Plant Stress ©2009 Global Science Books

Assessment of Salt Tolerance in *Vigna mungo* Revealed by Isoenzymes and RAPD Markers

Ajay Kumar Singh[1,2] • Avinash Mishra[1,3*] • Arvind Shukla[1]

[1] G.B. Pant University of Agriculture and Technology, Pantnagar-263145, India
[2] PPV & FR Authority, Department of Agriculture & Cooperation, Ministry of Agriculture, NAS Complex, New Delhi – 110012, India
[3] Discipline of Marine Biotechnology and Ecology, Central Salt and Marine Chemicals Research Institute, Bhavnagar- 364 002, India

Corresponding author: * **avinash@csmcri.org**

ABSTRACT

Twenty eight accessions of blackgram (*Vigna mungo* L. Hepper) were screened against salt stress under controlled conditions using five different parameters (germination percentage, plumule length, radicle length, plumule-radicle length ratio and dry matter weight) at four different salt concentrations (0.00 EC, 4.65 EC, 11.25 EC and 16.00 EC). Selected genotypes were further analyzed to assess salt stress-associated biochemical and RAPD markers. A band of peroxidase isoenzyme (Rm 0.38) was observed in tolerant genotypes at all four salt concentrations while in susceptible genotypes at high salt concentration (11.25 and 16.00 EC) only. A peroxidase band (Rm 0.50) was also observed in tolerant genotypes at 11.25 EC with faint intensity. Two bands of malate dehydrogenase (Rm 0.12 and 0.14) were observed under salt stress only. The RAPD banding pattern showed high polymorphism with several unique loci which can help in identification and discrimination of tolerant and susceptible genotypes. A 300 bp band, identified as a RAPD marker for discriminating tolerant from susceptible genotypes, was amplified by four primers (G 08, H02, H 06 and H 08) in the tolerant group only. The study results in categorization and listing of germplasm that can be explored while breeding for saline stress in blackgram.

Keywords: blackgram, malate dehydrogenase, peroxidase, salinity
Abbreviations: EC, electrical conductivity; **PAGE**, polyacrylamide gel electrophoresis; **P-R ratio**, plumule-radicle length ratio; **RAPD**, random amplification of polymorphic DNA

INTRODUCTION

Blackgram (*Vigna mungo* L. Hepper) is a tropical leguminous plant, domesticated in South Asia from *V. mungo* var. *silvestris* (Fuller 2002, 2007). It is widely cultivated in Indian subcontinent and to a lesser extent in Australia, Thailand, Asian and South Pacific countries (Poehlman 1991) for human consumption. Dry mature seeds contain three-times higher protein content in comparison to cereals and constitute an important source of protein for a vegetarian diet of common people (Lakhanpaul *et al*. 2000). Furthermore, it plays a crucial role in sustaining the productivity of a cropping system by adding atmospheric nitrogen to the soil. Blackgram is grown in various agro-ecological conditions and cropping systems with diverse agricultural practices. In various parts of India, a number of traditional landraces are cultivated as inter-crop between rice, sugarcane, cotton, groundnut and sorghum cultivating seasons and these landraces possess unique traits (e.g. disease tolerance, abiotic stress tolerance, pest tolerance), identified by the farmers (Sivaprakash *et al*. 2004).

Soil salinity, a major limiting factor for the proliferation of plants, inhibits metabolic activities and reduces agriculture productivity, affecting large terrestrial areas of the world. The majority of crops can not be grown on saline soils, however genetic mechanism of salinity tolerance can be used towards the economic utilization of saline soils. The ability of plants to tolerate varying salt concentration in the substrate is characteristic of the species to which they belong. Because of inadequate information available on species and varietal differences among the cultivated crops regarding salt injury and/or tolerance, there is a great possibility to tailor them to enhance adaptability in varying saline conditions. The extent of genetic diversity for salinity tolerance in blackgram germplasm has been explored very little and the scope of utilization of this crop in salt affected areas is being pursued in India. Salt responsive studies on blackgram are lacking. Therefore, in this study, an attempt has been made to assess blackgram genotypes for salt tolerance using biochemical and molecular markers.

MATERIALS AND METHODS

Experimental material

The experimental material comprised of 28 germplasm accessions of blackgram collected from different diversity pockets of India (Singh *et al*. 2009). The locations were reported to be rich in diversity of blackgram (Paroda and Arora 1991).

Screening of salt stress tolerant genotypes

Twenty eight genotypes were screened under incubator conditions (25°C and 80% RH) following International rules for seed testing (Anon 1976), using a combination of salts (NaCl, Na_2SO_4, KCl, K_2SO_4, $MgCl_2$, $MgSO_4$ and $CaCl_2$) in the ratio of 2:1:1:1:1:1:1, respectively, in deionized water to make a 100 ml solution. 0.075 g of NaCl and 0.037 g for the remaining salts were added for 4.69 dS/m solution while for a 11.25 dS/m solution, 0.175 g of NaCl and 0.0875 g of other salts were added. Similarly 0.25 g of NaCl and 0.125 g of remaining salts were dissolved for 16.00 dS/m solution, while no salt was added for control solution (i.e. 0.00 EC). Aqueous solution of four electrical conductivities (EC) viz. 0.00, 4.69, 11.25 and 16.00 dS/m were used for screening and EC was measured by a Systronic conductivity bridge (Model 305, Systronic, India).

Germination trend and early seedling behaviour viz. germination percentage, plumule length, radicle length, plumule-radicle

Received: 6 February, 2009. Accepted: 9 December, 2009.

Original Research Paper

length ratio and dry matter weight, were observed separately, after 8 days incubation with all four EC solution containing different salt concentrations. Genotypes showing the highest value for germination percentage, plumule length, radicle length, plumule-radicle length ratio and dry matter weight, both *per se* and in combinations (i.e. showing higher values for all the characteristics under study with an average of three replication), were selected and grouped as tolerant ones while those giving the lowest value were grouped in susceptible ones. The genotypes selected for these two groups were subjected to isoenzyme (viz. peroxidase and malate dehydrogenase) assay and RAPD.

