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Full Research Article

Molecular Analysis of *Rabi* Sorghum Genotypes Differing in Osmolytes Accumulation under Water Stress

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Abstract

Forty-eight sorghum accessions were screened for proline and glycine betaine osmolyte accumulation levels under-0.5 MPa PEG–6000 induced osmotic stress in the leaves of ten days old seedlings. Proline as well as glycine betaine accumulation was higher in stressed than unstressed condition. Under stressed conditions, tolerant sorghum genotypes exhibited highest increase in both the osmolytes, followed by stay-green and susceptible ones. Four each of drought susceptible, tolerant and stay-green sorghum genotypes differing in proline and glycine betaine accumulation potential under osmotic stress were analyzed with randomly amplified polymorphic DNA markers with a view to understand genetic diversity among the different types as well as proline and glycine betaine accumulation potential under water stress. Out of 43 primers screened, 26 amplified genomic DNA with 258 loci of which, 191 were polymorphic with 75.55% polymorphism. Among the random operon primers, thirteen showed twenty unique loci. The Dice similarity coefficient values based on RAPD data ranged from 0.70 to 0.91 with the minimum in a drought susceptible genotype RSV-1006 and the maximum in stay-green genotype M-35-1.The dendrogram revealed a separate major cluster of stay-green genotype, E-36-1. 2D scatter plot showed a separate group of all four stay-green genotypes closely placed with two high proline accumulating tolerant genotypes, RSLG-262 and RSV-1366. However, two high glycine betaine accumulating tolerant genotypes, RSV-458 and *Hadgaon* local, appeared to be distinct from the stay-green genotypes.

1. Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] forms the dietary staple of more than 500 millionpeople in more than 30 countries of the world (Dahlberg et al., 2011). The crop is the fifth most important cereal next only to rice, wheat, maize and barley in India. It is a self-pollinating, diploid (2n=2x=20) with a genome size of 1C=735 Mbp which is about 25% the size of maize, or sugarcane. The sorghum is grown during both the seasons of *kharif and rabi*. In Maharashtra, it is mostly grown on the residual soil moisture, which generally suffers from severe moisture stress during the later stage of crop growth in *rabi* season. Drought is a major abiotic stress factor limiting crop production. Sorghum is well adapted to hot and dry environments and is regarded as a model crop for studying drought resistance among the grasses. The drought resistance is a complex trait and gene expression depends on the action and interaction of different morphological, physiological and biochemical characters (Mitra, 2001; Dalal et al., 2012). Under stress condition, plant accumulates several kinds of compatible osmolytes such as proline, glycine betaine, sugar, alcohol and soluble protein (Delauney and Verma, 1993). The degree of stress tolerance has been positively correlated with the levels of organic solutes like proline and glycine betaine (GB) in a number of crop plants (Barnett and Naylor, 1996). Accumulation of proline under water deficit condition is considered to be an important character responsible for drought tolerance (Blum and Ebercon, 1976). It involves two major key regulatory enzymes such as pyrroline-5-carboxylate synthetase (P_5CS) and proline oxidase. The former is involved in proline biosynthesis, and the latter in its degradation (Reddy et al., 2015). The GB is synthesized from phosphatidylcholine which accumulates in plants in a large quantity. Genetic transformations have allowed the introduction of new pathways for biosynthesis of various compatible solutes in plants, resulting in the production of transgenic plants with

improved tolerance to stress. Transgenic plant accumulates GB at various levels and exhibit enhanced tolerance to several stresses (Sakamoto and Murata, 2002). The concentration of GB in such transgenic plant is generally low as compared to the levels observed in the stressed plant species that normally accumulate GB under stress. Two major factors have been identified that limit accumulation of glycine betaine in transgenic plants, the availability of endogenous choline itself (McNeil et al., 1999) and the transport of choline across the chloroplast envelope. Most sorghum plants show accelerated leaf senescence and premature death when drought occurs during grain-filling stage. Stay-green genotypes remain green due to a delay in leaf senescence and continue to fill grain under water-limited environments. Therefore, identification of genetic factors involved in plant responses to drought stress will provide the foundation to improve its drought resistance. Many studies have been carried outfor accessing patterns of sorghum genetic variation based on morphology (Appa-Rao et al., 1996; Shehzad et al., 2009), pedigree (Jorden et al., 1998) or phylogenetic diversity (Cheprot et al., 2013; Agrama et al., 2003). Various molecular and biochemical markers are available to differentiate variation in individuals. RAPD marker used in the present study because of it is one of the commonly used molecular technique based on PCR, their simplicity, speed and relatively low cost. However, the reproducibility of results is questioned, by using careful reaction components RAPD used extensively in various studies (Sharma et al., 2016). In the present study, both biochemical marker as well as molecular marker is used thoughtfully. Initially, drought susceptible and tolerant genotypes differing inproline and GB accumulation potential, under PEG-6000-induced osmotic stress i.e. high and low proline, and high and low GB containing genotypes were identified and further analyzed for RAPD polymorphism and compared with stay-green genotypes with view to find out relationship observed in biochemical parameters reflects in RAPD marker.

