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Influence of carbon dioxide enrichment, ozone and nitrogen fertilization on cotton (*Gossypium hirsutum* L.) leaf and root composition

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ABSTRACT

The objective of this study was to test whether elevated [CO_2], [O_3] and nitrogen (N) fertility altered leaf mass per area (LMPA), non-structural carbohydrate (TNC), N, lignin (LTGA) and proanthocyanidin (PA) concentrations in cotton (*Gossypium hirsutum* L.) leaves and roots. Cotton was grown in 14 dm^3 pots with either sufficient (0·8 g N dm^{-3}) or deficient (0·4 and 0·2 g N dm^{-3}) N fertilization, and treated in open-top chambers with either ambient or elevated (+ 175 and + 350 μmol mol^{-1}) [CO_2] in combination with either charcoal-filtered air (CF) or non-filtered air plus 1·5 times ambient [O_3]. At about 50 d after planting, LMPA, starch and PA concentrations in canopy leaves were as much as 51–72% higher in plants treated with elevated [CO_2] compared with plants treated with ambient [CO_2], whereas leaf N concentration was 29% lower in elevated [CO_2]-treated plants compared with controls. None of the treatments had a major effect on LTGA concentrations on a TNC-free mass basis. LMPA and starch levels were up to 48% lower in plants treated with elevated [O_3] and ambient [CO_2] compared with CF controls, although the elevated [O_3] effect was diminished when plants were treated concurrently with elevated [CO_2]. On a total mass basis, leaf N and PA concentrations were higher in samples treated with elevated [O_3] in ambient [CO_2], but the difference was much reduced by elevated [CO_2]. On a TNC-free basis, however, elevated [O_3] had little effect on tissue N and PA concentrations. Fertilization treatments resulted in higher PA and lower N concentrations in tissues from the deficient N fertility treatments. The experiment showed that suppression by elevated [O_3] of LMPA and starch was largely prevented by elevated [CO_2], and that interpretation of [CO_2] and [O_3] effects should include comparisons on a TNC-free basis. Overall, the experiment indicated that allocation to starch and PA may be related to how environmental factors affect source–sink relationships in plants, although the effects of elevated [O_3] on secondary metabolites differed in this respect.

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INTRODUCTION

It is well established that environmental factors can influence the production of secondary metabolites in plants (Waterman & Mole 1989). For example, in nutrient-deficient conditions, levels of non-nitrogenous metabolites derived from the shikimic acid pathway such as phenolic acids, lignin, hydrolysable tannins and proanthocyanidins (PA) usually increase in woody plants (Waterman & Mole 1989). This increase in C-based secondary metabolites frequently occurs when environmental conditions also promote an accumulation of non-structural carbohydrates (TNC) in plants (Herms & Mattison 1992). Elevated atmospheric [CO_2] often increases TNC concentrations in plants, and possibly stimulates secondary metabolism, although experimental results have not always indicated a relationship as predictable as that seen with nutrient deficiency (Herms & Mattison 1992; Lincoln 1993; Poorter *et al*. 1997). The air pollutant, tropospheric O_3, also affects secondary metabolism. Chronic O_3 exposure can stimulate defence responses involving phenylpropanoid and flavonoid metabolism (Sandermann 1996), although it often suppresses TNC levels (Miller 1988; Miller *et al*. 1989).

The objective of this study was to test whether elevated [CO_2], [O_3] and nitrogen (N) fertilization affected leaf mass per unit area (LMPA), TNC, N, lignin and PA concentrations in cotton (*Gossypium hirsutum* L.) leaves and roots. The levels of these components are important because they indicate how elevated [CO_2], [O_3] and N fertility affect allocation of assimilated resources. Non-structural carbohydrates, lignin and PA constitute major metabolic sinks for assimilated C (Lewis & Yamamoto 1989). In addition, elevated [CO_2], [O_3] and N fertility may also alter tissue chemistry in ways that affect plant resistance to microbial pathogens and pests (Lincoln 1993; Sandermann 1996). Proanthocyanidins, for example, act as antibiotics, antisporulants, feeding deterrents and enzyme denaturants (Bell, El-Zik & Thaxton 1992). In addition, microbial decomposition rates are affected by the levels of sugars,

protein and phenolic compounds, particularly polyphenols and lignin, in plant residues (Haynes 1986). Thus, it would be useful to understand how changing environmental conditions and agronomic practices might affect cotton leaf and root chemistry, which in turn could be used to better manage production.

MATERIALS AND METHODS

Plant growth conditions

The experiment was performed at a site 5 km south of Raleigh, North Carolina, USA (36°N, 79°W). Cotton (cv. Deltapine 51) seeds were planted on 23 May 1995 and 13 May 1996 and grown in pots containing 14 dm^3 of a 2 : 1 : 1 mixture of Appling (clayey, kaolinitic, thermic Typic Hapludult) soil : sand : Metro Mix 220 (Scotts Sierra Horticultural Products Co., Marysville, Ohio, USA) (pH 6·2). Pot temperature fluctuation was moderated by a sleeve of 0·6 cm-thick bubble wrap, coated on both sides with aluminium (Reflectix™, Reflectix Inc., Markleville, Indiana, USA), fit tightly around each pot and secured with aluminium tape. Plants were irrigated with drip tubes at 4 dm^3 per pot as needed to prevent visible signs of water stress. Insects and mites were controlled with applications of acephate (Valent USA Corp., Walnut Creek, California, USA) and bifenthrin (Whitmire Micro-Gen Research Laboratories Inc., St Louis, Missouri, USA). Root fungi were controlled with a single application of metalaxyl (Novartis Crop Protection, Inc., Greensboro, North Carolina, USA) and iprodione (Rhone Poulenc Inc., Research Triangle Park, North Carolina, USA).

