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ORIGINAL ARTICLE

Differentiation Between Drought Tolerance and Drought Susceptible Varieties of Barley Utilizing DNA Marker

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ABSTRACT
In present investigation RAPD polymorphism among 28 barley genotypes, using 30 decamer primers in PCR reaction was studied. 11 drought tolerant varieties and 17 drought susceptible were used in RAPD analysis. The amount of DNA obtained was 402- 1150 µg/ml. 25 markers showed amplification, producing 88 bands (76 - polymorphic ; 12 – monomorphic). The amplification obtained was of 2.93 bands / primer, with size of 300-2600 bp. At an arbitrary cut-off at approx. 65% similarity index on a dendrogram (hierarchical relationship), the barley accessions were characterized into two most important clusters. Average resemblance value of 0.71 was found among different genotypes, with maximum similarity (86%) between genotypes DWR 89 and DWRUB 52. RD 2552 and DWR 91 genotypes exhibited minimum similarity. Hence, the present research successfully distinguished drought tolerant and drought susceptible barley varieties that could prove useful forseed companies and national registration agencies.
Key words-Barley, Genetic diversity, similarity coefficient, molecular marker, RAPD

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INTRODUCTION

Barley is one of the chief cereals consumed globally. It ranks fifth among the cereals in worldwide production, belonging to the tribe Triticeae, family Poaceae. Barley is an important crop for direct human consumption and for animal feed. It is unique as a source of malt for beer and other products. It is easily grown in temperate zone [1]. The total numeral of barley accessions in Gene banks is around 0.2million. Barley had been the subject of intensive genome mapping (genome of 2n= 2x= 14). Appealing outcomes from previous research (using biochemical markers) exhibited considerably more variation in wild barley than the cultivated species [2, 3]. Six-rowed barley is derived from two-rowed ancestral forms [4, 5]. Furthermore, it possesses a lot of alleles that are very well adapted to particular environments. One isoform of barley was constrained to deserts. About 78% of the disparity in the occurrence of this isoform could be elucidated by site-of-origin of precipitation and hotness. In addition to this, β-amylase gene (*Bmy1*) has been mapped to the long arm of 4H chromosome. It was found to be firmly linked to a QTL for height [6] and *sh* for spring habit [7]. Thus, barley is enriched in many constructive characters such as drought avoidance, important biomass and yield for feed and food alimentation etc. [8-12], Preceding studies have further proved that drought tolerance is a polygenic trait and genetic constitution could be of great help to dissect the gene network(s) that regulate drought tolerance [13].
Molecular evidence showed evolutionary homology between barley, rye, and wheat. The productivity of barley is limited by major abiotic stresses including drought, fost, heat, chilling, high salinity and inorganic mineral toxicity. Because of large variations in precipitation, the crop suffers occasionally from drought. Among the various abiotic factors, drought stands to be the number one problem in major barley growing regions because the crop is grown on residual moisture and the crop is eventually exposed to terminal drought [14]. Drought decreases the water (-ve) potential in the cells and thus leads to decrease in mineral uptake. This results in decreased metabolism and hence retarded growth and reduced yield.
Genetic variability leads to survival and adaptability of a species. A species with diverse genetic variability among its interbreeding population will show more variations, most-fit alleles create a path for

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evolution. Healthy reproduction becomes more difficult with small genetic variation among member of species. Barley population exhibit vulnerability for a certain diseases is increase with reduction in genetic diversity. Thus barley germplasm is maintained at Barley germplasm centre in Okayama University, Japan and CGIAR in Syria. Thus there is a need to study diversity among the various genotypes as some of the desired traits such as yield, resistance to biotic and abiotic stresses can be accumulated in single genotype by employing new techniques of plant breeding and DNA marker technology. Trait based breeding, however requires trait dissection into components. Successful marker identification would facilitate integration of MAS (Marker Assisted Selection) procedures in breeding programs enabling the pyramiding of favorable alleles.

Conventional breeding programmes generated a segregating population that screened the phenotypes (morphology) of pooled or individual plants for desirable traits, which follows selfing, repeated backcrossing and testing. Use of molecular markers facilitates these breeding processes, since it can provide means of detecting and resolving complications, and accelerates the evolution of new varieties and allows recombination of phenotypic traits with gene locus [15]. Ideal molecular markers are quite stable, detectable and abundant in plant tissues regardless of cellular growth, tissue differentiation and body defence status. DNA markers are considered best for analysis of genetic variability and cultivar identification since they remain constant during development and are not affected by environmental changes. These markers are of 2 types: Hybridization based markers and PCR based markers. PCR-based markers have revolutionized molecular biology as they require only small amount of DNA, less labour intensive, cost-efficient and no use of harmful radioactivity. Markers like RAPD, SSR, RFLP, AFLP, ISSR, SNP etc. have numerous applications in plant biotechnology such as MAS, positional cloning of agronomically important genes and detection of locus linked to the desired gene. RAPD (Random Amplified Polymorphic) DNA based DNA analysis is a most powerful approach as no prior knowledge of sequence of genotype is required [16]. Use of dominant RAPD markers can be enhanced with identification of coupling and repulsion phase markers linked to the gene of interest [17, 18]. The availability of sufficient polymorphic markers is a prerequisite for successful linkage studies. (RAPD) shows high level of DNA polymorphism and is extensively used to construct genetic maps, to develop DNA fingerprinting and variety identification in many crops. These are dominant in nature and thus can be easily used for phylogenetic analysis of barley. Therefore, the present research work was carried out to study genetic diversity for drought tolerance in barley by using DNA markers.

