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Research Paper

A high protein recovery extraction method from buckwheat based on protein yield gel quality and 1-D gel electrophoresis

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ABSTRACT

Proteomics is an OMICS based technology used to functional characterization of gene expression. Its potential depends on recovery of proteins from tissues. A protocol that will lead to maximum recovery of proteins from any tissue is considered as the best method and efficient means to proceed for in depth proteome analysis. In this respect, we have tried various methods to establish best protein extraction method for recovery of proteins from different tissues of buckwheat plant. The tissues that we considered are shoot, root and seed. In case of Buckwheat, this is the first report in which we have established protein extraction methods from various tissues. Extracted proteins were furthered quantified using Bradford method and then resolved on SDS-PAGE. The results indicated that the efficacy of a specific protein extraction methodology is dependent on the structure and metabolic contents of different tissues. Among various methods tested phosphate -TCA acetone method showed high recovery of proteins from buckwheat shoots and roots having highest protein concentration of 3.31±0.05µg/µl and 1.20±0.04µg/µl in shoots and roots respectively. Whereas, by phenol-based protocol protein yield of 3.61±0.07 µg/µl was obtained from buckwheat seeds.

Keywords: 1D- PAGE, buckwheat, protein extraction methods, protein quantification

Buckwheat (*Fagopyrum spp*.) belonging to the Polygonaceae family is widely grown in Asia, the United States, Canada, Russia, and East Europe, and its human consumption is similar to cereals. The recent scientific evidence that it is packed with favorable combination of nutrient elements, vitamins, better protein quality and antioxidants so it is potential candidate crop for future nutritional security. Moreover, it is also rich in various bioactive components and nutraceuticals. In addition to being a nutritionally superior crop it is highly resilient and performs comparably better under low input farming systems.

Its antioxidative, antihemorrhagic, and blood vessel-protective characteristics is attributed to the presence of high content of phytochemicals such flavonoids, phenolic acids, condensed tannins, and phytosterols in buckwheat (Ahmed *et al*., 2014). According to Beitane and Krumina-Zemture (2017), buckwheat is high in the B group vitamins like thiamine, riboflavin, and pyridoxine as well as some macro- and microelements like sodium, potassium, copper, zinc, magnesium, iron, calcium, and manganese (Mota *et al*., 2016). Buckwheat is now a healthier alternative for making a variety of foods, including breads, cakes, casseroles, cookies, crepes, porridge, pancakes, pasta-noodle soups, and other confectionary products (Mohajan *et al*., 2019). Buckwheat is also notable for its high quantities of rutin and quercetin components as compared to other cereals. (Pongrac *et al*., 2016). Buckwheat had the highest level of total phenol in the seed extracts (323.4 mg GAE/100 g), and the levels fell in the following order: buckwheat > quinoa > wheat > amaranth. Buckwheat seed extract also had the highest antioxidant capacity when evaluated using the ferric ion reduction and radical 2,2-diphenyl-1-

