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# Plant Morphological and Biochemical Responses to Field Water Deficits

## I. RESPONSES OF GLUTATHIONE REDUCTASE ACTIVITY AND PARAQUAT SENSITIVITY

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### ABSTRACT

**The effects of water deficits on plant morphology and biochemistry were analyzed in two photoperiodic strains of field-grown cotton (*Gossypium hirsutum* L.). Plants grown under dryland conditions exhibited a 40 to 85% decrease in leaf number, leaf area index, leaf size, plant height, and total weight per plant. Gross photosynthesis decreased from 0.81 to 0.47 milligram $CO_2$ fixed per meter per second and the average midday water, osmotic, and turgor potentials decreased to −2.1, −2.4, and 0.3 megapascals, respectively.**

**There was a progressive increase in glutathione reductase activity and in the cellular antioxidant system in the leaves of stressed plants compared to the irrigated controls. The stress-induced increases in enzyme activity occurred at all canopy positions analyzed.**

**Irrigation of the dryland plots following severe water stress resulted in a 50% increase in leaf area per gram fresh weight in newly expanded leaves of both strains over the leaves which had expanded under the dryland conditions. Paraquat resistance (a relative measure of the cellular antioxidant system) decreased in the strain T25 following irrigation. Glutathione reductase activities remained elevated in the T25 and T185 leaves which were expanded fully prior to irrigation and in the leaves which expanded following the irrigation treatment.**

The midday closure of stomates in water-stressed crops (19) limits carbon assimilation, yet may optimize the water use efficiency of the plant on a daily basis (2). Stomatal closure results in a decrease in internal $CO_2$ concentration, and a concomitant decline in photosynthesis results from the diminished availability of $CO_2$ for carbon fixation. Because of the reduction in $CO_2$, toxic oxygen species within the leaf can increase as a consequence of the transfer of electrons from the photosynthetic electron transport chain, at the level of ferredoxin, to molecular oxygen (13). Excessive levels of these toxic oxygen species can damage plant tissues by several mechanisms, including the diversion of normal metabolic pathways into abnormal routes, the inactivation of enzymes, the formation of $H_2O_2$, lipid peroxidation, the formation of singlet oxygen, and production of oxygen-free radicals.

Chloroplasts are particularly sensitive to effects of oxygen toxicity. The process of light capture and conversion of the light energy into the chemical energy not only produces the driving force for carbon fixation but also can produce singlet oxygen, superoxide, $H_2O_2$, and hydroxyl radicals. Several chloroplast enzymes of the carbon fixation pathway lose activity upon reaction of essential sulfhydryl groups with activated $O_2$ species (11), and the highly unsaturated fatty acids of the thylakoids enhance the possibility of lipid peroxidation (12).

Plant cells contain high (mM) concentrations of GSH[2] that can protect enzyme thiol groups from oxidation. Because of the high cellular concentrations of this tripeptide, GSH is more accessible for reaction with $O_2$ than enzyme thiol groups, thereby protecting the enzymes from inactivation. GSH can also reactivate enzymes by reducing oxidized protein sulfhydryl groups. When GSH reacts with $O_2$, the glutathione is oxidized to form GSSG. The oxidation of GSH by $O_2$ occurs rapidly in plants at alkaline pH values where the reaction is catalyzed by metal ions and other cofactors (4, 18). The subsequent rereduction of GSSG to GSH is catalyzed by the enzyme glutathione reductase in an NADPH-dependent reaction. Glutathione reductase, therefore, plays an essential role in the protection of chloroplasts against oxidative damage by maintaining a high GSH/GSSG ratio.

It is thought that the mode of herbicidal action of paraquat results from the production of $H_2O_2$, superoxide, and other highly toxic free-radicals by reaction of molecular oxygen with the reduced paraquat radical formed in the chloroplast during photosynthesis (1, 3). Therefore, paraquat is a useful tool for studying the overall effectiveness of the chloroplast antioxidant system since increased resistance to paraquat may result from increases in the activities of the enzymes and substrates involved in the removal of the toxic oxygen products.

This study investigates the effects of water deficits upon plant morphology and the activity of glutathione reductase from two photoperiodic cotton strains. A relative measure of changes in the overall antioxidant system was obtained by analysis of leaf sensitivity to paraquat.

