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RESEARCH PAPER

Physiological and antioxidant responses of cotton and spurred anoda under interference and mild drought

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Abstract

The influence of plant interference and a mild drought on gas exchange and oxidative stress was investigated using potted plants of two cotton species (*Gossypium hirsutum* L. cv. Delta Pine 5415, and *Gossypium barbadense* L. cv. Pima S-7) and spurred anoda (*Anoda cristata* L. Schlecht.) of the Malvaceae. Without interference, cotton and spurred anoda had similar net photosynthesis (P_{net}) but different pigment profiles. Stomatal conductance (g_s) and transpiration rate (E) were greater in spurred anoda than cotton. Net photosynthesis and biomass in cotton were reduced more by spurred anoda interference than by intraspecific interference. With interference, the xanthophyll cycle conversion state and α-tocopherol levels increased in cotton, but remained unchanged in spurred anoda. Catalase, ascorbate peroxidase (APX) and glutathione reductase (GR) activities were not influenced by plant interference. Without interference, spurred anoda had lower APX, and similar catalase and GR activities compared with cotton. Mild drought increased APX activity more than 40% in cotton, and 26% in spurred anoda. Upon drought recovery, drought-induced APX activity was still higher in cotton, and GR activity was higher in previously drought-stressed cotton and spurred anoda plants compared with well-watered plants. The greater impact of spurred anoda interference than intraspecific interference on cotton biomass is due mainly to reduced carbon gain in cotton.

Key words: Environmental stress, gas exchange, oxidative stress, plant competition.

Introduction

Interference and drought are two major causes of limited productivity in plants. Investigations with annual cotton weeds have revealed that weed interference during the first 10 weeks after planting is most detrimental (Arle and Hamilton, 1973). Since weeds reduce the amount of water available to the crop, drought stress will probably alter crop performance under crop-weed interference differently from that under weed-free conditions. Among weeds commonly found in cotton, spurred anoda is also a member of the Malvaceae family with a growth pattern similar to cotton. These characteristics make spurred anoda more competitive and difficult to manage in cotton than many other weeds (Arle and Hamilton, 1973; Patterson, 1988).

The major cause of reduced plant productivity under abiotic or biotic stress is oxidative stress at the cellular level (Allen, 1995). Among other environmental stresses (for reviews see Inzé and Montagu, 1995; Noctor and Foyer, 1998; Arora *et al*., 2002), limited availability of soil moisture and nutrients which both occur under weed interference are well known to induce oxidative stress (Cakmak and Marschner, 1988; Yu *et al*., 1998; Bartoli *et al*., 1999). Under stress conditions that limit CO_2 fixation, the rate of reducing power production is greater than the rate of its use by the Calvin cycle. This accumulation of reductants in the photosynthetic electron

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transport system allows for excessive generation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH). These ROS react with lipids, proteins and nucleic acids resulting in lipid peroxidation, protein denaturation and DNA mutation (Bowler *et al*., 1992).

To minimize this oxidative damage, plants have evolved defence systems involving antioxidants and protective enzymes that either prevent the formation of or scavenge ROS. Antioxidants such as β-carotene and α-tocopherol minimize the formation of $^1O_2$ and lipid peroxidation, respectively. A major component of the enzymatic defence is the ascorbate–glutathione cycle that involves four enzymes including ascorbate peroxidase and glutathione reductase (Asada, 1999). The ascorbate–glutathione cycle and catalase scavenge H_2O_2, a product of dismutation of $O_2^{\cdot-}$ by superoxide dismutase thereby preventing the formation of highly reactive ·OH. Another important chloroplastic antioxidant defence is the xanthophyll cycle. Accumulation of H^+ in the chloroplast lumen activates violaxanthin de-epoxidase, and zeaxanthin is produced from violaxanthin via antheraxanthin (Yamamoto, 1979; Demmig-Adams, 1990). Binding of both H^+ and zeaxanthin to Photosystem II proteins is thought to switch the light-harvesting antenna into a conformation that allows for efficient non-photochemical quenching of excitation energy by thermal dissipation (Gilmore *et al*., 1998). Although these antioxidant and energy dissipation systems regulate the formation and destruction of ROS under normal physiological conditions, the generation of ROS can be in excess of their destruction, under stress conditions.

As far as is known, no study has investigated how interference among plants alters oxidative stress or has evaluated different gas exchange characteristics and antioxidant systems simultaneously. Although plant interference is a complex process involving both removal and addition of substances to the common environment, it influences the productivity of all agroecosystems. Given the impact of interference and any oxidative stress tolerance mechanisms on crop productivity, objectives of this study were: (1) to compare the basal levels of gas exchange and antioxidant systems of two cotton species and spurred anoda at different growth stages; (2) to understand whether oxidative stress tolerance mediates the effects of interference; and (3) to determine how plant interference alters the effect of a moderate drought. These findings may help strengthen the understanding of physiological mechanisms of interference. They may also provide information for developing models useful in crop management decisions in cotton such as weed thresholds, crop population densities, and the frequency of irrigation during the stage of crop growth that is most susceptible to weed interference.

Materials and methods

Plant material and interference treatments

Spurred anoda seeds were collected from an experimental plot at Leyendecker Research Farm of New Mexico State University. They were scarified by immersing in concentrated sulphuric acid (9.3 N) for 20 min, and rinsed with water for 30 min before germination. Cotton (Delta Pine 5415 and Pima S-7, hereafter referred to as Delta Pine and Pima, respectively) and spurred anoda seeds were germinated on moist paper towels at room temperature. Resulting seedlings were transplanted into 120 ml (4 cm diameter) pots containing Terra-Lite Metro Mix 360 (WR Grace & Co., Memphis, TN, USA). Plants with the emerging first true leaf were transplanted into 4.0 l (30 cm diameter) pots containing Metro Mix 360 and grown in the greenhouse under natural daylength in the autumn. An individual Delta Pine or Pima plant was grown alone, or three plants of each species were planted per pot for intraspecific interference studies. For interspecific interference, a single plant of each cotton species was grown with two plants of spurred anoda. When three seedlings per pot were planted, they were spaced *c*. 12 cm from one another at an approximate density of 428 000 plants ha^{-1}. A single plant of spurred anoda was maintained per replication, as well. These nine interference treatments were arranged in a randomized complete block design with six replications. Temperatures were maintained at approximately 30 °C and 18 °C during the day and night, respectively, and relative humidity was between 20% and 40%. Plants were watered daily to field capacity, and fertilized weekly with Technigro 20-18-18 (% N, P, K) fertilizer (Fisons Horticulture Inc., Warwick, NY). Measurements of net photosynthesis, quantum yield, enzyme activities, antioxidants, and pigments were taken 3 weeks after planting (WAP) before canopy closure for pre-interference, and 6 WAP after canopy closure for interference before subjecting the plants to the drought treatment. In each pot with three cotton plants, one plant was measured. In pots with one cotton plant and two spurred anoda plants, the cotton plant and one spurred anoda plant were measured. The same plant was measured each time.

Drought treatments and recovery

After measurements for interference were taken at 6 WAP, plants were re-randomized into three blocks on the same greenhouse benches so that each block had two pots with the same interference treatment. Water was withheld for 6 d at 7 WAP for one pot of each interference treatment within a block while the other pot served as a control and was watered daily. At the end of the 6 d drought period, the measurements mentioned previously, relative water content (*RWC*) and leaf water potential, were taken, and all plants were watered. Ten days after drought was terminated, the above-mentioned measurements were taken again to determine recovery from drought.

