PROLINE, P5CS ACTIVITY AND GLYCINE BETAINE CONTENT IN INTRA-HIRSUTUM (HXH), INTER-SPECIFIC (HXB) AND *G. ARBOREUM* CULTIVARS UNDER WATER STRESS

H.C. PAWAR, R.M. NAIK*, R.D. SATBHAI AND S.S. MEHETRE

Department of Biochemistry, MPKV, Rahuri, Maharashtra, India

Received on 29 Oct., 2009, Revised on 04 June, 2010

SUMMARY

Water stress created by withholding irrigation caused significant increase in free proline and glycine betaine content in the leaves of intra-hirsutum (HxH), inter-specific (HxB) and *G. arboreum* cultivars of cotton. The increase in the levels of proline is mainly attributed to *de-novo* synthesis of proline as evident from increased activity of P5CS specifically in *G. arboreum* cultivars. However, besides *de novo* synthesis of proline, loss of feed back inhibition of free proline appears to operate in regulating the levels of free proline in intra-hirsutum and inter-specific hybrids. Cotton is a natural glycine betaine accumulator and hence, a higher level of glycine betaine was recorded in control plants, which further increased on imposition of stress. A significant variation in the levels of free proline and glycine betaine was observed in all the cultivars tested. The levels of both the osmolytes increased simultaneously and can be used to select the drought tolerant segregating population involving breeding of cotton for drought tolerance.

Key words: **Cotton, glycine betaine, proline, P5CS, water stress**

INTRODUCTION

Rainfed cotton farming is a risky enterprise and is a challenge to sustain cotton productivity not only in India but also in similar agro-environments elsewhere. Yield fluctuation in cotton under rainfed condition varies significantly. Maharashtra is the largest cotton-growing state in the country which covers about 42% of total cotton area and contributes 17% to production (Anonymous 2007). The area under cotton has steadily increased with the introduction of Bt-cotton.

Water stress is one of the most important environmental variables affecting cotton growth and development. Diurnal changes in tissue water potential components, photosynthesis and specific leaf carbohydrates have been examined in water stress adapted and non-adapted cotton plants. Adapted plants exhibited lower daily minimum leaf water potentials and maintained turgor. Glucose and sucrose accumulation in response to decreasing water potential is also dependent on leaf age. Variability in leaf turgidity has also been observed among cotton strains grown under water deficit field conditions and proved to be useful in selecting germplasm with enhanced drought-tolerance (Quisenberry *et al.* 1985).

Increased accumulation of compatible osmolyte *viz.*, betaine and related compounds; polyols and sugar such as mannitol, sorbitol and trehalose; and an amino acid, proline has been reported in numerous crop plants as a most common metabolic response to stress in maintaining

*Corresponding author, E-mail: rajeevnaik2@rediffmail.com

cell turgor and the driving gradients for water uptake under stress (Rhodes and Hanson 1993, McNeil *et al.*1999, Balibrea *et al.* 2000).

Cotton genotypes subjected to short term drought stress recorded a dramatic increase in free proline content and the pattern of proline accumulation in segregating generation of cotton during hybrid development may prove to be useful criteria in selecting genotypes. The activity of Δ^1-pyrroline-5-carboxylate synthetase (P5CS), which catalyses the rate-limiting step in proline biosynthesis, has been shown to be increased substantially under stress conditions. The increase in P5CS activity was correlated with increase proline content and over expression of P5CS gene in transgenic tobacco caused increase in proline accumulation and osmotic tolerance (Kavi Kishor *et al.* 1995). Proline might play a role in reducing the oxidative stress brought about by osmotic stress (Nanjo *et al.* 1999). The cotton plant also has natural ability to accumulate glycine betaine and genotypic differences exist for this character. The level of glycine betaine accumulation in cotton is comparable with high betaine accumulating species such as spinach and barley (Rhodes and Hanson 1993). Glycine betaine is the predominant osmoprotectant and could be used as a reliable index of stress tolerance in cotton breeding programme.

