

Keywords: air pollution, carbon allocation, carbon partitioning, phloem loading, translocation.

Introduction

Ozone impacts on carbon assimilation and allocation

Ozone (O_3) impacts crop (Heck *et al.* 1988) and native (Davison and Barnes 1998) plant species, though the mechanism remains poorly characterized. In Pima cotton (*Gossypium barbadense* L.), biomass allocation to developing roots is inhibited, with direct effects on plant hydraulic conductance (Grantz and Yang 1996) and potential indirect effects on shoot gas exchange (Grantz *et al.* 1999). Ozone also accelerates leaf senescence (Alscher *et al.* 1997; Pell *et al.* 1997) and directly reduces photosynthetic carbon assimilation (A) (e.g. Reich 1983; Dann and Pell 1989; Farage *et al.* 1991; Kangasjarvi *et al.* 1994; Pell *et al.* 1997).

O_3 causes a shift in the allometric coefficient in many species (Reiling and Davison 1992), including upland cotton (*G. hirsutum* L.; Oshima *et al.* 1979) and Pima cotton (Grantz and Yang 1996, 2000). This alteration in the relative growth of roots and shoots is distinct from effects of O_3 on the rate of plant development, which may alter instantaneous root to shoot biomass ratios. Such reduced allocation of photosynthate to roots (Oshima *et al.* 1978 1979; Bennet *et al.* 1979; McCool and Menge 1983; Gorissen and van Veen 1988), or stolons (Wilbourn *et al.* 1995) is commonly observed (Cooley and Manning 1987; Darrall 1989; Taylor and Ferris 1996; but see Reiling and Davison 1992). Its mechanism is not well understood.

The changes in A, whether mediated directly by O_3 or indirectly through root hydraulic properties, could reduce the availability of soluble sugars in source leaves for phloem transport (Andersen *et al.* 1991). This reduced 'source strength' could itself alter carbon allocation between competing sinks (Minchin *et al.* 1993).

Abbreviations used: A, carbon assimilation; GM, Geiger–Muller; $k_{storage\text{-}transport}$, rate constant for efflux from storage compartment; $k_{transport\text{-}out}$, rate constant for efflux from transport compartment out of leaf; $Q_{transport}$, sugar content of labile, readily-translocated compartment.

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Carbohydrate transport

Our previous results (Grantz and Farrar 1999) do not support this proposed etiology. Pulse exposures of source leaves of Pima cotton to a range of O_3 concentrations inhibited both A and export of recent photoassimilate. However, a quantitative sensitivity analysis revealed the overwhelming dominance of the inhibition of export over the inhibition of A in reducing total carbohydrate availability to sink tissues. Such an inhibition of export would represent a somewhat different reduction of 'source strength', though also one that could mediate the altered allocation envisioned in the model of Minchin *et al.* (1993).

Similar observations have been made in other biological systems. Retention of ^{14}C in source leaves of tomato was increased by O_3 (McCool and Menge 1983). Translocation of ^{13}C was inhibited by O_3 in *Phaseolus vulgaris* (Okano *et al.* 1984). Velocities of carbohydrate transport along the stem were not significantly affected in either wheat (Mortensen and Engvild 1995) or loblolly pine (*Pinus taeda* L.; Spence *et al.* 1990), but they trended downward by about 11% in loblolly pine.

Here we extend our previous results, involving short duration efflux analyses, to a longer time scale consistent with a formal compartmental analysis. The resulting characterization of rapid and slower phases of assimilate export provides a probe of O_3 impacts on the plasmalemma and plasmodesmata, and on the tonoplast. Compartmental analysis has not previously been applied to the mechanism of O_3 phytotoxicity, nor has such analysis been presented for leaves of upland or Pima cottons.

Materials and methods

Plant growth

Plants were grown as reported previously (Grantz and Farrar 1999). Briefly, seeds of Pima cotton (*Gossypium barbadense* L.; cv. Pima S-6; J.G. Boswell Company, Corcoran CA, USA) were germinated in darkness on blotting paper wetted with distilled water. After germination, seedlings were transferred to hydroponic culture on continuously aerated, half-strength Long Ashton solution under artificial illumination (HQI/NDL/250 W; Sylvania HIS-TD; 1 mmol m^{-2} s^{-1}) at 28/22°C (18/6 h). Experiments were conducted when the second true leaf was fully expanded (about 15 d after transfer to hydroponics).

Gas exchange, exposure to O_3 and efflux analysis

Methods were those of Grantz and Farrar (1999) modified after Owera *et al.* (1983). Plants were acclimated to the measurement facility overnight (21°C). Leaves were sealed into the cuvette at least 2 h into the photoperiod, and allowed to acclimate for at least 1 h and until carbon assimilation attained steady state. Efflux kinetics were monitored until the second, slower phase of efflux was nearly complete (10–12 h).

The second true leaf was trimmed to create a 2-cm-wide leaf strap (see Grantz and Farrar 1999) from the terminal 4 cm of the mid-vein of the middle lobe. Modification of the leaf reduced total leaf area in the cuvette and increased ventilation between the leaf and a Geiger–Muller (GM) tube, (Mullard, ZP 140; Alrad Instruments, Newbury, UK) mounted in the bottom of the cuvette, both reducing a recurring problem with condensation on the surface of the GM tube. Direct tests revealed no effect of leaf trimming on A or ^{14}C efflux kinetics.