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picylhydrazyl scavenging assays (p 0.01). Sprouting was generally shown to enhance total phenol content and antioxidant activity, and bread making was found to reduce these levels. Phenolic acids, catechins, flavanol, flavone, and flavonol glycosides were all detected by liquid chromatography in combination with a diode array detector. In general, the seeds and sprouts of quinoa and buckwheat are potential sources of rich polyphenol compounds for increasing the nutritional qualities of foods like gluten-free breads (Alvarez *et al*., 2010). Furthermore, it is believed to offer high-quality protein, ranging from 8.5% to 18.8% depending on cultivar, source, and climate conditions (Dziadek *et al*., 2016). Buckwheat grains have a higher protein percentage than cereal grains (Bobkov, 2016). Buckwheat proteins are made up of globulins (43.3%-64.5%), albumins (12.5%-18.2%), prolamins (0.8%-2.9%), glutelins (8.0%-22.7%), and residual proteins (15%). (Chrungoo *et al*., 2016). The prolamine content in buckwheat proteins is very low, and proteins responsible for celiac diseases (30 kDa prolamins) are absent in buckwheat as observed from gel electrophoresis and enzyme-linked immunosorbent method by Petr *et al*. (2003). Buckwheat proteins contain 2S albumins, 8S and 13S globulins, which are related to legumin storage proteins (Bobkov, 2016). The protein content of buckwheat grains is 11.7%, compared to 21.6% in the bran and 10.6% in the flour. Several buckwheat species range from 11 to 15% in protein content. A buckwheat kernel has 35% of its protein in the endosperm, 55% of it in the embryo, and the remaining 10% in the hull (Leder, 2000). Only oat flour has lower protein content than buckwheat flour, which has much greater protein content than rice, wheat, millet, sorghum, and maize flour (Bonafaccia *et al*., 2003). The primary storage protein is 13 S globulin, with a hexameric structure with acidic (32-43 kDa) and basic (23-25 kDa) polypeptide subunits connected by disulfide bonding (Taylor *et al*., 2016). Buckwheat proteins provide well amino acid compositions and are enriched in arginine, lysine, and aspartic acids (Bhinder *et al*., 2020). Buckwheat's protein digestibility is reduced by the presence of tannins and protease inhibitors. The presence of lysine amino acid in buckwheat protein, on the other hand, boosts the protein digestibility-corrected amino acid scores of proteins in buckwheat over cereals (Zhang *et al*., 2012). Buckwheat allergy incidence rates are substantially lower than those associated with gluten ingestion (McAllister *et al*., 2019). Buckwheat grain value addition processing has grown in popularity (Luthar *et al*., 2021; Zhu, 2016), so its grain fractions and components, including the protein, must be used. For the creation of functional components and "healthy" food items, the buckwheat protein isolates, for instance, can be converted into bioactive peptides (Li *et al*., 2019).

Proteomics is an emerging field of omics for understanding the molecular regulations of any biological process. In recent past different gel based as well as gel free proteomics techniques have been developed for identification and quantification of low abundance proteins (Agrawal *et al*., 2013; Zargar *et al*., 2015). For the efficient proteome analysis, high recovery of protein from a tissue is an important step. For improved plant proteomic analysis, various protein extraction techniques have been developed in past (Faurobert, *et al*., 2007). For example, two fundamental protein extraction procedures were proposed for plant proteomics, one based on traditional TCA/acetone precipitation and the other on phenol extraction (Isaacson *et al*., 2006). A methodology by combining TCA/acetone precipitation and phenol extraction was devised to optimize an appropriate protein extraction from recalcitrant tissues (Wang *et al*., 2003). In order to establish a standard protocol for protein extraction from various tissues of buckwheat plant (Shoot, root and seeds), we have adopted different protein extraction protocols (Phenol extraction method, TCA-acetone and Phosphate-TCA-acetone). The extracted proteins were quantified and resolved on SDS-PAGE for confirmation.

MATERIALS AND METHODS

Plant Material: Nine Morphologically distinct genotypes of buckwheat (*Fagopyrum* sps) (BWZ1, BWZ2, BWZ33, BWZ41, BWZ69, BWZ92, BWM2, BWM4 and BWM57) (Figure 1) were grown in natural conditions in green house (23°C) and were harvested after 90 days of sowing. Various tissue samples (Shoot, root and seed) were then collected from the buckwheat plants for protein extraction. Root and seed were collected at time of harvesting were as shoot samples was taken after flowering. Harvested tissues were preserved at −80°C till further use.

Protein isolation methods: Protein extractions from different tissues were carried out using three different extraction/ buffers:

1. Phenol buffer Sucrose (0.7 M), Tris (tris hydroxymethyl aminomethane) (0.5 M), HCI (Hydrochloric acid) (30 mM), EDTA (Ethylene diamine tetracetic acid) (50 mM), KCl (0.1 M) (Singh *et al*., 2015)
2. TCA-Acetone buffer TCA (10%, w/v) in acetone with 2-mercaptoethanol (0.07%) (Singh *et al*., 2015).
3. Phosphate-TCA-Acetone buffer Phosphate buffer (0.1 M, pH 7.5), TCA (10%, w/v) (Trichloroacetic acid) in acetone with 2- mercaptoethanol (0.07%) (Singh *et al*., 2015).

