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SHORT COMMUNICATION

VARIATIONS IN PROLINE METABOLISM AND DNA POLYMORPHISM IN SORGHUM CULTIVARS DIFFERING IN OSMOTIC STRESS TOLERANCE

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Six drought tolerant and six drought susceptible sorghum cultivars were screened for proline accumulation and proline 5 carboxylate synthetase (P5CS) activity in relation to osmotic stress. The PCR based RAPD technique was employed to detect polymorphism in genomic DNA with eighteen operon primers in all twelve sorghum cultivars. Higher synthesis of proline and P5CS enzyme activity was observed in the roots of stressed plants of tolerant cultivars than susceptible ones. The primers OPC-19, OPE-03, OPE-09 and OPA-07 detected polymorphism in genomic DNA of six tolerant and six susceptible cultivars.

Key words: **Osmotic stress, PCR, Proline, RAPD, *Sorghum bicolor***

Sorghum bicolor L. Monech is the third important cereal crop in India, next only to rice, wheat and maize grown in India. The sorghum grains are an important source of dietary proteins, carbohydrates, minerals and B group vitamins, particularly to the vegetarian diets in India (Salunkhe *et al.* 1986). In India sorghum is cultivated on an area of about 9.10 million tonnes (Agriculture research data book 2006). Maharashtra ranks first in sorghum production in India, however, the national average productivity of sorghum is much higher than Maharashtra during *rabi* season due to recurrent moisture stress. Various biotic and abiotc factors affect the production of *rabi* sorghum (Chen and Murata 2002). Among these factors drought is the major limiting factor for crop growth and production under rainfed condition. Germplasm screening for drought tolerance under naturally occurring drought stress is less reliable and time consuming (Oritz *et al.* 2002). Under stress conditions, plant accumulates several kinds of osmolytes such as proline, glycine betaine, and soluble sugars (Delauney 1993). The present study is mainly focussed on proline accumulation and P5CS enzyme activity in relation to drought tolerance. Studying the various biochemical changes associated with moisture stress in sorghum will help to develop moisture stress tolerance varieties employing both conventional and molecular breeding (Maiti *et al.* 2000). Out of the several PCR based techniques, Random Amplified Polymorphic DNA (RAPD) offers a simple and economic means for rapid identification of a large number of accessions. The information obtained using RAPD is extensively used for identification of germplasm, varieties, assessing genetic diversity and monitoring the genetic stability of conserved germplasm. The present study is aimed to employ the RAPD approach to distinguish different varieties of sorghum in relation to drought tolerance.

The seeds of six drought susceptible cultivars *viz.* CSV-18, CSV-216, SPV-1502, RSLG-1119, RSV-423, SPV-1626 and six drought tolerant cultivars *viz*. M 35-1, RSLG-262, SPV-1546, RSV-651RSV-658, RSV-695 were obtained from the Sorghum Improvement Project,

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M P K V, Rahuri. The seeds of these cultivars were initially allowed to germinate for five days in petri plates at 27^0C in agar medium. After five days, stress was applied by adding PEG-6000 of osmotic stress of -1.0 Mpa after standardisation for stress study, and growth of seedlings were continued for another five days. The roots of both unstressed and stressed seedlings were analysed for contents of proline, P5CS activity, and soluble protein content while control seedlings were used for RAPD analysis. Proline content in roots was determined by using acid ninhydrin reagent as per the method described by Bates *et al.* (1973) and expressed on fresh weight basis as micromoles g^{-1} fresh wt^{-1}.

The activity of pyrroline-5-carboxylate synthetase was assayed according to the method of Garcia-Rios *et al.* (1997). Assay mixture contained 50 mM ã-glutamate, 10 mM ATP, 20 mM $MgCl_2$, 100 mM hydroxylamine hydrochloride, 50 mM Tris HCL buffer pH 7.0. Two ml of enzyme assay mixture was pipetted in test tube and 0.8 ml of crude enzyme was added to it. The reaction was terminated by addition of two ml of 2.5 M HCL containing 2.5% Ferric chloride and 6% TCA. The activity was expressed in terms of µmoles of ã-glutamyl hydroximate produced g^{-1} fresh wt. min^{-1}.

