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Protein profiling of adzuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi] genotypes grown under inorganic, organic and Zero Budget Natural Farming

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Abstract
Fifteen genotypes of adzuki bean grown under inorganic, organic and Zero Budget Natural Farming (ZBNF) condition of Himachal Pradesh were analyzed for protein banding pattern on polyacrylamide gel through SDS-PAGE. The organic, inorganic and ZBNF banding pattern showed significant variability in molecular weight and its ranged from size 11.8 kDa - 139.5 kDa, 11.7 kDa - 131.5 kDa and 10.6 kDa - 96.9 kDa, respectively. The protein banding profiles of adzuki bean genotypes was scored and subjected to cluster analysis using Unweighted Pair Group Method and Arithmetic Mean (UPGMA) for dendrogram grouping. The dendrogram generated from the SDS-PAGE profiles grouped the inorganic, organic and ZBNF genotypes into two major clusters i.e. Cluster-1 (genotypes grown under inorganic systems) and Cluster-2 (genotypes grown under organic and ZBNF systems). The present study demonstrates that genotypes grown under ZBNF system exhibited some distinct protein bands of low molecular weight (10.6 kDa, 12.2 - 12.5 kDa, 13.0 - 13.8 kDa and 17.3 - 17.7 kDa) belong to 11S and 2S globulins than genotypes grown under inorganic and organic system. Hence, the biochemical characterization revealed more specific discrimination among the genotypes grown under inorganic, organic and ZBNF system.

Keywords: Banding pattern, Organic, Protein, SDS-PAGE, ZBNF

1. Introduction
Adzuki bean [*V. angularis* (Willd.) Ohwi & Ohashi] commonly known as azuki and small red beans is an important food legume crop. It is mostly grown under a wide range of climate conditions including warm temperate, tropical regions, short growing season and dry conditions. The growth of the adzuki bean is similar to that of the other edible beans. It is native to the North- Eastern part of China and grown in more than 30 countries. Based on characteristics of seed size and colour, genetic factors, cultivar type, cultivation and harvest time, climate and region where it was cultivated, there are several numbers of varieties of adzuki beans present. In Japan it has been used as an ingredient in traditional confections and sweet desserts, such as *wagashi, youkan, manju and amanatto* (Gohara *et al*., 2016) [3].
Genetic diversity or genetic variation is one the most applied subjects being adapted by popular researchers of the world. The presence of genetic diversity /variation within organisms, especially in plants is key for the plant breeding purpose. It can be determined by using various approaches like morphology, electrophoresis and DNA based analysis. Among the various biochemical techniques, electrophoresis by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is one of the most convenient and consistent methods for exploring the protein patterns of plant because of the significantly independence of seed protein on various factors like environmental, rationality and adaptability (Sher *et al*., 2020) [11]. It is considered to be a universal technique for separation and estimation of proteins on the basis of size and molecular weight (Pavlova *et al*. 2017) [9]. Seed protein patterns obtained by SDS-PAGE have been effectively used to resolve the total proteins, to observe inter and intra specific diversity, phylogenetic relationships and to study the genetic similarity index of crops (Jukanti *et al*., 2017) [6].

In the present day agriculture imbalanced and indiscriminate use of chemical fertilizers, growth regulators, pesticides for enhancing crops has negatively affected the soil quality and crop ecosystem. Gradual increase in costs of crop production has become a major cause of distress in the farmers. Double the farmer's income by 2022 is a big challenge to the scientists and researchers, to achieve this objective of the Union Government recently; Zero Budget Natural Farming (ZBNF) is being known to be the best farming approach (Verma *et al*., 2018) [12]. Naturally grown organic food products without synthetic insecticides and fertilizers are being observed as more healthier and safer than the inorganic food products. Adzuki beans are also included in beans which are one of the foods that are produced under organic farming. Presently, adzuki beans have attracted attention because of their considerable health benefits and functional components. Major biochemical component of adzuki beans proteins which have beneficial effects on human health. The available literature lacks the information on the comparison of protein profiling of adzuki bean genotypes grown under inorganic, organic and ZBNF systems. Therefore, in the present study efforts were made to explore the genetic diversity and protein profiling of inorganic, organic and ZBNF adzuki bean genotypes by SDS-PAGE

