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SDS-PAGE profiling of dormant lines in mung bean (*Vigna radiata* L. Wilczek)

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ABSTRACT

Mung bean is an important pulse crop because of its high protein content. But it is very much susceptible to in-situ germination. An attempt was made to develop such lines which are resistant to in-situ germination by crossing a wild form of mung bean with a cultivated variety and a total seed protein profiling for such lines along with parents was done. The SDS-PAGE of 20 mung bean dormant lines was done. It includes one derivative of the four-way cross, thirteen derivatives of three-way crosses, five cultivated varieties and one wild form of mung bean i.e. BWM-29 (progenitor of cultivated mung bean). The study revealed 10-11 detectable peptides whose molecular weight was found to be in the range of 19 kDa to 117 kDa. The total seed protein was isolated using TCA-Acetone buffer and separated on 10% SDS-PAGE gel. The scoring of resolved proteins was done and a zygogram was generated. The zygogram revealed three genetic clusters. The first cluster contains nine genotypes and the second and third clusters group four and six genotypes respectively. Cluster I consist of 13 breeding lines and Cluster II consists of five breeding lines. Cluster III and Cluster IV are solitarily consisting of one genotype each. The wild form (BWM-29) was clearly isolated into a single cluster. The derivative of the four way cross SPS-10 was also separated into a single cluster. Further, it was also revealed that breeding lines in the same cluster based on total seed protein profiling have common agronomic characteristics and such clusters with unique morphological and yield-associated traits could be identified for genetic improvement.

Keywords: *SDS-PAGE, Protein profiling, Vigna radiata, Zygogram, Dormant lines, kDa, Cluster analysis*

INTRODUCTION

Pulses are best known as "poor man's meat". Among the pulses, mung bean is a well-known crop in Asian countries. It is the third most important pulse crop after Bengal gram and Red gram. It is largely known as mung bean or green gram. It is mostly cultivated in countries like India, Pakistan, Bangladesh, Sri Lanka, Thailand, Laos, Cambodia, Vietnam, Indonesia, Malaysia, South China and Formosa. In Africa and the U.S.A., it is probably recent. It has been estimated that annual production is 2.5-3 million tons per year [16]. It is a warm-season crop that can grow during hot, wet seasons and be cultivated in the arid and semi-arid tropics [9, 14]. Important mung bean growing states in India are Orissa, Andhra Pradesh, Maharashtra, Karnataka and Bihar. India ranks third with an area of about 23.63 million ha with a total production of 14.56 million tonnes [2]. Maharashtra contributes 16.19% area with 13.46% share in the production of the nation (average of last ten years). It is having desirable characterstics like short duration, high protein and fewer anti nutritional factors.

The seed storage proteins are non-enzymatic and have the sole purpose of providing proteins (nitrogen and sulfur source) required during germination and establishment of a new plant. Albumins and globulins comprise the storage proteins of dicots, whereas prolamins and glutelins are major proteins in monocots. Seed protein patterns obtained by electrophoresis have been successfully used to resolve the taxonomic and evolutionary problems of several plants [3, 15, 17]. Analysis of seed protein can also provide a better understanding of the genetic affinity of the germplasm [17]. This method can also be used as a promising tool for differentiating the cultivars of a particular plant species. SDS-PAGE is also considered to be a practical and reliable method for species identification because seed storage proteins are largely independent of environmental fluctuation. Polymorphism in seed storage proteins has been associated with the geographical origin [4]. Seed storage protein analysis represents a valid alternative and/or improved approach to varietals identification. The SDS-PAGE proved to be a powerful tool for the study of genetic diversity which was observed in studied breeding lines that are dormant to *in-situ* germination. The biochemical composition of mung bean has been studied by a number of workers. Since the composition is influenced by genetic constituents, cultural practices, and environmental factors. It is, therefore, important to evaluate the

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locally grown cultivars in order to assess their nutritional quality.

