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Physiological and antioxidant responses of cotton and spurred anoda (*Anoda cristata*) under nitrogen deficiency

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Spurred anoda is a major competitor with cotton in the southern United States. Physiological and antioxidant responses of two species of cotton (*Gossypium barbadense* L. cv. 'Pima S-7' and *Gossypium hirsutum* L., Delta and Pine Land Company cv. 'Delta Pine 5415') and two accessions of spurred anoda [New Mexico (NM) and Mississippi (MS)] were investigated under nitrogen (N) -sufficient and -deficient conditions in the greenhouse. Pima S-7 had the highest leaf N content of all the plants regardless of treatment. Biomass decreased in all species when N was withheld, with Pima S-7 exhibiting the least reduction and MS the greatest. Plant height decreased in cotton but not spurred anoda under N stress. Height:node ratio increased 9% in MS, but decreased 8% in DP 5415 when they were deprived of N. Withholding N reduced photosynthesis 45% regardless of species. Comparable decreases were found in stomatal conductance and transpiration, suggesting strong stomatal regulation of gas exchange under N stress. The quantum efficiency of photosystem II (dark-adapted F_v/F_m) decreased 4% under N deficiency. Alpha-carotene decreased for all species when N was withheld, except for the NM accession, in which the levels increased. Total chlorophyll and lutein decreased under N stress regardless of species, but alpha-tocopherol and the xanthophyll cycle conversion state increased. Pima S-7 had the most chlorophyll and lutein, and both cotton species had more alpha-tocopherol, anthocyanins, and free-radical scavenging capacity than spurred anoda. These enhanced pigment and antioxidant profiles of cotton, particularly Pima S-7, may contribute to cotton's ability to compete for N with spurred anoda.

Nomenclature: Spurred anoda, *Anoda cristata* (L.) Schlecht. ANVCR; cotton, *Gossypium barbadense* L. 'Pima S-7'; *Gossypium hirsutum* L. 'DP 5415'.

Key words: Antioxidants, competition, photosynthesis, stress.

Effective weed management depends on comprehensive understanding of specific crop–weed interactions. One important aspect of such knowledge is the insight into plant physiological and antioxidant responses under nutrient deficiency, a common result of crop–weed interference. Spurred anoda is one of the 10 most difficult weeds affecting cotton production (Dowler 1992; VanGessel and Westra 1997), and can cause large cotton yield reductions (Chandler 1977; Chandler and Meredith 1983; Chandler and Oliver 1979; Lambert and Oliver 1975). It is native to South America, Central America, and the southwestern United States, belongs to the family Malvaceae, and thus can be a major competitor of cotton, which is in the same plant family (Chandler and Oliver 1979). Growth characteristics of spurred anoda are similar to cotton, making infestations difficult to detect and manage, as control options are limited (Patterson 1988). Seed cotton yields have been reduced 30% by season-long interference from spurred anoda (Chandler 1977). Reduced yields can be attributed to reductions in both boll number and boll weight as spurred anoda densities increase (Molin et al. 2006). Because weeds often compete with crop plants for essential resources, including soil nutrients, a thorough understanding of how individual plant species respond to deficiencies is important in predicting how crops and weeds may respond to each other in a field setting.

The essential plant element absorbed from soil in the largest quantity is nitrogen (N). Nitrogen deficiency can severely impact plant growth and development. The addition of this nutrient often results in yield increases for crops (Patterson 1995). The timing of N deficiency can also affect cotton fiber quality, especially during the secondary wall thickening stage of fiber development (Reddy et al. 2004). However, weeds can often amass larger amounts of important plant nutrients than agricultural crops (Di Tomaso 1995), thus severely limiting nutrient availability to crop plants. Certain weeds may actually respond more favorably than crops when nitrogen fertilizers are applied, altering the dynamics of weed and crop competition (Blackshaw et al. 2003). Selective placement and timing of fertilizer applications may help reduce the competitive advantage that weeds generally have over crops (Blackshaw et al. 2004; Di Tomaso 1995; Harbur and Owen 2004). The source of nitrogen fertilizer may also affect weed and crop interactions (Di Tomaso 1995; Liebman and Gallandt 2002).

Environmental stresses such as deficiencies of water and nutrients, which occur under intense competition, can cause oxidative stress. Oxidative stress occurs when the plant's ability to use light energy is compromised. Energy absorbed from light is used in photochemistry, or is lost either as heat or fluorescence (Maxwell and Johnson 2000). If carbon fixation is limited by stress, excessive light energy and electrons from the photosynthetic electron transport system can build up, and allow oxygen to form free radicals that are injurious

to membranes and macromolecules such as proteins and DNA (Bowler et al. 1992). These reactive oxygen species (ROS) consist of a number of highly reactive oxygen molecules such as hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and superoxide ($O_2^{\bullet-}$) radicals.