2. Materials and Methods

2.1. Plant material and treatments

The field experiment was conducted in randomized block design (RBD) during *Kharif* season of 2018-2019 at the Experimental-Farm of the Department of Organic Agriculture and Natural Farming, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur (India). The fifteen adzuki bean genotypes were raised on Randomized Complete Block with three replications grown under three production systems i.e. inorganic (NPK was applied @20:40:20 kg/ha at the time of sowing), organic (*Vermicompost* (VC) was applied @5tonns/ha) and ZBNF (*Ghanajeevamrit* (A paste was prepared by using 100 kg mixture of local cow, buffalo and goat dung, 1 kg Jaggery, 2 kg of pulse flour, handful of soil from bund of farm and 5 liters of cow urine. The paste was allowed to ferment for 48 hrs. in shade. The fermented paste was then dried under sunlight with stirring using a wood stick to form fine particles) was applied as basal dose @40kg/ha along with the spray of 10% concentration of *Jeevamrit* (Fresh local cow dung (10 kg), aged cow urine (10 liters), Jaggery (2 kg), pulse flour (2 kg) and a handful of soil from the bund of farm were added in a barrel containing 200 liters of water and stirred in clockwise direction)). In the Zero Budget sowing *Jeevamrit* spray was done at the interval of 15 days till the physiological maturity of the crop. The sowing was done on 30th May, 2018. The plot size was 6m×30cm×2. Samples were used for protein profiling during the year 2018-2019.

2.2 Protein extraction and Protein profiling

Total seed proteins of 15 genotypes grown under three different production systems i.e inorganic, organic and ZBNF were extracted. Weighed 0.5g oven dried mature seed sample of adzuki bean transferred it in a pre-chilled pestle and mortar under ice-cold condition, added 10 ml Sodium Phosphate buffer (0.1 M, pH 7.2), 25µl β-mercaptoethanol and polyvinylpyrrolidone (12 mg) then ground it, the content was centrifuged at 18,000 rpm for 10 min. The supernatant was collected and kept at -20 °C for further protein profiling of adzuki bean genotypes by SDS-PAGE as described by Laemmli, 1970. The molecular weight of the proteins was estimated by using Benchmark^{TM} unstained protein Ladder (Thermo Fisher Scientific, Invitrogen Bioservice Pvt. Ltd., Bangalore, India) ranging from 10-220 kDa which was used as a standard. The electrophoretic gel was prepared on polyacrylamide gels. The mixture of polyacrylamide contained 5.0 ml 30% acrylamide, 5.0 ml of 1.5 M Tris- HCl buffer (pH 8.8), 0.2 ml of 10% SDS, 9.7 ml of double distilled water, 100 µl of 10% APS and 6.75 µl of TEMED. The protein was solubilized on 1x volume of reducing sample buffer (1.25 ml of 0.5 M Tris-HCl (pH 6.8), 1.0 ml of glycerol, 2.0 ml of 10% SDS, 0.2 ml of β-merchaptoethanol, 1.0 mg of bromophenol blue and final volume made up 5.0 ml with double distilled water). The 10µl protein sample of each genotype was loaded into the well of stacking gel contained 1.33 ml of 30% acrylamide, 2.50 ml of 0.5 M Tris buffer (pH 6.8), 0.2 ml of 10% SDS, 6.00 ml of double distilled water, 100 µl of 10% APS and 13.5 µl of TEMED. SDS-PAGE were run on constant 60 V for 3 hrs. and stained by Coomassie brilliant blue R-250 staining.