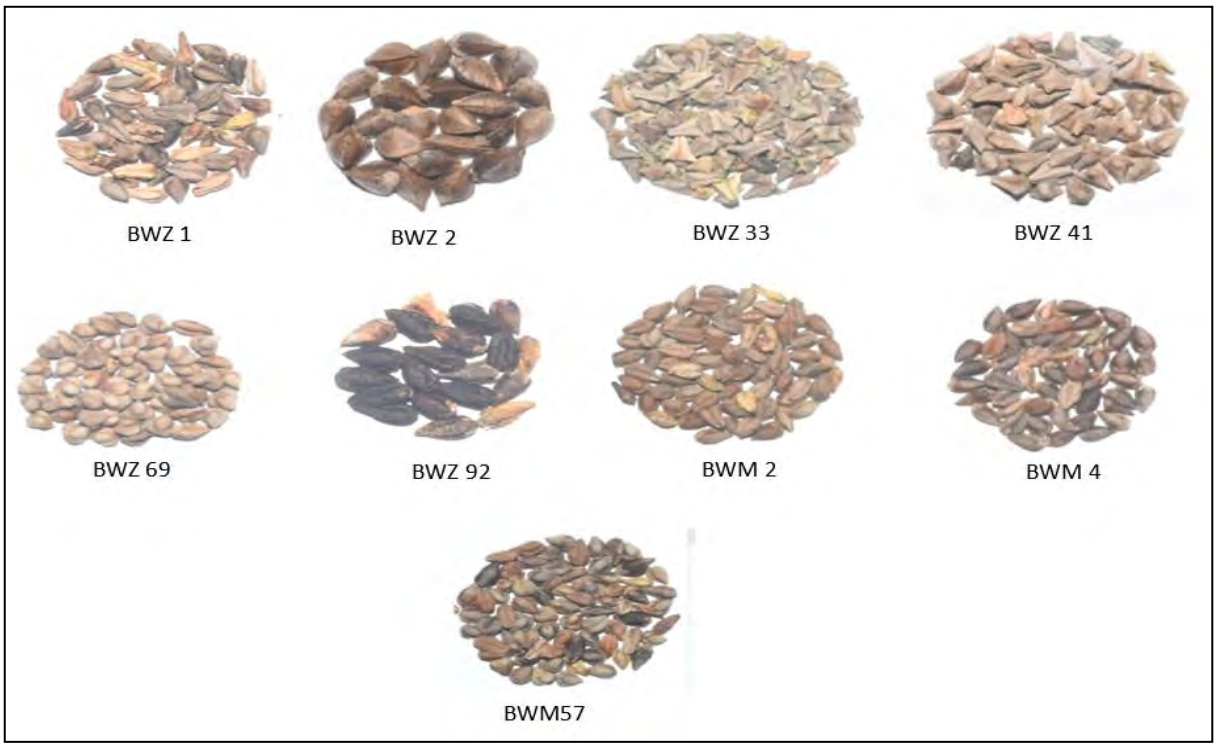


Fig. 1: Genotypes used for extraction of protein

Desirable tissues were weighed and homogenized before being subjected to various extraction techniques based on the buffers utilized (Protocol 1, 2 and 3). The flow diagram (Fig. 2) showed different steps involved for proteomics comprising protein precipitation, quantification and 1-D gel electrophoresis, gel staining, scanning and finally image analysis.