Plants were treated in 2·4 m tall × 3 m diameter open-top field chambers (Heagle, Body & Heck 1973). The main plot (chamber) treatments were mixtures of CO_2 and O_3 over a range of concentrations. Carbon dioxide gas was dispensed from a 12·7-t liquid receiver 24 h daily and monitored at canopy height with infra-red CO_2 analysers (model 6252, LiCor Inc., Lincoln, Nebraska, USA). The CO_2 analysers were calibrated bi-weekly with pressurized tank CO_2 over the range of concentrations used in this experiment. Carbon dioxide treatments were ambient air (AA), ambient air plus 175 μmol CO_2 mol^{-1} (+ 175) or ambient air plus 350 μmol CO_2 mol^{-1} (+ 350). Ozone was produced by electrostatic discharge in dry O_2 (model GTC-1A, Ozonia North America, Elmwood Park, New Jersey, USA) and monitored at canopy height using UV photometric O_3 analysers (model 49, Thermo Environmental Instruments Co., Franklin, Massachusetts, USA). The O_3 analysers were calibrated bi-weekly (model 49 PS calibrator, Thermo Environmental Instruments Co.). Ozone treatments were charcoal-filtered air (CF) or non-filtered air with O_3 added to 1·5 times the ambient $[O_3]$ 12 h daily (NF+). Average $[CO_2]$ and $[O_3]$ in each treatment are shown for each year in Table 1. Additional environmental parameters measured during the experiment also are shown in Table 1.

Soil medium was supplemented with 0·8, 0·4 or 0·2 g N dm^{-3} supplied as urea formaldehyde (38% N) for the high (HN), medium (MN) and low (LN) soil nitrogen treatments, respectively. Superphosphate and micronutrients (Scotts MicroMax) were added at 1·0 and 0·7 g dm^{-3} soil, respectively, to the medium. Plants were supplemented bi-weekly with 1 dm^3 per pot of a solution containing 1·4 g dm^{-3} of K_2SO_4. Four pots for each N fertilization treatment were placed as a group in the northern and southern halves of each chamber. It was anticipated that the different soil N fertilization treatments might lead to large differences in growth, and that large plants might shade smaller ones. To minimize shading, the MN treatment group was always placed between the HN and LN groups. The HN and LN treatment groups were randomly assigned to the eastern or western position in the northern chamber half, and to the opposite side in the southern chamber half (Heagle *et al*. 1999).

Table 1. Average daily $[CO_2]$ and $[O_3]$, mean daily and maximum air temperatures (daylight h), and average daily photosynthetic photon flux density (PPFD) from 24 May to 12 July 1995 and from 14 May to 2 July 1996

	1995	1996
$[CO_2]$ (μmol mol^{-1})[a]		
Ambient	367	372
+175	558	542
+350	730	726
$[O_3]$ (nmol mol^{-1})[b]		
Ambient	46	56
CF	21	26
NF+	77	83
Mean air temperature (°C)	33	26
Mean maximum air temperature (°C)	40	32
Mean PPFD (mol m^{-2} d^{-1})	41	49

[a]Daily 12 h (08·00–20·00 EST) average $[CO_2]$ in ambient $[CO_2]$ open-top chamber (Ambient) and in the elevated $[CO_2]$ open-top chambers (+ 175 and + 350 treatments). CO_2 was added to ambient air 24 h daily. [b]Daily 12 h (08·00–20·00 EST) average $[O_3]$ in ambient air, in charcoal-filtered air open-top chambers (CF), and in non-filtered air with added O_3 open-top chambers (NF+). Ozone was added to non-filtered air 12 h daily (08·00–20·00) at 1·5 times the ambient air concentration.

Tissue sampling

At 49 d after planting (DAP) in 1995 and at 50 DAP in 1996, one plant from each N fertilization treatment was removed from each group of four plants in the northern and southern halves of the chambers for measurements of leaf and root tissue chemistry. Five leaf disks (3 cm^2 each) were obtained from a 14-day-old main-stem canopy leaf on each plant from each treatment replicate and pooled (10 disks per sample). Washed fibrous roots from two plants from each treatment replicate were also sampled and pooled. Tissue samples were immediately frozen in liquid N_2 and stored at – 70 °C. Several days before being assayed, tissue samples were freeze-dried and ground to pass a 0·5 mm

mesh screen. Ground tissue samples were stored thereafter in a vacuum desiccator in the dark at 25°C. Samples were analysed with a CHN elemental analyser (model 2400, Perkin-Elmer, Inc., Analytical Services Laboratory, Department of Soil Science, North Carolina State University) to determine C, H and N concentrations.

Non-structural carbohydrate assay

Starch and soluble sugars were determined enzymatically by the UV method (R-Biopharm, Marshall, Michigan, USA). To solubilize starch, tissue samples (25 mg) were each mixed with 2·4 cm^3 of dimethylsulphoxide and 0·6 cm^3 of 8 N HCl in sealed polypropylene tubes for 60 min at 60 °C on a tube rocker. Samples were then neutralized with 0·6 cm^3 of 8 N NaOH and diluted to 15 cm^3 with 112 mmol m^{-3} citrate buffer (pH 4). Solutions were filtered (Whatman no. 1), and duplicate 50 mm^3 aliquots were assayed according to kit instructions. Results were expressed as D-glucose equivalents. Total non-structural carbohydrates (TNC) were determined by summing values derived from the starch and soluble sugar fractions.

Lignin assay

Tissue samples (100 mg) were freed of extractive components by washing with 1 cm^3 of methanol : chloroform : H_2O (2 : 1 : 0·8, by volume) (2 ×), liquefied phenol : acetic acid : H_2O (2 : 1 : 1, by volume) (3 ×) and 95% ethanol (4 ×), with centrifugation (16 000 *g*, 3 min) between washings (Friend 1992). Samples were dried at 70 °C and stored in a desiccator. Lignin concentration in the extractive-free tissue samples was measured by the thioglycolic acid method, as described previously (Booker & Miller 1998). Briefly, each extractive-free tissue sample was mixed with 0·9 cm^3 of 2 N HCl and 0·1 cm^3 of thioglycolic acid in a screw-top microcentrifuge tube. The microcentrifuge tubes were placed in pressure tubes and heated at 100 °C for 4 h. The residues were recovered by centrifugation, washed with 1 cm^3 of H_2O, resuspended in 1 cm^3 of 1 N NaOH and mixed overnight at 25 °C. Afterwards, the supernatants were recovered by centrifugation, the residues washed with H_2O, centrifuged at 14 000 *g*, and the supernatants pooled by sample. Supernatants were mixed with 0·1 cm^3 of concentrated HCl and incubated at 4 °C for 3 h. The resulting precipitates (lignothioglycolic acid, LTGA) were collected by centrifugation, washed with H_2O and centrifuged again at 14 000 *g*. The pellets were then dissolved in 1 cm^3 of 1 N NaOH. A 25 mm^3 aliquot of each sample solution was diluted to 1 cm^3 with 0·1 N NaOH, and absorbance of the diluted solutions was measured at 280 nm.