MATERIAL AND METHODS

The present investigation entitled “Molecular Characterization of Drought Tolerance in Barley using DNA Markers” carried out on twenty-eight genotypes of Barley (Table 1). The present investigation entitled “Molecular Characterization of Drought Tolerance in Barley (*Hordeum vulgare* L.) using DNA Markers” carried out on twenty-eight genotypes of Barley.

A. Plant material

Seeds of 28 genotypes of Barley (Table 1) were procured from Wheat & Barley section, Department of Genetics &Plant Breeding, CCSHAU, Hisar. Plants were raised in net house of the Department of Molecular Biology and Biotechnology using standard agronomic practices.

Table 1: List of Barley (*Hordeum Vulgare L.*) Cultivars Used in The Present Study

S. No.	Drought tolerant Genotype	S. No.	Drought susceptible Genotype
G1.	BH 08-34	G 15.	BH 932
G 2.	BH 07-14	G 16.	BH 07-18
G 3.	BH 08-24	G 17.	BH09-14
G 4.	K 551	G 18.	BH 933
G 5.	BH 393	G 19.	BH 09-6
G 6.	BH 08-20	G 20.	BH 08-16
G 7.	BH 08-19	G 21.	BH 09-46
G 8.	BH 08-18	G 22.	BH 07-34
G 9.	BH 08-05	G 23.	DWR 91
G 10.	RD 2800	G 24.	DWR 90
G 11.	RD 2552	G 25.	DWR89
	Drought susceptible Genotype		
G 12.	BH 05-2	G 26.	DWRUB 52
G 13.	BH 885	G 27.	BH 902
G 14.	BH 05-9	G 28.	RD 2801

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B. Chemicals
Taq DNA polymerase and dNTPs were obtained from Biolabs. All other chemicals used in the present investigation were of molecular/ analytical grade. A set of 30 randomly amplified polymorphic DNA (RAPD) markers procured from Sigma Aldrich chemical Pvt. Ltd .were used for molecular diversity studies(Table 2). Sigma primers RP series were used in the study.

Table 2: A BRIEF DESCRIPTION OF RAPD PRIMERS USED IN THE PRESENT INVESTIGATION

Sr. No.	Primer	Sequence (5' – 3')
1	RP1	CAGGCCCTTC
2	RP2	TGCCGAGCTG
3	RP5	GGTCCCTGAC
4	RP6	GAAACGGGTG
5	RP8	GGGTAACGCC
6	RP9	GTGATCGCCC
7	RP10	TCGGCGATAG
8	RP11	CAGCACCCAC
9	RP13	TTCCGAACCC
10	RP14	AGCCAGCGAA
11	RP16	AGGTGACCGT
12	RP17	CCAACGTCGG
13	RP18	GTTGCGATCC
14	RP19	CCCGGCATAA
15	RP20	CCCGTTGGGA
16	RP22	CTCCATGGGG
17	RP24	CCTCTCGACA
18	RP25	CATACCGTGG
19	RP26	TGAGCCTCAC
20	RP27	AAGCCCGAGG
21	RP30	TGTAGCAGGG
22	RP32	ACGCCAGTTC
23	RP35	GGTGCGGGAA
24	RP37	GTGACATGCC
25	RP38	TCAGGGAGGT
26	RP42	CACCAGGTGA
27	RP46	GTTTCGCTCC
28	RP47	TGCGCCCTTC
29	RP49	ACCCGGTACA
30	RP50	CAGCTCACGA

C. Experimental design
DNA Extraction
Leaf samples were taken (3-4 week old plants) and 5g of the fresh leaf tissue was hand homogenized in liquid nitrogen and was transferred to a 50 ml polypropylene tube containing 10 ml of preheated (at 65°C) CTAB buffer (2%). It is then mixed thoroughly and incubated at 65°C for 1 hour. Occasional mixing of the contents was done at an interval of 10-15 minutes. The samples were cooled to 25°C temperature followed by addition of equal volume of chloroform $CHCl_3$: isoamyl alcohol (24:1) solution and mixing thoroughly. The tubes were centrifuged at 10000 rpm for 24 min at 25°C temperature. After high speed centrifugation, the upper aqueous (polar) phase was transferred to a centrifuged tube (pre-sterilized) followed by adding 15 ml of chloroform ($CHCl_3$): isoamyl alcohol in 24:1 ratio of solution. The upper aqueous (polar) phase after 2nd centrifugation was transferred to another centrifuge tube (pre-sterilized). Equal volume of iso-propanol at low temp was subsequently added to precipitate DNA for 20 minutes at -20°C. DNA was spooled out. Pasteur pipettes and washed 2 times in 70% C_2H_5OH. DNA was then air dried overnight at 25°C temperature and subsequently dissolved in appropriate volume of T.E. (Tris-EDTA; 10:1) buffer. Samples were stored at -18°Cto -20°C till further use [19] and modified [20,21].
DNA purification
Samples of DNA were treated with 2 µl (micro litre) of DNase enzyme free RNase enzyme. A solution (10 µg/µl) per 100 µl of DNA sample and incubated in water bath at 37°C for 3-4 hours. The RNase and the protein were extracted with 500 µl (micro litre) of chloroform ($CHCl_3$): isoamyl alcohol in 24:1 ratio and solution was then homogenized briefly. The eppendorf tubes were centrifuged at 10,000 rpm for 6