The leaf strap was inserted into a well-stirred leaf cuvette (7 × 19 × 2.5 cm) and suspended about 0.3 cm above the GM tube. The GM tube was energized and interrogated with a rate meter (Model SR7; Nuclear Enterprises, Edinburgh, Scotland, UK) with analog output displayed on a chart recorder and captured every 60 s with a digital data logger (21 X, Campbell Scientific Inc., Logan UT, USA). Data were transferred to diskette for analysis in a laboratory computer.

Ozonation was initiated by energizing (0.75 h duration) an ultraviolet bulb (Hamamatsu Model 90-0001-01; Middlesex NJ, USA) permanently located in the gas stream. O_3 concentration was continuously monitored at the cuvette outlet (Model 8810; Monitor Labs). In the absence of power to the bulb there was no detectable O_3.

Introduction of $^{14}CO_2$ label into the cuvette was initiated 15 min after termination of ozonation by switching the air stream to bubble through 5 mL of lactic acid, to which 100 μCi (3.7 MBq) of $Na_2{}^{14}CO_3$ in 15 mL Na Tris (pH 7.0) was then added over 15 min with a perfusion pump. Air leaving the leaf cuvette was passed over soda lime to remove any residual $^{14}CO_2$.

Assimilation rate was monitored by infrared gas analysis (LI 6252, LiCor Inc., Lincoln NE, USA) operated in differential mode for CO_2, and recorded following the attainment of steady-state conditions before ozonation, and again following $^{14}CO_2$ exposure. A was depressed by the 0.75 h exposure to O_3 (see 'Results') and neither recovered nor declined further during the subsequent hours of these experiments. A of control plants often increased slightly (about 5% on average), following attainment of apparent steady state, during the 0.75 h 'exposure' to 0.0 μL L^{-1} O_3. Transpiration was not monitored.

Plants exposed to 0.0 or 0.2 μL L^{-1} O_3 and then returned to their growth environment exhibited no visible symptoms, while those exposed to 0.5 or 0.8 μL L^{-1} O_3 exhibited some discoloration or visible injury (respectively). The protocol of a single acute exposure (0.75 h) to high concentrations (up to 0.8 μL L^{-1}) of O_3 is clearly unrealistic but has considerable merit for physiological studies, as discussed previously (Tingey and Taylor 1982; Minchin and Gould 1986; Farage and Long 1995; Grantz and Farrar 1999).

Compartmental efflux analysis was performed on data retrieved from the digital files and normalized, taking 100% activity and initial time at the onset of the sustained reduction of ^{14}C activity. The efflux curves from individual leaves were fitted to a two-compartment model (Moorby and Jarman 1975; Bell and Incoll 1982; Owera *et al.* 1983) as a double exponential decay function with five parameters (Non-Linear Statistical Utility; SigmaPlot v. 3.0; SPSS; Chicago IL, USA):

$$y = ae^{-bt} + ce^{-dt} + e,$$

in which y is the percentage of the originally fixed ^{14}C label remaining at time t, b and d characterize the exponential decline of activity over time, e is the base of the natural logarithm, and e is the asymptote of non-translocated label. Quantities a, c and e are fitted parameters always totalling approximately 100%. This model is more complete than the single compartment model (Evans *et al.* 1963) applied previously to shorter-term efflux timecourses in Pima cotton (Grantz and Farrar 1999), while more complex models with additional compartments did not improve the fit to these data (e.g. Rocher *et al.* 1994).

Rate constants for rapid efflux from the transport compartment out of the leaf ($k_{transport\text{-}out}$) and for slower efflux from the storage compartment ($k_{storage\text{-}transport}$) were calculated using the equations of Atkins (1969) and Moorby and Jarman (1975).

Analysis of carbohydrates

Leaf disk samples were obtained with a #3 cork borer (0.33 cm diameter) from leaves inside the cuvette, prior to exposure and at the time corresponding to the end of the exposure to $^{14}CO_2$. Four disks were obtained from each leaf (1.36 cm^2 total) at each sampling time. Dry

weights were determined on parallel samples. Leaf disks were sampled directly into 5 mL hot aqueous ethanol (80°C; 95%) and extracted for 0.5 h prior to storage at 0°C.

Stored samples were warmed to room temperature and centrifuged (5 min, 6000 rpm; Model 5403; Eppendorf, Hamburg, FRG), and the pellet extracted twice more for 30 min in 5 mL aqueous ethanol (80°C; 95%) followed by centrifugation. The first two supernatants were combined and brought to 10 mL with 95% ethanol. The third extract was analysed only occasionally to confirm that no soluble sugars remained in the pellet.

Soluble sugars were analysed using the phenol–sulfuric acid method (Dubois *et al.* 1956) with aldose and ketose sugars determined by absorption at 490 nm (Cecil Spectrophotometer; Model CE 303; with flow through cuvette) against authentic sucrose standards (0–100 µg in 1 mL 95% ethanol). The major sugar, as expected (Hendrix and Grange 1991) was sucrose (about 50%), with stachyose and others constituting the remainder (Grantz and Yang 2000).

