

Two-dimensional Gel Analysis of Total Seed Protein of Horse Gram (*Macrotyloma uniflorum*) for Identification of their Proteomic Diversity

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Submission Date: 26-05-2022; Revision Date: 17-06-2022; Accepted Date: 29-07-2022.

ABSTRACT

Horse gram (*Macrotyloma uniflorum*) is a popular pulse in the Kumaun region of Uttarakhand, locally known as Gahat dal and Kulti. An experiment was conducted with 5 horse gram cultivars having different grain colors. Total seed protein was isolated and quantified with standard protocols. Five protein samples were subjected to one-dimensional (1D) and two-dimensional (2D) gel for proteomic analysis. 300g of each sample was loaded onto immobilized pH gradient (IPG) strips (3–10 pH Linear, 18 cm) and kept for Iso-Electric Focusing (IEF) and SDS-PAGE. The gels were scanned using a scanner and analyzed for proteomic diversity. Basically, two major clusters were constructed, and samples 1, 2, and 3 are in group one and 4 and 5 are in group two, and group one has two further sub-clusters. Sample one was separately clustered based on the number of spots presented on the 2D gel and based on its color. The analysis of variance revealed that the genotypes observed had highly significant differences. Further study is needed for the identification of unique peptides presented by protein sequencing. The proteomic analysis of total seed proteins could be useful for the development of nutraceuticals and food-to-food fortification. This study's current proteome and other proteomic information will be extremely useful to horse gram breeding programmes.

Keywords: SDS-PAGE, 2D gel, Protein, Horse gram, Kumaun.

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INTRODUCTION

Pulses are the second greatest source of food for mankind. Complex carbohydrates, essential vitamins, minerals, and phytochemicals are abundant, while lipids are scarce. Proteins of various legumes (beans, peas, lentils, cowpeas, chickpeas, pigeon peas, etc.) vary in composition and structure, which has implications for the appropriateness of finished products.^[1] In terms of nutraceuticals, horse gram (HG) is truly a super food. "Superfoods" are a special category of nutrient-packed foods that are high in antioxidants and

proteins. Pulses play a critical function in nutraceuticals and aid in the treatment of protein deficiency.^[2-3] *Macrotyloma uniflorum* (Horse Gram), formerly *Dolichos biflorus*, is a food legume that is underappreciated and underdeveloped. Proteomics, which includes gel-based techniques including two-dimensional gel electrophoresis (2D-GE), has taken a prominent role in quality assurance in recent years. Researchers have been able to address the safety concerns at the molecular scale with greater sensitivity and accuracy because of recent technological advances. *Macrotyloma uniflorum* (Horse Gram), formerly *Dolichos biflorus*, is a food legume that is underappreciated and underdeveloped. Proteomics, which includes gel-based techniques including two-dimensional gel electrophoresis (2D-GE), has taken a prominent role in quality assurance in recent years. Researchers have been able to address the safety concerns at the molecular scale with greater sensitivity and accuracy because of recent technological advances.

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DOI: 10.5530/ajbls.2022.11.45

^[4] Like any other pulse horse gram also consist of a good amount of different type of Carbohydrates, such as oligosaccharides, sucrose, maltose, glucose xylose, galactose, arabinose, and fructose having the approximate amount g/100g of 3.69 ± 0.24 , 1.21 ± 0.12 , 0.53 ± 0.06 , 0.00 ± 0.00 , 0.64 ± 0.09 , 0.08 ± 0.01 , 0.12 ± 0.03 , 0.03 ± 0.01 and 0.04 ± 0.00 also having total sugar 6.38 and total starch 36.0 ± 1.17 g/100g. Horse gram seed has a protein content of 18.5 to 28.5 percent.^[5] Enzyme inhibitors, phytosterols, phytic and tannic acids, oligosaccharides, and other nutrients found in pulses have been shown to have health-enhancing properties in humans. Pulses are an excellent source of these nutrients.^[1] 2D-GE is utilized to identify food authentication, food adulteration and the comparison of plant proteomes. The electrophoresis of 2D-GE has been widely employed for food authentication. 2DGE seems to be the only approach that itself is officially certified for identifying species, as per the AOAC. Comparing 2D protein profiles of various crops can aid in finding common and/or distinctive proteins produced by a species, their control, and the development of species-specific biomarkers that can aid in varietal recognition and food adulteration/contamination identification.^[6] 2DE is used for the segregation of proteins since it allows analysts to identify large-scale quantitative protein alterations. Thus, 2-DE is widely utilized to create scientific concepts for inquiry as an initial investigation. This method changed the field of proteomics and biomarkers in the detection of protein changes in differential expression and/or change.

