# MOLECULAR BIOLOGY AND PLANT PHYSIOLOGY

## Ozone Impacts on Carbon Dynamics in Cotton

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## ABSTRACT

Ozone ($O_3$) is a secondary atmospheric pollutant that causes widespread damage to vegetation, including cotton. The high temperatures and abundant sunlight that lead to good cotton yields also lead to production of $O_3$. The early crop loss experiments of the 1970s identified upland cotton as particularly sensitive to $O_3$. Exposure to ambient concentrations of $O_3$ resulted in a 10 to >15% loss in economic yield. More recent experiments with modern cultivars have confirmed damage to upland and Pima cultivars but have not assessed yield loss under agronomic conditions. It is likely that selection for yield in high $O_3$ environments has led to inadvertent selection for tolerance to $O_3$. Ozone reduces yield and fiber quality, mediated by $O_3$-induced changes in gross photosynthesis, respiration, and translocation of current photosynthate out of source leaves. Ozone typically reduces root development and the root-to-shoot biomass ratio, which has secondary effects on plant growth and development and on water relations. Ozone also reduces stomatal conductance as a primary defense against entry of $O_3$ into the plant, but this also restricts entry of $CO_2$ and therefore photosynthesis. Ozone is clearly phytotoxic and impacts cotton as well as other species. Recent research has led to understanding many aspects of the mechanism of $O_3$ damage and could lead to targeted approaches to improving $O_3$ tolerance, and therefore, yield in $O_3$-impacted production areas.

## WHY OZONE?

Ozone ($O_3$) is a secondary air pollutant, produced in the atmosphere by photochemical reactions among natural and anthropogenic precursors. Due to its widespread distribution, it is globally the most damaging air pollutant to vegetation (Booker et al., 2009) and is injurious to cotton, both *Gossypium hirsutum* L. and *G. barbadense* L.

Natural background concentrations of $O_3$ are low, but are currently increasing due to emissions of nitrogen oxides and organic compounds from industrial and transportation sources and increasingly from vegetation as the atmosphere warms (US EPA 2013). The spatial overlap between factors conducive to $O_3$ formation (Finlayson-Pitts and Pitts, 1986) and those promoting cotton production, particularly abundant sunshine and warm temperatures, suggest that $O_3$ impacts on yields are inevitable, as demonstrated by the global citrus crop (Grantz and Sanz, 2006). Elevated ambient $O_3$ is observed in major parts of the U.S. cotton belt (Fig. 1).
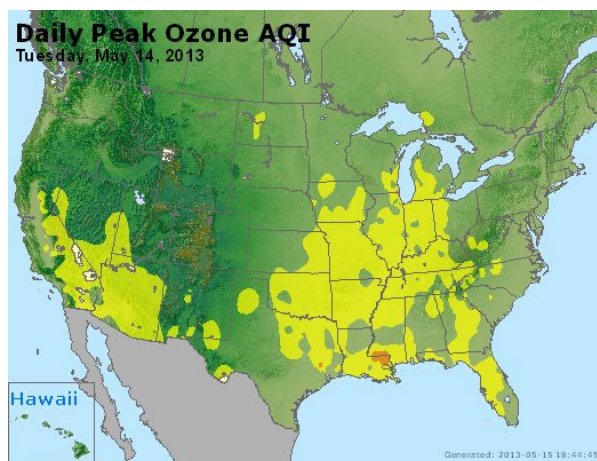


Figure 1. Ozone distribution on 14 May 2013 showing moderate (yellow; 60-75 ppb) and unhealthy (orange; 76-95 ppb) $O_3$ levels across many of the cotton production areas of the U.S. Areas in green had good $O_3$ air quality (< 59 ppb). Ozone levels even within the good range are likely damaging to cotton. Screen capture from: USEPA AIRNOW, at http://airnow.gov/index.cfm?action=airnow.mapsarchivedetail&domainid=53&mapdate=20130514&tab=2). Accessed August 2015.

Although $O_3$ is clearly phytotoxic, its former characterization as a harmful xenobiotic has been replaced slowly by a more nuanced understanding of its metabolic impact (e.g., Foyer and Noctor, 2005). Ozone is a strong oxidant and rapidly breaks down in the aqueous cell-wall space into a variety

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of reactive oxygen species, including hydrogen peroxide, superoxide, and hydroxyl radical. Breakdown products of $O_3$ can penetrate farther into the cell than $O_3$ itself. The new understanding is that these compounds are closely related or identical to reactive oxygen species that are involved in normal plant metabolism as byproducts of electron transfer reactions and as elements of signaling cascades. Despite the large number of systems and compounds that appear to be involved in cellular signaling (Assmann, 2010; Murata et al., 2015), a unifying feature appears to be these reactive oxygen species. Chemical interaction of $O_3$ or its breakdown products with biomolecular targets, for example, with ascorbate yielding oxalate (Sandermann, 2008), initiates plant response. Much of the damage, however, is associated with downstream signaling cascades that induce programmed responses (Baier et al., 2005). Plant response to acute $O_3$ exposure is similar to the hypersensitive response to pathogens (Vainonen and Kangasjarvi, 2015). However, the ensuing metabolic changes and programmed cell death in response to $O_3$ is a clearly nonadaptive cooptation of the effective response to microbial attack. The role of divalent cations in the $O_3$ response, particularly calcium, is consistent with this new paradigm (Castillo and Heath, 1990; McAinsh et al., 1996; Short et al., 2012).