In mung bean, SDS-PAGE cannot be used to identify genotypes on the basis of intra-specific variation because similar banding patterns were observed in some accessions that differed on the basis of characterization and evaluation; therefore, this technique might be more suitable to identify inter rather than intra-specific variation in Vigna spp. [4]. Keeping in the view of above facts, the present investigation was undertaken to determine the biochemical composition as well as storage protein profiling of twenty mung bean cultivars grown under rainfed conditions in the Badnapur region. Present results also depicted a high genetic diversity in the tested breeding lines based on electrophoresis of seed storage proteins as a dendrogram based on Electrophoretic data grouped the tested genotypes into four clusters.

Materials and Methods

Experimental materials

The material for the present study comprised 20 mung bean (*Vigna radiata* (L.) Wilczek) breeding lines were used which were found dormant to *in-situ* germination. The germination of these lines was tested consequently for two years both at field and laboratory conditions and those breeding lines found dormant for 15-25 days have been used for the study. Among the 20 mung bean breeding lines, five were the derivatives of three-way crosses ([(BM-4 × BWM-29)] × BM-2003-2), one was a derivative of four-way crosses ([(BM-4 × BWM-29) × BM-2003-2] × BM-2002-1), eight were derivatives of single crosses derived from an inter-specific cross (BM-4 × BWM-29) and five were checks and one wild form of mung bean i.e. BWM-29. The parental line BM-4, BM-2003-2 and BM-2002-1 are the cultivated varieties, whereas BWM-29 is a wild dormant parent. There was quick germination within five days in the cultivated varieties (parents) and no germination for 25-30 days in the wild parent (BWM-29). So there was a dormancy issue in BWM-29.

Table 1. List of Mung bean breeding lines used for SDS-PAGE

Sr. No.	Cross	Breeding lines
1	Derivatives of inter-specific four way cross (F_4 generation): [(BM-4 × BWM-29) × BM-2003-2] × BM-2002-1	SPS-10
2	Derivatives of inter-specific three way cross (F_5 generation):[(BM-4 × BWM-29)] × BM-2003-2	SPS-42-1-11-2, SPS-42-1-11-1, SPS-42-1-8, SPS-40-1-13, SPS-42-1-10
3	Derivatives of inter-specific single cross : (F_6 generation): BM-4 (*Vigna radiata*) × BWM-29 (*Vigna radiata* var. *sublobata*)	SPS-6-39-21, SPS-6-23-6, SPS-6-52-6, SPS-6-39-19, SPS-6-1-11, SPS-6-11-7, SPS-6-1-25, SPS-6-53-13
4	Checks	BM-2003-2, AKM-4, BM-4, BM-2002-1, BPMR-145

Protein isolation from seed: The protein was isolated from the mung bean seeds by using the TCA-Acetone buffer-based extraction method with some modifications as described by [19] as follows. For extraction of total seed proteins, the seeds were soaked in distilled water overnight and ground to a fine powder with the help of a mortar and pestle. 1 gm sample was taken, it was pulverized and then homogenized with 3ml extraction buffer comprising Tri Chloro Acetic acid (TCA) (10 %) in acetone with 2 Mercapto Ethanol (ME) (0.07 %). Then homogenized samples were centrifuged at 13000 rpm with a temperature of 4^{0}C for 10 min. After centrifugation, the collected supernatant in a fresh tube and removed the debris. The supernatant was kept as it is overnight. The total protein was precipitated overnight at -20^{0}C. The precipitate was vortexed and centrifuged at 13,000 rpm at 4^{0}C for 15 min. The pellet obtained was rinsed thrice with acetone supplemented with 2 ME (0.07 %), EDTA (2 mM) and 1 tablet of complete EDTA-free protease inhibitor. For every washing, 500 ml of chilled wash buffer was added and centrifuged at 13,000 rpm at 4^{0}C for 15 min. Final washing was carried out with pre-chilled acetone (100%). Air dried pellet was kept overnight at -80^{0}C for removing the remaining traces of acetone. The protein sample was dissolved in a dehydration buffer and stored at -80^{0}C. Stored protein samples were further used for SDS-PAGE. The sample to be run is first denatured in sample buffer by heating 95-99^{0}C for 5 min.

Procedure for SDS-PAGE: SDS-PAGE was carried on a vertical mini slab gel electrophoresis (BIORAD) with a gel of 1 mm thickness as per the procedure described by [10]. The internal surfaces of the gel plates were cleaned with 70 % ethanol. The two glass plates were mounted vertically into a cast. Prepared separating gel (10 % and 15 %) as below the given table 2. The best result of the resolution of bands was found for 10 % separating gel.