Oxidative stress is a major cause of reduction in plant productivity in plant cells (Allen 1995). Plants have evolved several protective systems to handle oxidative stress (Foyer et al. 1994). For example, antioxidant systems in plants help to alleviate membrane destruction by reacting and effectively neutralizing free radicals. Antioxidants such as beta-carotene and alpha-tocopherol are important in mediating cellular damage through scavenging free radicals. Furthermore, beta-carotene can quench triplet chlorophyll, preventing the formation of potentially damaging oxygen radicals. Alpha-tocopherol is the most active scavenger of all of the tocopherol analogs, and can deactivate singlet oxygen through the process of resonance energy transfer, and ultimately is sacrificed through an oxidation reaction (Fryer 1992). In addition to these important antioxidant molecules, oxidative stress can be alleviated through nonphotochemical quenching, which deactivates excited chlorophyll molecules through thermal dissipation of heat (Müller et al. 2001). The xanthophyll cycle, which involves the conversion of violaxanthin into zeaxanthin through de-epoxidation reactions, is an important mechanism for dissipating excess light energy as heat at Photosystem II (PSII) (Gilmore et al. 1998).

In previous work, cotton cultivars varied in tolerance to the PSII inhibitor, prometryn (Molin and Khan 1996; Waldrop et al. 1996). Prometryn tolerance was not explained by differences in absorption, translocation, metabolism, sequestration into lysigenous glands (Waldrop et al. 1996), or site of action (Khan and Molin 1996). Thus, we have hypothesized that this differential herbicide tolerance may be due to reduced sensitivity to oxidative stress. Therefore, cultivars with differential tolerance to prometryn were selected for studying environmental effects on oxidative stress tolerance mechanisms.

Though much is known about the effect of nitrogen on yield, growth, and physiology of cotton, little is known about nitrogen deficiency in spurred anoda and the impact of nitrogen fertility on the individual stress tolerance mechanisms in cotton and spurred anoda. Therefore, the objectives of this study were (1) to examine nitrogen-deficiency responses among two cotton species and two spurred anoda accessions by comparing gas exchange and whole plant growth characteristics, and (2) to determine how nitrogen deficiency affects mechanisms that protect against oxidative stress.

Materials and Methods

Plant material and nitrogen treatments

Cotton was germinated approximately 3 wk earlier than spurred anoda to target the same growth stage for cotton and spurred anoda. This allowed for the measurement of plants that were at approximately the same phenological age; regardless of plant types; plants of all species were beginning flowering. Cotton seeds from a prometryn-susceptible cultivar 'DP 5415', and a prometryn-tolerant cultivar 'Pima S-7' were germinated on moist paper towels at room temperature. Spurred anoda seeds were scarified by immersing in concentrated sulfuric acid (4.65M) for 20 min, rinsed with water for 30 min, and then germinated at room temperature as above. Seeds from two accessions of spurred anoda collected from different regions were used: The NM accession was collected at the Leyendecker Research Farm of New Mexico State University and the MS accession was provided by the USDA-ARS Southern Weed Science Research Unit in Stoneville, MS. Seedlings were planted into 120-ml (4-cm diameter) pots with Terra-Lite Metro Mix 360.[1] Two weeks after planting, seedlings were transplanted into Classic 400 (20-cm diameter) pots containing Metro Mix:sand (3:1). Plants were grown in a greenhouse throughout summer under natural daylight conditions at 20/35-C min/max temperatures. Plants were watered daily to field capacity.

Cotton and spurred anoda plants were placed in a randomized complete block design with six replications. Blocks were based on plant homogeneity within each block. Plants were fertilized weekly with 400 ml of Hoagland's nutrient solution containing nitrogen (Hoagland and Arnon 1950) until 6 wk after planting, when half of the plants were treated with Hoagland's nitrogen-free solution and the other plants were continuously treated with the nitrogen-containing solution. Four weeks after N treatments were imposed, photosynthesis and fluorescence measurements were taken, and then plants were destructively sampled for growth and biochemical measurements. The experiment was repeated.

Photosynthesis and fluorescence

The fourth leaf from the apex was used for measurement of both photosynthesis and fluorescence parameters. Net photosynthesis (P_{net}), transpiration (E), and conductance (g_s) were measured with an infrared gas-analyzer–based photosynthesis system.[2] All measurements were taken with photosynthetically active radiation inside the chamber of 500 μmol m^{-2} s^{-1}, and a constant internal chamber CO_2 concentration of 400 μmol mol^{-1}. A moderate light intensity was used for measurement, because the plants were acclimated to lowered light levels in the greenhouse; also we were targeting a subsaturated light intensity to detect endogenous differences between plant types and treatments without additional stress factors. Following photosynthesis measurements, light-adapted quantum yield was measured with a modulated chlorophyll fluorometer.[3] 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 quantum yield was computed as $[(F_{ms} - F_s)/F_{ms}]$. Dark adaptation for 5 min preceded measurements of ground-state fluorescence (F_0) and maximal fluorescence (F_m), and quantum efficiency of PSII, as given by the ratio of variable to maximal fluorescence (F_v/F_m), was calculated as $[(F_m - F_0)/F_m]$.