### MATERIALS AND METHODS

Two nonflowering (photoperiodic) cotton (*Gossypium hirsutum* L.) strains were planted under two soil-water regimes at Lubbock, TX. Three replications of each strain were planted on May 17, 1983. The two soil-water regimes consisted of fully irrigated and dryland tests located in the same field about 30 m apart. The soil type was an Acuff fine sandy loam soil (fine-loamy, mixed, thermic, Aridic Paleustolls). The strains in the dryland treatment were planted in a rainout shelter to prevent the addition of supplemental water by rainfall, while the irrigated plants were located in plots outside the confines of the rainout shelter. The irrigated plants were watered weekly to field capacity with a drip irrigation system. The Texas designations of the

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[2] Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; DAP, days after planting.

strains analyzed in this study are T25 and T185. The strains were planted in five 3 m rows with 1 m between rows. Plants were thinned after emergence to 11 plants/m. The three center rows were used for morphological and enzymic analyses.

**Determination of Leaf Water Status and Gross Photosynthesis.** All measurements were made on the same uppermost, fully expanded leaf. Data were collected between 1000 and 1600 CST and at light levels greater than 1500 $\mu$E $m^{-2}$ $s^{-1}$. Leaf water status was measured with Merrill leaf cutter psychrometers (17) calibrated with NaCl solutions at 30°C in a water bath controlled to ±0.1°C. All readings were made in the water bath. Leaf samples were punched from intact leaves, immediately sealed in the psychrometer chambers, and equilibrated for 4 h prior to measuring water potential. After the water potential was determined, the psychrometer chambers were placed in liquid $N_2$ for about 1 min then allowed to equilibrate for 1 h before the osmotic potential was estimated. Turgor pressure was estimated as the difference between leaf water potential and osmotic potential.

Gross photosynthetic rates were determined with $^{14}CO_2$ (16), the leaf discs were placed in glass liquid scintillation vials and digested in 0.2 ml of 70% (v/v) $HCO_4$ and 0.2 ml of 30% (v/v) $H_2O_2$ at room temperature. After 24 h, 15 ml of scintillation cocktail consisted of toluene (75% by volume), Triton X-100 (3:1, v/v) with 6.0 g/L 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene.

**Determination of Morphological and Enzymic Changes.** Leaf samples from both irrigated and dryland plots were harvested four times throughout the growing season for analysis of glutathione reductase activity. On the last harvest date, plants from both the irrigated and dryland plots were sampled for growth analyses. Measurements included fresh weight, dry weight, height, leaf area index, number of nodes, leaf number, and leaf size. After the last harvest date, the plots in the rainout shelter were irrigated with 10 to 12.5 cm of water. The stressed plants were allowed to recover for 2 weeks, at which time leaves were sampled for glutathione reductase activity and paraquat sensitivity. Following the irrigation of the rainout shelter, only those plants within the shelter were harvested for analysis. Tissue samples consisted of leaves that were either: (a) fully expanded prior to irrigation of the shelter; or (b) expanded after the irrigation.

To determine if leaf position in the canopy affected the activity of glutathione reductase or sensitivity to paraquat, leaves were excised from the base, middle, and top of the canopy. Three leaf samples were analyzed at each position on all of the sampling dates. Prior to leaf homogenization for subsequent enzyme analysis, leaf areas and fresh weights were recorded.

Leaf samples utilized for analysis of glutathione reductase activity were prepared by homogenization of leaves in a Waring Blendor for 30 s at 4°C. The grinding medium contained 0.1 M acetate buffer (pH 4.0), 20 mM 2-mercaptoethanol, and 0.1% (w/v) BSA. PVPP (2 g/2 ml of grind) added to the leaf samples to scavenge leaf phenolics. Homogenates were filtered through 4 and then 12 layers of cheesecloth and centrifuged at 17,000*g* for 15 min. The supernatant was subsequently assayed for glutathione reductase activity.

Glutathione reductase was assayed at 25°C in a 1-ml reaction mixture containing 0.1 mM NADPH, 0.18 M Tricine-NaOH (pH 7.8), and 50 to 100 $\mu$l of supernatant. The reaction was initiated by the addition of 50 mM GSSG and the rate of NADPH oxidation was monitored at 340 nm. Glutathione reductase activity was expressed as nmol NADPH oxidized $min^{-1}$ $cm^{-2}$.

