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Optimization of an efficient SDS-PAGE protocol for rapid protein analysis of *Brassica rapa*

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Abstract

SDS-PAGE plays a key role in the study of protein based variation among different brassica species. The present study aimed to develop an efficient Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) protocol for *B. rapa*. Ten diverse genotypes were used to study their electrophoretic protein profiling. A thoroughly precise protocol was developed for optimizing the conditions such as proper pH level, centrifugation time, sample size, ammonium per sulphate (APS) concentrations, staining and de-staining time period etc. By optimizing these factors maximum polymorphic proteins were recorded that sizes range from about 10-180 k Da. All the genotypes were classified into four major groups on the basis of similarity that exist. The similarity coefficient value ranges from 40 to 95.2%. The least (40%) and maximum (95.2%) similarity coefficient values were noted among Br-508/Br-728 and Br-695/Br-725 respectively. A robust and quick SDS-PAGE protocol was developed; it will be used to study genetic diversity of other crop species and to widen the agriculture breeding program.

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Introduction

Brassica species are commonly cultivated worldwide as oil seed crops. Genetic improvement of crops can be enhanced when broad genetic diversity and the information of these genetic resources are available. Research on SDS-PAGE analysis of important *Brassica* species is useful to investigate genetic variation and to classify plant varieties (Isemura *et al.,* 2001). However, a single SDS-PAGE protocol for complete protein profiling of different *Brassica* species is still a big problem (Rahman and Hirata, 2004).

SDS-PAGE method is used to study protein based variation among different organisms. It is used to detect various types of protein sub-units of different organisms (Zahhor *et al.,* 2015; Jiang *et al.,* 2016).

The critical protein based characterization of *Brassica* species is important to screen diverse genotypes (Semagn *et al.,* 2006). SDS-PAGE method give efficient and quick protein profiling of different crop species and it is safe from any environmental effect (Dhawale *et al.,* 2015; Das and Mukherjee, 1995).

Seed protein based variation is important by many reasons, as it give accurate genetic diversity among genotypes, help in plant domestication, for phylogenitic relationship, and also used as tool for crop improvement (Wadood *et al.,* 2016). Shinwari *et al.* (2013) identified maximum protein subunits at mass ranges from 15-220 KDa of important *Eruca sativa* L cultivars through SDS-PAGE method.

Zada *et al.* (2013) evaluated 94 different *Brassica carinata* L. (Ethiopian mustard) genotypes through this method. Both monomorphic and polymorphic proteins were noted in different experimental genotypes. Akbar *et al.* (2012) reported protein based polymorphism in different sesame (*Sesame indicum* L.) genotypes. Wadood *et al.* (2016) characterized 60 different genotypes of *Lens culinaris* from Malakand division, Khyber Pakhtunkhwa, Pakistan through this method.

Protein based polymorphism varies with type of specie (Dhawale *et al.,* 2015; Dudwadkar *et al.,* 2015). SDS-PAGE system gives accurate protein profile of all genotypes from all species. Therefore the present study was designed to develop an accurate protein profiling protocol for *B. rapa* genotypes.

Materials and methods

Experimental Materials

The present experiment was performed at Plant Genetic Resources Institute (PGRI), National Agricultural Research Centre (NARC), Islamabad, Pakistan. The mature seeds of 10 *Brassica rapa* genotypes were acquired from the gene bank of PGRI, NARC, Islamabad, Pakistan (Table 1).

Procedure of Protein Extraction

Fresh 10-15 seeds were finely ground with mortar and pestal. Crushed materials (0.02g) were transferred to each 1.5 ml eppendorf tube with addition of 400 µl protein extraction buffers (0.5M Tris-HCl (pH 8.0), 0.2% Sodium dodecyle sulphate (SDS), 5M urea, 1% 2-mercaptoethanol, and bromophenol blue dye). The samples were properly mixed by vertexes for 1-2 minutes and stored overnight in refrigerator at -20 ^{o}C.

Electrophoresis

Preparation of separation and stacking gels

The separation and staking gels were prepared by mixing chemicals in different concentrations (Table 2-7). The samples were then centrifuged at 12000 rpm for 10 minutes. 10 µL upper layer of each sample was loaded to each well along with protein marker at 100 V.

The moment of proteins were noted regularly until reach at the bottom of plates. The gels were then transferred into staining solution (Table 8) and kept for 2-3 hours on shaker.

The gels were then washed two times with distilled water and then transferred into fresh de-staining solution (Table 9) and kept on shaker for 24 hours.

The autoclave tissue paper was also kept on gel to remove excess of blue color. The bands patterns of all genotypes were noted. The clear bands were marked with score 1 and absence of bands with 0. Dedrogram was constructed by using UPGMA (Unweighted pair-group method with arithmetic averages) method (Sneath and Sokal, 1973). The NTSYS-pc, version 2.1 (Applied Biostatistics Inc., USA) software was used to analyse the data.

