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Biochemical markers for identification of cluster bean genotypes

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Abstract
Cluster bean or guar is important cash crop in rain-fed, especially in semi-arid and arid regions of India. India alone contributes more than 80% of global guar production followed by 15% in Pakistan. Its cultivation in India is concentrated in northwestern states namely Rajasthan, Haryana, and Gujarat. Guar is a natural source of nano-particles of hydrocolloids (substance that forms thick solution at low concentration with water). Adequate characterization is necessary to facilitate utilization of genotypes in crop improvement and to distinguish a variety from all other varieties. In addition, seed certification, which forms a link between variety registration and seed production, involves an assessment of both varietal identity and purity to assure the quality of seed marketed to farmer or grower. Finally, the ultimate consumers of the harvested seed also often need to be certain that they are purchasing the correct variety particularly. The present study attempts to characterize cluster bean genotypes based on biochemical markers viz. protein and esterase. The results of the electrophoretic banding pattern of the protein profile suggested that the specific genotype could be differentiated by either based on the position or intensity of bands. But not on the numbers as some of the genotypes expressed similar number of bands. In the genotypes having same number of bands, the location and intensity of bands varied, this is useful for clear differentiation of the cluster bean genotypes. Based on the zymograms of total soluble proteins, Region D (29.0 to 43.0 KD), E (20.0 to 29.0 KD), F (14.3 to 29.0 KD) and G (< 14.3 KD) were found more useful to distinguish most of the genotypes studied, as the banding pattern was distinct for each genotype in these regions. A total number of 5 bands were noticed among the 20 tested genotypes in the zymogram of esterase. The difference in number of bands, position and intensity in esterase zymogram has been considered to distinguish genotypes for characterization. From these results it is clear that there is enough variation in protein and esterase isozyme profile among genotypes and could be considered as a tool for genotype identification and screening for various traits.

Keywords: Biochemical markers, electrophoresis, SDS-PAGE, Protiens, esterase, guar

Introduction
Cluster bean [*Cyamposis tetragonoloba* (L.) Taub] or guar is a drought tolerant annual legume crop, mainly cultivated in India, adjoining Pakistan and South-eastern Asia as a vegetable and forage crop for a long time. Cluster bean seed has a spherical-shaped endosperm containing galactomannan gum, which forms a viscous gel in cold water. The crop little known elsewhere in the world is especially important to India owing to its high economic value as an export commodity. Guar gum obtained from seeds is used extensively in paper, mining, food, cosmetic, textile, oil and pharmaceutical industries around the world.

Descriptive characters for the assessment of varietal purity and identity are essential for quality seed production and certification programme. Varietal description for identification of crop varieties has attained a critical importance in national and international seed programmes and there is a need for the development of reliable methods and identifiable characters for identification purpose. As India conserves an enormous wealth of cluster bean along with a wide variability for morpho-physiological and other qualities, there is a strong need for appropriate addressing and well documentation of the guar germplasm. In the changing global scenario of the post-GATT era, large number of improved varieties and hybrids with respect to higher yield, resistant to biotic and abiotic stresses are under cultivation throughout the country. In recent years, both public and private institutions introduced several hybrids/varieties for commercial cultivation. So the maintenance of genetic purity of varieties is primarily important for preventing varietal deterioration during successive regeneration cycles and ensuring varietal performance at an expected level. The varieties could be identified or distinguished to a considerable degree of accuracy on the basis of consistent differences in one or more essential descriptive characters, which may be morphological, biochemical or DNA based markers.

It is necessary to develop better methods of characterization and evaluation of germplasm collections. Characterization of germplasm using biochemical techniques (storage proteins and isozymes) has received a great attention in the last decades. The electrophoresis of protein is a method to investigate genetic variation and to classify plant varieties (Isemura *et al* 2001) [7]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) is among the biochemical technique that is widely used due to its simplicity and effectiveness for describing the genetic structure of the germplasm accessions of plant species. Proteins have been used as genetic markers in identifying variation among the taxa of each species; screening the purity of the ever expanding number of cultivars; establishing genome relationships; exploiting the important traits of landraces and wild relatives to provide increasing crop production and stabilizing yield (Sammour 1991) [18], and using information on genetic diversity to make decisions regarding selection of superior genotypes for improvement yield of plants through breeding. Protein electrophoresis is considered a reliable, practical and reproducible method because proteins are the third hand copy of genomic DNA and largely independent of environmental fluctuations (Sammour, 1987 [17]; Iqbal *et al* 2005 [6]). Protein is not sensitive to environmental fluctuations; its banding pattern is very stable which advocated for cultivars identification purpose in crops. It has been widely suggested that such banding patterns could be important supplemental method for cultivars identification, particularly when there are legal disputes over the identity of a cultivar or when cultivars are to be patented (Tanksley and Jones, 1981) [22]. With this background a study involving the biochemical markers was taken up to characterize cluster bean genotypes.

Materials and methods

Characterization of cluster bean genotypes based on biochemical markers was conducted in the Section of Seed Science and Technology, Indian Institute of Horticultural Research, Hesaraghatta, Bengaluru. Freshly harvested elite genotypes of cluster bean (Table 1) were obtained from Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat. The seeds were cleaned and dried to safe level of moisture (< 9%) and graded to uniform size and used for studies.