To relate absorbance of the LTGA solutions to LTGA dry mass, aliquots of leaf and root LTGA solutions were pooled by treatment, precipitated, freeze-dried and used to construct standard curves. An $E_{1\%\ (w/v)\ 280\ nm}$ value was estimated from the slope of a linear regression model fitted to a standard curve for each treatment combination. Average LTGA $E_{1\%\ 280\ nm}$ values were 34 and 36 for leaf and root tissues, respectively. Results were expressed as mg LTGA g dry $mass^{-1}$ on both a total and TNC-free mass basis. TNC-free dry mass was estimated by deducting the dry mass contributed by TNC in each sample from the total dry mass of each sample used in the assay.

Proanthocyanidin assay

For the PA assays, leaf (50 mg) and root (25 mg) tissue samples were extracted with 1 cm^3 of 70% aqueous acetone (3 ×), with mixing for 10 min at 25 °C each time. Following each extraction, the insoluble material was pelleted by centrifugation (16 000 *g*, 3 min), and the supernatants were pooled by sample. Soluble and insoluble fractions were assayed immediately for PA concentration.

The efficacy of the extraction procedure was evaluated in preliminary experiments. Additional extractions of the insoluble fraction with 1 cm^3 of 70% acetone (2 ×), as well as recovery of purified PA added to leaf tissue samples before extraction, indicated that efficiency of the extraction procedure averaged 96%.

Proanthocyanidin concentration was determined by oxidative depolymerization to anthocyanidins in acid butanol (Porter, Hrstich & Chan 1986). Duplicate 0·1 cm^3 aliquots of the soluble fraction were each mixed in a 15 cm^3 polypropylene centrifuge tube with 0·9 cm^3 of methanol followed by 6 cm^3 of acid butanol (5% (v/v) concentrated HCl in *n*-butanol) and 0·2 cm^3 of 2% (w/v) $FeNH_4(SO_4)_2 \cdot 12\ H_2O$ in 2 N HCl. The insoluble material was resuspended in 1 cm^3 of *n*-butanol, centrifuged (16 000 *g*, 3 min), and the supernatant discarded. The insoluble residue was transferred to a 15 cm^3 polypropylene centrifuge tube and mixed with 0·9 cm^3 of methanol and the acid butanol assay reagents. To control for substances in the extracts that might interfere with the PA assay, a 0·1 cm^3 aliquot of each soluble fraction was mixed with 0·9 cm^3 of methanol followed by 6 cm^3 of *n*-butanol and 0·2 cm^3 of H_2O. The solutions and mixtures were incubated in sealed tubes in a water bath at 90 °C for 40 min and then cooled. Preparations containing the insoluble fraction were clarified by centrifugation (3400 *g*, 5 min). Absorbance of the solutions at 550 nm was measured immediately. Values were corrected for interfering substances by subtracting the absorbance at 550 nm of preparations containing the *n*-butanol and H_2O reagents from those containing the acid butanol reagents (Waterman & Mole 1994). A standard curve was constructed using PA purified from leaf tissue (see below), and assay results were expressed as PA equivalents. Total PA levels were determined by summing values derived from the soluble and insoluble fractions. The concentration of PA in the insoluble fraction ranged from 5 to 10% of the total PA concentration. Results were expressed on both a total and TNC-free dry mass basis.

Proanthocyanidins were purified from leaf tissue by a method adapted from Czochanska *et al*. (1980) and Hagerman & Butler (1980). At 70 DAP in 1996, duplicate leaf tissue samples were obtained from plants under the AA-CF-HN treatment. Samples were freeze-dried, ground

to pass a 0·5 mm mesh screen, and 5 g quantities were extracted with 40 cm^3 of absolute (100%) ethanol plus 0·1% (w/v) ascorbic acid in suspension (2 ×). The insoluble material was pelleted by centrifugation (3400 *g*, 5 min), and the supernatant discarded. The insoluble material from each sample was then extracted with 40 cm^3 of 70% acetone containing 0·1% ascorbic acid (3 ×). The insoluble material was pelleted by centrifugation (3400 *g*, 5 min), and the supernatants were pooled by sample. Acetone was removed from the soluble fraction by rotary evaporation at 40°C. The aqueous solution was extracted with an equal volume of diethyl ether (2 ×) and then ethyl acetate (2 ×) to remove pigments, lipids and low molecular weight phenolics, which included PA oligomers (dimers to tetramers). The aqueous fraction was reduced in volume in a rotary evaporator and freeze-dried. Crude product (0·5 g) was dissolved in 2 cm^3 of 50% methanol and applied to a column (5 cm long × 1·5 cm diameter) of Sephadex LH-20 previously equilibrated with degassed 50% methanol. The column was washed with 750 cm^3 of degassed 50% methanol, and the adsorbed PA eluted with 20 cm^3 of 50% acetone. The acetone was removed by rotary evaporation, and the aqueous solution was freeze-dried. Purified PA was stored in a desiccator at – 20 °C.

