

# Proline Accumulation in Water Stressed Cotton Leaves<sup>1</sup>

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## ABSTRACT

Greenhouse-grown cotton (*Gossypium hirsutum* L.) plants were subjected to a drying cycle when the plants had four true leaves and the proline content of the leaf tissue was determined. Free proline accumulated to about 100 times the concentration of the well-watered controls as the stress increased. The threshold of the pre-dawn leaf water potential for proline accumulation was about  $-15$  to  $-17$  bars. Total proline content also increased, which suggested that there was some proline synthesis. Free glutamic acid concomitantly decreased. Because proline did not accumulate until the water deficits were quite severe concentration of free proline is not a good indicator of the onset of plant water stress.

**Additional index words:** *Gossypium hirsutum* L., Plant water stress, Amino acid.

**B**ARNETT and Naylor (1) showed that free proline accumulates in wilted leaves of Bermudagrass [*Cynodon dactylon* (L.) Pers.]. Other investigators have also shown that free proline accumulates in leaf tissue of many other plant species in response to water stress (2, 4, 5, 10, 11, 15). Attempts have been made to relate the increase in free proline to drought tolerance or to use it as an indicator of the level of stress (2, 11, 14, 19). Their results indicate that proline does not accumulate appreciably before the leaves are visibly wilted (2, 22, 23), so proline concentrations may not be a useful indicator of levels of water stress. There is evidence, however, of a useful relationship between accumulation of free proline and drought tolerance (14, 19, 23). The large amount of accumulated free proline might also be a source of energy and N for the plant's use when stress is relieved (1, 2). The source of the accumulated free proline has also been investigated (20, 21). Protein hydrolysis during plant water stress releases some proline (11, 20, 21), although proline synthesis probably is also enhanced (2, 20).

The plant-water relations of cotton (*Gossypium hirsutum* L.) have been thoroughly studied (9, 12). Cotton routinely develops leaf water potentials lower than those of many other plants; predawn values of  $-10$  to  $-15$  bars are not uncommon early in a dry-

ing cycle. Proline accumulation has been studied in cotton also, but only in a preliminary fashion, (6). This work clearly established that proline does accumulate in cotton leaves during water stress, but did not establish the source or the water potential threshold for proline accumulations. The objective of this study was to confirm the results on proline accumulation in cotton leaves, to establish the threshold water stress level for proline accumulation, and to get some indication as to the source of the proline.

## MATERIALS AND METHODS

Cotton (cv. 'Deltapine 16') was grown in a commercial peat potting mixture (Jiffy-Mix<sup>®</sup>) in 20-cm clay pots. The pots were watered with a one-half strength Hoagland's solution (8) and tap water until the stress was imposed. Plants at the second to third true-leaf stage were thinned to two per pot.

When the plants had reached the fourth true-leaf stage, water stress was imposed by withholding water and nutrient solution for 4 days. The pre-dawn leaf water potentials, ( $\psi_R$ ), were measured each day during the drying cycle with a Scholander pressure bomb (16), and represent the highest leaf water potential achieved during that day. One plant per pot was used for leaf water potential measurements. Leaves from the second plant were used for chemical analysis. At the same time the leaf water potential was determined, the lowest three true leaves on the second plant were harvested and stored at  $-20$  C. Before analysis, the leaves were lyophilized, weighed, and ground to pass a 40-mesh sieve. Subsamples of 0.25 g were then taken and prepared for separate analyses of free and total amino acids according to methods described by Elmore and Leffler (7). A Beckman Model 121 Amino Acid Analyzer was used according to standard procedures for analysis of both physiological fluids and protein hydrolyzates. Duplicate N determinations were made with a Coleman N analyzer. Three pots were used for the first three sampling dates (0, 1, and 2 days after stress was begun) and six for the final two dates (3 and 4 days after stress was begun) when stress was highest. Plants in three pots were kept well-watered. These were harvested at the end of the experiment, along with six pots of plants that had been stressed for 4 days and then watered allowing the plants to recover turgidity. Leaf abscission was not observed to occur in any instance in the rewetted plants as was previously noted under stress conditions (12). This may have been due to the time element involved in stress induction. The plants used for this study were allowed to stress at a more gradual rate, thus possibly allowing for adaptation to the stress to occur, even though  $\psi_R$  values attained were comparable to those attained on Day 4 ( $-30$  bars). There may also have been a leaf age effect since the plants were only at the four-leaf stage.

