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GENETIC DIVERSITY ANALYSIS IN INDIAN MUSTARD [*Brassica juncea* (L.)] FOR SALINITY TOLERANCE USING SSR MARKERS

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Abstract: *Genetic diversity was performed among F_2 plants of the cross RH 30×CS 52 in Indian mustard (Brassica juncea) (CS 52 is salinity tolerant and RH 30 is salinity susceptible) using SSR markers. Out of 358 SSR markers, 42 were found polymorphic and 154 were monomorphic. A total of 225 alleles, ranging from 2 to 4 were amplified. The PIC (Polymorphic Information Content) value ranged from 0.427-0.730 of Jaccard's similarity coefficients was generated between these F_2 populations. F_2 population was grouped in 2 major clusters and 37 sub-clusters at a similarity coefficient of 0.58. The allelic information indicated that the genotypes used in the present study have distinct genetic patterns. It was concluded that SSR markers are more reliable and will be helpful in selecting diverse genotypes which can be used to identify QTL related to salinity.*

Keywords*: Brassica juncea, genetic diversity, SSR markers, dendrogram and salinity tolerance.*

Introduction: *Brassica juncea* commonly known as Indian mustard is an amphidiploid species that originated through the interspecific hybridization of *Brassica rapa* and *Brassica nigra* [1]. It is utilized as an oilseed, a condiment, vegetable, green manure, forage and cultivated primarily in tropical and sub-tropical countries [2]. *Brassica* oilseed crops have a very significant role in agriculture because almost each part of plant is consumed either by human beings or animals depending upon the crop and its growth stage.

Soil salinity is one of the serious abiotic stresses for crops due to which large areas of the agricultural lands are becoming unfertile. Three fourth of the total Earth surface is covered by saline water. Over 830 million hectares of land area in the entire earth is salt affected, either by saline water (403 million hectares) or by the conditions related with sodicity (434 million hectares) [3] and it is more than 6.0 per cent of the entire land area in the world. Excess amount of NaCl occurs as an abiotic environmental factor in many places such as salt deserts in the arid and semi-arid areas [4]. During the last decades, apart from the natural salinity, salinization of soils due to intensive agriculture and irrigation is also becoming a major concern in agriculture. Exposure of plants to high salt concentrations causes ion imbalance, ion toxicity and hyper osmotic stress [5, 4, 6], which causes a severe retardation growth and productivity. For most of the crops concentration of 150 mM NaCl is highly toxic though, for a few crops, as low as 25 mM NaCl is lethal. Does, it is the need of hour to develop plant varieties with enhance salt tolerance. Two main methods for providing the solution to salinity stress problem includes reclamation of saline soils by use of chemicals or by growing salt tolerant plants in the saline soils [7-10].

Molecular markers particularly the microsatellite markers (SSRs) provide a valuable tool for genetic analysis and produce salt tolerant plants [11]. It can help in the identification of suitable parental lines, diversity and pedigree analyses and is more suitable to fasten the selection and development of new improved genotypes, as it is independent of environmental factors and developmental stage of the plant [12].

At present, molecular markers are being successfully used to interpret genetic diversity among populations. Amongst the various types of molecular markers used, Simple Sequence Repeat (SSR) markers are generally used because of their higher reproducibility, co-dominance, abundance, wide distribution throughout the genome, easy scorability and multi-allelic variation [13]. SSR have been used for genetic diversity analysis in a number of crops including Indian bread wheat [14], rice [15] and maize [16]. A large number of SSR markers have been developed and extensively characterized in *B. rapa* [17-18] and *B. napus* [19-20]. However, only few SSR markers are reported in *B. juncea* [21-24]. Genetic diversity information forms the foundation of any breeding program and is of great importance to the sustainability of plant populations. An understanding of organized germplasm and relationship among genotypes provides an opportunity to develop improved crops by more efficient sampling of genotypes [25]. The present investigation was to evaluate the genetic diversity in Indian mustard genotypes for tolerance against salt stress. Identification of such genetic diversity will help in introgression of salinity tolerance into other high yielding cultivars.

Materials and Methods

Two parental lines (CS 52 and RH 30) and F_2 progeny lines of *Brassica juncea* were obtained from the Oilseeds Section, Department of Genetics & Plant Breeding, CCS HAU, Hisar during rabi, 2014-15.