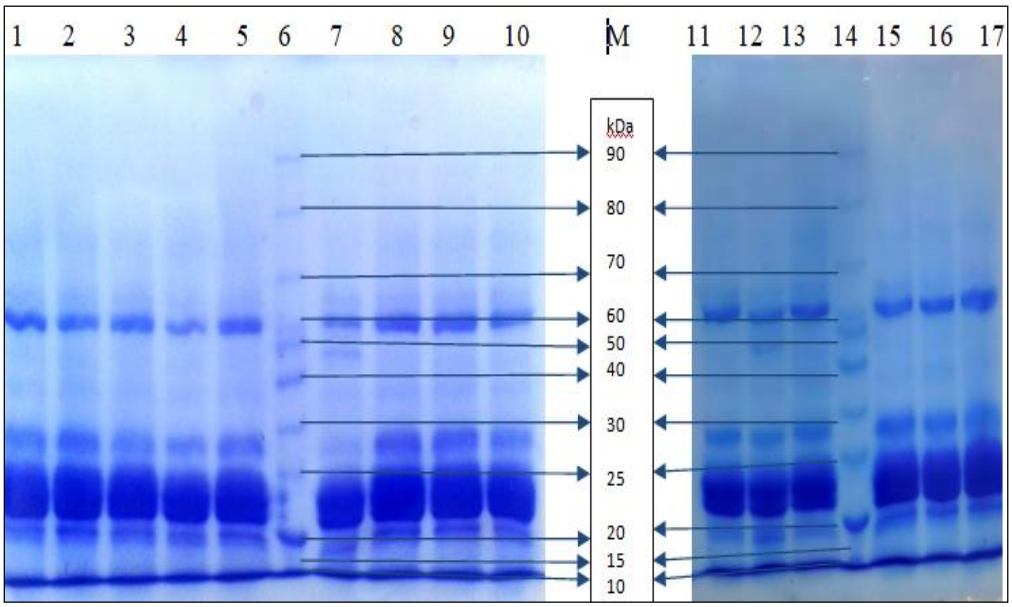
2.3 Phylogenetic data analysis

The protein data matrix and construction of a dendrogram was done according to 'Unweighted Pair Group Method and Arithmetic Mean' method using the online dendrogram construction utility, Dendro UPGMA (https://usuaris.tinet.cat/debb/UPGMA/ Garcia-Vallve and Puigbo 2002) [2]. Bands were marked as '1' and '0' based on their presence or absence respectively. The Jaccard similarity coefficient was calculated using a binary matrix generated by the SDS-PAGE profile.

3. Results

3.1 Protein profiling

The molecular weight of the proteins was calculated on the basis of relative mobility of the protein in the marker. The SDS-PAGE analysis showed remarkable variability in the banding pattern (Figure 1 to 3). The present study revealed the presence of low, medium as well as high range molecular weight proteins and phylogenetic relationship.



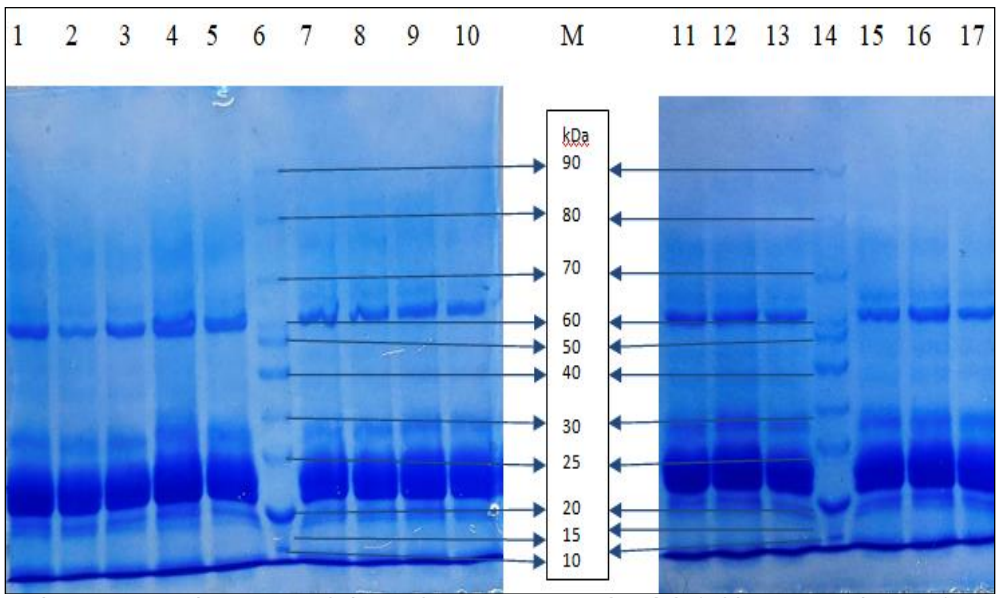
L1-ADHP-1	L5-ADHP-5	L9-ADHP-8	L13- EC-340286	L17- ADHP-10
L2-ADHP-2	L6- Marker	L10-ADHP-9	L14-Marker	
L3-ADHP-3	L7-ADHP-6	L11- HPU-51	L15- EC-030270	
L4-ADHP-4	L8-ADHP-7	L12- LOCAL TOTRU	L16- EC-340245	