Results

Factors Affecting SDS-PAGE System

In present study an efficient and quick SDS-PAGE protocol was established for important oil seed *B. rapa* species. The low concentration of APS causes no gel formation.

A very high concentration of APS make the gel very hard, thus retard the movement of proteins.

The 100 and 230 ul APS in stacking and separation gels increased the frequency of separation of both small and large size protein sub-units.

Table 1. List of Accessions and ecotypes of *B. rapa*.

Sr. No.	Accession	Source
1	Br-508	NARC, Islamabad, Pakistan
2	Br-554	NARC, Islamabad, Pakistan
3	Br-555	NARC, Islamabad, Pakistan
4	Br-568	NARC, Islamabad, Pakistan
5	Br-647	NARC, Islamabad, Pakistan
6	Br-695	NARC, Islamabad, Pakistan
7	Br-696	NARC, Islamabad, Pakistan
8	Br-705	NARC, Islamabad, Pakistan
9	Br-725	NARC, Islamabad, Pakistan
10	Br-728	NARC, Islamabad, Pakistan

Table 2. Composition of solution A.

Ingredient	Amount
Distilled water	100ml
Tris (hydroxymethyl) aminomethane	34g
SDS (Sodium dodecyl sulphate)	0.8g
pH	8.0

Stored in refrigerator.

The optimum pH of all types of solution is important for movement and separation of proteins.

The low or very high pH of different solutions and protein extraction buffer significantly effect on the movement and visibility of different sizes of protein.

The 2-3 hours of staining with shaker followed by 1-2 days of destaining gives clear bands of all sizes of protein.

The 0.02 gm seed sample and addition of 400 ul protein extraction buffer in these samples gave maximum polymorphic protein bands.

The soaking of gel with sterilised tissue paper also improved the visibility of clear proteins bands.

Cluster Analysis and Genetic Similarity Matrix

A total of 13 protein bands were recorded in which 10% are monomorphic while the rest of 90% are polymorphic.

Table 3. Composition of solution B.

Ingredient	Amount
Distilled water	100ml
Tris (hydroxymethyl) aminomethane	7g
SDS (Sodium dodycyl sulphate)	0.7g
pH	7.0

Stored in refrigerator

Table 4. Composition of solution C.

Ingredient	Amount
Acrylamide	31g
Bis (bis-acrylamide)	1g
Distilled water	100 ml

Stored in refrigerator.

All three types (small medium and large) protein sub-units were noted that size ranges from 10 to 180 kDa (Fig. 1). A genetic tree was constructed that classified all tested genotypes into four groups. The group I consisted three genotypes (Br-508, Br-568 and Br-728), followed by group II (Br-555, Br-696 and Br-705), group III (Br-647 and Br-725) and group IV (Br-554 and Br-695) (Table 10). The Br-508 and Br-568 are very close to each other in genetic tree (Fig. 2).

Table 5. Composition of APS.

Ingredient	Amount
Ammonium per sulphate (APS)	0.2g
Distilled water	1ml

Table 6. Composition of separation gel.

Ingredient	Amount
Distilled water	7.5 ml
Solution A	5 ml
Solution C	7.5ml
10% APS	230 µl
TEMED	60µl

The percent similarity coefficient values were also calculated for all genotypes. The maximum similarity coefficient value 9.52 (95.2%) was recorded among Br-508 and Br-728 followed by 9.47 (94.7%) in Br-568 and Br-728.

The lease similarity coefficient value 4 (40%) was noted for genotypes Br-695 and Br-725. The last two genotypes are very diverse from the rest of genotypes (Table 11). The other genotypes showed low to moderate level of diversity.

Discussion

An efficient SDS-PAGE protocol is important to screen diverse genotypes of *B. rapa* at protein levels. In present study an improved SDS-PAGE protocol was established for important *B. rapa* species by optimization of various factors.

A quick and efficient SDS-PAGE system is used to screen diverse genotypes of any crop species (Isemura *et al.,* 2001; Gepts and Bliss, 1988; Iqbal *et al.,* 2005; Javid *et al.,* 2004; Rahman and Hirata, 2004; Khan *et al.,* 2014).

Table 7. Composition of stacking gel.

Ingredient	Amount
Distilled water	6.0 ml
Solution B	3 ml
Solution C	2 ml
10% APS	100 µl
TEMED	50 µl

Table 8. Composition of Staining Solution.

Ingredient	Amount
Distilled water	470 ml
Acetic acid	70 ml
Methanol	460 ml
Coomassie brilliant blue (CBB) R250	2.10 g

Stored at room temperature.