Table 1: Cluster bean genotypes used for the study

Sl. No	Genotype	Sl. No	Genotype	Sl. No	Genotype	Sl. No	Genotype
1	GG-1	6	HG-2-20	11	IC- 116525	16	IC-116612
2	GG-2	7	IC- 102828	12	IC- 116577	17	IC-116626
3	PNB	8	IC-102853	13	IC- 116594	18	IC-116627
4	HG-365	9	IC- 103060	14	IC- 116601	19	IC-116633
5	HG-75	10	IC- 116523	15	IC-116609	20	IC-116645

Protein profiling

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of protein was carried out by using 15 per cent polyacrylamide gel according to the method prescribed by Laemeli (l970) [11] with slight modifications. The electrophoresis was done in vertical slab gel of 16 cm x 14 cm x 1 mm dimension. The detailed procedure was as follows:

(a) Sample preparation

Ten days old seedlings of each genotype was crushed by pestle and mortar using 200µl of 0.1 M Tris extraction buffer. The ground sample was kept kept at 4 ^{0}C overnight for protein extraction. Then the suspension was centrifuged under refrigeration at 10,000 rpm for 10 min and the clear supernatant was collected. To this supernatant, double volume of chilled acetone was added and centrifuged. The pellet was formed and dissolved in 30 µl of 0.1 M Tris buffer. This protein extract was dissolved in an equal amount of working buffer and kept in boiling water at 90 ^{0}C for 10 minutes and kept at 4 ^{0}C for cooling and loaded in to the gel.

(b) Preparation of gel for electrophoresis

i) Resolving gel (15%) was prepared by mixing 12.5 ml of 30 per cent acrylamide solution, 6.3 ml of resolving gel buffer, 5.7 ml of double distilled water, 0.25 ml of 10 per cent SDS, 0.25 ml of 10 per cent APS and 0.06 ml of TEMED quickly poured into the gel plates leaving a margin of 2.0 to 3.0 cm on upper side the gel. Overlay with water and left for polymerization for about 30 minutes.

ii) Stacking gel (5%) was prepared by mixing 1.0 ml of 30 per cent acrylamide, 1.5 ml of stacking gel buffer, 3.4 ml distilled water, 0.07 ml 10% APS and 0.02 ml TEMED. Top of the resolving gel was thoroughly cleaned before pouring the stacking gel, then the stacking gel solution was poured on to the top of the resolving gel solution and immediately a comb was inserted to form the wells of 1.5 cm depth taking care not to trap the air bubbles underneath the comb. The gel was allowed to polymerize for 30 minutes, then the comb was removed carefully and the wells were rinsed with distilled water.

(c) Electrophoresis

The upper and lower reservoirs of electrophoretic unit were filled with electrode buffer. Then 50 to 60 µl of protein extract was loaded into the wells of stacking gel by layering them under electrode buffer using micropipette. A current of 1.5 mA per well with a voltage of 80 was applied until the tracking dye crossed the stacking gel. Later the current was increased to 2 Ma per well and voltage up to 120. The electrophoresis was stopped when the tracking dye reached the bottom of the resolving gel, which took six to eight hours.

(d) Staining of gel

Silver staining was done for gel to get protein profiles. The reagents used for staining are Solution A (It was prepared by mixing 100 ml of methanol, 25 ml of Acetic acid, 100 ml of formaldehyde in 200 ml of double distilled water, Solution B (0.04 g gram of sodium thiosulfate was dissolved in 200 ml distilled water), Solution C (It was prepared by dissolving 0.4 g silver nitrate, 150 µl formaldehyde in 200 ml double distilled water) and Solution D (It was prepared by dissolving 12 g sodium carbonate and 1 ml of Solution B in 200 ml of double distilled water). Gel is soaked in 200 ml of solution A kept on shaker for one hour. After staining, the gel is washed with 50% methanol thrice for 10 minutes. Solution B is added to the gel and kept on shaker for 5 minutes. Then the gel is

washed thrice with doubled distilled water for 20 seconds. Solution C is poured to the gel and again kept on shaker for 20 minutes. The gel is washed with doubled distilled water for 20 seconds. To the washed gel solution D is added and gel is kept on shaker till the bands are developed. Once the bands are developed, the gel is washed with doubled distilled water for 20 seconds and the gel is fixed using acetic acid.

Electrophoretic analysis of esterase

Esterase (EST) was analyzed for isozymic pattern as described by Glaszman *et al* (1988) [2] with slight modification. Seedlings were raised at 25^0C and five days old seedlings were used for this purpose.

a) Sample preparation

Five days old seedling was ground thoroughly in a pestle and mortor with 50µl of extraction buffer under ice condition. The supernatant was collected and 10 µl of tracking dye (1% bromophenol blue containing 0.05% glycerol) was added into each tube. 50 µl of sample extract was used for loading.

b) Preparation of gel (8%)

Running and spacer gel solutions were prepared by using the solutions I, II and III as given below:

Solution I

Acrylamide	30.08 g
Bisacrylamide	0.8 g
Distilled water	to make 100 ml

Solution II

Prepare 1.5 M Tris by dissolving 18 g in 80 ml distilled water and adjust the pH to 8.8 with 1 N HCl, make up the volume to 100 ml.

Solution III

Prepare 0.5M Tris by dissolving 6 g in 80 ml distilled water and adjust the pH to 6.8 with 1 N HCl, make up the volume to 100 ml.

i) Resolving gel (8%): It was prepared by mixing 9.9 ml of solution I, 7.5 ml of solution II, 11.1 ml of double distilled water, 900 µl of ammonium per sulphate, 34 µl of TEMED. The solution was shaken and poured quickly into the gel plates leaving a margin of 2.0 to 3.0 cm on upper side. The gel was allowed to polymerize for 15 minutes.

ii) Stacking gel (5%): It was prepared by mixing 2.8 ml of solution I, 1.5 ml of solution III, 7.2 ml of double distilled water, 150 µl ammonium per sulphate (APS) and 30 µl TEMED. Pour the solution on to the top of the resolving gel and immediately a comb was inserted to form the wells of 1.5 cm depth taking care not to trap the air bubbles underneath the comb. The gel was allowed to polymerize, then the comb was removed carefully and the wells were rinsed with distilled water.

