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Protein stability study with respect to mutation in *Brassica juncea, Brassica rapa* and *Brassica napus*

Simran Kotwal and SN Malode*

Government Vidarbha Institute of Science and Humanities, Amravati 444604, Maharashtra, India
**Corresponding author: satishmalode17@gmail.com*
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Abstract

In the present study *Brassica juncea, B. rapa, B. napus* and their accessions lines of M_5 generation were analyzed in M_6 generation for protein profiling through sodium dodecyle sulphate (SDS)–PAGE. SDS–PAGE with some modifications the resolution capacity of small molecular mass protein less than 20 kDa were separable with the used technique. The banding pattern of protein of rapeseed-mustard and their accessions have variable molecular, weight bands extend from 9 to 65 kDa. The total 50 bands obtained in which 43 bands were polymorphic, 7 bands were unique and 7 were monomorphic in rapeseed-mustard and their accessions. The *B. juncea* var. Bio-902, 3321, 666, GPT1 has unique protein bands. The value of similarity among different cultivars fluctuates between 0.26 to 1.0. The highest similarity value was observed 0.68 in *B. napus*. The lowest similarity value was recorded 0.26 in *B. juncea* accession (427-2). After studying the M_6 generation of rapeseed-mustard soluble seed protein revealed some differences among the species in terms of the number, position and intensity of bands, while accession discrimination based on SDS-PAGE was also done. Based on polymorphic and unique bands there was 86 % polymorphism among the rapeseed-mustard and their accessions.

Keywords: *Mutation, rapeseed-mustard, polymorphism, SDS-PAGE, Stability*

Introduction

Rapeseed is either diploid (*Brassica rapa)* or allotetraploid (*B. juncea* and *B. napus*) globally economical important oilseed crop. Globally, India holds second and third position in rapeseed-mustard area under cultivation and production, respectively and it contributes about 12.64 % of the share in global rapeseed-mustard production (Choudhary *et al*., 2023). The breeding of canola for protein in feed and food, in response to this interest, scientists have been tasked with altering and optimizing the protein production chain to ensure canola proteins are safe for consumption and economical to produce. Specifically, the role of plant breeders in developing suitable varieties with the necessary protein profiles is crucial to this interdisciplinary endeavor (So and Duncan, 2021). Rapeseed-mustard seed storage protein has a unique class of proteins which were especially exhibits in the developing stage of seeds. The predominant seed storage proteins in rapeseed-mustard are cruciferin and napin that have nutritive and functional properties (Schwenke, 1990). The seed storage protein allied detected in vegetative tissues of canola, role of these proteins in nitrogen storage (Wanasundara, 2011; Bieker *et al*., 2019). The SDS–PAGE procures advantages of molecular size, shape and charge. Electrophoresis of seed storage protein has proved economical and more reliable for the identification and substantiation of accessions. During the plant breeding and mutation breeding, crop improvement occurs but there is necessity to evaluate and characterize genetic diversity. For the understanding and distinguishing genetic diversity, it is crucial to develop genetic variability in different genotypes for the improvement in plant breeding. The genetic variation is randomly investigated by electrophoresis of seed storage proteins which significantly helps in classifying plant accessions. The vigour of mutant varies exceptionally over environment conditions revealed by mutational breeding experiments (Gustafsson, 1951). The mutations may lead to changes in protein which causes change in morphological and biochemical parameters. Protein profiling of mutants do act as one of the essential components, in proving its diversity. For this reason, attempts have been made to determine the stability of proteins to retain their native conformation in accessions as well as factors to explain protein stabilization with respect mutagenesis. It was important to perform biochemical analysis to know about the total protein content. The aim of this study was to analyze and compare the M_5 generation in M_6 generation for protein stability analysis, and to identify the genetic

relationship among the *Brassica* species and in their accessions by SDS-PAGE.