2. Materials and Methods

2.1. Plant material

The seeds of twenty-two drought tolerant and susceptible along withfour stay-green sorghum genotypes were collected from different locations of Maharashtra, however of same agro-ecological zone of Deccan plateau, hot semi-arid eco-region. The sterilized petri-dishes of uniform size and shape were used to grow the sorghum seedlings. A fixed volume of sterilized liquid agar solution (0.8%) was pouredinto petri-dishes and allowed to cool for solidification. The clean and viablesorghum seeds were surface-sterilized with 0.1% (w/v) $HgCl_2$ and washed 4-5 times with distilled water. The seeds were puton the agar medium. The petri dishes were kept in an incubator already maintained at 27±1 °C for seven days to obtain better initial germination and growth.The seedlings were then treated with an optimized osmotic stress of -0.5 MPa PEG-6000 solution, kept for three more days and unstressed without PEG served as control. The leaves of the ten days old seedling, given water stress with -0.5 MPa PEG-6000 and the control (only 0.8% agar) were cut with a pair of scissors, weighed and used for proline and GB content.

2.2. Plant analysis

The leaves of the forty-eight genotypes were separately weighed and analyzed for proline (Bates et al., 1973) and glycine betaine (Stumpf, 1984). Based on proline and glycine betaine accumulation potential eight genotypes were selected, in which two each from high (RSLG-262 and RSV-1366) and low (SPV-504 and RSV-1006) proline accumulators and two each from high (RSV-458 and *Hadgaon* local) and low (RSV-1045 and CSV-18) glycine betaine accumulators, in addition based on the data reported by the ICRISAT, India on % green leaf area of several stay-green genotypes (Mahalakshmi and Bidinger, 2002), the most suitable four stay-green genotypes, B-35, E-36-1, M-35-1 and Sel-3 were selected in the present study for comparison (Table 1).

2.3. DNA extraction and PCR assay

Table 1: Characteristics of drought tolerant, susceptible and stay-green *rabi* sorghum genotypes

Sl. No.	Genotypes	Pedigree
A.	Tolerant genotypes	
1.	RSLG-262 (*Maulee*)	Selection from local germplasm
2.	RSV-1366	SPV-1587×Phule *Maulee*
3.	RSV-458 (*Anuradha*)	RSLG-559×RSLG-1175
4.	*Hadgaon* local (H. local)	Selection from local collection
B.	Susceptible genotypes	
5.	SPV-504 (*Swati*)	SPV 86×M-35-1
6.	RSV-1006 (*Revati*)	CSV-216×SPV-1502
7.	RSV-1045	RSV-214×RSFR-9509
8.	CSV-18 (SPV-1596)	CR-4×IS-18370
C.	Stay-green genotypes	
9.	B-35	USA origin
10.	E-36-1	Ethiopian origin
11.	M-35-1	Selection from local *Maldandi* population
12.	Sel-3	Selection from local *Bedar*