Enzyme extraction

Eight days-old, 1.0 g healthy seedlings of each accession were homogenized in ice cold 5.0 ml extraction buffer (50 mM Tris–HCl with 1% v/v β-mercaptoethanol, pH 7.0). The extract/ homogenate was centrifuged at 12,000 rpm for 30 min at 4°C and supernatant fluid was used for isoenzyme assay (Li *et al*. 2007; Singh *et al*. 2009). Sample mix (25 µl), containing isoenzyme extract, glycerol (50%) and bromophenol blue (0.05 mg/ml in dH_2O) in the ratio of 5:3:2 (Upadhyay *et al.* 2002), was electrophoresed on 7% native-PAGE at 80 V for 4–5 h at 4°C until the tracking dye migrated 1 cm above the anodal end (Sambrook *et al*. 1989).

Peroxidase

Gel was incubated in fresh solution containing 0.1% (w/v) tetra methyl benzidine (TMBZ), 50% (v/v) methanol and 0.5 M Na-acetate (pH 4.7) for about 5 min in dark thereafter H_2O_2 (30%) was added drop wise till the bands become visible. Gel was transferred to a solution of 7% acetic acid for 5 min to fix bands (Song *et al*. 2007; Zhou *et al*. 2007; Singh *et al*. 2009).

Malate dehydrogenase

Gel was stained in reaction buffer containing 0.1 M Tris-HCl (pH 7.5), 30 mg NAD^+, 4 mg phenyl methyl sulfonate (PMS), 20 mg MTT and 3 ml 1 M L-malate (pH 7.5) by incubating in dark at 30°C for 15 to 60 min until blue bands appeared (Schuelter *et al*. 1999; Bhandary *et al*. 2006), thereafter bands were fixed by soaking in 7% acetic acid for 5 min.

Randomly amplified polymorphic DNA (RAPD)

The genomic DNA was isolated, using SDS method (Dellaporta *et al*. 1983), purified (Sambrook *et al*. 1989) and quantified by using both UV spectrophotometer (DU640B spectrophotometer, Beckman) and DyNA Quant 2000 flourimeter (Hoefer). Eight decamer random primers (all 5′-3′, OPC-07: GTCCCGACGA, OPC-13: AAGCCTCGTC, OPC-05: GATGACCGCC, OPC-04: CCGCAT CTAC, OPAA-18: TGGTCCAGCC, H-04: TGGTGACTGA, H-06: TGCTCACTGA and H-08: TGGTCACGGA) were used for RAPD. PCR was carried out in 25 µl of a reaction mixture containing 20 ng of genomic DNA, 200 µM each dNTPs, 1.25 U *Taq* DNA polymerase, 10 mM Tris-Cl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin and 0.2 µM random decamer primer. The PCR amplification was conducted in Biometra thermal cycler programmed as an initial denaturation at 94°C for 5 min; remaining 40 cycles with 94°C denaturation for 1 min., 37°C annealing for 1 min and 72°C extension for 2 min. Final extension was given at 72°C for 5 min. PCR amplified products were analyzed on 1.5% agarose gel in TAE (pH 8.0) buffer (Sambrook *et al*. 1989).

Data analysis

Morphological data obtained from the genotypes grown under controlled germination tests were subjected to analytical and statistical analysis. Two factorial Analysis of variance (ANOVA) (Gomez and Gomez 1984) was calculated using the Statistical Package for Agricultural Research Data Analysis (SPAR 2.0). Simple linear correlation coefficient was computed to determine the association among the characteristics at different salt concentrations. The genotypic and phenotypic correlations were computed according to the method given by Searle (1961).

For biochemical analysis, polyacrylamide gels of isoenzyme assay were analyzed and zymograms were prepared using relative mobility of each band visualized on gel. Molecular analysis was done based on the expression of banding pattern of genomic DNA of selected accessions and bulked DNA analysis of tolerant and susceptible accessions. RAPD bands electrophoresed on agarose and PAGE gels were scored twice, independently and manually. All monomorphic and unique bands were also scored and included in the analysis.

RESULTS AND DISCUSSION

Similar to other leguminous crops, blackgram is sensitive to salinity and by comparing different developmental stages it was observed that seedling stage is more sensitive to salt stress (Promila and Kumar 2000; Dash and Panda 2001). The literature on salt tolerance of blackgram is scanty and present study may be the first report so far. Twenty eight genotypes of blackgram were subjected to four different salt concentrations (0.00EC, 4.65 EC, 11.25 EC and 16.00 EC) and response of five different parameters (germination percentage, plumule length, radicle length, plumule- radicle length ratio and dry matter weight) were studied (**Tables 1-5**). Analysis of variance reveals that mean squares among the genotypes at four salt concentrations were significant for all five parameters. Coefficient of variation was found maximum for radicle length (9.762) followed by dry matter weight (7.533), plumule length (6.826). PR length ratio (3.618) and germination percentage (2.708), inferring that genotypes gave a differential response towards varying salinity (**Table 6**). The critical difference (CD) for all the characters as shown in **Table 6**, compared different factorial plots and subplots with their interactions. Simple linear correlation coefficients, determined for above five characters at different salt concentrations (**Table 7**), reveal that germination percentage was positively correlated with plumule length and radicle length, however negatively correlated with P-R length ratio at 4.65 and 11.25 dS/m salt concentration. Radicle length was observed positively correlated to

Table 1 Mean percentage germination for blackgram genotypes at different levels of salt concentration.