c) Electrophoresis

The upper and lower reservoirs of electrophoretic apparatus were filled with electrode buffer. Then 50 µl of esterase extract was loaded into the wells of stacking gel using micropipette. A current of 1.5 mA per well with a voltage of 80 was applied until the tracking dye crossed the stacking gel. Later the current was increased to 2 mA per well and voltage up to 100. The electrophoresis was stopped when the tracking dye reached the bottom of the resolving gel, which took five to six hours.

d) Staining of gel

Immediately after removing the gels from the electrophoresis unit, gels were presoaked in 0.5 M phosphate buffer for one hour. Staining solution was prepared by dissolving 200 mg fast blue RR salt per 250 ml of 0.5 M phosphate buffer. This solution was stirred continuously for 30-45 minutes on magnetic stirrer. While stirring, the beaker was covered with brown paper cover to avoid exposure to the light. Two hundred milligram of α-napthyl acetate was dissolved in 2-3 ml of chilled acetone and it was stored in refrigerator until the use. Just before staining, already prepared α-napthyl acetate solution was added to fast blue RR salt buffer by constant stirring and immediately this reaction mixture was added to gel tray and shaking continued for 1-2 hours till the appearance of the dark brown bands. Gel was preserved in 30 per cent glycerol till documentation.

Result and discussion

Protein Electrophoregrams

Electrophoretic methods differ in the manner of protein extraction and in the subsequent conditions of separation. Although traditional method of varietal characterization by the assessment of descriptors based on UPOV guidelines and the grow out test for genetic purity testing, it is cumbersome, tedious and time consuming process. Besides, this method usually record expression of morphological characters at different growth stages of the crop. The expression of these morphological characters is not stable over the season or year or location, due to high interaction with environment. The phenological characters are influenced significantly by environmental fluctuations (Higgins *et al* 1981) [4]; whereas, qualitative traits are limited and require specific time to judge the expression for distinctness. Moreover, it becomes difficult to distinguish closely related varieties by morphological characters alone as the phenotypic differences within the species are too minute to discriminate. Ideally the differences between cultivars should be based on gene differences, but direct comparison of genes is difficult and time consuming. The differences can be measured by comparing the product of gene activity i.e. by using protein as genotype marker. Proteins being the direct gene product reflect the genomic composition of genotypes accurately to some extent and therefore, are ideal for genotyping distinctness. Seed proteins are the product of structural genes and present comparatively in large amount and could be easily and rapidly extracted. Therefore, the electrophoretic identification of seed proteins provides a powerful and convenient way of plant genotype identification. (Sharma and Maloo, 2006) [20].

Protein was extracted and separated by SDS– PAGE method and attempted to characterize cluster bean genotypes based on the electrophoretic banding pattern. The detailed electrophorograms of proteins of genotypes (1 to 10) is presented in Fig. 1 and while for genotypes (11 to 20) in Fig. 2.

Entire protein banding profile was divided into seven regions (A to G) based on its decreasing order of molecular weight (Region A: > 97.4 KD; Region B: 66.0 to 97.4 KD; Region C: 43.0 to 66.0 KD; Region D: 29.0 to 43.0 KD; Region E: 20.0 to 29.0 KD; Region F: 14.3 to 29.0 KD; Region G: < 14.3 KD). The presence or absence of specific band or group of bands, their relative intensities were taken as the criteria to characterize the genotypes. The banding pattern thus obtained

exhibited qualitative variation (a particular band as designated by its Rm values was present in the electrophorogram of one genotype but absent in that of another) quantitative variation (a particular band was observed in the electrophorogram of two or more different genotypes but differs in staining intensity). A significant differences was observed in the pattern of protein bands of studied genotypes. The genotypes differed in the number of bands, their relative mobility (Rm) and intensity. The proteins separated on acrylamide gel could be distinguished and grouped based on the number of bands, their relative mobility and intensity (Table 2 and 3). By using SDS-PAGE, the total soluble protein could be fractioned into 16 bands, which showed heterogeneity among different genotypes. The Rm values for 16 bands scored ranged between 0.160 and 0.933. The consistent difference in the protein profile suggests that each genotype has a reproducibly stable profile as a consequence of its specific gene arrangement (Ladizinsky, 1975) [10]. The differences were either in the total number of bands present, location of bands or intensity of bands or it can be even presence or absence of three categories of bands namely dense, medium light. The total number of bands present in different genotypes ranged from 5 to 13. The genotypes GG-1, GG-2 exhibited maximum number of bands (13 and 12) followed by HG-75 and IC-103060 (11). Least number of bands (5) exhibited in IC-116577 genotype. Band number 10 was present in all genotypes except genotype PNB and HG-75.

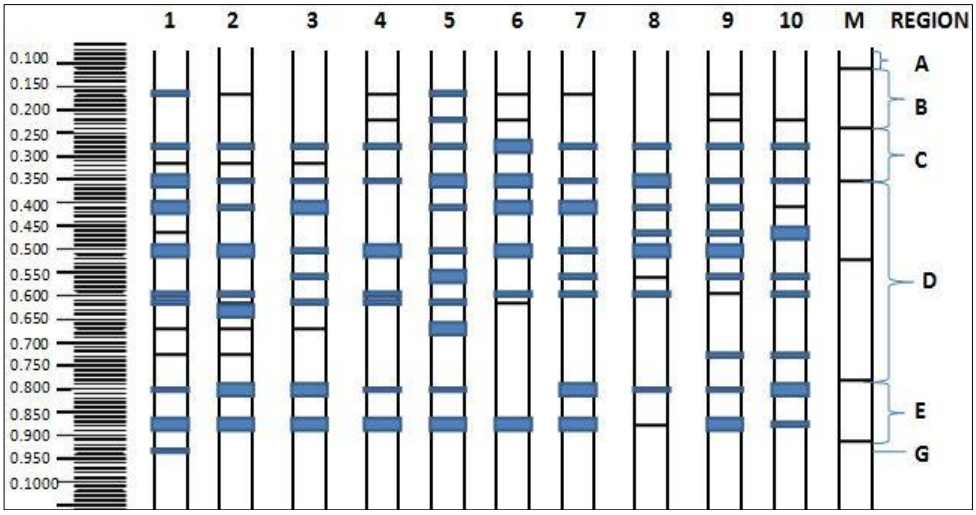

Fig 1: Zymogram of total soluble protein of cluster bean genotypes (1-10)