Soluble proteins from the roots were extracted in 0.5 M Tris HCL buffer (pH 6.8) and determined by the colourimetric method described by Lowry *et al.* (1951). Genomic DNA of all cultivars under study was isolated from 15 days old normally grown whole seedlings using the method of Murray and Thompson (1980). Purification and quantification of DNA was also carried out as described in Sambrook and Russell (2000). For RAPD analysis 18 random decamer primers (Operon Technologies, Inc. U S A.) were used to amplify genomic DNA. The PCR of genomic DNA of the cultivars was carried out as per the protocol described by Naghia *et al.* (2002). PCR reactions were conducted in volume of 25 µl containing 2.5 µl 10 X Taq buffer, 2.0 µl of dNTPs (0.5 µl each), 2.0 µl primer, 1.5 µl Taq DNA polymerase , 15.0 µl sterile water and 2.0 µl of genomic DNA (25 ng). The PCR reaction was performed in a thermal cycler (Techne U.K.). Amplified DNA fragments were electrophoresed on a 1.2% (w/v) agarose gel and visualised under UV- transilluminator and the gel was photographed on ALPHAIMAGER gel documentation system.

The results obtained in this study indicate differences in root proline content in susceptible and tolerant cultivars under unstressed condition (Table 1). After inducing the osmotic stress of -1.0 mpa it was observed that proline content was increased in both tolerant and susceptible cultivars. In tolerant cultivars proline accumulation ranged from 15.44 to 19.69 µmoles g^{-1} fw with mean of 17.43 µmoles g^{-1} fw (7 to 8 folds) increase while in susceptible type it varied from 5.99 to 8.82 µmoles g^{-1} fw with a mean of 7.51 µmoles g^{-1}fw (4 to 5 fold) increase. Based on the proline content under osmotic stressed condition and fold increase over control the cultivars M 35-1 and SPV-1546 appeared to be more drought tolerant than the rest of cultivars. In addition, the cultivar RSLG-1119 showed drought susceptibility based on the amount of proline accumulation. The higher magnitude of proline accumulation may help plants to

Table 1. Effect of osmotic stress on proline content (µmol g^{-1} fresh wt.) in sorghum cultivars

Sr. No.	Cultivar	Control	Stress
A)	**Tolerant**		
1.	M-35-1	2.23	19.69
2.	RSLG-262	2.81	18.43
3.	RSV-651	2.31	17.32
4.	SPV-1546	2.11	17.17
5.	RSV-658	2.71	16.54
6.	RSV-695	1.83	15.44
	Mean	2.33	17.43
	SEm ±	1.20	3.08
	CD at 5%	3.6	9.24
B)	**Susceptible**		
7.	CSV-216	2.38	8.82
8.	CSV-18	2.11	8.35
9.	SPV-1502	1.66	7.75
10.	SPV-1626	1.56	7.40
11.	RSV-423	1.48	6.77
12.	RSLG-1119	1.81	5.99
	Mean	1.83	7.51
	SEm ±	1.08	2.20
	CD at 5%	3.6	6.60

tolerate the dehydration by maintaining cell turgidity as reported earlier by Siva Kumar *et al.* (1998) and may protect plants against singlet oxygen and free radical induced damages. Several investigators have reported positive relationship between free proline content in leaves with drought tolerance in sorghum (Bhaskaran *et al.* 1985, Mulla *et al.* 2004).