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minutes at 25°C temperature. The aqueous supernatant was pipetted out and transferred to fresh pre-sterilized eppendorf tubes. DNA was ppt. out by adding up 1/10th volume of 3M CH_3COONa (sodium acetate at pH 5.2) and two volumes of ice-cold C_2H_5OH and incubated at -70ºC for one hour. Centrifugation (10000 rpm) for 15 minutes at 4ºC makes DNA pelleted. The supernatant was removed carefully and pellet was washed with C_2H_5OH of 70 percent concentration. Sample tubes were kept open to remove last traces of C_2H_5OH. The DNA pellets were dissolved in appropriate volume of TE buffer and stored at -20ºC till further use.

Qualitative and quantitative estimation of DNA

Quantity and quality of DNA was estimated by UV spectrophotometer (absorbance (A) was determined at 260 nm and 280 nm wavelength) and agarose gel electrophoresis on 0.8 % agarose.

Quantity of DNA

Quantity of DNA was estimated from the following formula:

Concentration of DNA (µg/ml) = A_{260}X 50 X dilution factor

Quality of DNA

Samples with a ratio of 1.8 were considered of good quality.

Polymerase Chain Reaction (PCR) Amplification

PCR was performed according to the protocol of Williams *et al.* (1990). PCR reactions were carried out in Biometra ThermoCycler. Based on the results of pilot experiments run for PCR conditions, 50 ng of genomic DNA,1.2U Taq DNA polymerase, 1X PCR buffer, 0.7 µM primer, 300 µM of dNTPs mix and 40°C for primer annealing were used in experiments. Therefore, the genomic DNA of all samples was adjusted to the concentration of 50ng/µl. The volume was made up to 25 µl with sterile distilled water. PCR tubes containing the above components were short spinned to allow proper mixing of reaction mixture. Amplified products were stored at -20°C till further use. The experiments were repeated two times.

Agarose Gel electrophoresis

The PCR amplified products were analyzed with horizontal gel electrophoresis using 1.5% (w/v) agarose gel. Gel casting tray was washed, air dried and its ends were sealed with cello tapes or rubber stopper. Agarose was melted by boiling in 1X TBE buffer, cooled to 50-55ºC. Ethidium bromide at a concentration of (1µg / 50ml gel) (1 µl of 10 mg/ml EtBr in 50 ml gel) was added after cooling the gel to 40ºC. Gel solution was poured into gel casting plate with an appropriate comb with required number of wells and size inserted. Gel was allowed to solidify for 30 min. After solidification, rubber stopper or sealing tapes were removed to allow conduction and gently comb was removed. Plate was submerged in 1 X TBE buffer. Samples were prepared by adding 2 µl of 6x loading dye and were spin briefly in a micro-centrifuge for proper mixing. DNA samples were loaded in the wells and electrophoresis was carried out at a steady voltage (3Volt/cm of gel) (80 V, 60 mA) till bromophenol blue (loading dye) migrated at a speed of 2-3 cm in the gel. PCR amplified products were visualized under UV trans-illuminator and photographed using Chemilager TM 440 chemiluminescence gel documentation system (Alpha Innotech Corporation).

Allele Scoring/Gel Scoring

Each amplified product was considered as a DNA marker/ allele (DNA segment) and was designed across all samples. These DNA bands were designed into a binary character matrix, with '1' for the presence and '0' for the absence of band at particular position in gel. Only clearly, bright, broad, distinguishable bands patterns were used in genetic analysis. Molecular weights of the bands were estimated by using Gene Ruler 1k bp plus DNA ladder (Biolabs) as standard.

D. Data analysis

Similarity coefficient

The data set of cultivars and reproducible bands were used to evaluate pair-wise similarity index(coefficient) following Jaccard (1908). It represents frequency of presence (+) and absence (-) of RAPD bands in i^{th} and j^{th} genotypes

I^{th}\\j^{th}		J	
		+	-
I	+	A	B
	-	C	D

Where,

m = a+d (number of matches)

µ = b+c (number of unmatches)

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n = μ+m (total RAPD markers)

Jaccard's similarity coefficient (J) = a/n-d

Dendrogram

The matrix of similarity coefficient was subjected to unweighted pair group method for arithmetic mean (UPGMA) to create a dendrogram using average linkage procedure. The standardized data matrix was used to evaluate correlations among variables. These correlations were subjected to 'Eigen' vector analysis to extract the first three most informative Principal Components. All the numerical or algebraic taxonomic analysis were analyzed by using the computer based programmer NTSYS-PC, version 2.0 (Exeter Software, New York) based on the expertise [22].

2D and 3D principal component analysis

Principal Component Analysis (PCA) for 2D and 3D was made using the 'EIGEN' sub-programmer of NTSYS-PC software.

RESULTS

The present investigation was undertaken to study polymorphism among 28 different genotypes of barley (11 drought tolerant and 17 drought susceptible) using RAPD markers. The polymorphic data was used to analyze genetic relationship/diversity among different genotypes of barley.