Genotypes	Salt concentration			
	0 EC	4.6 EC	11.25 EC	16 EC
ShU 9503	80.0	76.6	78.3	83.0
ShU 9505	80.0	71.66	68.3	80.0
ShU 9508	93.4	81.6	76.6	68.0
ShU 9511	88.4	70.0	70.0	71.6
ShU 9519	81.7	81.6	70.3	76.7
ShU 9525	93.0	76.6	66.7	71.6
ShU 9532	86.7	83.3	70.0	76.7
ShU 9536	74.0	60.0	73.3	80.0
ShU 9601	85.0	70.0	83.0	75.0
ShU 9603	86.0	75.0	70.0	66.7
ShU 9611	70.0	73.3	75.0	81.7
ShU 9612	78.3	73.3	83.0	83.4
ShU 9614	71.6	70.0	73.3	78.3
ShU 9619	75.0	80.0	86.6	70.0
ShU 9621	88.0	56.0	88.3	78.3
ShU 9626	78.4	61.6	83.3	66.7
ShU 9632	71.6	81.6	70.0	60.0
ShU 9633	73.3	78.3	73.3	71.6
ShU 9636	78.4	80.0	90.0	63.3
ShU 9641	96.0	75.0	68.3	60.0
ShU 9642	78.0	63.0	80.0	88.3
ShU 9682	83.4	76.6	80.0	86.0
ShU 96110	95.0	76.0	68.3	53.3
ShU 9720	73.4	65.0	61.6	66.0
ShU 9725	78.0	76.6	76.7	71.6
ShU 9737	68.0	56.0	76.7	72.0
ShU 9797	85.0	70.0	75.0	84.0
ShU 9901	95.0	70.0	66.7	75.0
Grand Mean	82.59	78.42	75.9	71.9

dry matter weight at all salt concentrations and P-R length ratio had a negative correlation with radicle length and dry matter weight.

Mean values were calculated for observations recorded for each of the 28 selected accessions to assess the tolerance strength of accessions under saline conditions (**Tables 1-5**). Genotypes having the highest and lowest values for all characteristics at different salt concentrations were selected for further evaluation. Overall effect of the saline environment on the expression of the characteristics was observed

Table 2 Mean plumule length (cm) for blackgram genotypes at different levels of salt concentration.

Genotypes	Salt concentration			
	0 EC	4.6 EC	11.25 EC	16 EC
ShU 9503	20.72	12.7	11.36	7.7
ShU 9505	17.6	15.4	8.84	8.2
ShU 9508	21.4	16.8	10.6	7.4
ShU 9511	17.4	16.6	11.8	6.4
ShU 9519	21.0	13.6	10.7	7.9
ShU 9525	19.4	15.5	8.8	8.5
ShU 9532	18.2	14.3	10.2	9.4
ShU 9536	18.9	11.9	8.8	9.5
ShU 9601	19.8	12.0	11.5	7.4
ShU 9603	20.8	15.5	12.6	8.6
ShU 9611	16.6	14.0	11.7	8.4
ShU 9612	24.6	13.3	12.08	8.9
ShU 9614	22.0	17.1	12.3	9.7
ShU 9619	18.4	14.0	11.7	6.9
ShU 9621	21.5	12.7	12.3	7.1
ShU 9626	20.7	15.6	10.3	7.2
ShU 9632	18.5	17.3	10.7	8.7
ShU 9633	21.3	15.7	12.5	7.9
ShU 9636	19.8	15.2	11.9	7.9
ShU 9641	19.8	13.8	11.8	7.7
ShU 9642	17.5	15.5	11.2	8.1
ShU 9682	21.1	16.4	12.8	7.6
ShU 96110	18.0	11.0	11.7	7.3
ShU 9720	17.7	14.7	11.3	8.0
ShU 9725	17.2	15.6	11.4	8.0
ShU 9737	18.1	10.6	9.4	7.3
ShU 9797	23.6	14.7	10.2	7.5
ShU 9901	19.3	14.4	10.7	7.0
Grand Mean	19.67	14.49	11.18	8.01

Table 3 Mean radicle length (cm) for blackgram genotypes at different levels of salt concentration.

Genotypes	Salt concentration			
	0 EC	4.6 EC	11.25 EC	16 EC
ShU 9503	10.1	9.8	9.4	7.3
ShU 9505	9.5	5.8	6.9	5.6
ShU 9508	8.0	6.5	9.6	6.7
ShU 9511	10.4	8.7	7.3	5.2
ShU 9519	7.2	7.9	12.8	7.4
ShU 9525	9.0	6.7	5.3	6.7
ShU 9532	8.9	5.7	6.3	6.3
ShU 9536	7.9	5.9	5.1	5.5
ShU 9601	10.2	6.7	8.2	6.6
ShU 9603	10.3	7.2	7.56	6.6
ShU 9611	8.6	5.2	4.9	5.2
ShU 9612	8.4	6.4	7.2	7.9
ShU 9614	7.9	8.7	6.5	6.5
ShU 9619	10.3	9.6	7.3	4.9
ShU 9621	9.8	5.7	7.9	6.3
ShU 9626	9.1	5.7	6.6	5.0
ShU 9632	8.9	9.7	6.7	4.7
ShU 9633	9.5	10.0	6.6	6.3
ShU 9636	11.7	10.9	8.7	5.3
ShU 9641	10.1	10.7	8.4	4.6
ShU 9642	8.5	8.3	6.7	6.7
ShU 9682	7.9	5.1	9.3	6.5
ShU 96110	9.72	4.6	8.1	4.3
ShU 9720	10.2	4.1	8.1	5.2
ShU 9725	10.8	7.4	6.5	5.6
ShU 9737	8.5	2.3	6.6	4.8
ShU 9797	9.2	7.7	8.2	5.1
ShU 9901	8.8	6.1	8.3	6.0
Grand Mean	9.24	6.72	7.53	5.88

Table 4 Mean plumule-radicle length (P-R) ratio for blackgram genotypes at different levels of salt concentration.

