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CHARACTERIZATION OF CASTOR BEAN GENOTYPES UNDER VARIOUS ENVIRONMENTS USING SDS-PAGE OF TOTAL SEED STORAGE PROTEINS

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Abstract

Castor bean (*Ricinus communis* L.) is an indeterminate, non-edible oil seed crop grown in low rainfall regions of semi-arid tropics and sub-tropics. The present work was conducted to see the feasibility of electrophoresis for intra-specific characterization of castor bean on the basis of their total seed storage proteins. The protein profiles of all the four accessions were very much alike, both in number of bands and in their distribution on the gel, even though accessions represented a wide range of geographic origin. Occasionally, variation was observed in the density or sharpness of bands. The seed protein patterns were also uniform among various accessions grown under various environments. On the basis of banding pattern, polypeptides could be divided into three regions, A to C, equivalent to increasing Rf value and decreasing molecular weight. The present investigation revealed no variation in different accessions and under different environments with regards to their total seed protein profiles. The results clearly showed that it was impossible to discriminate various genotypes from each other, as they were characterized by same banding patterns. However, it might be useful to distinguish diverse forms of it from one another. The present investigation revealed very limited variation in castor bean genotypes in Pakistan.

Introduction

Electrophoresis is the process of moving charged molecules in solution by applying an electric field across the mixture. Molecules in an electric field move with a speed dependent on their charge, shape and size, electrophoresis has been extensively developed for molecular separations. As an analytical tool, electrophoresis is simple and relatively rapid. It is used chiefly for analysis and purification of very large molecules such as proteins and nucleic acids, but can also be applied to simpler charged molecules. Polyacrylamide is the most common matrix for separating proteins.

In Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separations, migration is determined by molecular weight of polypeptides. Sodium dodecyl sulphate (SDS) is an anionic detergent that denatures proteins by wrapping the hydrophobic tail around the polypeptide backbone. For almost all proteins, SDS binds at a ratio of approximately 1.4g SDS per gram of protein, thus conferring a net negative charge to the polypeptide in proportion to its length. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions and substantially unfolds the protein molecules, minimizing differences in molecular form by eliminating the tertiary and secondary structures. The proteins can be totally unfolded when a reducing agent is employed. The SDS denatured and reduced polypeptides are flexible rods with uniform negative charge per unit length. Thus, because molecular weight is essentially a linear function of peptide chain length, in sieving gels the proteins separate by molecular weight.

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The castor plant is an endospermic dicot (Kermode *et al*., 1985). In mature castor seed, 90-95% of the total seed protein is in the endosperm. In the endosperm, crystalloid proteins comprise 70 to 80% of the total protein and are insoluble in water. The residual soluble protein fractions are the lectins (ricin and RCA) and albumins. Castor seeds contain two toxins called ricin and *Ricinus communis* agglutinin (Hartley & Lord, 2004). Ricin is a ribosome inactivating protein that is manufactured in the endosperm. It is a small dipeptide molecule (mol wt approx. 65kDa) containing both an A chain (-32kDa) and a B chain (~32-34kDa) linked together by a disulphide bond (Kumar *et al*., 2004). The A chain of ricin is a ribosome-inactivating protein (Lord *et al*., 1994). It prevents protein synthesis by irreversibly altering the ribosomal RNA subunits involved in translation. The A chain specifically binds to 28S ribosomal subunits, permanently altering its structure. The lectin portion of ricin is the B chain. Ricin's B chain specifically binds glycoproteins and glycolipids on the cell surface terminating in galactose or N-acetylgalactosamine (Lord *et al*., 1994). The B chain attaches to the eukaryotic cell and the intact toxin enters cell by receptor mediated endocytosis (Bilge *et al*., 1994). *Ricinus communis* agglutinin (RCA), a hemagglutinin lectin, is also found in the endosperm of castor seeds. It is less toxic than ricin, but causes increased agglutination of red blood cells. RCA is a 120kDa polypeptide composed of 4 subunits, two similar to the A chain of ricin and two similar to the B chain. In cotyledons of mature castor seed, the distribution of protein differs with the insoluble fraction comprising only 10% of the total protein. The soluble protein portions found in the cotyledons are almost entirely albumins. Of the total protein in the protein bodies of castor bean, approximately 40% is represented by a group of closely related albumins localized in the matrix of the organelle. This group of albumins has a sedimentation value of 2S and is resolved into several proteins of molecular weight around 12kDa by sodium dodecyl sulfate-acrylamide gel electrophoresis. It has a high content of glutamate/glutamine and undergoes rapid degradation during the early stage of germination (Youle & Huang, 1978).

