

Proline Accumulation by Water and Nitrogen Stressed Cotton¹

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ABSTRACT

Germinating and developing cotton (*Gossypium hirsutum* L.) seedlings were examined for proline accumulation under severe water stress and N deficiency to examine the relationship of N to proline accumulation. Seeds of 'Coker 201' were dark-germinated for 3 days with one-half desiccated during the last 12 hours. In another experiment, plants were light grown under well watered conditions with or without nutrient solution containing NO₃-N. On the 12th day they were subjected to severe water stress. Dry weight, free amino acids, and plant water potentials were determined after stress developed. Three-day desiccated cotyledons accumulated 28% more proline than the unstressed controls. Axes of these stressed seedlings, however, did not accumulate free proline. The older water stressed cotyledons and leaves accumulated free proline, but only when the seedlings had an exogenous source of N. Thus under severe N deficiency, proline accumulation ability is impaired in cotton leaf tissue. Free histidine, on the other hand, accumulated in N deficient leaves, but not cotyledons. The results indicate that proline accumulation by water stressed tissue is related to the N status of the tissue as well.

Additional index words: Free amino acids, *Gossypium hirsutum* L., Cotyledons, Histidine.

FREE proline accumulation by water-stressed leaf tissue is well established (1, 11) and has been shown to occur in cotton (*Gossypium hirsutum* L.) leaves (14). Tissues other than leaves of higher plants also accumulate free proline during water stress (2, 28). There are, how-

ever, reports of cases where water stress does not induce proline accumulation (5). The stress mode may take several forms, some of which may simply be different ways of producing water stress. This is surely the case for salinity and may in part be the case for cold stress (9, 11). Mineral deficiency, which causes proline accumulation in certain cases (8, 9, 17, 21, 26), is not easily interpreted as a water stress phenomenon (9). Nitrogen stress (deficiency) in particular has been shown to induce proline accumulation in maize shoots and roots (9), but not in turnips (*Brassica rapa* L.) (26). Others have suggested, based on work with diatoms, that a N source is necessary for proline accumulation (21). These reports of mineral effects on proline accumulation and the reported inability of cotton cotyledons to accumulate proline when desiccated (5) prompted us to investigate the situation in cotton further. Our objectives were to (a) confirm and extend the results of Capdevila and Dure (5) on cotton cotyledons by investigating very young germinating seedlings as well as older more mature cotyledons, (b) to examine proline accumulation in water-stressed cotton tissue which is also N deficient, and (c) to compare responses of three cotton cultivars to water and N deficits, at least on a preliminary basis.

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MATERIALS AND METHODS

Experiment 1. Seeds of cotton (cv. 'Coker 201') were germinated under the conditions described by Capdevila and Dure (5) for the dark-germinated seedling experiment. After 60 hours, the seedlings for the desiccated treatment were transferred to dry filter paper and the petri dish covers removed. The controls were not disturbed. After desiccation for 12 hours, axes were separated from the cotyledons, and the tissues were frozen, lyophilized, ground to pass a 40 mesh screen and stored in glass vials until analyzed. Free amino acids were determined using the procedure of McMichael and Elmore (14) on two replicates of 25 seedlings.

Experiment 2. Proline accumulation in response to water stress in combination with N deficiency was tested using the three cultivars 'Delfos 9169,' 'STV 213,' and 'DY 5167.' These cultivars were selected to represent low, medium, and high protein content seed, respectively, as previously determined (15). Seed were selectively graded in sucrose solutions and those that floated in 5% (w/v) sucrose or sank in 25% (w/v) sucrose were discarded (6). The selected seed were planted in washed sand moistened with nutrient solution containing 5 mMNO₃ or 0 N (19) in flats (36 × 50 × 9 cm) in the greenhouse (100

seeds/flat). Four samples of 20 seedlings each, taken from different flats, were taken on the 3rd, 6th, 9th, and 12th days for dry weight and leaf area determinations. After one sampling the remaining seedlings in a flat were discarded. Moisture stress was imposed by withholding nutrient solution. After the plants had become severely stressed (1 day), water potential was measured with the pressure bomb (14) and samples of 20 seedlings each were harvested from the stressed and unstressed controls for dry weight determination and amino acid analysis (14).

Five determinations of water potential were made for each treatment. Dry weight was determined on four replications of 20 seedlings taken from four flats. Amino acid analyses were determined in duplicate using the lyophilized material.