Growth parameters, nitrogen content, and tissue sampling

Plant height and number of nodes were measured prior to all other measurements. Leaf tissue was collected from the top of the plant, because nitrogen is mobilized to younger tissues, and individual leaves were sampled at the same nodal position on all plants to achieve a consistent age for leaf samples. Because leaves were sampled over a 2-d period, differences between individuals were minimized. All leaf tis-

sue above the fourth fully expanded leaf was excised, air-dried, pooled, and approximately 1 g for each sample was analyzed for Kjeldahl nitrogen content (Soil, Water, and Agricultural Testing Laboratory, New Mexico State University, Las Cruces, NM). Approximately 60 leaf discs were cut with a standard paper-hole puncher (0.6-cm diameter) from the fourth fully expanded leaf from the apex, the same leaf used to measure photosynthesis and fluorescence. Leaf discs were immediately frozen on dry ice, and stored at −20 C until biochemical analyses. The fifth, fully expanded leaf from the apex was harvested to measure leaf area with the use of a portable leaf area meter.[4] Remaining leaves and stems were dried separately in an oven at 70 C to constant weight.

Antioxidant and pigment analysis

Chlorophyll pigments and antioxidants were analyzed according to Gilmore and Yamamoto (1991) with some modification. Five leaf discs were homogenized in 350 μl of acetone in dim light, and extracted in the dark for 30 min by stirring on ice. Extracts were centrifuged at 7000 × *g* and at 4 C and for 5 min. Supernatant was filtered through 0.2-μm nylon syringe filters, and analyzed with the use of HPLC.[5] The mobile phase was composed of two solvents: Solvent A, acetonitrile:methanol:Tris HCl buffer 0.1M pH 8.0 (72:8:3) and Solvent B, methanol:ethyl acetate (68:32). The solvent program consisted of an isocratic elution of Solvent A for 6 min, followed by a 10-min linear gradient to 100% B. This was followed by a 1-min linear gradient to A, and finally 5 additional min of an isocratic elution of Solvent A to re-equilibrate the column before the next sample was injected. The flow rate was 1 ml min^{-1}, the injection volume was 20 μl, and autosampler temperature was 4 C. The stationary phase was a Spherisorb ODS-1 column (5-μm particle size, 250 × 4.6-mm I.D.) with a C_{18} guard column.[6] A photodiode array detector (A_{445} nm) was used for detection of chlorophylls and carotenoids, and alpha-tocopherol was detected with the use of a fluorescence detector with excitation at 295 and emission at 340 nm. The conversion state of the xanthophyll cycle (VAZ) was calculated as (Z + A)/(V + A + Z) where Z, zeaxanthin; A, antheraxanthin; V, violaxanthin. Chlorophyll and carotenoid standards were obtained to confirm HPLC peak identities, and to quantify chlorophylls, beta-carotene, and alpha-tocopherol.[7,8]

DPPH antioxidant assay

The colorimetric 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Yu et al. 2002) was used to determine the free-radical scavenging in vitro. It is known that 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) oxidizes many antioxidant compounds such as cysteine, glutathione, ascorbate, alpha-tocopherol, polyhydroxy aromatic compounds, and aromatic amino acids (Blois 1958). As $DPPH^{\cdot}$ becomes $DPPH_2$ it changes from deep purple to light yellow, which can be monitored by measuring the decline in absorbance of $DPPH^{\cdot}$ at 515 nm. Because $DPPH^{\cdot}$ is stable in solution, oxidizes common, naturally occurring antioxidants, and is not interfered with by glucose, it can be conveniently used for estimating antiradical activity of biological materials (Blois 1958).

Five leaf discs were homogenized in 800 μl of dimethyl sulfoxide (DMSO) and extracted by stirring for 10 min, centrifuged for 5 min at 7000 × *g*, and 500 μl of the supernatant was collected. A solution of 800 μM DPPH in 95% ethanol was combined with each plant sample (3:1, v/v, respectively) for a total of 200 μl per sample; each sample was blanked using 95% ethanol and absorbance was measured in triplicate. Standards of alpha-tocopherol and ascorbic acid were used as positive controls to monitor the effectiveness of the assay. Absorbance was measured at 515 nm on a microplate reader.[9] The DPPH scavenging activity was calculated on a fresh weight basis as $(C - S)/W$ where C is the mean A_{515} of the controls for each plate, S represents the mean A_{515} for each individual plant sample, and W is the fresh weight of each sample.

Anthocyanin assay

Total anthocyanin content was measured according to Schmidt and Mohr (1981). Ten leaf discs were homogenized in 1 ml water:1-propanol:HCl (81:18:1 by volume). The samples were placed in a boiling water bath for 3 min, incubated at room temperature overnight, and stored in the dark for 24 h. Extracts were then centrifuged for 20 min at 7000 × *g*. The supernatant was collected and absorbance read at 535 and 650 nm with the use of a spectrophotometer.[10] Data were corrected by subtracting the absorbance at 650 nm from that at 535 nm (A_{535}–A_{650}) to obtain total anthocyanin content.

