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Characterization of seed storage proteins of different varieties of *Brassica napus* seeds

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Abstract

Brassica napus (Canola), an important cash crop in Pakistan, has rich oil (40%) and protein (15%) content. The present study was designed to investigate nine different varieties of *B. napus* (Tarch, Altex, Dasi, Dunkled, V-248, Bulb 98, Rainbow, Asker and Habib-98), for various physiochemical parameters (moisture content, thousand seed weight [TSW] and electrical conductivity [EC]), and also to investigate the genetic relationships among these genotypes using Sodium dodecyl sulphate-polyacralamide gel electrophoresis (SDS-PAGE). The maximum amount of moisture content was 6.31% for variety Dasi and minimum of 4.26% for variety Dunkled. The maximum TSW value was found for V-248 (4.22 g) and the minimum value for Dunkled variety (2.11 g). Electrical conductivity for different varieties was estimated at time intervals of 10 min, 20, 30, 40, 50, 60 and 70 minutes. The EC was determined by conductometer and the mean value ranged from 3.0-6.5 µ sec. Seeds protein analyzed by SDS-PAGE showed a significant genetic variation among the nine different varieties of *B. napus*, and they were clustered into two distinct clades with significant genetic distances. Our results demonstrate that seed storage protein profiles could be useful marker in the studies of genetic diversity and classification of adopted cultivars, thereby improving the efficiency of *Brassica* breeding programs in the cultivar development.

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Introduction

Brassica napus L. (known as Canola) consists of 41 species, which are placed in genus *Brassica* (Gladis and Hammer, 1990) and produces seeds with approximately 40% oil and 15% protein. *B. napus* contains 35% crude protein basis, having amino acids like Alanine, Histidine, Arginine, Leucine, Cystein, Lysine, Glutamate, Methionine, Glycine, Metionine, cystein, Phenylalanine, Tryptophane, Proline, Tyrosine, Serine, Valine, and Threonine (Bell *et al.*, 2000).

Research done by Morinaga (1934) has revealed the triangular genetic relationships among the six economically most important *Brassica* species (Figure 1). *B. napus* (2*n*=38, AACC), *B. juncea* (2*n*=36, AABB), and *B. carinata* (2*n*=34, BBCC) are amphidiploids species resulting from combining chromosome sets of the low chromosome number species *B. nigra* (2*n*=16, BB), *B. oleracea* (2*n*=18, CC), and *B. rapa* (2*n*=20, AA). The diploid species are thought to have originated from a common ancestor with the basic chromosomes number x= 6 (Prakash and Hinata, 1980), and the allotetraploid species to have arisen from spontaneous hybridization between the diploid species (UN, 1935).

For an efficient breeding program, information concerning the extent and nature of genetic diversity within a crop species is useful for characterizing individual accessions and cultivars in the selection of parents for hybridization (Rabbani *et al.*, 1998). There are various methods for studying the genetic variability of crop germplasm, including morphological traits, total seed proteins, isozymes and various types of molecular markers (Rabbani *et al.*, 1998). Molecular markers are the best tools for determining genetic relationships. Different types of molecular markers have been used for biodiversity analysis. The electrophoresis of seed storage proteins is a method to investigate genetic variation and to classify plant varieties (Tankesley and Jones, 1986). However, the information on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of different varieties of *B. napus* for genetic diversity is still limited (Mukhlesur *et al.*, 2004; Rabbani *et al.*, 2001)

Polyacrylamide gel electrophoresis in the presence of SDS has become one of the most widely used techniques to separate and characterize proteins. SDS-PAGE offers two distinct advantages. Polypeptides migrate according to their molecular weight on the gel, so their molecular weight may be easily and rapidly estimated. At the same time many insoluble proteins are solubilized by SDS, so SDS-PAGE has become the method of choice for resolving mixtures of insoluble proteins, especially membrane proteins (Zia *et al.*, 2008).

