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Perform cluster analysis to assess the differences in the cowpea mutants

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Abstract
The study was carried out on 38 mutants derived from EMS (0.5%) mutagenesis of two cowpea varieties RC-19 and RC-101 for determine the variation in their profile of seed storage protein subunits through sodium dodecyl sulphate - polyacryl amide gel electrophoresis (SDS-PAGE). The protein bands of region I and II were monomorphic and intensely stained and identified to have a MW between 97.4 kD to 43 kD. Only certain bands of region III and IV were polymorphic. The band of region V was monomorphic. The binary data generated from the polymorphic bands over the genotypes were used to compute Jaccard's similarity coefficients using NTSYS-pc software. The similarity matrix thus prepared was used to construct a dendrogram by UPGMA. The dendrogram distributed the 40 genotypes in 11 clusters. About 50% mutants were in one cluster and their parents in another clusters. One of the mutants (number 30) was placed in a unique cluster. Clustering seemed to be independent of the seed attributes studied. The protein content of the mutants was invariably reduced as compared to their parents and ranged from 21-30.3%. It was concluded that results of the studies may be useful in selection of mutants for hybridization programme for possible improvement of the quality of seed storage proteins in cowpea.

Keywords: Cowpea mutant, seed storage protein, SDS-PAGE, clustering, protein bands

Introduction
Cowpea [*Vigna unguiculata* (L.) Walp] is an annual, self-pollinated, leguminous crop (Mackie and Smith, 1935) [4] with a chromosome number of 2n=2x=22 (Darlington and Wyile, 1955) [2] and belongs to family Fabaceae (earlier Leguminoceae). Cowpea is native to India (Vavilov, 1949) [13] but tropical and Central Africa is also considered as secondary centers of origin where wild races are found even now (Ng and Marechal, 1985) [5].

There are several diverse uses of cowpea due to which the varietal requirement in terms of plant type, seed type, maturity, pattern of use and growth are diverse from region to region. Therefore, cowpea breeding programme becomes more complex and no single variety can be suitable for all objectives (Barrett, 1987) [1]. Thus, there is need to develop varieties suitable for a specific region and /or use. However, production is constrained by low and variable grain yield, grain quality, susceptibility to diseases and pests and the absence of improved cultivars.

The genetic diversity in cowpea seems to be narrow in spite of substantial variation in seed color, seed proteins, plant type, pod type and seed size among cultivated cowpeas (Panella and Gepts, 1992; Vaillancourt *et al.* 1993; Pannella *et al.*, 1993) [6, 12, 7]. For an effective breeding programme the characterization of genetic diversity for making choice of parents for hybridization is important. While this aspect is routinely addressed in most crop breeding programmes, the nutritional aspect of food legumes, such as, cowpea is equally important. While the seed storage protein profile, on one hand, is an important consideration to be taken in account when drawing inferences from genetic diversity studies based only on morphological traits, such a protein profile, on the other hand, directly refers to its nutritional status.

The research work related to mutant characterization in cowpea using SDS-PAGE of storage seed protein is very scanty. The objective of the present investigation was therefore, to perform cluster analysis to assess the differences in the mutants of cowpea varieties RC-19 and RC-101 for this storage seed protein profile.

Material and Methods
The present investigation was carried out at the Central Laboratory, S.K.N. College of Agriculture, Jobner. Jobner is situated at an elevation 420 meters above mean sea level at 20^0 6' N and 75^0 25 'E.

The details of material and methods used in the present investigation are given below under separate heading.

Experimental material

A total of 40 genotypes of cowpea (*Vigna unguiculata*) comprising 38 mutants and two of their parents RC-101 and RC-19 were evaluated in the present study. These mutants were obtained from the Department of Plant Breeding and Genetics, at S.K.N. College of Agriculture, Jobner. The list of mutants of cowpea and their parents along with their seed characters are presented in (Table 1, Fig 1.).

Methods

Test weight (g)

A random sample of 100 seeds was drawn from each genotype and weighed on sensitive electronic balance and expressed in gram (g).

Seed volume (ml)

A random sample of 100 seeds was drawn from each genotype and immersed in a 100ml measuring cylinder, containing 10ml of distilled water. Rise in the meniscus in milliliters was recorded and divided to determine volume of a single seed.