Statistical analysis

Data for each experiment were analyzed individually using a general linear model (Proc GLM) with the use of the SAS® version 8.02 (SAS® Institute, Cary, NC) to determine if the experiment interacted with other factors. Because no significant interactions were detected, data were pooled across experiments and analyzed using the SAS® GLM procedure to calculate means and standard errors, then mean comparisons were performed for all parameters with least significant differences (LSD) pairwise comparisons at the significance level of $\alpha = 0.05$.

Results and Discussion

Physiological and growth responses

Because there was no species by N interaction only the main effects are reported. On the average, the leaf N content was reduced 33% in the nitrogen-deficient plants (Table 1). Among the plant types, leaf nitrogen allocation to the youngest leaves was highest in Pima S-7 cotton (Table 2). In other work studying nitrogen accumulation in different cotton varieties, Fritschi et al. (2004) reported that Pima cotton had higher N-use efficiency than Acala cotton. In addition, Pima cotton absorbed more nitrate from solution than Acala cotton under N limited conditions, possibly due to greater root surface area per unit of biomass, resulting in more uptake sites in roots (Aslam et al. 1997). Nitrogen assimilation also was greater in Pima than Acala because of increased nitrate reduction under limited N (Aslam et al. 1997). Weeds typically can accumulate more nutrients than crops (Di Tomaso 1995), but the opposite result occurred in this experiment, because Pima S-7 cotton had the highest leaf N content.

TABLE 1. Physiological responses and leaf area in cotton and spurred anoda under N deficiency. Data were pooled across experiments and plant types; $n = 48$.

Treatment	Leaf nitrogen	Gas exchange				Fluorescence				Node no.	Leaf area
							Dark adapted[a]				
		P_{net}	g_s	E	C_i	Quantum yield	F_v/F_m	F_0	F_m		
	g g^{-1}FW	μmol m^{-2} s^{-1}	mol m^{-2} s^{-1}	mmol m^{-2} s^{-1}	μmol mol^{-1}	$(F_{ms} - F_s)/F_{ms}$	$(F_m - F_0)/F_m$				cm^2
N+	27.6a[b]	13.84a	0.37a	6.41a	285a	0.67a	0.82a	235b	1286a	19a	98.2a
N−	18.4b	7.55b	0.20b	4.13b	295a	0.64b	0.79b	264a	1263a	18b	104.5a
P values	<0.0001	<0.0001	<0.0001	<0.0001	0.2659	0.0117	0.0002	<0.0001	0.3586	<0.0001	0.1826

[a] Abbreviations: FW, fresh weight; F_v, variable fluorescence; F_0, ground-state fluorescence; F_m, dark-adapted maximal fluorescence; F_s, steady-state fluorescence; F_{ms}, maximal fluorescence.
[b] Means followed by different letters in a column are significantly different, according to LSD test at the 5% probability level.

TABLE 2. Physiological responses and leaf area in cotton and spurred anoda under N deficiency. Data were pooled across experiments and nitrogen treatments; $n = 24$.

Plant type	Leaf nitrogen	Gas exchange				Fluorescence				Node no.	Leaf area
							Dark adapted[a]				
		P_{net}	g_s	E	C_i	Quantum yield	F_v/F_m	F_0	F_m		
	g g^{-1}FW	μmol m^{-2} s^{-1}	mol m^{-2} s^{-1}	mmol m^{-2} s^{-1}	μmol mol^{-1}	$(F_{ms} - F_s)/F_{ms}$	$(F_m - F_0)/F_m$				cm^2
Cotton											
Delta pine 5415	22.4b[b]	10.55a	0.24b	4.64b	288a	0.68a	0.81a	235b	1215b	14b	138.9b
Pima S-7	25.9a	10.59a	0.25b	5.06b	277a	0.69a	0.81a	239b	1261b	13c	173.5a
Spurred anoda											
Mississippi	23.1b	12.06a	0.39a	6.36a	300a	0.63b	0.80a	261a	1339a	24a	39.8d
New Mexico	20.6b	9.56a	0.27b	5.02b	296a	0.62b	0.80a	262a	1284ab	24a	53.3c
P values	<0.0001	0.0847	0.0006	0.0442	0.3262	0.0013	0.5954	<0.0001	<0.0001	<0.0001	<0.0001

[a] Abbreviations: FW, fresh weight; F_v, variable fluorescence; F_0, ground-state fluorescence; F_m, dark-adapted maximal fluorescence; F_s, steady-state fluorescence; F_{ms}, maximal fluorescence.
[b] Means followed by different letters in a column are significantly different, according to LSD test at the 5% probability level.

TABLE 3. Plant growth and alpha-carotene concentration in response to N treatment. Data were pooled across experiments; $n = 12$.

