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STUDY OF GENETIC DIVERSITY AMONG FIVE ACCESSIONS OF *BRASSICA JUNCEA* L. CZERN & COSS. USING PROTEIN PROFILING AND RAPD MARKER

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ABSTRACT

Indian mustard (*Brassica juncea* L.) is an important oil seed crop. The present investigation was undertaken to explore and detect genetic diversity of five mustard accessions *i.e.* Maya, Kranti, Poosa Bold, RH- 30, and RGN-73 by SDS-PAGE for protein profiling and Randomly Amplified Polymorphic DNA (RAPD) for assessment of genetic variation at the molecular level. Gel were scored for the presence (1) and absence (0) for polymorphic band. Molecular diversity studies were also done to find out the maximum polymorphism of DNA. The most efficient amplification and polymorphism of DNA was found with random primer OPA-11, 5'- CAA TCG CCG T- 3'.Total 8 polypeptide bonds were distinguished among five accession. Out of these 4 band was found polymorphic with a polymorphism of about 0.83% similarity coefficient. The average polymorphism was calculated 50%. The similarity matrix computed with Jaccard's coefficient revealed the maximum similarity between accessions Poosa bold with RH-30 and Maya with Kranti, while distantly related varieties where observed with RH-30 and RGN-73. Brassica cultivars RH-30 and RGN-73 were found to produce hybrid vigour during breeding programs because they are genetically distinct from other cultivars of *Brassica* and have minimum genetic similarity.

Keywords: *Brassica juncea*, Genetic diversity, protein profiling, SDS-PAGE, RAPD, UPGMA.

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INTRODUCTION

Brassica juncea (L.) Czern & Coss (Indian mustard) is major *Rabi* season oilseed crop of India. India is the fourth producer of mustard seed contributing around 11 per cent of world's total production.It contributes nearly 27 percent of total edible oil production of India. *B. juncea* is the second highest cultivated oilseed crop in India after soyabean (*Glycine max*). Indian mustard is mostly grown in various states like Rajasthan, Gujarat, Uttar Pradesh, Madhya Pradesh, Haryana, Bihar, Punjab and West Bengal of India [1].

The family Brassicaceae is one of the major groups of the plant kingdom, comprising of 340–360 genera and over 3,700 species distributed worldwide [2-3]. It is natural amphidiploid having chromosome number (2n=36). It is self-pollinated but certain amount of cross pollination (2-15 %) occurs due to insect and other factors. The place of origin of mustard is China and from there, it was introduced to India. At present time, mustard has been identifies as a good crop for supporting bee keeping activity. The oil of mustard possesses a sizable amount of erucic acid (38-57 %), together with linolenic acid (4.7 to 13.0 %). The oleic and linoleic acids, which have a higher nutritive value, together constitute about 27

% [4].

Knowledge about germplasm diversity and genetic relationship among breeding material were supposed to be an invaluable aid in crop improvement strategies. Various methods are currently available for analysis of genetic diversity in germplasm accessions, breeding lines and segregating populations. These methods have relied on pedigree data, morphological data, agronomic performance data, biochemical data and more recently molecular (DNA based) data [5].

A variety of molecular markers including Random amplified polymorphic DNA, (RAPD) Amplified fragment length polymorphism (AFLP) and Restriction fragment length polymorphism (RFLP) have been used to find the extent of genetic variation among the diverse group of important test crop species [6].

Verma *et al.,* (2016) used RAPD markers to access the genetic integrity of Indian mustard *i.e.* NRCDR-2. Out of 40 RAPD marker screened only 27 RAPD markers produced a total (198) clear, distinct and reproducible amplicons. All RAPD markers produced a total 198 bands, ranging 50 to 8000 bp length and the number of scorable bands varied 3 to 15 per primer with an average of 7.33 bands per primer [7]. Yousuf *et al*., (2013) detected genetic diversity among five mustard varieties with the help of random primers. A total 20 bands were scored and the level of genetic polymorphism was in the range of 0 to 66.66 % [8]. They were found maximum similarity of variety Kranti with Varuna (similarity indices 0.9677) while distantly related varieties were Basanti and Rohini.

Genetic diversity were important for knowing varied among accessions of *Brassica* where protein profiling is one of the basic and important technique. Genotyping of different species is necessary for characterization of different accessions of crop germplasm, testing of varietal purity and registration of newly developed cultivars [9]. Among numerous techniques available for assessing the genetic variability and relatedness, seed storage protein analysis represents a valid alternative to varietal identification [10]. It is useful tool for studying genetic diversity via Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Seed storage protein markers are highly polymorphic and had environmental influence as their electrophoretic pattern is limited [11].