## RESULTS AND DISCUSSION

The change in concentration of free proline with increasing plant water deficit is shown in Fig. 1. Analysis of the data by regression techniques indicated an exponential relationship between proline concentration and  $\psi_R$ . Proline concentrations remained at or near trace levels ( $<2.4 \mu\text{M/g}$  dry weight) at pre-

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Table 1. Free amino acid and N composition of water-stressed cotton-leaf tissue.

Component	Days after stress initiation							
	0	1	2	3	4	8		SE†
						Rewatered	Control	
μmoles·g dry wt <sup>-1</sup>								
Lysine	0.14	0.40	2.50	0.36	2.25	0.06	0.08	ns
Histidine	—‡	0.14	0.28	0.37	1.80	—	Tr	0.17**
Ammonia	9.63	5.12	8.76	10.47	44.37	2.21	Tr	ns
Arginine	—	—	0.48	Tr	0.92	—	—	ns
Aspartic acid	14.10	15.44	16.73	16.29	14.24	10.49	10.91	1.61**
Threonine	1.02	2.07	2.27	3.00	4.06	0.68	1.04	0.72**
Serine	1.31	1.34	1.31	1.79	2.72	1.16	1.16	0.32**
Glutamic acid + glutamine	19.91	16.56	20.67	24.27	17.44	11.82	16.24	3.91*
Proline	—	Tr	2.77	13.70	86.26	Tr	Tr	21.12**
Glycine	—	Tr	0.46	0.36	0.89	Tr	Tr	0.20**
Alanine	2.61	3.60	3.72	5.39	5.54	2.96	2.97	0.78**
Valine	—	0.60	1.04	2.11	5.34	Tr	0.18	1.20**
Methionine	0.14	—	Tr	0.11	0.16	Tr	—	ns
Isoleucine	—	0.61	0.75	1.71	2.68	Tr	0.16	0.45**
Leucine	—	0.35	0.46	1.09	1.37	Tr	Tr	0.38**
Tyrosine	—	—	0.14	0.63	0.37	Tr	Tr	ns
Phenylalanine	—	0.16	0.49	1.34	5.05	Tr	Tr	1.31**
asparagine	12.01	5.42	9.30	19.48	43.03	1.16	1.06	3.72**
Glucosamine	0.98	1.17	0.93	0.98	0.98	0.76	0.20	ns
Ethanolamine	3.73	2.35	2.47	2.38	4.06	2.68	1.66	ns
γ-aminobutyric acid	3.83	8.80	5.65	9.03	9.83	6.74	4.28	1.87*
Soluble N	82	70	95	126	305	42	41	35.8**
N (%)	5.98	5.43	5.38	5.35	5.66	4.82	3.84	0.20**
N content, mg	27.5	35.3	34.4	36.4	32.3	45.8	39.6	
Avg. dry weight (g)	0.46	0.65	0.64	0.68	0.57	0.95	1.03	0.08**
Avg. $\psi_R$ (bars)	-9.6	-11.3	-11.5	-21.4	-31.2	-12.5	-11.8	
SE of $\psi_R$	0.7	1.4	1.7	2.0	2.8	0.9	0.1	

\*, \*\* F values significant at the 0.05 and 0.01 levels, respectively.

† The SE of the difference of two means, for comparison of data in column 3, 4, or 8R with data in columns 0, 1, 2, or 8C. To compare data among columns 0, 1, 2, and 8C, multiply the given SE  $\times 1.15$ ; and to compare data among columns 3, 4, and 8R, multiply the given SE  $\times 0.816$ .

‡ None detected.