RESULTS

Experiment 1. Water-stressed germinating cotton seedlings accumulated very little free proline (Table 1), as was reported by Capdevila and Dure (5). However, we found significantly higher free proline concentrations in the stressed cotyledons, but not in the axes, when compared to unstressed controls. The percentage changes in proline were similar to that reported by Capdevila and Dure (5) who found a 23% increase in free proline concentration in the stressed germinating seedling while we found a 28% increase in the stressed cotyledons. While free proline increases in these two studies were in the same order of magnitude, they were far below the nearly 100-fold increase in free proline for stressed cotton leaves we reported previously (14).

Concentrations of the other free amino acids in the stressed cotyledons changed very little. Only aspartic acid, serine, and glycine differed significantly from the controls. These three amino acids also were affected in stressed cotton leaves (14).

Experiment 2. Nitrogen availability significantly affected growth of the seedlings by Day 12 (Table 2). Both leaf and cotyledon area and dry weight of all parts were reduced by the lack of N. The effect on dry weight was noticeable by Day 9, but the effect on cotyledon area was apparent by Day 6. All cultivars were similarly affected.

Plant water potentials for water-stressed plants are shown in Table 3. Water-stressed plants with N had lower water potentials than those without N. The controls were typical for cotton under well-watered conditions in mid-afternoon on a warm sunny day in the greenhouse (14).

Table 1. Free amino acid and ammonia concentrations in 3-day-old stressed and nonstressed Coker 201 cotton seedlings.†

Amino acid	Cotyledons		Axes
	Control	Stressed	Control
	μmoles/g dry wt		
Aspartate	6.0	4.3†	30.5
Threonine	5.6	6.0	22.2
Serine	8.6	11.5†	49.8
Asparagine	104.7	76.4	583.3
Glutamate	8.1	8.4	145.8
Glutamine	11.7	13.3	9.9
Proline	2.9	3.7†	5.4
Glycine	2.0	1.6†	8.6
Alanine	2.7	5.6	18.0
Valine	2.0	3.3	5.3
Isoleucine	1.0	1.4	5.6
Leucine	1.2	1.4	5.6
Tyrosine	2.5	3.0	5.2
Phenylalanine	1.0	1.5	1.9
γ-aminobutyrate	6.8	8.7	17.4
Ethanolamine	3.0	4.1	7.5
Lysine	5.9	7.6	17.9
Histidine	10.3	11.9	11.8
Arginine	16.4	25.7	20.2
Ammonia	16.4	12.9	36.9

† Significantly different from control at 95% level as determined by analysis of variance. ‡ Stressed axes were not different from the nonstressed controls.

Table 2. Dry weight and leaf area of cotton seedlings during germination and early seedling development as affected by N availability. Mean of the three cultivars.

		Day									
Tissue	N Treatment	0	SE	3	SE	6†	SE	9†	SE	12†	SE
g/20 seedlings											
Cotyledon	+N	1.33	0.06	1.05	0.05	0.98	0.09	1.30	0.05	1.72	0.12
	-N			1.04	0.07	0.88	0.04	1.06	0.06	1.28	0.08
Axes	+N			0.26	0.01	0.51	0.02	1.08	0.03	1.80	0.10
	-N			0.24	0.01	0.52	0.02	0.93	0.04	1.28	0.07
Total	+N			1.31	0.05	1.49	0.10	2.52	0.06	4.22	0.18
	-N			1.27	0.06	1.40	0.05	2.10	0.10	2.96	0.17
dm ² /20 seedlings											
Cotyledons	+N			0.88	0.05	3.28	0.17	4.76	0.24	4.78	0.27
	-N			0.89	0.04	2.54	0.14	2.67	0.05	3.19	0.37
Leaves	+N			-	-	0.03	-	0.84	0.18	2.92	0.18
	-N			-	-	0.02	-	0.40	0.02	1.55	0.06

† Dry weight totals on Days 6, 9, and 12 include epicotyl and leaf weights.

Table 3. Plant water potentials attained at sampling time for three cotton cultivars grown under two watering and two N regimes.

Cultivar	Stress level	N level	Ψ (- Bars) [†]
STV 213	+	+	34.5 (0.1)
	+	-	24.0 (0.9)
	-	+	13.2 (0.5)
	-	-	13.1 (0.5)
DY 5167	+	+	34.5 (0.3)
	+	-	26.6 (0.8)
	-	+	12.8 (0.4)
	-	-	12.6 (0.2)
Delfos 9169	+	+	34.5 (0.3)
	+	-	22.1 (0.7)
	-	+	15.4 (0.6)
	-	-	12.8 (0.4)

[†] Each value of Ψ represents the mean of five determinations, standard errors are given in parenthesis.