Changes in P5CS activity in relation to proline accumulation was studied in root tissues under normal and stress conditions. Upon application of osmotic stress, the P5CS activity was found to increase markedly in tolerant types. The activity was found to range from 0.37 to 0.84 with a mean value of 0.54 µmoles of ã-glutamyl hydroximate produced g^{-1} fw root tissue min^{-1} (4 folds) in tolerant types while it was found to vary from 0.23 to 0.32 µmoles of ã-glutamyl hydroximate produced g^{-1} fw root tissue min^{-1} with a mean value of 0.28 (2 folds) in susceptible types (Table 2). These results clearly indicate that proline accumulation was found to be positively related with P5CS activity (Cheng *et al.* 1998, Anon 2006 b). So the increase in proline accumulation due to feedback activity of P5CS enzyme can be used as a most reliable biochemical marker to screen the sorghum genotypes of segregating populations of hybridisation programme to identify drought tolerant genotypes in sorghum.

The changes in root soluble protein contents in response to osmotic stress were monitored to study its relationship with proline accumulation. Upon application of stress the soluble protein was found to increase from 26.20 to 39.99 mg g^{-1} fw with a mean of 33.65 mg g^{-1} fw in tolerant cultivars (3 folds) while it was found to vary from 19.44 to 24.02 mg g^{-1} fw with a mean of 21.58 mg g^{-1} fw in susceptible types (2 folds) (Table 3). These indicated that osmotic stress induces protein biosynthesis and increased proline content is due to protein degradation. Mulla (2004) reported increase in soluble protein irrespective of tolerance and susceptible cultivars from leaves of sorghum due to water stress.

Table 2. Effect of osmotic stress on root P5CS activity (µmol γ-glutamyl hydroximate g^{-1} fw min^{-1}) in sorghum cultivars

Sr. No.	Cultivar	Control	Stress
A)	**Tolerant**		
1.	M-35-1	0.23	0.84
2.	RSLG-262	0.16	0.59
3.	RSV-651	0.14	0.37
4.	SPV-1546	0.14	0.51
5.	RSV-658	0.13	0.45
6.	RSV-695	0.14	0.49
	Mean	0.16	0.54
	SEm ±	0.26	0.40
	CD at 5%	0.78	1.20
B)	**Susceptible**		
7.	CSV-216	0.19	0.26
8.	CSV-18	0.16	0.32
9.	SPV-1502	0.20	0.30
10.	SPV-1626	0.16	0.24
11.	RSV-423	0.14	0.31
12.	RSLG-1119	0.13	0.23
	Mean	0.16	0.28
	SEm ±	0.21	0.34
	CD at 5%	0.63	1.02

Table 3. Effect of osmotic stress on root soluble protein content (mg g^{-1} fresh weight) in sorghum cultivars

Sr. No.	Cultivar	Control	Stress
A)	**Tolerant**		
1.	M-35-1	12.40	39.99
2.	RSLG-262	11.58	26.20
3.	RSV-651	18.08	38.88
4.	SPV-1546	16.26	33.60
5.	RSV-658	13.50	35.12
6.	RSV-695	10.30	28.12
	Mean	13.69	33.65
	SEm ±	1.04	2.12
	CD at 5%	3.12	6.36
B)	**Susceptible**		
7.	CSV-216	11.30	24.02
8.	CSV-18	13.28	21.08
9.	SPV-1502	10.69	19.44
10.	SPV-1626	15.10	21.42
11.	RSV-423	17.22	20.60
12.	RSLG-1119	12.26	22.90
	Mean	15.31	21.58
	SEm ±	1.10	1.72
	CD at 5%	3.30	5.16