Table 2. Composition of Separating gel (10 % and 15 %) of SDS-PAGE

Sr. No.	Components	For 10 % gel	For 15 % gel
1	1.5 M Tris-HCl (pH 8.8)	7.5 ml	8.0 ml
2	Water	12.3 ml	11.4 ml
3	Acrylamide: Bisacrylamide	9.9 ml	20.0 ml
4	10% SDS	0.15 ml	0. 4 ml
5	10% APS	0.225 ml	0.2 ml
6	TEMED	0.02 ml	0.02 ml
Total volume		**30 ml**	**40 ml**

The 10 % separating gel was loaded into a vertical slab between two glass plates and allowed to polymerize for 30 min. The gel was loaded up to the ¾th portion of the plates and the remaining ¼th portion was added the stacking gel. A layer of Isopropanol was added over the separating gel for an even surface of the gel and also prevented drying of the gel. Isopropanol was removed with filter paper after gel was polymerized. While the separating gel was getting polymerized, stacking gel (4%) was prepared as given in the Table 3.

Table 3. Composition of Stacking gel (4%) of SDS-PAGE

Sr. No.	Components	4% stacking gel
1	0.6 M Tris- HCl (pH 6.8)	1.25 ml
2	Stock Acrylamide	0.67 ml
3	Water	3.075 ml
4	10% SDS	0.025 ml
5	10% APS	0.025 ml
6	TEMED	0.005 ml
Total volume		**5.0 ml**

Stacking gel (4 %) was sprayed over the separating gel. Sample wells were made at one end of the gel by placing comb-shaped jig into stacking gel before it sets of polymerized. The stacking gel was allowed to polymerize for about 30 min so that the wells can be cast side by side. After the gel was properly set, the comb was removed carefully. The wells were washed with water and cleaned by keeping vertical small pieces of tissue paper. Assembled the cassette in the electrophoresis tank. Equal quantities of samples along with protein molecular weight marker (117, 85, 49, 34, 25, 19 kDa bands) were carefully loaded into hives of the comb using the micro syringe into 10% gels. Now running buffer was poured into lower and upper buffer reservoirs. The two terminal anodes and cathode were connected with the anode to the lower reservoir and the cathode to the upper reservoir. The instrument was switched on with a current of 60 volt. After the dye was displaced to the separating gel, the current was increased to 80 volts. All proteins have travelled to the anode (+). Electrophoresis was carried out for 2 or 3 hrs. until the bromophenol blue dye reached the bottom of the gel. Dismantled the gel apparatus, and tried to open the gel plates. At end of electrophoresis, gels were fixed in a solution containing 10% Acetic acid and 40% Ethanol for 15 min. with constant agitation on a shaker. After fixing gel was washed with distilled water for 15 min. with changing the water every 5 min. Gels were then stained with Coomassie blue G-250 dye and Destaining in water overnight.

Preparation of reagent

1. 30% Acrylamide - Dissolved 29.8 gm Acrylamide and 0.8 gm of Bis-acrylamide in 100 ml of water.
2. 1.5M Tris HCl buffer (pH 8.8) - Dissolved 9 gm of Tris (Hydroxyl methyl) amino methane in 50 ml of water and adjusted pH to 8.8 with 1N HCl.
3. 1.0M Tris HCl buffer (pH 6.8) - Dissolved 6 gm of Tris (Hydroxyl methyl) amino methane in 50 ml of water and adjusted pH to 6.8 with 1N HCl.
4. 10% SDS - Dissolved 100 mg of Sodium Dodecyl Sulfate (SDS) in 1 ml of double distilled water.
5. 10% APS - Dissolved 100 mg of Ammonium Persulfate in 1 ml of water.
6. TEMED (N, N, N', N Tetramethylethylene diamine).
7. 2% Agar - Dissolved 2 gm of agar in 50ml of water.
8. Sample buffer - The components were mixed as follows, 1 ml of 1M of Tris HCl, (pH 6.8), 1 ml of Beta Mercaptoethanol, 4 ml of 10 % Sodium Dodecyl Sulfate (SDS), 20 mg of Bromophenol blue and 2 ml of Glycerol. Finally, the volume was made upto 20 ml with distilled water.
9. Electrode buffer /Running buffer – Tris-Glycine buffer (pH 8.3) - Prepared 5X stock solution by dissolving 15.1 gm of Tris, 94 gm of Glycine, 50 ml of 10% SDS in about 80 ml of water and finally made the volume up to 1 liter. This was diluted one in five times and used.