Genotypes	Salt concentration			
	0 EC	4.6 EC	11.25 EC	16 EC
ShU 9503	2.05	1.29	1.20	1.05
ShU 9505	1.85	2.6	1.28	1.46
ShU 9508	2.67	2.5	1.10	1.10
ShU 9511	1.67	1.90	1.61	1.23
ShU 9519	2.91	1.72	0.83	1.06
ShU 9525	2.15	2.31	1.66	1.26
ShU 9532	2.04	2.50	1.61	1.49
ShU 9536	2.39	2.01	1.72	1.72
ShU 9601	1.94	1.79	1.40	1.12
ShU 9603	2.01	2.15	1.66	1.32
ShU 9611	1.93	2.69	2.38	1.61
ShU 9612	2.92	2.07	1.67	1.02
ShU 9614	2.78	1.96	1.89	1.49
ShU 9619	1.78	1.45	1.60	1.40
ShU 9621	2.19	2.22	1.55	1.12
ShU 9626	2.27	2.73	1.56	1.44
ShU 9632	2.07	1.78	1.89	2.27
ShU 9633	2.24	1.57	1.89	1.25
ShU 9636	1.69	1.39	1.36	1.67
ShU 9641	1.96	1.28	1.40	1.67
ShU 9642	2.05	1.87	1.67	1.20
ShU 9682	2.67	3.21	1.37	1.16
ShU 96110	1.85	2.39	1.44	1.69
ShU 9720	1.68	3.58	1.39	1.53
ShU 9725	1.59	2.10	1.75	1.42
ShU 9737	2.12	4.60	1.42	1.52
ShU 9797	2.56	1.90	1.24	1.47
ShU 9901	2.41	2.36	1.28	1.16
Grand Mean	2.15	2.20	1.50	1.30

Table 5 Mean dry matter weight (g) for blackgram genotypes at different levels of salt concentration.

Genotypes	Salt concentration			
	0 EC	4.6 EC	11.25 EC	16 EC
ShU 9503	0.20	0.17	0.15	0.12
ShU 9505	0.14	0.16	0.19	0.20
ShU 9508	0.08	0.14	0.15	0.13
ShU 9511	0.13	0.10	0.13	0.09
ShU 9519	0.11	0.14	0.14	0.21
ShU 9525	0.10	0.13	0.12	0.12
ShU 9532	0.09	0.13	0.11	0.16
ShU 9536	0.10	0.10	0.13	0.13
ShU 9601	0.10	0.13	0.18	0.15
ShU 9603	0.12	0.16	0.12	0.13
ShU 9611	0.08	0.14	0.14	0.14
ShU 9612	0.11	0.14	0.12	0.16
ShU 9614	0.10	0.11	0.12	0.13
ShU 9619	0.08	0.10	0.16	0.12
ShU 9621	0.14	0.13	0.09	0.13
ShU 9626	0.08	0.12	0.10	0.13
ShU 9632	0.08	0.12	0.18	0.12
ShU 9633	0.18	0.11	0.14	0.16
ShU 9636	0.14	0.15	0.18	0.15
ShU 9641	0.09	0.18	0.10	0.16
ShU 9642	0.09	0.11	0.09	0.11
ShU 9682	0.11	0.14	0.13	0.14
ShU 96110	0.09	0.10	0.13	0.11
ShU 9720	0.10	0.12	0.13	0.14
ShU 9725	0.12	0.13	0.13	0.12
ShU 9737	0.12	0.12	0.12	0.11
ShU 9797	0.08	0.10	0.13	0.14
ShU 9901	0.12	0.14	0.16	0.11
Grand Mean	0.11	0.12	0.13	0.14

Table 6 Analysis of variance of different characters and various salt concentrations in blackgram genotypes.

Characters		Genotypes (27)	Salt concentrations (3)	Interaction (81)	Error (240)	Total (335)	Coefficient of variation (CV)
Germination (%)	MS	181.37**	1207.06**	164.35**	4.28	22791.75	2.708
	SEM	0.59	0.22	1.19			
	CD	2.19	0.83	4.39			
Plumule length (cm)	MS	9.27**	2072.66**	6.30**	0.83	7165.23	6.826
	SEM	0.26	0.09	0.52			
	CD	0.96	0.36	1.93			
Radicle length (cm)	MS	8.95**	164.97*	6.19**	0.52	1356.61	9.762
	SEM	0.20	0.07	0.41			
	CD	0.76	0.29	1.53			
Plumule Radilce length ratio	MS	0.60**	14.27**	0.620**	0.42	110.25	3.618
	SEM	0.01	0.007	0.03			
	CD	0.06	0.02	0.13			
Drymatter weight (g)	MS	0.34**	0.12 **	0.15**	0.92	0.27	7.533
	SEM	0.002	0.001	0.005			
	CD	0.01	0.003	0.02			

*, ** significance at 1 and 5 percent level respectively
MS: Mean Standard; SEM: Standard Error of Mean; CD: Critical Difference

Table 7 Simple linear correlation coefficients among 5 characters in 28 genotypes of blackgram at different salt concentrations

Characters	Salt concentrations (EC)	Plumule length (cm)	Radicle length (cm)	Plumule radicle length ratio	Dry matter weight (g)
Germination percentage	0.00	0.142	0.110	0.061	-0.080
	4.69	0.364	0.487**	-0.433*	0.241
	11.25	0.289	0.194	-0.046	-0.107
	16.00	0.124	0.021	0.158	0.127
Plumule length (cm)	0.00		-0.0261	0.559**	+0.127
	4.69		0.403	-0.182	-0.061
	11.25		0.141	0.328*	-0.003
	16.00		0.023	0.600**	0.189
Radicle length (cm)	0.00			-0.775**	0.320*
	4.69			-0.882**	0.151
	11.25			-0.881**	0.213
	16.00			0.745**	0.322
Plumule Radicle length ratio	0.00				-0.155
	4.69				-0.099
	11.25				-0.147
	16.00				-0.091
Drymatter weight (g)	0.00				
	4.69				
	11.25				
	16.00				