The isolation and purification of DNA from seedlings of various sorghum genotypes was performed as per the method described (Dellaporta and Wood, 1983) with some modifications. Concentration of purified DNA was measured using UV-visible spectrophotometer (Nanodrop, ND-1000 USA) at 260 and 280 nm wavelengths. The ratio of absorbance at 260/280 was calculated which was ~ 1.8. Two µl of all DNA extracts were electrophoresed (Bio Rad sub cell model 96, USA) on 0.8% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide at 6 Vcm^{-1} in TBE buffer. After electrophoresis, the band intensity of genomic DNA was visualized on gel documentation unit (FluorChem TM Alpha Innotech, USA) and compared with standard DNA. These gels also provided a visual measure of purity and integrity of DNA. RAPD was performed using 43 random decamer primers obtained from Operon Biotechnologies, GmbH, Germany. Amplification was performed in a 0.2 ml PCR tubes as described (Arya et al., 2006) with some modifications. The 25 µl reaction volume containing 1 U Taq DNA polymerase, 2.5 µl 10×PCR buffer and 0.2 µM dNTPs mix. The PCR cycle consisted of initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94 °C for one min, annealing at 37 °C for one min and a final extension at 72 °C for 10 min.

2.4. Data analysis

The data on biochemical characters were analyzed using completely randomized block design, whereas the UPGMA based dendrogram of twelve sorghum genotypes were generated with NTSYSpc 2.02i programme.

3. Results and Discussion

3.1. Proline and glycine betaine accumulation under osmotic stress

The data presented in Table 2 showed the effect of -0.5 MPa PEG–6000 -induced osmotic stress on proline and GB accumulation levels in the leaves of 10 days old sorghum seedlings. Screening of forty-eight sorghum genotypes revealed that tolerant genotypes, RSLG-262 and RSV-1366 were found to accumulate the highest proline of 0.962 and 1.263 µmoles g^{-1} fr.wt. respectively, whereas the genotypes, SPV-504 and RSV-1006 were the lowest proline accumulating ones from susceptible group under osmotic

Table 2: Effect of PEG-6000–induced osmotic stress on proline and glycine betaine content

Sl. No.	Genotypes	Proline (µ moles g^{-1} fr.wt.)			Glycine betaine (µ moles g^{-1} fr.wt.)		
		Unstressed	Stressed	% increase	Unstressed	Stressed	% increase
A.	Tolerant genotypes						
1.	RSLG-262	0.154	0.956	521	6.30	18.48	193
2.	RSV-1366	0.147	1.263	759	7.68	21.78	185
3.	RSV-458	0.145	0.562	288	8.40	33.48	299
4.	*Hadgaon* local	0.177	0.324	83	9.48	37.14	291
	Mean	0.156	0.776	413	7.96	27.72	242
B.	Susceptible genotypes						
5.	SPV-504	0.140	0.181	29	7.62	16.80	120
6.	RSV-1006	0.142	0.233	64	6.60	14.40	118
7.	RSV-1045	0.161	0.291	81	5.34	11.28	111
8.	CSV-18	0.164	0.288	76	5.20	10.56	103
	Mean	0.152	0.248	62.5	6.19	13.26	113
C.	Stay–green genotypes						
9.	B-35	0.159	0.309	94	12.30	28.74	133
10.	E-36-1	0.179	0.350	96	12.24	28.08	130
11.	M-35-1	0.164	0.339	107	11.52	27.78	141
12.	Sel-3	0.147	0.293	99	11.04	22.98	108
	Mean	0.162	0.323	99	11.78	26.90	108
	Comparison	S.E.±	CD (p=0.05)		S.E.±	CD (p=0.05)	
1.	Genotypes	0.041	0.113		1.11	2.66	
2.	Treatments	0.008	0.023		0.40	1.20	
3.	Genotypes×Treatments	0.058	0.16		1.51.	3.85	

stress condition. The tolerant genotypes, RSV-458 and *Hadgaon* local accumulated the highest glycine betaine of 33.48 and 37.14 µmoles g^{-1} fr.wt. respectively, whereas the genotypes, RSV-1045 and CSV-18 were the lowest glycine betaine accumulating ones from the susceptible group under osmotic stress condition.The mean proline and glycine betaine contents were the higher in tolerant genotypes followed by stay-green and susceptible ones. Thus based on the proline and glycine betaine accumulation under standardized osmotic stressed condition, these twelve genotypes were used further for RAPD analysis.