Photosynthesis and fluorescence measurements

Net photosynthesis (P_{net}) was measured with an infrared gas analyzer-based photosynthesis system (LI-6400, Li-Cor Inc., Lincoln, Nebraska, USA). Maximum P_{net} of the fourth leaf from the apex was recorded by observing the current P_{net} on the display. All measurements were taken at an internal photon flux of 500 μmol m^{-2} s^{-1}, a flow rate of 400 μmol s^{-1} and an internal CO_2 concentration of 400 μmol mol^{-1}. Light-adapted quantum yield was measured using the same leaf with an OS5-FL modulated chlorophyll fluorometer (Opti-Sciences Inc., Tyngsboro, MA, USA). Steady state (F_s) and maximal (F_{ms}) fluorescence values were measured at a modulation intensity of 200 and saturation pulse intensity of 230, and the quantum yield was computed as $[(F_{ms}–F_s)/F_{ms}]$.

Tissue sampling

Leaf discs (0.6 cm diameter) were taken using a paper hole puncher from the same leaf used to measure $P_{net.}$ Discs were frozen immediately on dry ice and stored at –20 °C until used for biochemical assays.

Lipid peroxidation assay

Lipid peroxidation was determined according to Albro *et al*. (1986) based on thiobarbituric acid reactive substances (TBARS). Five discs (*c.* 30 mg) were homogenized in 1400 µl ethanol with 0.05% (w/v) butylated hydroxytoluene, followed by centrifugation at 7000 *g* for 5 min at 4 °C. An aliquot of 500 µl supernatant was added to 500 µl of either (i) plus TBA (+TBA) solution comprised of equal volumes of 20% (w/v) trichloroacetic acid and 0.65% (w/v) thiobarbituric acid, or (ii) minus TBA (–TBA) solution containing equal volumes of 20% (w/v) trichloroacetic acid and water. Both samples were heated at 95 °C for 30 min, and cooled to room temperature. Absorbance of +TBA was read at 532 nm and 600 nm using the corresponding –TBA as the blank. *TBARS* (µmol g^{-1} FW) were calculated as $[10^6 \times (Abs_{532} – Abs_{600})/155]/FW$.

Determination of antioxidant enzyme activities and total protein

Ten leaf discs (*c.* 60 mg) were homogenized in 700 µl of a solution containing 50 mM PIPES buffer, 6 mM cysteine hydrochloride, 10 mM D-isoascorbic acid, 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone (PVP-10), 0.3% (v/v) Triton X-100, pH 6.8, and centrifuged at 7000 *g* for 15 min at 4 °C, as modified from Foster and Hess (1980). Before assaying total protein and enzyme activities, the supernatant was desalted by elution through a 5 ml Sephadex G-25 column that was pre-equilibrated with 100 mM TRIS (pH 7). Total protein content was determined with the protein-binding Coomassie Plus Reagent 23236 following the manufacturer's microplate protocol (Pierce Inc., Rockford, IL, USA). Absorbance was read at 595 nm with an Emax Precision Microplate reader (Molecular Devices, Sunnyvale, CA, USA). Catalase activity was determined by monitoring the disappearance of H_2O_2 by recording the decrease in absorbance at 240 nm of a reaction mixture containing 0.059 M H_2O_2 in 50 mM $KHPO_4$ (pH 7, 4.5 mM H_2O_2 in final assay mixture), water, and leaf extract (Beers and Sizer, 1952). Ascorbate peroxidase (APX) activity was assayed by monitoring the ascorbic acid-dependent reduction of H_2O_2 at 265 nm in a reaction mixture comprising 106 mM HEPES-KOH (pH 7), 0.11 mM EDTA, 0.06 mM Na ascorbate, 0.17 mM H_2O_2, and extract (Anderson *et al*., 1992). Glutathione reductase (GR) activity was determined by monitoring the glutathione (GSSG)-dependant oxidation of NADPH at 340 nm in a reaction mixture containing 0.5 mM GSSG, and 3 mM $MgCl_2$ in 50 mM TRIS (pH 7, 0.42 mM GSSG, 2.5 mM $MgCl_2$ in final assay mixture), 0.007 mM NADPH, and extract (Schaedle and Bassham, 1977). For APX and GR, the values were corrected for the non-specific activities of H_2O_2 and NADPH, respectively, by subtracting the activity (ΔOD) of reaction mixture and protein extraction buffer from the activity of reaction mixture and the plant extracts. All reactions for enzyme activities were initiated at 24 °C, and absorbance was monitored using an HP 8453 UV-Vis spectrophotometer automated with Chemstation A.02.05 (Hewlett-Packard GmbH, Germany). One unit of catalase and APX was defined as the amount necessary to decompose 1 µmol substrate min^{-1}. One unit of GR decomposed 1 nmol substrate min^{-1}. Specific activities of enzymes were expressed as units mg^{-1} of protein.

Antioxidants and pigments

Antioxidants and chlorophylls were assayed according to García-Plazaola and Becerril (1999) with some modifications. Ten leaf discs were homogenized in 300 µl acetone in dim light and extracted by stirring for 30 min in dark at 4°C. Each homogenate was centrifuged at 7000 *g* for 5 min at 4 °C, and the supernatant was filtered through 0.2 µm filters, and injected into HPLC (Agilent 1100 automated with Chemstation A.08.03, Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of two components: A, acetonitrile:-methanol:water (84:9:7, v : v) and B, methanol:ethyl acetate (68:32, v:v). A linear gradient from 100% A to 100% B during the first 12 min was followed by an isocratic elution for 6 min. A 1 min linear gradient from 100% B to 100% A, and an isocratic elution with 100% A for the next 6 min were used for re-equilibrating the column before the next injection. Flow rate was 1.2 ml min^{-1}, injection volume was 25 µl, and the autosampler was set at 4°C during the entire chromatographic analysis. Chlorophylls and carotenoids were detected at 445 nm using a photodiode array detector, and α-tocopherol was detected using a fluorescence detector with excitation at 295 nm and emission at 340 nm. Xanthophyll cycle conversion state was computed as (Z+A)/(V+A+Z) where Z, A and V are zeaxanthin, anthiraxanthin and violaxanthin, respectively. Carotenoid standards were purchased from DHI Water and Environment, Hørsholm, Denmark. Chlorophylls and α-tocopherol were from Sigma-Aldrich, St Louis, MO, USA.

Plant height:node, RWC*, water potential and biomass*

Plant height:node ratios were recorded at 3, 6 and 10 WAP. Relative water content was measured using the fifth leaf from the apex as $(FW–DW)/(TW–DW)$ where *FW* is fresh weight, *DW* is dry weight, and *TW* is turgid weight (weight after the leaf was kept immersed in de-ionized water overnight). Water potential of the sixth leaf from the apex was measured using a pressure chamber (PMS Instrument Co. Corvallis, OR, USA). Total biomass was determined by drying the total shoot mass at 70 °C to a constant weight at 10 WAP.

Statistical analysis

StatView (Feldman and Gagnon, 1986) and JMP (ver. 2.0.1; SAS Institute) were used to analyse the results of the response variables. For well-watered plants at 3 and 6 WAP, species and interference levels were considered treatments. For 8 WAP and 10 WAP, drought was also included as a treatment. When treatment main effects were significant in ANOVA, Fisher's protected LSD was used for mean comparison for each treatment combination at $P < 0.05$. When interference or drought effects were not significant, data were pooled to show species effects, or age effects within species.