*, ** significance at 1 and 5 percent level respectively

and a grand mean of observations was recorded for all the genotypes under four different salt concentrations. Grand mean value of germination at different salt concentrations indicates that application of saline water reduced the percentage of germination compared to control treatment. Significant reduction in germination percentage with increased salinity was also observed in mungbean (Shekhar 1994) and blackgram (Sarkar and Shukla 1997; Dash and Panda 2001). This may be due to the osmotic imbalance, ion toxicity and alteration in mineral nutrient acquisition (Rogers *et al*. 1995). The grand mean of plumule length decreased with increasing salinity and at maximum salt concentration (16.00 EC), it reduced to more than half as compared to control. Similar pattern was also observed for P-R length ratio, while root length increased at 11.25 EC as compared to 4.65 EC, there after decreased at 16.00 EC. The grand mean value of dry matter weight increased concomitantly with salinity. These findings may be justified as, with an increase in salt concentration, ions get accumulated and shoot length are more affected with ion toxicity than root length. It was observed in maize by Maiti *et al*. (1996) and they explained it as a mechanical resistance for maintaining osmoregulation while increase in dry matter weight with salinity was also noticed by Shekhar (1994) in mungbean seedlings.

It was suggested that radicle length is the most useful parameter to evaluate salt tolerant cultivars of crop plants (Kuhad and Sheoran 1987; Freitas and Camargo 1988; Shekhar 1994) and a positive correlation of germination and radicle length at higher salt concentrations also support the possible selection criteria for salt tolerance in blackgram. Based on these suggested criteria and comparing the mean values for all characters studied, three genotypes viz. ShU9503, ShU9519 and ShU9612 were found tolerant and have more than 80% germination and also had higher radicle length at 16.00 dS/m salt concentration. Four genotypes (ShU9632, ShU9636 ShU9641 and ShU96110) were designated as susceptible against different level of salt concentrations. These seven genotypes (3 tolerant and 4 susceptible) were selected for further studies to identify markers associated with tolerance or susceptibility using isoenzyme analysis and RAPD banding pattern. Selected genotypes were subjected for the study of isoenzymic banding pattern of peroxidase and malate dehydrogenase under different salinity levels. The isoenzymes were selected from previous reports (Mittal and Dubey 1995; Ritambhara and Dubey 1995; Converso *et al*. 1997; Dash and Panda 2001).

Isoenzyme bands were categorized in four zones (A, B, C and D) and characterized by number, relative mobility and intensity of bands (**Figs. 1, 2**). A band of peroxidase (**Fig. 1**) with Rm 0.38 was observed in tolerant genotypes in all four salt concentrations compared to control, while in susceptible genotypes at high salt concentration (11.25 and 16.00 EC) only. Apart from this, a peroxidase band (Rm

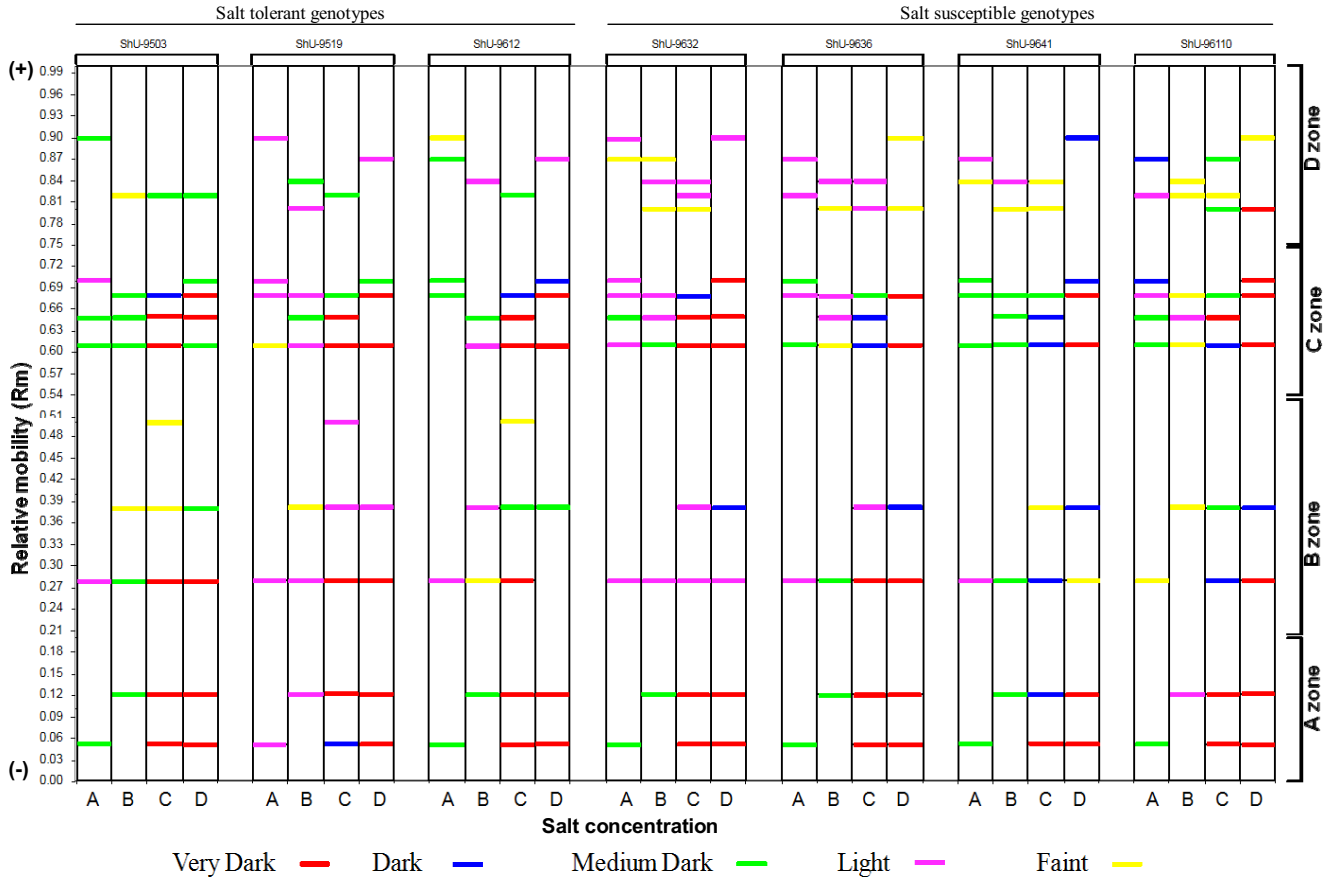

Fig. 1 Zymogram of electrophoretic banding pattern for peroxidase isoenzyme of salt tolerant and susceptible blackgram genotypes at four different salt concentrations. (**A**) Control (0 dS/m), (**B**) 4.65 dS/m, (**C**) 11.25 dS/m, (**D**) 16.0 dS/m.