Significantly, positive correlation was observed between the free proline content in leaves and PEG -induced osmotic stress in various crops. PEG -induced osmotic stress resulted in higher proline accumulation in sorghum (Jafar et al., 2004, Johnson et al., 2015). In severely stressed sorghum plants, proline accounted for more than 60% of the total free amino acids pooland it goes up to 80% in many plants (Wood et al., 1996; Matysik et al., 2002). Such accumulation of proline proposed to play an important role in the osmoadaptation or osmoprotectant in the plant cell (Savoure et al., 1995, Gill et al., 2014). Proline accumulation initiated in the leaves of all sorghum cultivars as the leaf water potential was reduced from -14 to -16 bars and higher correlation observed between relative water content and proline in wheat (Blum and Ebercon, 1976, Keyvan, 2010). Similar results were obtained in the present study, which may be due to multifunctional role of proline as a signaling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression, which can be essential for plant to recover from stress (Szabados and Savoure, 2009).

Another osmolytes GB alsoaccumulated at higher level in the drought tolerant and stay-green genotypes than the susceptible ones. The large number of sorghum germplasm of 240 lines showed the genetic differences in GB content (Yang et al., 2003). Glycine betaine level under water deficit condition, increased by 26-fold in sorghum, while by doublein sugarcane (Wood et al., 1996, Abbas et al., 2014). Water deficit and salinity can lead to denaturation of proteins and disruption of membrane structures. Glycine betaine maintains the activity of enzymes under a variety of unfavorable conditions including high temperature, extremes of pH and high salt concentrations (Mickelbart et al., 1999). An increase in GB content on imposition of osmotic stress by PEG may be due to a role of GB in an adaptive response to abiotic environmental stress (Yang et al., 2003), by the enzyme choline monooxygenase activity, the rate-limiting step for GB synthesis and expression of gene induced under osmotic stress (Rathinasabapathi et al., 1997).

3.2. RAPD evaluation of genomic DNAs in sorghum genotypes

The genomic DNA were isolated from the selected four drought susceptible genotypes (viz., SPV-504, RSV 1006, RSV-1045, CSV-18), four drought tolerant genotypes (viz., RSLG-262, RSV-1366, RSV-458, *Hadgaon* local)which were also categorized into low and high proline and GB accumulating ones and fourstay-green genotypes (viz., B-35, E-36-1, M-35-1 and Sel-3). On RAPD analysis, out of 43 random decamer primers used only 26 primers amplified generating a total of 258 loci by amplification in the size range of 0.26 to 5.42 kb., Out of them 191 loci were polymorphic with an average polymorphism of 75.55% (Table 3). Each primer thus produced on an average of 7.34 polymorphic loci. The % polymorphic bands with different primers ranged from 33.33 to 100%, with OPA 04, OPC 20, OPD 04 and OPD 15 recording 100% polymorphism. The mean number of bands per accession ranged from 1.0 (with OPD 15 primer) to 12.25

Table 3: Polymorphism, size of loci as amplified by 26 RAPD primers in 12 sorghum genotypes

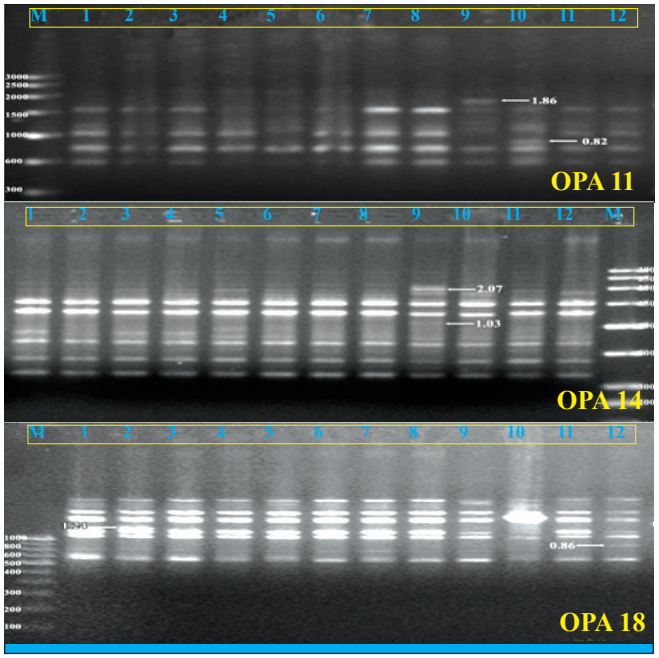
Sl. No.	Random primer	Total number of bands	No. of band position (locus)	Mean no. of bands accession^{-1}	Poly-morphic loci including unique loci*	Mono-morphic loci	% Poly-morphic bands	Fragment size (kb)
1.	OPA 01	102	9	8.50	3	6	33.33	0.54 to 2.77
2.	OPA 04	48	6	4.00	6	0	100.00	0.54 to 2.28
3.	OPA 05	106	10	8.83	3 (1)	7	30.00	0.42 to 3.12
4.	OPA 09	66	8	5.50	7	1	87.50	0.99 to 2.80
5.	OPA 10	48	5	4.00	3	2	60.00	0.62 to 3.08
6.	OPA 11	53	8	4.42	7 (2)	1	87.50	0.54 to 2.41
7.	OPA 12	81	9	6.75	4 (1)	5	44.44	0.84 to 2.22
8.	OPA 13	118	17	9.83	13	4	76.47	0.26 to 3.55
9.	OPA 14	132	16	11.0	10 (2)	6	62.50	0.33 to 2.88
10.	OPA 17	90	11	7.58	10	1	90.90	0.82 to 4.38
11.	OPA 18	97	11	8.08	8 (2)	3	72.72	0.65 to 2.23