Variety	Nitrogen	Dry weight	Height	Height:node	Specific leaf area	Alpha carotene peak area
		g plant^{-1}	cm	cm node^{-1}	cm^2 g^{-1}	g^{-1} FW[a]
Delta pine 5415	+	53.2a[b]	86d	6.0b	15.6b	3319b
Pima S-7	+	54.6a	104b	7.9a	15.3b	4587a
Mississippi	+	52.9a	105b	4.2e	14.8bc	1489c
New Mexico	+	52.0ab	119a	4.9d	16.4ab	1598c
Delta pine 5415	−	44.9c	72e	5.5c	13.2c	2339bc
Pima S-7	−	49.1b	95c	7.5a	13.3bc	2028c
Mississippi	−	36.2d	109b	4.6d	18.1a	1347c
New Mexico	−	42.8c	117a	5.0d	15.5b	1663c
P values		0.0003	0.0037	0.0012	0.0002	0.0076

[a] FW, fresh weight.
[b] Means followed by different letters in a column are significantly different, according to LSD test at the 5% probability level.

The reduction in leaf N levels influenced all physiological parameters evaluated (Table 1). Photosynthesis decreased 45% in response to N deficiency regardless of plant type. Similarly g_s and E were reduced 46 and 36%, respectively. Ground-state fluorescence (F_0) increased 11%, quantum yield decreased 4.4%, and PSII efficiency decreased 3.7% in all plant types under N deficiency.

Although photosynthesis and dark-adapted quantum PSII efficiency did not differ among species, regardless of nitrogen stress, species responded differently for other physiological parameters (Table 2). Conductance and T_s were greatest in MS spurred anoda. Quantum yield was lower in spurred anoda than cotton regardless of N status. Ground-state fluorescence was 9% greater in spurred anoda. Maximal fluorescence was the highest in MS, and was 9 and 6% higher than DP 5415 and Pima S-7, respectively.

Although photosynthesis was reduced by as much as 45% in plants without N, fluorescence did not increase comparably. The increase in F_0 may provide an indication of thermal damage of PSII, or photoinhibition (Krause and Weis 1984) in nitrogen-deficient plants. The individual fluorescence parameters, F_0 and F_m, were lower in cotton, suggesting that the proportion of excitation energy quenched through dissipation as heat is greater in cotton than spurred anoda. The decrease in photosynthetic efficiency in N-deficient plants, as measured with fluorescence, was approximately 4%, which suggests that reduced energy harvesting by oxidized PSII reaction centers in light was not the main cause of photosynthetic reduction. However, the decrease of F_v/F_m indicates that there was some photoinactivation of PSII reaction centers under N deficiency. This decrease may also be due, in part, to the reduced amount of pigment exhibited in plants where N was withheld, resulting in less light capture overall.

The decreased photosynthesis across species was associated with a large reduction in g_s, which would limit the potential for carbon fixation due to inadequate access to CO_2. These results are consistent with other reports with effects of nitrogen nutrition on photosynthesis in Pima cotton (Reddy et al. 1996) and in a previous experiment investigating spurred anoda interference on physiological and antioxidant properties of cotton (Ratnayaka et al. 2003). Pima cotton has high rates of g_s and photosynthesis (Cornish et al. 1991), and typically has a higher E rate than upland cotton cultivars during early and late season growth (Munk et al. 2004). However, significant differences in E between cotton cultivars were not evident in this experiment, or in previous work examining prometryn tolerance (Waldrop et al. 1996). Additionally, other studies have demonstrated that RuBP carboxylase activity decreases under low nitrogen levels, causing reduced carbon fixation and lower rates of photosynthesis in Pima cotton (Reddy et al. 1996), apple (*Malus domestica* Borkh) (Cheng and Fuchigami 2000), and a variety of other C_3 plant species (Evans 1989). Because of this decreased photosynthetic efficiency, light energy can become excessive (Chen and Cheng 2003), ultimately leading to a buildup of harmful reduced oxygen molecules (Logan et al. 1999), thereby increasing oxidative stress across species.

In addition to the effects on physiological responses, N deficiency affected plant growth and structural features. There were no N treatment by plant type interactions for node number and leaf area variables; therefore only the main effects are presented. Regardless of plant type, node number decreased from 19 to 18 under N deficiency, although leaf size was not affected (Table 1). Both spurred anoda accessions had a greater number of nodes than either cotton cultivar, with MS having the greatest number, and Pima S-7 the least. The leaf size was the largest in Pima S-7 and the smallest in MS.

Interactions between N treatment and plant type were significant for dry weight, height, height:node ratio, and specific leaf area (Table 3). Total dry weight was reduced in all plant types under N deficiency, in which MS showed the largest reduction, 32% and Pima S-7 the least, 10%. Species differences for photosynthesis were not significant (Table 2), indicating that some other factor may have been responsible for the decreased biomass accumulation in MS. Mississippi spurred anoda had considerably higher rates of g_s and E compared to other species, along with the least biomass accumulation, suggesting that MS was the least water-use efficient. However, instantaneous water-use efficiency values were not different among species (data not shown).