Staining and Destaining

The gel was stained in Coomassie brilliant blue staining solution for overnight with constant shaking on the shaker. Excess stain was removed by washing the gel in a Destaining solution and later with distilled water.

1. Staining solution - Coomassie brilliant blue (0.1%) - 0.1 gm, Methanol - 50 ml, Glacial acetic acid - 10 ml and double distilled water - 40 ml.

2. Destaining solution - Acetic acid -10 ml, Methanol - 50 ml and double distilled water - 40 ml.

Gel documentation and analysis

Finally gels were photographed using the Alpha Innotech gel documentation system. The binary data matrix of the presence (1) / absence (0) of polypeptide bands was analyzed following the NTSYS software program to estimate Jaccard's similarity coefficient [8] values. Genetic diversity among the test breeding lines was assessed based on similarity coefficients calculated using 10 polypeptide bands using Unweighted Paired Group Method with Arithmetic Averages (UPGMA)-phenograms [21] employing Sequential Agglomerative Hierarchic and Non-overlapping clustering (SAHN) and a dendrogram was constructed.

Result and Discussion

SDS-PAGE was done for the total protein content in the seeds. In the present investigation, genetic diversity among test genotypes was assessed based on similarity coefficients calculated using 10 polypeptide bands of protein followed by [8], and UPGMA dendrogram was constructed as per [21]. In the present study, 15 most dormant lines with five cultivated parents were used to see the protein profiling pattern. The seed protein was isolated using TCA-Acetone buffer and separated on 10% SDS-PAGE gel as described in the materials and method. The bands were scored using a 117 Kda protein marker. The SDS study revealed 10-11 detectable peptides whose molecular weight was found to be in the range of 19 kDa to 117 kDa. The similarity matrix was given in Table 4.

Zygogram (Fig 1) based on Electrophoretic data grouped 20 breeding lines into four clusters with 75% similarity. The scoring of resolved proteins was done with a known protein marker and a zygogram was generated. The zygogram differentiated the selected sample into four clusters and the wild variety (BWM-29) was found to be out group (Figure 1). Cluster I consist of 13 breeding lines and Cluster II consists of five breeding lines. Cluster III and Cluster IV is solitarily consisting of one genotype each. A total of 13 breeding lines were grouped together in Cluster I which are SPS-42-1-10, SPS-6-1-25, SPS-6-53-13, SPS-40-1-13, SPS-42-1-8, SPS-6-1-11, SPS-6-11-7, SPS-42-1-11-2, and SPS-42-1-11-1. Here in cluster I all the single crosses and three-way crosses had grouped. Out of 13 breeding lines, eight lines are single crosses and five lines are three-way crosses, which covers most of the traits of cultivated species and they have accommodated in the same cluster. Cluster II consisted of five derivatives. It consists of BM-2003-2, BM-4, BM-2002-1, BPMR-145, and AKM-4. The genotypes BM-2003-2, BM-4 and BM-2002-1 are parents of three-way and four-way crosses, whereas genotypes BPMR-145 and AKM-4 are the cultivated checks. The protein banding pattern is similar for all cultivated types and grouped into single a cluster. Cluster III consisted of one derivative only. Breeding line SPS-10 is a four-way cross, which occupied in cluster III. SPS-10 covered different traits of the cultivated parent with good dormancy. It is accommodated in a separate cluster. The genotypes in cluster III and cluster IV are both mostly divergent than others. Cluster IV is solitary and it consists of a single wild genotype i.e. BWM-29, which is out-grouped based on protein profile. Genotype BWM-29 which is a wild donor parent of dormancy used in all single crosses, three-way crosses and four-way crosses. Due to it's wild behaviour like spreading plant growth habit, small seed size, etc., it was found out grouped in cluster IV. The protein banding pattern for this genotype was totally unique and was different from the other derived breeding lines and cultivated checks. Thus it has out-grouped into the separate distinct cluster. Based on the protein banding pattern or SDS-PAGE study, out of 20 dormant breeding lines including parents, those lines found most similar at the protein level are BM-2003-2, BM-4, BM-2002-1, SPS-6-52-6, SPS-6-39-19, SPS-40-1-13, SPS-42-1-8, SPS-6-1-25 and SPS-6-53-13, whereas lines which found most divergent at the protein level are SPS-10 and BWM-29.