Genotypes					
1	:	GG-1	6	:	HG-2-20
2	:	GG-2	7	:	IC- 102828
3	:	PNB	8	:	IC-102853
4	:	HG-365	9	:	IC- 103060
5	:	HG-75	10	:	IC- 116523

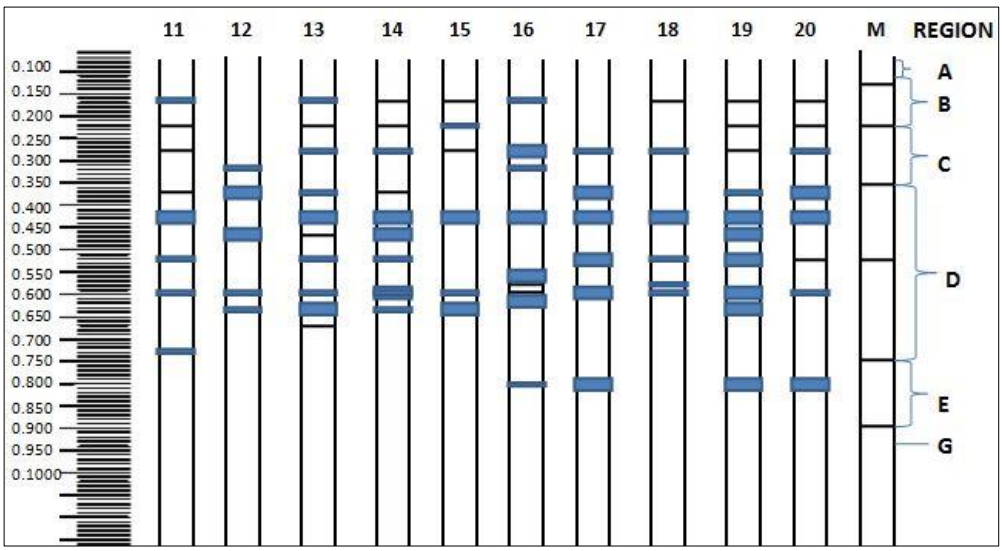

Fig 2: Zymogram of total soluble proteins of cluster bean genotypes (11-20)

Genotypes					
11	:	IC- 116525	16	:	IC-116612
12	:	IC- 116577	17	:	IC-116626
13	:	IC- 116594	18	:	IC-116627
14	:	IC- 116601	19	:	IC-116633
15	:	IC-116609	20	:	IC-116645

Table 2: Band intensity and relative mobility of total soluble proteins in cluster bean (genotypes 1-10)

Region	Rm Value	Band No.	Cultivars									
			1	2	3	4	5	6	7	8	9	10
A	-	-	-	-	-	-	-	-	-	-	-	-
B	0.160	1	++	+	+	+	++	+	+	+	+	-
	0.220	2	-	-	-	+	++	+	-	-	+	+
C	0.293	3	++	++	++	++	++	+++	++	++	++	++
	0.327	4	+	+	+	-	-	-	-	-	-	-
D	0.360	5	+++	++	++	++	+++	+++	++	+++	++	++
	0.420	6	+++	++	+++	-	++	+++	+++	++	++	+
	0.467	7	+	-	-	-	-	-	-	++	++	+++
	0.513	8	+++	+++	++	+++	++	+++	++	+++	+++	-
E	0.567	9	-	-	++	-	+++	-	++	+	++	++
	0.600	10	++	++	-	++	-	++	++	++	+	++
	0.620	11	++	+++	++	++	++	+	-	-	-	-
	0.673	12	+	+	+	-	+++	-	-	-	-	-
	0.733	13	+	+	-	-	-	-	-	-	++	++
F	0.800	14	++	+++	+++	++	++	-	+++	++	++	+++
	0.867	15	+++	+++	+++	+++	+++	+++	+++	+	+++	++
G	0.933	16	++	-	-	-	-	-	-	-	-	-

Band intensity: Absent (-); Low intensity (+); Medium intensity (++); High intensity (+++)

1: GG-1	2: GG-2	3: PNB	4: HG-365	5: HG-75
6: HG-2-20	7: IC- 102828	8: IC-102853	9: IC- 103060	10: IC- 116523

Table 3: Band intensity and relative mobility of total soluble proteins in cluster bean (genotypes 11-20)

Region	Rm Value	Band No.	Cultivars									
			11	12	13	14	15	16	17	18	19	20
A												
B	0.160	1	++	-	++	+	+	++	-	+	+	+
	0.220	2	+	-	+	+	++	-	-	-	+	+
C	0.293	3	+	-	++	++	+	+++	++	++	+	++
	0.327	4	-	++	-	-	-	++	-	-	-	-
D	0.360	5	+	+++	++	+	-	-	+++	-	++	+++
	0.420	6	+++	-	+++	+++	+++	+++	+++	+++	+++	+++
	0.467	7	-	+++	+	+++	-	-	-	-	+++	-
	0.513	8	++	-	++	++	-	-	+++	++	+++	+
E	0.567	9	-	-	-	-	-	+++	-	++	-	-
	0.600	10	++	++	++	+++	++	+++	+++	++	+++	++
	0.620	11	-	++	+++	++	+++	-	-	-	+++	-
	0.673	12	-	-	+	-	-	-	-	-	-	-
	0.733	13	++	-	-	-	-	-	-	-	-	-
F	0.800	14	-	-	-	-	-	++	+++	-	+++	+++
	0.867	15	-	-	-	-	-	-	-	-	-	-
G	0.933	16	-	-	-	-	-	-	-	-	-	-

Band intensity: Absent (-); Low intensity (+); Medium intensity (++); High intensity (+++)

11: IC- 116525	12: IC- 116577	13: IC- 116594	14: IC- 116601	15: IC-116609
16: IC-116612	17: IC-116626	18: IC-116627	19: IC-116633	20: IC-116645

Region A (> 97.4 KD; phosphorylase b)
There were no bands in the region A in all the genotypes.