Effects of $O_3$ on plasma membrane function are observed quickly. These membrane effects do not reflect nonspecific oxidant damage but are mediated by regulated control of specific ion transport channels, including unidentified calcium channels that mediate a signaling function, as well as slowly deactivating anion channels and potassium channels (Torsethaugen et al., 1999; Vahisalu et al., 2008; Vainonen and Kangasjarvi, 2015). The oxidizing potential of $O_3$ is readily communicated to the cytoplasm, resulting in altered expression of a large number of genes (Li et al., 2006), reduction of chloroplastic pH gradients, and changes in mesophyll enzyme activities (Dann and Pell, 1989; Evans and Ting, 1973; Heath, 1987; Heath and Frederick, 1979).

A general treatment of responses to air pollutants in cotton has been presented by Temple and Grantz (2010). Descriptions and color photographs of $O_3$ injury to many species can be found in Recognition of Air Pollution Injury to Vegetation: A Pictorial Atlas (Flagler, 1998). The following discussion considers the effects of $O_3$ on yield, development, and the underlying carbon dynamics.

## COTTON YIELD AND QUALITY

Ozone was identified as the vegetation damaging component of Los Angles-type smog in the 1950s. Foliar $O_3$ injury on field-grown cotton has been observed widely in experimental settings and production fields in polluted areas such as the San Joaquin and Coachella valleys in California; Phoenix, AZ; Raleigh, NC; and elsewhere (Brewer and Ferry, 1974; Heagle et al., 1986, 1999; Taylor and Mersereau, 1963; Temple et al., 1988a). Unfortunately, visible injury is not well correlated with impact on yield and depressed yield can occur without any foliar indication.

By the 1970s, quantitative measurements had shown that upland cotton, cultivar SJ-1, was sensitive to this newly described air pollutant. Charcoal-filtered (i.e., $O_3$-free) air improved yield by 20% relative to cotton grown in nonfiltered chambers across a gradient of air quality in the southern San Joaquin Valley (Brewer and Ferry, 1974). The resulting proportionality between yield loss and ambient $O_3$ concentration strongly suggested causality. Early concerns that closed or open-top chambers could overestimate yield impacts of $O_3$ have proven unfounded (Temple, 1990a; Temple and Grantz, 2010; USEPA, 2013). In open-top chambers designed to minimize potential effects on plant growth and development caused by the closed chambers used previously (Temple et al., 1985), cotton yields in the San Joaquin Valley similarly were reduced by 15 to 20% relative to charcoal-filtered controls. In the higher $O_3$ of Riverside CA, cultivar SJ-2 yield was reduced by 26.2% (Temple et al., 1988a). Exposed to the lower ambient $O_3$ in Raleigh, NC, cultivars Stoneville 213 and McNair 235 declined by 11 to 15% in boll number and 20% in seed cotton relative to charcoal-filtered chambers (Heagle et al., 1986). A free-air exposure technology with no chamber reduced yield of another $C_3$ crop, soybean, by 15 to 25% in Illinois (Morgan et al., 2006). This is similar to or greater than the approximately 10% average yield loss observed across more than 30 open-top chamber exposure experiments with this species (Ashmore, 2002).

As expected in agronomic trials, yield estimates in these studies were variable with year, cultivar, and location. Across five studies in the National Crop Loss Assessment Network (NCLAN) (Heck et al., 1988), SJ-2 exhibited both the lowest loss in the first year and the greatest loss in the second year

(Temple et al., 1985). Stoneville 213 and McNair 235 were intermediate. An effort to define phytotoxic $O_3$ levels in Europe used $O_3$ responses of U.S. cotton to define a single linear yield response function (Mills et al., 2007). With acceptable precision (e.g., $r^2 = 0.69$ with 17 observations among five diverse cultivars), a single relationship could be fitted between relative yield loss and mean daytime $O_3$ exposure. Using relationships developed by Mills et al. (2007), $O_3$ concentrations between 50 and 60 ppb, common in many cotton growing regions of the US (Lefohn, 1992; Fig. 1), can cause yield losses of 10 to 15% for cotton cultivars similar in $O_3$ susceptibility to those used in the NCLAN studies. A serious limitation of these and other available data is that they were obtained with now obsolete cultivars (Heagle, 1989; Heck et al., 1988). It is clear from the progression of Pima cultivars in the San Joaquin Valley in recent years that selection for yield in this $O_3$-rich environment has selected inadvertently for enhanced tolerance to $O_3$, a process that can be limited by the narrow germplasm base in many breeding programs.