The present investigation was undertaken to screen one month old potted plants of eight intra-hirsutum (HxH) with two checks, five inter-specific (HxB) hybrids along with two checks and eight *G. arboreum* cultivars for evaluating the levels of proline and glycine betaine and to monitor the activity of P5CS a key regulatory enzyme in proline biosynthesis under stress.

MATERIAL AND METHODS

The seeds of eight intra-hirsutum (HxH) *viz.,* RHH-196, RHH-202, RHH-211, RHH-216, RHH-222, RHH-507, RHH-520, RHH-523 along with two checks NHH-44, Phule-492 and five inter-specific (HxB) hybrids *viz.,* RHB-206, RHB-208, RHB-204, RHB-302, RHB-488 along with two checks i.e. Phule-388, DCH-32 were obtained from the Cotton Improvement Project, MPKV, Rahuri.

The seeds of eight *Gossypium arboreum* cultivars *viz.,* Turab, PA-183, AKA-8401, AKA-7, Sarvottum, Jawahar Tapti, Y-1, JLA-794 were obtained from ARS, Jalgaon, Maharashtra.

The present experiment laid out in FCRD with three replications. The seedlings were grown in pots filled with 10 kg of soil. Soil moisture was maintained close to field capacity until the 3rd leaf stage and in another replication plants were subjected to water stress by withholding water for 5 days. The leaf samples were collected from each of these plants at field capacity (control) and from water stressed plants. The control and stressed leaf samples were analyzed for the contents of free proline, glycine betaine and the activity of Δ^1-pyrroline-5-carboxylate synthetase. Proline content in leaf tissues of both stressed and unstressed seedlings was determined by using the acid ninhydrin reagent as per the method described by Bates *et al.* (1973). The proline content was expressed as μ mole g^{-1} fresh weight.

Glycine betaine content in leaves of both the control and stress seedlings was determined by using the Dragendorff reagent as per the method described by Stumps (1984). The glycine betaine content was expressed as $\mu g\ g^{-1}$ fresh weight. The activity of Δ^1-pyrroline-5-carboxylate synthetase was assayed according to the method of Hayzer and Leisinger (1980).

RESULTS AND DISCUSSION

Effect of water stress on free proline content and P5CS activity under control and stressed condition in intra-hirsutum (HXH) hybrids, inter-specific (HxB) hybrids and *G. arboreum* cultivars is presented in Tables 1-3, respectively. The proline content of control (unstressed) plants varied significantly from 17.70 in RHH-216 to 65.60 in RHH-507 with a mean value of 36.37 $\mu mol\ g^{-1}$ FW. However, the free proline content increased significantly under stress condition, which ranged from 284.31 to 907.71 with a mean value of 518.47 $\mu mol\ g^{-1}$ FW. Thus, there was 15.55-fold increase in mean proline content under stress. The highest fold increase of 24.35 was recorded in RHH-216 and the lowest of 8.48 in Phule-492. The hybrids RHH-202 (24.03-fold) and RHH-520 (23.51-fold) also appeared to

 Indian J. Plant Physiol., Vol. 15, No. 2, (N.S.) pp. 125-130 (April-June, 2010)

be promising drought tolerant ones based on the proline accumulation potential under stress condition (Table 1).