Region B (66.0 to 97.4 KD; Bovine Serum Albumin)
It was categorized into two bands (band No. 1 and 2) with Rm values of 0.160 to 0.220.

Band No. 1: characterized into two forms of intensity. Medium intensity band was appeared in GG-1, HG-75, IC-116525, IC- 116594, IC-116612 while, low intensity band was appeared in GG-2, HG-365, HG-2-20, IC- 102828, IC-103060, IC- 116601, IC-116609, IC-116627, IC-116633, IC-116645. However, it was not appeared in PNB, IC-102853, IC- 116523, IC- 116577 and IC-116626.

Band No. 2: composed of two types of intensity. Medium intensity band was present in HG-75, IC-116609 whereas, low intensity band was noticed in HG-365, HG-2-20, IC- 103060, IC- 116523, IC- 116525, IC- 116594, IC- 116601, IC-116633, IC-116645 and absent in GG-1, GG-2, PNB, IC- 102828, IC-102853, IC- 116577, IC-116612, IC-116626, IC-116627.

Region C (43.0 to 66.0 KD; Ovalbumin)
In this region two bands (band No. 3 and 4) were observed with Rm values from 0.293 to 0.327.

Band No. 3: categorized into three forms of intensity. Low intensity band was appeared in IC- 116525, IC-116609, IC-116633 while, medium intensity band was present in GG-1, GG-2, PNB, HG-365, HG-75, IC- 102828, IC-102853, IC-103060, IC- 116523, IC- 116594, IC- 116601, IC-116626, IC-116627, IC-116645. Band three of high intensity was present in HG-2-20, IC-116612. However, it was absent in IC-116577.

Band No. 4: categorized into two forms. Low intensity band was present in GG-1, GG-2, PNB while, medium intensity band was appeared in IC- 116577, IC-116612 absent in HG-365, HG-75, HG-2-20, IC- 102828, IC-102853, IC- 103060, IC- 116523, IC- 116525, IC- 116594, IC- 116601, IC-116609, IC-116626, IC-116627, IC-116633, IC-116645.

Region D (29.0 to 43.0 KD; Carbonic Anhydrase)
It was composed of four bands (band No. 5, 6, 7, 8) with the Rm value 0.360, 0.420, 0.467 and 0.513 respectively.

Band No. 5: characterized into three forms of intensity. Low intensity band was appeared in IC- 116525, IC- 116601 while, medium intensity band was present in GG-2, PNB, HG-365, IC- 102828, IC- 103060, IC- 116594, IC-116633, IC- 116523. However the genotypes, GG-1, HG-75, HG-2-20, IC-102853, IC- 116577, IC-116626, and IC-116645 exhibited high density band. Band number five was absent in IC-116609, IC-116612 and IC-116627.

Band No. 6: categorized into three forms of intensity. Low intensity band was present in genotype IC- 116523 while, medium intensity band was appeared in genotypes GG-2, HG-75, IC-102853 and IC- 103060. While genotypes, GG-1, PNB, HG-2-20, IC- 102828, IC- 116525, IC- 116594, IC-116601, IC-116609, IC-116612, IC-116626, IC-116627, IC-116633 and IC-116645 exhibited high density band. It was not appeared in genotypes HG-365 and IC- 116577.

Band No. 7: band number seven varied with three forms of intensity. Genotypes GG-1 and IC- 116594 exhibited low intensity band while, medium intensity band was appeared in IC-102853 and IC- 103060. Band number seven of high intensity was present in genotypes IC- 116523, IC- 116577, IC- 116601 and IC-116633. It was absent in genotypes GG-2, PNB, HG-365, HG-75, HG-2-20, IC- 102828, IC- 116525, IC-116609, IC-116612, IC-116626, IC-116627 and IC-116645.

Band No. 8: characterized into three forms of intensity. Low intensity band was marked in only one genotype IC-116645. Medium intensity band was appeared in PNB, HG-75, IC-102828, IC- 116525, IC- 116594, IC- 116601 and IC-116627 while, genotypes GG-1, GG-2, HG-365, HG-2-20, IC-102853, IC- 103060, IC-116626 and IC-116633 exhibits high intensity bands. It was absent in IC- 116523, IC- 116577, IC-116609 and IC-116612.

Region E (20.0 to 29.0 KD; Soybean Trypsin Inhibitor)
It was characterized in to five bands (9, 10, 11, 12, and 13) with Rm value 0.567, 0.600, 0.620, 0.673 and 0.733 respectively.