**Analysis of Leaf Antioxidant Protection Levels.** Paraquat sensitivity was determined 3 times during the growing season using the leaf spot test. A minimum of nine leaves of each strain under dryland and irrigated conditions were analyzed to determine if stress-induced changes had occurred in the minimum effective paraquat concentration. The minimum effective paraquat concentration was defined to be the lowest concentration required to produce paraquat-induced necrotic spots. The concentrations of paraquat used in this study were $10^{-2}$ M, $5 \times 10^{-3}$ M, $10^{-3}$ M, $5 \times 10^{-4}$ M, and $10^{-4}$ M. Paraquat was applied to the abaxial surface of the leaf using Q-tips[3] dipped in the appropriate paraquat concentration. Necrosis at the point of paraquat application was evaluated 6 h after the initial application.

## RESULTS AND DISCUSSION

The effects of water deficits on the water status and development of two photoperiodic cotton strains were monitored throughout the growing season by measurements of: (a) changes in leaf water status; and (b) reduction in plant growth. In conjunction with these analyses, measurements of glutathione reductase activity and the overall effectiveness of the chloroplast antioxidant system were determined.

**Changes in Leaf Water Status and Gross Photosynthesis.** The midday leaf water status of the two photoperiodic cotton strains was monitored at 50, 78, 92, and 106 DAP. The water status data are presented in Table I. Approximately a 2-fold difference in leaf water potential occurred between the irrigated and dryland treatment at 50, 78, and 92 DAP. Throughout the course of this experiment, the effects of the soil water deficits on leaf water status were enhanced in the T185 material compared with T25.

The T25 turgor potentials for both the irrigated and dryland treatments remained relatively constant between treatments, with a reduction in the turgor potential from 0.82 to 0.45 MPa from 50 to 106 DAP (Table I). The T185 turgor potentials exhibited a greater decline between 50 and 106 DAP with an average decrease from 0.75 to 0.12 MPa. The leaf water status data show the progressive development of water stress on the two photoperiodic cotton strains.

Analysis of gross photosynthesis at 50 and 106 DAP also illustrates the severity of the water stress. At 50 DAP, the irrigated and dryland treatments of both T25 and T185 had photosynthetic rates of 0.72 to 0.81 mg $CO_2$ fixed $m^{-2}$ $s^{-1}$. Similar measurements at 106 DAP showed photosynthetic rates in the irrigated treatment between 0.78 and 0.97 mg $CO_2$ fixed $m^{-2}$ $s^{-1}$. The dryland treatments, however, had photosynthetic rates between 0.36 and 0.50 mg $CO_2$ fixed $m^{-2}$ $s^{-1}$ at 106 DAP. These results demonstrate that the water stress was severe enough by the end of the growing season to result in a 50% decrease in the rate of leaf photosynthesis in both strains.

**Stress-Induced Changes in Plant Morphology.** The second parameter used to identify the severity of the soil water deficit was analysis of changes in the growth parameters of the two photoperiodic cotton strains at 106 DAP. All of the parameters exhibited dramatic declines under the stress conditions (Table II). Plant height decreased 65% between the irrigated and dryland treatments of T25, while T185 showed a 80% reduction in plant height. The magnitude of the reduction in plant height, leaf area index, plant dry weight, and leaf number between irrigated and dryland was greater in T185 than T25 treatments. Leaf size, however, showed a similar decline in both strains with T25 and T185 exhibiting a 34% and 36% decline, respectively.

**Stress-Induced Changes in Glutathione Reductase Activity.** The activity of the antioxidant enzyme, glutathione reductase, in plants from both irrigated and dryland treatments was monitored from 50 DAP until 106 DAP. The changes in glutathione reductase activity associated with soil water deficits are presented in Figure 1. T25 and T185 irrigated samples showed a decline in

[3] Mention of a trade name does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Table I. *Midday Water Status of Two Photoperiodic Cotton Strains under Irrigated and Dryland Conditions*