In mung bean, SDS-PAGE cannot be used to identify genotypes on the basis of intra-specific variation because similar banding patterns (Fig 2, 3, 4) were observed in some accessions that differed on the basis of characterization and evaluation; therefore, this technique might be more suitable to identify inter rather than intra-specific variation in *Vigna* spp. [4]. Present results also depicted a high genetic diversity in the tested breeding lines based on electrophoresis of seed storage proteins as a dendrogram based on Electrophoretic data grouped the tested genotypes into four clusters. In the present investigation, the multi-variety clusters was dissociated rapidly into several distinct sub-clusters, each containing a few genotypes. Thus, it was possible to see the difference between breeding lines grouped in different clusters or sub-clusters at different phenon levels. The breeding lines were distributed into four divergent clusters. A dendrogram based on Electrophoretic data grouped 20 lines into four clusters with 75% similarity.

[11] Also found four clusters. [13] Analyzed 74 mung bean land races, three wild accessions and a popular cultivated variety 'Jyoti' for total seed storage protein. They also observed that these genotypes had clustered into seven groups at 70% phenon level. They reported that, three wild accessions were found to be divergent from other genotypes. This finding is similar to the present study. In the present pursuit, one wild form of mung bean i.e. BWM-29 was separated out and formed one cluster. It may be due to its unique polypeptide banding pattern. Similar findings were also found by [22, 23]. This wild parent is morphologically distinct from the rest of test genotypes owing to its viny growth habit with basal branching, and unique leaflet shape. Remaining all characters like stem, leaf, and pod of all inter-specific derivatives are covered most of the desirable traits like cultivated varieties or checks. It showed a typical characteristic polypeptide banding pattern. This is considered to be quite divergent from the rest of the genotypes and could serve as valuable breeding material for the improvement of mung bean agronomical and quality traits. [26] also found three clusters and they also grouped the wild and cultivated accession into two different clusters. Similar results were also reported by [1, 18, 24, 25] and [12] in soybean and [24] in urd bean. The genotypes in the same cluster based on seed protein profiling have some common agronomic performance. Again these results are in accordance with the earlier workers [4, 5, 6, 7]. Such clusters with unique morpho-economic traits could be identified for genetic improvement. Seed storage proteins are considered as biochemical markers and have become a powerful tool in studying genetic variation within and among genotypes in crop plants and also distinguishing cultivars of a particular crop species.

Conclusion

The results of SDS-PAGE (Electrophoretic study) revealed that, 10-11 detectable peptide with molecular weight of 19 kDa to 117 kDa. Zygogram based on Electrophoretic data grouped 20 breeding lines into four clusters with 75% similarity. The zygogram differentiated the selected sample into four clusters and the wild genotype (BWM-29) i.e. fourth cluster was found out group. Further, it was also revealed that breeding lines in the same cluster based on total seed protein profiling have common agronomic characterstics and such clusters with unique morphological and yield-associated traits could be identified for genetic improvement. Seed storage proteins are considered biochemical markers and have become a powerful tool in studying genetic variation within and among genotypes in crop plants and also distinguishing cultivars of a particular crop species.

Implications of the research for future mung bean breeding program

The lines which have shown seed dormancy with high yield are SPS-42-1-11-1, SPS-6-53-13, SPS-42-1-11-2, SPS-40-1-13, SPS-6-39-19 and SPS-42-1-10. Breeding lines selected through inter-specific hybridization have covered most of the desirable agronomic traits along with high yield with a shorter period of dormancy definitely useful in future hybridization programmes as a parental lines or generation of breeding material and development of improved cultivars having high seed yield with a shorter period of seed dormancy.