Results

Plant interference and species effects at different growth stages under well-watered conditions

Plant biomass was reduced by interference in all three species (Fig. 1). In Delta Pine, intraspecific and interspecific interference reduced biomass 26% ($P < 0.05$) and 64% ($P < 0.001$), respectively, compared to Delta Pine grown alone. In Pima, biomass was reduced 28% ($P < 0.05$) and 53% ($P < 0.001$) due to intraspecific and interspecific interference, respectively, compared to Pima grown alone. In spurred anoda, interference by another spurred anoda plant and either a Delta Pine or Pima plant reduced biomass more than 28% ($P < 0.01$), compared to spurred anoda grown alone. Plant height:node ratio increased by more than 11% due to interference in both cotton species, and 7% in spurred anoda, regardless of interfering species (Fig. 1, $P < 0.05$). However, plant height was not affected

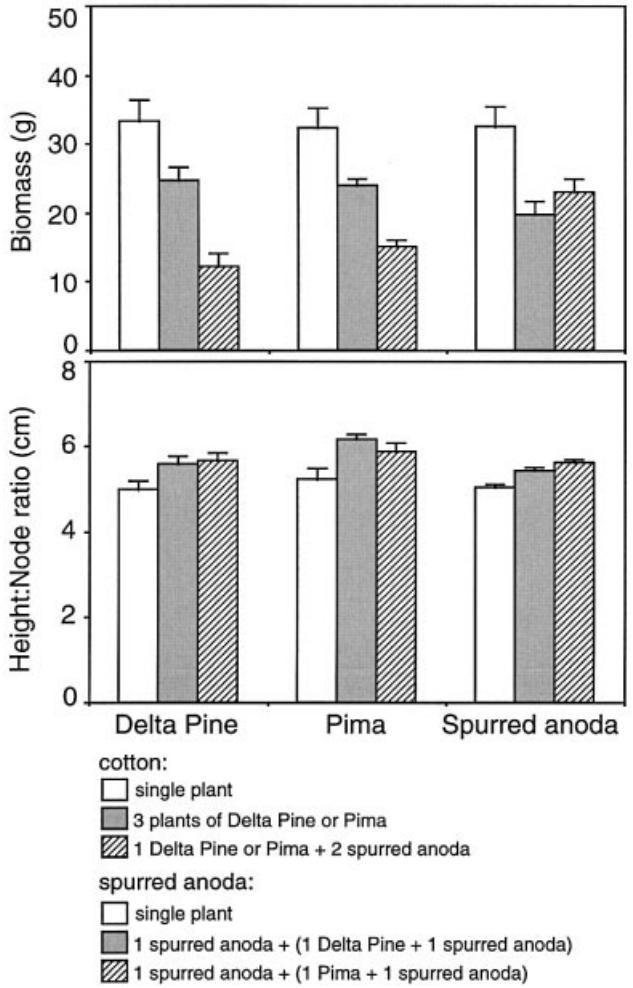

Fig. 1. Plant biomass and height:node ratio of the two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda at the end of experiment (10 WAP, n=6). Error bars are SE of the mean.

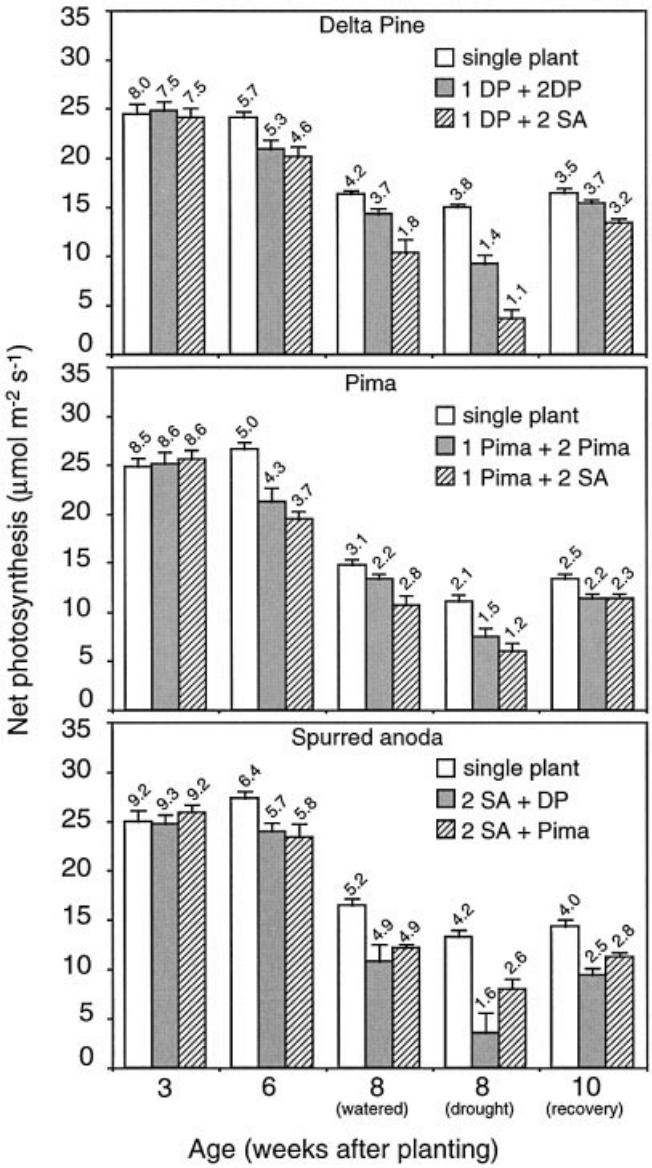

Fig. 2. Net photosynthesis of two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda under different types of interference at different growth stages. DP and SA are Delta Pine 5415 and spurred anoda, respectively. Error bars are SE of the mean (n=6, except for 8 WAP where n=3). Numbers above the bars are transpiration rates (mmol m^{-2} s^{-1}).

by interference in any species, except for a 19% reduction in Delta Pine with interspecific interference (P <0.05) compared to intraspecific interference or no interference. Plant height of cotton species was similar. However, the height of spurred anoda, 99 cm, was 80% greater than either cotton species (P <0.001). The 6 d drought imposed at 7 WAP (Fig. 1) had no effect on biomass or height:node ratio at 10 WAP; therefore, data were pooled across drought treatment.

At 3 WAP, before the canopy closure, P_{net} was similar in all three species and was unaffected by interference (Fig. 2). However, at 6 WAP, P_{net} was reduced by *c.* 13%, 25% and 15%, respectively, in Delta Pine, Pima and spurred anoda due to interference without regard to interfering species (P <0.01). By 8 (watered plants) WAP, P_{net} was reduced more by interspecific interference by spurred anoda than by intraspecific interference in both cotton species (P <0.001, Fig. 2). In spurred anoda, mixed interference caused a 25% reduction in P_{net} at 8 (watered plants) WAP (P <0.001). Furthermore, P_{net} decreased by more than 30% in all three species from 6 WAP to 8 (watered plants) WAP or later, regardless of interference. At 10 WAP, for the data pooled across drought treatments, P_{net} was still lower in plants under interference compared to the plant grown alone in all three species (P <0.001), although there was no difference between the type of interference. For the data pooled across interference levels at 10 WAP, Delta Pine had *c.* 32% higher P_{net} than Pima or spurred anoda (P <0.001).