Total starch was analysed following the methods of Madore (1990) and Hendrix (1993). The pellet remaining after the third ethanolic extraction of soluble sugars was dried to constant weight (55°C), resuspended in 2 mL of 2 N KOH, and subjected to partial alkaline hydrolysis and tissue disintegration at 100°C for 1 h. Following cooling to room temperature, the suspension was adjusted with 2 mL of 2 N acetic acid to pH 4.5. Starch was then fully hydrolysed to glucose using amyloglucosidase (Fluka 10115; Rankonkoma NY, USA) dissolved in 50 mM Na Acetate buffer, pH 4.5. Glucose was assayed with the hexose kinase method (Sigma Diagnostics, Inc., St. Louis MO, USA; Procedure 16-UV) in a microplate reader (Model 3550-UV; BioRad) by absorption at 340 nm. Authentic glucose served as the standard. Glucose determinations were converted to starch concentrations as [0.9 × glucose] to reflect the addition of 1 H_2O per glucose residue during hydrolysis.

All carbohydrates are expressed as g C m^{-2}. Specific leaf weight was about 44 g m^{-2}.

Calculation of percentage changes

A and leaf sugar contents changed by a few percent, and starch contents by almost 30%, following the attainment of apparent steady-state conditions, during the 0.75 h exposure of control leaves to 0.0 µL L^{-1} O_3. To preserve this information, and because O_3 induced a dose-specific change in the rate of starch accumulation, all percentage changes in these parameters are presented in the figures relative to initial, rather than final, values. On occasion in the text these changes are evaluated relative to the final values attained by the controls following the 0.75 h exposure period.

Results

Carbon assimilation

A in the low PPFD-grown Pima cotton plants (about half of full sunlight) was about 10 µmol m^{-2} s^{-1} (about 400 mg C m^{-2} h^{-1}). Although plants were allowed to achieve apparently constant *A* before the start of O_3 exposure, a slight increase in *A* (about 5%; Fig. 1) was often observed in the control plants during the 0.75 h exposure to O_3-free air. In contrast, even the lowest non-zero O_3 concentration tested (0.2 µL L^{-1}) inhibited *A* by over 10%, relative to the final value of control *A*, and by about 7% relative to initial values (Fig. 1). Further increase in O_3 concentration elicited a dose-dependent inhibition of *A* (Fig. 1), by about 25% relative to final control values at the highest O_3 concentration tested (0.8 µL L^{-1}).

Carbohydrate content

Soluble sugar contents of these source leaves, determined with the phenol–sulfuric acid reagent, were about 1.2 g C m^{-2} when *A* first achieved steady state early in the photoperiod.

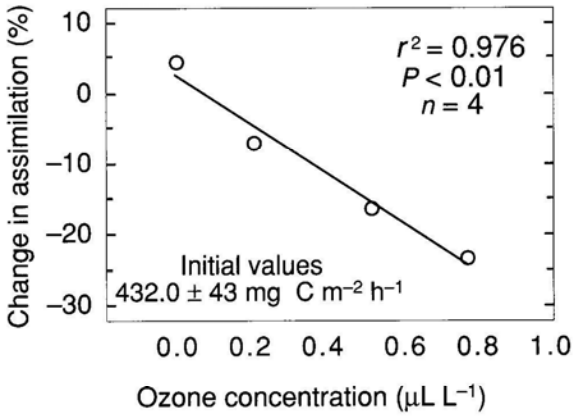

Fig. 1. Effect of exposure to O_3 (0.75 h) on net carbon assimilation 1.3 h after onset of ozonation relative to values prior to exposure, in source leaves of Pima cotton. Initial values are means ± standard errors with $n = 16$ leaves.

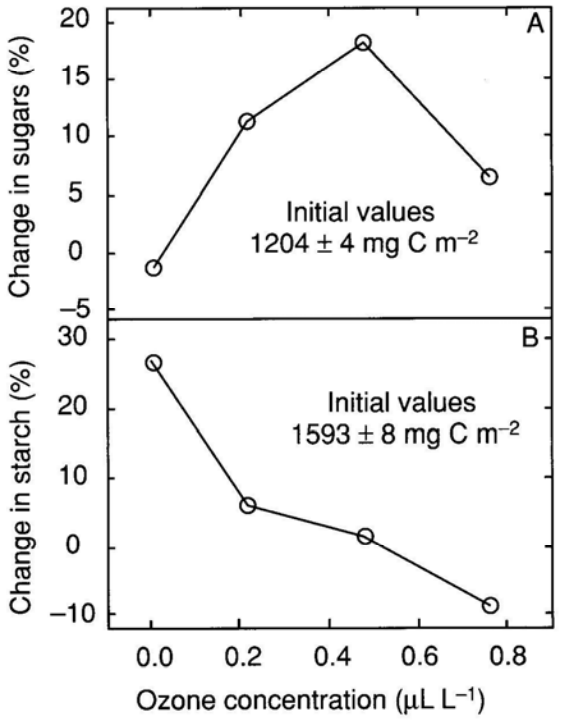

Fig. 2. Effect of exposure to O_3 (0.75 h) on (*A*) total soluble sugar contents and (*B*) starch contents 1.3 h after onset of ozonation relative to values prior to exposure, in source leaves of Pima cotton. Initial values as in Fig. 1.

Sugar contents were constant or declined slightly during 0.75 h exposure to O_3-free air (Fig. 2*A*), but increased by about 10% following 0.75 h exposure to 0.2 μL L^{-1} O_3 and by nearly 20% at 0.5 μL L^{-1}. Additional increase in O_3 concentration to 0.8 μL L^{-1} reduced sugar accumulation to about 6% above initial levels.