Screening of Parental Genotypes under Salinity Stress Condition in Laboratory: The crop was raised during *rabi* season (2014-15) in the laboratory of the Department of Molecular Biology, Biotechnology and Bioinformatics, CCS HAU. The seeds of parents i.e. RH 30 and CS 52 were shown at three salinity levels *i.e.* control, 6 dS/m, 8 dS/m and 10 dS/m in petri plates containing MS medium. Seed germination rate was observed [26].

Genomic DNA Isolation, Purification and Quantification: Genomic DNA was isolated from young leaves using CTAB method [27]. The precipitated DNA was washed with 70% ethanol and dried overnight at room temperature. The dried pellets were dissolved in T.E. buffer (1M Tris, 0.5M EDTA and pH 8.0). The DNA quality and concentration was checked by electrophoresis in 0.8% agarose gel and UV spectrophotometer.

PCR Amplification: SSR markers were used to evaluate genetic variability among the Indian mustard genotypes. PCR amplifications were performed using T100^{TM} thermocycler. The total volume of PCR reaction was 20 µl per sample, containing 1 µl DNA, 2 µl of 10X PCR buffer with $MgCl_2$, 0.4 µl of 10 mM each forward and reverse primers, 0.4 µl of 10 mM dNTP and 0.25 µl of 1.25U *Taq* DNA polymerase. The PCR tubes were set on the wells of the thermocycler plate. Then, the machine was run according to the following setup: Initial denaturation at 95°C for 3 min; Denaturation at 94°C for 1 min; Annealing at 50-60°C for 1 min; Extension at 72°C for 1 min; completion of cycling program (40 cycles); Final extension at 72°C for 7 min and reaction were held at 4°C. The amplified products were separated on 6% polyacrylamide gels containing ethidium bromide [28]. Molecular weight marker of 20 bp was run with the PCR products. DNA bands were observed on UV-trans-illuminator in the dark chamber of the Image Documentation System.

Data Analysis: All distinct bands (SSR markers) were thereby given identification numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each primer. The scores obtained using all markers in the SSR analysis were then pooled to create a single data matrix and to construct a UPGMA dendrogram using NTSYS–pc software [29].

Results and Discussion

Seed germination was studied using three salinity levels *i.e.* control, 6 dS/m, 8 dS/m and 10 dS/m in petri plates containing MS medium. The results revealed a significant effect of salinity on seed germination rate and length of shoot and root in Indian mustard (Figure1). The germination percentage of parental genotypes of CS 52 and RH 30 were 60% and 40% under salinity concentration at 10 dS/m, which was lower than control and treatments. *Brassica* was classified as moderately salt tolerant with a superiority of amphidiploids species over the diploid species [30]. Reported that the amphidiploids species are more salt tolerant as compared to diploid species [31].

The decrease in growth under the saline condition by the reduction in leaf area, yield and decreased water uptake along with decline in photosynthesis [32]. It was detected that significant variances were given among all genotypes and NaCl-treatment with respect to shoot length, root length and biomass production.

Observed that shoot length decreased by the increasing salt concentrations [33]. It is well established that adverse effects of salinity affect plants in various ways: oxidative stress, water stress, ion toxicity, genotoxicity, nutritional disorders, alteration in the metabolic processes, membrane disorganization, reduction of cell division and expansion [34-35]. These mechanisms declined plant growth and persistence [36-37]. Our investigations are supported with the previous results of other experiments [38-41].

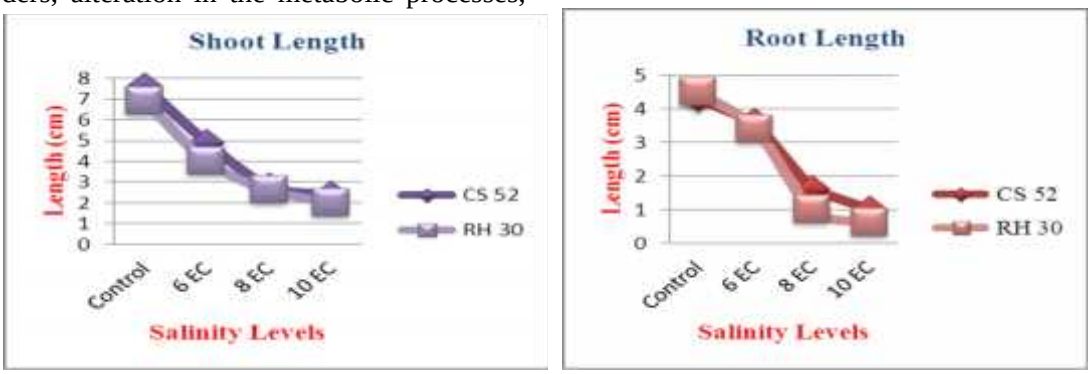

Figure 1: Effect of salinity levels on length of shoot and root in parental genotypes *i.e.* CS 52 and RH 30 under laboratory conditions.