Table 4 . Similarity matrix of 20 dormant lines including parents in Mung bean breeding lines by SDS-PAGE

Lines	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.000																			
2	0.571	1.000																		
3	0.286	0.333	1.000																	
4	0.286	0.333	0.500	1.000																
5	0.286	0.333	1.000	0.500	1.000															
6	0.286	0.333	1.000	0.500	1.000	1.000														
7	0.429	0.286	0.750	0.400	0.750	0.750	1.000													
8	0.286	0.600	0.500	0.500	0.500	0.500	0.400	1.000												
9	0.333	0.167	0.250	0.250	0.250	0.250	0.200	0.250	1.000											
10	0.750	0.444	0.222	0.375	0.222	0.222	0.333	0.222	0.250	1.000										
11	0.750	0.625	0.222	0.222	0.222	0.222	0.333	0.375	0.250	0.778	1.000									
12	0.750	0.625	0.222	0.222	0.222	0.222	0.333	0.375	0.250	0.778	1.000	1.000								
13	0.500	0.571	0.125	0.125	0.125	0.125	0.250	0.286	0.000	0.556	0.750	0.750	1.000							
14	0.500	0.571	0.125	0.125	0.125	0.125	0.250	0.286	0.000	0.556	0.750	0.750	1.000	1.000						
15	0.714	0.571	0.286	0.286	0.286	0.286	0.250	0.286	0.333	0.750	0.750	0.750	0.500	0.500	1.000					

16	0.500	0.375	0.286	0.500	0.286	0.286	0.250	0.286	0.333	0.750	0.556	0.556	0.333	0.333	0.714	1.000			
17	0.500	0.375	0.286	0.500	0.286	0.286	0.429	0.286	0.143	0.750	0.556	0.556	0.500	0.500	0.500	0.714	1.000		
18	0.333	0.167	0.250	0.250	0.250	0.250	0.500	0.250	0.000	0.250	0.250	0.250	0.333	0.333	0.143	0.143	0.333	1.000	
19	0.625	0.500	0.429	0.250	0.429	0.429	0.571	0.250	0.125	0.667	0.667	0.667	0.625	0.625	0.625	0.444	0.625	0.286	1.000
20	0.625	0.500	0.429	0.250	0.429	0.429	0.571	0.250	0.125	0.667	0.667	0.667	0.625	0.625	0.625	0.444	0.625	0.286	1.000	1.000

1.	SPS-6-39-21	8.	SPS-42-1-11-1	15.	SPS-42-1-10
2.	SPS-42-1-11-2	9.	BWM-29	16.	SPS-6-1-11
3.	BM-2003-2	10	SPS-6-23-6	17.	SPS-6-11-7
4.	AKM-4	11.	SPS-6-52-6	18.	SPS-10
5.	BM-4	12.	SPS-6-39-19	19.	SPS-6-1-25
6.	BM-2002-1	13.	SPS-40-1-13	20	SPS-6-53-13
7.	BPMR-145	14.	SPS-42-1-8		

List of 20 breeding lines used for SDS-PAGE Electrophoretic study

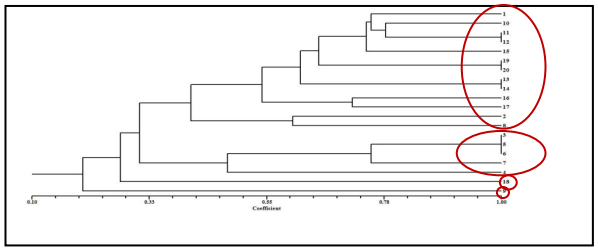

Fig 1. Zygogram showing different clusters based on polymorphism in total seed proteins