Materials and Methods

Plant material and growth conditions

The mature seeds of rapeseed-mustard and their 14 accessions were selected. The selection of accessions was merely based on superior characteristics like high oil, bold seed, early flowering, dwarf, shattering resistance, bi-lobed leaf, good plant type, appressed pod and high yield. These accessions were developed by the experiment of induced mutagenesis, at Government Vidarbha Institute of Science and Humanities, Amravati. The three *Brassica* species and their accessions were analyzed morphologically and biochemically for their stabilization through successive generations M_5 to M_6. To know the impact of different environmental conditions; germplasm was planted in different geographic conditions. In one year, two generations were taken from 2020 to 2021. The M_5 generation was taken in the departmental field, Amravati, Maharashtra. Crop was sown on 15 October 2020. The M_6 generation was sown on 18th April 2021 in Batote district of Jammu and Kashmir. The *Brassica* species and their accessions have been summarized in Table 1.

Table 1: List of *Brassica* species and their stabilized accessions

Taxonomy	Place of collection
B. juncea (control)	Punjabrao Deshmukh Krishi Vidhyalaya, Maharashtra
Mutant-666	GVISH, Maharashtra
Mutant-171	GVISH, Maharashtra
Mutant-bold Seeds	GVISH, Maharashtra
Mutant-427	GVISH, Maharashtra
Mutant-426	GVISH, Maharashtra
B. rapa (control)	GVISH, Maharashtra
Mutant-GPT_1	GVISH, Maharashtra
Mutant-bilobed leaf	GVISH, Maharashtra
Mutant-GPT_2	GVISH, Maharashtra
B. napus (control)	GVISH, Maharashtra
Mutant-R7 – 3	GVISH, Maharashtra
Mutant-R9 – 5	GVISH, Maharashtra
Mutant-R10 -5	GVISH, Maharashtra
Mutant-R6-2	GVISH, Maharashtra
Mutant-R8-4	GVISH, Maharashtra

Protein extraction

Seed was used for protein extraction. The 100 mg seeds were grounded with pre-chilled mortar and pestle to fine powder for defatting process in sohxlet apparatus by using petroleum ether as solvent. The defatted thimbles were usually dried at room temperature and used for protein extraction. SDS–PAGE was performed as described by Laemmli UK (1970). 2 ml of tris–sucrose buffer (1 M Tris chloride, 4 M sucrose, 10 mM KCl, 1mM $MgSO_4$), 1mM EDTA, 1mM PMSF (phenylmethylsulfonyl fluoride), 100 µl â-mercaptoethanol [Tris ph 7.4] was added for the homogenization. The crude homogenized material was then transferred to eppendrof microtubes and centrifuged at 12,000 rpm at 4^0C for 15 min. Thereafter 15 micro litre of the extract was analyzed by using resolving gel (10%) and stacking gel (5%) using puregene genetix standard low molecular weight markers in a Bio-rad (GX–SC22) vertical gel electrophoresis apparatus. The electrophoresis was carried out at 75 volts and 40 volts for staking and resolving gel. The loading dye bromophenol blue (0.1%) was used as loading dye.

Staining and de-staining

Coomassie brilliant blue was used for the purpose of staining (CBB–2 %, 45 % methanol, 10 % glacial acetic acid) after the electrophoresis run was over. For overnight the gels were placed in solution (15 % TCA) for fixing. The gels were de-stained with the solution (45 % methanol, 10 % glacial acetic acid). The bands get clearly visible due to the disappearance of excess coomassie brilliant blue. The gels were then photographed in Bio-rad (Gel Doc™ EZ), Rf value was used for the calculation of molecular weight protein bands. The Jaccard similarity coefficient then used to construct dendrogram tree by UPGMA using NTSYS-pc. The polymorphism percentage was calculated by (No of Polymorphic band/Total number of band) × 100.

Results and Discussion

In the present study different isolated, *B. juncea*, *B. rapa*, *B. napus* and their accessions, lines of M_5 generation were analyzed in M_6 generation for protein profiling through SDS–PAGE. Electrophoretic banding pattern of seed storage protein from M_5 generation were observed in M_6 generation were illustrated by SDS–PAGE (Fig. 1 & 2). The molecular weight protein bands obtained from protein gel electrophoresis, and polymorphic and monomorphic bands were summarized in Table 2. By UPGMA the dendrogram was derived and illustrated in Fig. 4. The Jaccard similarity coefficient matrix of *Brassica* species and their accessions was based on SDS-PAGE (UPGMA) (Table 3).