Fig 1: SDS-PAGE profiling of adzuki bean genotypes grown under inorganic condition

The analysis by SDS-PAGE with CBB R-250 staining detected 14 protein bands in adzuki bean genotypes grown under inorganic and organic production systems while 18 protein bands appeared in genotypes grown under ZBNF production method. The molecular weight of the detected band varied from 11.8 kDa to 139.5 kDa, 11.7 kDa to 131.5 kDa and 10.6 kDa to 96.9 kDa grown under inorganic, organic and ZBNF conditions, respectively.

Based on SDS-PAGE analysis, several protein bands were identified in some genotypes. The genotypes grown under an inorganic production system detected two specific banding patterns in 10 genotypes (ADHP-1, ADHP-2, ADHP-5, ADHP-7, ADHP-8, ADHP-9, ADHP-10, EC-340245, EC-030270 and HPU-51) out of 15 with molecular weight range from 95.0 kDa to 139.5 kDa, however, in genotypes Local-Totru and ADHP-6 also detected two distinct bands (Figure.1) with molecular weight varied from 42.2 kDa to 47.2 kDa that was not observed in other genotypes.

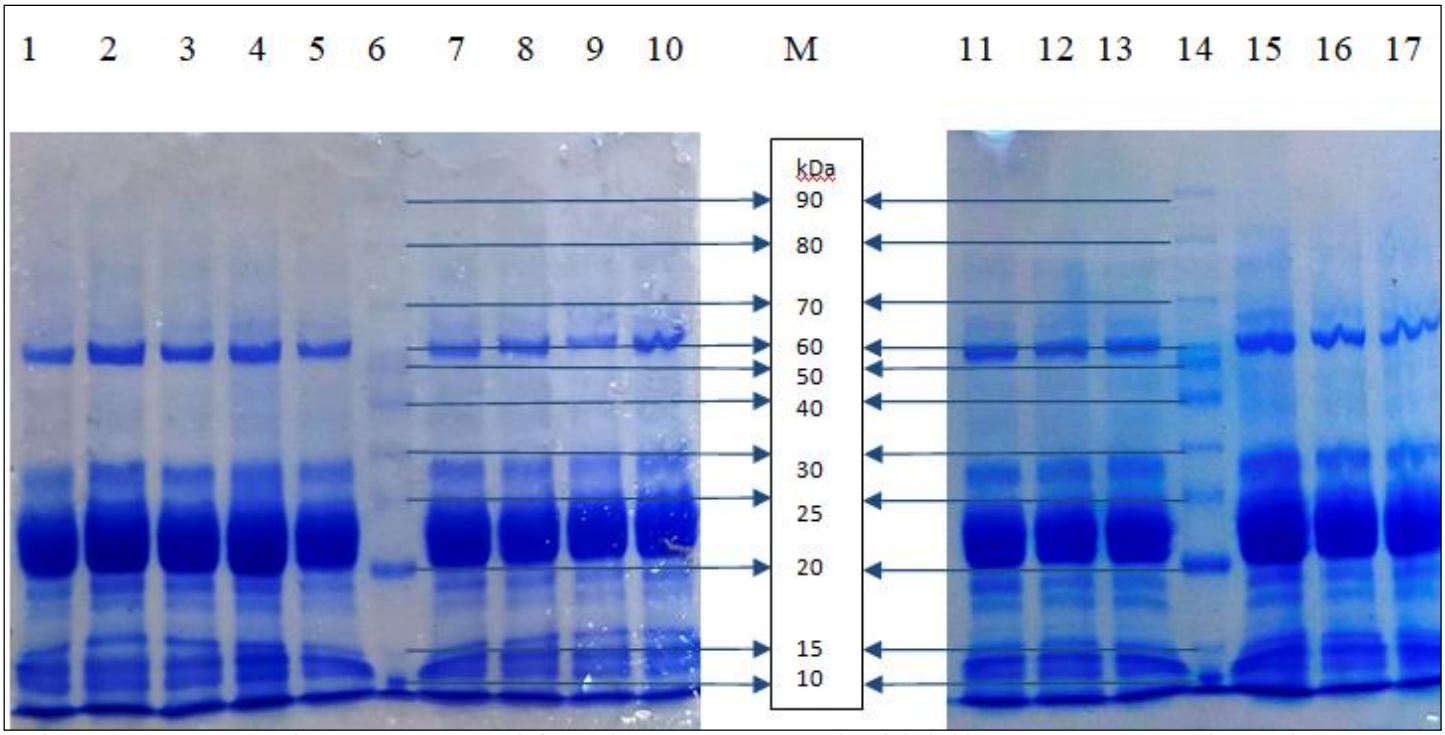
In case of an organic condition six genotypes (ADHP-10, EC-340245, EC-030270, EC-340286, Local-Totru and HPU-51) were detected two specific bands having 109.4 kDa and131.5 kDa molecular weight, while protein band of molecular weight ranging from 51.1 kDa to 55.8 kDa detected only in 11 genotypes (ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADHP-8, ADHP-9, ADHP-10 and EC-030270). In two genotypes ADHP-10 and EC-030270 detected one distinct protein band (Figure.2) with molecular weight 47.2 kDa that was absent in the rest of genotypes.