Protein content (%)

For protein estimation of seeds, the total nitrogen content of the seed was determined by micro Kjeldahl method described by Peach and Tracey (1956) [8]. The total N content so estimated was multiplied by factor 6.25 to predict the protein content per 100 mg of dry weight of the seed. The resultant solution was titrated with N/28 HCL and amount of HCL required to neutralize the ammonia present in distilled solution was recorded. The nitrogen content was calculated as described by Sadasivam and Manickam (1996) [9].

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to procedure of Laemmli (1970) [3] with minor modification described by Tripathy *et al.* (2010) [11]. For protein extraction, seed coat and embryo were removed and cotyledons were ground and sieved to get a fine powder. Proteins were extracted by grinding first in 1ml of water followed by subsequent grinding in 1ml of 1M Nacl, respectively as described by Sharma (2012) [10]. Extracted protein samples (1ml) were transferred into Eppendrof tubes and centrifuged for 3 minutes at 10,000 rpm. One half milliliter of (0.5) supernatant was transferred into a fresh Eppendorf tube (1.5ml tube) and denatured with 0.5ml cracking buffer (0.2M Tris Hcl buffer P^H 6.8, 10% SDS, 20% glycerol, 10 Mm mercaptoethanol, 0.05% bromophenol blue). These samples were loaded into the wells of the polyacrylamide gel slab prepared for electrophoresis.