The free amino acid concentration of 13-day old cotton leaf and cotyledon tissue is shown in Table 4. Cotyledons responded differently from leaves for asparagine and histidine composition and showed slight differences in degree of response for other amino acids (e.g., threonine, glutamine, proline, glycine, isoleucine, leucine, phenylalanine, γ -aminobutyrate, ethanolamine, and ammonia). Stressed leaves and cotyledons with N had high levels of asparagine and proline. When N was absent from the nutrient solution, stress had no significant effect on those two amino acids. Histidine is an anomalous case. In the absence of N (regardless of water stress treatment), histidine level is high. This tendency is more pronounced in leaves than in cotyledons.

Cultivars differed in free amino acid composition of cotyledons and leaves (Table 5). There were differences between cotyledons and leaves for at least one of the cultivars for each amino acid except glutamate, glycine, and alanine. Cultivars also differed from each other for concentrations of free amino acids in the cotyledons for all except glutamate and ammonia, and in the leaves for all except glycine, isoleucine, lysine, and arginine. These cultivar differences were not surprising since the cultivars picked represent extremes in protein content (14). That there are differences between cotyledons and leaves within the cultivars is interesting and informative. These cotyledons are fully functional, photosynthetically active structures that will persist for weeks (13, 20). They also do not show any differences from leaves ultrastructurally (unpublished data). These data indicate, however, that cotyledons retain subtle biochemical and compositional differences from leaves.

Free proline accumulation for each treatment combination is shown in Table 6. The stressed plants with N accumulated more free proline than any other treatment. Within the stressed plants with N, leaves accumulated more proline than the cotyledons; and cultivars differed greatly in their ability to accumulate free proline. Proline accumulation ranking of the cultivars had no relationship to the protein content of the seed. The low protein cultivar Delfos 9169 had consistently higher levels of free proline than either the high protein cultivar DY 5167 or the intermediate cultivar STV 213. There were no differences in proline accumulation in any of the N deficient treatments or the nonwater stressed treatment with N.

DISCUSSION

Levels of free proline accumulations by the dark-germinated cotyledons were quite low. We did not measure actual water stress levels in these seedlings, but the tissue was severely desiccated, probably beyond the point of recovery. It could be that more time was needed for proline to accumulate. Yet, Stewart et al. (24) reported maximum proline accumulation in detached, wilted leaves during the first 24 hours. Also, both of the known common precursors of proline, glutamate, and arginine (3, 4, 27), are present in relatively high concentrations in germinating cottonseed. Therefore, lack of substrate, except as it may be related to cellular location, should not be an obstacle to proline accumulation in young, germinating cotyledons.

Photosynthesis is not necessary for proline accumulation, because detached leaves in the dark accumulate proline (24). Two factors probably explain the lack of extensive proline accumulating ability by young cotton cotyledons. One is the extensive metabolic reaction sequence set off by the onset of germination. Because of this accelerated metabolic activity, the accumulation of new storage products may be reduced. In the case of older cotyledons, leaves, and tubers, the tissue is relatively mature with little cell division, expansion, or growth occurring, which is certainly not the case with germinating cotyledons. Another aspect which may play a role is the lack of an exogenous N source. Schobert (21) has shown that stressed diatoms accumulate proline only when an external source of N is present, and that added amino acids not in the proline synthetic pathway can serve as N donors. Our dark-germinated and light-grown, N deficient cotyledons had no such supply of N. Also, Stewart et al. (24) reported that detached turnip leaves stressed without added N did accumulate proline. Apparently no general conclusion can be reached without further investigation, but the mineral status and age of the tissue are probably related to inherent proline accumulating ability.

Cultivar differences for proline accumulation are not unusual (2, 7, 10, 23), but relating the observed differences to a useful screening technique for such a trait as drought tolerance is another matter (7, 10). Although initial progress was reported for barley (*Hordeum vulgare* L.) (23), Hanson et al. (10) indicated that there was little hope for using the technique in any beneficial way in a breeding program. The cultivars used in these experiments represent a considerable range of the *Gossypium* germplasm from old, no longer used cultivars (Coker 201 and Delfos 9169) to a contemporary cultivar (STV 213) to exotic germplasm (DY 5167) (15). The range of protein content in the seed was expected to be useful in providing clues to the relation between plant N metabolism and proline accumulation. The relationship is not obvious if it exists. The low protein cultivar (Delfos 9169) has the greatest amount of free proline when stressed, followed by DY 5167, the high protein cultivar. This may occur because there is no relationship between leaf protein concentration and seed protein concentration. At any rate there remain unanswered questions concerning genotypic influence upon proline accumulation under water stress

Table 4. Free amino acid concentration of 13-day-old cotton cotyledons and leaves as affected by water stress and N availability. Mean of three cultivars.