Starch contents, about 1.6 g C m^{-2} in leaves prior to O_3 exposure, increased at all levels of O_3 except the highest tested (Fig. 2*B*). The relative accumulation of starch declined in a dose-dependent manner, with a maximum of almost 30% in O_3-free controls, no net change at 0.5 μL L^{-1} and a 10% decline at 0.8 μL L^{-1} O_3.

Transport compartment

Kinetics

The initial phase of export of ^{14}C label from the source leaf was rapid in the absence of O_3 exposure. The value of $k_{transport\text{-}out}$ was about 2 h^{-1} (Fig. 3*A*). This is equivalent to a half time for exchange of about 20 min. O_3 reduced the rate of export at the lowest O_3 concentration tested (k = 1.75 h^{-1} at 0.2 μL L^{-1}), and substantially restricted efflux at the highest concentration tested (k = 1 h^{-1} at 0.8 μL L^{-1}). This doubled the half time for export from this rapidly exchanging compartment to about 42 min. The rate constant declined linearly and highly significantly with increasing O_3 concentration (Fig. 3*A*).

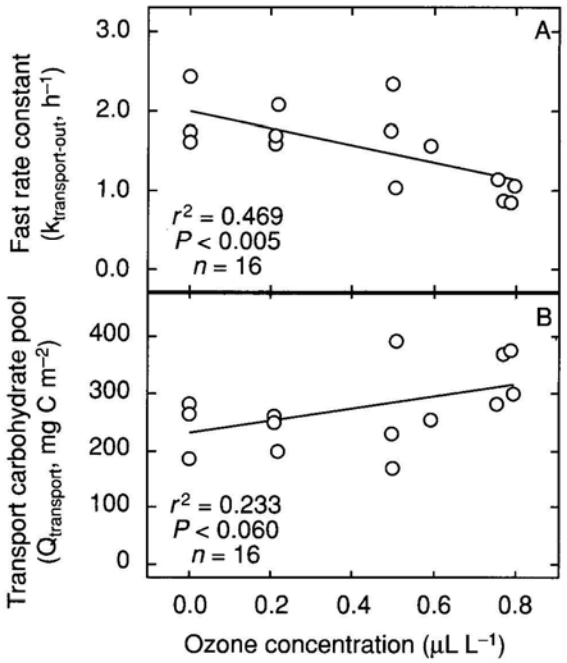

Fig. 3. Effect of exposure to O_3 (0.75 h) on (*A*) the rate constant ($k_{transport\text{-}out}$) for the fast phase of efflux of ^{14}C-labeled photoassimilate and (*B*) the calculated sugar content of the labile, transport carbohydrate pool in source leaves of Pima cotton.

Pool sizes

The calculated sugar content of the labile, readily translocated compartment ($Q_{transport}$; Fig. 3*B*) was somewhat variable, perhaps reflecting the complex response of measured sugar contents to O_3 exposure (*cf.* Fig. 2*A*). Exposure to O_3 induced a marginally significant ($P < 0.06$) increase in the size of this transport pool, by up to 50% (Fig. 3*B*), in leaves exposed to the highest O_3 concentration. The calculated values of $Q_{transport}$ of 0.2–0.3 g C m^{-2} were consistent with measured total leaf contents (*cf.* Fig. 2*A*).

Storage compartment

Kinetics

The two-compartment model used to analyse these efflux data incorporates a storage compartment (putative vacuole) that communicates with the leaf exterior only through the transport compartment (putative cytoplasm). Exposure to O_3 did not consistently impact the rate constant for transport of storage carbohydrate to the transport compartment. This rate constant ($k_{storage\text{-}transport}$) was about 0.02 h^{-1} at all concentrations of O_3 tested (Fig. 4*A*). In general, the parameters describing the slower component of ^{14}C efflux were more variable than those characterizing the fast phase, reducing

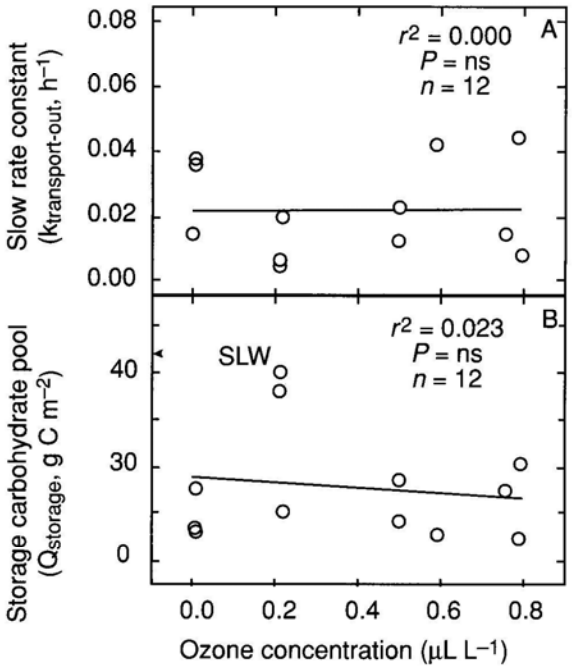

Fig. 4. Effect of exposure to O_3 (0.75 h) on (*A*) the rate constant ($k_{storage\text{-}transport}$) for the slow phase of efflux of ^{14}C-labeled photoassimilate and (*B*) the calculated sugar content of the slowly mobilized, storage carbohydrate pool in source leaves of Pima cotton. The horizontal arrow in (*B*) refers to the specific leaf weight, including starch and soluble sugars.

the power of this method to resolve an effect of O_3 on mobilization of storage carbohydrates.