In upland cotton cultivar SJ-2, $O_3$ had little effect on fiber quality or on the ratio of lint to seed (Temple et al., 1985). This was supported by a greenhouse study (Oshima et al., 1979). However, in the $O_3$-sensitive Pima cultivar S-6 (Grantz and McCool, 1992), exposure to $O_3$ in closed-top chambers to ambient $O_3$ levels in Riverside CA reduced both boll yield and fiber quality (micronaire, length, and length uniformity). Minor changes in fiber quality also were observed at high $O_3$ concentrations in both Stoneville 213 and McNair 235 (Heagle et al., 1986). In cultivar Deltapine 51 (Heagle et al., 1999), fiber grade was not affected by $O_3$, but only because increases in fiber length and brightness were offset by decreases in micronaire and uniformity.

## DEVELOPMENT AND MORPHOLOGY

Yield reflects plant carbon balance and the allocation of that carbon. An important impact of $O_3$ on cotton is through the leaf area display upon which carbon acquisition depends (Miller et al., 1988). In general, $O_3$-exposed cotton plants exhibit decreased leaf area due to accelerated senescence and abscission (Temple et al., 1988b), as well as reduced leaf area production during early development (Grantz and Yang, 1996). Leaf abscission averaged over four cultivars was 51 to 75% at 10 to 90 ppb (Temple,

1990b). Specific leaf weight declines along with leaf area and number of leaves (Grantz and Shrestha, 2006). Leaf area duration of McNair 235 was reduced by 13 to 28% at 51 to 73 ppb daylight mean $O_3$, relative to plants grown in charcoal-filtered air (Miller et al., 1988).

Abscission does not fully account for $O_3$-induced losses in yield, as there is a contribution from reduced efficiency of $CO_2$ assimilation in the remaining leaves (Miller et al., 1988). Effects on canopy carbon acquisition depend on the interaction of total leaf number, area per leaf, and photosynthetic activity per unit leaf area. There is an additional effect of $O_3$ on carbon translocation that appears to impact plant morphology independently of light interception and carbon assimilation (Grantz and Farrar, 2000; Grantz and Yang, 1996).

Ozone, in common with shading, herbivory, and other above-ground stressors reduces root growth more than shoot growth (Chapin, 1991). The resulting root-to-shoot ratio of $O_3$-injured plants is often lower than in controls (Cooley and Manning, 1987; Reiling and Davison, 1992). Inhibition of root growth and decreased root-to-shoot ratio has been observed in both upland (Oshima et al., 1979) and Pima (Grantz and Yang, 1996) cottons exposed to chronic $O_3$. In a field study with SJ-2, root biomass was reduced 20% more than stem biomass and the root-to-shoot ratio decreased from 0.13 to 0.09 at 111 ppb. However, in a field study with McNair 235, root growth was reduced but was balanced by leaf abscission, yielding no significant effect of $O_3$ on root-to-shoot ratio (Miller et al., 1988).

Biomass allocation is not well understood in general, and $O_3$ impacts on biomass allocation even less. A variety of experimental manipulations in Pima cotton (Grantz and Yang, 2000) ruled out two hypotheses for $O_3$-induced effects on root-to-shoot allocation. First, source limitation did not explain $O_3$-induced changes in the root-to-shoot ratio. When leaf area reduction due to $O_3$ was matched by leaf area reduction by incremental mechanical removal of leaves under $O_3$-free conditions, the two responses were clearly different. Whole plant biomass and leaf area were reduced to the same extent in both treatments, but root-to-shoot allocation was much more sensitive to leaf area following $O_3$ exposure (Grantz and Yang, 1995, 1996, 2000). Second, plants with the same total biomass were obtained by varying plant age or by varying $O_3$ exposure. Although plant size and leaf area were

the same, effects on root-to-shoot allocation were, again, more pronounced following $O_3$ exposure. Thus, $O_3$-induced slowing of plant development did not explain $O_3$-induced changes in the root-to-shoot ratio. Because root-to-shoot ratio is an imperfect metric that changes with plant development, allocation below ground was re-assessed by meta-analysis, using a development-independent root-to-shoot allometric coefficient (Grantz et al., 2006). In cotton and in a broad range of other herbaceous dicotyledonous plants, the allometric coefficient was reduced 3.1% by $O_3$, whereas whole plant relative growth rate was reduced by 8.2%.

Plant morphology can have direct effects on yield and its response to $O_3$ exposure. Cultivars of elite cotton differ in compensatory responses (Pell et al., 1994), providing avenues to plant improvement based on these traits. In general, loss of photosynthetic capacity can be compensated (Pell et al., 1994) by increased stomatal conductance and photosynthesis in leaves that emerge and expand following $O_3$-induced abscission of older leaves (Beyers et al., 1992; Greitner et al., 1994). This compensatory photosynthesis in newly emerging leaves can restore much of the whole-plant carbon assimilatory capacity (Pell et al., 1994). The area of individual leaves might increase, though they might be thinner than the control, with reduced specific leaf mass (Grantz and Shrestha, 2006; Miller et al., 1988). Yield in cotton is a strong function of the number of sympodial branches (Oosterhuis and Urwiler, 1988). The indeterminate cultivar, Acala SJ-2, increased branching following $O_3$ exposure (Oshima et al., 1979), whereas the determinate cultivars GC510 and SS2086 increased main-stem leaf production and shortened vegetative internodes, but did not increase branching (Temple, 1990b). The absence of additional sympodial branches in determinate cultivars can favor indeterminate cultivars in areas with high ambient $O_3$. However, reduced carbohydrate availability and its retention in leaves can lead to abscission or poor development of these additional bolls, particularly if the growing season is not long enough to mature them. Despite its indeterminate growth habit, yield losses in SJ-2 were generally caused by reduced boll number rather than lower individual boll weight (Temple et al., 1988a). In both Stoneville 213 and McNair 235, losses were attributable both to fewer bolls and reduced boll weight (Heagle et al., 1986).