In RAPD analysis study of tolerant and susceptible cultivars, the maximum number of bands obtained with each primer varied from a minimum of 7 with OPA-04 to a maximum of 14 with OPD-06 primer. The molecular size of amplicons varied from 0.28 to 2.76 kb and the % of polymorphism ranged from a minimum of 22.22% with OPE-06 to a maximum of 90.0% with OPC-19 (Table 4). The presence of one or two prominent amplicons in resistant type and absent in susceptible one is important. Such amplicons can be used as an innovative marker to design specific primers for monitoring the segregating populations in appropriate crosses. In this context, the amplification pattern observed with OPE-03 and OPC-19 in tolerant types or with OPE-09 and OPA-07 in susceptible types, seems to be interesting in selecting appropriate parents for hybridisation. With OPC-19 the amplicons of 0.76 kb was observed to be quite distinct and prominent in three tolerant cultivars, similarly the amplicons of 0.29 kb is observed in all tolerant types with OPE-03. Several investigators have used RAPD analysis using variety of primers to study the genetic diversity in sorghum (Dalhberg *et al.* 2002, Agrama and Tunistra 2003).

Table 4. Percentage polymorphism shown by different RAPD primers in sorghum cultivars

Sr. No.	Primer	Total No. of bands (T)	Total No. of polymorphic bands (P)	Percent polymorphism P/T x 100
1	OPE-06	9	2	22.22
2	OPB-07	14	5	35.71
3	OPD-15	8	3	37.50
4	OPA-11	9	4	44.44
5	OPA-12	8	4	50.00
6	OPD-08	11	6	54.54
7	OPB-08	11	6	54.54
8	OPA-04	7	4	57.54
9	OPE-09	8	5	62.50
10	OPA-09	11	7	63.63
11	OPA-13	14	9	64.28
12	OPE-14	9	6	66.66
13	OPB-09	9	6	66.66
14	OPD-03	13	10	76.92
15	OPE-03	9	7	77.77
16	OPD-06	14	11	78.57
17	OPA-07	8	7	87.50
18	OPC-19	10	9	90.00

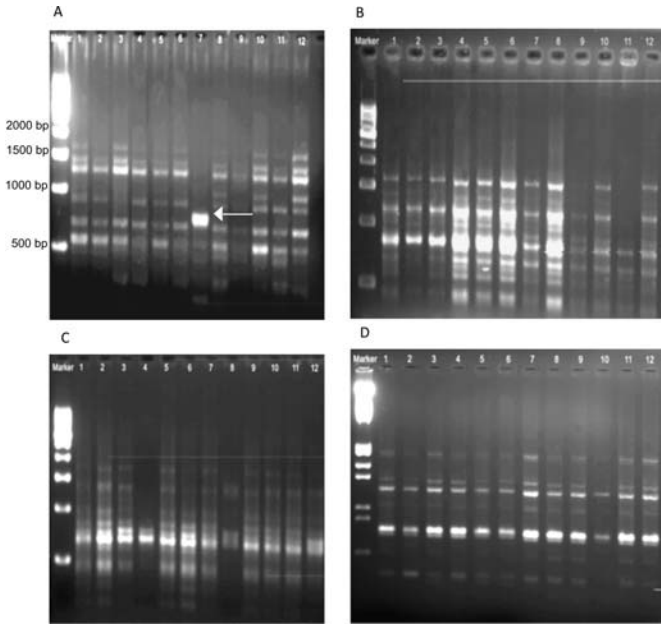

Plate 1. Amplification Profile of six drought tolerant and six drought susceptible cultivated varieties of sorghum with OPA-07 (A), OPC-19 (B), OPE-03 (C) and OPE-09 (D)
M: γ 500 bp DNA ladder: 1. M-35-1, 2. RSLG-262, 3. SPV-1546, 4. RSV-651, 5. RSV-658, 6. RSV-695, 7. CSV-18, 8. CSV-216, 9. SPV-1502, 10. RSLG-1119, 11. RSV-423, 12. SPV-1626

This study concludes that large increase in proline and P5CS activity in tolerant sorghum cultivars may be considered as an important adaptive characteristic under water stress. RAPD analysis of genomic DNA revealed that the primers OPE-03 and OPC-19 synthesised unique fragments only in tolerant cultivars and can be used in further study for selection of segregating populations.

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