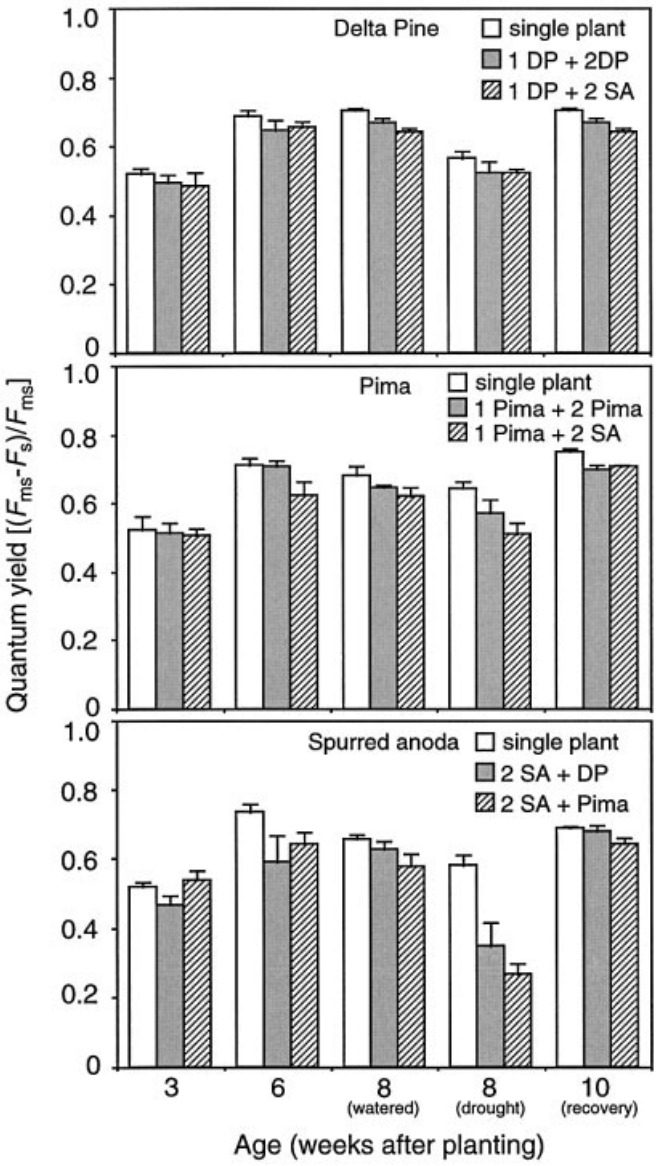

Fig. 3. Quantum yield of two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda under different types of interference at different growth stages. DP and SA are Delta Pine 5415 and spurred anoda, respectively. Error bars are SE of the mean (n=6, except for 8 WAP where n=3).

Stomatal conductance (g_s) was unaffected by interference in all three species at 3 WAP, but was highest in spurred anoda, 0.85 mol H_2O m^{-2} s^{-1}. Pima had greater g_s, 0.64, than Delta Pine, 0.49 mol H_2O m^{-2} s^{-1} (P <0.01, data not presented). Transpiration rate (E) showed the same pattern as g_s except that differences between Pima and spurred anoda were not significant (P=0.13, Fig. 2). Intrinsic water use efficiency (WUE, P_{net}/E) was highest in Delta Pine, 3.2, followed by Pima, 2.9, and spurred anoda, 2.6 μmol $mmol^{-1}$ at 3 WAP (P <0.05, data not presented). At 6 WAP, although g_s and E were still highest in spurred anoda, Pima had the lowest g_s and E (P <0.01).

Table 1. *Pigment concentrations of two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda at different growth stages* Data were pooled across interference levels at all ages, and across drought levels at 8 WAP since interference and drought effects were not significant (P >0.05). Means (n=18) followed by different letters are statistically different for a given variable across ages (a or b), and across species (x or y) (P <0.05).

Age	Delta Pine				Pima				Spurred anoda			
	chl. *a* (μg g^{-1} FW)	chl. *a:b*	Lutein (HPLC peak area mg^{-1} FW)	β-carotene (μg g^{-1} FW)	chl. *a* (μg g^{-1} FW)	chl. *a:b*	Lutein (HPLC peak area mg^{-1} FW)	β-carotene (μg g^{-1} FW)	chl. *a* (μg g^{-1} FW)	chl. *a:b*	Lutein (HPLC peak area mg^{-1} FW)	β-carotene (μg g^{-1} FW)
3 WAP	1355 a,x	3.3 a,y	72 a,x	186 a,x	1124 a,x	3.3 a,y	67 a,x	149 a,x	2368 a,y	2.6 a,x	153 a,y	191 a,x
6 WAP	3522 b,x	3.8 ab,y	234 b,x	408 b,x	2751 b,x	3.6 a,y	200 b,x	379b,x	3652 b,y	2.4 a,x	290 b,y	373 b,x
8 WAP	2738 b,x	4.2 b,y	143 b,x	413 b,x	2460 b,x	3.9 a,y	130 b,x	421 b,x	3524 b,y	3.3 b,x	166 a,y	346 b,x
10 WAP	3264 b,x	3.7 ab,y	163 b,x	482 b,x	2791 b,x	3.5 a,y	166 b,x	561 b,x	2894 ab,x	2.7 ab,x	169 a,x	278 ab,x

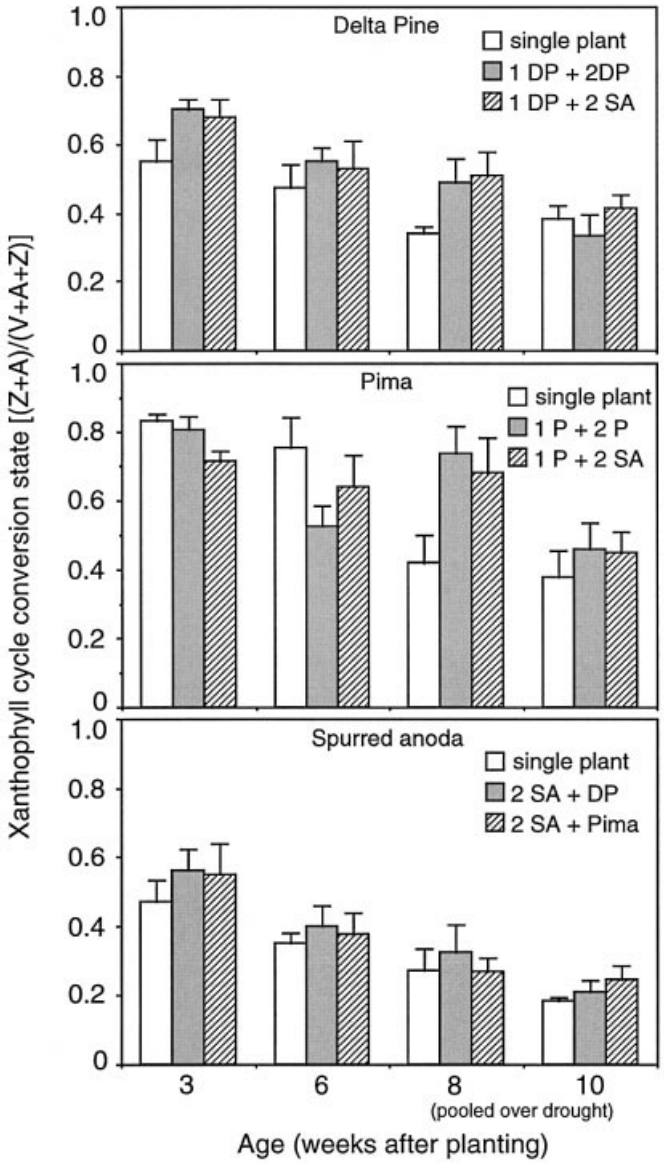

Fig. 4. Xanthophyll cycle conversion state [(Z+A)/(V+A+Z), Z, zeaxanthin; A, antheraxanthin; V, violaxanthin] of two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda under different types of interference at different growth stages. DP, P and SA are Delta Pine 5415, Pima S-7 and spurred anoda, respectively. Error bars are SE of the mean (n=6).