Seed storage protein profiling based on SDS-PAGE were employed for various purposes such as characterization of germplasm [12], varietal identification, biosystematic analysis, determination of phylogenetic relationship between different species [13]. Genetic diversity, of seed storage proteins has also been reported in Mustard [14].

Jan *et al.*, (2017) studied twenty genotypes of *B. rapa* through SDS-PAGE method and data were analyzed by UPGMA where they hand noted small, medium, and large size of proteins [15]. They had reported total 12 bands which were obtained among 10 genotypes *i.e*.83.33 % and were highly polymorphic while the rest two 15.38 % were found to be monomorphic. The protein size base polymorphism was divided into different groups on the basis of molecular weight that ranged from ~10 kDa to ~ 180 kD as.

In present investigation, the extent of genetic diversity among five accessions of *B. juncea* by using protein profiling and RAPD marker. The information were used to identify genetically diverse genotypes that can be utilized in future breeding programs aimed to create genetic diversity in *B.juncea*.

MATERIALS AND METHODS

The objective of the present study was to analyze the genetic diversity among the given five accession of *B.juncea* L. namely Maya, Kranti, Poosa Bold, RH-30 and RGN-73. All accessions were collected from ICAR, New Delhi, India.

Protein profiling

The isolation of proteins was conducted at IBRC (Institute of Biotechnology & Research Center) Agra U.P. Grounded1g of seedwere kept chilled with pestle mortar by maintaining 4^oC temperature. Then addition of 10ml per gram of seed of protein extraction buffer (pH 7.6). Acid washed sand used as an abrasive for a finer grinding and incubated at 4^oC for half an hour. Then transferred the homogenate in a sterilized test tube and centrifuged the homogenate at 15,000 rpm for 15 min. Now transferred the supernatant into another 1.5 ml eppendorf tube and store at 4^oC till further use.

Preparation of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic procedure was carried out by using slab type SDS-PAGE with 12% Polyacrylamide gel. A 12% resolving gel(3.0 M Tris HCL, pH 8.8, 10% SDS and 4.5% stacking gel) was prepared and polymerized chemically by addition of 17 ml of N,N,N,N Tetramethylenediamine and 10% Ammonium per sulphate (APS). Electrode buffer solution was poured into the bottom pool of the apparatus. Gel plates were placed in the apparatus carefully so as to prevent bubble formation at the bottom of the gel plates. The electrode buffer (0.025 M Tris, 1.29M Glycine, 0.125%SDS) was added to the top of the apparatus. 200µl of the extracted protein was loaded with the help of micropipette in to each well of the gel. The apparatus was connected with constant electric supply (50 V) till the tracking dye Bromophenolblue (BPB) reaches the bottom of the gel. Gels were then stained with staining solution comprising 0.2 %(W/V), Coomassie Brilliant Blue (CBB) R 250 dissolved in 10 % (V/V), acetic acid and 40 % (V/V) methanol for about an hour at room temperature. Gels

were detained in a solution containing 5% (V/V) acetic acid and 20% (V/V) methanol. 10µl of protein extract were loaded in each well of SDS PAGE gel. The Gels were shaked using double Shaker Mixer gently under the background of the gel became clear and protein bands were clearly visible. After detaining the gels were photographed using gel documentation system.

Data Analysis

The bands were scored in Gel for presence (1) and absence (0) for each protein band. Similarity matrices as computed by the programme were used to construct the dendrogram using UPGMA (un-weighted pair group method with arithmetic average) method to elucidate the diversity among five accessions of *B. juncea* [16].

Isolation of genomic DNA

DNA was extracted from seeds of the plant by slight modification of CTAB protocol [17]. For each accessions of *B.juncea* 2 g of seed was ground in liquid N_2 to make a fine powder. This powder was taken in a centrifuge tube and 15 mL of 2 x CTAB (DNA extraction buffer) was added in each tube separately. Extraction buffer contained (per L) 2 g CTAB 1M Tris pH-8 (10 mL), 5 M NaCl (28 mL), 0.5 MEDTA (4 mL) with sterile distilled water (57 mL) and 1 mL β – mercaptoethenol were used. This was incubated at 65 ^{o}C water bath for 30 min with intermittent shaking. The mixture was centrifuged at 13,000 rpm/min for 15 min at 4 ^{o}C to pellet the seed. Supernatant was taken in a fresh Oakridge tube and an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added with 2-3 min slow inversion. The mixture was again centrifuge at 13,000 rpm/minute for 15 min at 4 ^{o}C. The aqueous supernatant was taken in a fresh tube and added 0.6volume isopropanol and was incubated at –20 ^{o}C overnight. After incubation, it was again centrifuged at 13,000 rpm/min for 20 min at 4 ^{o}C temperature. The supernatant was discarded and pellet was washed with 70 % ethanol. The pellet was dissolved in 500 µl of T.E. buffer and was used for PCR.