The results obtained here are in agreement with the analysis of cotton genotypes subjected to short term drought stress which recorded a dramatic increase in free proline content to the extent of 41-fold in a drought tolerant cultivar (GM 090304) and 21-fold in Ca/H 631, a drought susceptible cultivar (Parida *et al.* 2007). Similarly, an increase in free proline content of approx 100 times the concentration of well watered control plants has been recorded in *G. hirsutum* (McMichael and Elmore 1977) with concomitant decrease in glutamic acid indicating the acceleration of proline synthesis. The significant increase in proline content under stress could mainly be attributed to increased rate of its synthesis. Over expression of gene coding for an enzyme "[1] pyrroline-5-carboxylate synthetase, a rate limiting enzyme in proline biosynthesis has been reported to confer stress tolerance in tobacco and rice (Kavi Kishor *et al.* 1995, Zhu *et al.* 1998). The activity of P5CS was therefore, determined from the leaf tissues of control and stress seedlings of cotton. The P5CS activity in the control leaf samples ranged from 16.75 in RHH-523 to 25.88 in RHH-196 with a mean value of 19.20 µmol ã-glutamyl hydroxamate g^{-1} h^{-1}, which increased more than 50% under stress condition (Table 1). An increase in the P5CS activity under PEG-6000 induced osmotic stress has been reported in sorghum and chickpea (Dalvi *et al.* 2007). Cotton hybrid RHH-216 recording highest fold increase in free proline content under drought also recorded a 3.35 fold increase in P5CS activity which was maximum.

In inter-specific (HxB) hybrids along with two checks *viz.*, DCH-32 and Phule-388, the proline content of control plants varied significantly from 9.32 in DCH-32 to 30.87 in RHB-208 with a mean value of 23.93 µmol g^{-1} FW. Upon imposition of water stress by withholding irrigation, the free proline content increased significantly ranging from 62.96 to 508.83 with a mean value of 265.02 µmol g^{-1} FW, with an overall fold increase of 11.82 (Table 2). The highest fold increase of 18.51 was recorded in RHB-302 and the lowest of 2.14 in RHB-488. The hybrids RHB-208 (16.48-fold) and DCH-32 (13.80-fold) also appeared to be promising drought tolerant ones based on the proline accumulation potentials under stress condition.

Table 1. Evaluation of intra-hirsutum (HxH) hybrids for proline, P5CS activity and glycine betaine content under control and stressed condition.

Hybrids	Proline (µmol g^{-1} FW)			P5CS activity (µmol ã- glutamyl hydroxamate formed g^{-1} h^{-1})			Glycine betaine (µg g^{-1} FW)		
	Control	Stress	Fold increase	Control	Stress	Fold increase	Control	Stress	Fold increase
RHH 196	36.39	456.41	12.54	25.88	27.54	1.06	99.78	122.11	1.22
RHH 202	21.96	527.77	24.03	17.17	46.33	2.69	53.65	80.35	1.49
RHH 211	25.59	422.70	16.51	19.44	44.09	2.26	63.43	72.15	1.14
RHH 216	17.70	431.12	24.35	14.58	48.83	3.35	38.06	83.26	2.19
RHH 222	24.85	284.31	11.44	21.60	43.69	2.02	64.49	79.03	1.23
RHH 507	65.60	907.71	13.83	20.52	28.04	1.36	129.51	170.48	1.32
RHH 520	35.02	823.61	23.51	19.44	45.43	2.33	33.70	66.87	1.98
RHH 523	31.81	331.94	10.43	16.75	18.26	1.09	103.74	136.78	1.32
Nanded-44	57.52	595.28	10.40	18.36	19.44	1.06	45.59	84.84	1.86
Phule-492	47.58	403.82	8.48	18.36	19.44	1.06	54.44	53.65	0.98
Mean	36.37	518.47	15.55	19.20	34.11	1.82	68.64	94.95	1.47
SE	0.85	5.43	-	1.08	2.10	-	0.35	0.34	-
C.D.	2.50	15.97	-	3.17	6.19	-	1.04	1.01	-

Table 2. Evaluation of inter- specific (HxB) hybrids for proline, P5CS activity and glycine betaine content under control and stressed condition.