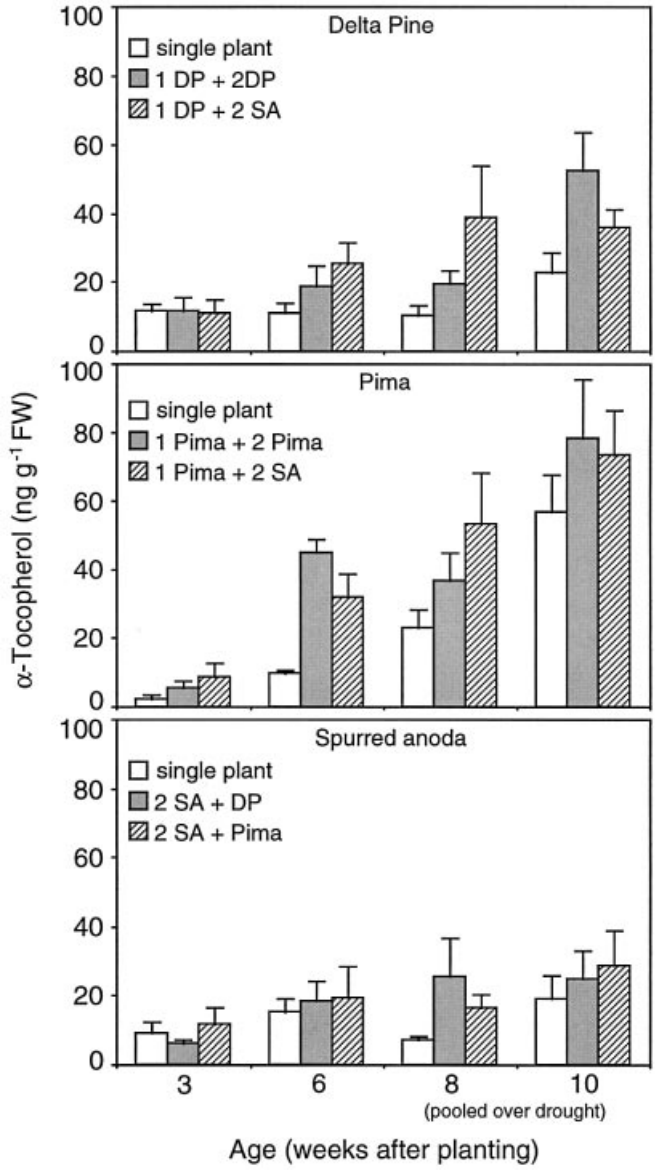

Fig. 5. Leaf α-tocopherol content of two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda under different types of interference at different growth stages. DP and SA are Delta Pine 5415 and spurred anoda, respectively. Error bars are SE of the mean (n=6).

Within a species, g_s and E followed the same pattern as P_{net} with a significant interference effect (P <0.01) at this growth stage. Although unaffected by interference, *WUE* was highest in Pima (P <0.05) and similar in Delta Pine and spurred anoda at 6 WAP.

Quantum yield was not affected by interference either, and was similar in all three species at 3 WAP (Fig. 3). At 6, 8 (watered plants) and 10 WAP, the main effect of interference on quantum yield was significant (P <0.05). However, only the *c.* 12% reduction in quantum yield in spurred anoda at 6 WAP, and 6% reduction in Pima at 10 WAP due to interference was significant for a given species (P <0.05). Furthermore, quantum yield was *c.* 20% less during pre-canopy closure than at canopy closure (6 WAP) and at late growth phases in all three species.

In all three species, concentrations of leaf chlorophyll *a* and *a*:*b* ratio, lutein and β-carotene were not influenced by interference at any growth phase. Therefore, the data were pooled across interference treatments (Table 1). However, levels of these pigments increased after 3 WAP (P <0.01) in all species and remained at high levels throughout the experiment, except for lutein concentration in spurred anoda which decreased after 6 WAP to the levels of 3 WAP. Chlorophyll *a* concentration was 73% and 133% greater in spurred anoda than Delta Pine and Pima,

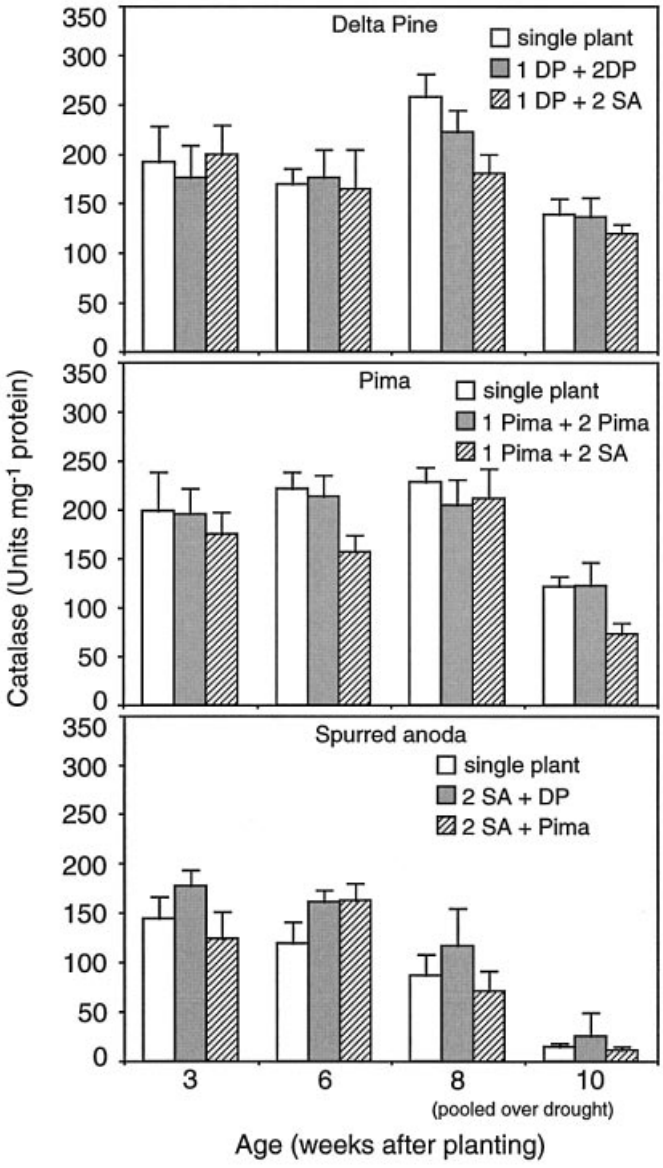

Fig. 6. Catalase activity of two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda under different types of interference at different growth stages. DP and SA are Delta Pine 5415 and spurred anoda, respectively. Error bars are SE of the mean (n=6).

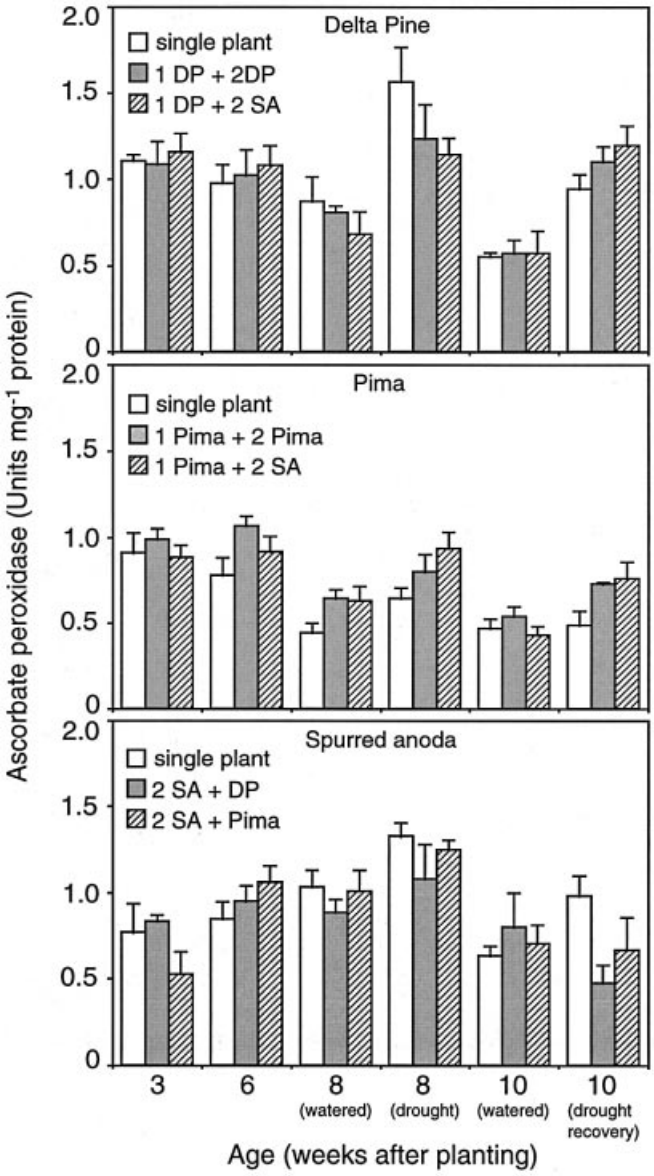

Fig. 7. Ascorbate peroxidase activity of the two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda under different types of interference at different growth stages. DP and SA are Delta Pine 5415 and spurred anoda, respectively. Error bars are SE of the mean (n=6, except for 8 and 10 WAP where n=3).