Various studies have been done on characterization of *B. napus* germplasm to assess the genetic and biochemical diversity of different varieties in Pakistan (Ali *et al.,* 2011; Ali *et al.*, 2007). However, these studies were about the genetic diversity through molecular markers like rapid amplification of polymorphic DNA (RAPD) and simple sequence repeats (SSR) (Ali *et al.,* 2011). Assessing the genetic diversity of different varieties is very important as it gives information regarding the genetic background and the total gene pool available. This information could further be utilized for future breeding or other crop improvement strategies.

The current study was designed to characterize and compare the *B. napus* seeds of different varieties for various physiochemical parameters and to evaluate their genetic diversity.

Materials and methods

Plant materials

The seeds of nine different varieties of *B. napus* were obtained from Agriculture Research Station, Swat, Khyber Pakhtunkhwa (KP) and National Agriculture Research Centre (NARC) Islamabad, and were analyzed for various physiochemical parameters like seed moisture content, electrical conductivity (EC) and thousand seeds weight (TSW). The seeds storage proteins were extracted and used for molecular characterization using SDS-PAGE.

Analysis of physiochemical parameters (moisture content, thousand seed weight and electrical conductivity).

Moisture content was determined by International Seed Testing Association method. Electric conductivity was determined by imbibition method. In this method 10 seeds for each variety were placed in a conical flask and with the help of calibrated conductive meter, electrical conductivity was determined at time intervals of 10 min, 20, 30, 40, 50, 60, 70 and 80 minutes. For TSW, thousand seeds were counted and their mass was measured with electrical balance.

Protein analysis using SDS-PAGE

Brassica seeds proteins were analyzed by SDS-PAGE as described by Shuaib *et al.*, 2010. Proteins were extracted using protein extraction buffer. For the preparation of protein extraction buffer (100 ml), Tris (0.6057 g), SDS (0.2 g) and Urea (30 g) were dissolved in distilled water and adjusted the pH at value 8. Mercaptoethanol (1 ml) was added and diluted to 100 ml. An electrode buffer (500 ml) was prepared by dissolving 15.15 g Tris, 0.5 g SDS and 7.2 g glycine in distilled water. Staining solution was prepared by mixing methanol 440 ml, acetic acid (glacial) 60 ml and coomassie brilliant blue (CBB R-250) 2.25 g in distilled water. Destaining solution was prepared by mixing ethanol, acetic acid and distilled water at the ratio of 20: 5: 75, respectively. For extraction of proteins, grains were ground to a fine powder and added the protein extraction buffer (400 µL/0.01 g of seed flour of *B. napus)* and vertex thoroughly to homogenize, kept overnight at 40°C. The samples were centrifuged at 13000 rpm for 10 min and the supernatant was transferred to a new 1.5 ml eppendorf tubes. The proteins were run on the polyacrylamide gel. After electrophoresis the gel was transferred to a tray containing staining solution and was shaken gently for 40 min, followed by destaining until the background color of the gel disappeared.

Data Analysis

All the visible monomorphic and polymorphic bands were scored and only unambiguously scored bands were used in the analysis. Each band was given a score of 1 for presence and 0 for absence. Genetic Distance Estimates (GDEs) based on seed protein profile were calculated. The Unweighed Pair Group Method of Arithmetic averages (UPGMA) (Nei and Lie, 1979) was used to construct a dendrogram using computer programme "Popgene32" version 1.31 (http://www.ualberta.ca./~fyeh/fyeh). Data were analyzed using standard formulas as described below and presented graphically using Microsoft word files version 2003.

$GDxy = 1 - Nxy / Nx + Ny - Nxy$

Where GDxy = Genetic distance between two genotypes

Nxy = Total numbers of common bands in the two genotypes.

Results

Physiochemical analysis of seed storage protein

The moisture content and TSW values for the nine different varieties of *B. napus* seeds are shown in Table 1. The percentage of moisture content for these samples are; Tarch has 4.72 %, Altex 5.08, Dasi 6.31, Dunkled 4.26, V-248 4.97, Bulb-98 4.32, Rainbow 5.10, Asker 5.60 and Habib 6.20 %. TSW values for these varieties are; Tarch has 2.75g, Altex 3.54g, Dasi 2.69g, Dunkled 2.11g, V-248 4.22g, Bulb-98 3.24g, Rainbow 2.31g, Asker 2.85g and Habib 3.51g. The mean of electrical conductivity values for *B. napus* seeds determined at different time intervals are shown in Table 2. Variety Habib-98 showed a maximum EC value at each interval of time.