Nitrogen deficiency reduced plant height in cotton, but not in spurred anoda (Table 3). Height was reduced 16% in DP 5415 and 9% in Pima S-7. Plant height is typically reduced under environmental stress (Kerby et al. 1997). Although spurred anoda height was unchanged by N level; the NM accession was taller than the MS accession. This observation is consistent with the typical growth habits of these

two plant accessions when grown in a common garden (VanGessel et al. 1998).

Height:node ratio increased by 9% for MS spurred anoda but decreased 8% for DP 5415 due to low N. Both cotton species had higher height:node ratios than either spurred anoda accession, and Pima S-7 cotton retained the largest height:node ratio of all the species under deficient nitrogen conditions. Increases in height:node ratio in MS under N deficiency may provide a competitive advantage over cotton under field conditions, if they grow taller and shade developing cotton. Mississippi spurred anoda had the lowest height:node ratio compared to other plant types. Increased height:node ratio measurements can provide an indication of increased crop vigor early in the season (Kerby et al. 1997). Variations in height:node ratio can also provide a measure of induced environmental stress, and can give an indication of the source/sink balance in plants as they shift from vegetative to reproductive growth (Kerby et al. 1998).

Specific leaf area also exhibited an interaction between nitrogen treatment and plant type. Specific leaf area decreased 15% in DP 5415, but increased 18% in MS as a result of decreased nitrogen. Specific leaf area was determined to be inversely related to starch content in cotton leaves (Reddy et al. 1989). Under nitrogen deficiency, a higher percentage of starch was found to contribute to cotton leaf mass (Radin and Eidenbock 1986).

Plant pigments and antioxidants

Interactions between species and N were not significant for any of the plant pigments or antioxidants studied except alpha-carotene, so only main effects of nitrogen and plant type will be discussed. When N was withheld, chlorophyll a and b were lowered regardless of plant type by at least 39% (Table 4). A reduced chlorophyll level under low N is typical in leaves of C_3 plants (Evans 1989). With lower chlorophyll, accompanied by less efficient photochemistry, light absorption can decrease, helping to minimize potential photosystem damage, even under moderate light, which may result in photoinhibition and further degradation of photosensitive chlorophyll due to a buildup of reactive oxygen species (Müller 2001). Similar to the response observed in this study under N deficiency, prometryn application reduced chlorophyll a:b ratio in Pima and DP 5415 cotton (Hernández-Ríos 2004). Chlorophyll a:b, however, was unaffected by the effects of cotton and spurred anoda interference and mild drought in a previous experiment (Ratnayaka et al. 2003). Chlorophyll a and b were interconvertible in cucumber (Ito et al. 1993), which could give plants a dynamic ability to control chlorophyll content. Therefore, stomatal regulation coupled with less available chlorophyll is most likely responsible for the decrease in photosynthesis of the plants studied.

Xanthophyll de-epoxidation and alpha-tocopherol concentration increased by 30% and 17%, respectively, with nitrogen deficiency, but lutein, violaxanthin, and antheraxanthin levels decreased 31%. Levels of beta-carotene, anthocyanin, and DPPH-scavenging ability were not altered by N stress. Similar responses of chlorophyll content and antioxidants were found in grape (*Vitis labrusca* L. cv. Concord) leaves (Chen and Cheng 2003) and 'Fuji' apple leaves (Cheng 2003) under nitrogen deficiency, in which chlorophyll content decreased and VAZ increased.

Leaf antioxidant and pigment concentrations varied among plant types (Table 5). Pima S-7 cotton had 22, 26, and 35% more total chlorophyll than DP 5415, MS, and NM, respectively. The lowest chlorophyll a:b ratio, 2.01, was found in NM. On the other hand, VAZ was highest in NM with a value of 0.631, which was 19, 27, and 32% higher than MS, Pima S-7, and DP 5415, respectively. Thus, NM spurred anoda had the lowest violaxanthin and the highest zeaxanthin concentrations, regardless of nitrogen status. Cotton had lower levels of antheraxanthin than spurred anoda. Lutein levels were the highest in Pima S-7 cotton, containing 22, 29, and 30% more lutein than MS, NM, and DP 5415, respectively. Compared to spurred anoda, cotton had at least two and three times more alpha-tocopherol and DPPH scavenging ability, respectively.

Alpha-carotene was the only antioxidant to exhibit differential species responses as a result of N deficiency (Table 3). Regardless of N stress, both cotton cultivars had higher levels of alpha-carotene than either accession of spurred anoda. Nitrogen deficiency reduced alpha-carotene in Pima S-7 cotton by 56%, but had no effect on the other plant types studied. Alpha-carotene is a precursor in the biosynthetic pathway of lutein; thus the sharp drop in alpha-carotene concentration in Pima S-7 as a result of N deficiency may be responsible for the concomitant rise in lutein levels.