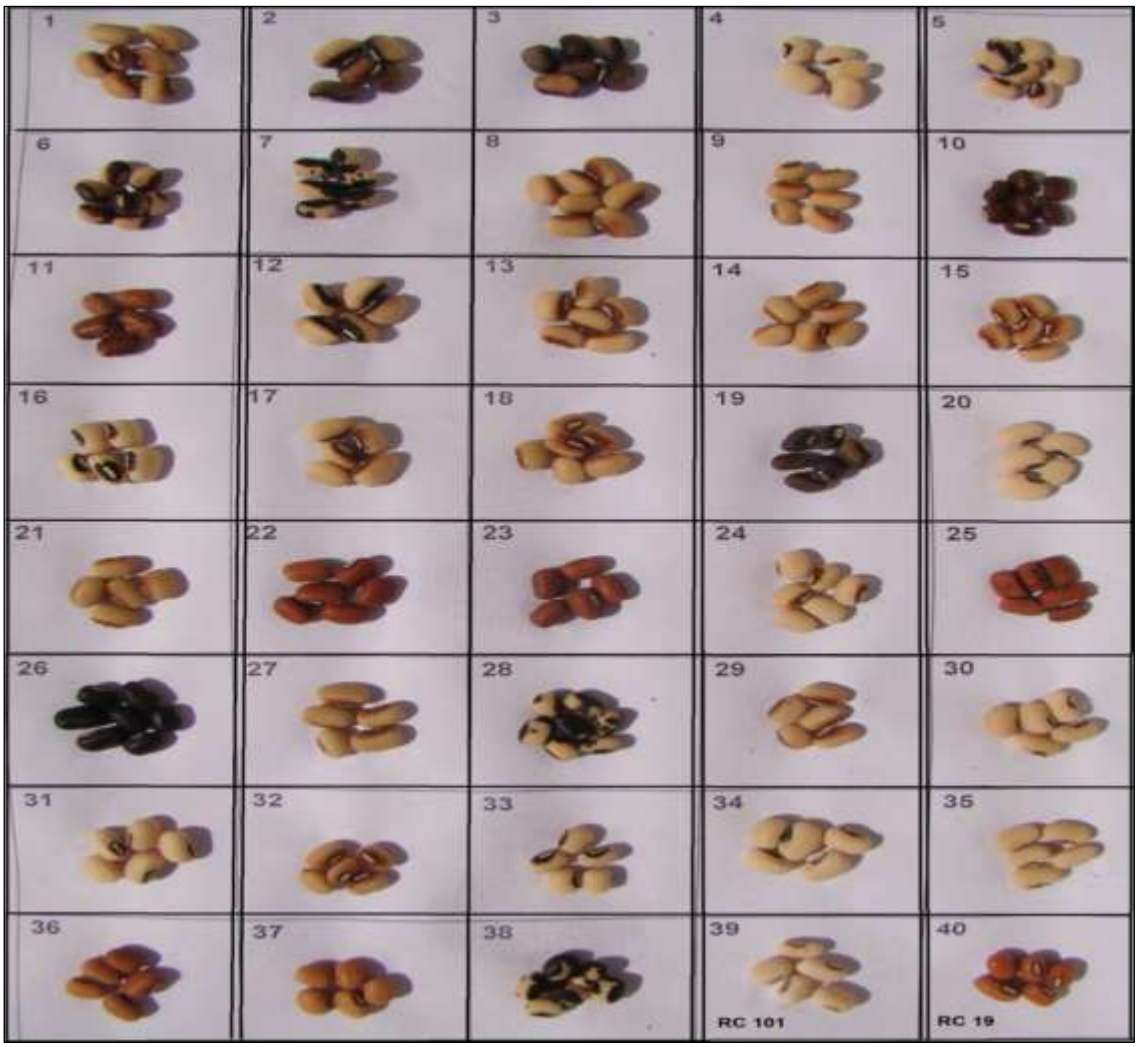

Fig 1: A view of seeds of different cowpea mutants of RC-101 and RC-19.

Results and Discussion

The present investigation employing SDS-PAGE of seed storage proteins was carried out on different mutants of cowpea variety RC-101 (white seeded) and RC-19 (light brown seeded). On account of their distinct seed coat color/ seed shape/ plant type these mutants have been investigated for variations in seed storage protein profile.

The protein extracts from the cotyledons of 40 genotypes (i.e. parent and 38 mutants) were prepared as described above and 14 samples loaded on a gel plate at a time along with marker protein in the first lane. The comb used in these experiments could develop 15 wells for loading the samples.

Win *et al.* (2011) [14] have also described a similar picture of electrophoregram in cowpea accessions of Myanmar and have identified 5 regions on the basis of banding pattern within the similar molecular weight range of 97kD to 15 kD. However, on the basis of results of protein band polymorphism, the results of the present study are at variance from those of Win *et al.* (2011) [14]. The marker protein has invariably shown 5 distinct protein bands of 205, 97.4, 66.0, 43.0 and 29.0 kD MW.

The Jaccard's similarity co-efficient between different accessions ranged between 0. 2 to 1.00 with a mean of 0.54. Considerable number of genotypes showed absolute similarity. Among the 40 genotypes (38 mutants + 2 parents), minimum genetic similarity (maximum diversity) value was associated with 38 cases of pairs whereas maximum similarity co-efficient values were associated with 171 cases of pairs. It is also seen that 34.26% of the pairs showed similarity coefficient values within the range of 0.2 to 0.3 indicating these genotypes carry deviations from the parents or mutants.

A dendrogram was constructed using Jaccard's similarity coefficients obtained for protein band binary data observed on the 40 genotypes of cowpea employing NTSYS-pc programme (Fig 2&3). The cluster analysis on the accessions revealed 11 distinct clusters. The salient finding of the clustering are described as follows:

1. At 0.5 similarity coefficient, three clusters could be identified, namely 1, 2 and 3. Cluster 1 included half of the mutants (mutants of both the parents). Cluster 2 included only one mutant i.e. 30 whereas in cluster 3 represented the rest of the mutants including both the parents.
2. At 0.7 similarity coefficient, 11 clusters could be seen. Mutant 17 was similar to RC-101 and the mutant 33 was similar to RC-19.
3. A comparison of the mutant's seed appearance with clusters showed no association between them. Even protein content / seed volume/ 100 seed weight seemed to have no relation with clustering because higher or lower magnitude for these traits were observed with the mutants in all the clusters.
4. Storage seed proteins seemed to be independent of seed characteristics studied.

The results thus demonstrate that the two parents are quite close to each other on the basis of seed storage protein banding pattern but about 50% of the mutants are quite distinct from the parents and similar among themselves. A separate dendrogram for mutants of RC-19 and that of RC-101 were prepared (Figs 2 and 3). It can be seen that, in case of 8 mutants of RC- 19 studied, all the mutants fell in five clusters where as in case of 30 mutants of RC-101 ten distinct clusters were visible (table 3). In case mutants of RC-19 there seemed an association between clusters and seed attributes studied (table 2).

When the banding pattern of the protein subunits of the mutants were compared among themselves and with other seed characters studied, for the three mottled seed coat color mutants, namely, 7, 28 and 38 there seemed to be a distinct relatively thick band of 97.4 kD protein associated (Fig.4). Speculatively, this protein may be associated with the mottled seed coat color. However, more studies are required to confirm this finding. Such studies may involve crossing these mutants with other and analyze for the presence of this band in the seed protein profile of F_2 individuals.