## CARBON BALANCE

Net carbon assimilation is the difference between carbon acquisition through photosynthesis and carbon expenditures through respiration, for maintenance and repair. The remainder is available for allocation among plant organs for growth. Under abiotic stress, including drought and $O_3$, net carbon assimilation is reduced by decreased gross photosynthesis and often by increased dark respiration in leaves. The role of stomatal conductance as cause or consequence of impaired gas exchange remains unclear and can vary with type and severity of stress.

**Respiration**. Acute $O_3$ exposure, particularly when it induces visible leaf injury, increases leaf respiration in a broad range of species (Amthor, 1988; Barnes et al., 1990; Darrall, 1989; Dizengremel and Petrini, 1994; Dugger and Ting, 1970; Lehnherr et al., 1988; Pell and Brennan, 1973). Cross tolerance and similarity of response have been observed to drought and $O_3$ (Iyer et al., 2013). In field-grown cotton, drought slightly decreased the magnitude of dark respiration but increased the ratio of respiration to assimilation due to reductions in carbon assimilation (Chastain et al., 2014). On some sample dates there was little effect. Similar to drought, chronic exposure to $O_3$ can either increase or decrease leaf respiration (Miller, 1988; Runeckles and Chevone, 1992). The response under specific physiological conditions can reflect the net availability of carbohydrate in leaves, which itself reflects a complex interaction of $O_3$-impaired photosynthesis, increased repair costs, and altered translocation.

Ozone impacts on root and rhizosphere respiration are similar to those observed in leaves. Chronic exposure of cotton to $O_3$ increased respiration of fine roots (Grantz et al., 2003) (Fig. 2). Similar results have been observed in many species (Andersen and Scagel, 1997), although in some cases, reduced root respiration has been reported (Edwards, 1991; Hofstra et al., 1981). Respiration can respond bimodally to abiotic stress (Flexas et al., 2005), decreasing as growth and maintenance respiration are down-regulated by mild stress, then increasing with further severity of stress as repair metabolism increases. These complex patterns were observed in cotton following $O_3$ exposure (Grantz et al., 2003). As with leaves, these variable responses likely reflect the net carbohydrate balance, in this case between reduced phloem transport of carbohydrates to roots, reduced root growth, and increased root repair metabolism.
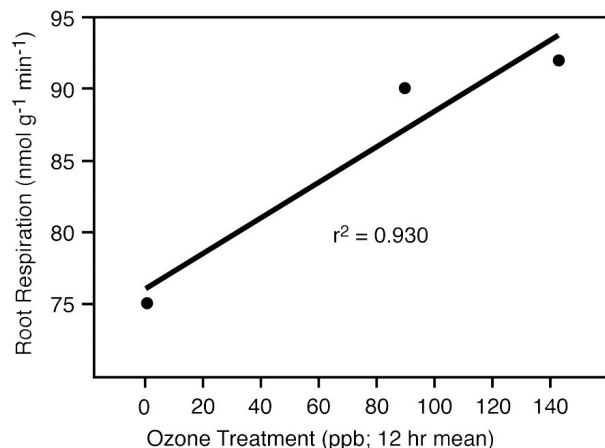
**Figure 2. Response of fine root respiration of cotton to chronic exposure to O$_3$. Adapted with permission from Grantz et al., 2003.**
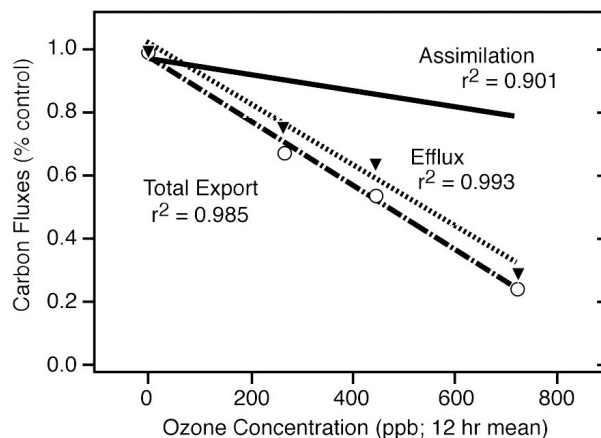


**Figure 3. Effect of 45 min exposure to O$_3$ on net carbon assimilation (solid circles), carbon translocation from source leaves (solid triangles), and the product of the two, total inhibition of carbon export to sink tissues (open circles), measured 80 min following onset of O$_3$ exposure, relative to values prior to exposure (100%) in source leaves of Pima cotton. Adapted with permission from Grantz and Farrar, 2000.**