A total of 50 soluble protein bands, ranging from the molecular weight of 9 to 65 kDa were experimentally determined. The molecular weight was calculated on the basis of Rf value. Among 50 bands, 43 bands were polymorphic, 8 bands were unique and 7 were

monomorphic in *Brassica* species. The most important one is unique bands because of its presence in only certain genotypes that was completely absent in another genotypes. *B. juncea* cv. Bio-902 has four unique bands while its accessions 3321 and 666 have two and one unique bands respectively. Only one accession (GPT1) of *B. rapa* var. BCY-2 has one unique band; whereas none of the accessions of *B. napus* cv. Excel have unique band. On the basis of number of polymorphic bands there is 86 % polymorphism among the *Brassica* species and their accessions. However, by observing the Table 3 the highest similarity value was 0.68 in *B. napus* cv. Excel. Lowest similarity value was 0.26 in *B. juncea* accession (427). The value of similarity among different cultivars fluctuates between 0.26 to 1.0. Similarity matrix index was calculated on the basis of band homology. The studied protein analysis shows the relationship within the accessions and between the *Brassica* species. The differences explored which was founded with in the accessions by SDS-PAGE. However, it can be used to find out intra and inter-specific genetic diversity.

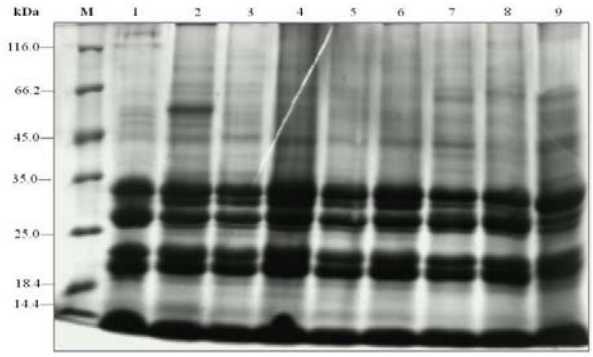

Fig. 1: Electrophoretic patterns of seed storage protein
Where; L1 = *B. juncea*, L2 = GPT2, L3 = *B. campestris*, L4= GPT1, L5 = Bilobed Leaf, L6 = APM, L7 = 666, L8 = 171, L9= *B. napus*, L = Lane, M = molecular weight marker

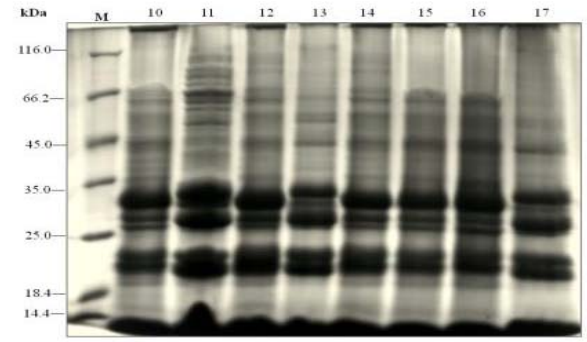

Fig. 2: Electrophoretic patterns of seed storage protein
Where; L10 = R7-3, L11 = 3321, L12 = R9-5, L13 = 427-6, L14= R10-5, L15 = R6-2, L16 = R8-4, L17 = 427-7