Continue...

Sl. No.	Random primer	Total number of bands	No. of band position (locus)	Mean no. of bands accession^{-1}	Poly-morphic loci including unique loci*	Mono-morphic loci	% Poly-morphic bands	Fragment size (kb)
12.	OPA 19	54	11	4.50	10 (3)	1	90.90	0.27 to 2.91
13.	OPC 05	118	17	9.83	13	4	76.47	0.33 to 2.93
14.	OPC 09	38	8	3.17	7 (3)	1	87.50	0.76 to 2.73
15.	OPC 11	67	8	5.58	7	1	87.50	0.91 to 2.30
16.	OPC 19	35	8	2.92	7 (1)	1	87.50	0.73 to 2.62
17.	OPC 20	68	12	5.67	12 (1)	0	100.00	0.33 to 2.43
18.	OPD 04	50	6	4.17	6	0	100.00	0.63 to 2.53
19.	OPD 08	109	13	9.08	8 (1)	5	61.54	0.55 to 3.67
20.	OPD 11	127	13	10.58	6	7	46.15	0.37 to 2.77
21	OPD 15	12	2	1.00	2 (1)	0	100.00	1.87 to 2.45
22.	OPD 18	147	15	12.25	11	4	73.33	0.27 to 3.19
23.	OPE 06	109	13	9.08	12 (1)	1	92.30	0.55 to 3.62
24.	OPE 08	57	7	4.75	5	2	71.42	0.78 to 2.83
25	OPE 12	49	6	4.08	4 (1)	2	66.66	1.77 to 5.42
26.	OPE 14	64	9	5.33	7	2	77.77	0.82 to 3.64

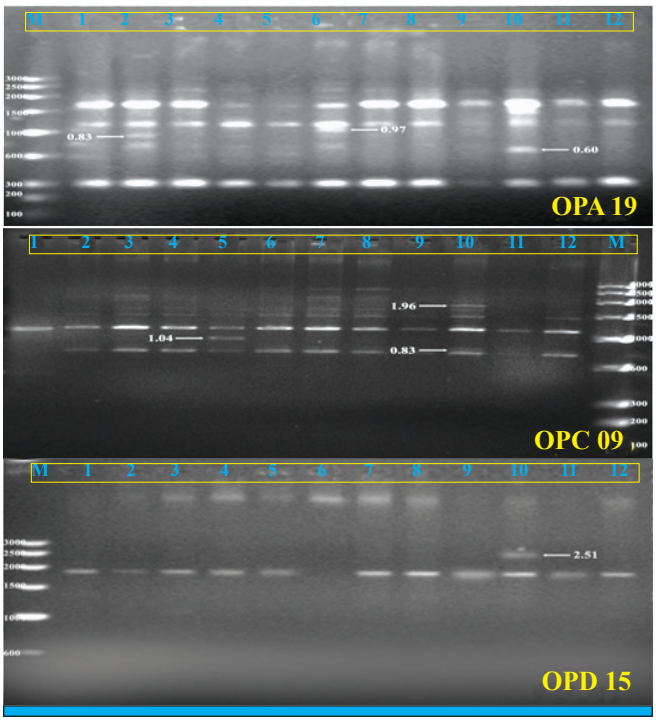
*Numbers given in parenthesis indicate unique loci.

