Identification of Cotton Genotypes By Polyacrlamide Gel Electrophoresis

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Abstract- *Varietal development and its identification is one of the most important aspects of seed industry and seed trade. Due to continuous breeding programme by using elite lines, it is difficult to characterize and identify the varieties on the basis of morphological characters alone. This has led to the exploration of new stable characters including genetic makeup to be used as markers for varietal identification. The present study includes the identification of three cotton hybrids and its parents and four varieties of cotton genotypes on the basis of their protein profile. Protein was extracted from the seeds using electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After fixation and coomaasie blue staining, varieties were identified according to their differences in the banding pattern and staining intensities.*

Keywords- cotton, electrophoresis, seed protein, SDS-PAGE

I. INTRODUCTION

Cotton (*Gossypium spp.*) is an important cash crop in India. In recent years new hybrids and varieties have been developed and released for commercial cultivation. Characterization and identification of different cotton cultivars is an indispensable activity and identification based on morphological characters is time consuming and influenced by environment. In this, seed protein electrophoresis can effectively used for characterization of cultivars. Electrophoresis is a process of separation of different biomolecules under the influence of electric field and has been successfully applied for the identification of varieties. Sample characterized by different proteins bands are considered to differ genetically while sample having the same protein bands may be of the same variety. Numerous studies have already been conducted for examining the protein pattern in important crops for varietal identification (Cooke, 1984; Gilliland, 1989). In 1986, ISTA adopted a standard reference method of PAGE for identification of varieties of wheat and barley into its international rules, involving separation of gliadin from wheat and hordein from barley (Cooper, 1987). A number of new varieties are available in cotton are very difficult to characterize based on morphological characters due to continuous breeding programme. Therefore, the present study was undertaken with the objective of developing Polyacrylamide Gel Electrophoresis (PAGE) for identification of cotton cultivars.

II. MATERIALS AND METHODS

Seed source

Three cotton hybrids and its parents and four varieties of cotton were used for characterization based on protein profile (Table 1).

Table 1 List of cotton cultivars used for identification

Sl. No	Hybrid	Male parent	Female parent
1.	HB 224	LRA 5166	P 4
2.	Surya	T 13	M 12
3.	Savitha	T 7	M 12
4.	**Varieties**		
	Surabhi, Anjali, Supriya, SVPR-2		

The seeds of three cotton hybrids *viz.*, HB 224, Surya and Savitha and its parents LRA 5166 X P4, T13X M12 and T7 X M12 respectively were obtained from the Central Institute For Cotton Research, Coimbatore, India. The cotton varieties *viz.*, Surabhi, Anjali and Supriya were obtained from Central Institute for Cotton Research, Coimbatore and SVPR-2 was obtained from Regional Research Station, Srivilliputhur,Tamil Nadu.

Electrophoretic technique of total soluble seed proteins

SDS-PAGE of total soluble seed proteins was carried out by using 15 per cent gels according to the methods prescribed by Dadlani and Varier, (1993) with slight modifications.

Defatting the seed

Ten seeds of each cultivar were de-coated and powdered in a pre-chilled pestle and mortar. The seed powder was defatted in test tubes with a mixture of chloroform, methanol and acetone (2:1:1) at intervals of four hours.

Protein extraction

The defatted seed material was then transferred to eppendorf tube for protein extraction. To each tube 0.3 ml of protein extraction solution was added. The contents were shaken and left overnight for extraction in a refrigerator and was heated in boiling water for five minutes and cooled and centrifuged at 10,000 rpm for 30 minutes and clear supernatant solution was collected. The extraction was repeated with 2 ml buffer each time until no further protein was extracted. The supernatant was pooled together and used for gel electrophoresis.

Preparation of stock solution

Extraction buffer (Tris-HCl pH 7.5): Tris base (1.21 g) was dissolved in 50 ml distilled water and pH adjusted with concentrated HCl to 7.5 and then the volume made upto 100 ml.

Protein sample buffer (5x concentration)

Tris-HCl buffer (pH 6.6) 0.6M: Tris- base (7.2g) was dissolved in 50 ml of distilled water and pH adjusted with concentrated HCl to 6.6 and volume made upto 100 ml with distilled water.

Stock-sample buffer

Tris-HCl buffer (pH 6.6) 0.6M	:	10.4 ml
Distilled water	:	7.9 ml
Sodium dodecyl sulphate	:	4 g
Glycerol	:	10.0 ml

The above mentioned components were mixed thoroughly.