A. Qualitative and quantitative estimation of DNA

The amount of DNA isolated from various genotypes of barley ranged from 402 -750 µg/ml (Table 3). The genotype BH 08-34 yielded the highest amount of DNA (750µg/ml). On the other hand, lowest amount of DNA was 402µg/ml from genotype DWRUB 52.

A ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) of various DNA samples ranged from 1.7 to 1.80 which indicated that DNA was free from contaminants like polysaccharides, proteins and RNA. A single discrete band near the wells was observed in all genotypes (Fig.1) showing that genomic DNA was intact and of high molecular weight.

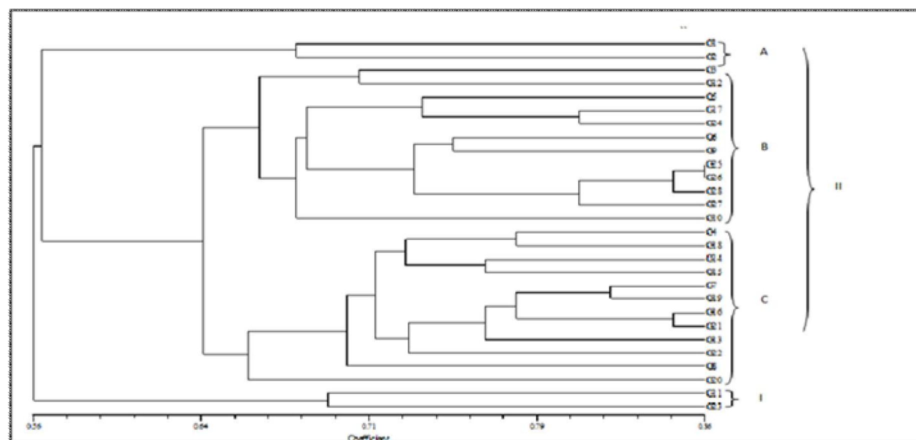

Figure 1: UPGMA dendrogram for the 28 genotypes of barley based on RAPD analysis using similarity coefficient.

Table 3: A_{260}/A_{280} AND QUANTITY OF TOTAL GENOMIC DNA OF 28 GENOTYPES OF BARLEY

Sr. No.	Genotype	Quantity (µg/ml)	Absorbance ratio (260/280)
1.	BH 08-34	750	1.86
2.	BH 07-14	672	1.83
3.	BH 08-24	640	1.85
4.	K 551	462	1.88
5.	BH 393	496	1.85
6.	BH 08-20	671	1.82
7.	BH 08-19	603	1.88
8.	BH 08-18	616	1.83
9.	BH 08-05	561	1.86
10.	RD 2800	456	1.85
11.	RD 2552	507	1.87
12.	BH 05-2	482	1.84

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13.	BH 885	554	1.85
14.	BH 05-9	626	1.86
15.	BH 932	506	1.83
16.	BH 07-18	486	1.89
17.	BH09-14	561	1.84
18.	BH 933	474	1.87
19.	BH 09-6	629	1.82
20.	BH 08-16	478	1.83
21.	BH 09-46	538	1.84
22.	BH 07-34	591	1.85
23.	DWR 91	679	1.83
24.	DWR 90	428	1.86
25.	DWR89	642	1.83
26.	DWRUB 52	402	1.85
27.	BH 902	626	1.83
28.	RD 2801	471	1.87

B. Random amplified polymorphic DNA (RAPD) analysis

For evaluating molecular diversity among 28 genotypes a total of 30 random decamer primers (RAPD) were used.

C. Polymorphism among fourteen genotypes of barley using RAPD primers

30 RAPD primers were used for the present investigation, out of which only 25 primers showed amplification. Using a total of 25 primers, 88 amplified bands were obtained of which 76 were polymorphic. The DNA amplification and polymorphism generated among various genotypes of *H. vulgare* using these RAPD primers are presented in Table 4. The total number of bands observed for every primer was recorded separately and polymorphic bands percentage was calculated subsequently. The number of amplified DNA bands varied between 1 (RP 9) and 5 (RP 16 and RP17) with an mean value of 2.93 bands/ primer. The polymorphism percentage ranged from as low as 66.7% to as high as 100%. Mean polymorphism across all the 28 barley genotypes was found to be 72.72%. Overall size of PCR amplified products ranged between 300and 2600bp. RP 38primer has unique band of 960 bp in all drought tolerant except in BH 08-18(G8).

Table 4: Dna Polymorphism Generated Using 25 RAPD Primers in 28 Genotypes Of Barley

S. No.	Primer	Total bands	Polymorphic Bands	Monomorphic Bands	polymorphism (%)	Band Size (bp)
1	RP1	3	3	0	100	300-1800
2	RP2	5	5	0	100	500-2200
3	RP5	4	3	1	75	1400-2000
4	RP8	3	3	0	100	800-1500
5	RP9	1	1	0	100	400-1400
6	RP10	4	3	1	75	300-1800
7	RP11	3	3	0	100	700-2600
8	RP13	3	2	1	66.67	900-1400
9	RP14	4	4	0	100	400-2200
10	RP16	5	4	1	80	400-1900
11	RP17	5	4	1	80	450-1700
12	RP18	3	2	1	66.67	900-1850
13	RP19	5	4	1	80	400-2100
14	RP22	3	2	1	66.67	1000-1900
15	RP24	3	3	0	100	500-1950
16	RP25	4	4	0	100	700-1600
17	RP26	2	2	0	100	400-1300
18	RP27	4	3	1	75	400-2500
19	RP30	3	3	0	100	350-1550
20	RP37	3	2	1	66.67	800-1500
21	RP38	4	4	0	100	300-1000
22	RP46	4	3	1	75	450-2100
23	RP47	2	2	0	100	380-1600
24	RP49	4	3	1	75	580-1500
25	RP50	4	4	0	100	650-1600
	Total	**88**	**76**	**12**		
	Mean	2.93	2.53	0.4	72.72	