The rhizosphere is impacted as well. O$_3$ does not penetrate into the soil (Turner et al., 1973), so root and rhizosphere responses are initiated in the shoot. Reduced carbohydrate partitioning below ground alters root density and the production of root exudates. These are required to recruit and support endosymbionts and free-living soil microbes. In legumes, O$_3$ reduced root nodulation by *Rhizobium* (Blum et al., 1983), whereas in tomato, O$_3$ reduced endomycorrhizal infection (McCool et al., 1982). In pine trees, the ratio of fungal to bacterial soil microbial biomass increased with O$_3$, though their combined biomass was highest at intermediate O$_3$ (Scagel and Andersen, 1997). These changes, too, are mediated by reduced pools of available carbohydrate in roots (McCool, 1988). The resulting reduction in rhizosphere activity can inhibit water and nutrient scavenging—additional deleterious impacts of O$_3$ exposure.

**Assimilation**. Carbon assimilation is reduced following exposure to O$_3$ (Dann and Pell, 1989; Darrall, 1989; Runeckles and Chevone, 1992; Wiese and Pell, 1997). Chronic exposure of cotton, SJ-2, to O$_3$ in open-top chambers reduced net photosynthesis of youngest, fully expanded leaves (Temple et al., 1988b). The reduction was proportional to O$_3$ exposure, helping to establish causality, although no visible O$_3$ injury was observed. In Pima S-6, acute exposures to high O$_3$ caused a rapid reduction in net carbon assimilation (Fig. 3, solid circles; Grantz and Farrar, 1999), prior to appearance of any visible injury. This type of pulse exposure and reduced carbon assimilation has proven uniquely informative regarding mechanisms of O$_3$ effects on gas exchange (Farage et al., 1991; Guidi et al., 2010).

Both chronic (moderate concentration, prolonged exposure) and acute (high concentration, periodic exposure) to O$_3$ are observed in field environments and have overlapping but distinct response signatures (Chen et al., 2009; Martin et al., 2000). Acute exposures can lead to programmed cell death and visible leaf injury, whereas chronic exposures are associated with long-term growth inhibition and reduction in photosynthetic capacity (Vainonen and Kangasjarvi, 2015). Ozone impacts on carbon assimilation can reflect a combination of these effects. Acute and chronic exposure to O$_3$ had similar effects on the magnitude of net photosynthesis in soybean (Chen et al., 2009), but different underlying mechanisms of effects on photosystem II reaction centers.

Direct effects of O$_3$ on photosynthesis are initiated by oxidative attack on the plasmalemma by O$_3$ or reaction products of O$_3$ that penetrate or are generated in the cell wall apoplast. Attack on the plasmalemma leads to increased membrane permeability and loss of essential cations. Ozone eventually causes irreversible photosynthetic downregulation, chlorosis (Hassan and Tewfik, 2006), and loss of the pH gradient across the chloroplast membrane (Heath, 1987). Carboxylation efficiency is reduced, along with activity and quantity of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) as turnover is enhanced and mRNA abundance for resynthesis is reduced. The dark reactions are impacted in response to O$_3$ prior to reductions in light harvesting, electron

transport, and ribulose-1,5-bisphosphate (RuBP) regeneration (Farage et al., 1991; Pell et al., 1992). The chlorophyll *a* fluorescence kinetics are impacted and ultrastructure of the thylakoid membranes undergoes visible change (Heath, 1988). Over time, accelerated leaf senescence and foliar abscission are observed (Pell et al., 1992).

**Translocation**. Under normal conditions, translocation of recent photoassimilate does not limit carbon acquisition in cotton or growth of sink tissues (Baker and Baker, 2010). However, this situation is altered by exposure to $O_3$, which impairs phloem loading (Friend and Tomlinson, 1992; Grantz and Farrar, 1999; Gunthardt-Goerg et al., 1993; Okano et al., 1984; Spence et al., 1990). High $O_3$ inhibited carbon assimilation by 19%, but reduced carbohydrate export by 71% in Pima cotton (Fig. 3, triangles). Therefore, the 77% reduction in availability of carbohydrate from recent photosynthate for root or boll development was almost entirely due to inhibited phloem loading and much less due to reduced assimilation (Fig. 3; open circles; Grantz and Farrar, 1999, 2000).

The retention of newly fixed carbon in source leaves of plants exposed to $O_3$ has been demonstrated by carbon isotope studies. In bean (*Phaseolus vulgaris* L.), carbohydrate export was reduced by more than 50% (McLaughlin and McConathy, 1983). Lower leaves retained greater amounts of ^{13}C-labelled carbohydrates, whereas upper leaves increased export to shoot apices (Okano et al., 1984).