| Strain | Treatment | Potential | Days after Planting | | | |
|---|---|---|---|---|---|---|
| | | | 50 | 78 | 92 | 106 |
| | | | *MPa* | | | |
| T25 | Irrigated | Water | −0.45 ± 0.03 | −0.62 ± 0.09 | −0.68 ± 0.11 | −1.34 ± 0.12 |
| | Dryland | Water | −0.83 ± 0.03 | −1.29 ± 0.09 | −1.48 ± 0.12 | −1.89 ± 0.08 |
| | Irrigated | Osmotic | −1.29 ± 0.06 | −1.24 ± 0.06 | −1.03 ± 0.14 | −1.73 ± 0.07 |
| | Dryland | Osmotic | −1.63 ± 0.07 | −1.90 ± 0.11 | −1.76 ± 0.11 | −2.38 ± 0.11 |
| | Irrigated | Turgor | 0.84 ± 0.05 | 0.62 ± 0.06 | 0.34 ± 0.05 | 0.39 ± 0.07 |
| | Dryland | Turgor | 0.81 ± 0.05 | 0.60 ± 0.09 | 0.28 ± 0.10 | 0.49 ± 0.08 |
| T185 | Irrigated | Water | −0.67 ± 0.07 | −0.75 ± 0.11 | −0.87 ± 0.08 | −1.60 ± 0.12 |
| | Dryland | Water | −0.94 ± 0.05 | −1.62 ± 0.11 | −2.01 ± 0.11 | −2.38 ± 0.17 |
| | Irrigated | Osmotic | −1.51 ± 0.06 | −1.43 ± 0.10 | −1.10 ± 0.11 | −1.72 ± 0.04 |
| | Dryland | Osmotic | −1.64 ± 0.09 | −1.96 ± 0.13 | −2.13 ± 0.17 | −2.51 ± 0.19 |
| | Irrigated | Turgor | 0.84 ± 0.06 | 0.68 ± 0.09 | 0.23 ± 0.05 | 0.12 ± 0.10 |
| | Dryland | Turgor | 0.65 ± 0.04 | 0.34 ± 0.06 | 0.12 ± 0.08 | 0.12 ± 0.08 |

Table II. *Morphological Responses of Two Photoperiodic Cotton Strains to Irrigated and Dryland Conditions*

| | | T25 | T185 |
|---|---|---|---|
| Height (cm) | Irrigated | 108.4 ± 8.7 | 135.0 ± 1.9 |
| | Dryland | 38.0 ± 3.3 | 27.0 ± 3.6 |
| LAI | Irrigated | 5.2 ± 0.6 | 5.2 ± 0.3 |
| | Dryland | 1.3 ± 0.2 | 0.8 ± 0.1 |
| Dry wt (g plant$^{-1}$) | Irrigated | 57.6 ± 8.3 | 57.2 ± 3.2 |
| | Dryland | 18.0 ± 3.2 | 9.5 ± 2.5 |
| Leaf size (cm$^2$) | Irrigated | 62.0 ± 5.3 | 132.0 ± 9.5 |
| | Dryland | 41.0 ± 1.7 | 85.0 ± 2.7 |
| Leaf no. | Irrigated | 82.0 ± 8.0 | 37.0 ± 6.3 |
| | Dryland | 32.0 ± 5.2 | 7.0 ± 1.7 |

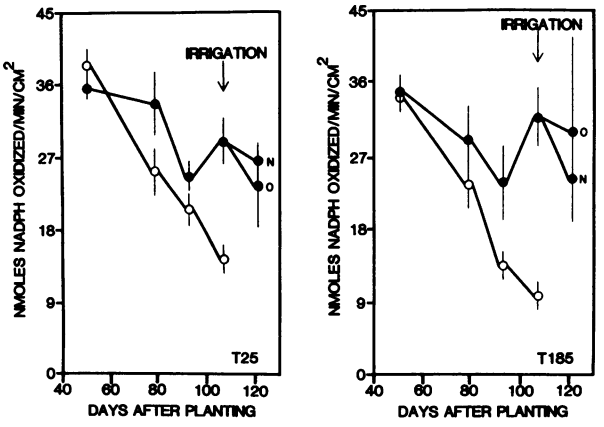


FIG. 1. Changes in glutathione reductase activity in the leaves of two photoperiodic cotton strains under irrigated (O) and dryland (●) conditions. Enzyme activity is expressed as a function of leaf area. Each data point represents the mean of nine samples ± SE.