Band No. 9: it is characterized as three differential forms. Low intensity band was appeared in only one genotype IC-102853. The genotypes PNB, IC- 102828, IC- 103060, IC-116523 and IC-116627 exhibited medium intensity band whereas, high intensity band was present in HG-75 and IC-116612. Band number nine was absent in GG-1, GG-2, HG-365, HG-2-20, IC- 116525, IC- 116577, IC- 116594, IC-116601, IC-116609, IC-116626, IC-116633 and IC-116645

Band No.10: It exhibited three differential forms of intensity. Only one genotype IC- 103060 marked low intensity band whereas, genotypes GG-1, GG-2, HG-365, HG-2-20, IC-102828, IC-102853, IC- 116523, IC- 116525, IC- 116577, IC-116594, IC-116609, IC-116627 and IC-116645 exhibited medium intensity band. Band number ten of high intensity was present in IC- 116601, IC-116612, IC-116626 and IC-116633 and absent in PNB and HG-75.

Band No.11: It is characterized into three forms of intensity. Genotype HG-2-20 exhibited low intensity band while, genotypes GG-1, PNB, HG-365, HG-75, IC- 116577 and IC-116601 recorded medium intensity band. Band number eleven of high intensity was present in genotypes GG-2, IC- 116594, IC-116609 and IC-116633. It was absent in IC- 102828, IC-102853, IC- 103060, IC- 116523, IC- 116525, IC-116612, IC-116626, IC-116627 and IC-116645

Band No.12: It was composed of two different forms of intensity. Low intensity band was appeared in genotypes GG-1, GG-2, PNB and IC- 116594 while, only one genotype HG-75 exhibited high intensity band. It was absent in genotypes HG-365, HG-2-20, IC- 102828, IC-102853, IC- 103060, IC-116523, IC- 116525, IC- 116577, IC- 116601, IC-116609, IC-116612, IC-116626, IC-116627, IC-116633 and IC-116645.

Band No.13: it was classified into three different forms of intensity. Low intensity band was appeared genotypes GG-1 and GG-2 while, medium density band was present in genotypes IC- 103060, IC- 116523 and IC- 116525. It was absent in PNB, HG-365, HG-75, HG-2-20, IC- 102828, IC-102853, IC- 116577, IC- 116594, IC- 116601, IC-116609, IC-116612, IC-116626, IC-116627, IC-116633 and IC-116645.

Region F (14.3 to 29.0 KD; Lysozyme)
This region was characterized by two bands (band No. 14 and 15) with Rm value of 0.800 and 0.867.

Band No.14: It was composed of two forms of intensity. Genotypes GG-1, HG-365, HG-75, IC-102853, IC- 103060, IC-116612 exhibited medium intensity bands while, genotypes GG-2, PNB, IC- 102828, IC- 116523, IC-116626, IC-116633 and IC-116645 showed high density bands. However, it was absent in HG-2-20, IC- 116525, IC- 116577, IC- 116594, IC- 116601, IC-116609 and IC-116627.

Band No.15: it varied with three forms of intensity. Genotypes GG-1, GG-2, PNB, HG-365, HG-75, HG-2-20, IC- 102828 showed high density band in this region while, genotypes IC-102853 and IC- 116523 exhibited low and medium intensity band respectively. It was absent in genotypes IC- 116525, IC- 116577, IC- 116594, IC- 116601, IC-116609, IC-116612, IC-116626, IC-116627, IC-116633 and IC-116645.

Region G (< 14.3 KD; Daltons)
This region consists of only one medium intensity band (band No. 16) with Rm value 0.933 and present only in one genotype GG-1 and absent in remaining all genotypes.
In the genotypes having same number of bands, the location and intensity of bands varied, this is useful for clear differentiation of the cluster bean genotypes. Electrophoretic differences in protein banding pattern of different genotypes enable us to identify a particular genotype with the presence or absence of specific position of band and also the intensity of band which could be used as genetic marker (Liang *et al* 2004) [13]. These specific protein bands migrating to the same distance gave some evidence of homology in molecular

structure and function (Javaid *et al* 2004) [9]. Thus, the results of the electrophoretic banding pattern of the protein profile suggested that the specific genotype could be differentiated by either based on the position or intensity of bands. But not on the numbers as some of the genotypes expressed similar number of bands. In conclusion, this electrophoresis of soluble seed protein was able to distinguish all the 20 genotypes and can be employed effectively for characterization of cluster bean genotypes. Many of the research workers demonstrated use of protein electrophoresis as a powerful tool to identify the crop cultivars (Ismet Berber and Fikret Yasar, 2011[8] in bean; Rao *et al.,* 2011[15] in groundnut; Sossah *et al.,* 2014[21] in sweet potato).

Isozymes polymorphism

Since the discovery of isozyme techniques (Hunter and Markert, 1957) [5] and the isozyme variation within populations of Drosophila (Lewontin and Hubby, 1966) [12], the technique has been successfully applied to various related fields of genetic studies such as germplasm management (Bretting and Widrlechner 1995) [1]. Electrophoretic separation and visualization of isozyme polymorphism has also been the most commonly used procedures to estimate plant genetic diversity in several plant genera.

In general, the isozyme loci for a given enzyme are highly conserved in a given taxa. The number of loci which are polymorphic (% polymorphic loci) and the mean number of alleles per polymorphic loci are two of the genetic diversity parameters which give an estimate of the extent of diversity maintained by plant species (Hamrick and Godt 1997) [3]. Esterases (EST) isozymes have been considered as an effective tool for characterization as this isozyme is known to exhibit high polymorphism (Varier *et al*., 1999) [23]. In the present study also polymorphism in esterase isozyme profiles with respect to number, position and intensity of bands was noticed among genotypes. Based on these results, all the 20 cluster bean genotypes could be differentiated. The zymogram of esterase (EST) is presented in Fig. 3 and Fig. 4. The difference in number of bands, position and intensity in esterase zymogram has been considered to distinguish genotypes for characterization. A total number of 5 bands were noticed among the 20 tested genotypes in the zymogram of esterase (Table 4).