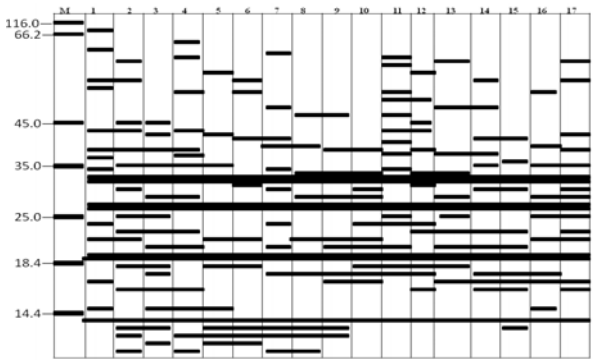

Fig. 3: Summarized spectrum of molecular weight proteins

By analyzing the dendrogram tree, the cluster analysis was carried out which keeps the genotypes into three clusters (Fig. 4). Cluster I was outrooted from main cluster II and represented by *B. juncea* cv. Bio-902 brown seeded. Cluster II was the biggest among three sub-clusters, represented by 14 genotypes. Sub-clusters IIa and IIb contained four genotypes for each. Sub-cluster IIa contained (a) GPT2, 427-7, (b) *B. rapa* and R9-5. Sub-cluster IIb contained (a) R7-3, 427-6 (b) R10-5 and R6-2. Sub-cluster IIc represented by only *B. campestris* accession (GPT1). Sub-cluster IId contained five accessions (bilobed leaf, APM, 666, 171, R8-4 and *B. napus* cv. Excel. Cluster III comprised only 3321 accession of *B. juncea* was genetically different from the genotypes, which came under main cluster II.

The technique SDS-PAGE was performed to quantify seed storage protein which has been proven to be very useful. The experiments for assessing protein samples before that purity and integrity are the very first priority. SDS-PAGE was successfully used for the characterisation of storage proteins derived from different genotypes (Ehsanpour *et al*., 2010). After summarizing the obtained

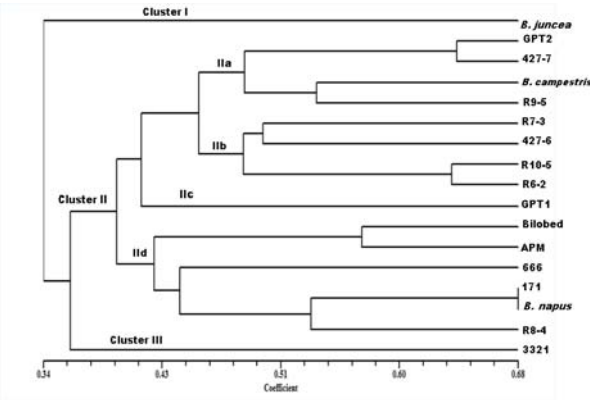

Fig. 4: Dendrogram showing inter and intra-specific protein based variation among *Brassica* species and their accessions

Table 2: The molecular weight bands obtained by SDS gel electrophoresis in investigated samples of *Brassica* species

No. of bands	M.W.	Taxa																	
		B. juncea	GPT2	*B. campestris*	GPT1	Bilobed	APM	666	171	*B. napus*	R7-3	3321	R9-5	427-6	R10-5	R6-2	R8-4	427-7	P,M,U
1	65.4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	U
2	62.8	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	U
3	59.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	U
4	56.8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	U
5	55.6	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	U
6	54.0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	P
7	53.4	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	U
8	52.4	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	P
9	51.3	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	P
10	50.3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	U
11	49.3	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	P
12	48.3	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	P
13	47.4	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	P
14	46.4	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	P
15	45.6	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	P
16	43.7	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	P
17	42.8	0	0	1	0	1	0	0	0	0	1	1	1	1	1	0	0	1	P
18	41.1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	P
19	40.3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	U
20	39.5	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	P
21	38.7	1	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	1	P
22	37.2	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	P
23	36.5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	P
24	35.7	0	1	1	1	1	0	0	0	0	0	0	0	0	1	0	1	1	P
25	34.3	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	P
26	33.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	M
27	32.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	M
28	31.0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1	0	P
29	30.4	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	P
30	29.2	0	1	0	0	0	0	1	0	0	1	0	0	0	1	1	0	1	P
31	28.6	0	0	1	1	0	0	0	1	1	1	0	0	1	0	0	1	1	P
32	27.5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	M
33	26.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	M
34	25.4	0	1	1	0	0	0	0	0	1	0	1	0	1	0	0	1	1	P
35	24.4	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	P
36	23.9	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	1	P
37	22.5	1	1	0	0	1	1	0	1	1	1	0	0	0	0	0	1	1	P
38	21.2	0	0	1	1	0	0	1	0	1	1	1	0	1	1	1	0	0	P
39	20.7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	M
40	19.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	M
41	18.4	0	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	P
42	17.7	0	0	1	0	0	0	1	1	1	1	1	1	0	1	1	1	0	P
43	16.0	1	0	0	0	0	0	0	0	1	1	0	0	1	1	1	1	1	P
44	15.3	0	1	1	1	0	0	0	0	0	0	0	1	0	1	1	0	1	P
45	14.0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	P
46	13.6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	M
47	12.5	0	1	1	0	1	1	1	1	1	0	0	0	0	0	1	0	0	P
48	11.3	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	P
49	10.7	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	P
50	9.5	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	P