The morphological, biochemical and molecular based variation play a key role for identification of improves genotypes of different crop species for further breeding program (Nawaz *et al.,* 2015; Arif *et al.,* 2015).

Various factors that affect this process were optimized. The addition of 230 and 100 ul APS in separation and stacking gels give best results. The high or very low concentrations of these two ingredients affect gel formation.

Table 9. Composition of Destaining Solution.

Ingredient	Amount
Distilled water	700 ml
Methanol	250 ml
Acetic acid	50 ml

Stored at room temperature.

Table 10. Grouping of *Brassica rapa* genotypes through cluster analysis.

Clusters	No. of genotypes	Genotypes
I	3	Br-508, Br-568 and Br-728
II	3	Br-555, Br-696 and Br-705
III	2	Br-647 and Br-725
IV	2	Br-554 and Br-695

The 0.02g sample size, addition of 400 ul protein extraction buffer in sample, centrifuge at 12000 rpm for 10 mins were found optimum for this method. Other factors such as proper staining and destaining with shaker increase the visibility of clear protein bands. Similar type of protocol was optimized by Jiang *et al.* (2016) for separation of proteins by using mini gel electrophoresis system.

The maximum protein bands with sizes range from 1-30 kDa were recorded via this method. They also reported that 10% glycrol and 4.2 M urea in gel increase the resolution for separation of small proteins. Hossain *et al.* (2014) developed an efficient SDS-PAGE protocol for important crop species *Brassica oleracea*. A total of 13 expressed proteins were reported by optimizing various factors.

Table 11. Comparison of similarity coefficient of different *B. rapa* germplasm.

	Br-508	Br-554	Br-568	Br-555	Br-647	Br-695	Br-696	Br-705	Br-725	Br-728
Br-508	1.00									
Br-554	8.42	1.00								
Br-568	9.00	8.24	1.00							
Br-555	9.00	7.06	7.78	1.00						
Br-647	8.42	6.25	8.24	7.06	1.00					
Br-695	7.37	8.75	7.06	5.88	6.25	1.00				
Br-696	9.17	7.62	8.18	8.18	7.62	7.62	1.00			
Br-705	9.00	7.06	8.89	7.78	8.24	5.88	8.18	1.00		
Br-725	7.78	5.33	7.50	7.50	8.00	4.00	7.00	7.50	1.00	
Br-728	9.52	8.89	9.47	8.42	7.78	7.78	8.70	8.42	7.06	1.00

The proteins extracted from seeds were subjected SDS-PAGE analysis and maximum of 13 proteins bands were recorded (Fig 1). Both monomorphic and polymorphic proteins were noted. All these proteins were sorted into 4 different diverse groups on the basis of their close relationship with each other. The similar protocol was also used by Zada *et al.* (2013) for *Brassica carinata* genotypes and a total of 31 loci were recorded. Shinwari *et al.* (2013) reported 17 diverse polymorphic and 1 monomorphic protein sub-units in *Eruca sativa*. Turi *et al.* (2010) recorded four new types of protein for important brassica species. The protein based polymorphism varies with type of protocol and genotypes used (Rabbani *et al.,* 2001).