The UV spectrum of purified PA dissolved in water showed λ_{max} at 206 and 278 nm, and a λ_{sh} at 240 nm, which was similar to the UV spectrum for a PA preparation containing both procyanidin and prodelphinidin units (Czochanska *et al*. 1980). Purified PA dissolved in methanol yielded an average $E_{1\%\ 280\ nm}$ value of 89. The acid butanol assay of purified PA dissolved in 70% acetone yielded an average $E_{1\%\ 550\ nm}$ value of 514. These $E_{1\%}$ values were similar to those reported by Porter *et al*. (1986) for PA purified from cotton flower buds ($E_{1\%\ 280\ nm}$ = 84, $E_{1\%\ 550\ nm}$ = 465). Combustion analysis of purified PA gave results consistent with a polyflavan-3-ol structure, although slight contamination, probably by protein, was indicated (found: C, 53·4; H, 5·3; N, 0·08%; $C_{15}H_{12}O_{6\cdot6}\cdot3\ H_2O$ requires C, 51·2; H, 5·2%) (Czochanska *et al*. 1980; Chan 1985). The ^{13}C-NMR spectrum of purified PA dissolved in D_2O:acetone-d_6 (1 : 1, by volume) was measured at 75 MHz on an NMR spectrometer (model AC-300, Bruker Instruments, Inc., Billerica, Massachusetts, USA) (Fig. 1). The spectrum resembled closely the ^{13}C-NMR spectrum of PA purified from cotton flower buds (Chan 1985). The ^{13}C-NMR spectrum of PA can be fully interpreted on the basis of C4 to C8 (or C6)-linked polymeric pro-cyanidins and prodelphinidins consisting mostly of epigallocatechin-4 and epicatechin-4 units, plus a small number of gallocatechin-4 and catechin-4 units, with the terminal unit being (+)-catechin (Chan 1985). The number-average degree of polymerization, P_n, obtained by NMR was 9·3. The UV spectrum, $E_{1\%}$ values, combustion analysis and ^{13}C-NMR spectrum of the purified PA indicated that it was reasonably free of contaminants and acceptable for use as a standard.

Statistics

Data were analysed by analysis of variance and correlation (SAS Institute 1985). Main plot treatments were assigned to chambers in a completely randomized factorial design. For each year of the experiment, the design required 16 chambers to provide three randomized replicates for all main plot treatment combinations except for treatments containing the mid-level CO_2 treatments, which had two replicates. Soil N treatments were treated as subplots. Runs of the experiment (1995 and 1996) were treated as sub-subplots within the experiment. Thus, the results were analysed as a split–split plot design, with [CO_2] and [O_3] as the whole-plot treatments, N fertilization as the subplot treatment, and runs of the experiment as the sub-subplot treatment. By combining the 1995 and 1996 runs of the experiment in this way, there were six replicates of each treatment combination, except for the mid-level CO_2 treatments, for which there were four replicates. Data were tested for homogeneity of variance and normality prior to analysis.

RESULTS

Leaf mass per area

Average LMPA was 36 and 51% higher in leaf samples obtained from plants under the + 175-CF and + 350-CF

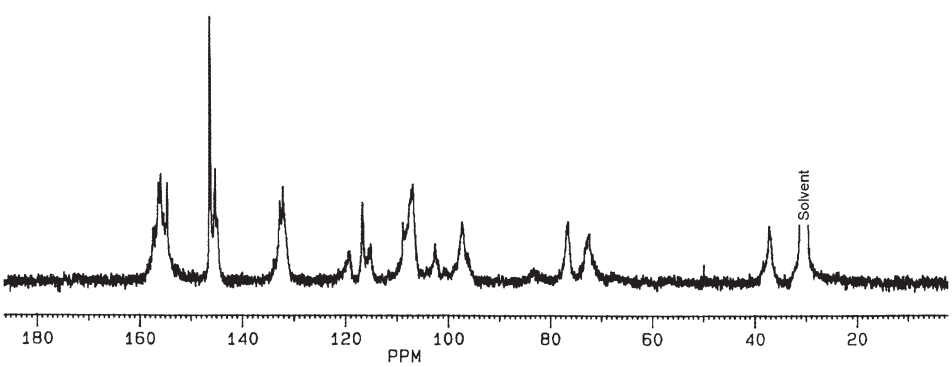

Figure 1. ^{13}C NMR spectrum at 75 MHz of proanthocyanidin (PA) polymer from cotton leaves. The PA polymer was purified from canopy leaves on plants treated with ambient [CO_2] in charcoal-filtered air and optimum N fertilization 70 days after planting.

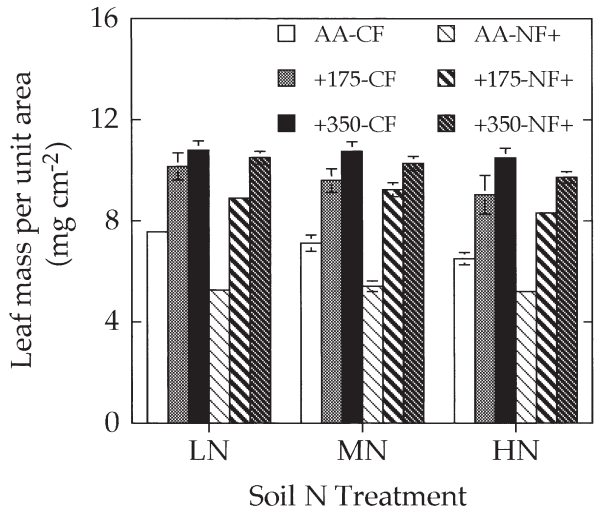

Figure 2. Average (± SE) leaf mass per unit area in 14-day-old canopy leaves at about 50 days after planting. Cotton plants were treated with either ambient [CO_2] (AA), ambient plus 175 (+ 175) or ambient plus 350 (+ 350) μmol CO_2 mol^{-1} in combination with either charcoal-filtered air (CF) or non-filtered air plus O_3 (NF +) with either low (LN), medium (MN) or high (HN) nitrogen fertilization. Values are the means of six replicates, except for the + 175 treatments, which are the means of four replicates.

treatments, respectively, compared with leaf samples obtained from plants under the AA-CF treatments (Fig. 2, Table 2). The NF+ treatments generally suppressed LMPA, although a CO_2–O_3 interaction was indicated. For example, when averaged among N fertilization treatments, LMPA was 25% lower in leaf samples from plants under the AA-NF+ treatments compared with samples from the AA-CF treatments. However, LMPA was only 5% lower in leaf samples from the + 350-NF+ treatments compared with samples from the + 350-CF treatments. Leaf mass per area was 6–8% higher for the MN and LN treatments compared with the HN treatments. LMPA was highly correlated with leaf starch concentration ($r = 0{\cdot}8$, $P < 0{\cdot}001$).