The results of present study have demonstrated that a large number of mutants of cowpea have deviated from their parents in the seed storage protein profile. This was substantiated by the dendrogram which revealed 5 clusters for mutants of RC-19 (Fig.2, table-2) and 10 clusters for mutants of RC-101 (Fig.3, table-3) which may be indicative of different loci which have been mutated.

Table 1: Two seed attributes of mutants of cowpea varieties RC-101 and RC-19

S. No.	Designation	100 Seed Weight (g)	Seed Volume (ml)	Parents
1.	A	9.43	9.4	RC-101
2.	B	8.98	10.4	RC-101
3.	C	8.18	8.4	RC-101
4.	D	8.95	9.4	RC-101
5.	E	6.18	6.4	RC-101
6.	F	4.75	5.4	RC-19
7.	G	5.91	6.4	RC-101
8.	H	10.10	9.4	RC-101
9.	I	5.96	6.9	RC-101
10.	J	4.99	5.4	RC-19
11.	K	5.82	6.4	RC-19
12.	L	9.24	8.4	RC-101
13.	M	8.67	7.4	RC-101
14.	N	7.45	6.4	RC-101
15.	O	7.20	7.4	RC-101
16.	P	9.54	9.4	RC-101
17.	Q	9.91	9.4	RC-101
18.	R	7.76	7.4	RC-101
19.	S	6.13	6.4	RC-101

20.	T	10.18	9.4	RC-101
21.	U	8.41	8.4	RC-101
22.	V	8.92	6.4	RC-19
23.	W	8.12	7.4	RC-19
24.	X	8.66	7.9	RC-101
25.	Y	4.56	5.4	RC-19
26.	Z	7.72	8.4	RC-101
27.	Aa	7.44	8.4	RC-101
28.	Bb	6.22	5.4	RC-101
29.	Cc	7.32	7.4	RC-101
30.	Dd	8.65	7.9	RC-101
31.	Ee	10.09	10.4	RC-101
32.	Ff	7.69	7.9	RC-101
33.	Gg	7.25	7.4	RC-101
34.	Hh	10.06	10.4	RC-101
35.	Ii	8.28	8.4	RC-101
36.	Jj	7.53	7.9	RC-19
37.	Kk	7.25	8.6	RC-19
38.	Ll	7.19	9.4	RC-101
39.	RC-19	7.20	6.4	RC-19
40.	RC-101	8.92	7.4	RC-101

Table 2: Seed attributes of mutants of cowpea variety RC-19

Cluster	No. of mutants	Seed attributes		
		100 Seed weight (g.)	Seed volume (ml.)	Protein content (%)
I	6	4.75	5.4	25.66
	10	4.99	5.4	25.74
	11	5.82	6.4	21.57
	25	4.56	5.4	28.43
II	22	8.92	6.4	21.62
	23	8.12	7.4	25.19
III	36	7.53	7.9	21.81
IV	37	7.25	8.6	24.58
V	RC-19	8.92	7.4	30.3

Table 3: Seed attributes of mutants of cowpea variety RC-101

Cluster	No. of mutants	Seed attributes		
		100 seed weight (g)	Seed volume (ml)	Protein content (%)
I	1	9.43	9.4	24.09
	2	8.98	10.4	24.55
	5	6.18	6.4	22.75
	7	5.91	6.4	23.62
	12	9.24	8.4	22.75
	34	10.06	10.4	26.25
	13	8.67	7.4	26.10
	15	7.20	7.4	27.56
	28	6.22	5.4	24.10
	24	8.66	7.9	22.50
	26	7.72	8.4	24.41
	19	6.13	6.4	21.78
	16	9.54	9.4	21.93
II	30	8.65	7.9	28.44
III	3	8.18	8.4	25.32
	4	8.95	9.4	26.24
	21	8.41	8.4	25.46
	27	7.44	8.4	21.81
IV	18	7.76	7.4	21.78
V	8	10.10	9.4	26.30
	38	8.28	8.4	26.26
	14	7.45	6.4	23.18
VI	9	5.96	6.9	28.99
VII	33	7.25	7.4	21.84
VIII	17	9.91	9.4	22.70
	RC-101	7.20	6.4	29.28
	31	10.09	10.4	24.94
	32	7.69	7.9	23.40