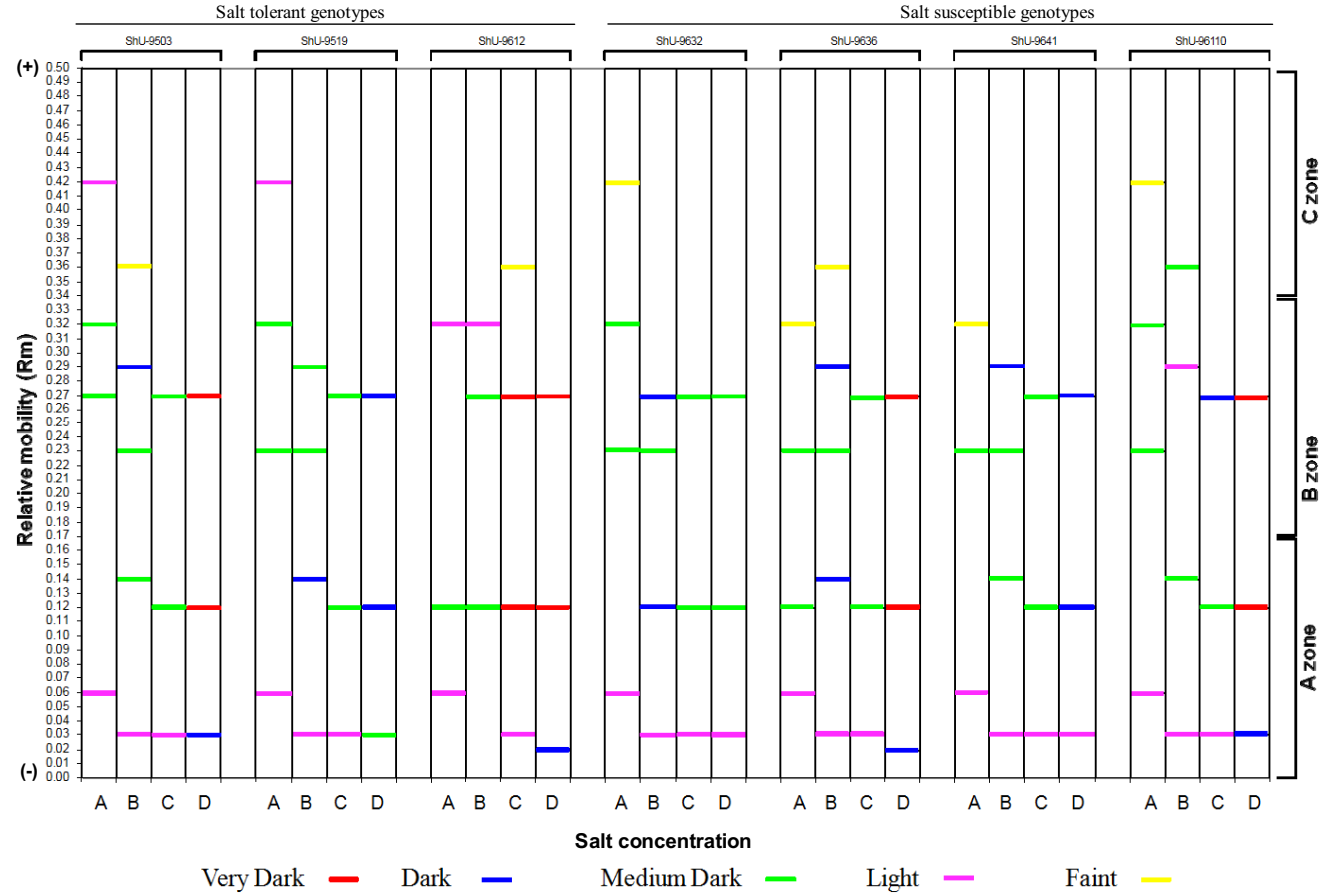

Fig. 2 Zymogram of electrophoretic banding pattern for malate dehydrogenase isoenzyme of salt tolerant and susceptible blackgram genotypes at four different salt concentrations. (**A**) Control (0 dS/m), (**B**) 4.65 dS/m, (**C**) 11.25 dS/m, (**D**) 16.0 dS/m.

Table 8 RAPD analysis of unique loci obtained by different primers on agarose and polyacrylamide gel.

Primer code	Agarose gel			Polyacrylamide gel		
	Unique loci	Size (kb)	Genotypes possessing loci	Unique loci	Size (kb)	Genotypes possessing loci
OPC-07	2	0.8	S2, S3, S4	3	0.7	S1, S2, S3, S4
		0.7	S3		0.3	S2, S3, S4
					0.2	S2, S3, S4
OPC-13				1	0.75	R1, R2, R3
OPC-05	5	0.7	S3, S4	5	0.9	R1, R2, R3
		0.6	S1, S2, S3		0.65	R1, R2, R3
		0.5	S1, S2, S3		0.45	S1, S2, S3
		0.4	R1, R2, R3		0.37	S1, S2, S3
		0.1	S2, S3, S4		0.30	S1, S2
OPAA-18	3	0.7	S2	4	0.75	R1, R2, R3
		0.35	S2, S3, S4		0.10	S1, S2, S3, S4
		0.30	S1, S2, S3, S4		<0.10	S1, S2, S3, S4
					<0.10	S1, S2, S3, S4
H04	3	1.0	S2	2	0.50	S2, S3, S4
		0.75	R1, R2		0.35	S2, S3, S4
		0.6	S2, S3, S4			
H06				6	2.0	S1, S2, S3, S4
					1.8	S1, S2, S3, S4
					0.8	S1, S2, S3, S4
					0.5	S1, S2, S3, S4
					0.42	S1, S2, S3, S4
					0.3	S1, S2, S3, S4
H08				2	0.35	S1, S2, S3, S4
					<0.10	S1, S2, S3, S4