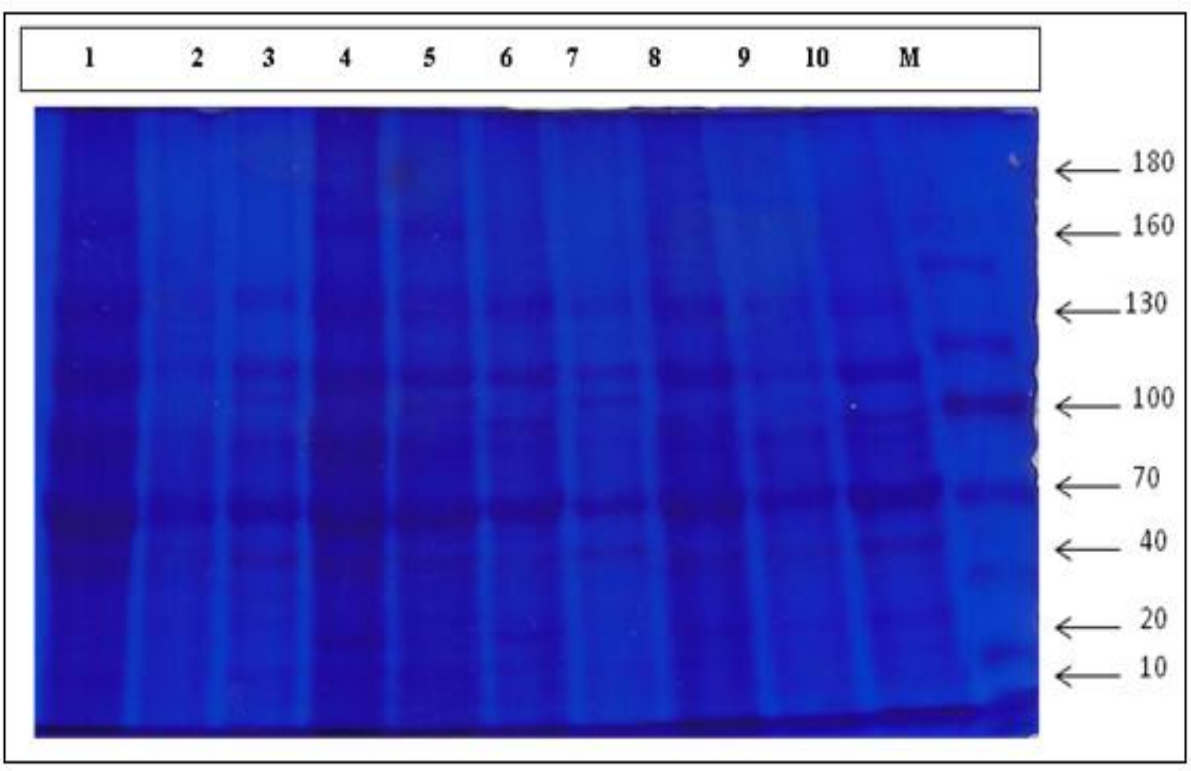

Fig. 1. Gel documentation of *B. rapa* genotypes generated through SDS-PAGE of total seed storage proteins. M represents molecular size marker, while numbers from 1-10 represent accessions Br-508, Br-554, Br-568, Br-725, Br-555, Br-728, Br-696, Br-705, Br-695 and Br-647, respectively.

The genetic similarity coefficient values were calculated for all tested genotypes that range from 40 to 95.2% (Table 11). Our findings show maximum dissimilarity among Br-695 and Br-725 genotypes. These two genotypes are highly diverse from rest of the genotypes.

Our results are not in line with the findings of Turi *et al.* (2010) that observed 98% similarity coefficient value among different Brassica genotypes. Our results are contradictory to Shinwari *et al.* (2013) who recorded 60% to 100% similarity values for different *Eruca sativa* genotypes.

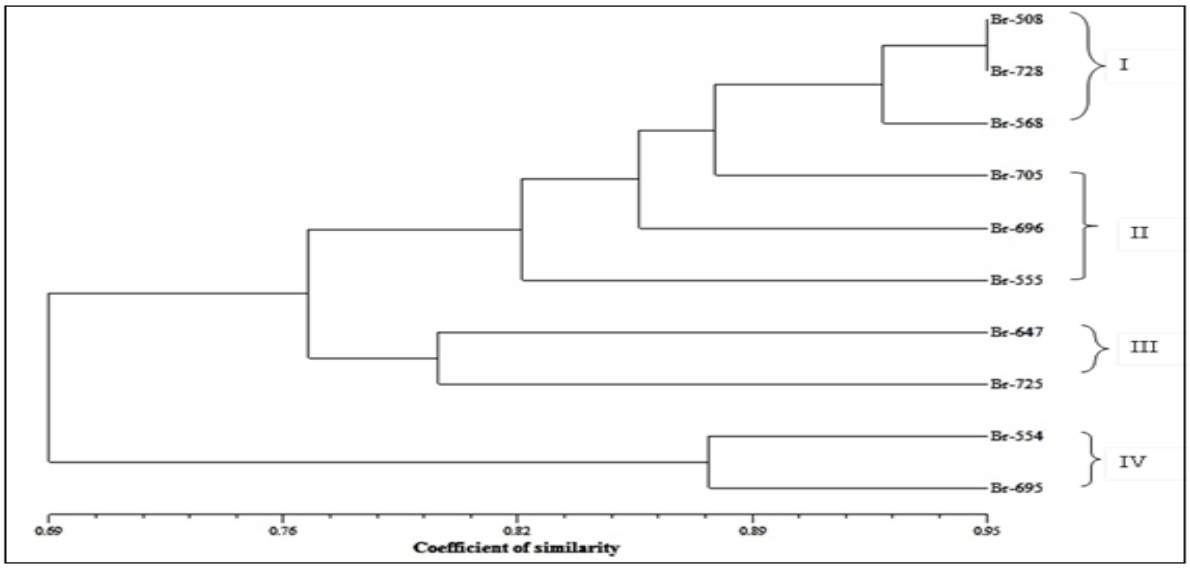

Fig. 2. Dendrogram showing the intra-specific protein based variation among different *B. rapa* genotypes.

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