MATERIALS AND METHODS

Sample Collection

M. uniflorum seeds were collected from farmers in Uttarakhand's various districts for the identification of their biochemical, and molecular diversity. The samples were harvested as per the protocol of Mehra, 2013.^[7] All chemicals and instruments were purchased from HiMedia, SRL Laboratories and Merck. All the basic instruments used in the study are present in our laboratory (Earthworm biotechnology and molecular diagnostic Laboratory, Nainital). The difference in colour and appearance of collected seeds can be seen in Table 1 and Figure 1.

Total Protein Isolation

1 g seeds of five cultivars were selected, ground to a fine powder in a pestle-mortar. The chloroform-defatted powder was placed into centrifuge tubes. 0.1M PMSF (100 μ l) and 2 ml of chilled extraction buffer (5mM-EDTA, 50 mM - NaCl, 25 mM-Sodium phosphate, pH 7.2) were added in centrifuge tubes. The tubes were then vortexed by hand tabbing, and all samples were centrifuged at 10,000g for 15 min at 4°C. The supernatant was collected in fresh centrifuge tubes and stored at -20°C.

1D gel Analysis

Total protein was quantified according to Bradford assay^[8] and 25 μ g/well (3 μ L) protein in 12% SDS-PAGE gel run according to Laemmli, 1970.^[9] The gel documentation (Bio-Print, Eppendorf) system was used

Table 1: List of Horse Gram germplasm collected from different elevations of Kumaun and Garwal of Uttarakhand.

Sl.No.	Location	Local Name	Amount collected	Seeds colour	Given Name
1.	Kumaun Elevation:1556m N-29°51.172';E-079°36.219'	Gahat	~500g	Dark brown	SPNP-1
2.	Kumaun Elevation:1803m N-29°30.406';E-079°45.815'	Gahat and Kulti	~250g	Red and Black, non-spotted	SPNP-2
3.	Kumaun Elevation:2178m N-29°27.034';E-079°46.210'	Gahat and Kulti	~250g	Red and Black, non-spotted	SPNP-3
4.	Garwal Elevation:2032m N-30°18.029';E-078°25.342'	Gahat	~250g	Dark brown and spotted	SPNP-4
5.	Garwal Elevation:2032m N-30°18.029';E-078°25.342'	Gahat	~250g	Dark brown and non-spotted	SPNP-5

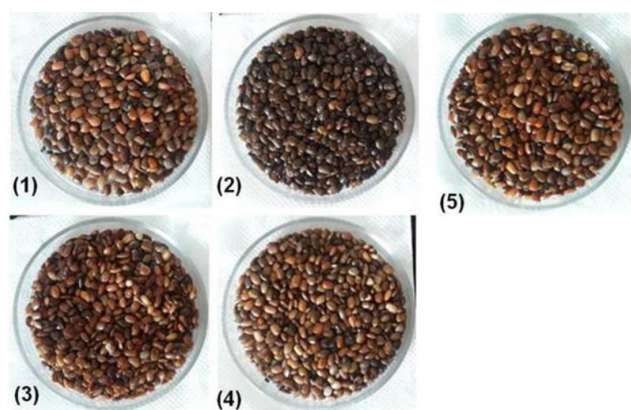


Figure 1: Horse gram (*Macrotyloma uniflorum*) seeds collected from different locations in Uttarakhand.

to observe the banding pattern of the Total Protein in a 12.5% SDS-PAGE gel.

Gel Staining

After electrophoresis, the gel was stained with silver stain according to Chevallet *et al.*, 2006^[10] with minor modifications, the gel was fixed by drenching in the fixing solution (50% Methanol in water and 10% acetic acid) for 30 min and washed with the wash solution (5% Methanol in water) for 15 min. Then the gel was equilibrated using water in three subsequent steps for 15 min, with shaking. Sensitization was done with (1.5 g potassium fericyanide, 3.0 g sodium thiosulfate and 0.5 g sodium carbonate in water) for 2 min. Washing of the gel with HPLC grade water was done for four times (10 min each) then staining was done with 1 g silver nitrate and 50 μ L 37% formaldehyde (formalin) in 1 L water for 30 min, with intermediate shaking. Afterwards, the gel was washed for 1 to 2 min, in water. The gel was developed by covering it with 25g sodium carbonate and 200 μ L 37% formaldehyde (formalin) in 1 L water (formaldehyde was added just prior to using the developing solution) until the protein bands were achieved. After achieving the desired stain, the developer solution was replaced with a stop solution (10 mL reagent-grade acetic acid in 1 L water).