Acute exposure of Pima S-6 to high $O_3$ (Grantz and Farrar, 2000) inhibited transport of sugars across the plasma membrane (the fast phase of ^{14}C efflux; Fig. 4A), which increased cytosolic sugar content (Fig. 4B). This could cause end-product inhibition and potentially induce downregulation of photosynthetic enzymes (Dickson et al., 1997; Stitt et al., 1987). Ozone did not appear to impact the slower transport of ^{14}C across the tonoplast of mesophyll cells (Fig. 4C) and caused only a slight decline in the much larger pool of storage sugars in the vacuole (Fig. 4D). Over a range of lower $O_3$ concentrations in the youngest, fully expanded leaf of Pima cotton (Grantz and Yang, 2000), total leaf starch content declined while soluble sugar content increased linearly with $O_3$ exposure. The inhibition of sugar transport across the plasma membrane from mesophyll cells (Fig. 4A, B) can be related causally to whole-plant developmental effects mediated by altered carbohydrate translocation (Cooley and

Manning, 1987; Grantz and Yang, 1996; Grantz et al., 2006). Reduced translocation in cotton is also associated with potassium deficiency and is associated with foliar symptoms that are similar to those induced by $O_3$ exposure.
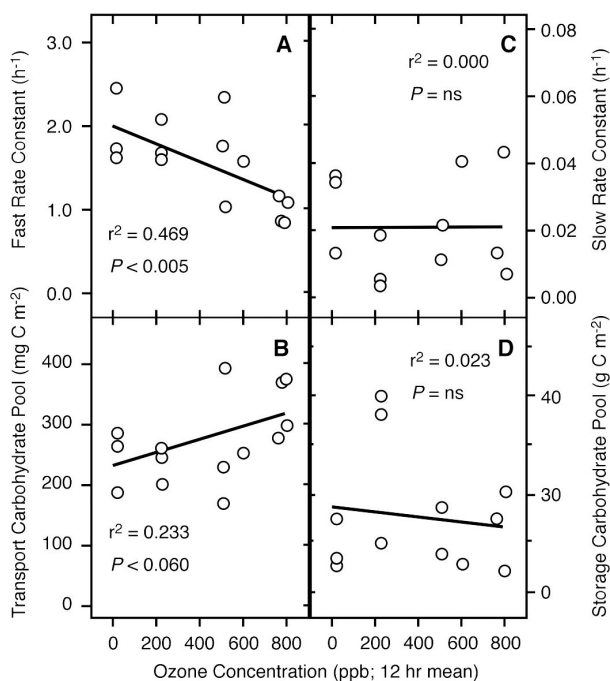


Figure 4. Effect of 45 min exposure to $O_3$ on (A) the rate constant for the fast phase of efflux of photoassimilate; (B) the calculated pool of labile, transport carbohydrate; (C) the rate constant for the slow phase of efflux of photoassimilate; and (D) the calculated pool of slowly mobilized, storage carbohydrate in source leaves of Pima cotton. SLW (specific leaf weight), reflecting all leaf components, was 42 g m-2. Adapted with permission from Grantz and Farrar, 2000.

**Stomatal Conductance.** Stomata play a major role in mediating $O_3$ impacts on cotton. Ozone damage follows entry of $O_3$ to the leaf interior. The stomatal pathway dominates $O_3$ removal from the atmosphere by plants. In upland cotton, non-stomatal uptake was approximately 25% of the total (Massman and Grantz, 1995), whereas in barley non-stomatal uptake ranged from approximately 40 to 60% of total uptake (Gerosa et al., 2004). Stomatal regulation is key to determination of total $O_3$ uptake (Baldocchi et al., 1987; Wesely, 1989), which can be predicted from models of stomatal conductance and ambient $O_3$ concentrations (Emberson et al., 2000; Fuentes et al., 1992; Grantz et al., 1994, 1997; Musselman and Massman, 1999). On a moderately $O_3$-polluted day in the San Joaquin Valley (mean $O_3$ 45 ppb), field cotton removed about 330 g/ha/day of

$O_3$ (Grantz et al., 1994) based on aircraft measurements using the eddy covariance technique, higher than other crops growing nearby. This is attributed to the larger stomatal conductance of well-watered cotton relative to other vegetation, as shown by the close relationship between deposition velocity (total surface conductance to $O_3$ deposition) and measured stomatal conductance scaled to the canopy under wet and dry surface conditions (Fig. 5A, B).