L1-ADHP-1	L5-ADHP-5	L9-ADHP-8	L13- EC-340286	L17- ADHP-10
L2-ADHP-2	L6- Marker	L10-ADHP-9	L14-Marker	
L3-ADHP-3	L7-ADHP-6	L11- HPU-51	L15- EC-030270	
L4-ADHP-4	L8-ADHP-7	L12- LOCAL TOTRU	L16- EC-340245	

Fig 2: SDS-PAGE profiling of adzuki bean genotypes grown under organic condition

Genotypes grown under ZBNF system only 9 genotypes (ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADHP-8 and ADHP-9) have specific protein band (Figure. 3) with molecular weight 89.5 kDa which was missing in leftover six genotypes.



L1-ADHP-1	L5-ADHP-5	L9-ADHP-8	L13- EC-340286	L17- ADHP-10
L2-ADHP-2	L6- Marker	L10-ADHP-9	L14-Marker	
L3-ADHP-3	L7-ADHP-6	L11- HPU-51	L15- EC-030270	
L4-ADHP-4	L8-ADHP-7	L12- LOCAL TOTRU	L16- EC-340245	

Fig 3: SDS-PAGE profiling of adzuki bean genotypes grown under ZBNF condition

Variability in intensity was observed in some bands that indicated the quantity of proteins at a particular molecular weight. In case genotypes grown under inorganic production method at 4 number band highly intense with molecular weight 17.8 to 19.3 kDa was observed followed by bands 5, 10, 2 and 3 with molecular weight 26.3 to 25.4 kDa, 51.3 to 51.9 kDa, 14.7 to15.0 kDa and 15.5 kDa, respectively.