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D. Genetic relationship and cluster tree analysis

Similarity Matrices

Out of 25 primers, 13 primers produced 100 percent polymorphism. The percentage polymorphism ranged from 66.67 % to 100 % among the 28 genotypes of barley with mean of 72.72%. Size of amplified alleles obtained in present investigation ranged from 300-2600 bp. Number of DNA bands ranged from 1 to 5 for all the primers, with an average of 2.93 bands per primer. Maximum number of bands (5) was obtained with primer RP 16 and RP 17.

RAPD similarity matrices of barley genotypes revealed the relationship among them (Table 5). The similarity coefficient between different genotypes ranged from 0.56 to 0.86.Maximum similarity value of 0.86was observed between genotypes G25 (DWR 89) and G26 (DWRUB 52). Genotypes G11 (RD 2552) and G23 (DWR 91) and also genotypes G1 (BH 08-34) and G2 (BH 07-14) were found to be genetically most diverse with similarity value of 0.56.The similarity index across all the genotypes was to be 0.71, indicating a high genetic similarity among the different genotypes.

Cluster Tree Analysis

The hierarchical cluster analysis showed that the genotypes were divided into two clusters at a similarity coefficient of 0.56 (Fig. 1). Cluster–I was divided at the similarity coefficient of 0.685 and found two genotype RD 2552 (G11) and DWR 91(G23). Cluster-2 was further divided into three subclusters in which subcluster-A had two genotypes, BH 08-34(G1) and BH07-14(G2) (drought tolerant at a similarity coefficient of 0.661). 12 genotypes were present in subcluster-B that was also further divided into three sub-subclusters. Subcluster-A was further divided into two groups. Group-1included 2 genotypes BH 08-24(G3) and BH 05-2(G12) were drought tolerant and drought susceptible respectively at a similarity coefficient of 0.693. Group-2 was further divided into sub groups and this group has 6 genotypes.

Subcluster-B included 10 genotypes, at a similarity coefficient of 0.671 made a class in which BH 392(G5), BH 09-14(G17) and DWR 90 (G24) were drought tolerant and G17 & G 24 were drought susceptible and at a similarity coffeicient of 0.714.Two groups were made, groupA1 and groupA2 in which groupA1 had two drought tolerant genotypes BH 08-20(G6) and BH 08-05(G9). Similarly groupA2 has three drought susceptible genotypes-DWR89(G25),DWRUB 52(G26) and RD2801(G28).

Similarly, sub-subcluster-C had two groups; group B2 and group B2'. GroupB2 had four genotypes BH 05-9(G14),BH 932(G15) & BH 933(G18) and K551(G4) were drought susceptible and drought tolerant respectively. Group B2' had six genotypes at a similarity coefficient 0.723 and genotypes were BH 885(G13), BH 07-18(G16), BH 09-6(G19), BH 09-46(G21),BH 07-34(22) and BH 08-19,in which first five genotypes were drought susceptible and last one was drought tolerant.

Table 5: SIMILARITY MATRIX OF 28 BARLEY GENOTYPES USING DATABASE GENERATED BY RAPD MARKERS

genotype	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24	G25	G26	G27	G28
G1	1.00																											
G2	0.68	1.00																										
G3	0.59	0.58	1.00																									
G4	0.50	0.56	0.70	1.00																								
G5	0.44	0.45	0.62	0.69	1.00																							
G6	0.51	0.52	0.63	0.68	0.68	1.00																						
G7	0.51	0.61	0.66	0.76	0.65	0.69	1.00																					
G8	0.55	0.62	0.65	0.72	0.55	0.56	0.73	1.00																				
G9	0.56	0.61	0.69	0.68	0.68	0.75	0.66	0.59	1.00																			
G10	0.65	0.55	0.69	0.73	0.68	0.61	0.63	0.59	0.69	1.00																		
G11	0.51	0.66	0.47	0.56	0.56	0.47	0.58	0.51	0.50	0.58	1.00																	
G12	0.52	0.65	0.70	0.63	0.61	0.59	0.65	0.50	0.65	0.56	0.59	1.00																