R1- ShU 9503, R2- ShU 9519, R3- ShU 9612, S1- ShU 9632, S2- ShU 9636, S3- ShU 9641, S4- ShU 96110
R: Resistant/ Tolerant; S: Susceptible

0.50) was observed in tolerant genotypes in 11.25 EC with faint intensity, absent in the susceptible group. It is suggested that change in peroxidase banding pattern is related to salinity tolerance as the number of bands increased in tolerant genotypes and showed a positive correlation between salt damage and peroxidase activity, providing a protective action against salinity. Two bands of malate dehydrogenase (**Fig. 2**) with Rm 0.12 and 0.14 were observed under salt stress only. In general, the intensity of isoenzyme bands increased with salinity according to Upadhyay and Baijal (1988) in mungbean and Ritambhara and Dubey (1995) in rice.

Selected genotypes were also assessed for RAPD markers, associated with salt tolerance using arbitrary random primers (decamer). In this study, genomic DNA, isolated from selected individuals, was subjected to PCR amplification in two ways. First, genomic DNA of individual genotype was independently and separately subjected to amplification and analysis was done. Secondly, an equal amount of genomic DNA, isolated from individual salt tolerant genotypes, were bulked to form one pool and similarly DNA of salt susceptible genotypes were bulked to form another pool. These two pool samples were also subjected to PCR amplification using same random primers. RAPD banding pattern was studied and similar strategy was followed by Liu (1996), for evaluation of genetic diversity and relationship among hyacinth bean. Miklas *et al*. (1993) used same strategy for identification of molecular markers for rust resistance in common bean while for salinity resistance in rice by Erickson *et al*. (1995), Wang *et al*. (2000) and in maize by Abdel-Tawab *et al*. (1997).

The RAPD banding pattern of tolerant and susceptible genotypes showed high polymorphism. A total of 12 RAPD loci were obtained with primers H 06 and H 08 on agarose gel while 18 on PAGE with primer H 06. Apart from polymorphic loci several unique loci were also present (**Table 8**) which could help in identification and discrimination of tolerant and susceptible genotypes.

Primer G 08 gave a unique RAPD locus of 300 bp size in all tolerant genotypes on agarose gel. Similarly, a unique RAPD locus of size 750 bp was also observed with primer H 04 in genotypes ShU9503 and ShU9519 only. A unique RAPD locus of 750 bp was observed with primers G 06, H 02 and H 04 while a band of 900 bp was detected with primer G 08 in all tolerant genotypes on PAGE.

For susceptible genotypes, on agarose gel, primer G 04 amplified 5 RAPD loci along with two unique loci of 800 bp and 700 bp. Primer G 08 gave three unique loci of 700 bp, 600 bp and 100 bp while two unique loci of 1 kb and 600 bp were observed with primer H 04. Whereas on PAGE, the number of unique RAPD loci was more for susceptible genotypes. Primer G 04 gave three unique loci of 200, 300 and 700 bp while two unique loci of 370 and 450 bp were observed with primer G 08. Three unique loci of 100 and <100 bp were amplified with primer H 02; however, primer H 06 gave the maximum number of unique loci of 350 bp, 500 bp, 800 bp, 1.8 kb and 2 kb in size.

Primers G 04, G 06 and H 04 gave a distinct RAPD pattern for susceptible genotypes with large number of unique RAPD loci thus it can be used to discriminate tolerant and susceptible genotypes, since a large number of unique loci were also present hence these are more helpful in varietal differentiation.

To avoid this confusion, another strategy of RAPD using pooled genomic DNAs as template was followed and it was observed that a 300-bp band was amplified by four primers (G 08, H02, H 06 and H 08) in the tolerant bulk only. This band may be identified as a RAPD marker for discriminating tolerant genotypes from susceptible genotypes. It is similar to the findings of Abdel-Tawab *et al*. (1997), who found a 2-kb band in tolerant maize lines while Wang *et al*. (2000) detected a 1.1 kb band in salt-tolerant rice genotypes through RAPD technique.

The strategy of bulked segregant analysis applied for this analysis is an indicative that it may concentrate efforts and resources towards the identification of those RAPDs which are tightly linked to the target locus. The probability of having no false positive will increase, as the number of individuals comprising a bulk increases (Michelmore *et al*. 1991). Thus, bulked samples formed with three or more than three individuals may be warranted for such studies. The present study results in the categorization, evaluation and listing of germplasm that can be explored further while breeding for salt tolerance in blackgram.