The rate constant for loading of the putative vacuolar storage compartment (generally about 0.8 h^{-1}) was also unaffected by O_3 except at the highest O_3 concentration. At 0.8 μL L^{-1} O_3, $k_{transport\text{-}storage}$ was reduced to about 0.6 h^{-1}, significantly below the other values (not shown). Although intriguing, this result requires further substantiation due to variability in the calculated values.

Pool sizes

Calculated sugar contents of the storage compartment (Fig. 4*B*) were also unchanged by exposure to O_3. Calculated pool values were generally about 10 g C m^{-2}. These calculated values for this slowly exchanging pool were severalfold greater than directly measured values for soluble sugars in the whole leaf (*cf.* Fig. 2*A*). In a few cases these values approached the specific weight of the leaf (arrow, Fig. 4*B*).

Non-transported carbohydrate

The inhibited efflux from the transport compartment suggested that the amount of ^{14}C retained by the source leaves in a non-exportable pool might increase with increasing exposure to O_3. The calculated asymptote in the double exponential efflux relationships (*e*; Fig. 5) was highly conserved across all O_3 concentrations tested. This parameter ranged from 30 to 40%, averaging about 35% of recently assimilated $^{14}CO_2$ incorporated into structural compounds or other nonlabile compartments.

Carbohydrate budget

The effect of O_3 on the change in total C in these source leaves at 1.3 h after onset of O_3 exposure can be estimated from the separate effects of O_3 on *A* and on the two rate constants for ^{14}C efflux. Two simplifying assumptions are required. Carbon assimilation measured at 1.3 h is assumed to have been constant over the course of the 0.75 h exposure and afterwards, and values of $k_{transport\text{-}out}$ and $k_{storage\text{-}transport}$ determined from the entire 10–12 h efflux time course are assumed to be characteristic of the initial 1.3-h period. Both are qualitatively reasonable assumptions, but quantitative agreement with actual values cannot be determined from available data. Calculated in this way for the 1.3-h time point, total carbon accumulation in the source leaves declined linearly and significantly with increasing exposure to O_3 (Fig. 6*A*; ●).

Comparison of these calculated values with the measured changes in total C (starch plus sugars; Fig. 2) relative to the original contents in individual leaves (Fig. 6*A*, Δ) indicates a general agreement except at the highest O_3 concentration. At this 0.8 μL L^{-1} O_3 exposure, measured C content declined by about 5%, whereas the calculated value increased by about 9%.

The effect of O_3 on C available for export to sink tissues can be similarly estimated as the difference between cumulative *A* over 1.3 h and the calculated change in C content in the source leaf at this time. The amount of carbohydrate translocated from the source leaves declined linearly, highly

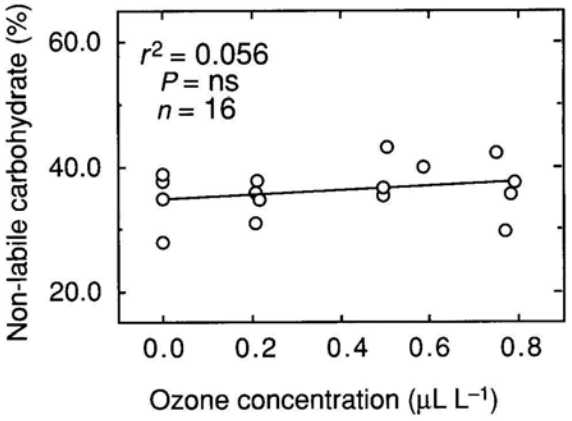

Fig. 5. Effect of exposure to O_3 (0.75 h) on the calculated fraction of ^{14}C-labeled photoassimilate that is not exported from source leaves of Pima cotton.

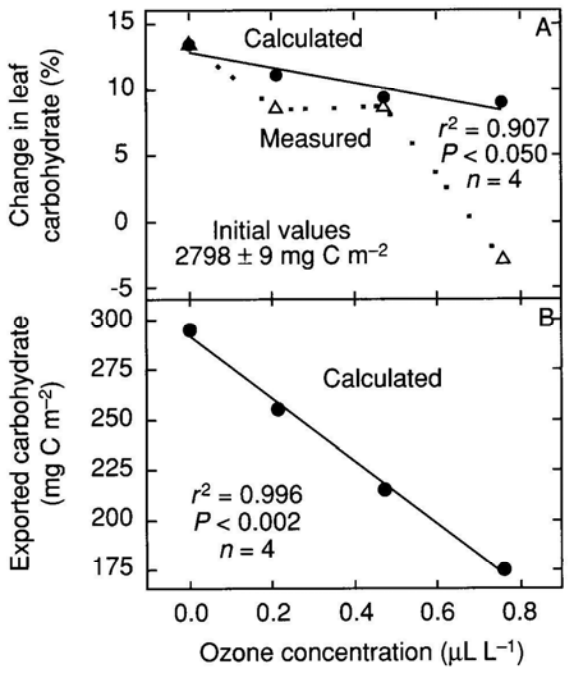

Fig. 6. Effect of exposure to O3 (0.75 h) on (*A*) total carbohydrate contents in source leaves (starch plus soluble sugars) relative to values prior to exposure, and (*B*) total carbon exported from the source leaves to sink tissues, both at 1.3 h after onset of ozonation in Pima cotton. Initial values in (*A*) as in Fig. 1. ● and regressions refer to values calculated from effects of O_3 on assimilation (Fig. 1) and on export (Figs 3*A* and 4*A*). Δ refers to measured values.

significantly, and by about 40%, over this range of O_3 concentrations (Fig. 6*B*).