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G13	0.58	0.65	0.65	0.72	0.63	0.56	0.79	0.75	0.65	0.65	0.59	0.69	1.00															
G14	0.51	0.55	0.52	0.76	0.59	0.58	0.72	0.73	0.66	0.58	0.52	0.48	0.68	1.00														
G15	0.52	0.56	0.56	0.75	0.61	0.68	0.76	0.69	0.68	0.68	0.54	0.55	0.69	0.76	1.00													
G16	0.62	0.58	0.58	0.76	0.59	0.66	0.83	0.76	0.63	0.69	0.55	0.51	0.79	0.77	0.81	1.00												
G17	0.47	0.54	0.62	0.72	0.69	0.65	0.73	0.61	0.62	0.65	0.59	0.61	0.63	0.70	0.61	0.70	1.00											
G18	0.52	0.59	0.62	0.77	0.69	0.70	0.76	0.66	0.65	0.65	0.51	0.66	0.72	0.70	0.69	0.73	0.77	1.00										
G19	0.52	0.56	0.65	0.63	0.52	0.65	0.81	0.72	0.54	0.56	0.54	0.55	0.72	0.65	0.66	0.76	0.61	0.69	1.00									
G20	0.58	0.56	0.56	0.61	0.55	0.51	0.76	0.66	0.51	0.45	0.51	0.50	0.63	0.62	0.58	0.68	0.63	0.61	0.77	1.00								
G21	0.52	0.51	0.54	0.75	0.61	0.68	0.79	0.63	0.62	0.73	0.62	0.52	0.75	0.68	0.72	0.84	0.69	0.69	0.72	0.66	1.00							
G22	0.45	0.50	0.52	0.73	0.70	0.58	0.75	0.59	0.55	0.66	0.72	0.59	0.70	0.66	0.70	0.75	0.70	0.62	0.65	0.62	0.79	1.00						
G23	0.48	0.55	0.52	0.54	0.62	0.52	0.61	0.59	0.50	0.52	0.69	0.51	0.59	0.52	0.54	0.55	0.56	0.54	0.70	0.65	0.59	0.69	1.00					
G24	0.52	0.51	0.70	0.63	0.77	0.59	0.70	0.52	0.68	0.70	0.56	0.66	0.63	0.59	0.61	0.62	0.80	0.69	0.63	0.66	0.61	0.65	0.65	1.00				
G25	0.61	0.54	0.73	0.66	0.66	0.73	0.73	0.61	0.70	0.68	0.56	0.72	0.69	0.59	0.66	0.70	0.75	0.72	0.61	0.61	0.69	0.68	0.54	0.77	1.00			
G26	0.69	0.59	0.68	0.66	0.61	0.65	0.68	0.61	0.73	0.68	0.54	0.69	0.63	0.68	0.69	0.73	0.72	0.72	0.63	0.61	0.63	0.65	0.56	0.75	0.86	1.00		
G27	0.68	0.69	0.72	0.65	0.59	0.72	0.66	0.59	0.77	0.69	0.52	0.62	0.56	0.61	0.73	0.69	0.68	0.65	0.68	0.62	0.59	0.58	0.61	0.73	0.76	0.81	1.00	
G28	0.68	0.63	0.72	0.73	0.70	0.75	0.72	0.65	0.77	0.72	0.47	0.68	0.70	0.69	0.73	0.77	0.68	0.70	0.65	0.59	0.65	0.66	0.50	0.73	0.84	0.84	0.83	1.00

Principal Component Analysis (PCA)

Two and three dimensional PCA based on RAPD data (Fig. 2 and 3 respectively) showed similar clustering of the 28barleygenotypes as was evident from cluster tree analysis. The genotypes tended to group in many clusters, but three clusters showed clear distinction between drought tolerant and sensitive genotypes of barley. The first cluster had all drought tolerant genotypes, BH 08-24(G3), BH 393(G5), BH 08-20(G6) and BH 08-18(G8) except BH 05-9(G14). Genotypes BH 885(G13), BH 933(G19), BH 08-16(G20), BH 09-46(G21) and BH 07-34(G22) were drought susceptible and lying in second cluster while genotypes G25, DWRUB 52(G26), BH 902(G27)and RD 2801(G28) formed third cluster which also had drought sensitive genotypes.

Figure 2: 2 D PCA of 28 genotypes of barley based on RAPD data

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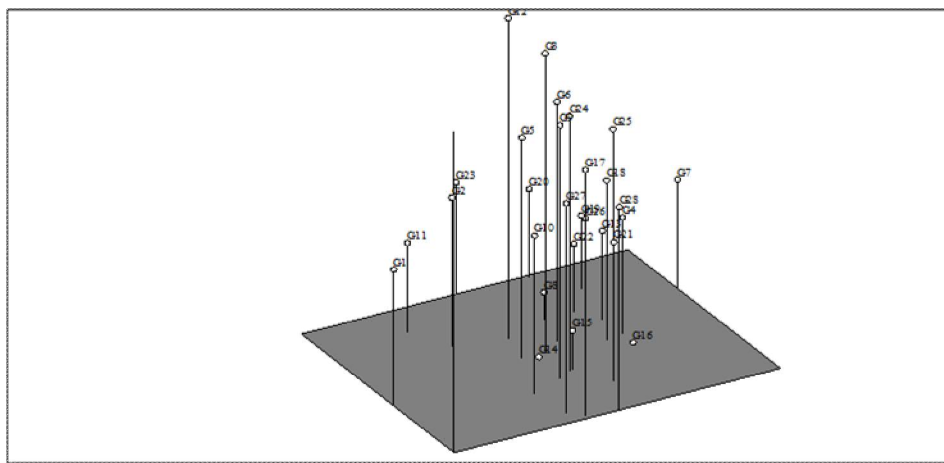

Figure 3: 3D PCA of 28 genotypes of barley based on RAPD data

BH 08-34(G1) occupied the unique positions in both 2-D and 3-D analyses. Genotypes, DWR-89(G25) and DWRUB-52(G26) were genetically very similar and were placed close to each other and had their unique position in both 2-D and 3-D plot. Genotypes BH 07-18 and BH 09-46 were also found to be close to one another.