PCR amplification

The germplasm was used for RAPD analysis to analyze the genetic diversity among five accession of *B.juncea*. PCR was carried out in a final volume of 25 µl, containing 10 mM of oligonucleotides primer (1 µl) 20 mM of each of the four deoxynucleotide triphosphates (0.75 µl), 25 mM $MgCl_2$ (1.0 µl), 0.35 µl of Taq DNA polymerase 10 X Assay buffer (2.5 µl) and 1.0 µl template DNA (60 ng/ µl). Primer OPA-11, 5'- CAA TCG CCG T-3', were chosen after preliminary screening of fifty primers. The reaction mixture was overlaid with 10 µl of mineral oil and the tube was microfused at 13,000 g for 10 second. Amplification was carried out in a gradient thermal cycler with initial denaturation of 94 ^{o}C for 4.0 min and 40 cycles of 94 ^{o}C for 1 min, 34 ^{o}C for 2 min and 72 ^{o}C for 2 min.

Electrophoresis

Twenty µl of PCR product were mixed with 6 µl of gel loading buffer (0.25%) bromophenol blue, (0.25%) xylene cyanol and(30 %) glycerol were dissolved in 1xTAE buffer) and subjected to electrophoresis at 65 V for 3 h in 1.2 % agarose gel prepared in 1x TAE buffer (40 mMTris acetate 1 mM EDTA, pH 8.0). Ethidium bromide was added to the agarose gel at 6 µl/100 mL for staining. A ladder (lambda DNA/Eco RI + Hind III) was used as a size standard. DNA was visualized by trans-illuminator with u.v. light and photographed. For each accession individual RAPD marker were scored for their presence (value = 1) or absence (value = 0).

Statistical analysis

RAPD scores were used for similarity analysis using Jaccord co-efficient and clustering using UPGMA (Unweighted Pair Group Method of Arithmetic Average). It was constructed using numerical taxonomy and multivariate analysis system software [18].

RESULT AND DISCUSSION

Genetic diversity through protein profiling

The SDS-PAGE of seed proteins of five genotypes of *B.juncea* was carried out to investigate the genetic diversity at biochemical and molecular level. Total 8 polypeptide bands were distinguished among five accessions. Out of 8 polypeptide bands calculated, only 4 bands were polymorphic and the rest 4 were monomorphic. The average polymorphism was calculated 50%. The accession B1 (Maya) and B2 (Kranti) showed the maximum number of bands 6 while as the minimum number of bands 5 were present in the accession B3 (Poosa Bold), B4 (RH-30) and accessionB5 (RGN-73). 6 bands were observed in B1 and B2 and 5 bands were calculated in B3, B4, and B5. (Fig. 1)The protein band with highest molecular weight *i.e.* 80 KDa was generated in all the five accessions while as band for lowest molecular weight *i.e.* 07 KDa was generated in B5. Polypeptide bands with molecular weights of 80, 55, 44, 26, 18, 10, 09 and 07 KDa were present in all. Jan *et al.*, (2016) estimated the total seed protein among different *B. rapa* ecotypes [15]. They were analyzed through SDS-PAGE and data were analyzed through UPGMA. The polymorphism in protein size was investigated and were divided into four main groups on the basis of molecular weight ranging from~ 10 kDa to ~ 180 kDa. Group A comprised of large size protein (~136 kDa to ~180 kDa), group D consist of small size proteins (~ 10 kDa to ~ 19 kDa) whereas group B and C consisted of medium sized proteins (~26 kDa to ~115 kDa).