The role of stomata in $O_3$ uptake is demonstrated by the effects of dew on the leaf (Fig. 5C). Uptake is reduced by leaf wetness, which occludes some adaxial stomatal pores. Significantly, this was observed in amphistomatous cotton (Grantz et al., 1997), but not in hypostomatous grape (Grantz et al., 1995), because dew accumulates on upward-facing leaf surfaces.
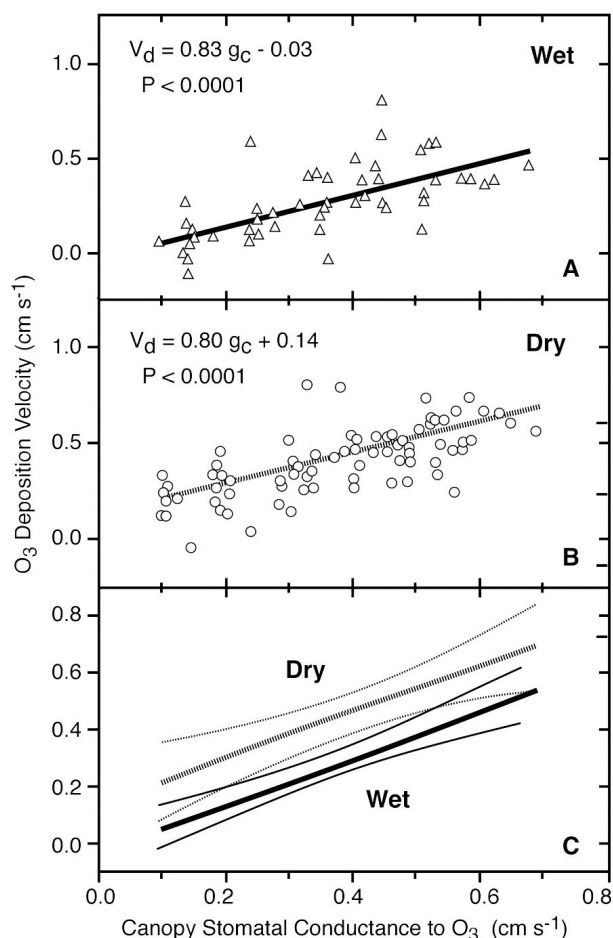


**Figure 5. The close relationship between $O_3$ deposition velocity and canopy stomatal conductance in field-grown cotton, under dew-wetted (A) and dry (B) canopy conditions. (C) The relationships are well constrained, as shown by the 95% confidence intervals (curved lines around each relationship). Leaf surface wetness reduced uptake of $O_3$ in amphistomatous cotton. Adapted with permission from Grantz et al., 1997.**

It has been recognized for some time that dependence of $O_3$ uptake on stomatal conductance could provide an effective mitigation strategy. Limiting $O_3$ entry could limit $O_3$ damage. Differences in prevailing stomatal conductance between cultivars partially explained variance in $O_3$ tolerance (Ting and Dugger, 1968; Turner et al., 1972). Similarly, the impacts of the same ambient $O_3$ concentration are greater in cool, humid growing seasons relative to hot, dry seasons, due to stomatal closing responses to soil water deficit and low humidity. Stomata close in response to $O_3$, which limits further entry of $O_3$, and reduces $O_3$ damage. This has been observed in upland cotton (SJ-2) growing in the field (Temple, 1986; Temple et al., 1988a) and in chamber experiments (Grantz and McCool, 1992) and in Pima cotton (S-6) growing in greenhouse chambers (Grantz and Yang, 1996; Grantz et al., 2015).

A management technique that might be feasible when high $O_3$ is forecast is regulated deficit irrigation (Grantz et al., 2015). Drought closes stomata in cotton, and drought-stressed cotton exhibited smaller relative yield losses due to $O_3$ than well-watered cotton (Brewer and Ferry, 1974; Heagle et al., 1986, 1988; Miller et al., 1988; Temple et al., 1985, 1988a). Foliar abscission was reduced by drought in SJ-2 in California (Temple et al., 1985, 1988a) but not in McNair 235 in North Carolina (Miller et al., 1988). Drought mitigated the $O_3$-induced reduction in reducing sugars, sucrose, and starch throughout the plant in McNair 235 (Miller et al., 1989). Soil drought, in common with other below-ground stressors, favors production of root over shoot (Chapin, 1991) and can counteract $O_3$ inhibition of root proliferation. In severely drought-stressed plants, root-to-shoot ratio decreased from 0.26 to 0.18 in the high-$O_3$ treatment (Temple et al., 1988b), but the change was from 0.13 to 0.09 in well-watered plants. Unfortunately, in many cases, direct inhibition by drought of yield and biomass production can be greater than the protective benefits against $O_3$-damage. Furthermore, the time scale of imposing meaningful soil moisture deficit might not be compatible with the dynamics of $O_3$ episodes.

The mechanism of stomatal closure following $O_3$ exposure remains unclear. This reflects the complexity of stomatal regulation in general. Stomatal conductance and stomatal guard cells have become model systems for studying signal

transduction cascades (Assmann, 2009) and particularly the abscisic acid/calcium system (Kim et al., 2010; Murata et al., 2015), two features that are relevant to $O_3$ impacts on stomatal regulation. Encoded in this elaborate signaling system (Murata et al., 2015) is the mechanism of coordination of stomatal and mesophyll function that produces homeostasis in photosynthetic regulation even in the presence of xenobiotic factors such as $O_3$. It can also reflect the interplay of three contrasting models of stomatal control.

Stomata can respond to altered mesophyll metabolism (Martin et al., 2000). Early observations suggested that $O_3$-induced stomatal closure was a secondary response to inhibition of photosynthesis (Pell et al., 1992), mediated by increased intercellular $CO_2$ concentration (Morison and Jarvis, 1983) or by yet unspecified metabolic linkages between mesophyll and guard cell function (Farage et al., 1991; Martin et al., 2000). In intact leaves exposed to $O_3$, the intercellular concentration of $CO_2$ ($C_i$) initially declined, and stomatal limitation of carbon assimilation increased (Farage et al., 1991; Pell et al., 1992). With prolonged exposure, stomatal limitation declined, restoring the generally observed homeostasis in $C_i$ that reflects the usual balance between assimilation and stomatal conductance, albeit at a reduced rate of carbon assimilation under (e.g.) drought or phosphorous limitation (Singh et al., 2013), and exposure to $O_3$.