the activity of glutathione reductase with age. Enzymic activity in T25 irrigated samples declined from 39 nmol NADPH oxidized min$^{-1}$ cm$^{-2}$ at 50 DAP to 14 nmol NADPH oxidized min$^{-1}$ cm$^{-2}$ at 106 DAP, while T185 irrigated samples showed a decline from 34 nmol NADPH oxidized min$^{-1}$ cm$^{-2}$ at 50 DAP to 9 nmol NADPH oxidized min$^{-1}$ cm$^{-2}$ at 106 DAP. T25 and T185 dryland samples did not show the dramatic decline in glutathione reductase activity with minimum activities of 25 and 26 nmol NADPH oxidized min$^{-1}$ cm$^{-2}$, respectively. At 106 DAP, glutathione reductase activity in the dryland plants was 2 to 3 times greater than the activity in the irrigated controls (Fig. 1). The elevation in glutathione reductase activity in the dryland samples was maintained in leaves that had expanded fully prior to subsequent irrigation at 107 DAP (O). This elevated activity was also observed in leaves that had expanded fully during the 2 weeks following the rainout shelter irrigation (N).

Leaves harvested from the basal, middle, and apical portion of the canopy exhibited a 2- to 4-fold increase in glutathione reductase activity in response to water stress (Table III). These results are in contrast to what has been observed in leaf blades of field grown winter wheat (*Triticum aestivum* L.) grown under irrigated and dryland conditions (8). Flag leaves from dryland plants sown at 60 kg/ha showed no change in either glutathione reductase or catalase activities, expressed as a function of leaf area, while leaves from the basal portion of the canopy exhibited a 273% increase in glutathione reductase activity and a 60% increase in catalase activity. Glutathione reductase activity in dryland wheat sown at 120 kg/ha increased 25% in flag leaves and 225% in basal leaves. No change in catalase activity was observed in either flag or basal leaves from these same plants (8). This difference between the influence of canopy position on changes in glutathione reductase activity in wheat and cotton may be related to the markedly different plant microclimates (15).

Environmental factors have been reported to cause increases in the level of glutathione reductase activity in plants. Increased levels of glutathione reductase activity were observed in leaf extracts from spinach (*Spinacia oleracea* L.) in response to frost-hardening (9, 10). In addition, leaves from both corn (*Zea mays* L.) and cotton (*Gossypium herbaceum* L.) exposed to a 75% $O_2$ concentration showed a 2- to 3-fold increase in glutathione reductase activity within 48 h (5, 6). Foster and Hess (5) have proposed that increases in glutathione reductase activity in response to elevated $O_2$ concentrations suggest a prominent role for this enzyme in the protection of leaf tissue against oxidative damage.

**Analysis of Leaf Antioxidant Protection Levels.** The analysis of the effect of soil water deficits on the overall antioxidant system of the cotton leaves is presented in Table IV. For evaluation of paraquat sensitivity, the minimum effective paraquat concentration was defined to be the lowest concentration required to produce paraquat-induced necrotic spots. On 106 DAP, both T25 and T185 irrigated treatments had a minimum effective paraquat concentration of $5 \times 10^{-4}$ M. However, under dryland conditions the two strains differed in resistance to paraquat with

Table III. *Glutathione Reductase Activity in Leaves from Irrigated and Dryland Cotton Plants Collected on the Last Sampling Date (106 DAP)*

Data are means of three replications ± SE.

| Strain | Top Leaf | | Middle Leaf | | Base Leaf | |
|---|---|---|---|---|---|---|
| | Irrigated | Dryland | Irrigated | Dryland | Irrigated | Dryland |
| | *nmol NADPH oxidized $min^{-1}$ $cm^{-2}$* | | | | | |
| T25 | 19.57 ± 3.00 | 39.53 ± 3.87 | 13.04 ± 1.31 | 24.67 ± 1.20 | 10.61 ± 0.70 | 22.97 ± 1.68 |
| T185 | 9.01 ± 6.91 | 31.55 ± 3.47 | 8.27 ± 2.26 | 40.41 ± 6.96 | 12.01 ± 0.62 | 23.77 ± 4.23 |

Table IV. *Measurement of the Effect of Water Deficits on the Sensitivity of Two Photoperiodic Cotton Strains to Paraquat*

| Strain | Paraquat Concentration | | | | |
|---|---|---|---|---|---|
| | $10^{-2}$ M | $5 \times 10^{-3}$ M | $10^{-3}$ M | $5 \times 10^{-4}$ M | $10^{-4}$ M |
| | *% effectiveness*[a] | | | | |
| T25 | | | | | |
| Dry | 100 | 25 | 12 | 0 | 0 |
| Irrigated | 100 | 100 | 100 | 30 | 0 |
| D/I[b] | 100 | 100 | 67 | 0 | 0 |
| T185 | | | | | |
| Dry | 100 | 100 | 71 | 14 | 0 |
| Irrigated | 100 | 100 | 100 | 30 | 0 |
| D/I | 100 | 100 | 89 | 0 | 0 |

[a] Percent effectiveness is defined as the percentage of all leaves treated that exhibited paraquat-induced necrotic spots.