Leaf composition

Average starch concentration was 39 and 59% higher in leaf samples from plants grown under the + 175-CF and +350-CF treatments, respectively, than in leaf samples from the AA-CF treatments (Fig. 3, Table 2). For starch concentrations in leaf samples from the O_3 treatments, however, a CO_2–O_3 interaction was indicated. When averaged among N fertilization treatments, starch concentration was 48% lower in leaf samples from the AA-NF+ treatments compared with samples from the AA-CF treatments, whereas it was only 7% lower in samples from the + 350-NF+ treatments compared with samples from the + 350-CF treatments. Leaf starch concentration was about 10% higher in samples from plants under the LN and MN treatments compared with samples from plants under the HN treatments. However, starch concentrations were 40% higher in leaf samples from the AA-CF-LN and AA-CF-MN treatments compared with samples from the AA-CF-HN treatments. Differences in leaf starch concentration between the + 175 and + 350 treatments were less than 12% when compared among the N fertilization treatments.

There was no statistically significant effect of either the CO_2 or O_3 treatments on leaf soluble sugar concentration (Fig. 3, Table 2). Average soluble sugar concentration was 7

Table 2. Probability of treatment differences in leaf and root components in cotton plants treated with either ambient [CO_2], ambient plus 175 or ambient plus 350 μmol CO_2 mol^{-1} in combination with either charcoal-filtered air or non-filtered air plus O_3 and either sufficient or deficient N fertilization.

Parameter	LMPA	Starch	Sugars	N	$N_{TNC\text{-}free}$	LTGA	$LTGA_{TNC\text{-}free}$	PA	$PA_{TNC\text{-}free}$
Leaf									
CO_2	0·001[a]	0·001[a]	0·1	0·001[a]	0·001[a]	0·001[b]	0·2[b]	0·004[b]	0·001[b]
O_3	0·001	0·001	0·4	0·001	0·003	0·7	0·14	0·4	0·1
N	0·001[d]	0·001[d]	0·03	0·001[c]	0·001[c]	0·06	0·03[d]	0·001[c]	0·001[c]
$CO_2 \times O_3$	0·04	0·001	0·4	0·001	0·08	0·4	0·8	0·04	0·3
$CO_2 \times N$	0·7	0·02	0·8	0·001	0·03	0·8	0·4	0·9	0·9
$O_3 \times N$	0·13	0·4	0·9	0·3	0·1	0·13	0·2	0·9	0·6
Root									
CO_2		0·02	0·3	0·11	0·14	0·4	0·3	0·005[a]	0·006[a]
O_3		0·4	0·7	0·7	0·7	0·9	0·9	0·7	0·6
N		0·5[d]	0·01[d]	0·001[c]	0·001[c]	0·6	0·6	0·007[d]	0·01[d]
$CO_2 \times O_3$		0·3	0·5	0·3	0·3	0·8	0·8	0·5	0·6
$CO_2 \times N$		0·4	0·3	0·13	0·3	0·9	0·8	0·5	0·6
$O_3 \times N$		0·4	0·4	0·8	0·7	0·9	0·9	0·7	0·8

Analysis of variance P values for leaf mass per unit area (LMPA), starch, soluble sugars, nitrogen concentration (N), lignothioglycolic acid (LTGA), and proanthocyanidins (PA) are shown. Analysis of variance results for plant components expressed on TNC-free mass basis are also shown ($N_{TNC\text{-}free}$, $LTGA_{TNC\text{-}free}$, and $PA_{TNC\text{-}free}$) [a]+ 175 versus + 350 treatments, $P \le 0{\cdot}05$; [b]+ 175 versus + 350 treatments, $P > 0{\cdot}05$; [c]MN versus LN treatments, $P \le 0{\cdot}05$; [d]MN versus LN treatments, $P > 0{\cdot}05$.