IX	20	10.18	9.4	24.50
	29	7.32	7.4	26.56
X	35	8.28	8.4	26.26

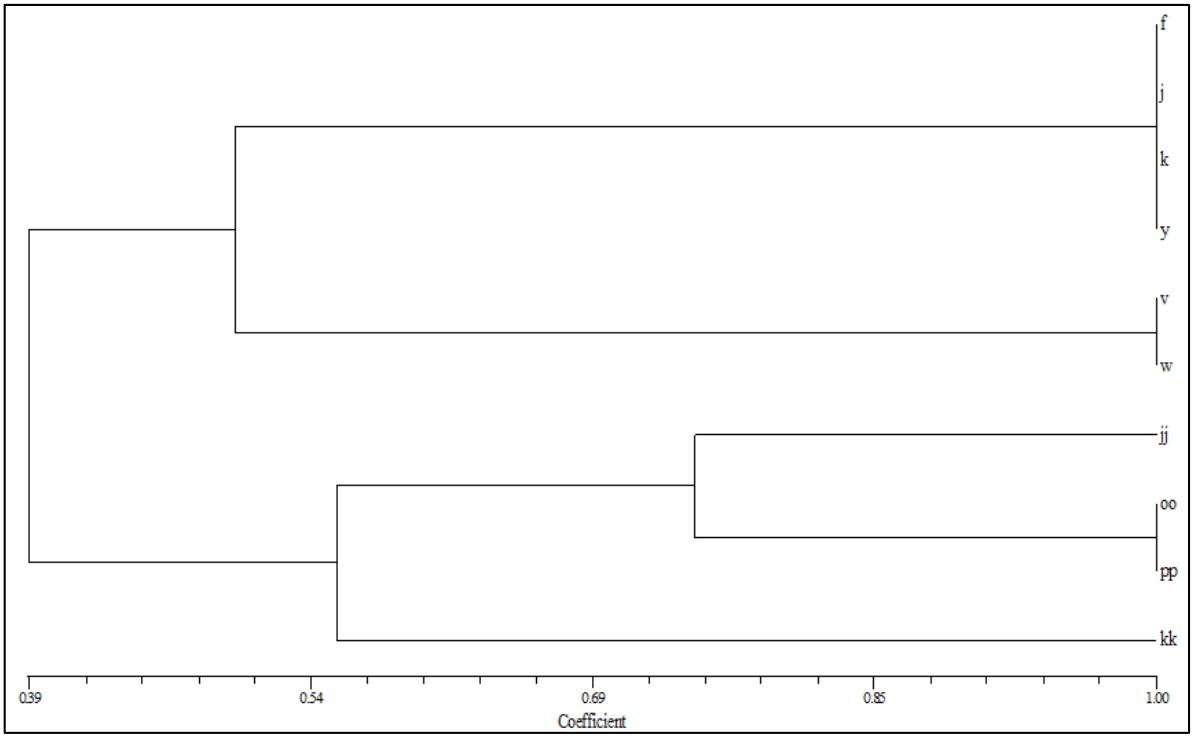

Fig 2: Dendrogram of the 8 cowpea mutants of RC-19 revealed by UPGMA cluster analysis of SDS-PAGE based genetic similarity estimates