Discussion

Carbon assimilation

A of recently mature leaves of Pima cotton declined in a dose-dependent manner, by up to 25%, over the range of O_3 exposures tested here. Photosynthetic inhibition is a common response to O_3. Brief (1 h) exposure to 0.6 μL L^{-1} O_3 reduced *A* in tomato (*Lycopersicon esculentum)* by about 43%, and in bean (*Phaseolus vulgaris)* by about 29%. Similar (1.5 h) exposure to 0.4 μL L^{-1} O_3 reduced *A* in tobacco (*Nicotiana tabacum)* by 78% (Hill and Littlefield 1969). Over these relatively short exposures the effects are likely to reflect rapid and direct attack of O_3 on the biochemical components of the photosynthetic apparatus (e.g. Farage and Long 1995). Over longer time periods the inhibited export of C to sink tissues such as roots (Grantz and Yang 1996, 2000) may exert further indirect feedback effects on *A* (e.g. Grantz *et al.* 1999). Regardless of impacts on carbohydrate efflux and allocation, these impacts of O_3 on *A* reflect a substantial impact on plant productivity.

Efflux kinetics

Transport compartment

Increasing exposure to O_3 resulted in a significant decline in the fast rate constant from about 2 to 1 h^{-1}. These values of $k_{transport\text{-}out}$ are somewhat greater than those reported in the absence of O_3 for the C_3 species, barley (*Hordeum vulgare* L.; 0.4–1.0 h^{-1}; Farrar and Farrar 1986) and tomato (0.8 h^{-1}; Moorby and Jarman 1975), and for the C_4 species, *Amaranthus caudatus* (0.4 h^{-1}; Moorby and Jarman 1975). It is important to note that, while they are also considerably above the values previously reported for Pima cotton (Grantz and Farrar 1999), these previous values were derived using a simpler, one-compartment model and a shorter period of ^{14}C efflux (0.1–0.3 h^{-1}). The various models available each adequately describe the biological system, given certain assumptions regarding its complexity, and all yield meaningful treatment comparisons. However, they do not yield compatible estimates of the fast rate constant, though this parameter is common to all.

One of the early events in oxidant damage to these source leaves of Pima cotton (at least within 1 h of exposure) is the disruption of one or more of the processes leading to removal of ^{14}C activity in the source leaf from the vicinity of the GM tube (Grantz and Farrar 1999). This is within the same time frame as rapid impacts of O_3 on photosynthetic mechanisms (Farage *et al.* 1991). Since a build-up of sugars in the mesophyll, due to inhibition of export, can quickly inhibit carbon assimilation (Krapp *et al.* 1993), the sequence of causal events in O_3 phytotoxicity remains to be fully elucidated.

The rapid phase of efflux corresponds to movement of the labile pool of carbohydrate (Moorby and Jarman 1975; Farrar and Farrar 1985). While these data cannot distinguish between damage to cell membranes of exporting mesophyll cells and damage to those of importing phloem companion cells, the O_3 impact on these rapid kinetics is likely to be associated with impairment of plasmalemma function along the transport pathway. Potentially sensitive sites of action could be the plasmodesmata (Lucas *et al.* 1996) and the membrane-bound sucrose translocator system (Bush 1999). These potential targets of O_3 action have not been evaluated in this regard.

Storage compartment

There was no indication of an impact of O_3 on mobilization of storage carbohydrate from the slowly mobilized pool. There was similarly little evidence for inhibition of transport of current assimilate into the storage compartment. A potential impact on vacuolar loading at the highest O_3 concentration is suggested by these data but requires confirmation. Effects of O_3 on translocation seem likely to be restricted to the plasmalemma-based functions suggested above.

These conclusions must be tempered by the observation that estimates of the slow rate constants are less accurate than for the rapid decay parameters. Furthermore, compartmental analysis (Zierler 1981; Bell and Incoll 1982; Farrar and Farrar 1985; Cheeseman 1986) is sensitive to assumptions that diurnal variations in rate constants are negligible, that the efflux kinetics are truly first order, and that the soluble carbon pool sizes are at steady state.

The latter assumption is particularly questionable following a perturbation such as O_3 exposure. In grasses such as barley (Farrar and Farrar 1986) and wheat (Balaguer *et al.* 1995), sucrose contents in source leaves may reach maximum values late in the photoperiod. In dicots such as cotton (Hendrix and Grange 1991) and sugarbeet (Geiger *et al.* 1983), however, these pools fill more rapidly and stabilize within 2 h. The present measurements were thus performed at steady-state sugar contents in control leaves, as reflected in the measured soluble sugar contents in the O_3-free control. O_3-induced perturbations of both *A* and efflux, however, altered the measured sucrose contents and undoubtedly biased the calculation of pool sizes. Despite these potential shortcomings of the method, the previous efflux analyses with and without a non-exchangeable compartment (Grantz and Farrar 1999) and the two-compartment analysis performed here, suggest an O_3 impact on components associated with phloem loading.