Biomass accumulation diminished in each of the four plant types under N deprivation, indicating that N deficiency reduced plant productivity. Pima S-7 showed the least decrease in biomass compared to other plant types. One possible explanation for this is that chlorophyll levels and lutein concentrations were highest in Pima S-7 cotton. Lutein is an abundant carotenoid pigment typically found in the light-harvesting complex of PSII (Siefermann-Harms 1987), which has either a direct or indirect effect on heat dissipation of PSII (Niyogi et al. 1998; Pogson et al. 1998). Other carotenoid pigments such as violaxanthin and beta-carotene may substitute for lutein at PSII (Pogson et al. 1996), but evidence suggests that this substitution could result in decreased efficiency in nonphotochemical quenching of excess energy (Gilmore 2001). Even though lutein may not be absolutely essential to photosynthesis in plants (Pogson et al. 1996; Taylor 1996), deficiency of this pigment decreases nonphotochemical quenching of PSII (Pogson et al. 1998). Thus the smaller size of the light-harvesting complex, as indicated by lowered lutein and chlorophyll, and also supported by the decrease in F_v/F_m resulted in diminished photosynthetic efficiency.

Xanthophyll pigments have important roles in light-harvesting complexes in plants including light absorption, quenching of excited triplet chlorophyll, structural stabilization of the light-harvesting apparatus (Peterman et al. 1997), and also dissipation of excess energy as heat. When carbon fixation is limited by environmental stresses, hydrogen ions accumulate in the thylakoid lumen. This low-lumen pH activates violaxanthin de-epoxidase, which converts the xanthophyll pigment violaxanthin into zeaxanthin via antheraxanthin (Bugos and Yamamoto 1996; Demmig-Adams and Adams 1992). Excess excitation energy is then quenched through thermal dissipation (Cheng 2003; Gilmore 2001) as a result of conformational changes in PSII due to binding of zeaxanthin and antheraxanthin to its proteins (Gilmore 1997; Müller et al. 2001). Thermal dissi-

TABLE 4. Plant pigment and antioxidant responses to nitrogen treatment. Data were pooled across experiments and plant type; $n = 48$.

	Chlorophyll		Carotenids						Antioxidants		
Treatment	Total	a:b	Beta carotene	Lutein (×1000)	Violaxanthin (×1000)	Antheraxanthin (×1000)	Zeaxanthin (×1000)	VAZ[a]	Alpha-tocopherol	Anthocyanins ($A_{535} - A_{650}$)	DPPH (A_{515})
	μg g^{-1} FW		μg g^{-1} FW	PA g^{-1} FW					ng g^{-1} FW	g^{-1} FW	g^{-1} FW
N+	3408a[b]	2.22a	1621a	338a	111a	30a	47a	0.418b	65b	19.7a	82a
N−	2070b	2.04b	1533a	232b	50b	23b	51a	0.598a	79a	19.7a	83a
P values	<0.0001	<0.0001	0.3744	<0.0001	<0.0001	0.0015	0.3390	<0.0001	0.0235	0.0855	0.9562

[a] Abbreviations: PA, HPLC peak area; FW, fresh weight; VAZ, xanthophyll cycle conversion state; DPPH, 2,2-diphenyl-1-picrylhydrazyl.
[b] Means followed by different letters in a column are significantly different, according to LSD test at the 5% probability level.

TABLE 5. Plant pigment and antioxidant profiles in cotton and spurred anoda. Data were pooled across experiments and N treatment; $n = 24$.

	Chlorophyll		Carotenoids								
Variety	Total	a:b	Beta carotene	Lutein (×1000)	Violaxanthin (×1000)	Antheraxanthin (×1000)	Zeaxanthin (×1000)	VAZ[a]	Alpha-tocopherol	Anthocyanins ($A_{535} - A_{650}$)	DPPH (A_{515})
	μg g^{-1} FW		μg g^{-1} FW	PA g^{-1} FW					ng g^{-1} FW	g^{-1} FW	g^{-1} FW
Cotton											
Delta pine 5415	2712b[b]	2.20a	1406a	249b	85ab	22b	37b	0.433b	100a	23.5a	113a
Pima S-7	3462a	2.14a	1591a	356a	102a	23b	50a	0.459b	100a	15.9b	118a
Spurred anoda											
Mississippi	2549b	2.15a	1642a	279b	79b	29a	49a	0.509b	50b	ND[c]	45b
New Mexico	2233b	2.01b	1667a	255b	55c	31a	61a	0.631a	37b	ND	34b
P values	0.0003	<0.0001	0.2372	0.0018	0.0003	0.0047	0.0018	<0.0001	<0.0001	<0.0001	<0.0001

[a] Abbreviations: PA, HPLC peak area; FW, fresh weight; VAZ, xanthophyll cycle conversion state; DPPH, 2,2-diphenyl-1-picrylhydrazyl.
[b] Means followed by different letters in a column are significantly different, according to LSD test at the 5% probability level.
[c] Not detected.

pation of excess heat through xanthophyll cycle conversion has been correlated with a decrease in F_v/F_mmeasured in apple leaves (Cheng 2003). This phenomenon was evident in this study. Regardless of plant type, nitrogen deficiency decreased $F_v/F_m$by 4%, whereas VAZ increased by 30%.