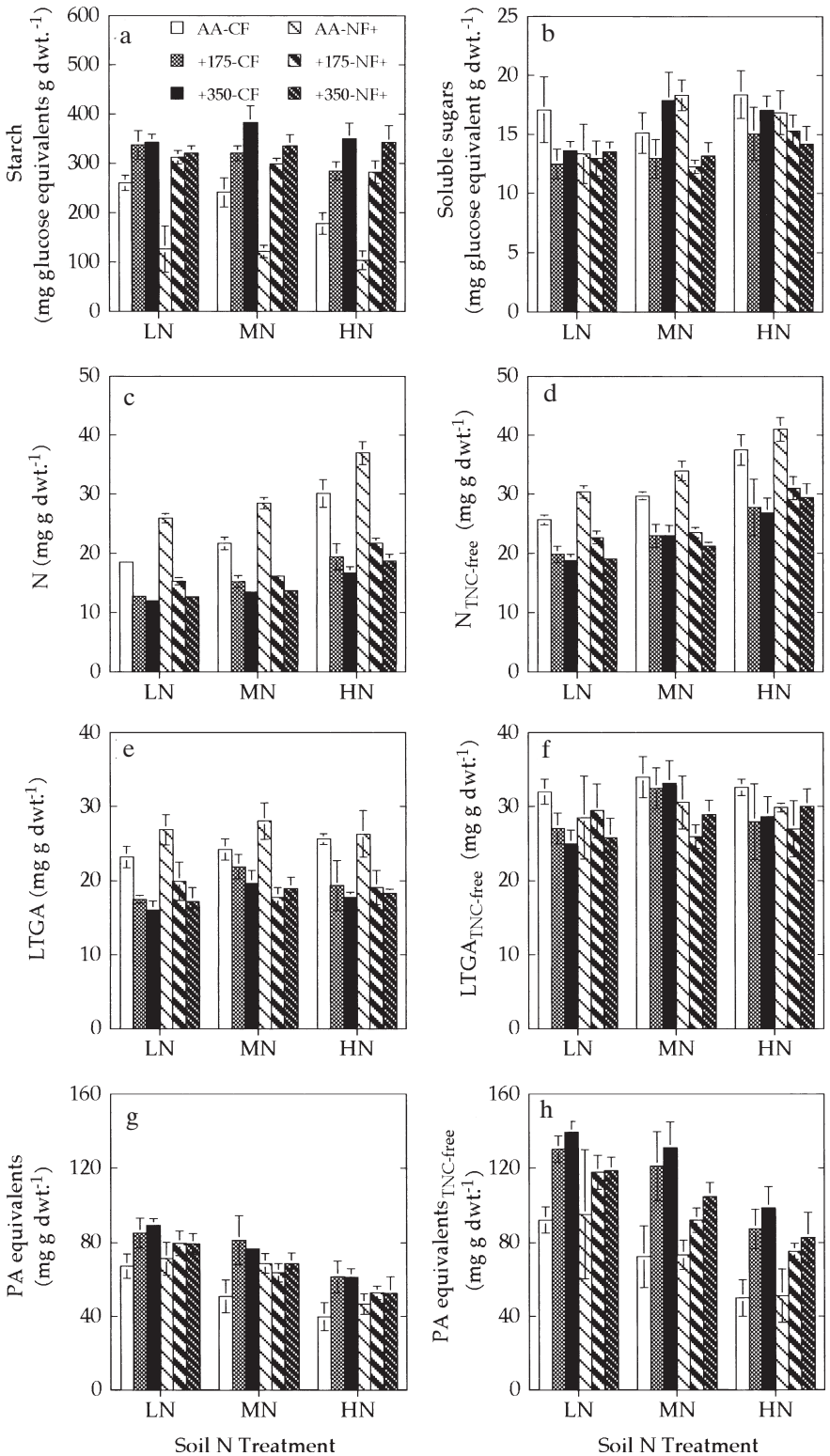

Figure 3. Average (± SE) starch (a), soluble sugar (b), N (c,d), lignothioglycolic acid (LTGA) (e,f) and proanthocyanidin (PA) (g,h) concentrations in 14-day-old canopy leaves at about 50 days after planting. Cotton plants were treated with either ambient [CO_2] (AA), ambient plus 175 (+ 175) or ambient plus 350 (+ 350) μmol CO_2 mol^{-1} in combination with either charcoal-filtered air (CF) or non-filtered air plus O_3 (NF+) with either low (LN), medium (MN) or high (HN) nitrogen fertilization. Nitrogen, LTGA and PA concentrations are expressed on a total dry mass basis (c, e, g, respectively) and on a TNC-free dry mass basis ($_{TNC\text{-}free}$) (d, f, h, respectively). Values are the means of six replicates, except for the + 175 treatments, which are the means of four replicates.

and 13% lower in leaf samples from the MN and LN treatments, respectively, compared with samples from the HN treatments.

Average leaf N concentration expressed on a total dry mass basis was 33 and 40% lower in samples from plants in the + 175-CF and + 350-CF treatments, respectively, compared with samples from plants in the AA-CF treatments (Fig. 3, Table 2). On a TNC-free dry mass basis, average leaf N concentration was 24 and 29% lower in samples from the + 175-CF and + 350-CF treatments, respectively, compared with samples from the AA-CF treatments. For N concentrations in leaf samples from the O_3 treatments, however, a

CO_2–O_3 interaction was indicated. When averaged among N fertilization treatments, N concentration was 30% higher in leaf samples from the AA-NF+ treatments compared with samples from the AA-CF treatments, whereas it was only 7–12% higher in samples from the +CO_2-NF+ treatments compared with samples from the +CO_2-CF treatments. On a TNC-free mass basis, however, leaf N concentration was only 3% higher in plants from the NF+ treatments. Leaf N concentration on either a total or TNC-free dry mass basis was about 32 and 22% lower in samples from plants under the LN and MN treatments, respectively, compared with samples from plants under the HN treatments. Although the CO_2–N interaction was statistically significant, the relative effect of the elevated [CO_2] treatments on leaf N concentration among the N fertilization treatments was less than 3%.

On a total mass basis, the concentration of LTGA was 19–26% lower in leaf samples obtained from plants under the + 175-CF and + 350-CF treatments compared with leaf samples from plants under the AA-CF treatments (Fig. 3, Table 2). However, on TNC-free mass basis, LTGA concentrations in leaf samples from plants in the CO_2 treatments were not significantly different. There was no statistically significant effect of the O_3 treatments on leaf LTGA concentrations on either a total or TNC-free mass basis. On a total mass basis, leaf LTGA concentrations were not significantly different among N fertilization treatments. On a TNC-free mass basis, LTGA concentrations in leaf samples differed by 10% or less among the N fertilization treatments.

Leaf PA concentration, on both a total and TNC-free mass basis, increased in the elevated [CO_2] and sub-optimum soil N treatments (Fig. 3, Table 2). In leaf samples from the + 175-CF and +350-CF treatments, average PA concentration was 44% higher on a total mass basis compared with leaf samples from the AA-CF treatments. On a TNC-free mass basis, PA concentration was 58 and 72% higher in leaf samples from plants in the + 175-CF and + 350-CF treatments, respectively, compared with leaf samples from plants in the AA-CF treatments. The O_3 treatments had no statistically significant effect on leaf PA concentration on a total mass basis, although a CO_2–O_3 interaction was indicated. On a total mass basis, leaf PA concentration was 18% higher in samples from the AA-NF+ treatments compared with samples from the AA-CF treatments, but it was 12% lower in samples from the + 350-NF+ treatments compared with samples from the + 350-CF treatments. However, on a TNC-free mass basis, the O_3 treatments had no statistically significant effect on leaf PA concentration and no interactions were indicated. On both a total and TNC-free mass basis, leaf PA concentration was about 32 and 53% higher in samples from the MN and LN treatments, respectively, compared with samples from the HN treatments.

Root composition

Average root tissue starch concentrations were extremely low, and differences among treatments were small (Fig. 4, Table 2). Root soluble sugar, N, LTGA and PA concentrations were not significantly different among either the CO_2 or O_3 treatments, except that PA concentration was about 12% higher in samples from the +350 treatments compared with samples from the AA treatments. Root soluble sugar and N concentrations were 20–30% lower in the MN and LN treatments compared with tissue samples from the HN treatment, whereas PA concentrations were 7–13% higher in samples from the MN and LN treatments compared with samples from the HN treatment. Root tissue LTGA concentration was not significantly affected by the N fertility treatments.

Nitrogen versus pro-anthocyanidin concentrations

Linear correlations between N and PA concentrations, on a TNC-free mass basis, were highly significant ($P < 0{\cdot}001$) for both leaf and root tissue samples (Fig. 5). Correlation coefficients were – 0·67 and – 0·64 for leaf and root samples, respectively.