respectively, at 3 WAP (P <0.001). Furthermore, lutein concentration was 113% and 126% greater in spurred anoda than Delta Pine and Pima, respectively, at 3 WAP (P <0.01). However, chlorophyll a:b ratio was more than 23% greater in either cotton species than spurred anoda, at 3 WAP (P <0.05). The concentration of β-carotene was constant in all species at all growth phases.

Although the xanthophyll cycle conversion state was not influenced by interference in any species at 3 or 6 WAP, plants with interference had *c.* 65%, and 44% greater proportion of Z+A in the total xanthophyll pool compared to single plants in Pima and Delta Pine, respectively, at 8 WAP (Fig. 4). The xanthophyll cycle conversion state was greater in cotton species than spurred anoda at any growth stage (P <0.01). At pre-canopy closure, leaf α-tocopherol content was not affected by interference in any species (Fig. 5). However, there was more α-tocopherol in both cotton species at 6 and 8 WAP, and in spurred anoda at 8 WAP, under interference than in plants grown alone (P <0.01). Although there was no difference in α-tocopherol content between the three species during early vegetative growth (3 WAP), Pima had a greater α-tocopherol content than Delta Pine and spurred anoda from 6 WAP to the end of the experiment (P <0.001).

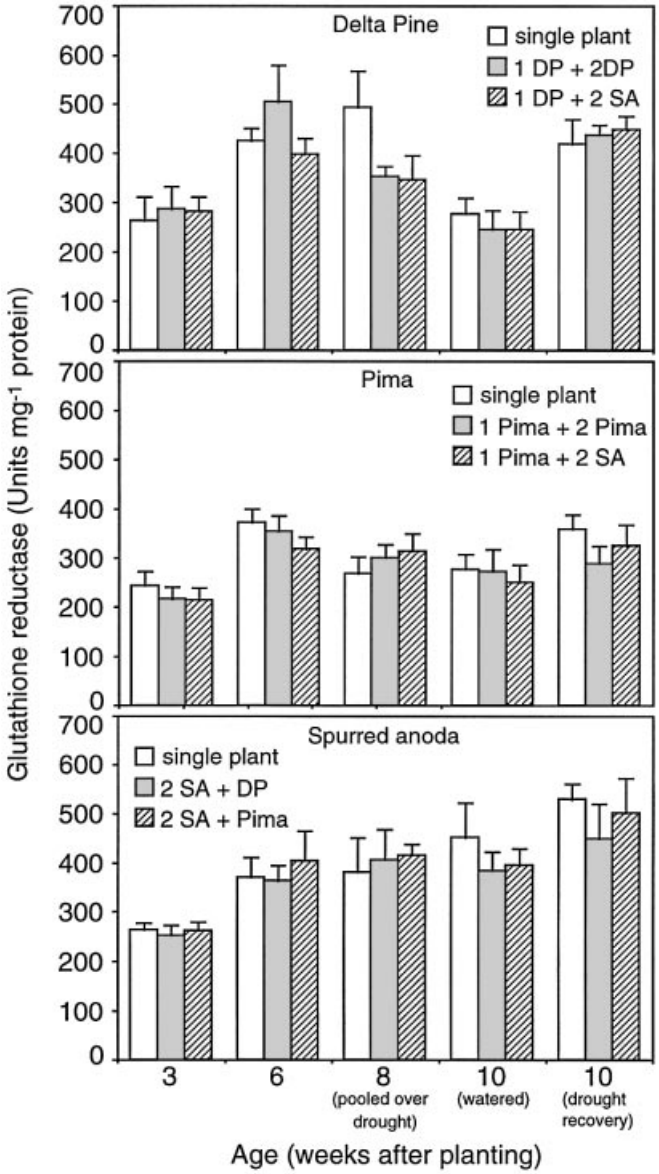

Fig. 8. Glutathione reductase activity of two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda under different types of interference at different growth stages. DP and SA are Delta Pine 5415 and spurred anoda, respectively. Error bars are SE of the mean (n=6, except for 10 WAP where n=3).

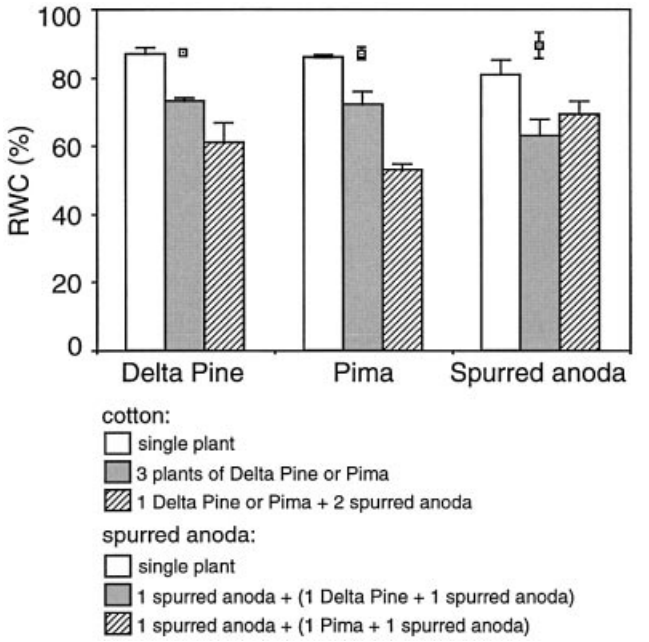

Fig. 9. Leaf relative water content (*RWC*) of two cotton species (cultivars, Delta Pine 5415 and Pima S-7) and spurred anoda at the end of 6 d drought imposed at 7 WAP (n=3). Single mean (small open square) indicates *RWC* of watered plants for the respective species (n=9). Error bars are SE of the mean.

Catalase activity was not affected by interference in any species at a given growth stage (Fig. 6). Although, all three species had similar catalase activity at 3 WAP, Pima had 33% greater catalase activity than spurred anoda at 6 WAP (P <0.05). At 8 and 10 WAP, both cotton species had greater catalase activity than spurred anoda (P <0.001). At a given growth stage, catalase activity was similar between the two cotton species. Catalase activity declined after 6 WAP in spurred anoda, and after 8 WAP in the two cotton species.

APX activity was not influenced by interference in any species at a given growth stage (Fig. 7). However, unlike catalase activity, APX activity of Delta Pine was 20% and 57% greater than Pima and spurred anoda, respectively, at 3 WAP (P <0.001). Furthermore, Pima had 30% greater APX activity than spurred anoda (P <0.001) at 3 WAP. Although, APX activity dropped in Delta Pine and Pima by 29% and 38%, respectively, from 3 to 8 WAP (watered plants), a 38% increase of APX activity was observed in spurred anoda during this period (P <0.001). Thus, by 8 WAP (watered plants) spurred anoda had 38% and 92% greater APX activity than Delta Pine and Pima, respectively (P <0.001). Delta Pine had 39% greater APX activity than Pima at 8 WAP (P <0.001). At 10 WAP, spurred anoda still had 55% greater APX activity than Pima (P <0.001).