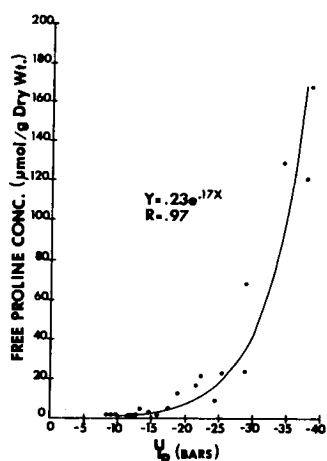


Fig. 1. Accumulation of free proline as a function of leaf water potential recovery values ( $\psi_R$ ).

dawn leaf water potential recovery values of less than -15 bars, and then increased exponentially as water deficits increased. The general shape of the curve is similar to that reported for other species (2, 17, 18, 20, 23), but the threshold potentials for the initial rise in proline concentrations (-15 to -17 bars) is higher than those reported for other species (22, 23).

The increase in soluble N (Table 1) (at day 4 of stress, almost 3.5 times the original value) reflects an increase in the total free amino acids. About 40% of this increase was contributed by proline; 28% by

asparagine, which has two moles of N per mole; and 15% by ammonia. Smaller increases in other amino acids and amides accounted for the remainder of the increase in soluble N. Free proline values returned to trave levels upon relief of the stress. These levels were the same as unstressed control plants.

The concentration of free glutamic acid plus glutamine increased and then decreased as the stress developed, as was reported in barley (*Hordeum vulgare* L.) (17). Conversion of glutamic acid to proline could account for the decrease in glutamic acid (11) and for part of the increase in free proline. The concentrations of all the amino acids in the free pool returned to levels similar to control plants upon rewatering. The drastic decrease in proline levels when stress is relieved may contribute to the ability of a plant to recover from stress (2). Barnett and Naylor (1) and Blum and Ebercon (2) both suggested that the large amounts of proline present may be used as a rapid energy source for the recovering plant.

Growth of the leaves during the drying cycle complicated the interpretation of the results. Dry weight increased from the 1st day to the 2nd (0 vs. 1 in Table 1) and then leveled off. When stress was relieved, growth began again, but dry weight in stressed rewatered plants remained slightly lower than that in controls. The total N content (percent N times dry weight) followed a similar pattern although percent N declined during the experiment. The declining percent N probably represents dilution of the N present as leaves grew, because the total N content remained stable. The percent N and the total N

Table 2. Amino acid composition of water-stressed cotton-leaf tissue.

Component	Days after stress initiation							SE†
	0	1	2	3	4	8		
						Rewatered	Control	
	g/100 g protein							
Lysine	7.59	7.39	7.25	7.30	6.90	7.49	7.19	0.21*
Histidine	1.93	1.97	2.17	2.00	2.00	2.14	2.04	0.07*
Ammonia	6.26	4.98	6.81	6.35	5.88	4.69	3.27	0.82*
Arginine	6.02	5.90	6.03	5.80	5.38	6.41	5.90	0.28*
Aspartic acid	10.67	10.97	10.93	11.04	11.77	10.26	10.67	0.24**
Threonine	3.40	3.81	3.71	3.56	3.48	3.66	3.80	ns
Serine	2.63	2.73	2.62	2.52	2.51	2.62	2.66	ns
Glutamic acid	13.98	14.50	14.18	13.93	13.21	13.78	14.52	ns
Proline	4.62	4.57	4.61	4.91	6.55	4.94	4.91	0.44**
Glycine	6.01	6.12	5.91	5.90	5.81	5.87	6.06	ns
Alanine	6.05	6.22	6.00	5.97	5.79	6.04	6.20	ns
1/2 cystine	0.88	0.77	0.86	0.90	0.80	0.30	0.18	0.09**
Valine	6.36	6.44	6.51	6.43	6.60	6.55	6.64	ns
Methionine	0.69	0.98	0.76	0.83	1.04	1.18	1.31	0.33**
Isoleucine	4.98	5.00	4.87	4.85	4.76	5.04	5.12	0.10*
Leucine	9.98	10.09	9.73	9.66	9.35	10.01	10.22	0.17**
Tyrosine	2.49	2.08	1.61	2.66	2.74	3.44	3.58	0.16**
Phenylalanine	5.46	5.50	5.45	5.41	5.44	5.58	5.72	0.09*

\*,\*\* F values significant at the 0.05 and 0.01 levels, respectively.