E. Unique bands for drought tolerance in barley

RP 38 primer showed unique band of 960 bp in all drought tolerant except in BH 08-18(G8).

DISCUSSION

A.DNA isolation

CTAB extraction method is used for isolating genomic DNA from leaves of 28 genotypes of barley [19] modified by expertise [20, 23]. Best DNA preparations were obtained with 0.2 M Tris buffer (pH 8.0), containing CTAB (2.0%), 1.4 M NaCl and β-mercaptoethanol (2%) with an incubation period of 90 min at 65ºC.

Different extraction methods have been proposed for DNA isolation. However, CTAB DNA extraction method given by Murray and Thompson [20] and modified by expertise has been a method of selection for several years and was reported in a number of plant species including barley. Mann *et al.*, 2010 used the DNA extraction buffer containing 200 mM (milli molar) Tris-HCl, pH-7.5; 200 mM (milli molar) NaCl; 25 mM (milli molar) $EDTANa_2$, pH-8.0; 2 % mercaptoethanol and 2 % CTAB and it was added to 1 g lyophilized tissue and the homogenate was incubated at 65˚C in water bath for one and half hours. Zangenberg [24] used 2% of CTAB and obtained good quality of DNA. Ciulca [25] used PCRreaction mixture (25µL) contained approximately 50 ng (nano) of barley DNA, 0.5 µM (micro) of primer, 0.2 mM (milli) of each dNTP, 10 mM (milli) Tris-HCl (pH 8.0), 50 mM KCl (Potassium Chloride), 2 mM $MgCl_2$ and 1U of Taq DNA polymerase.

B.PCR amplification conditions

To obtain reproducible banding patterns, the PCR reaction conditions need to be well defined. For RAPD analysis, reproducible and clear banding patterns were obtained in a reaction mixture of 25 µl containing 50ng (nano) of template DNA, 300 µM (micro) of each dNTP, 1X PCR buffer solution, 1.5 mM (milli) $MgCl_2$ (Magnesium, Chloride) 0.7µM (micro) primer and 1.2 U Taq (Thermus Aquaticus) DNA polymerase. At low conc. of DNA,, low intensity bands were observed whereas with high concentration of template DNA, there was not much difference in band intensity. The conc. of $MgCl_2$ is reported to be the most important factor for unambiguous and reproducible DNA amplification. $MgCl_2$ conc. has been found to alter the quantity and kind of the final product formed in PCR reaction. Zangenberg [24] observed that suboptimal conc. of $MgCl_2$ can result in low yield.

C. Molecular marker analysis

The characterization of genetic variability (diversity) within a closely related crop germplasm is an essential tool for coherent use of genetic resources. The study of genetic variation (diversity) in inter breeding resources is main focus for plant breeders, as it contributes to genomic or traits selection, monitoring of germplasm and prediction of potential genetic material. Molecular markers have the potential to detect genetic variability (diversity) and to aid in the management of plant genetic resources. Among various molecular markers currently available, RAPD markers are used as variety of applications in breeding because of their multiple allelic nature, co-dominance, relative abundance, and extensive

genome coverage. To evaluate genetic diversity among 28 genotypes of barley, a total of 30 RAPD primers were used. Out of 30 primers only 25 primers showed amplification. A total of 88 sharp and reproducible bands were obtained out of which 76 were polymorphic and 12 were monomorphic resulting in 72.72 % polymorphism among the genotypes. The percentage polymorphism ranged from 66.67 % to 100 % among the 28 genotypes of barley with mean of 72.72 %. In present investigation size of amplified DNA fragment (alleles) ranged from 300-2800 bpb (base pair). Number of DNA bands ranged from 1 to 5 for all the primers, with mean value of 2.93 bands / primer. Primer RP 16 yield maximum no. of bands [5].

Ciulca [25] studied three RAPD primers in barley. Amplified fragments of genome had sizes between 180 and 1550 bp (base pair) for primer S18, and 150-1150 bp (base pair) for S39 primer respectively, while primer S32 represented small quantity of the amplified DNA fragment of genomic size (400 and 1500 bp). Number of DNA bands ranged from 10 to 14 for all the primers, with an mean value of 11.33 bands/ primer. Considering all 3 RAPD primers in he presented study, resulted an mean number of 10 polymorphic bands/primers of the 34 bands generated by RAPD primers included in the research study, 30 were polymorphic and 4 monomorphic, ensuing an 88.% average value of polymorphism. Ahmed [26] found that the number of bands ranged from 2 to 4 with the averagevalue of 3 bands by using 16 RAPD primers in barley. Agrawal and Srivastava [27] found 50 polymorphic RAPD primers out of 60 and loci per marker were found to be 3.55 in barley genotypes and with size ranging from 200 to 1000bp.