Band number 1 (Rm: 0.136): It was present in all studied genotypes with varying intensity. The genotypes GG-1, GG-2, PNB, HG-365, HG-75, HG-2-20, IC- 102828, IC-102853, IC-103060, IC- 116523 exhibited medium intensity band with Rm value 0.136 and genotypes IC- 116525, IC- 116577, IC-116594, IC- 116601, IC-116633, IC-116645 recorded high intensity bands, while genotypes IC-116609, IC-116612, IC-116626 exhibited low intensity bands.

Band number 2 (Rm: 0.300): It was categorized into two differential intensities of bands. The genotypes namely GG-1, GG-2, PNB, HG-75, IC- 116525, IC- 116577, IC- 116594, IC- 116601, IC-116609, IC-116612, IC-116626, IC-116627, IC-116633, IC-116645 exhibited high intensity band, whereas genotype IC-102853 showed medium intensity band at Rm value 0.300 and it was absent in genotypes HG-365, HG-2-20, IC- 102828, IC- 103060, IC- 116523.

Band number 3: It does not vary with respect to banding intensity and present in genotypes GG-1, HG-75, IC- 116525, IC- 116577, IC-116627, IC-116633, IC-116645 at Rm value 0.355 and absent in rest of the genotypes.

Band number 4 and 5 were monomorphic bands, present in all genotypes with Rm values ranging from 0.409 to 0.527. The band number 4 (Rm value 0.409) does not varied with banding intensity and low intensity bands appeared in all the genotypes. The intensity of band number 5 (Rm: 0.527) was medium and present in all genotypes.

In conjunction with these, the findings of castor (Varier *et al* 1999) [23], and grass pea (Roy *et al.,* 2001) [16] were also explored the possibility of use of esterase isozyme to differentiate the cultivars. Thus, the present investigation suggested that esterase isozyme can be used as an effective tool for characterizing genotypes particularly in those cases where morphological distinction is not possible. Similar results were reported by Pratibha Brahmi *et al* (2004) [14] in cluster bean and Shantharaja (2011) [19] in chickpea.

From these results, it is clear that there is enough variation in protein and esterase isozyme profile among genotypes and could be considered as a tool for genotype identification and screening for various traits. Characterization of genotypes for protection, identification and varietal purity testing has attained much importance in seed production programme of almost all-major agricultural crops. All sectors of seed industry benefits from the ability to assess cultivar purity and identity. Based on the zymograms of total soluble proteins, Region D (29.0 to 43.0 KD), E (20.0 to 29.0 KD), F (14.3 to 29.0 KD) and G (< 14.3 KD) were found more useful to distinguish most of the genotypes studied, as the banding pattern was distinct for each genotype in these regions. The isozyme electrophoresis of cluster bean genotypes for esterase produced marked differences in the banding pattern and their intensities that could be used for the identification of cluster bean genotypes. The electrophoretic banding pattern of proteins and esterase isozyme served as potential tool in characterizing and identification of all the selected cluster bean genotypes.

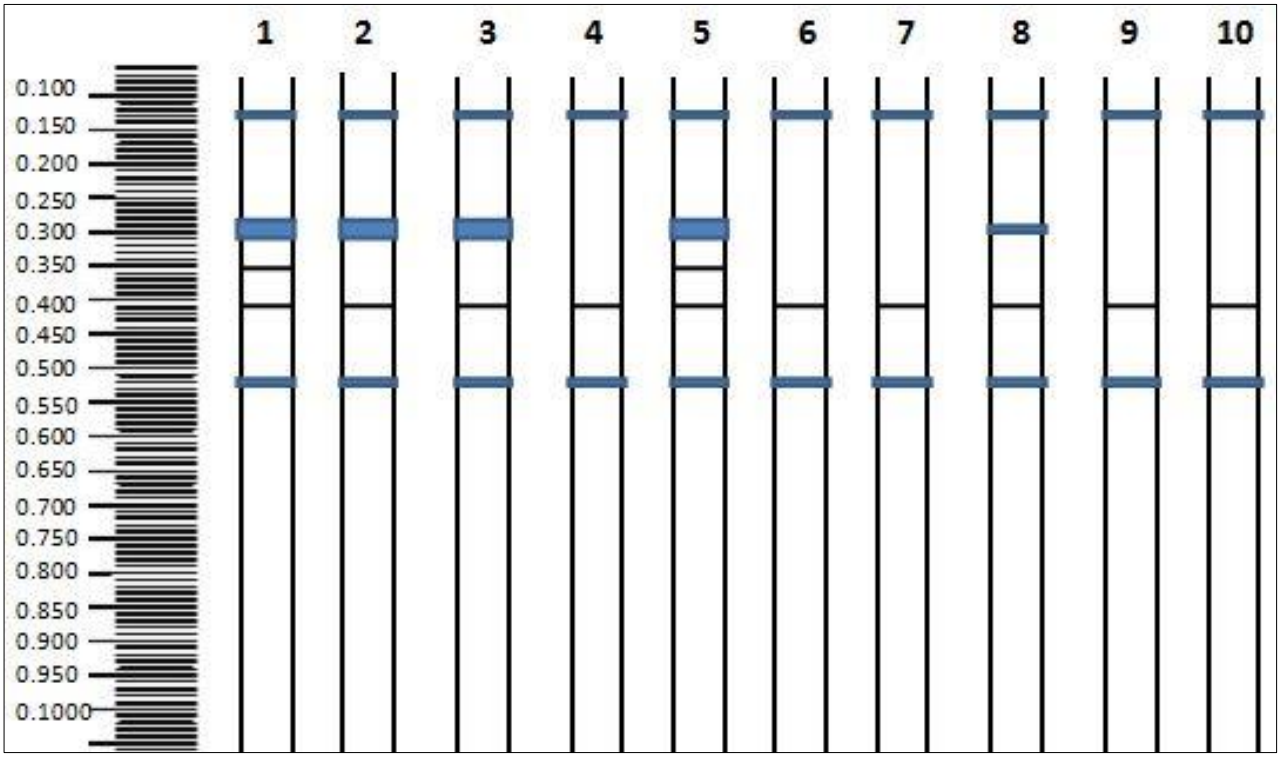

Fig 3: Zymogram of esterase isozyme of cluster bean genotypes (1-10)