In case genotypes grown under organic conditions, variations in intense band was also observed at 4 number band highly intense 19.1 to19.3 kDa molecular weight protein was found followed by bands 5, 10, 2 and 3 of molecular weight 26.5 to 35.0 kDa, 52.4 to 82.0 kDa, 14.9 to15.3 kDa and 15.4 to16.5 kDa, correspondingly

In case genotypes grown under ZBNF system more number of intense bands were observed, at band number 8 most intense band of protein of molecular weight ranged from 21.2 to 21.3 kda was observed, followed by bands 10,14, 6, 7, 2, 3 and 4 (28.9 to 30.2 kDa), (50.3 to 55.4 kDa), (15.7 to 16.3 kDa), (17.3 to17.7 kDa), (11.3 to11.7 kDa), (12.2 to12.5 kDa) and (13.0 to 13.8 kDa), in that order.

3.2. Phylogenetic relationships

The phylogenetic analysis revealed that the fifteen genotypes grown under inorganic, organic and ZBNF condition were broadly clustered into two major clusters: (i) Cluster1- genotypes grown under an inorganic system (ii) Cluster 2- the other genotypes grown under organic and ZBNF system (Figure 4). Among the organic and ZBNF system genotypes, two genotypes (ADHP-10 and ADHP-12) grown under organic system; nine genotypes (ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADHP-8, ADHP-9) grown under ZBNF system and six genotypes (HPU-51, Local-Totru, EC-340286, EC-030270, EC-340245 and ADHP-10) grown under ZBNF system were clustered into two sub clusters, while rest of thirteen genotypes grown under organic system were clustered together.