Seed protein electrophoresis has been used as a tool to resolve taxonomic and evolutionary problems, varietal and species identification in many crops and has a range of applications in breeding programs. Numerous seed protein profile studies have been done with various plant species. This strongly supports the hypothesis that molecular polymorphisms are neutral in natural selection. Seed material is relatively easy to handle with respect to protein extraction and more important, the seed may be regarded as a fixed physiological state. In taxonomic studies, it is critical to compare organs at the same stage as well as morphology. In this sense, the seed and its proteins may be regarded as a "conservative" unit, little affected by the environment, geographic origin, seasonal fluctuations and chromosomal rearrangements (Ladizinsky & Hymowitz, 1979).

In the recent decades considerable interest has been focused on the use of biochemical methods for plant variety discrimination and identification. Seed protein patterns are considered to be particularly reliable, as seed storage proteins are largely independent of environmental factors. Seed protein patterns obtained by electrophoresis have been successfully used to resolve the taxonomic and evolutionary problems of several crop plants (Ladizinsky & Hymowitz, 1979; Sammour, 1989; Khan, 1992; Das & Mukherjee, 1995). They have also provided a promising tool to distinguish cultivars of a particular crop species (Cooke, 1984; Ferguson & Grabe, 1986; Gardiner & Forde, 1988; Gadgil *et al*., 1989; Koranyi, 1989; Moller & Spoor, 1993; Jha & Ohri, 1996). However, few studies indicated that cultivar identification was not possible with the SDS-PAGE method, as the electrophoretic patterns of the proteins were similar among the cultivars (Ladizinsky & Adler, 1975; Raymond *et al*., 1991; Ahmad & Slinkard, 1992; de Vries, 1996). As for castor bean, however, there is paucity of data regarding seed protein electrophoresis, and relatively little attention has been paid to distinguish the cultivars of a particular species on

the basis of their seed storage protein patterns obtained by electrophoresis. Varier *et al*., (1999) characterized four F_1 hybrids of castor bean and their parents on the basis of seed shape, mottling on the seed coat, shape and size of micropyle using electron microscopy and electrophoresis. They found that hybrids were more similar to their female parents in seed morphology, whereas the soluble proteins of the 2 hybrids were identical to their male parents and different from their female parents.

In the present study an attempt has been made to investigate the feasibility of using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of total seed storage proteins to characterize few accessions of castor plant at intra-specific level and determine the effects of environment on seed storage patterns of these lines.

Materials and Methods

Plant materials: Plant materials consisted of a total of four accessions of castor bean, which were grown under three environments of Potohar region including Attock, Chakwal and NARC-Islamabad (Table 1).

Protein extraction: For the extraction of proteins, whole individual grains of castor bean were taken, removed seed coat, crushed and ground to fine powder in a mortar and pestle. Castor grain powder (0.01g) was defatted using 500µl n-Hexane in each sample, centrifuged for 15,000 rpm for 10 minutes at room temperature. Supernatant was discarded; the pellet was resuspended in 800µl of individual seed protein extraction buffer solution (0.5M NaCl, 10mM Tris-HCl pH 6.8), sonicated for 15 minutes and centrifuged. This procedure of extraction was repeated for 10 times. This procedure was replicated and the supernatant was combined. The combined supernatant was added with pre-cooled acetone, frozen, thawed and centrifuged to get individual seed proteins. The extracted crude proteins were recovered as clear supernatant, transferred into new 1.5 ml eppendorf tubes and stored at -20 ^{o}C until electrophoresis (Table 2).