D.Genetic similarity and cluster tree analysis

Based on this data, cluster analysis was done to estimate relationship among genotypes. A maximum genetic similarity value of 0.86 was observed between G25 (DWR 89) and G26(DWRUB 52) genotypes, which revealed a high degree of similarity to the extent of 86% existing between them. A minimum genetic similarity value of 0.65 was experiential l found in genotype BH 08-16(G20). Similar studies were conducted by different investigators using RAPD markers [25]. It was assumed that such a high level of genetic compatibility (similarity) may be the result of biased selection of the material in the previous breeding programs, which ultimately narrowed the genetic base of the barley germplasm. It is further suggested that more polymorphic barley could be used for efficient screening of the germplasm by saturating more regions of the barley genome.

In present investigation we found the genotypes tended to group in many clusters, but three clusters showed clear distinction between drought tolerant and susceptible genotypes of barley. The first cluster had all drought tolerant genotypes, BH 08-24(G3), BH 393(G5), BH 08-20(G6) and BH 08-18(G8) except BH 05-9(G14). Genotypes BH 885(G13), BH 933(G19), BH 08-16(G20), BH 09-46(G21) and BH 07-34(G22) were drought susceptible and lying in second cluster while genotypes DWR 89 (G25), DWRUB 52(G26), BH 902(G27)and RD 2801(G28) formed third cluster having drought susceptible genotypes.BH 08-34(G1) occupied the unique positions in both 2-D and 3-D analysis. Genotypes, DWR-89(G25) and DWRUB-52(G26) were genetically very similar and are placed close to each other and have their unique position in both 2-D and 3-D plot. Genotypes BH 07-18 and BH 09-46 were also found to be close to one another.

Ciulca [25] found that the Romanian cultivars Andrei and Dana present a genetic similarity (compatibility) index of approx 76-77 % and composed a first subgroup of the first cluster, collectively with Tas cultivar. The second subgroup is composed of Salemer,, Regal and Madalin cultivars showing an average genetic variability of approx 32-33 %. A second cluster comprise of cultivars: Gerbel, Dina, Orizont, Center Lyric, Nelly,Plaisant, Majestik, records a genetic similarity of approx 65 % between them, and an average diversity of approx 63 % toward the first cluster. The third cluster included the cultivars Landi, Malwinta, Turul,Secura, Rezi, , which has 65% common alleles. The collection of Hungarian cultivars Kunsagi Metal, Viktor, Judy represent an average genetic variability of 43 %. The very last cluster composed of 7 cultivars showed an average genetic compatibility of 57 % between them . Karim [28] found that in RAPD analysis, out of 93 bands, 69 bands (74%) were polymorphic with the mean of 4.6 / primer. For every primer, the digit of bands ranged from 4 to 10, with an average of 6.2.

Eshghi [29] used bulked segregant analysis (BSA) was to identify RAPD markers associated with $-glucan in barley. RAPD markers linked to $-glucan was identified in two DNA pools (high and low $-glucan), which were established using selected F individuals. The analysis of gene actions indicated that a large part of the total genetic variation observed for $-glucan contentwas in the form of dominance genetic effects. In addition, duplicate epistasis observed in the expression of this trait. Estimating gene number by different formulae representing several QTL was involved in the genetic control of this trait. In bulked segregant analysis two separate RAPD fragment (S39-150 bp and WE013-1750 bp) was found among the 3968 amplified bands that was related to the $-glucan content.

L. Nazari [30] worked on 16 cultivars of barley for different effect of drought but these tests involved 5 tolerant and 5 sensitive types. The results of RAPD investigation signified its suitability for evaluating polymorphism among the samples. Among 30 primers used with RAPD-PCR technique, primers No. 3, 26 and 28 did not represent amplification of any definite DNA bands and primers No. 24, 25 did not generate any scoreable polymorphic DNA bands. In all the 25 selected primers amplified a total of 275 amplicons from 16 barley genotypes were detected, among them, 65 fragments (24%) were monomorphic and the rest (76%) were polymorphic between one or more genotypes.

CONCLUSION

Barleyis not justan important crop worldwide moreover an excellent system for genome mapping and genome-based analysis because its chromosomes are homologous to cultivated wheat and rye, respectively. It is well adapted to low soil fertility, drought, high salinity, low pH (acidic) and high temperatures among tropical cereals and is grown on >28 million hectares in arid and semiarid regions of Africa (15 million hectares) and Asia (11 million hectares). This crop provides basic sustenance to resource farmers and can grow in the poorest soil.The plant material for the present research comprised 11 drought tolerent and 17 drought susceptible genotypes of barley. The performance of these markers was evaluated by means of different parameters such as percentage polymorphism, similarity matrix data and clusters formed in the dendrogram. The experiment was performed out in two stages. In the first stage, DNA isolated from 28 genotypes of barley was used for PCR (*in vitro*) amplification technique using 30 RAPD primers. In the second stage, the selected primers showing amplification were used to evaluate genetic varieties among 28 barley genotypes and to identify drought stress specific molecular marker/s.The amount of DNA obtained was 402- 1150 µg/ml. 25 markers showed amplification, producing 88 bands (76 - polymorphic ; 12 – monomorphic). The amplification obtained was of 2.93 bands/primer; with size of 300-2600 bp. Average similarity value of 0.71 was found between different genotypes, with maximum similarity (86%) between genotypes DWR 89 and DWRUB 52. RD 2552 and DWR 91 genotypes were show minimum similarity matrix. The study has special value since seed companies and national registration agencies have an interest in DNA fingerprinting because the technology can differentiate between drought tolerant and drought susceptible.

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