Hybrids	Proline (µmol g^{-1} FW)			P5CS activity (µmol ã- glutamyl hydroxamate formed g^{-1} h^{-1})			Glycine betaine (µg g^{-1} FW)		
	Control	Stress	Fold increase	Control	Stress	Fold increase	Control	Stress	Fold increase
RHB-204	25.88	338.04	13.06	37.26	48.06	1.29	21.80	82.46	3.78
RHB-206	26.19	258.71	9.88	34.56	44.28	1.28	22.99	77.97	3.39
RHB-208	30.87	508.83	16.48	26.46	36.72	1.38	16.52	67.40	4.07
RHB-302	15.50	286.88	18.51	16.74	31.32	1.87	14.40	80.61	5.59
RHB-488	29.33	62.96	2.14	31.86	35.10	1.10	25.50	70.04	2.74
DCH-32	9.32	128.64	13.80	27.00	47.52	1.76	18.50	84.05	4.54
Phule-388	30.44	271.12	8.91	31.32	37.26	1.19	24.97	86.03	3.44
Mean	23.93	265.02	11.52	29.31	40.03	1.41	20.66	78.36	3.93
SE	1.47	2.69	-	1.27	1.17	-	3.28	0.33	-
C.D	4.45	8.09	-	3.86	3.55	-	9.93	0.99	-

The P5CS activity in the control leaf samples ranged from 16.74 in RHB-302 to 37.26 in RHB-204 with a mean value of 29.31 µmol ã-glutamyl hydroxamate formed g^{-1} h^{-1} in the stressed leaf samples, the P5CS activity was found to increase from 31.32 in Phule-388 to 48.06 RHB-204 with a mean value of 40.03 µmol ã-glutamyl hydroxamate formed g^{-1} h^{-1} (Table 2). The highest fold increase in free proline content was recorded in cultivar RHB- 302 under drought which also recorded a 1.87-fold increase in P5CS activity and was the maximum. Similar increase in the level of the P5CS was recorded in *Vigna* roots under salt stress. Removal of the feedback inhibition of P5CS followed by overproduction of such a mutant enzyme in transgenic plants has been reported to cause higher of accumulation of proline (Rudulier *et al.* 1984).

The free proline content and P5CS activity in the leaves of stressed and unstressed plants of *G. arboreum* cultivars (Table 3) showed that the proline content of control plants also varied significantly from 40.59 in AKA-8401 to 137.60 in AKA-7 with a mean value of 108.61 µmol g^{-1} FW. The free proline content increased significantly under stress condition and ranged from 146.20 to 627.45 with a mean value of 451.02 µmol g^{-1} FW (Table 3). The highest fold increase of 5.75 was recorded in JLA-794 and the lowest of 2.91 in Jawahar Tapti. The cultivars Y-1 and PA-183 which showed 5.23 and 4.85 fold increase, respectively, also appeared to be promising drought tolerant ones based on their proline accumulation potentials under stress condition. The endogenous proline levels under unstressed situation were higher in *G. arboreum* which is widely grown under rainfed conditions in Maharashtra.

The activity of Δ^1-pyrroline-5-carboxylate synthetase also increased substantially under stress condition. The P5CS activity in control leaves ranged from 8.07 in Sarvottum to 19.23 in Jawahar Tapti with a mean value of 13.58 µmol ã-glutamyl hydroxamate g^{-1} h^{-1} and from 18.32 in Sarvottum to 56.59 in PA-183 with mean value of 35.47 µmol ã-glutamyl hydroxamate g^{-1} h^{-1} (Table 3). The fold increase in the P5CS activity is correlated with the corresponding increase in proline content. The maximum fold increase of 3.51 was recorded in JLA-794 whereas, the minimum folds increase of 2.13 was in Jawahar Tapti.

Similar increase in free proline content in ragi leaves ranging from 71 to 87 µg g^{-1} FW under normal condition to a very large increase in free proline under stress condition has been reported. The stressed leaves had 6.5

Table 3. Evaluation of *G. arboreum* for proline, P5CS activity and glycine betaine content under control and stressed condition.