2D analysis

Proteomics analysis was performed on five protein samples using a 2D gel. IPG strips 3-10 pH Linear, 18cm were loaded with 300g of each sample and kept for Iso-Electric Focusing. After the IEF run, the strip was equilibrated in Equilibration Buffer and the second dimension was carried out on a 10% SDS-PAGE. The gels were Coomassie-stained to observe the protein spots and were scanned using Epson Expression 11000XL Scanner. Instruments used for the analysis

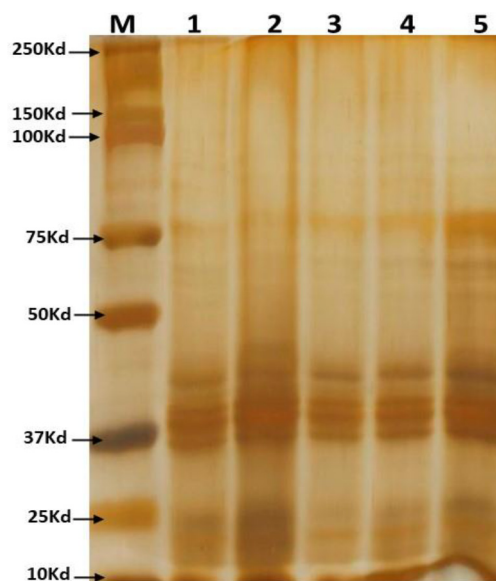


Figure 2: SDS-PAGE for TCA precipitated protein and stained with silver staining of five selected samples, Loading, M; protein molecular weight marker, well 1 to 5 sequentially samples 1 to 5.

were, Ettan IPGphor3 from GE; Ettan Dalt Six Gel Electrophoresis Unit from GE; Epson Expression 11000XL Scanner (Cytogene, Lucknow, India).

Statistical Analysis

Graph and histogram were constructed with the help of Microsoft Excel and UPGMA clustering was constructed with SPSSpc21. The 2D gels were analysed with the online software Melanie viewer 7.0.

RESULTS AND DISCUSSION

1D analysis

Samples were run in 12% SDS-PAGE gel for analyzing the diversity. All the samples showed multiple bands and diversity in one-dimensional gel electrophoresis (Figure 2). Sample 1 and then were subjected to 2D analysis.

2D analysis

Protein obtained for all flour samples was used for protein profiling by 2D-GE. Using the optimized 2D-GE protocol, better separation of proteins with clear resolution of spots and minimal streaking was obtained. The gels were Coomassie stained (Figure 3) to observe the protein spots and were scanned using Epson Expression 11000XL Scanner. 2D gel electrophoresis data (Table 2) clearly revealed that peptide-based diversity would be analyzed or established clearly (Figure 3). In UPGMA clustering, major two clusters were constructed and samples 1, 2, and 3 are in group

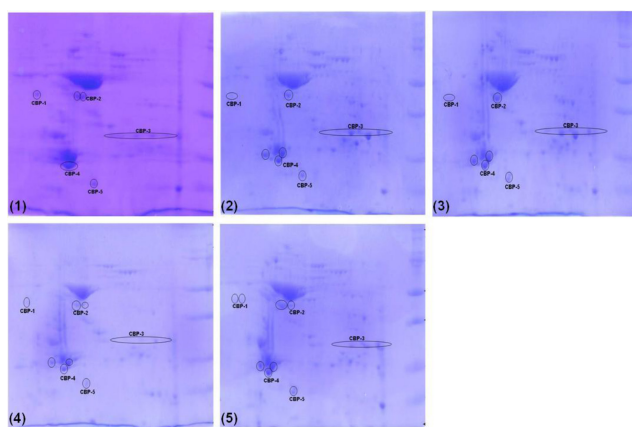


Figure 3: 2D gel electrophoresis of total buffer soluble protein of horse gram seed protein for identification of calcium-binding proteins (CBPs) peptides.