Carbohydrate content

Starch

Cotton leaves exhibit a large range of starch contents under varying environmental conditions, from 0.4 to 16 g C m^{-2}

(Radin *et al.* 1987; Miller *et al.* 1989; Wong 1990; Hendrix and Grange 1991). Control starch contents in the present study were about 1.5 g C m^{-2} prior to exposure to O_3, with values of 1–2 g C m^{-2} across the full range of O_3 exposures.

Unlike the contents of soluble sugars, starch was not at steady state even in the control leaves. Starch accumulated during the experiment at all but the highest O_3 concentration. However, the amount of accumulation declined with increasing exposure. In a previous study of chronic exposure of Pima cotton to O_3, starch content declined relative to O_3-free controls at high O_3 concentrations (Grantz and Yang 2000), though at moderate O_3 exposure little difference was observed. A similar decline in starch was observed in aspen (*Populus tremuloides*; Coleman *et al.* 1995).

In cucumber (*Cucumis sativus* L.) and pinto bean (*Phaseolus vulgaris* L.), exposure to O_3 increased the starch content measured after a dark period (Hanson and Stewart 1970). In wheat, carbon efflux in the dark was inhibited by O_3 (Balaguer *et al.* 1995). These observations probably reflect an inhibition of starch remobilization. The current study was performed during a single photoperiod, and thus cannot reveal any information about O_3 impacts on carbohydrate metabolism in the dark.

Sugars

Soluble sugar contents were about 1.2 g C m^{-2} in unexposed leaves of Pima cotton. Total sucrose contents of unexposed upland cotton leaves in other studies range from about 0.4 g C m^{-2} (Miller *et al.* 1989; Hendrix and Grange 1991) to about 1 g C m^{-2} (Wong 1990). Some glucose is also present in cotton leaves (0.1–0.5 g C m^{-2}; Miller *et al.* 1989; Hendrix and Grange 1991), though as a non-transport sugar its contribution to the kinetically determined compartments in the present study is unknown.

The inhibition of *A* following exposure to O_3 could lead to depletion of the transport pool in source leaves, and a consequent inhibition of export. In a few biological systems (Tingey *et al.* 1973; Bennett *et al.* 1979), foliar pools of soluble sugars have been reduced by exposure to O_3. For example, sucrose content declined in wheat leaves from about 1.2 to about 0.8 g C m^{-2} (Balaguer *et al.* 1995), despite the greater inhibition of efflux than *A*. This result was attributed to O_3-induced increases in dark respiration.

More commonly, as in aspen (Coleman *et al.* 1995), soluble sugar contents have increased following exposure to O_3. Chronic exposure of upland cotton to O_3 (Miller *et al.* 1989) caused small and inconsistent changes in both glucose and sucrose contents. In Pima cotton, chronic exposure to O_3 (Grantz and Yang 1996, 2000) increased the leaf content of sucrose (about 50% of soluble sugars) on a dry weight basis. A similar increase, following acute exposures in the present study, was observed on a leaf area basis. Increasing leaf contents of transport sugars such as sucrose do not support the hypothesis that substrate availability in individual source leaves limits phloem loading.

Fast pool sizes

Sugar contents determined from whole leaf extractions cannot distinguish distinct pools. Within the constraints of the method, compartmental analysis provides estimates of the contents of each of the kinetically identified compartments. The calculated transport pool of soluble sugars in the present study increased from about 0.2 to more than 0.3 g C m^{-2} following exposure to O_3.

The transport pool of source leaves contains a variable fraction of the soluble sugars. In barley and spinach about 20–30% (Gerhardt and Heldt 1984; Farrar and Farrar 1986) of sugars reside in the transport pool. In starch-storing dicotyledonous species such as sugar beet, up to 60–80% (Geiger *et al.* 1983) may be located in the rapidly mobilized pool. The present results suggest that only a few percent of the total contents were in the transport pool in Pima cotton. However, uncertainties in the estimation of the large storage pool make these conclusions regarding intracellular partitioning highly tentative.

Slow pool sizes

The calculated soluble sugar content of the slow compartment appeared unrealistically large across all concentrations of O_3, particularly at the highest concentrations. Calculated values of about 10 g C m^{-2} for this compartment alone were higher than the measured sugar contents in the whole leaf. The control leaves, and leaves prior to exposure to O_3, had osmotic potentials of 1.17 ± 0.04 MPa at midday (Grantz and Farrar 1999), equivalent to over 15 g C m^{-2} if all osmotic activity is attributed to sucrose. Thus the calculated sugar contents are not constrained by osmotic considerations. However, the specific leaf weight of the leaves in the present study was about 44 g m^{-2}, independent of O_3 exposure. The calculated storage contents were high relative to this value, and relative to the calculated fast pool sizes.

Non-transported carbohydrate

The present compartmental analysis did not consider starch storage as a discrete compartment, and the method cannot identify the location or identity of non-labile material. The steady-state assumption underlying the method (Atkins 1969) implies that $k_{transport\text{-}storage}$ and $k_{storage\text{-}transport}$ are equal. However, the former rate constant must absorb any diversion to starch that is otherwise hidden from the two-compartment model. A large ratio of starch accumulation to *A* will lead to overestimation of the soluble pool contents. Thus the measured changes in starch content may have contributed to the overestimation of the storage pool. A model including diversion of current assimilate to starch might improve these estimates.