Molecular analysis of seed storage protein

Molecular characterization of seed protein was done on SDS-PAGE using a protein ladder in a range of 10 kDa to 140 kDa. Molecular weight and the corresponding band numbers of each variety are shown in Figure 2 and Table 4. Protein banding pattern of the samples showed a high range of variation. The proteins were resolved into clear distinguishable bands. The protein bands were represented by "0" for the absence of the band and by "1" for the presence of the band (Table 3). The digits 1,

2, 3, 4, 5, 6, 7, 8 and 9 in Figure 2 represent the *B. napus* varieties Tarch, Altex, Dasi, Dunkled, V-248, Bulb-98, Rainbow, Asker and Habib-98, respectively. The results of cluster analysis are shown in dendrogram (Figure 3). Cluster analysis sorted the *B. napus* varieties into two major groups (lineages) A and B. The group A consists of varieties Tarch, Dunkled, Rainbow and Dasi while group B consists of varieties Altex, V-248, Asker, Bulb-98 and Habib-98. The maximum genetic distance of 0.95 was found among varieties Altex, V-248 and Asker. The minimum genetic distance of 0.63 was found among variety Tarch and Altex, and Tarch and V-248 (Table 5).

Table 1. Thousand seed weight and percent moisture content of nine different varieties of *Brassica napus*.

S.NO	VARIETIES	THOUSAND SEED WEIGHT (TSW) IN GRAMS	% MOISTURE CONTENT
1	Tarch	2.75	4.72
2	Altex	3.54	5.08
3	Dasi	2.69	6.31
4	Dunkled	2.11	4.26
5	V-248	4.22	4.97
6	Bulb-98	3.24	4.32
7	Rainbow	2.31	5.10
8	Asker	2.85	5.60
9	Habib-98	3.51	6.20

Table 2. Electrical conductivity of nine different varieties of *Brassica napus*.

S. No	VARIETIES	10 MIN	20 MIN	30 MIN	40 MIN	50 MIN	60 MIN	70 MIN	MEAN
1	Tarch	2.8	3.1	3.3	3.4	4.2	4.7	4.8	3.7
2	Altex	3.1	3.4	3.8	4.3	4.7	7.1	5.3	4.5
3	Dasi	2.7	3.2	3.5	4	4.4	4.8	4.9	3.9
4	Dunkled	3.0	3.4	4	4.3	4.7	5.2	5.2	4.2
5	V-248	3.9	4	4.3	4.8	5	5.4	5.4	4.7
6	Bulb-98	3.3	3.7	3.7	4.2	4.6	5.9	6.2	4.6
7	Rainbow	4.4	3.8	4.3	5	5.3	5.6	5.7	4.8
8	Asker	1.8	2.2	2.6	3.1	3.6	3.8	4	3.0
9	Habib-98	4.9	5.8	6.4	6.8	7.1	7.3	7.3	6.5

Discussion

Genetic diversity exists among the different varieties of a crop. Identification of a particular trait requires searching for a specific marker. There are various methods for identifying the genetic variability of a crop germplasm, including morphological traits, and various types of molecular markers. To search for these variations within a crop species are useful for an efficient breeding program (Rabbani *et al.*, 1998). The current study was started with the same objectives in mind to characterize and evaluate the genetic diversity of *B. napus* seeds of different varieties.

Moisture content of different varieties of B. napus

The moisture content of different varieties of *B. napus* studied here ranges from 4.26 to 6.31%. Moisture content can vary in the seed from <5% to >15% (Mailer, *et al.*, 1998). The moisture content of a sample is the loss in weight when it is dried. It is expressed as a percentage of the weight of the original sample and is calculated based on the fresh weight of the seeds. Moisture influences seed storage potential, shipment, viability, and vigor levels over time. It can help in determining the appropriate harvest time.