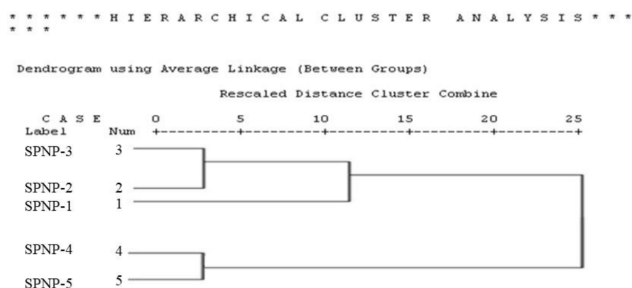


Figure 4: UPGMA clustering of 2D gel analysis on the basis of total spots counted with NTSYSp21 software.

one and 4 and 5 in group two and group one has further two sub-clusters. Sample one was separately clustered based on the number of spots presented on 2D gel and based on its color (Figure 4).

Based on molecular weight and 1D gel stained with stains-all the CNPs were showing no similar banding pattern and based on the elevation of the sampling site its dominance was increased.

The summary analysis of all 2D gel has summarized in Table 2. Sample one has the lowest count of 33 spots but a maximum average size (219.212). The total area was maximum in sample two but percent Area was maximum in sample three.

The intensity of areas A and B is highest in sample three, while the intensity of region C is highest in sample two, based on the 3-D analysis of horse gram seed protein located in 2D-GE (Figure 5 and Table 3).

DISCUSSION

To promote intestinal health and appetite changes, seed coat portions of legumes with high fibre and low protein may be effective in food product compositions.^[11] Minimize diarrhoea, modification of blood sugar levels,

Table 2: Summary of 2D gels.				
Slice	Spot Count	Total Area	Average Size	%Area
One	33	7234	219.212	3.109
Two	93	8649	093.000	3.636
Three	93	8381	090.118	4.031
Four	69	4910	071.159	2.480
Five	76	5805	076.382	3.066

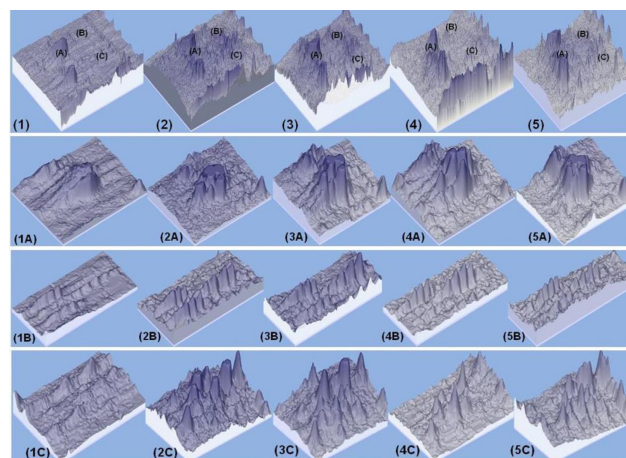


Figure 5: 3D images of 2D gel electrophoresis for identification of spots diversity of peptides. Image created by software Melanie viewer 7.0.

cholesterol reduction, prebiotic effects, avoidance of some malignancies, cardiovascular diseases (CVD), diverticular disease, overweight, and lowers blood pressure are the main bioactive properties linked to dietary fibres^[5] In India, *Macrotyloma uniflorum* has long been utilized as an anti-obesity natural dietary supplement.^[12] The horse gram has astringent, diuretic and antioxidant effects. The horse gram plant is beneficial in treating menstruation disorders, leucorrhoea, and bleeding during pregnancy. Urolithiasis is known popularly as a renal stone and in the last twenty years has had a significant impact on public health.^[13] Crystallization inhibitors included in horse gram seed extract are soluble in water, steady in temperature, polar, non-tannin and non-protein, and are therefore employed in the treatment of renal stone.^[14] Drought, which is regarded as a primary abiotic load on the fauna and flora world, is caused by a shortage of environmental resources like water. The drought resistance of horse gram is ascribed in part to several processes like as antioxidant and osmolyte production, making it tough enough to tolerate long dry periods with no maintenance.^[15] It can withstand drought, salt, and heavy metal exposure. Horse gram is a type of grain that is mostly farmed in India, Africa, Australia, Burma, Malaysia, Mauritius, and the West Indies. The Ancient World Tropics, particularly India

Table 3: 2D gel spot analysis by Melanie viewer 7.0 software.