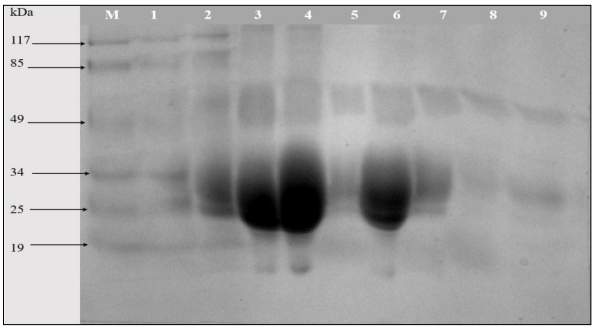

Fig 2. Protein profiling of nine mung bean genotypes through SDS-PAGE

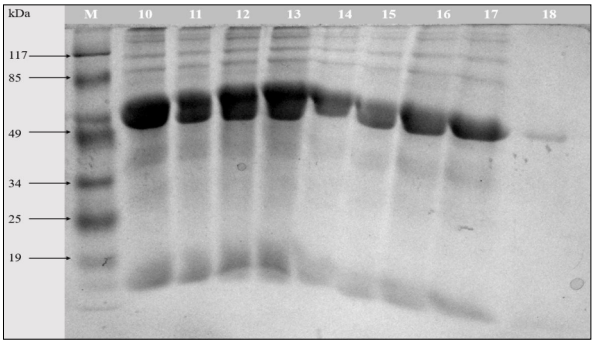

Fig 3. Seed storage protein profiles of mung bean genotypes using SDS-PAGE

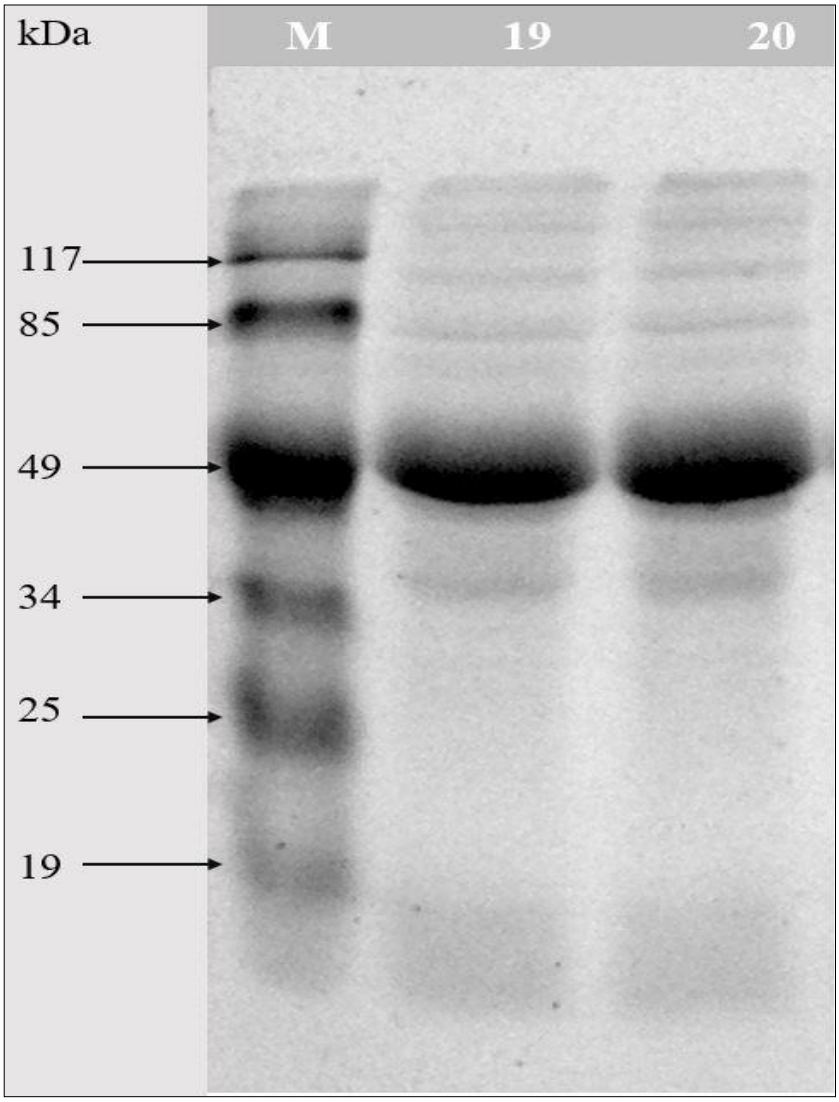

Fig 4. Protein profiling of remaining two mung bean genotypes through SDS-PAGE

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