Amino acid	Cotyledons				Leaves				L.S.D. _{0.05}
	+N-S†	+N+S	-N-S	-N+S	+N-S	+N+S	-N-S	-N+S	
					μmoles/g dry wt				
Aspartate	1.98	1.37	1.28	1.27	2.36	1.74	1.07	1.52	0.49
Threonine	0.65	2.06	0.85	0.98	1.31	1.60	1.08	1.61	0.35
Serine	1.51	1.33	1.28	0.93	1.84	1.03	1.01	1.11	0.64
Asparagine	2.56	7.83	3.34	2.99	1.01	4.21	0.15	0.38	2.17
Glutamate	1.51	1.03	0.89	0.93	1.22	1.09	0.85	1.06	0.55
Glutamine	0.66	2.66	0.16	0.53	2.49	3.13	0.63	1.45	0.91
Proline	0.20	4.29	0.20	0.27	0.42	7.15	0.30	0.66	0.94
Glycine	0.56	0.26	0.87	0.56	0.72	0.38	0.14	0.32	0.50
Alanine	1.79	2.97	1.15	1.81	2.16	3.20	0.75	1.61	0.62
Valine	0.21	2.66	0.32	0.56	0.60	2.60	0.63	0.87	0.69
Methionine	0.58	0.14	0.28	0.15	0.32	0.14	0.18	0.09	0.25
Isoleucine	0.15	1.48	0.25	0.49	0.15	1.96	0.28	0.88	0.13
Leucine	0.21	0.94	0.36	0.55	0.24	1.68	0.20	0.57	0.16
Tyrosine	0.07	0.29	0.10	0.17	0.12	0.45	0.16	0.26	0.14
Phenylalanine	0.17	2.04	0.19	0.44	0.16	1.36	0.04	0.15	0.25
γ-aminobutyrate	2.38	7.53	2.31	3.42	5.37	9.19	2.90	3.54	1.47
Ethanolamine	0.62	1.02	0.84	0.91	1.21	1.22	1.24	1.19	0.38
Ammonia‡	1.50	1.90	1.22	1.51	3.40	3.39	1.38	2.22	0.91
Lysine	0.23	1.03	0.51	0.74	0.40	1.37	0.47	0.64	0.22
Histidine	0.30	1.63	0.96	1.11	0.31	1.43	3.96	4.90	0.77
Arginine	0.93	1.43	2.74	2.79	2.49	3.32	0.34	0.66	1.79

† Treatment identification is as follows: +N = nutrient solution with N, -N = nutrient solution without N; -S = unstressed controls, +S = severely water stressed.

‡ The data on ammonia, which is not an amino acid, are included for information.

Table 5. Free amino acid composition of cotton leaves and cotyledons for three cultivars. † Means over stress and N treatments.

Amino acid	Cotyledons			Leaves		
	DY 5167	STV 213	Delfos 9169	DY 5167	STV 213	Delfos 9169
	μmoles/g dry wt					
Aspartate	1.53b*	1.26c	1.63ab	1.86a	1.47bc	1.69ab
Threonine	1.60a	0.87d	0.95d	1.55ab	1.41bc	1.24c
Serine	1.63a	1.23bc	0.94c	1.16c	1.54ab	1.04c
Asparagine	5.93a	3.04c	4.18b	1.48cd	0.97d	2.08c
Glutamate	1.00ab	1.06ab	1.21ab	1.24a	0.91b	1.01ab
Glutamine	1.50b	0.57c	0.93c	2.08a	1.53b	2.17a
Proline	1.02bc	0.77c	1.93b	2.41a	1.26b	2.74a
Glycine	0.82a	0.57ab	0.30b	0.41ab	0.44ab	0.34ab
Alanine	2.30a	1.52c	1.97b	2.26ab	1.34c	2.12ab
Valine	1.24a	0.56d	1.02bc	0.79cd	0.83cd	1.91a
Methionine	0.37a	0.21bc	0.28ab	0.14c	0.30ab	0.10c
Isoleucine	0.72b	0.49d	0.57c	0.82a	0.81a	0.83a
Leucine	0.63b	0.44c	0.47c	0.63b	0.73a	0.66ab
Tyrosine	0.21bc	0.11d	0.15cd	0.28a	0.21bc	0.25ab
Phenylalanine	0.97a	0.45cd	0.72b	0.39d	0.37d	0.53c
γ-aminobutyrate	3.83d	3.98cd	3.93cd	4.89b	6.19a	4.66bc
Ethanolamine	0.91c	0.67d	0.96c	1.19b	1.41a	1.05bc
Ammonia‡	1.71cd	1.29d	1.60cd	2.52b	3.30a	1.98c
Lysine	0.85a	0.43d	0.61c	0.71bc	0.78ab	0.67bc
Histidine	1.46c	0.55e	0.99d	2.97a	2.60ab	2.38b
Arginine	3.31a	1.06b	1.55b	1.81b	1.72b	1.57b

* Values for an amino acid followed by identical letters are not different as determined by Duncan's Multiple Range Test at the 0.05 level of probability.