Hybrids	Proline (µmol g^{-1} FW)			P5CS activity (µmol ã- glutamyl hydroxamate formed g^{-1} h^{-1})			Glycine betaine (µg g^{-1} FW)		
	Control	Stress	Fold increase	Control	Stress	Fold increase	Control	Stress	Fold increase
Y-1	115.58	605.44	5.23	12.94	40.71	3.14	63.44	97.13	1.53
JLA-794	90.81	522.88	5.75	11.46	40.24	3.51	90.49	58.21	1.74
Turab	134.84	412.80	3.06	10.97	23.90	2.18	71.36	93.56	1.31
PA-183	129.34	627.45	4.85	18.55	56.59	3.05	45.59	75.59	1.65
AKA-8401	40.59	146.20	3.60	12.33	26.45	2.14	61.72	77.31	1.25
AKA-7	137.60	566.91	4.12	15.13	36.56	2.41	59.47	80.61	1.35
Sarvottum	118.83	429.31	3.62	8.07	18.32	2.27	80.61	89.86	1.11
Jawahar Tapti	101.82	297.21	2.91	19.23	41.06	2.13	75.33	82.60	1.09
Mean	108.61	451.02	4.14	13.58	35.47	2.60	68.50	94.36	1.37
SE	7.34	12.07	-	0.17	0.23	-	0.29	0.29	-
C.D.	22.19	36.53	-	0.50	0.70	-	0.87	0.87	-

to 65-fold more proline as the stress prolonged and attained the maximal level after the polyethylene glycol treatment (Kandpal *et al.* 1981). The activity of proline metabolizing enzyme, ã-glutamyl kinase was found to increase under water stress in *Abelmoschus esculentus*. The ã-glutamyl kinase activity increased significantly in leaf tissue of JK Haritha variety by 81 and 33% over control and the lowest accumulation was observed in Mahyco, by 64 and 7% over control at 50 and 70 DAS under drought stressed conditions (Sankar *et al.* 2007).

The changes in leaf glycine betaine content in response to water stress in intra-hirsutum (HxH), inter-specific (HxB) and *G. arboreum* cultivar are depicted in Table 1- 3. The glycine betaine contents in the leaves of stressed plants increased significantly in all the three groups of cotton cultivars. The highest fold increase of 2.19 in glycine betaine was recorded in RHH-216 followed by RHH-520 with 1.98 and Nanded-44 with 1.86 fold increase under stress condition in HxH hybrids (Table 1). In case of HxB hybrid the highest fold increase of 5.59 in glycine betaine level was recorded in RHB-302 (Table-2) whereas, *G. arboreum* cultivars (Table-3) JLA-794 recorded 1.74 fold increase followed by PA-183 with 1.65 and Y-1 with 1.53 under stressed condition as compared to control. The endogenous level of glycine betaine in the leaves of *G. arboreum* was maximum, it appears that *G. arboreum* cotton cultivars have the inherent ability to withstand or tolerate stress. The fold increase in glycine betaine in *G. arboreum* varied from 1.09 to 1.74 against the intra-hirsutum 0.98 to 2.19 and in inter-specific 2.74 to 5.59.

Water deficit resulted in a significant increase in glycine betaine content in cotton cultivars compared to its levels at field capacity. A significant variation was recorded for glycine betaine level among cotton cultivars, cultivar Tamcot HQ 95 and Tamcot sphinx, bred for rainfed and water stress conditions recorded the highest levels of glycine betaine (Naidu *et al.* 1998).

The *G. arboreum, a desi* cotton, is suitable for cultivation under dry land condition and is widely cultivated in *Khandesh* and *Vidarbha* regions of Maharashtra. The endogenous levels of glycine betaine in the leaves of *G. arboreum* cultivars were maximum as compared to intra-hirsutum (HxH) and inter-specific (HxB) hybrids. It appears that the *G. arboreum* cotton has the inherent trait to withstand or tolerate stress. The highest fold increase in the glycine betaine contents in *G. arboreum* cultivars varied from 1.09 to 1.74 as against the glycine betaine contents in intra-hirsutum (0.98 to 2.19) and inter- specific (2.74 - 5.59) hybrids.

Evaluation of HxH hybrids revealed that the endogenous levels of leaf glycine betaine varied significantly from 33.7 to 129.5 under unstressed which increased from 53.65 to 170.48 under water stress (Table 1-3). Hybrids RHH-216 and RHH-520 recorded fold increase in glycine betaine levels of 2.19 and 1.98 respectively which is better than Nanded-44.