Rabbani *et al.*, (2001) also observed less polymorphism in Indian mustard having different

geographical origin [14]. Mukhlesur and Hirata (2004) evaluated various *Brassica* spp and obtained 21.2% of polymorphism in *B. rapa*, followed by 18.8% polymorphism in *B. napus* and 3.2% polymorphism in *B.juncea* [19]. Mukhlesur *et al.*, (2004), recorded 18.8% polymorphism in 32 genetypes of *B. rapa* collected from Bangladesh, japan and China [20]. Keeping in view, our result of SDS-PAGE were in conformity with the results of El-Beltagi *et al.*, (2011) and Toosi *et al.*, (2011) [21-22]. Mukhlesur *et al.*, (2004), Ahmad, (2012) and Rabbani *et al.*, (2001) who found low level of genetic diversity in Brassica on protein level. The low level of protein polymorphism could be attributed due to the conservative nature of the seed proteins [14, 20, 23]. The information on the SDS-PAGE on different species of Brassica for genetic diversity is still limited [19].

Different workers who worked on different plant species and reported almost similar pattern of polymorphism for example Salimi and Abdola (2013) studied the genetic variation of seed protein by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for 19 genotypes of soybean (*Glycine max)* and reported 60% polymorphism [24].

The present study of SDS-PAGE in five accessions of *B. juncea* showed low magnitude of diversity which might be due to the conservative nature of seed proteins. The results clearly showed that it was quite difficult to discern closely related oilseed collection and cultivars used however, seed proteins were useful to distinguish different types of mustard from each other. Therefore. it is concluded that protein provided a sound basis on biosystematics relationships among different species and diverse forms of same species. It is necessary to conduct further analysis of protein diversity using a number of local strains from vegetable and condiments mustard of diverse origin to further elucidate the experiment on a wider level.

Genetic diversity through RAPD

Molecular studies were carried out to find diversity in five accession of *B. juncea* By RAPD-PCR. One random primer (OPA-11, 5'- CAA TCG CCG T- 3') were selected for the study based on preliminary screening of 50 random primers because of their reproducibility results of polymorphism between the individuals. These primers were used to find the genetic diversity within a collection of five accession of *B. juncea*. The genomic DNA have presented for five accession in Fig. 3.The polymorphism were detected by molecular markers, therefore reveals differences among genotypes which were not influenced by the environment. Availability of many sources of DNA probes and primers enables screening of a large population of genome, both coding and non-coding, thereby facilitating differentiation of even closely related genotypes. Random amplified polymorphic DNA (RAPD) has been shown to reveal a significant level of DNA polymorphism in different plant species. Out of these 1 band was found polymorphic with a polymorphism of about 08.33%. Pairwise comparison between the tested genotypes were used to calculate the genetic similarity. The similarity matrix computed with Jaccard's coefficient revealed the maximum similarity between accessions B3 (Poosa bold) with B4 (RH-30) and B1 (Maya) with B2 (Kranti) while distantly related varieties were B4 and B5 (RGN-73). Primer OPA-11 generated a total of twelve bands out of which one band was scored as polymorphic. (Fig. 4 & 5)

The data obtained from RAPD analysis was used for constructing dendrogram. The five accessions were grouped into two clusters. First comprises B5 and second cluster comprises B1, B2, B3 and B4 cultivars.The cluster analysis revealed that B5 and B4 distinctly related to each other (Fig. 4).

The results obtained were in agreement with previous report by Demir *et al.* (2010) who reported a total of 100 amplified bands by using 11 RAPD primers out of which 29 bands were polymorphic indicating 29% polymorphism in case of eggplant accessions, while Biswas *et al. (*2009) also demonstrated RAPD makes as a potential tool for eggplant accession using four decamer RAPD primers [25-26]. Nosrati *et al.,* (2012) found polymorphism in range of 20.6% in German type and 60.30% in Polish accessions of *Lathyrus sativus* L. using 5 RAPD primers [27].

CONCLUSION

Different workers who worked on different plant species and reported almost similar pattern of polymorphism e.g. such as Szilagyi *et al. (*2011) found 51.78% polymorphism in *Phaseolus vulgaris* by utilizing four random primers [28], while Biswas *et al.(*2009) also demonstrated RAPD markers as a potential tool for eggplant improvement and found 57.89% polymorphism among 10 eggplant accession using four decamer RAPD primers [26]. On the other hand 49.40% polymorphism was reported by Panwar *et al.* (2010) in 52 finger millet genotypes upon utilizing 18 random decamer primers. Tahir and Nawroz (2008) reported 40% and 35.7% polymorphism respectively in 11 durum and bread wheat genotypes by using 10 decamer primers [29]. Behera *et al.* (2012) also observed the Polymorphism of 41.34% in 52 bitter ground genotypes by using 17 random decamer primers [30].