SSR Markers Based Analysis: In the present research work, genetic diversity was assessed among F_2 plants of the cross RH 30×CS 52 along with both the parents in Indian mustard (*Brassica juncea*). Out of 358 SSR markers, 42 were clear, distinguishable and unambiguous bands were chosen for genetic diversity evaluation and 153 were monomorphic; did not generate any amplification product or stable banding patterns. A total of 225 alleles were amplified and the number of alleles ranged 2-4 per primers (Figure 2 &3 and Table 1). The average number of alleles in the present study was lower than those reported in most other studies on Indian mustard using SSR markers. Using 134 SSR markers, [22] detected 2-8 alleles per primers; [42] observed 1-8 alleles with a mean of 2.79 and found 2-5 alleles with an average of 2.9 per primer [24].

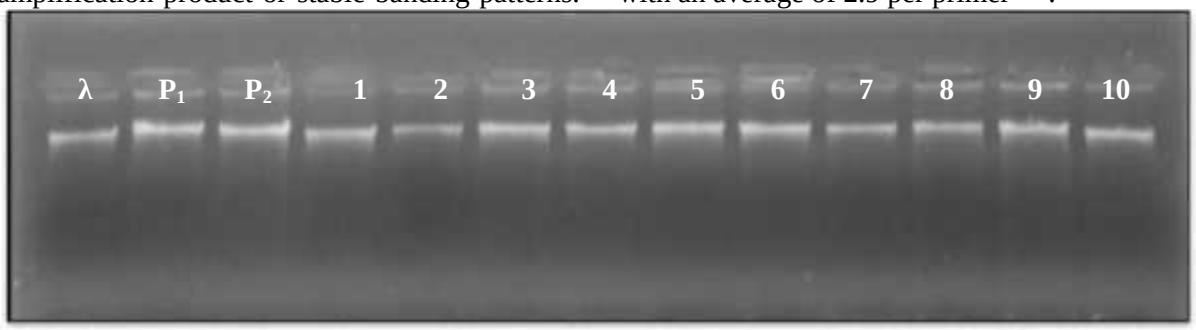

Figure 2: Agarose gel electrophoresis showing genomic DNA of parents P_1 and P_2 (P_1-CS 52, P_2- RH 30). Where 1-10 plants showing F_2 (RH 30 × CS 52) population, λ -lambda DNA (50 ng/µl)

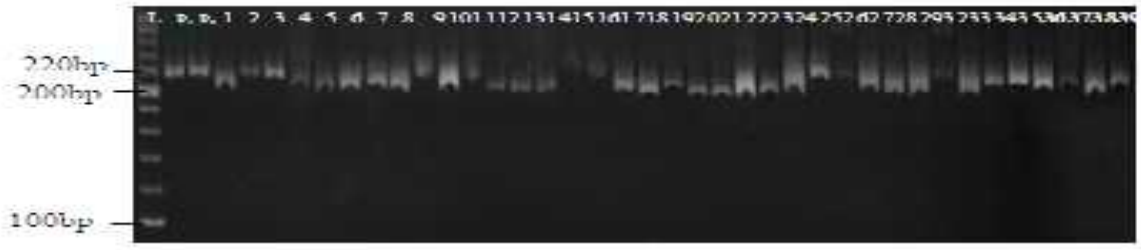

Figure 3: Polyacrylamide gel showing allelic polymorphism among F_2 plants using marker GOL 3, P_1 –CS 52, P_2-RH 30, L- Ladder (20bp) and 1-39 F_2 plants

Genetic Diversity Evaluation Studies: The mean polymorphic information content (PIC) values provide an estimate of the discriminatory power of a locus by taking into account the number of alleles that were expressed, as well as, the relative frequencies of the alleles. In our study, the PIC values ranged from 0.427-0.730 with an average of 0.555. SSR Ni2-A01 was found to be the most informative marker depicting the highest PIC value of 0.730; source