Where; P = Polymorphic, M = Monomorphic, U= Unique, M.W. = Molecular weight

Table 3: The Jaccard similarity coefficient matrix of *Brassica* species by SDS–PAGE (UPGMA)

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
B. juncea		1															
GPT1	0.36	1															
B. rapa	0.29	0.53	1														
GPT2	0.32	0.46	0.48	1													
Bi-lobed	0.33	0.44	0.52	0.39	1												
APM1	0.34	0.46	0.37	0.31	0.57	1											
666	0.31	0.36	0.33	0.32	0.33	0.40	1										
171	0.30	0.42	0.38	0.37	0.45	0.47	0.54	1									
B. napus	0.38	0.44	0.52	0.39	0.41	0.43	0.44	0.68	1								
R7-3	0.40	0.36	0.48	0.36	0.44	0.34	0.40	0.41	0.56	1							
3321	0.31	0.28	0.37	0.40	0.33	0.30	0.35	0.31	0.37	0.44	1						
R9-5	0.35	0.46	0.53	0.36	0.38	0.34	0.31	0.30	0.33	0.46	0.44	1					
427-6	0.28	0.39	0.52	0.39	0.36	0.32	0.34	0.33	0.47	0.50	0.40	0.38	1				
R10 –5	0.31	0.41	0.48	0.41	0.33	0.34	0.46	0.30	0.38	0.46	0.35	0.40	0.56	1			
R6-2	0.33	0.39	0.46	0.39	0.36	0.37	0.50	0.39	0.47	0.50	0.33	0.38	0.41	0.63	1		
R8-4	0.38	0.34	0.46	0.44	0.47	0.37	0.33	0.52	0.54	0.50	0.37	0.28	0.41	0.38	0.41	1	
427-7	0.40	0.64	0.53	0.41	0.38	0.34	0.26	0.36	0.50	0.52	0.27	0.40	0.56	0.58	0.44	0.50	1

results of *Brassica* genotype, it was clearly observed that all the studied *Brassica* species show huge genetic variability. The difference that was visible in banding pattern may be because the *Brassica* species and their accessions were stabilized due to induced mutagenesis (Fig. 2 & 3). The polymorphic and unique bands were obtained during SDS-PAGE analysis, helps in distinguishing *Brassica* species and their accessions among each other. Which shows there presence in certain genotypes and completely absent in other, can be used for identification in taxonomy (Raboin *et al*., 2005). The unique bands can prove a landmark, if it will be targeted, marks the nonexistence of significant protein bands definitely benefits in taxonomy and plant breeding study (Helaly *et al*., 2017). The appearance of certain bands 11 and 13 kDa molecular weight bands was polymorphic in rapeseed and in their accessions. The protein band of molecular weight 14 kDa was polymorphic renowned as napin not present in all accessions as shown in (Table 2) contrasted with the results of (Venkatesh and Rao, 1988). The protein band 27 kDa was represented as monomorphic band for all studied *Brassica* species and their accessions it is dimer of napin contrasted with the findings of (Krzyzaniak *et al*., 1998). The additional band 9.5 kDa was present as polymorphic bands after the dissociation of dimer napin. The cruciferin protein bands 20 kDa molecular weight protein is monomorphic and from 21 to 24 kDa these are polymorphic molecular weight proteins as shown in (Table 2). It is possible to discriminate between two varieties up to 96-99%, if the varieties have 80-90 % of unique banding pattern (Smulders *et al*., 1996).

Conclusion

This study was carried out to follow the genetic diversity and identify genetically the relationship among all rapeseed genotypes by using SDS–PAGE. Hence, it is useful for the discrimination among *Brassica* genotypes based on the banding pattern and similarity index. After studying the M_6 generation of rapeseed-mustard and their accession, it was concluded that with huge polymorphism and diverse genetic diversity existed among them.

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