(with OPD 18 primer). These primers amplified 20 unique loci in nine sorghum genotypes (except RSV-458, Hadgaon local and M-35-1) (Figure 1 and 2). The stay-green genotype E-36-1 recorded eight unique loci with the primers, OPA 5, OPA 11, OPA 12, OPA 19, OPC 9, OPD 8 and OPD 15. The



M: Marker, →: Arrow indicates unique band

Figure 1: RAPD amplification pattern of tolerant, susceptible and stay-green sorghum genotypes using primers, OPA11, OPA 14 and OPA 18.



M: Marker, →: Arrow indicates unique band

Figure 2: RAPD amplification pattern of tolerant, susceptible and stay-green sorghum genotypes using primers, OPA 19, OPC 9 and OPD 15.

primers OPA 19 and OPC 9 produced three each unique loci in twelve sorghum genotypes. Among the markers generated by these random primers, a few putative genotype specific

amplification products were generated which could be useful for germplasm classification and introgression studies.

3.3. Genetic diversity analysis by RAPD markers

Among the sorghum studied the pair wise similarity coefficient values genotypes ranged from 0.70 to 0.91 (Table 4), the maximum similarity noticed between drought susceptible genotypes, RSV-1006 and RSV-1045 and the minimum between drought susceptible genotype RSV-1006 and stay-green genotype M-35-1. However, the genetic diversity detected using molecular markers in the present investigation indicates moderate discrimination capacity of RAPD markers.

The UPGMA based dendrogram of 12 sorghum genotypes generated presented in Figure 3 revealed two major clusters. Second major cluster consisted of a single stay-green genotype E-36-1, whereas the first major cluster consisted of two sub-clusters. The second sub-cluster consisted of two stay-green genotypes, B-35 and M-35-1, however the first sub-cluster comprised of remaining four susceptible, four tolerant and one stay-green genotype. Further, in the first sub-cluster, one group comprised of one high proline genotype RSLG-262 and one stay-green genotype Sel-3. Another group consisted of both the high glycine betaine genotypes, RSV-458 and *Hadgaon* local. The genotype E-36-1 of the stay-green type was found to be the most divergent among the twelve dissimilar sorghum genotypes studied.

In the present investigation, genetic diversity was observed among 12 sorghum genotypes using RAPD markers that indicated its high discrimination capacity. From the similarity coefficient and consensus tree, it may be concluded that crossing between four drought tolerant, four drought susceptible and four stay-green genotypes with Dice similarity

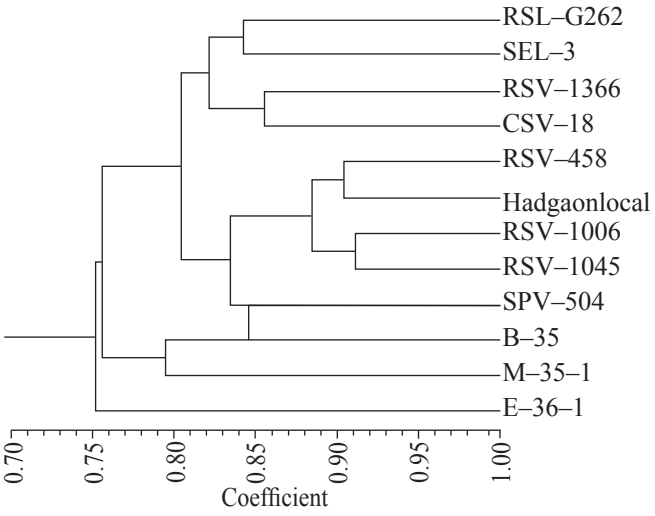


Figure 3: Consensus tree showing clustering of tolerant, susceptible and stay-green sorghum genotypes using RAPD analysis with NTSYSpc 2.02i software

coefficient ranging from 0.70 to 0.91 fell in different clusters and sub clusters resulting in obtaining heterocyst in relation to drought tolerance. The maximum similarity coefficient of 0.91 was observed between two susceptible genotypes, RSV-1006 and RSV-1045. The similarity coefficient of 0.90 was observed in two drought tolerant genotypes, RSV-458 and *Hadgaon* local, accumulating higher levels of GB osmolyte. The lowest similarity coefficient of 0.70 was observed in susceptible RSV-1006 and stay-green M-35-1 indicating their divergence.