of this marker is *Brassica nigra*. Several researchers have used SSR markers for diversity analysis in *Brassica species* [43-44]. In our study, the average PIC values were found to be higher than that of reported [45] in *B. juncea* (0.46). Reported low PIC value 0.281 [46]; PIC values ranged from 0.12-0.61 [47] with an average to 0.314 but lower than that, observed [48]. The PIC values (0.38-0.96) observed [24] were found to be higher than that of our study. Lower number of alleles per locus and lower PIC values may be attributed either to the use of less informative SSR markers, or the presence of lesser genetic diversity among the tested genotypes. The Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrogram was constructed using Jaccard's similarity coefficients based on SSR marker data generated on F_2 population with parents (RH 30 and CS 52) (Figure 4). UPGMA grouped into two main clusters at a similarity coefficient of 0.51. Both clusters were further divided into sub clusters i.e. 37 sub-clusters at a similarity coefficient of 0.58. The highest populations for genetic similarity coefficient were found near to CS 52 (salinity tolerant). The least similarity coefficient populations were found near to RH 30 (salinity sensitive); this showed that presence of maximum genetic diversity lie between both the genotypes. However, observed eight clusters with a range of 1-18 genotypes in each cluster (cluster III being the largest) in the genetic diversity study on forty six Indian mustard genotypes [4]. The grouping of accessions based on SSR marker data seems to be more reliable than morphological (phenotypic) data as it is free from environmental fluctuations. Suggested that a large portion of variation detected by molecular markers is non-adaptive [49] and is, therefore, not subject to either natural or artificial selection as compared with phenotypic characters, which in addition to selection pressure are influenced by the environment. Principal component analysis (PCA) explained variation based on the molecular data by phenotypic data (Figure 5). In their study PCA revealed that the grouping of genotypes based on the SSR marker data was more convincing than the phenotypic data. Therefore, the genetic diversity analyzed in the present study based on SSR markers is more authentic than the phenotypic data. Genetic diversity analysis is helpful in selecting diverse genotypes which can be used to identify QTL related to salinity.

Table 1 : Summary of SSR amplified products

S.No.	SSR amplified products	values
1	Total number of primer pairs tested	358
2	Number of polymorphic primers	42
3	Total number of polymorphic bands	42
4	Total number of monomorphic bands	153
5	Size of amplified products (bp)	115-300
6	Range of alleles	2-4
7	Range of PIC value	0.427-0.730
8	Average of PIC value	0.555

Conclusion: The present study revealed that genetic diversity between the genotypes and F_2 population can be exploited through hybridization to recover the segregants possessing high yield potential with improved seeds quality characteristics. However, genetic diversity analysis at molecular level by SSR markers that covers the whole genome can be helpful for the sequencing of whole genome and also to identify the diverse parents. Molecular markers are also help in construction of a linkage map and QTL identification for salinity tolerance, which will lead to the development of salt tolerant cultivars with improved yield.

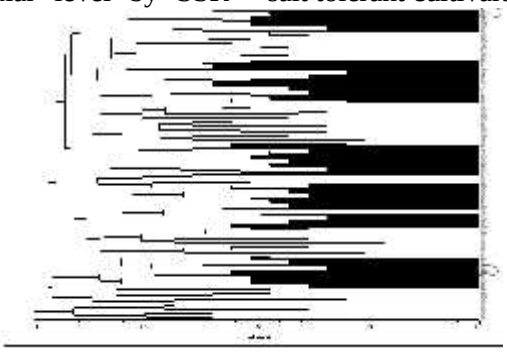

Figure 4: Dendrogram (NTSYS-pc) displaying diversity among 156 F_2 population (RH 30 × CS 52) and parental genotypes using allelic diversity data at 42 SSR loci

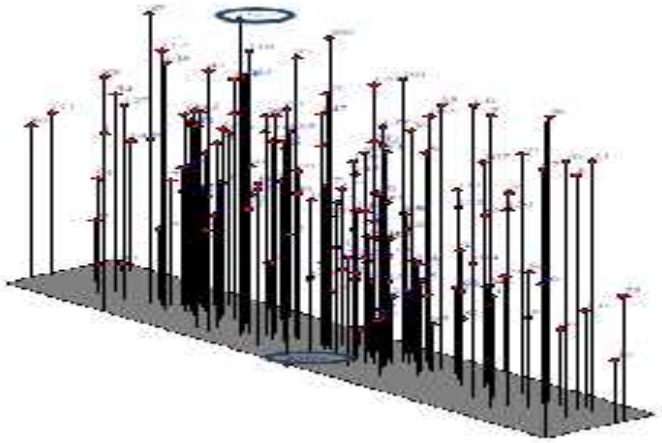

Figure 5: Three dimensional PCA scaling display diversity of 156 F_2 progeny lines (RH 30 × CS 52) and parental genotypes using allelic diversity data at 42 SSR loci

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