† The SE of the difference of two means, for comparison of data in columns 3, 4, or 8R with data in columns 0, 1, 2, or 8C. To compare data among columns 0, 1, 2, and 8C, multiply the given SE  $\times$  1.15; and to compare data among columns 3, 4, and 8R, multiply the given SE  $\times$  0.816.

content were higher in rewatered plants than in the controls, perhaps because of changes in metabolism during stress and relief of stress.

The total amino acid composition of the leaf tissue is shown in Table 2. The major change that occurs here is an increase in the proline concentration between the 3rd and 4th day into the stress cycle. Proline concentrations remained fairly constant until the stress became severe during the 3rd day and then increased markedly. The increase in free proline with water stress is caused partly by hydrolysis of leaf proteins as the stress develops (11, 13, 20, 21). Others have observed that the increase in free proline far exceeds that found in proteins and thus synthesis of proline occurs under stress (10, 17, 20). In our studies leaf proline content, calculated from the hydrolysate data, is consistent with these interpretations as shown in Fig. 2. There is an increase in free proline and total proline. Protein proline, calculated by difference, decreases, but the decrease in protein proline is less than the increase in free proline, suggesting hydrolysis and synthesis as the sources of free proline in water stressed cotton leaves.

Leaves of stressed, rewatered plants were higher than those of control plants in both proline and N, but the higher proline resulted from the higher protein level rather than from an increase in proline concentration in the protein. Our findings with water stressed cottonleaf tissue indicate that proline accumulates and that other amino acids, such as glutamic acid, behave much as reported previously for other species (17). Water stress delays accumulation of dry matter and may have a hysteretic effect on protein concentration in leaves.

The pattern of proline accumulation in cotton may prove useful in the study of differences in drought tolerance among genotypes and differences in drought adaptation within a particular genotype. However, since factors such as environment influence proline

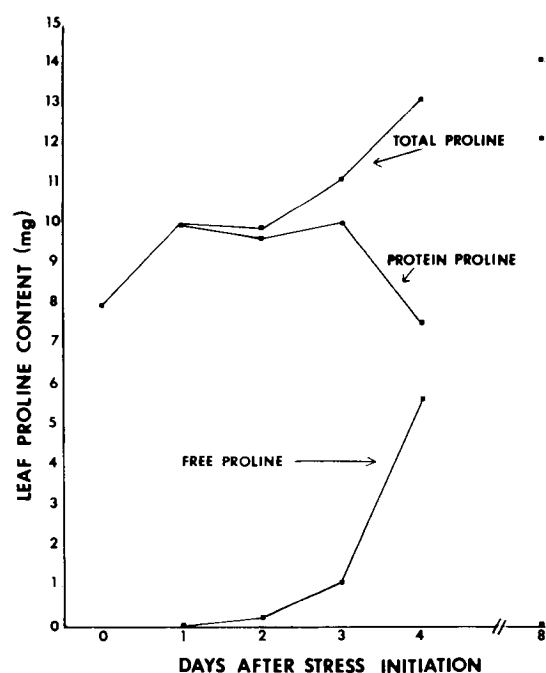


Fig. 2. Proline accumulations in water stressed cotton leaves. Proline content is shown as mg of proline per harvested leaf sample. Protein proline is determined by the difference between total and free. Plants were rewatered on day 4. (R = rewatered, C = control).

levels among genotypes these factors must be accounted for in a selection program regarding proline and drought stress. There is also some evidence on the effects of drought "hardening" on proline accumulations in plants. Barley plants subjected to repeated water deficits to "harden" them exhibited an increased tendency to accumulate proline after each successive stress cycle (18). Significant differences were also

noted between genotypes in proline levels under the same stress conditions (19). It has also been well documented that when plants are drought "hardened" other phenomena, such as altered stomatal action (the stomata close at much lower water potentials when hardened) occur (3). These changes may be associated with proline accumulation and may aid in determining the role of proline in drought stress. These aspects, however, remain to be investigated in cotton.

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