Electrical conductivity of different varieties of B. napus

The mean EC values were in the range of 3.0 µ sec (Asker) to 6.5 µ sec (Habib-98). Electrical conductivity reported by Stephen *et al.*, (2001) was 2.2 µ sec for nutrient seeds of *B. napus* which shows resemblance to the present study. In contrast to

nutrient seeds when saline water was used the value recorded was 11.2-24.9 µ sec. This variation could be due to increase in Ion concentration. The EC of some other varieties of *Brassica* seeds studied by Zia *et al.*, (2008) ranged from 5-8 µ sec, validate the present study.

Table 3. Bivariate 0/1 data scoring matrix based on the results of SDS-PAGE.

Brassica Varieties								
1 (17)	2 (20)	3 (17)	4 (18)	5 (20)	6 (21)	7 (19)	8 (20)	9 (21)
Tarch	Altex	Dasi	Dunkled	V-248	Bulb-98	Rainbow	Asker	Habib -98
1	0	0	1	0	1	1	0	1
0	1	0	0	1	1	0	1	1
0	1	0	0	1	1	0	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
0	1	1	0	1	1	1	1	1
0	1	0	0	1	1	0	1	0
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
0	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	0	1	1	1	1	1	1
1	0	1	1	0	0	1	0	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1

Table 4. Molecular weight banding range of the seeds of nine different *Brassica napus* varieties.

S/NO	Variety	Molecular weight range (kDa)	No. of Protein Bands	Total Bands
01	Tarch	10-40	4	17
		40-80	5	
		80-120	8	
		120-140	0	
02	Altex	10-40	7	20
		40-80	6	
		80-120	7	
		120-140	0	
03	Dasi	10-40	4	17
		40-80	6	
		80-120	7	
		120-140	0	
04	Dunkled	10-40	4	18
		40-80	6	
		80-120	8	
		120-140	0	
05	V-248	10-40	7	20
		40-80	6	
		80-120	7	
		120-140	0	
06	Bulb-98	10-40	8	21
		40-80	6	
		80-120	7	
		120-140	0	
07	Rainbow	10-40	5	19
		40-80	6	
		80-120	8	
		120-140	0	
08	Asker	10-40	7	20
		40-80	6	
		80-120	7	
		120-140	0	
09	Habib-98	10-40	7	21
		40-80	6	
		80-120	8	
		120-140	0	

Thousand seed weight of different varieties of B. napus

The maximum TSW value was 4.22 g (V-248) and minimum 2.11 g (Dunkled). Zia *et al.*, (2008) reported TSW for the oil seed rape varieties ranging from 3.5 - 4.5 g, which is in agreement with the current findings. Akbar *et al.*, (2007) found the TSW between 3.12 -4.10 g, Kricka *et al.*,(1999) between 2.92-4.66 g and Farahbakhsh *et al.*, (2006) between 2.34-3.54 g which is also in accordance with the present notion**.**

Table 5. Genetic distances (GD) among nine *Brassica napus* varieties.

	1	2	3	4	5	6	7	8	9
1	-								
2	0.63	-							
3	0.73	0.71	-						
4	0.88	0.68	0.79	-					
5	0.63	0.95	0.71	0.68	-				
6	0.68	0.90	0.68	0.73	0.90	-			
7	0.84	0.81	0.84	0.89	0.73	0.77	-		
8	0.71	0.95	0.68	0.68	0.95	0.90	0.73	-	
9	0.73	0.82	0.76	0.81	0.82	0.86	0.86	0.82	-

Here, 1=Tarch, 2=Altex, 3=Dasi, 4=Dunkled, 5=V-248, 6=Bulb-98, 7=Rainbow, 8=Asker, 9=Habib-98.

Molecular analysis of seed storage protein of different varieties of B. napus

SDS-PAGE analysis of the total protein of nine different varieties showed maximum number of bands in a range of 80-120 kDa (Table 4). This notion is in contrast to Zia *et al.*, (2008), who showed maximum number of bands in a range of 10-50 kDa. Minimum numbers of bands were found in range of 120-140 kDa. A maximum of 21 bands were found for Bulb-98 and Habib-98 while a minimum of 17 bands were found for Dasi and Tarch varieties.