[b] D/I, leaves from dryland plants 2 weeks after irrigation of the rainout shelter.

T25 showing paraquat-induced necrotic spots in all plants tested only at the highest paraquat concentration ($10^{-2}$ M), while all of the T185 plants were damaged by $5 \times 10^{-3}$ M (Table IV). The difference in resistance was also evident at $10^{-3}$ M paraquat with 12% of the T25 plants being damaged compared with 71% damaged in T185. Following the irrigation of the dryland treatment at 107 DAP, T25 showed a dramatic increase in sensitivity to paraquat, resulting in a paraquat sensitivity distribution intermediate to the irrigated controls and dryland plants. Little change was observed in T185 following irrigation of the dryland treatments, nor were irrigated controls much different from the dryland plants in this strain.

Harper and Harvey (14) have shown elevated levels of superoxide dismutase, catalase, and peroxidase in Paraquat-tolerant lines of perennial ryegrass (*Lolium perenne* L.). However, since chloroplasts contain little if any, catalase or 'nonspecific' peroxidase activity, other protective mechanisms located within the chloroplast must dispose of the $H_2O_2$ formed by the reduction of paraquat. Foyer and Halliwell (7) have suggested that the $H_2O_2$ is disposed of by an ascorbate-glutathione cycle in which glutathione reductase is a key enzyme. The increased resistance to paraquat in water-stressed plants is not entirely due to the elevation in glutathione reductase activity, however, since both T25 and T185 dryland samples exhibited a 2- to 3-fold increase in glutathione reductase activity (Fig. 1), but showed a differential response to paraquat sensitivity (Table IV). Changes in other protective mechanisms within the chloroplast antioxidant system, such as superoxide dismutase and ascorbate peroxidase, might be occurring in T25 and not in T185 to account for the increased paraquat resistance observed in T25. Another possible mechanism allowing for the increased paraquat resistance in the dryland plants may be a change in cuticular waxes on the leaf surface which is affecting the uptake of the herbicide. To answer this question, further studies using [$^{14}$C]paraquat are needed.

Two weeks after irrigation of the dryland plots, T25 and T185 plants exhibited a 50 to 60% increase in leaf area/g fresh weight. The leaf area of T25 increased from 29.7 to 45.5 $cm^2$/g fresh weight, and T185 increased from 34.2 to 53.6 $cm^2$/g fresh weight. During this change in leaf morphology, T25 dramatically increased its sensitivity to paraquat, while T185 showed no change in paraquat sensitivity. The changes in the leaf area/g fresh weight in response to the irrigation of the stressed plants suggests that gross changes in leaf morphology are not directly affecting paraquat resistance.

## CONCLUSIONS

The two photoperiodic cotton strains analyzed in the present study exhibited the classic symptoms of severe water stress. Dry matter, leaf area, plant height, and photosynthesis declined in plants experiencing soil water deficits. Canopy temperatures increased as the stress developed, reaching a maximum of 42°C by the end of the study.

Analysis of glutathione reductase, an enzyme of the cellular antioxidant system, showed that the decline in enzyme activity associated with plant development under irrigated conditions was inhibited by water stress. The stressed plants exhibited 2- to 3-fold higher levels of activity at 106 DAP than their irrigated counterparts. This inhibition in the decline of glutathione reductase activity in the leaves of the water stressed cotton may serve to protect chloroplasts from reactive oxygen species produced under conditions of severe water stress. Glutathione reductase may serve to ensure the availability of $NADP^+$ to accept electrons from photosynthetic electron transport, thereby directing electrons away from oxygen and minimizing the production of superoxide (12) during stress-induced midday stomatal closure. Glutathione reductase may also function in the ascorbate-glutathione cycle to remove $H_2O_2$ generated within the chloroplasts (11).

The increase in glutathione reductase activity in response to water stress is not the sole modification of the cellular antioxidant system. The changes in sensitivity to the herbicide paraquat clearly indicate that other components of the antioxidant system are also responding to the stress stimuli. The characterization of specific changes of this integrated antioxidant system to the numerous and varied stress events requires further research.

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