Electrophoresis: SDS-PAGE of total seed protein was carried out in polyacrylamide slab gels in a discontinuous buffer system according to the method of Laemmli (1970). Vertical gel slabs were prepared in a glass sandwich which was tightened by a set of plastic clips lined with a band of foamed silicon rubber. Separation gel was put into the space between a set of glass plates (up to 2 cm from the top). Small amount of distilled water (120 µl) was added on separation gel gently to prevent gel surface from air and promote fixation. The set up was left for 30 minutes so that gel was fixed. The separating gels contained 15% by weight of acrylamide and 0.135% by weight of N.N-methylene-bis-acrylamide in 1 M Tris-HCl buffer (pH 8.8) with 0.27% SDS. The gels were polymerized chemically by the addition of 20µl by volume of tetramethylethylene-diamine (TEMED) and 10% ammonium persulfate (APS). During the fixation of separation gel, stacking gel was prepared. Stacking gel consisted of 4.5%. The stacking gel was polymerized chemically in the same way as for the separation gel. When separation gel was fixed, distilled water was removed from its top and stacking gel solution poured on it. Combs were fixed into the stacking gel. Combs were put with special care and it was confirmed that there was no air bubble at the bottom of the combs. The set up was left for 15 minutes so that the stacking solution became gel. Combs, clips and gaskets were removed from glass plates carefully and confirmed that there was no any air bubble at this stage. Gel plates were freshly used for electrophoresis but it was also possible that these would be wrapped in aluminum foil and could be used even for one week.

Table 1. Material used during present study.

Sr. No.	Variety	Location	Sr. No.	Variety	Location
1.	V1	Attock	7.	V3	Chakwal
2.	V2	Attock	8.	V4	Chakwal
3.	V3	Attock	9.	V1	NARC
4.	V4	Attock	10.	V2	NARC
5.	V1	Chakwal	11.	V3	NARC
6.	V2	Chakwal	12.	V4	NARC

Table 2. Protein extraction buffer.

Reagent	Final Conc.	100 ml
Tris (Hydroxymethyl) Aminomethane	0.05 M	0.6057 g
Sodium Dodecyl Sulphate (SDS)	0.2%	0.2 g
Urea	5 M	30.3 g
Autoclaved Distilled water		70 ml
HCl (Conc.)	Adjust to pH 8.0	
2-Mercaptoethanol	1%	1 ml
Total Volume of		**100 ml**

Add a little bit Bromophenol blue (BPB). Store buffer solution in a refrigerator

The electrode buffer contained Tris-glycine (9.0 g Tris HCl and 43.2 g glycine per 3 liters buffer solution at a pH 8.9) with 3.0 g (0.1%) SDS. Fifteen µl of protein supernatant were applied into the stacking gel sample wells with a micro syringe, followed by 20 µl of reservoir buffer containing bromophenol blue which served as the tracking dye. Electrophoresis was carried out at 70mA until the bromophenol blue marker reached the bottom of the gel (approximately two and a half hour). In order to check the reproducibility of the method two separate gels were run under similar electrophoretic conditions.

Solution-A (3M Tris-HCl/pH 9.0; 0.4% SDS):

Reagent	Final Conc.	100 ml
Tris (hydroxymethyl) aminomethane	3 M	36.3 g
Sodium Dodecyl Sulphate (SDS)	0.4%	0.4 g
Autoclaved Distilled water		70 ml
HCl (Conc.)	Adjust to pH 8.8	
Total volume of		**100 ml**

Store in a refrigerator

Solution-B (0.493M Tris-HCl/pH 7.0; 0.4% SDS):

Reagent	Final Conc.	100 ml
Tris (Hydroxymethyl) Aminomethane	0.493 M	5.98 g
Sodium Dodecyl Sulphate (SDS)	0.4%	0.4 g
Autoclaved Distilled water		80 ml
HCl (Conc.)	Adjust to pH 7.0	
Total volume of		**100 ml**

Store in a refrigerator

Solution-C (30% Acrylamide; 0.8% Acrylamide/Bis = 30:0.8):

Reagent	Final Conc.	100 ml
Acrylamide*	30%	30 g
Bis (Bis-Acrylamide)*	0.8%	0.8 g
Autoclaved distilled water to total volume of		**100 ml**

Store in a refrigerator; *Acrylamide and Bis-acrylamide are highly toxic and carcinogenic. Gloves were used while preparing solution using these reagents.

10% APS:

Reagent	Final Conc.	100 ml
Ammonium Per Sulfate (APS)	10%	0.1 g
Autoclaved distilled water to total volume of		**1 ml**

Can be stored in a refrigerator for several days but it is preferred to prepare fresh all the times for better performance.