REFERENCES

Anonymous. (2007). Performance of Bt-cotton cultivars in Maharashtra. *In*: Report of State Department of Agriculture, Pune- 411 001, Maharashtra, India.

Balibrea, M.E., Deli Amico, J., Bolarin, M.C. and Perez-Alfocea, F. (2000). Carbon partitioning and sucrose metabolism in tomato plants growing under salinity. *Physiol Plant*. **110:** 503-511.

Bates, L.S., Waldren, R.P. and Teare, I.D. (1973). Rapid determination of free proline for water stress studies. *Plant Soil* **39:** 205-207.

Dalvi, U.S., Chavan, U.D., Kachare, D.P. and Naik, R.M. (2007). Proline metabolism in sorghum and chickpea cultivars during water stress. *Indian J. Plant Physiol.* **12:** 287-289.

Hayzer, D.J. and Leisinger, T. (1980). The gene enzyme relationships of proline biosynthesis in *E. coli*. *J. Gen. Microbiol.* **118:** 287-293.

Kandpal, R.P., Vidyanathan, C.S., Udaykumar, M., Krisnasastry, K.S. and Appaji-Rao, N. (1981). Alterations in the activities of the enzymes of proline metabolism in ragi (*Eleusine coracana*) leaves during water stress. *J. Biol. Sci*. **3:** 361-370.

Kavi Kishor, P.B., Hong, Z., Miao, G. and Verma, D.P.S. (1995). Over expression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol* **108:** 1387-1394.

McMichael, B.L. and Elmore, C.D. (1977). Proline accumulation in water stressed cotton leaves. *Crop Sci.* **17:** 905-908.

McNeil, S.D., Nuccio, M.L., Hanson, A.D. (1999). Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. *Plant Physiol*. **120:** 945-949.

Naidu, B.P., Cameron, D.F. and Konduri, S.V. (1998). Improving drought tolerance of cotton by glycine betaine application and selection. CSIRO Tropical Agricultural, Cunningham Laboratory, St Lucia, Old. 4067. *Proc. Aust. Agro. Conf, Aust. Soc. Agron*., Australia.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukaya, H., Kukubari, Y., and Yamaguchi-Shinozaki, K. (1999). Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. *Plant J*. **18:** 185-193.

Parida, A.K., Dagaonkar, V.S., Phalak, M.S., Umalkar, G.V. and Aurangabadkar, L.P. (2007). Alterations in photosynthetic pigments, protein and osmotic components in cotton genotypes subjected to short-term drought stress followed by recovery. *Plant Biotechnol. Rep.* **1:** 37-48.

Quisenberry, J.Z, Wendt, C.W., Berlin, J.D. and McMichael, B.L. (1985). Potential for using leaf turgidity to select drought tolerance in cotton. *Crop. Sci*. **25:** 294-299.

Rhodes, D. and Hanson, A.D. (1993). Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **44:** 357-384.

Rudulier, D.L., Strom, A.R., Dandekar, A.M., Smith, L.T. and Valentine, R.C. (1984). Molecular biology of osmoregulation. *Science* **224:** 1064-1068.

Sankar, B., Jaleel, C.A., Manivannan, P., Kishorekumar, A., Somasundaram, R. and Panneeselvam, R. (2007). Drought-induced biochemical modifications and proline metabolism in *Abelmoschus esculentus* (L.) Moench. *Acta Bot. Croat*. **66:** 43-56.

Stumps, D.K. (1984). Quantification and purification of quaternary ammonium compounds from halophyte tissue. *Plant Physiol*. **75:** 273-274.

Zhu, B., Su, J., Chang, M., Verma, D.P.S., Fan, Y.L. and Wu, R. (1998). Over expression of Δ^1-pyrroline-5- carboxylate synthetase gene and analysis of tolerance to water and salt stress in transgenic rice. *Plant Sci*. **139:** 41-48.