Working sample buffer

Stock-sample buffer	:	4.25 ml
β-mercapto ethanol	:	75 ml

These components were mixed thoroughly and the volume was made up to 10 ml and to this a pinch of Bromophenol blue was added to act as a tracking dye.

Stock gel solution (30% acrylamide)

Acrylamide	:	30.08 g
Bis-Acrylamide	:	0.80 g

Dissolved in distilled water and volume made up to 100 ml.

Defatting solution

Chloroform	:	2 parts
Methanol	:	1 part
Acetone	:	1 part

Mixed thoroughly and used for defatting.

Separating gel buffer (pH 8.8) 0.5 M: Tris-base (22.69 g) was dissolved in 70 ml distilled water. The pH was adjusted to 8.8 with KOH and volume made up to 100 ml with distilled water.

Stacking gel buffer (pH 6.8) 0.5 M: Tris-base (6.05 g) was dissolved in 70 ml of distilled water, pH adjusted with concentrated HCl to 6.8 and volume made up to 100 ml distilled water.

SDS 10%: SDS (Sodium Dodecyl Sulphate) 1.0 g was dissolved in 6 ml of distilled water and volume made up to 10 ml.

Preparation of separating or running gel mixture

Solutions	:	**Gel concentration (10%) for 15 ml**
Acrylamide solution (C)	:	5.0 ml
Separating gel buffer	:	3.75 ml
Distilled water	:	6.25 ml
10% SDS (G)	:	100 µl
10% APS (H)	:	100 µl
Tetramethyl Ethylene Diamide (TEMED)	:	20 µl

The separating gel mixture was poured to the glass sandwich assembly to a level of about 4 cm from the notch. One ml of butanol saturated with distilled water was gently applied over the separating gel. After polymerization, (10 min) the outer layer of butanol was washed off by inverting the casting gel and washed 3 to 4 times with distilled water to remove traces of butanol. The water droplets were removed using a filter paper without touching the separating gel.

Preparation of stacking gel mixture (10 ml)

Stock acrylamide solution (C)	:	1.3 ml
Stacking gel buffer (F)	:	2.5 ml
10% SDS (G)	:	100 ml
10% APS (H)	:	50 ml
Distilled water:6	:	1 ml
Tetramethyl Ethylene Diamide (TEMED)	:	10 µl

The above solution were mixed thoroughly and poured on the top of the separating gel. An acrylic well forming comb was inserted, ensuring no air bubble trapped beneath. The top portion of the gel solution was overlayed with 1 ml butanol saturated with distilled water. The gel was allowed to polymerize for 45 min. Then the acrylic comb was removed carefully not to distort the wells and resultant wells were cleaned with distilled water. The excess water was sucked out using a micro syringe and each well was cleaned carefully using bits of filter paper. The gel was then installed by removing the gasket in the electrophoresis apparatus to which electrode buffer was poured and pre-run for 10 min.

Electrophoresis

Five seeds were ground in centrifuge tube by using micro pestle and 200 µl Tris HCl extraction buffer (25mM, pH 8.8) was added. The mixture was agitated thoroughly and kept at 8ºC for overnight for protein extraction. Then the mixture was centrifuged at 10,000 rpm for 15 minutes and the supernatant was collected. This protein extract was dissolved in an equal volume of working buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue) and incubated at 60-70ºC for 10 minutes, cooled immediately for 5 minutes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was used for loading on to the gel. A current of 25 mA per well with a voltage of 80 V was applied until the tracking dye crossed the stacking gel. Later the current was increased to 60 mA per well and voltage up to 120 V. The electrophoresis was stopped when the tracking dye reached the bottom of the resolving gel. Then the gel was stained using coomaasie blue.

Staining

The gels were fixed over night in 15% TCA rinsed with distilled water and stained in a mixture of 2% comassie blue and 15% TCA (1:8) for two days. The gels were washed in distilled water and photographed.

The gel sticks with protein bands were analyzed for intensities of banding and Rm values with a UV-is computerized spectrophotometer fitted with gel scanner. A blank gel without seed proteins was analyzed first for standardization and references, and the percent transmissions were recorded for each gel at 620 nm.

Peaks from the start of run indicated different bands with their intensities on a recorder sheet. From the position of peaks, Rm values were determined by the relation:

$$Rm = \frac{\text{Distance travelled by the protein}}{\text{Distance travelled by the tracking dye}}$$

III. RESULT AND DISCUSSION

The total seed protein was extracted and separated by SDS-PAGE method. The detailed profile of 12 cotton cultivars studied has been presented in Fig 1.