Separation Gel with 1mm Thickness (For two mini gels):

Solution	12.25%	15%
Solution A	5 ml	5 ml
Solution C	8.17 ml	10 ml
10% APS	200 µl	200 µl
Autoclaved distilled water	6.83 ml	5 ml
TEMED	15µl	15 µl

Add TEMED (N-N-N-N-Tetramethylethylenediamine) at the end and shake well.

Stacking Gel (For two mini gels):

Solution	4.5%
Solution B	2.5 ml
Solution C	1.5 ml
10% APS	70 µl
Autoclaved distilled water	6.0 ml
TEMED	17 µl

Add TEMED (N-N-N-N-Tetramethylethylenediamine) at the end and shake well.

Electrode Buffer Solution (0.025M Tris; 0.129M Glycine; 0.125% SDS):

Reagent	Final Conc.	1000 ml
Tris (Hydroxymethyl) Aminomethane	0.025 M	3.0 g
Glycine	0.129 M	14.4 g
SDS	0.125%	1.25 g
Autoclaved distilled water to total volume of		**1000 ml**

Stored at room temperature

Staining and destaining: After electrophoresis, the gels were stained with 0.2% (w/v) coomassie brilliant blue R250 dissolved in a solution containing 6% (v/v) acetic acid, 44% (v/v) methanol and water in the ratio of 6:44:50 (v/v) for one hour. Gels were then destained by washing with a solution containing 5% (v/v) acetic acid, 20% (v/v) methanol and water in the ratio of 5:20:75 (v/v) until the color of background disappeared and electrophoresis bands were clearly visible. After destaining, the gels were dried using Gel Drying Processor for about 100 minutes.

Staining Solution (Acetic Acid:Methanol:Water = 6:44:50):

Reagent	**1000 ml**
Acetic Acid	60 ml
Methanol	440 ml
Autoclaved distilled water	500 ml
Coomassie Brilliant Blue (CBB)*R250	2.25 g
Autoclaved distilled water to total volume of	**1000 ml**

Stir solution for 30 min and then filter, store at room temperature. *CBB is a protein staining dye.

Destaining Solution (Acetic Acid:Methanol:Water = 5:20:75):

Reagent	**1000 ml**
Acetic Acid	50 ml
Methanol	200 ml
Autoclaved distilled water	750 ml
Autoclaved distilled water to total volume of	**1000 ml**

Stored at room temperature

Results

Ricin is a highly toxic protein from the seeds of the castor bean plant. Under non-reduced condition, ricin gives single band. When ricin is treated with P-mercaptoethanol (1%), it gives two bands, which represent A, and B subunits of the ricin. The molecular weight of the ricin has been found to be 65kDa approx. The molecular weight of its two peptides was estimated to be 34kDa and 32kDa. Variations have been reported in the literature regarding molecular weight of ricin and its subunits. Ricin having molecular weight from 60 to 65kDa by SDS-PAGE technique has been reported (Nicolson *et al*., 1974). The ricin subunits, having molecular weight 29.5kDa and 34kDa after P-mercaptoethanol treatment have been described. Under reducing condition, the ricin appeared to have two subunits, corresponding to the molecular masses of 30kDa and 32kDa (Woo *et al*., 1998). The subunit structure of ricin on treatment with P-mercaptoethanol (1%) at molecular level revealed that the reducing agent converts ricin into two peptides.

In order to obtain good resolution of proteins of low mol wt by gel electrophoresis, we used polyacrylamide gels of high density (15% gel). Such a gel system gave good resolution of proteins of low mol wt, but was not suitable for the identification of proteins. The electrophoretic seed protein profiles of all the accessions of castor bean were very much alike, both in number of bands and in their distribution on the gel, even though accessions represented a wide range of geographic origin. Occasionally, variation was observed in the density or sharpness of bands. The electrophoretic seed protein patterns were also uniform among various accessions grown under various environments. On the basis of banding pattern, polypeptide banding patterns could be divided into three regions, A to C, equivalent to increasing Rf value and decreasing molecular weight (Fig. 1). The protein bands at region-A (upper portion of the gel) represent the RCA having 120kDa molecular weight, all of which are monomorphic containing relatively weakly stained protein bands. The polypeptide bands at region-B (middle portion) correspond to Ricin. It ranged from 34 to 32 kDa, with dark stained bands in various protein type accessions. Region C included the lowest weight protein subunits in 12kDa molecular weight at the cathode end. The protein bands at the lower portion of the gel were identified as the 2S albumin proteins. Banding pattern in region C was almost uniform between all protein types.