REFERENCES

Abdel-Tawab FM, Fahmy EM, Bahieldud A, Eissa HF (1997) Molecular markers for salt tolerance in source inbreds of maize (*Zea mays* L.). *Arab University Journal of Agriculture Science* **5**, 387-417

Anon (1976) International rules for seed testing. International Seed Testing Association. *Seed Science and Technology* **4**, 3-39

Bhandary YP, Krithika KN, Kulkarni S, Reddy MVR, Harinath MVR (2006) Detection of enzymes dehydrogenases and proteases in *Brugia malayi* filarial parasites. *Indian Journal of Clinical Biochemistry* **21**, 1-7

Converso DA, Fernandez ME, Tomaro ML (1997) Effect of development and salt stress on the activity and expression of wheat peroxidases. *Phyton (Buenos Aires)* **61**, 147-154

Dash M, Panda SK (2001) Salt stress induced changes in growth and enzyme activities in germinating *Phaseolus mungo* seeds. *Biologia Plantarum* **44**, 507-589

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. *Plant Molecular Biology Reporter* **1**, 19-21

Erikson J, Rogers WJ, Perera ALT (1995) Genetic screening of rice varieties using RAPD markers for indication of salt sensitivity/resistance. *Srilankan Journal of Agriculture Science* **32**, 16-29

Freitas JGde, Camargo CEdeO (1988) Tolerance of rice and wheat cultivars to salinity. *Bragantia* **47**, 125-135

Fuller DQ (2002) Fifty years of archaeological studies in India: laying a solid foundation. In: Settar S, Korisettar R (Eds) *India Archaeology in Retrospect* (Vol III), Archaeology and interactive disciplines, Indian Council of Historical Research, Manohar, India pp 247-364

Fuller DQ (2007) Contrasting patterns in crop domestication and domestication rates: Recent archaeobotanical insights from the old world. *Annals of Botany* **100**, 903-924

Gomez KA, Gomez AA (1984) *Statistical Procedures for Agricultural Research* (2nd Edn), Wiley Interscience, New York, 680 pp

Kuhad MS, Sheoran IS (1987) Influence of simulated drought and salt stress at iso-osmotic level on seedling growth on pigeonpea genotype. *International Pigeonpea Newsletter* **6**, 48-50

Lakhanpaul S, Chadha S, Bhat KV (2000) Random amplified polymorphic DNA (RAPD) analysis in Indian mung bean (*Vigna radiata* (L.) Wilczek) cultivars. *Genetica* **109**, 227-234

Li Y, Feng HY, Chen T, Yang XM, An LZ (2007) Physiological responses of *Limonium aureum* seeds to ultra-drying. *Journal of Integrated Plant Biology* **49**, 569-575

Liu CJ (1996) Genetic diversity and relationship among *Lablab purpureus* genotypes evaluated using RAPD as markers. *Euphytica* **90**, 115-119

Maiti RK, Amaya LED, Cardona SI, Dimas AMO, Ibarra M de la R, Castillo H de L (1996) Genotypic variability in maize cultivars (*Zea mays* L.) for resistance to draught and salinity at the seedling stage. *Journal of Plant Physiology* **148**, 741-744

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method of detect markers in specific genomic regions by using segregating population. *Proceedings of the National Academy of Sciences USA* **88**, 9828-9832

Miklas PN, Stavely JR, Kelly JD (1993) Identification and potential use of a molecular marker for rust resistance in common bean. *Theoretical and Applied Genetics* **85**, 745-749

Mittal R, Dubey RS (1995) Influence of sodium chloride salinity on polyphenol oxidase, indole-3-acetic acid oxidase and catalase activities in rice seedlings differing in salt tolerance *Tropical Science* **35**, 141-149

Paroda RS, Arora RK (1991) *Plant Genetic Resources: Conservation and Management*, IBPGR, Rome

Poehlman JM (1991) History, description, classification and origin. In: Poehlman JM (Ed) *The Mungbean*, West View, Boulder, pp 6-21

Promila K, Kumar S (2000) *Vigna radiata* seed germination under salinity. *Biologia Plantarum* **43**, 423-426

Ritambhara GK, Dubey RS (1995) Influence of NaCl salinity on the behaviours of malate, isocitrate and glucose 6-phosphate dehydrogenases in growing rice seedlings in relation to salt tolerance. *Indian Journal of Plant Physiology* **38**, 48-53

Rogers ME, Noble CL, Halloran GM, Nicolas ME (1995) The effect of NaCl on the germination and early seedling growth of white clover (*Trifolium repens* L.) populations selected for high and low salinity tolerance. *Seed Science and Technology* **23**, 277-287

Sambrook T, Fritsh EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour, New York

Sarkar B, Shukla A (1997) Selection criteria for salt tolerance in lentil germplasm. I. Germination and seedling traits. *Indian Journal of Plant Genetic Resources* **10**, 35-40

Schuelter AR, Casali VWD, Finger FL (1999) Inheritance of malate dehydrogenase in wild pepper. *Bragantia* **58**, 1-6

Searle SR (1961) Phenotypic, genotypic and environmental correlation. *Biometrics* **17**, 477-480

Shekhar MR (1994) Salt tolerance of mungbean (*Vigna radiata* L. Wilczek) at germination stage. *Annals of Agriculture Research* **15**, 90-91

Singh AK, Mishra A, Shukla A (2009) Genetic assessment of traits and genetic relationship in blackgram (*Vigna mungo*) revealed by isoenzymes. *Biochemical Genetics* **47**, 471-485

Sivprakash KR, Prashanth SR, Mohanty BP, Parida A (2004) Genetic diversity of black gram (*Vigna mungo*) landraces as evaluated by amplified fragment length polymorphism markers. *Current Science* **86**, 1411-1416

Song NH, Yin XL, Chen GF, Yang H (2007) Biological responses of wheat (*Triticum aestivum*) plants to the herbicide chlorotoluron in soils. *Chemosphere* **68**, 1779-1787

Upadhyay AK, Baijal BD (1988) Influence of salt stress on respiratory enzymes in germination mungbean (*Vigna radiata* L. Wilczek) seeds. *Indian Journal of Plant Physiology* **3**, 288-289

Upadhyay SR, Shukla A, Gaur AK (2002) Measurement of genetic diversity in hybrid (f_1) population of urdbean using isoperoxidase patterns. *Theoretical and Applied Genetics* **105**, 1215-1219

Wang Z, Zheng JY, Wang ZY, Zheng JY (2000) Studies on RAPD and POD isozymes of salt tolerant and salt sensitive rice. *Chinese Journal of Applied Environmental Biology* **6**, 106-111

Zhou ZS, Huang SQ, Guo K, Mehta SK, Zhang PC, Yang ZM (2007) Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. *Journal of Inorganic Biochemistry* **101**, 1-9