Sl. No	File Name	Match set	C	Cols	Rows	MaxValue	Sports	MaxGray	Size	Modified	Comment	Staining
1	One			524	444	241	0	241	456	16/05/2019 10:52:08		Coomassie Blue
2	Two			537	444	198	0	198	467	16/05/2019 11:00:33		Coomassie Blue
3	Three			502	418	215	00	215	411	16/05/2019 11:04:18		Coomassie Blue
4	Four			478	417	215	0	215	391	16/05/2019 11:04:53		Coomassie Blue
5	Five			456	418	243	0	243	374	16/05/2019 11:05:26		Coomassie Blue

and the Himalayas, are thought to have the most genetic variation.^[5] In the future, a seed protein-based marker could be employed to identify local varieties. For Protein content, SDS-PAGE could be used to detect genetic diversity and similarity in cultivars at a low cost.^[16] According to a previous study by Sharma *et al.*, 2018, one band of high molecular weight protein (~43KDa) and three bands ranging between (~25.1 to ~17 KDa) were found in horse gram and the similar results have come out in the present investigation where spots of the ~45.6, ~46.7, ~41.1, ~39.1 and ~37.4 KDa were found which can conclude that the proteomic diversity helps in health benefits. The horse gram genome included a lot of bHLH, ERF, C2H2, WRKY, and NAC transcription factors, according to a protein-protein homology search against a plant TFs database. In several crops, especially horse gram, these TFs offer tolerance to biotic and abiotic stressors.^[17-21]

This study could be a defining point in the domain of *M. uniflorum* proteomics, and it will be helpful to practitioners and farmers for additional investigation and prospective cash cropping and nutraceutical farming in Uttarakhand's hills. The results of this study's proteome and proteomic analysis will be extremely useful to horse gramme breeding efforts. Furthermore, in light of the growing need for food with nutraceutical properties, these proteomic findings offer the opportunity to investigate the utilization of horse gramme as a source of food and nutraceuticals.^[22]

CONCLUSION

Proteomics is an omics scientific subject that focuses on analysing the entire set of proteins generated in a cell, tissue, or organism at any particular time, i.e., proteomes. The study found that the diversity seen depending on the colour and elevation of the sample size was significant in the context of proteomics and its usefulness for the investigation of various biological situations. This data

will be used with findings from other proteomic studies to learn more about the mechanisms underlying the elevation responsive peptides of the proteome and the modification of biological processes in response to seed colour.

ACKNOWLEDGEMENT

The authors wish to acknowledge the Science Engineering and Research Board, Department of Science and Technology, New Delhi, Govt. of India. Vide letter no. PDF/2016/001883. The authors are also thankful to farmers of Uttarakhand for providing the seed samples of the cultivar analysed in the present study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

1D: One-dimensional; **2D:** Two-dimensional; **IPG:** Immobilized pH gradient; **IEF:** Iso-Electric Focusing; **HG:** Horse gram; **2D-GE:** Two-dimensional gel electrophoresis; **AOAC:** Association of Analytical Communities; **SRL Laboratories:** Sisco Research Laboratories; **EDTA:** Ethylenediamine tetraacetic acid; **PMSF:** Phenylmethylsulfonyl fluoride; **SDS-PAGE:** Sodium dodecyl-sulfate polyacrylamide gel electrophoresis; **HPLC:** High Performance Liquid Chromatography; **IEF:** Isoelectric focusing; **IPG:** Immobilized pH gradient; **UPGMA:** Unweighted Pair Group Method with Arithmetic Mean; **SPSS:** Statistical Package for the Social Sciences.

SUMMARY

Five different horse gramme cultivars with different grain colours were used in an experiment. Standard

protocols were used to isolate and quantify total seed protein. For proteomic analysis, these five protein samples were run through one-dimensional (1D) and two-dimensional (2D) gels. 300g of each sample was loaded onto immobilised pH gradient (IPG) strips (3–10 pH Linear, 18 cm) and stored for Iso-Electric Focusing (IEF) and SDS-PAGE. Essentially, two major clusters were formed, with samples 1, 2, and 3 belonging to group one and 4 and 5 belonging to group two, and group one having two further sub-clusters. Sample one was clustered separately based on the number of spots visible on the 2D gel as well as its colour. The analysis of variance revealed that the genotypes observed had highly significant differences. More research is needed to identify the distinctive peptides revealed by protein sequencing. Protein analysis of total seed proteins could aid in the development of nutraceuticals and food-to-food fortification. The current proteome and other proteomic information presented in this study will be extremely beneficial to horse breeding programmes.

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Cite this article: Sharma N, Bisht SS, Gupta S, Dewali S, Parveen N, Pant R. Two-dimensional Gel Analysis of Total Seed Protein of Horse Gram (*Macrotyloma uniflorum*) for Identification of their Proteomic Diversity. *Asian J Biol Life Sci*. 2022;11(2):334-9.