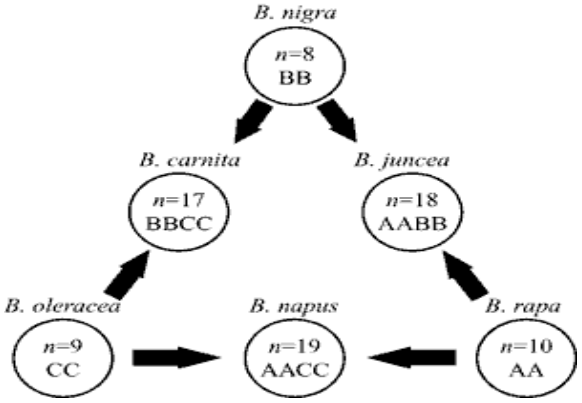

Fig. 1. Triangle representing the genomic relationships among *Brassica* species.

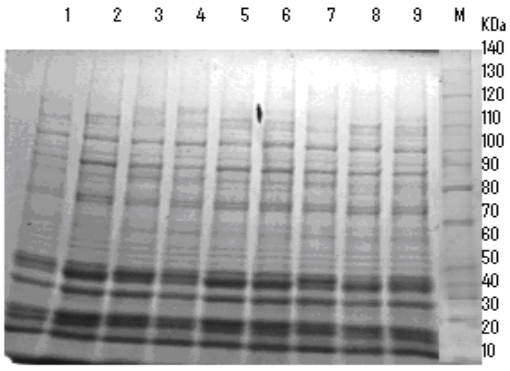

Fig. 2. Electrophorogram showing banding pattern of B*rassica napus* proteins and molecular weight marker.

1=Tarch, 2=Altex, 3=Dasi, 4=Dunkled, 5=V-248, 6=Bulb-98, 7=Rainbow, 8=Asker, 9=Habib-98, M= Molecular marker, KDa= Kilodalton.

The interpreted Euclidean distances and a dendogram made on the basis of the results of the SDS–PAGE showed the maximum genetic distance of 0.95 and minimum genetic distance of 0.63 put these

nine varieties into two major clades (Figure 3, Table 5). The study highlights that variation exists in both the physiochemical and protein banding patterns of seeds of different varieties of *B. napus*. These variations in seed protein profiles could be exploited for an efficient breeding program especially for the selection of parents for hybridization.

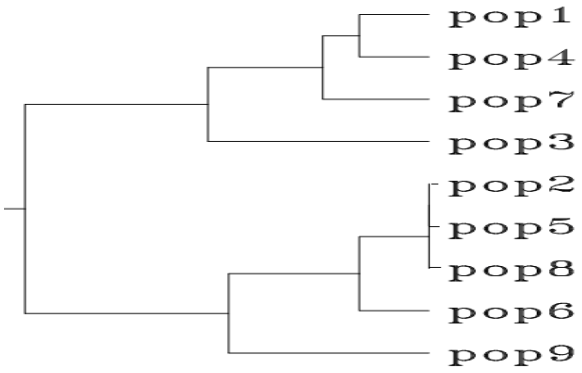

Fig. 3. Dendogram showing clusters of different varieties of *Brassica napus* based on SDS-PAGE (UPGMA).

Pop 1 = Tarch, pop 4 = Dunkled, pop 7 = Rainbow, pop 3 = Dasi, pop2 = Altex,

pop5 = V-248, pop 8 = Askar, pop 6 =Bulb 98, pop 9 = Habib-98.

Conclusion

All the nine varieties of *B. napus* studied showed a significant variation, both physiochemical and genetic variability (in term of protein expression profiles), and they were clustered into two distinct clades with significant genetic distances. Thus seed storage protein profiles could be useful marker in cultivar identification, classification, registration of new varieties, pedigree analysis and in the studies of genetic diversity of the adopted cultivars.

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