C_4 grasses may export more than 80% of newly assimilated label in 2 h (Hartt 1965; Hofstra and Nelson 1969*a*) but

C_3 species export less rapidly and less completely (Hofstra and Nelson 1969*b*). Source leaves of cotton retain from 10% (Benedict and Kohel 1975) to 40% (Ashley 1972) of ^{14}C-labeled recent photoassimilate after 24 h. Our preliminary estimates of a non-labile pool of about 65% (Grantz and Farrar 1999) were consistent with the 60% retention observed at 2 h in main stem leaves of upland cotton (Ashley 1972). The present analysis over much longer time periods found about 35% of recent ^{14}C label was retained asymptotically after a long photoperiod, consistent with the 40% estimated by Ashley (1972) over longer time periods. Neither the current value nor the previous estimates (Grantz and Farrar 1999) were affected by O_3 concentration.

Relationship between A *and efflux*

Exposure to O_3 inhibited both A and efflux. The generally linear decline of both A and efflux with increasing O_3 concentration led to considerable covariance of mean data (not shown). While this suggested a possible mechanistic relationship between these inhibitory responses, this was not supported by examination of data from individual leaves. There was no significant correlation between A and efflux, nor between inhibition of A and inhibition of efflux (not shown). This is consistent with previous results (Grantz and Farrar 1999) and with the independence of ^{11}C-efflux and A in the C_4 species maize (*Zea mays*) in the presence and absence of CO_2 (Minchin and Gould 1986). In upland cotton (Hendrix and Grange 1991), altering the photoperiod changed carbon export without affecting the relative export of newly assimilated carbon (i.e. the rate constants).

The relative sensitivity of A and export depends on leaf maturity. In aspen (Coleman *et al.* 1995), O_3 reduced export in older leaves while in younger leaves, export was unaffected. In primary leaves of *Phaseolus vulgaris* (Okano *et al.* 1984), A was reduced more than export, but in younger trifoliate leaves A was reduced less than export. Source–sink transition may have a role in this phenomenon, as well as the role of O_3 in accelerating senescence in the older leaves.

The general relationship between A and carbohydrate export may be mediated indirectly through increased sugar concentrations. In tomato (Ho 1976) and upland cotton (Hendrix and Peelen 1987), export of newly assimilated carbon in the light was proportional to leaf sucrose contents. In spinach (Servaites *et al.* 1989) there was only a poor correlation. This is consistent with the sequestration of most of the sucrose in the storage compartment in spinach (Gerhardt and Heldt 1984). In wheat (Balaguer *et al.* 1995) efflux and soluble sugar content covaried across a range of treatments except those involving O_3. This suggests that O_3 could have directly impacted translocation with a consequent effect on sugar accumulation. This could inhibit A through a number of potential feedback and end-product inhibitory effects on enzyme activities or through down-regulation of transcription of the small subunit of Rubisco (Krapp *et al.* 1993). O_3 impacts on A could reflect the O_3-induced build-up of sugars rather than, or in addition to, direct oxidative damage to the photosynthetic apparatus.

Carbohydrate budget

O_3 impacts on total non-structural carbohydrate (starch plus sugars) in source leaves of Pima cotton were dominated by the response of starch, with an increase in total pool size observed at all but the highest O_3 concentration (0.8 $\mu L\ L^{-1}$). The decline at this extreme O_3 concentration was anomalous, relative to the other measured contents and to the calculated pool sizes, which increased at all O_3 concentrations and agreed with measured values except at 0.8 $\mu L\ L^{-1}$. The increase in the calculated contents declined linearly with increasing O_3 concentration, one aspect of O_3-reduced productivity.

The other aspect of O_3-reduced productivity is inhibition of export to heterotrophic sink tissues such as roots, reproductive structures and shoot meristems. Retention of newly assimilated carbon in source leaves is associated with reduction in sugar content of sink tissues (e.g. Balaguer *et al.* 1995). In Pima and upland cottons it is associated with reduced development of root systems and impaired hydraulic function (Oshima *et al.* 1978; Grantz and Yang 1996). The present results indicate a linear decline in carbohydrate export to sinks with increasing exposure to O_3. This decline was dominated by effects of O_3 on carbohydrate export rather than on A.

The reduced flux of C associated with the inhibition of phloem transport could result in apparent preferential transport to adjacent sinks (e.g. stems) at the expense of distant sinks (e.g. roots), as modeled by Minchin *et al.* (1993). This could explain the continued transport of carbon to stems following exposure to O_3 (Grantz and Yang 1996) and SO_2 (Jones and Mansfield 1982), despite inhibition of export from source leaves. Further work on the mechanisms underlying whole plant carbon allocation will be required to fully elucidate oxidant effects on plant growth, development, and productivity. The current results support our previous conclusions (Grantz and Farrar 1999) that effects of O_3 on carbon transport may dominate O_3 effects on carbon assimilation. The substantial impact of O_3 on the fast phase of export over long time periods in Pima cotton is ameliorated considerably by the lack of a significant impact on the slower efflux of the much larger storage pool.

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