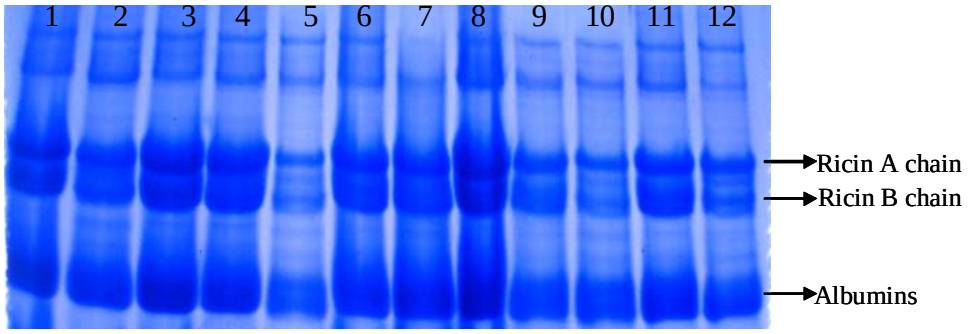

Fig. 1. Seed protein profiles of castor cultivars grown under three environments of Potohar - Pakistan. Numbers indicate the cultivars codes given in Table 1.

Discussion

Seed protein analysis by SDS-PAGE has proved to be an effective way of revealing the differences and relationships between taxa. The high stability of the seed protein profile and its additive nature make seed protein electrophoresis a powerful tool in elucidating the origin and the evolution of cultivated plants (Ladizinsky & Hymowitz, 1979). The present investigation revealed no variation in different accessions and under different environments with regards to their total seed protein profiles. The genetic affinities among the castor bean accessions from Pakistan determined by the seed protein profile study here did not corroborate the morphological analysis. Phenotypically, most of the oilseed accessions showed considerable variability. This uniformity of seed protein profiles agreed with the findings of Ladizinsky & Alder (1975) and Ahmad & Slinkard (1992), who examined different cultivars of chickpea and concluded that seed protein was a very conservative trait in chickpea. Similarly, Raymond *et al*., (1991) and de Vries (1996) also reported similar electrophoretic patterns of protein among the cultivars of sunflower and lettuce, respectively. Likewise, Tomooka *et al*., (1992) observed similar patterns in the local strains of mung bean collected from southeast Asia using SDS-PAGE analysis. Ladizinsky & Hymowita (1979) also stated that taxonomic categories below the species level, despite morphological and ecological differences, still possess basically the same seed protein profiles.

The protein profile of castor bean observed in the present work was similar to the earlier findings of Kour & Singh (2004) who also observed three zones of banding profiles using Indian mustard genotypes. However, banding patterns of mustard accessions were divided into four zones on the basis of Rf values of different bands (Rabbani *et al*., 2001). The present work was initiated to see the feasibility of electrophoresis for intra-specific characterization of castor bean on the basis of their total seed protein. The results clearly showed that it was impossible to discriminate various genotypes from each other, as they were characterized by same banding patterns. However, it might be useful to distinguish diverse forms of it from one another. The present investigation revealed very limited variation in castor bean genotypes in Pakistan. A low level of intra-specific variation has been reported in groundnut using SDS-PAGE of seed storage proteins (Javaid *et al.*, 2004). Similarly among 52 accessions of oilseed mustard from Pakistan, very close relationship was found between the oilseed collections as well as cultivars (Rabbani *et al*., 2001).

However, a considerable amount of variation was observed in pea based on SDS-PAGE (Ghafoor *et al*., 2008). Previous research on castor bean seed using SDS-PAGE of seed storage proteins focused on inbred lines and hybrids discrimination (Varier *et al*., 1999). They found that soluble proteins of 2 hybrids were identical to their male parents and different from their female parents. In four genotypes of castor bean, no polymorphism was detected by SDS-PAGE. Similarly, genotypes from different environmental conditions did not differ in protein types. In conclusion, it was impossible to differentiate the closely related accessions/cultivars of castor bean on the basis of seed storage proteins under diverse environments.

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