DISCUSSION

Higher LMPA values in the elevated [CO_2] treatments were due in large part to higher starch levels, which increased from 23% of leaf dry mass in the AA-CF treatments to 31 and 36% of leaf dry mass in the + 175-CF and + 350-CF treatments, respectively. Large increases in leaf starch concentration in response to elevated [CO_2] have been reported previously for cotton (Wong 1990; Hendrix 1992) and a number of other herbaceous and woody species (Bazzaz 1990; Lindroth 1996; Fischer *et al*. 1997; Kinney *et al*. 1997; Poorter *et al*. 1997; Sims, Luo & Seemann 1998; Carter, Theodorou & Morris 1999). In addition, LMPA and leaf starch concentrations were higher in samples from the sub-optimum N fertilization treatments, which has also been reported previously (Herms & Mattison 1992; Fischer *et al*. 1997). Increased starch levels would be expected if photosynthate production exceeded the capacity to transport and utilize photosynthate for growth and maintenance (Stitt 1991; Fischer *et al*. 1997). However, the effect of elevated [CO_2] on carbohydrate allocation might be related in part to suppressed photorespiration, which reduced P_i availability and thus promoted starch biosynthesis (Sims *et al*. 1998). In addition, C and N sink limitations might increase photosynthate utilization for the production of C-based secondary metabolites such as lignin and soluble phenolics (Herms & Mattison 1992).

In this study, however, leaf LTGA concentrations on a total dry mass basis were lower in plants treated with elevated [CO_2]. Chu, Field & Mooney (1996) found either no significant difference or small decreases in shoot and root lignin concentrations in two grasses (*Avena fatua* L. and *Plantago erecta* E. Morris) following treatment with elevated [CO_2], depending on nutrient status and species. Lignin concentration was lower in leaves from elevated [CO_2]-treated *Eucalyptus tereticornis* (Smith) seedlings

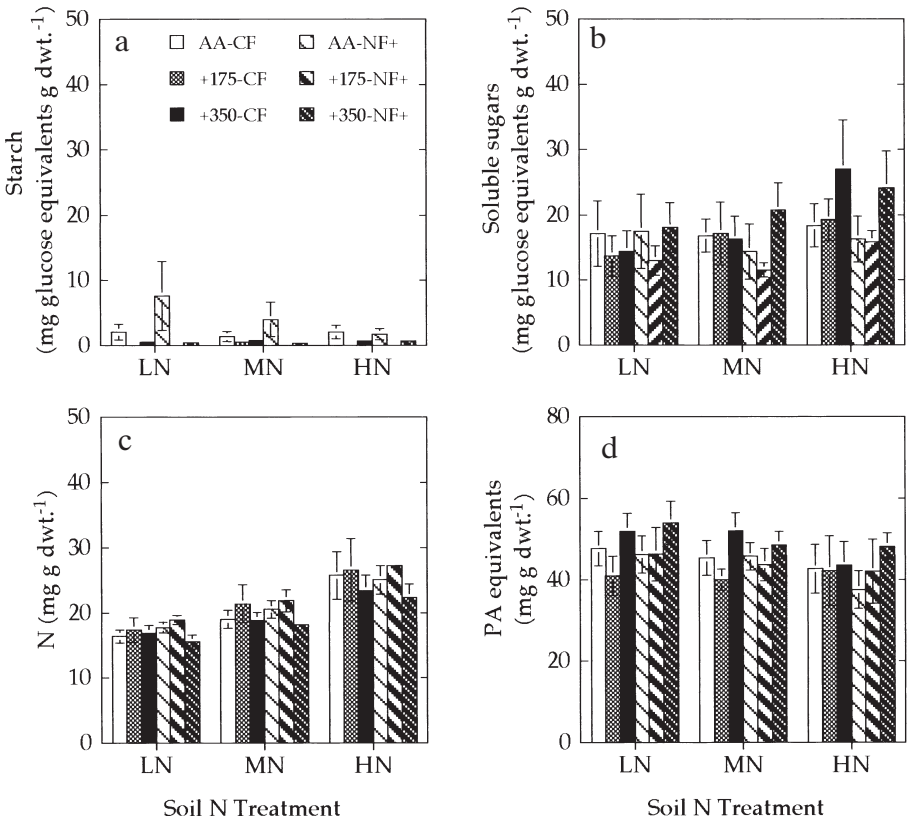

Figure 4. Average (± SE) starch (a), soluble sugar (b), N (c), and proanthocyanidin (PA) (d) concentrations in root tissue at about 50 days after planting. Cotton plants were treated with either ambient [CO_2] (AA), ambient plus 175 (+ 175) or ambient plus 350 (+ 350) μmol CO_2 mol^{-1} in combination with either charcoal-filtered air (CF) or non-filtered air plus O_3 (NF+) with either low (LN), medium (MN) or high (HN) nitrogen fertilization. Values are the means of six replicates, except for the + 175 treatments, which are the means of four replicates.

(Lawler *et al*. 1997). In contrast, no statistically significant difference in leaf lignin concentration was found in cotton or 26 other herbaceous and woody species (Poorter *et al*. 1997) or in three prairie grasses (Kemp *et al*. 1994) following treatment with elevated [CO_2]. In addition, leaf and stem lignin concentrations in sudan grass (*Sorghum bicolor* L. Moench) and wheat (*Triticum aestivum* L.) were generally unaffected by treatment with elevated [CO_2] in free-air [CO_2] enrichment studies (Akin *et al*. 1994, 1995).

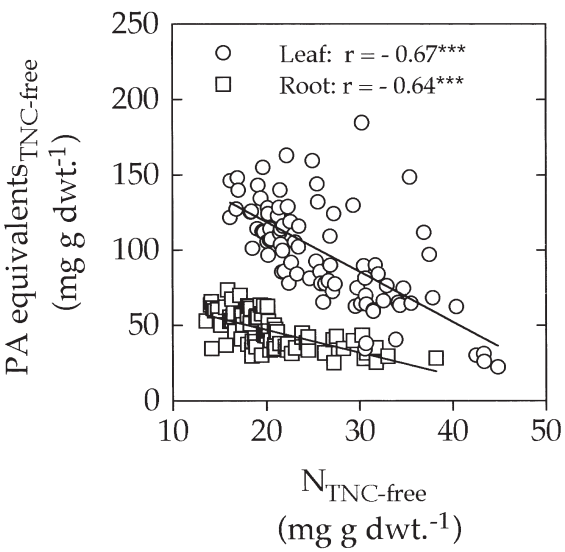

Figure 5. Nitrogen versus PA concentrations on a TNC-free basis for leaf and root tissues following growth for about 50 days in combinations of CO_2, O_3 and N fertilization treatments.