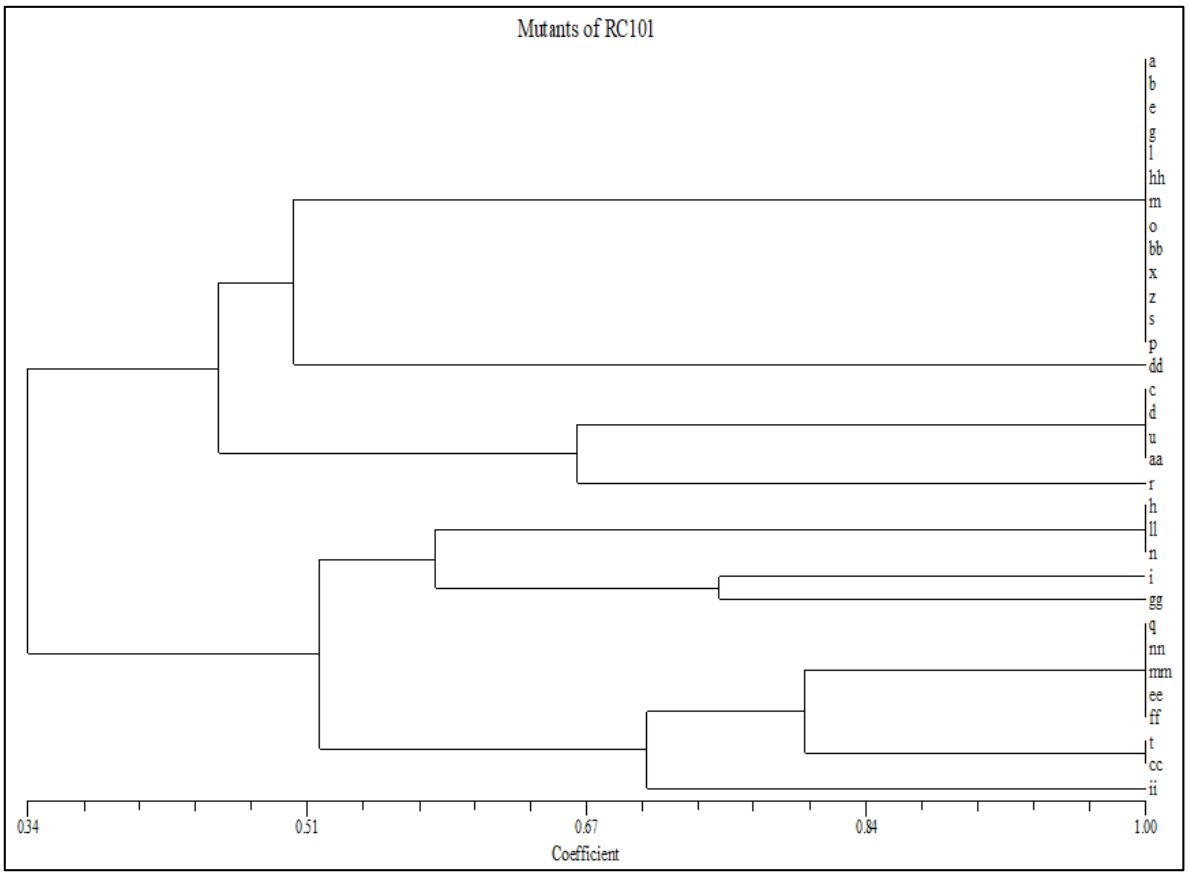

Fig 3: Dendrogram of the 30 cowpea genotypes (mutants of RC-101) revealed by UPGMA cluster analysis of SDS-PAGE based genetic similarity estimate

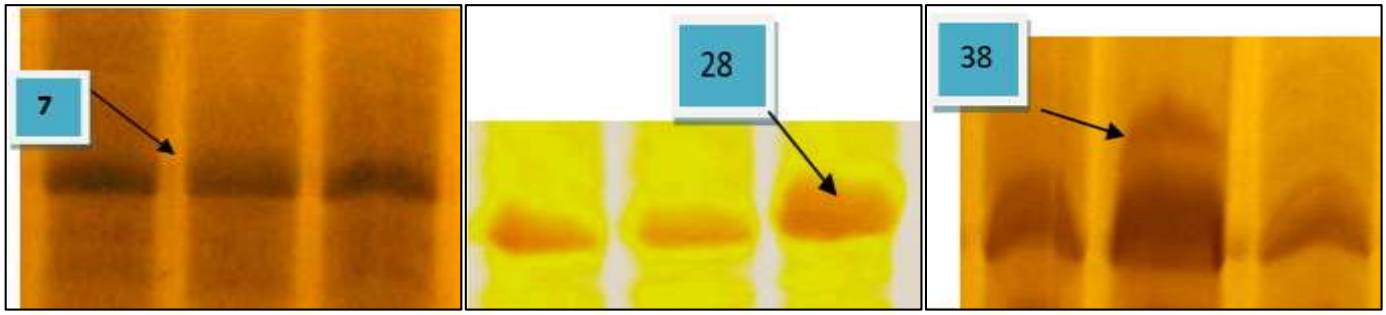

Fig. 4: Comparative view of 97.4 kD protein band of the three mutants with mottled seed coat color

Conclusion

On account of convincing discrete mutational changes that have occurred in the mutants studied, it would be plausible to further characterize these mutants for their nutrient contents and perform hybridization between the selected ones to explore the possibility of improving the nutritional quality in the recombinants.

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