Interference did not influence GR activity either, in any species at all four measurement times (Fig. 8). There were no differences in GR activity among the three species at 3 WAP. However, averaged over interference levels, GR activity increased dramatically in all the species from 3 WAP to 6 WAP with a maximum of 58% in Delta Pine (P <0.001). GR activity of Delta Pine was *c.* 25% greater (P <0.01) than Pima, but was similar to spurred anoda, at 6 and 8 WAP. Glutathione reductase activity remained unchanged in spurred anoda, but declined in both cotton species from 6 to 10 WAP.

Effect of mild drought

Withholding water for 6 d at 7 WAP reduced leaf relative water content (*RWC*) in plants under interference only

(Fig. 9). In Delta Pine, *RWC* was 0.73 and 0.58 in plants with intraspecific and interspecific interference, respectively, compared with 0.88 of the well-watered control. In Pima, *RWC* was 0.72 and 0.53 under intraspecific and interspecific interference, respectively, compared to 0.87 of the well-watered plants. In spurred anoda *RWC* was *c*. 0.65 under interference compared to 0.89 for the well-watered plants.

Although drought reduced P_{net} compared with the well-watered controls, plants under interference had the greatest reduction with a maximum of 66% decline in Delta Pine with interspecific interference compared to a single plant (Fig. 2). Polynomial fits of P_{net} versus g_s for drought-stressed plants pooled across interference levels were Delta Pine, $P_{net}=0.98+184.06g_s-503.49(g_s)^2$, $r^2=0.91$; Pima, $P_{net}=1.02+156.69g_s-504.73(g_s)^2$, $r^2=0.86$; spurred anoda, $P_{net}=-1.43+151.23g_s-349.37(g_s)^2$, $r^2=0.91$. Similarly, quantum yield was reduced due to drought at all interference levels in all species with a maximum of *c*. 47% decline in spurred anoda plants with interference (Fig. 4). Two weeks after the termination of drought (at 10 WAP), plants that underwent drought had the same P_{net} and quantum yield as the well-watered plants in all three species (Figs 2, 3).

Levels of chlorophyll *a*, chlorophyll *a*:*b* ratio, lutein, β-carotene, xanthophyll cycle conversion state, α-tocopherol, and TBARS were unaffected by the 6 d drought (Table 1; Figs 4, 5; data pooled across drought treatments, TBARS data not shown). However, of the antioxidant enzymes, APX activity increased in drought-stressed plants 74%, 41% and 26% in Delta Pine, Pima and spurred anoda, respectively, compared to the well-watered controls ($P<0.001$, Fig. 7, 8 WAP). Drought-induced APX activity was still present two weeks after recovery from drought in the two cotton species, but not in spurred anoda. Although, unaffected during drought, GR activity was 62%, 21% and 20% greater in the plants that underwent drought, compared with well-watered plants of Delta Pine, Pima and spurred anoda, respectively, during recovery from drought 10 WAP ($P<0.01$, Fig. 8).

Discussion

Plant interference alters the availability of resources, and influences the capability of plant to acquire them. Therefore, the degree of damage caused, and the type or level of defence induced in a given plant after exposure to a certain level of abiotic stress will be different under specific types and severities of interference compared to no interference. In this study, severe interference was imposed on plants growing with two other plants as revealed by reduced biomass and increased height:node ratio at 10 WAP, and lower P_{net} and quantum yield at or after 6 WAP. Furthermore, although spurred anoda shares the same family and biomass accumulation pattern with cotton, spurred anoda caused more dramatic reductions in cotton biomass and P_{net} than intraspecific interference. In this paper, evidence of clear differences between cotton and spurred anoda in their relative response to interference is reported and discussed. These differential responses found in gas exchange characteristics, pigment profiles and antioxidant systems should help expand understanding of the impacts of intraspecific and interspecific interference in plants.

Reductions in biomass, P_{net} and quantum yield under interference suggest that stress was imposed, and the hypothesis was that antioxidant defence systems would be enhanced in response. Of the measured antioxidant responses, α-tocopherol content in all three species, and the xanthophyll cycle conversion state in cotton were increased under interference. Besides its structural role in membrane stability (Salgado *et al*., 1993; Havaux, 1998), α-tocopherol is a powerful antioxidant that prevents formation of, and scavenges $^1O_2$ and lipid peroxy radicals, thus preventing lipid peroxidation and the resulting damage to thylakoid and chloroplast membranes (Fryer, 1992; Pogson *et al*., 1996). Thylakoids are especially susceptible to degradation by oxygen free radical attack due to their high proportion of polyunsaturated fatty acid residues (*c*. 90% linolenate alone) (Fryer, 1992). Furthermore, α-tocopherol is involved in long-term protection of photosynthetic pigments (Wise and Naylor, 1987) and in slowing ageing (Dhindsa *et al*., 1982; Thompson *et al*., 1987). Thus, increased α-tocopherol levels and reduced P_{net} in cotton could be considered acclamatory to interference stress, and α-tocopherol could have prevented a measurable increase in lipid peroxidation.

High xanthophyll cycle conversion state caused by increased formation of antheraxanthin and zeaxanthin via de-epoxidation of violaxanthin, allows quenching of excess energy from chlorophyll before it reaches reaction centres (Demmig-Adams and Adams, 1996; Niyogi *et al*., 1998). Reduced P_{net} and quantum yield in the plants under interference, compared with plants grown alone, are evidence that these plants were unable to utilize all the incident light. Although, main stems of spurred anoda plants were taller than cotton plants, shading of cotton plants under interspecific interference was minimal due to the smaller leaf size and the spreading architecture of spurred anoda (branches projecting outward from the plant base), and adequate spacing between pots allowing no contact between plants of different pots. Furthermore, although a constant photon flux density (PFD) of 500 μmol m^{-2} s^{-1} was supplied by the LI-6400 during all the gas exchange measurements, maximum ambient PFDs of *c*. 1100 μmol m^{-2} s^{-1} were recorded in the greenhouse during the measurements at 8 WAP when the xanthophyll cycle conversion of cotton plants under interference was greater than for the respective single plants. These ambient light

levels recorded by LI-6400 were not different among interference levels. Thus, photoinhibition may have occurred to some degree in both cotton species during interference, and the xanthophyll cycle conversion state could have prevented permanent damage to photosystems.

In spurred anoda, however, xanthophyll cycle conversion state was constitutively lower than cotton at all times of measurement, and was not induced under interference. On the other hand, spurred anoda had much higher contents of lutein and chlorophyll than either cotton species, without interference. While the role of lutein as a PSII light-harvesting complex binding carotenoid has been known for some time (Demmig-Adams *et al*., 1996), Pogson *et al*. (1996), using lutein-deficient *Arabidopsis* mutants, showed that levels of xanthophyll cycle pigments increase to compensate for the lack of lutein's function. Thus, these results suggest that cotton and spurred anoda have different strategies for protecting photosystems during photoinhibition. Cotton resorts to the xanthophyll cycle at PFDs of around 1000 μmol m^{-2} s^{-1}, but spurred anoda plants with their high constitutive levels of lutein still avoid permanent damage to photosystems without appreciable increases in the xanthophyll cycle conversion state. Although not observed in this study, loss of chlorophyll under severe photoinhibitory conditions is well-known (Maxwell *et al*., 1999; Balaguer *et al*., 2002). Higher chlorophyll content in spurred anoda may compensate for such losses, and allow for maintaining a greater carbon gain than cotton during a more severe stress (this was observed to be true in a progressive drought experiment, data not shown). Despite lower chlorophyll and lutein levels, greater chlorophyll *a*:*b* ratio and Z+A in cotton, which are indicative of adaptation to high light intensity, may underlie P_{net} rates as high as spurred anoda when interference was absent.