However, the concentration of lignin, N and PA should also be expressed on a TNC-free dry mass basis because it corrects for the contribution of TNC to total dry mass. The correction is needed because the effect of elevated [CO_2] on total dry mass is exaggerated to some extent by the accumulation of a large quantity of TNC that has not been incorporated into structural tissues (Wong 1990). In this experiment, when leaf LTGA concentration was calculated on a TNC-free mass basis, there was no significant difference in leaf LTGA concentration among the CO_2 treatments. Root LTGA concentrations were not significantly different among the CO_2, O_3 or N fertility treatments regardless of the denominator used.

By contrast, leaf PA concentrations were consistently higher in plants treated with elevated [CO_2] and suboptimum N fertilization, particularly when expressed on a TNC-free mass basis. Average PA concentration increased from 7% of leaf TNC-free dry mass in the AA-HN treatments to 12% of TNC-free dry mass in the + 350-LN treatments. Previous studies with herbaceous and woody species also found that leaf PA concentrations increased with [CO_2] enrichment or low N fertility (Waring *et al*. 1985; Bryant *et al*. 1987; Lavola & Julkunen-Tiitto 1994; Lindroth 1996; Kinney *et al*. 1997; Lawler *et al*. 1997; Carter *et al*. 1999). In addition, Poorter *et al*. (1997) found that concen-

trations of soluble phenolics, when expressed on TNC-free basis, were higher in plants treated with elevated [CO_2].

The effects of elevated [CO_2] and N fertility on source–sink relationships and regulation of C partitioning are not well understood, and are influenced by the species, developmental stage and environmental conditions (Stitt 1991; Herms & Mattison 1992). Sink limitations could occur if growth-related metabolic activity other than the photosynthesis pathway was not similarly enhanced in plants grown in elevated [CO_2], possibly because C availability was not the factor limiting high-[CO_2]-grown plants (Wong 1990; Poorter *et al*. 1997). Allocation of photosynthate to C-based secondary metabolites might be a mechanism for shunting fixed C and recycling N when demands for growth are constrained (Herms & Mattison 1992; Lambers 1993).

With regard to O_3, however, the effect on photosynthesis is opposite that of elevated [CO_2]. Chronic O_3 exposure typically suppresses net photosynthesis and hence C availability (Miller 1988; Pell, Schlagnhaufer & Arteca 1997). In this experiment, lower LMPA and leaf starch concentrations, as well as lower net photosynthesis rates and biomass (Heagle *et al*. 1999), in elevated [O_3]-treated cotton plants supported this conclusion. Therefore, it might be expected that C allocation to lignin and PA would be reduced in O_3-treated plants. However, LTGA and PA concentrations expressed on a TNC-free mass basis were not affected by added O_3.

In a previous study, lignin concentration was higher in sugar maple (*Acer saccharum* Marsh.) leaves following treatment with elevated [O_3], but it was not significantly affected in leaves from similarly treated black cherry (*Prunus serotina* Ehrh.) and yellow poplar (*Lireodendron tulipifera* L.) seedlings (Boerner & Rebbeck 1995). Other studies also found that O_3 did not affect lignin content even though enzymes and mRNA transcripts of genes in the lignin pathway were stimulated (Tingey, Wilhour & Standley 1976; Booker, Anttonen & Heagle 1996; Sandermann 1996; Booker & Miller 1998). Higher concentrations of phenolic acids, stilbenes, flavonoids and related compounds have been found in leaf and needle tissues in response to O_3 (Booker *et al*. 1996; Sandermann 1996; Booker & Miller 1998). However, in plants such as cotton that produce large amounts of starch in their leaves, higher levels of soluble phenolics in O_3-treated plants might be related to lower levels of TNC, as seen in this study. For example, PA concentrations were higher in leaf tissues from the AA-NF+ treatments compared with leaf tissues from the AA-CF treatments on a total mass basis, whereas calculation of PA concentrations on a TNC-free mass basis showed that PA concentrations were not significantly different among samples from the AA-NF+ and AA-CF treatments. Consideration of [O_3] effects on TNC concentrations is thus warranted when evaluating [O_3] effects on other metabolites.

The suppression by O_3 of LMPA and leaf starch concentration was prevented by [CO_2] enrichment. This response is likely to be related to increased C availability and to CO_2-induced reduction in stomatal conductance and hence reduced O_3 flux (Allen 1990). Net photosynthesis was higher and stomatal conductance was lower in cotton plants treated with elevated [CO_2] in a related experiment (Heagle *et al*. 1999), suggesting that C available for detoxification and repair processes was increased and that O_3 flux was decreased. Although the relative importance of these, and possibly other components, in preventing O_3 injury in the presence of elevated [CO_2] has yet to be fully demonstrated, stomatal exclusion clearly has a role in the protective effect of elevated [CO_2] against O_3 damage (Fiscus *et al*. 1997; McKee, Eiblmeier & Polle 1997).

In conclusion, this experiment indicated that environmental factors that resulted in C and N sink limitations stimulated C allocation to starch and PA. The carbon–nutrient balance hypothesis of plant growth proposes that the relative availability of resources in the environment influences the biosynthesis of secondary metabolites by mediating plants' carbohydrate stores (Bryant, Chapin & Klein 1983). Increased carbohydrate availability due to elevated [CO_2] or N deficiency might thus be used for C-based secondary metabolites such as PA. Decreased C availability, due to an inhibition of photosynthesis by O_3, should suppress PA biosynthesis. However, C-source limitation caused by O_3 stress had no significant effect on allocation to either lignin or PA, although C allocation to starch decreased. Overall, the experiment indicated that allocation to starch and PA may be related to how environmental factors affect source–sink relationships in plants, although the effect of elevated [O_3] on secondary metabolites differed in this respect.

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