The detection of high level genetic diversity observed in Brinjal genotypes confirms the findings of Welsh and McClelland (1990) who demonstrated that RAPD markers are effective for visualizing high level of polymorphism in plant species [31-34].

Fig. 1Protein profile of five accession of *B.juncea* produced by using SDS-PAGE. Distance similarity matrix generated using UPGMA Jaccards Method

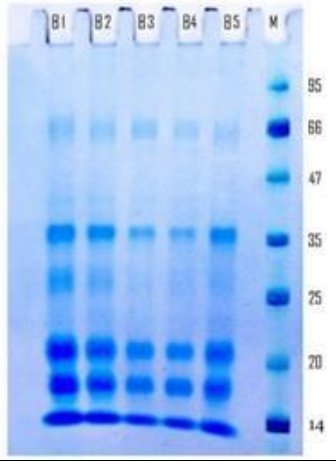


Fig. 2 UPGMA dendrogram showing genetic relationship among five accession of *B.juncea* based on SDS-PAGE.

	B1	B2	B3	B4	B5
B1	1.00000				
B2	0.96666	1.00000			
B3	0.83333	0.83333	1.00000		
B4	0.83333	0.83333	0.96666	1.00000	
B5	0.83333	0.83333	0.70000	0.70000	1.00000

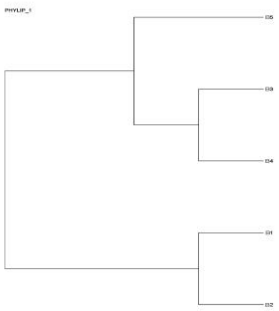


Fig. 3 Genomic DNA of five accession of *B. juncea*

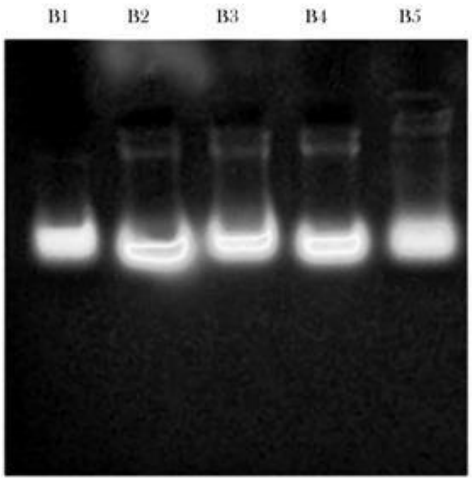


Fig. 4 RAPD profile of five accession of *B. juncea* produced by using OPA-11 (5'-CAATCGCCGT-3').

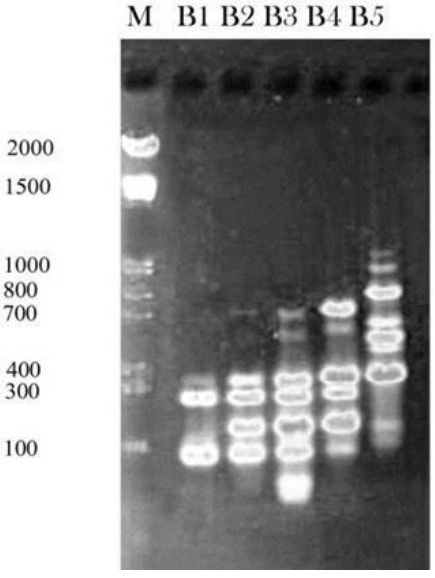
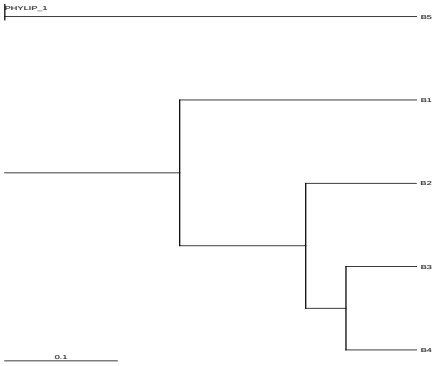


Fig. 5 UPGMA dendrogram depicting phylogenetic relationships among five accession of *B. juncea* based on RAPD.

	B1	B2	B3	B4	B5
B1	1.00000				
B2	0.66667	1.00000			
B3	0.50000	0.75000	1.00000		
B4	0.57143	0.85714	0.87500	1.00000	
B5	0.18182	0.25000	0.30769	0.33333	1.00000



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CONFLICT OF INTEREST

Nil

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