A second potential mechanism of stomatal response is that $O_3$ can directly impact stomatal guard cells (Torsethaugen et al., 1999) and thereby affect stomatal conductance directly. This could reduce $C_i$ at least in the short term. A number of types of ion channels are involved in the regulation and mechanics of membrane transport. Stomatal opening and closing are closely associated with $O_3$ effects on these systems, including calcium channels (Evans et al., 2005), inward rectifying $K^+$ channel that mediate guard cell swelling and stomatal opening and slowly deactivating anion channels (Vahisalu et al., 2008). In *Vicia faba* L. (Torsethaugen et al., 1999) exposure to 100 ppb $O_3$ for 4 hr reduced steady-state stomatal conductance and slightly inhibited the rate of stomatal opening without affecting net carbon assimilation at saturating intercellular $CO_2$. Further increase in the $O_3$ concentration inhibited both net carbon assimilation and stomatal conductance.

Direct reduction of stomatal conductance by $O_3$ increases substrate $CO_2$ limitation of rubisco and reduces net carbon assimilation, as observed with drought and other abiotic stresses. Water deficit in cotton reduced gross photosynthesis (Chastain et al., 2014), mediated mostly by stomatal closure that reduced intercellular $CO_2$, limited $CO_2$ fixation by rubisco, and increased photorespiration as the ratio of intercellular $O_2$ to $CO_2$ increased. Long term suppression of substrate $CO_2$ could lead to downregulation of photosynthetic enzymes (Stitt et al., 1987).

A third potential mechanism is hydraulic. Reduced epidermal cell water potential can depress stomatal conductance as demonstrated by analysis of experimental studies (Fig. 6) and by a simulation model (Fig. 6; Grantz et al., 1999). This model simulated $O_3$ impacts on single leaf stomatal conductance mediated by reduced root proliferation caused by impaired carbon translocation. It did not incorporate direct $O_3$ effects on either guard cell or mesophyll function. Impaired root development limits water acquisition (Grantz and Yang, 1996) but also might reduce root capacity for hormonal signaling. Scaling these observations to the canopy level using a comprehensive soil-plant-atmosphere transport model reproduced $O_3$ fluxes observed in the field (Grantz et al., 1999). Although a complete understanding of the mechanism of $O_3$ action on whole plants remains elusive, it is clear that a holistic, integrated view of the plant will ultimately be required.
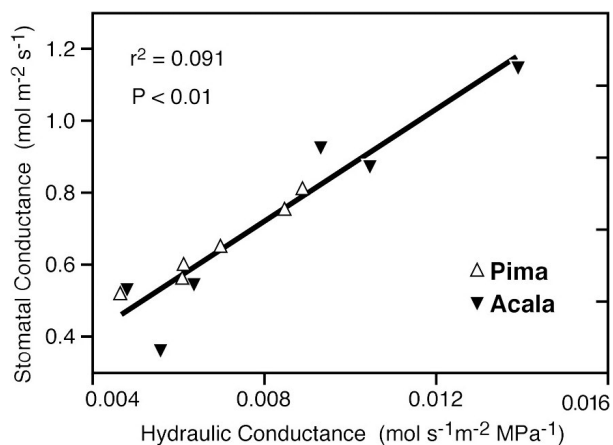


**Figure 6. Relationship between midday stomatal conductance of youngest fully expanded leaves (vapor phase) and whole plant hydraulic conductance (liquid phase) in both upland and Pima cottons obtained in a variety of experimental settings. Adapted with permission from Grantz et al., 1999.**

## CONCLUSIONS

Much of the research on $O_3$ impacts on crops occurred some time ago. It is clear that many conclusions require updating with modern cultivars and modern experimental techniques at both canopy and molecular scales. Knowledge to date indicates that cotton and other crop species are seriously affected by current and potential future concentrations of ambient $O_3$. This is ultimately reflected in yield, but plays out in the various components of the carbon dynamics of a developing cotton plant. Stomatal control of uptake is both impacted by $O_3$ exposure and serves to limit such exposure. $O_3$ that enters the stomata impacts all components of the photosynthetic process, beginning with the enzymes of the dark reactions, but eventually impacts electron transport and pigments as well. The carbon that is assimilated is retained in source leaves due to an inhibition of phloem loading. This reduces carbohydrate available for growth of sink tissues such as vegetative meristematic tissues, and particularly roots and bolls. The reduction in root proliferation might feed back through plant hydraulics on stomatal conductance itself, which might further impact carbon assimilation. Ozone has become a useful probe of plant regulatory networks, including in stomatal guard cells. In future, the complexity of carbon dynamics in cotton will be further elucidated and the knowledge applied to crop improvement.

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