The detailed electrophoregram of total soluble seed protein has been presented in Table 2.The frequent occurrences of insufficient varietal discrimination by grow out test and the consequent inability to confirm distinctness encouraged to investigate complementary methods of describing varieties for comparison with conventional methods. One approach was to use protein electrophoresis. Many workers have attempted to characterize crop plants by electrophoretic analysis of seed protein. In present study attempt was made to characterize 12 cotton cultivars by total soluble seed proteins separated by SDS-PAGE. A wide variation was observed in the pattern of protein bands of studied cultivars. The cultivars differed in the number of bands, their relative mobility and intensity. The proteins separated on twelve per cent acrylamide gel could be distinguished and grouped based on the standard marker. By using SDS-PAGE, the total soluble seed protein could be fractionated into 19 bands, which showed heterogeneity among different cultivars.

Banding patterns and the corresponding drawings of tris- Hcl soluble seed proteins of cotton hybrids, their parents and varieties were given in plate Fig. 1 & Table 2. Some of the bands which were faint on the gel are not visible on the photograph and have been drawn in the zymogram (Table 3). Based on the Rm values, 35 bands could be observed in all the genotypes put together. The banding patterns of different samples of the seed lot of particular variety were identical. The number of protein bands detected in different cultivars varied and many of the protein bands were common to more than one cultivar.

The hybrid savitha and its male parent M 12 had a similar banding pattern but both are different from the female parent T 7. The hybrid HB 224 and its male parent P 4 had similar banding pattern. The hybrid Surya and its male parent M 12 had a similar banding pattern.

The hybrid Savitha could be identified by the absence of bands at Rm values 0.25, 0.30, 0.41, 0.42, 0.53, 0.53, 0.66, 0.75 and 0.80. The male parent of hybrid Savitha, M 12 could be identified by the absence of bands at Rm values 0.25, 0.30, 0.42, 0.55, 0.63 and 0.66.

The hybrid HB 224 could be identified by its male parent P 4 by the absence of bands at Rm values of 0.37, 0.20, 0.50 and 0.86. The hybrid HB 224 could be identified from its female parent LRA 5166 by the absence of bands at Rm values 0.32, 0.43, 0.50, 0.70, 0.72, 0.75 and 0.92.

The hybrid Surya could be identified from its female parent T 13 by the absence of bands at Rm values 0.35, 0.45, 0.55, 0.60, 0.67, 0.68 and 0.80 and could be identified from its male parent T 13 by the absence of bands at Rm values 0.51, 0.60, 0.63, 0.68 and 0.78.

The four varieties *viz.*, Supriya, Surabhi, Anjali and SVPR-2 could be identified by the differences in bands at Rm values. The variety Anjali is identified by the presence of a prominent dark band at Rm values of 0.85. The remaining varieties *viz.*, Supriya, Surabhi and SVPR-2 did not have the prominent band at Rm value 0.85. The variety Supriya could be identified by the presence of light bands at Rm values of 0.60 and 0.65. The variety Surabhi could be identified by the presence of two light bands at Rm values of 0.60 and 0.65. The variety Surabhi could be identified by the presence of two light bands at Rm values of 0.35 and 0.62.

The variation in number and intensity of the bands might be due to differential extraction or difference in solubility of protein or lack of separation of several proteins having similar migration rates Ladizinsky and Hymowitz, 1979). Similar observations based on band intensity were reported by Asghar*et al*. (2003) and Varma *et al.* 2005) in maize genotypes, Devi, (2000) in sunflower, Vijayan, (2005) in rice, Paul and Datta, (2006) in celery and ajowan, Nisha, (2007) in wheat and Sumathi, (2007) in oats.

IV. CONCLUSION

Using this method, it is possible to prepare electrophoretic patterns of all of the cultivars of Cotton and such a catalogue would be useful reference for identification of unknown varieties.

Table 2: Electrophoregrams of seed proteins of cotton genotypes.

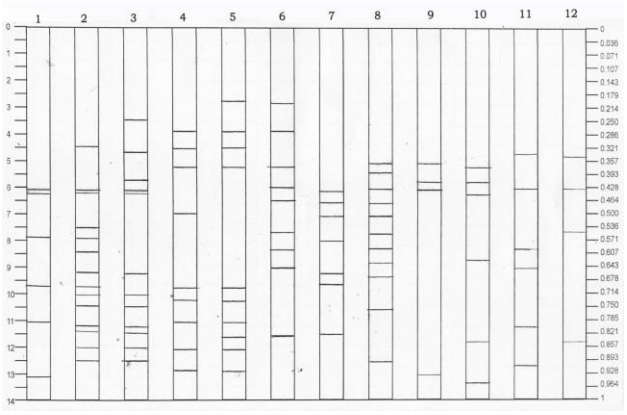

Table 3: Electrophoretic profiles of seed proteins (Tris-HCl soluable) of hybrids and inbreds