Molecular analysis using 20 RAPD primers detected 121 loci, among which a total of 8 primers showed polymorphism, while 12 primers produced monomorphic pattern in sorghum genotypes.

Out of these amplified products, 33% were polymorphic, each primer produced on an average of 6 loci with the size of amplified product ranging from 2.5 to 3.0 kb with 59.58% polymorphism (Tabasam et al., 2006). However, the higher polymorphism of 75.59% was observed in the present investigation. Twenty pearl millet genotypes were analyzed using 30 different 10-mer primers, however 12 primers revealed scorable polymorphism between genotypes of pearl millet, out of which 12 primers produced 99 polymorphic bands at an average of 8.25 polymorphic bands primer^{-1}. Thus, the results obtained in the present investigation are in accordance with these reports (Govindaraj et al., 2009). In the present study, a total of 191 polymorphic bands with an average polymorphism of 7.34 bands were produced per primer. Genetic diversity among four wheat accessions analyzed by RAPD technique using 15 RAPD primers generated a total of 75 RAPD bands, 37 of these bands were found to be polymorphic. The number of amplification products per primer varied from 3 to 6 with a mean value of 5. These primers produced fragments, which fell in the range of 250 to 2000 bp in size. The calculated coefficient of similarity between all accessed genotypes varied between 0.627 and 0.76 (Iqbal and Bano, 2009). In the present study, analyses of genomic DNA of twelve sorghum genotypes using 26 RAPD primers depicted moderate diversity. The pair wise similarity coefficient values ranged from 0.70 in RSV-1006 and RSV-1045 to 0.91 in M-35-1 and RSV-1006. The genotype E-36-1 appeared to be the most divergent and thus grouped in a separate cluster. Based on clustering pattern wide genetic diversity reported among the grain and forage type sorghum accessions (Sinha et al., 2014).

2D scatter plot showed a separate group of all four stay-green genotypes closely placed with two high proline accumulating tolerant genotypes, RSLG-262 and RSV-1366 (Figure 4) which may be due to high proline accumulated for the stabilization of proteins and protein complexes in

the chloroplast and cytosol, protection of the photosynthetic apparatus and enzymes involved in ROS detoxification.

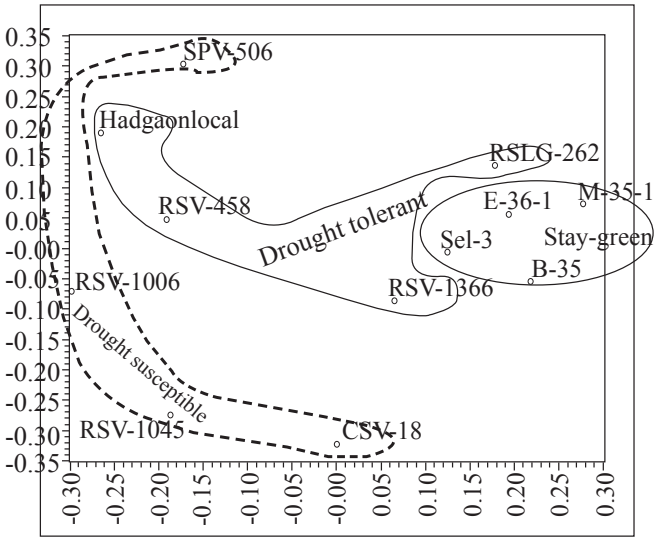


Figure 4: RAPD 2D PCO scatter plot of twelve sorghum genotypes

However, two high glycine betaine accumulating tolerant genotypes, RSV-458 and *Hadgaon* local appeared distinct from the stay-green genotypes, probably because of low level of induction of anti-oxidative enzymes.

4. Conclusion

The genotypes accumulating osmolytes under osmotic stress were divergent, however high proline accumulating and stay greengenotypes havemore similarity thus, could be further utilized in the breeding programme for enhancing drought tolerancecharacter in sorghum.

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