In this study, except for the 6 d of water stress treatment at 7 WAP, the application of fertilizer and water was the same for all pots regardless of the number of plants per pot. No effect of interference was found on pigment concentrations and *RWC* (in well-watered plants) indicating that a major nutrient or water deficit was not imposed on the plants with interference compared with single plants. However, given the reduced biomass of plants under interference (especially by interference of spurred anoda on cotton) and adequate availability of PFD for all plants, the possibility cannot be excluded that some water stress could have been imposed on the plants with interference between daily waterings, although it was not sufficient to show a difference in *RWC*. Of the three species, spurred anoda had the highest g_s and *E* during all the gas exchange measurements suggesting that spurred anoda uses more water than cotton at a given growth stage. Furthermore, g_s followed the same pattern of P_{net} at 6 WAP and later (highest in single plants in all three species, and in cotton, least in plants with interspecific interference, data not presented). This evidence supports the finding that stomatal activity was restricted in plants under interference. Thus, it is proposed that plants under interference sensed a mild moisture deficit during the day and signalled leaves to restrict stomatal opening which reduced P_{net} through effect on C_i (intercellular CO_2 concentration, data not shown). The involvement of root-originated ABA in restricting g_s during the initial stages of soil drying before a measurable change in leaf water status occurs is known in many species (Blake and Ferrel, 1977; Blackman and Davies, 1985; Zhang *et al*., 1987). This is also consistent with the result that the mild drought stress imposed at 7 WAP reduced leaf *RWC* only in plants with interference, but not the single plants, and with Patterson (1988) that well-watered spurred anoda had significantly greater leaf area and whole plant water loss than *G. hirsutum* cotton at 4 WAP. Even a minor water stress occurring daily must have hampered nutrient acquisition as well to some degree, in the plants with interference. Although insufficient to reduce pigment levels, this possibility may also have contributed to reduced P_{net}.

Thus, limited carbon gain from around 6 WAP to the time of harvesting the biomass (10 WAP), caused mainly by restricted stomatal activity, is the major factor underlying the reduced biomass in plants with interference compared with single plants, and greater biomass reduction in cotton with interference by spurred anoda compared with intraspecific interference. Furthermore, given that the decrease in P_{net} was not linked to a comparable decrease in PSII activity $[(F_{ms}–F_s)/F_{ms}]$, photorespiration may have been an alternative carbon sink in plants with interference causing reduced biomass compared with single plants. Some Malvaceous weeds contain allelochemicals (Sterling and Putnam, 1987), and given that the biomass of singly grown plants of spurred anoda and cotton was the same, but spurred anoda interference reduced cotton biomass more than intraspecific interference, the possibility cannot be excluded of a harmful allelopathic effect of spurred anoda on cotton.

When watering was withheld for 6 d, *RWC* was lower in plants under interference by spurred anoda than under intraspecific interference in both cotton species. As mentioned above, the greater g_s and *E* that allowed for greater water loss by spurred anoda than cotton explains this. Although P_{net} and quantum yield were reduced due to drought (more severely under interference than without interference), pigment levels were not affected by this mild drought indicating that photosynthesis was reduced by stomatal regulation. Medrano *et al*. (2002) reviewed gas exchange data of many species under drought and showed that although a good correlation is often observed between leaf water status and g_s, due to the responsiveness of g_s to all the external (e.g. soil water availability, *VPD*) and internal (e.g. ABA, xylem conductivity, leaf water status) factors related to drought, g_s is a more integrative basis for

drought effects than leaf water status. A close relationship was found ($r^2 \approx 0.9$) between P_{net} and g_s for the water-stressed plants of all three species.

Although the 6 d drought did not affect lipid peroxidation (data not shown) or the levels of antioxidants measured (α-tocopherol, β-carotene and xanthophyll cycle pigments), APX activity markedly increased in all three species during drought, and increased levels of APX were maintained in cotton during recovery from drought. APX isoenzymes play a pivotal role in scavenging H_2O_2 and are distributed in at least four cell compartments, namely, stroma and thylakoid membrane of chloroplast, microbodies and cytosol (Noctor and Foyer, 1998; Yoshimura *et al*., 2000; Asada, 1992; Miyake and Asada, 1992). Transgenic cotton overexpressing stromal APX, showed decreased chilling-induced photoinhibition of PSII (Dmytro *et al*., 2001), and drought-resistant maize plants possessed greater APX and GR activities than drought-sensitive plants (Pastori and Trippi, 1992). It is increasingly evident that the generation of H_2O_2, the primary signal for induction of APX activity (although differences exist in expression of different APX isozymes in different species; see reviews by Shigeoka *et al*., 2002; Neill *et al*., 2002) and therefore an essential regulator of ROS-scavenging ascorbate-glutathione cycle, is rapid and transient (Foyer *et al*., 1997; Shigeoka *et al*., 2002). Furthermore, H_2O_2 levels in plant cells increase under many environmental stresses (Foyer *et al*., 1997; Yoshimura *et al*., 2000), and are linked with other metabolic pathways (Kovtun *et al*., 2000; Samuel *et al*., 2000). Accordingly, the activities of APX isoforms in different cellular compartments are important in the overall stress response, but this was not studied here. It is also apparent that APX activity generally increased with other antioxidant enzymes such as GR and superoxide dismutase. In this study, levels of GR activity were maintained higher in drought-stressed plants of all three species during recovery although not during drought. Mittler and Zilinskas (1994) found that transcript levels of cytosolic APX increased more dramatically during drought recovery than during drought (15-fold versus 4-fold) in pea plants.

There is commonality among oxidative stress tolerance mechanisms and they are co-regulated (Neill *et al*., 2002; Shigeoka *et al*., 2002). Thus, elevated levels of APX and GR levels in drought-stressed plants during recovery strongly suggest that drought stress in cotton and spurred anoda may lead to acclimation tolerance to a more severe drought stress and/or cross-tolerance (see Neill *et al*., 2002, for a review) to other stresses later in growth. Interestingly, TBARS levels, although unaffected by drought or interference, were elevated in previously drought-stressed plants during recovery from drought (data not shown). TBARS are widely regarded as indicators of oxidative stress damage to cellular membranes, but not as signals of stress. However, TBARS represent a wide array of lipid peroxidation products such as malondialdehyde, alkanals, alkenals, and dienals (Knight *et al*., 1988). Since no literature was found on the testing of these groups of compounds as signalling molecules, no further insight can be gained into whether increased TBARS levels during drought recovery serve as signals.

In summary, although cotton and spurred anoda share the same family and growth habits they have clear differences in (1) pigment profiles (greater chlorophyll and lutein levels but lower chlorophyll *a*:*b* ratio in spurred anoda), (2) gas exchange characteristics (greater g_s and E in spurred anoda), and (3) levels of antioxidant defences (lower xanthophyll cycle conversion state throughout growth and APX during early juvenile growth in spurred anoda). Under the same levels of resource availability spurred anoda interference restricts cotton biomass more than intraspecific interference. Evidence has been provided suggesting that this greater competitiveness is due to higher g_s and E of spurred anoda than cotton. This greater loss of water by spurred anoda creates a temporary but recurring drought which leads to stomatal-regulated reduction in carbon gain by cotton plants between waterings. Except for the possible roles of higher chlorophyll and lutein levels of spurred anoda than cotton, in carbon gain under stress, antioxidant defences may not underlie this competitive advantage. It is proposed that a mild drought be tested as an inducer of acclimation and cross-tolerance in cotton in site-specific field research and used to increase productivity under stress. Furthermore, the results of protective mechanisms in cotton, upon appropriate field-testing, may serve as comparisons or variables in modelling crop/weed success in a given cropping system.

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