Band No.	Rm value of band	Genotypes											
		T 7	M12	Savitha	HB 224	P4	LRA 5166	Surya	T 13	Surabhi	Anjali	Supriya	SVPR2
1.	0.20	-	-	-	-	+	+	-	-	-	-	-	-
2.	0.25	-	-	+	-	-	-	-	-	-	-	-	-
3.	0.27	-	-	-	+	+	+	-	-	-	-	-	-
4.	0.30	-	-	-	-	-	-	-	-	-	-	-	-
5.	0.32	-	+	-	+	+	-	-	-	-	-	-	-
6.	0.05	-	-	+	-	-	-	-	+	-	+	-	-
7.	0.37	-	-	-	+	+	+	-	+	-	+	-	+
8.	0.38	-	+	-	-	-	-	-	+	+	-	-	-
9.	0.41	-	-	+	-	-	-	-	-	-	+	-	-
10.	0.42	-	+	+	-	-	-	-	-	+	-	-	-
11.	0.43	+	+	+	-	-	-	-	-	-	-	-	-
12.	0.45	-	-	-	-	-	-	-	+	-	+	+	+
13.	0.47	-	-	-	-	-	+	+	+	-	-	-	-
14.	0.50	-	-	-	+	-	-	-	-	-	-	-	-
15.	0.51	-	-	-	-	-	-	+	+	-	-	-	-
16.	0.53	-	+	-	-	-	-	-	-	-	-	-	-
17.	0.55	+	-	-	-	-	-	-	-	-	-	-	+

Band No.	Rm value Of band	Genotypes											
		T 7	M12	Savitha	HB 224	P4	LRA 5166	Surya	T 13	Surabhi	Anjali	Supriya	SVPR2
18.	0.57	-	+	-	-	-	+	+	+	-	-	-	-
19.	0.60	-	+	-	-	-	+	-	+	-	-	+	-
20.	0.62	-	-	-	-	-	-	-	-	-	-	-	-
21.	0.63	-	-	-	-	-	-	-	+	-	-	-	-
22.	0.65	-	-	-	-	-	+	-	-	-	+	+	-
23.	0.66	-	+	+	-	-	-	-	-	-	-	-	-
24.	0.67	-	-	-	-	-	-	-	+	-	-	-	-
25.	0.68	+	+	-	-	-	-	+	-	-	-	-	-
26.	0.70	-	+	+	+	+	-	-	-	-	-	-	-
27.	0.72	-	+	+	+	+	-	-	-	-	-	-	-
28.	0.75	-	+	+	+	+	-	-	-	-	-	-	-
29.	0.78	+	+	+	-	-	-	-	+	-	-	-	-
30.	0.80	-	+	+	+	+	-	-	-	-	-	-	-
31.	0.81	-	-	-	-	-	-	-	-	-	+	+	-
32.	0.82	-	-	+	+			-	-	-	+	-	+
33.	0.86	-	+	+	+	+	-	-	+	+	+	+	-
34.	0.92	+	-	+	+	-		-	-	-	-	-	-
35.	0.93	-	-	-	-	-	-	-	-	-	-	-	-

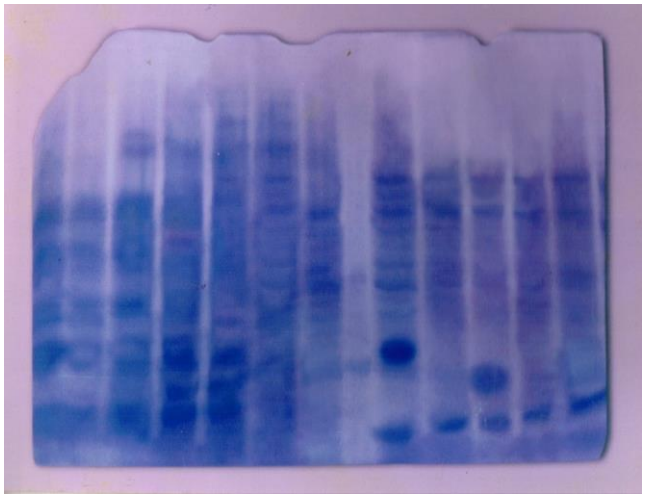

Fig. 1 SDS – Page profile of tris soluble seed proteins of cotton genotypes

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