Genotypes					
1	:	GG-1	6	:	HG-2-20
2	:	GG-2	7	:	IC- 102828
3	:	PNB	8	:	IC-102853
4	:	HG-365	9	:	IC- 103060
5	:	HG-75	10	:	IC- 116523

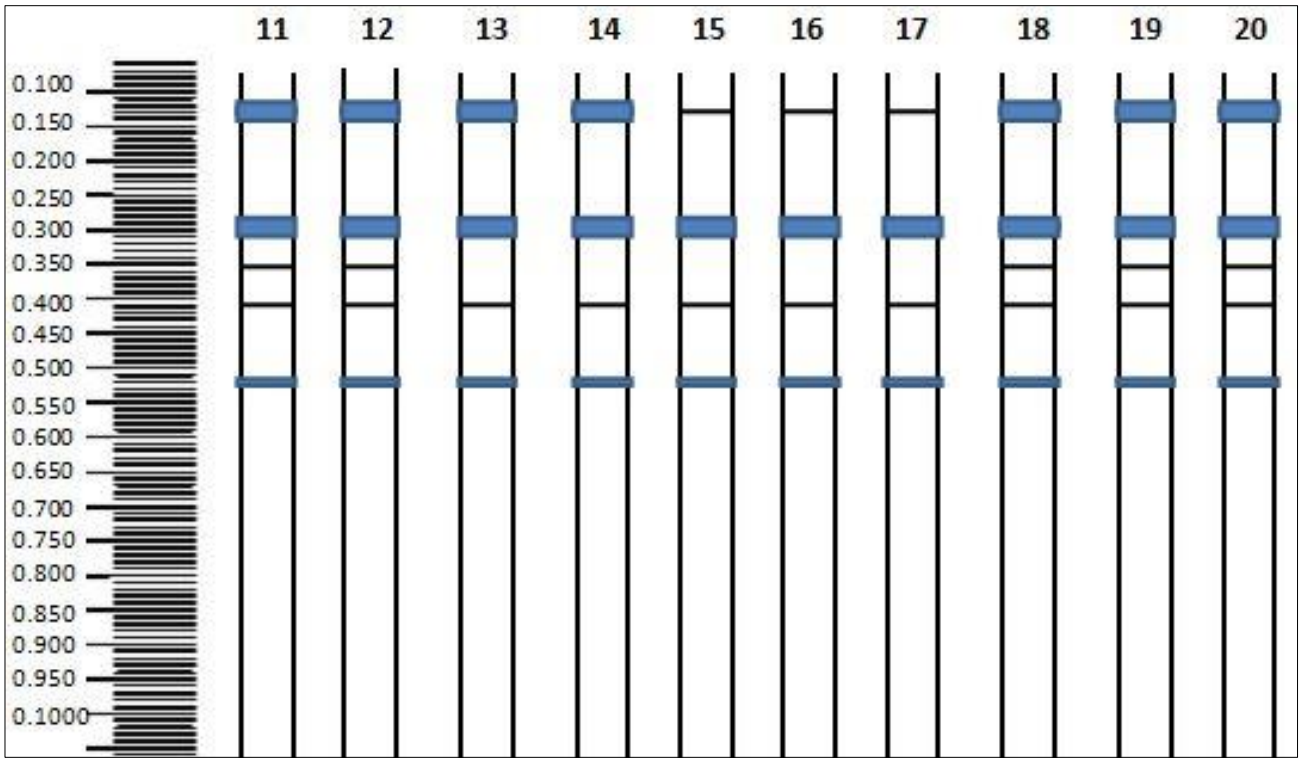

Fig 4: Zymogram of esterase isozyme of cluster bean genotypes (11-20)

Genotypes					
11	:	IC- 116525	16	:	IC-116612
12	:	IC- 116577	17	:	IC-116626
13	:	IC- 116594	18	:	IC-116627
14	:	IC- 116601	19	:	IC-116633
15	:	IC-116609	20	:	IC-116645

Table 4: Band intensity and relative mobility of esterase isozyme in cluster bean genotypes

	Genotypes (1 to 10)										
Band No	**RM Value**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0.136	++	++	++	++	++	++	++	++	++	++
2	0.300	+++	+++	+++	-	+++	-	-	++	-	-
3	0.355	+	-	-	-	+	-	-	-	-	-
4	0.409	+	+	+	+	+	+	+	+	+	+
5	0.527	++	++	++	++	++	++	++	++	++	++
6	0.673	-	-	-	-	+	-	-	-	-	-
	Genotypes (11 to 20)										
Band No	**RM Value**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	0.136	+++	+++	+++	+++	+	+	+	+++	+++	+++
2	0.300	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3	0.355	+	+	-	-	-	-	-	+	+	+
4	0.409	+	+	+	+	+	+	+	+	+	+
5	0.527	++	++	++	++	++	++	++	++	++	++
6	0.673	-	-	-	-	-	+	+	+	+	+

Band intensity: Absent (-); Low intensity (+); Medium intensity (++); High intensity (+++)

1: GG-1	2: GG-2	3: PNB	4: HG-365	5: HG-75
6: HG-2-20	7: IC- 102828	8: IC-102853	9: IC- 103060	10: IC- 116523
11: IC- 116525	12: IC- 116577	13: IC- 116594	14: IC- 116601	15: IC-116609
16: IC-116612	17: IC-116626	18: IC-116627	19: IC-116633	20: IC-116645

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