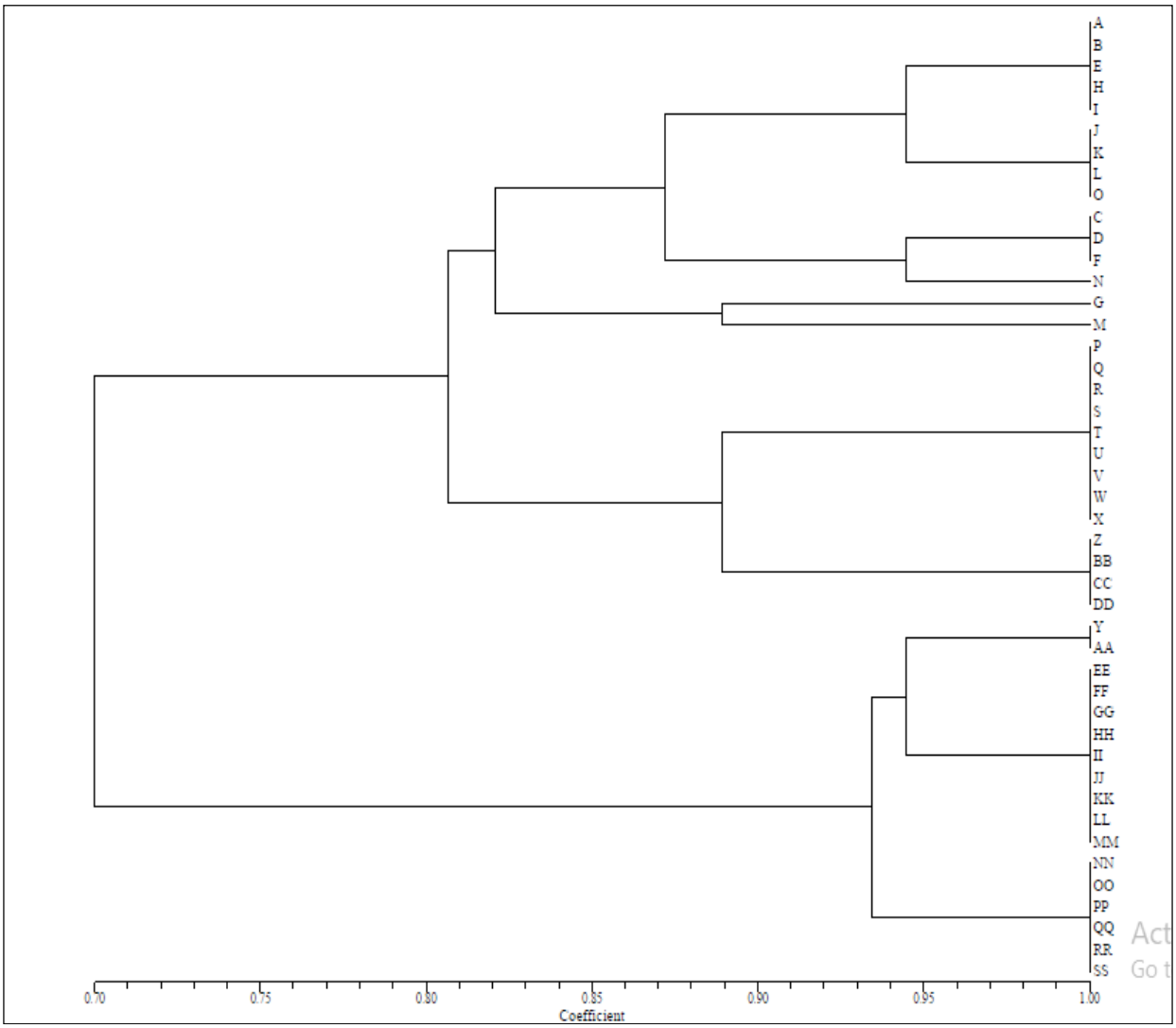


Fig 4: Phylogenetic relationships among adzuki bean genotypes grown under Inorganic (A to O), organic (P to DD) and ZBNF (EE to SS) condition

4. Discussion

The present study revealed that among three different systems (inorganic, organic and ZBNF) in adzuki bean genotypes. Genotypes grown under organic system, one new distinct protein band (51.1 to 55.8 kDa) was detected (Figure 2), however it is missing in genotypes grown under inorganic condition, this result was accordance with Selvakumar *et al*., (2012) [10] who have reported one new induced protein band detected in black gram in response of bio-fertilizers, whereas in grown under ZBNF system, detected some distinct protein bands of low molecular weight (10.6 kDa, 12.2 to 12.5 kDa, 13.0 to 13.8 kDa and 17.3 to 17.7 kDa), which were not found in genotypes grown under either in inorganic or organic system (Figure 3). These different resolved protein bands mainly belong to 11S and 2S globulins as seed storage protein (Malviya *et al*., 2008 and Hammed *et al*., 2012) [10, 4]. Similarly, based on the banding pattern grown under the ZBNF system detected a total number of 18 bands as compared to adzuki bean genotypes grown under inorganic and organic conditions.

Phylogenetic analysis in present study demonstrates that dendrogram generated from the SDS-PAGE profiles of adzuki bean genotypes grown under inorganic, organic and ZBNF condition formed two major clusters i.e Cluster-1 (genotypes grown under inorganic system) and Cluster-2. The second cluster (including the genotypes grown under organic and ZBNF system) was further sub-divided into two sub-clusters. Genotypes grown under ZBNF system total 7 numbers of intense bands detected, although inorganic and organic system genotypes had a total number of intense bands 5 in each. Chen *et al*., (2017) [1] have reported 4 intense bands in adzuki bean as molecular weight of 59, 46, 28, and 25 kDa grown under inorganic system and present investigation more or less similarity with this findings as five intense bands with molecular weight ranged from 17.8 to 19.3 kDa, 26.3 to 25.4 kDa, 51.3 to 51.9 kDa, 14.7 to 15.0 kDa and 15.5 kDa.

5. Conclusions

Based on present study it can be concluded that SDS-PAGE technique if done efficiently can be a useful tool to understanding the genetic diversity and phylogenetic relationships among genotypes grown under three different production systems i.e. inorganic, organic and ZBNF. The present study also demonstrates that genotypes grown under ZBNF system exhibited some distinct protein bands of low molecular weight (10.6 kDa, 12.2 to 12.5 kDa, 13.0 to 13.8 kDa and 17.3 to 17.7 kDa)) belong to 11S and 2S globulins than genotypes grown under inorganic and organic system.

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7. Conflict of Interest
There is no conflict between the authors and the contribution of the authors is clear and unquestionable. Both declare that they have no conflict of interest. Therefore, authors are allowed to publish the article

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