Both cotton species were superior over either spurred anoda accession at scavenging free radicals as evidenced by the DPPH assay. The presence of considerably higher constitutive levels of alpha-tocopherol may have increased the antioxidant capabilities of DP 5415 and Pima S-7 compared to spurred anoda. Alpha-tocopherol scavenges membrane-damaging ROS and helps prevent lipid peroxidation (Fryer 1992; Pogson 1996). Alpha-tocopherol is also important in protecting photosynthetic pigments (Wise and Naylor 1987). There were no differences in beta-carotene concentration among the species studied.

The concentration of anthocyanins was 32% higher in DP 5415 than in Pima S-7, but undetectable in spurred anoda. Anthocyanins are a group of compounds that shield plant leaves from damaging UV radiation, and thus help prevent photoinhibition (Deikman and Hammer 1995; Hoch et al. 2003). Higher concentrations of anthocyanins usually occur early in the development of the plant, or under environmental conditions such as high light, low temperature, and nutrient deficiencies of phosphorus and nitrogen (Steyn et al. 2002). Anthocyanins are thought to provide photoprotection before other defenses, such as the xanthophyll cycle, become fully functional (Manetas et al. 2002; Steyn et al. 2002). The presence of detectable levels of anthocyanins may have increased the antioxidant capabilities of cotton compared with spurred anoda. There was no change in anthocyanin levels due to nitrogen deficiency in cotton, indicating that their synthesis was not impacted by N deficiency.

Although anthocyanins were not detected, in spurred anoda leaves exhibited a reddish-purple coloration under N deficiency. Because the uniquely colored betalain pigments occur exclusively in the plant order Caryophyllales (Nielson and Harley 1996), these pigments are not likely the cause of the red coloration of spurred anoda. The possibility that anthocyanins were degraded during extraction from spurred anoda because anthocyanins can be unstable under high pH conditions (Mazza and Brouillard 1987) cannot be ruled out, although the acidic conditions used during extraction should have stabilized any red color. Although the red, cationic form is predominant under extremely acidic conditions, pH increases could have caused an equilibrium shift toward the colorless carbinol pseudobase, which is the favored neutral analog (Mazza and Brouillard 1987). Furthermore, given that there are many different anthocyanins and analogs found in plants, the specific anthocyanins present in cotton may be different from those in spurred anoda. However, because we did successfully extract anthocyanins from cranberries (*Vaccinium sp.*) and blueberries (*Vaccinium sp.*), species with large anthocyanin levels (data not shown), it is likely spurred anoda does not possess detectable levels of anthocyanins.

Because greater biomass was accumulated in Pima S-7 cotton relative to the other plant types tested under N deficiency, Pima S-7 demonstrated a potential advantage. Protective mechanisms that help to minimize oxidative stress may play a role in mediating some of the damage to plant productivity that occurs due to environmental stress. Pima S-7 had the highest levels of leaf N, chlorophyll, and lutein, maintained the largest height:node ratio, and exhibited the least biomass reduction in response to nitrogen deficiency. Both cotton species had considerably higher levels of alpha-tocopherol and greater antiradical activity compared to spurred anoda under the conditions of cropping systems. These characteristics may provide cotton an advantage over spurred anoda by alleviating the effects of oxidative stress imposed through the effects of competition.

Sources of Materials

[1] Metro-Mix 360, Scotts-Sierra Horticultural Products Co., 14111 Scottslawn Rd., Marysville, OH 43041.

[2] LI-6400 photosynthesis meter, Li-Cor Inc., 4421 Superior Street, Lincoln, NE 68504.

[3] OS5-FL modulated chlorophyll fluorometer, Opti-Sciences Inc., 164 Westford Rd. No. 4, Tyngsboro, MA 01879.

[4] LI-3000 leaf area meter, Li-Cor Inc., 4421 Superior Street, Lincoln, NE 68504.

[5] Agilent 1100 HPLC automated with Chemstation Rev A.08.03 [847], Agilent Technologies, 395 Page Mill Rd., Palo Alto, CA 94306.

[6] Spherisorb ODS-1 column, (5-μm particle size, 250 by 4.6-mm I.D.) with a C_{18} guard column, Alltech Associates Inc., 2051 Waukegan Rd., Deerfield, IL 60015.

[7] Sigma-Aldrich Corporation, P.O. Box 14508, St. Louis, MO 63178.

[8] DHI Water and Environment, Agern Allé 5. DK-2970 Hørsholm, Denmark.

[9] Emax Precision microplate reader, Molecular Devices, 1311 Orleans Dr., Sunnyvale, CA 94089-1136.

[10] HP 8453 UV-Vis spectrophotometer equipped with Chemstation A.02.05, Hewlett-Packard Company, 20555 SH249, Houston, TX 77070.

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