† Cultivar DY 5167 is the high protein selection, STV 213 the intermediate selection and Delfos 9169 the low protein selection.

‡ The data on ammonia, which is not an amino acid, are included for information.

Table 6. Free proline concentration of water and N stressed cotton leaves and cotyledons of three cultivars. †

Tissue	Cultivar	Mean free proline content			
		-N‡		+N	
		-S	+S	-S	+S
		μmoles/g dry wt			
Cotyledons	DY 5167	0.20e*	0.20e	0.20e	3.49c
	STV 213	0.20e	0.20e	0.20e	2.47d
	Delfos 9169	0.20e	0.42e	0.20e	6.90b
Leaves	DY 5167	0.44e	1.09e	0.33e	7.76b
	STV 213	0.20e	0.38e	0.41e	4.04c
	Delfos 9169	0.27e	0.51e	0.53e	9.65a

* Means followed by the same letter or no letter are not different according to Duncan's Multiple Range Test at the 0.05 level of probability.

† Cultivar DY 5167 is the high protein selection, STV 213 the intermediate selection and Delfos 9169 the low protein selection.

‡ Treatment identifications is as follows: +N = nutrient solutions with N; -N = nutrient solution without N; -S = unstressed controls; +S = severely water stressed.

conditions and its relationship to leaf protein metabolism.

The role of free proline accumulation in water-stressed tissue is not well understood. The accumulated proline could be a source of readily available reduced N for use upon stress relief (1). It could be an osmoticum (16). Stewart and Lee (25) and Schobert (22), however, have

suggested alternate roles for free proline accumulation in stressed plants and in halophytes. In halophytes, Stewart and Lee (25) suggest that free proline accumulation functions as the solute for osmotic adjustment in plants exposed to high salinity. Schobert (22) carried the suggestion further and proposed that proline might function in osmotic regulation, not as an osmoticum, but, along with certain polyols, as a replacement for water in the maintenance of hydration of the biopolymers of plant cells. Recent work by Goring and Thien (9) suggest that proline induced by nutrient deficiency accumulates in the cytoplasm. This would be in accord with the hypothesis of Schobert (22), but is certainly far from definitive.

Our results with N deficiency agree in part with those of Thompson et al. (26). They showed that under N deficiency proline did not accumulate in turnip, whereas it did with some other mineral deficiencies. They did not

see the histidine response we found for N deficiency but did for deficiencies of P and S. Others have shown that a N deficiency results in proline accumulation (9). Perhaps the severity of deficiency and state of tissue reserves of N are different enough to result in varied responses. That proline does not accumulate in N deficient tissue even when water stressed is supportive of such a conclusion. Evidently a great deal more research needs to be conducted to clarify the role of proline accumulation in stressed, deficient leaf tissue.

Radin and Parker (18, 19) have shown that the water relations of N deficient plants differ from unstressed plants. They show in their experiments that low N level plants tend to resist dehydration more than plants with adequate N based on changes in relative water content during dehydration. Their N deficient plants thus exhibited many of the traits associated with drought adaptation and drought resistance. These findings are in agreement with those shown in Table 3 where the Ψ of N deficient plants were 8 to 12 bars higher for each cultivar when water was withheld for identical periods of time. That interpretation is confounded in our experiment, however, because the N deficient plants had smaller leaf area. Thus in the same time period they lost less water per unit of time. The water potential may have been different for that reason. These differences were not evident under well-watered conditions. Radin and Parker (19) also demonstrated that threshold Ψ for stomatal closure was much higher in low N plants which could also help to account for the maintenance of higher Ψ we observed in the water stressed, N deficient plants.

In contrast to our experiment Radin and Parker (18, 19) used low N nutrient medium to produce their N deficient plants which were older than the ones we used. We basically had zero N treatments to produce N deficient plants. It would be instructive to use a range of levels of N and investigate the N metabolism, protein metabolism, etc. in stressed plants. Proline is such a general response to stress in plants that its role in stress avoidance and tolerance should be studied further. Perhaps continued studies along the lines of Humphrey et al. (12), Goring and Thein (9), Radin and Parker (18, 19), Schobert (21, 22), and Stewart and Lee (25) would be most helpful in elucidating the role of proline.

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