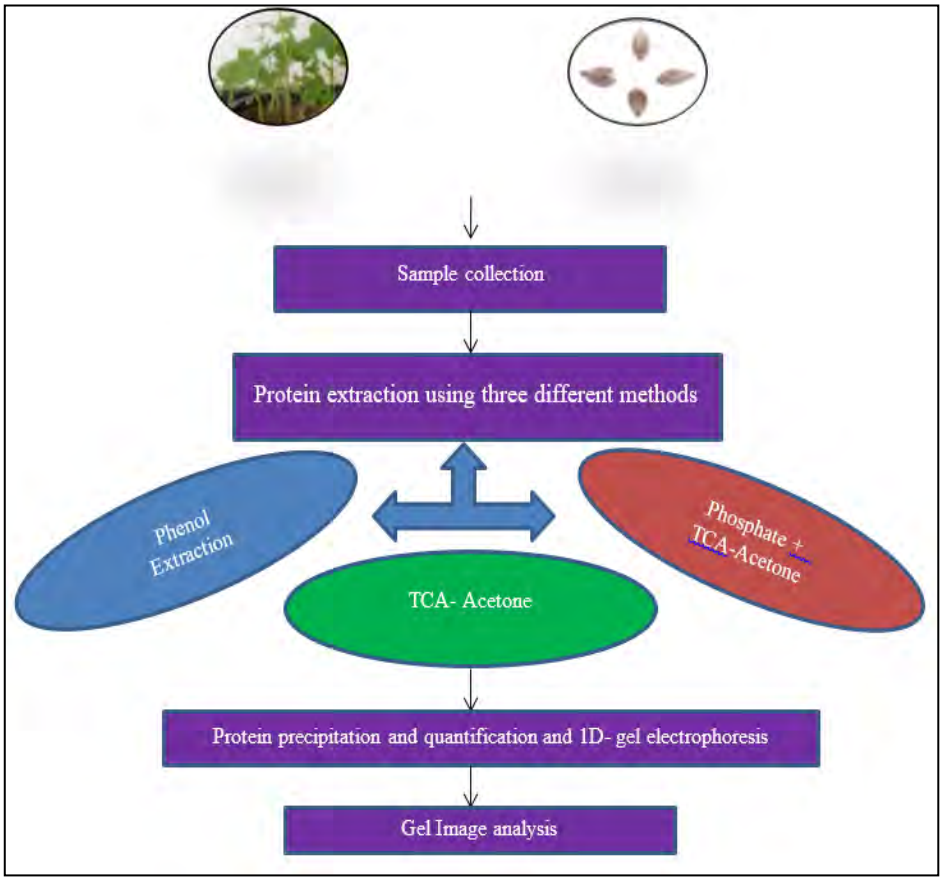


Fig. 2: Flow chart depicting protein extraction from leaves and root of buckwheat

Protein Extraction Protocol 1 (Phenol Extraction Method): Fresh tissues (shoot, root and seeds) weighing 1 gm each were ground using liquid nitrogen in a pre-chilled pestle and mortar. The powder was then re-dissolved in 3 ml of extraction buffer and kept at 4

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°C. 2-ME (2-Mercaptoethanol) and 1 mM PMSF (phenylmethylsulfonyl fluoride) were added to the pre-chilled solution right before usage. The suspension was incubated at 4°C for 10 min while being constantly shaken. Following the addition of an equal volume of Tris-buffered phenol, the mixture was once more shaken for 10 min at 4°C. To separate the aqueous and organic phases the solution was centrifuged for 15 min. at 4 °C at 13000 rpm. Carefully recovering the phenolic phase, it was then extracted again using an equal volume of extraction buffer. Phase separation was achieved by vortexing and centrifuging the materials (Singh *et al*., 2015).

Protocol 2 (Trichloroacetic Acid-Acetone Buffer): Liquid nitrogen was used to grind the samples it was then homogenized using a 3ml solution of TCA (10%) in acetone with 2 ME (0.07%). Further the samples were kept for overnight at -20°C, for protein precipitated. The precipitate was vortexed, then centrifuged for 15 minutes at 13000 rpm at 4 °C. The pellet was rinsed three times with acetone, 2 β ME (0.07%) (2-β mercaptoethanol), 2 mM EDTA, and 1 tablet of complete EDTA-free protease inhibitor was added. Each washing involved adding 500µl of chilled wash buffer, vortexing vigorously, then centrifuging for 15 minutes at 13000 rpm at 4°C. Acetone (100%) that had been pre-chilled was then used for the last wash. For the purpose of eliminating any last residues of acetone, the air dried pellet was then dissolved in rehydration buffer and stored overnight at -80°C (Singh *et al*., 2015)

Protocol 3 (Phosphate-TCA-Acetone Buffer): The tissue samples were finely grounded using liquid nitrogen. After that, phosphate buffer was used to homogenize the powdered materials (0.1 M, pH 7.5). In order to precipitate the proteins, the supernatant was transferred to a new tube containing 2 ml of TCA (10%) in acetone and 2 ME (0.07%). After 15 minutes of centrifugation at 13000 rpm and 4°C, a protein pellet was produced. The pellet was purified by washing three times with cold acetone that also contained 1 tablet of a complete EDTA-free protease inhibitor, 2 ME (0.07%), and 2 mM EDTA. Final washing was done using pure acetone. Pellet was kept at -20°C for overnight. Acetone-free pellet was dissolved in rehydration buffer (7 M urea, 2 M thiourea and 2% (w/v) CHAPS and quantified (Singh *et al*., 2015).

Protein Quantification: Proteins isolated from different tissues using these protocols were quantified as described by (Bradford, 1976).The method involves use of 1ml of Bradford reagent and taking spectrophotometric readings at 595 nm. The standard curve obtained is represented in fig. 3.

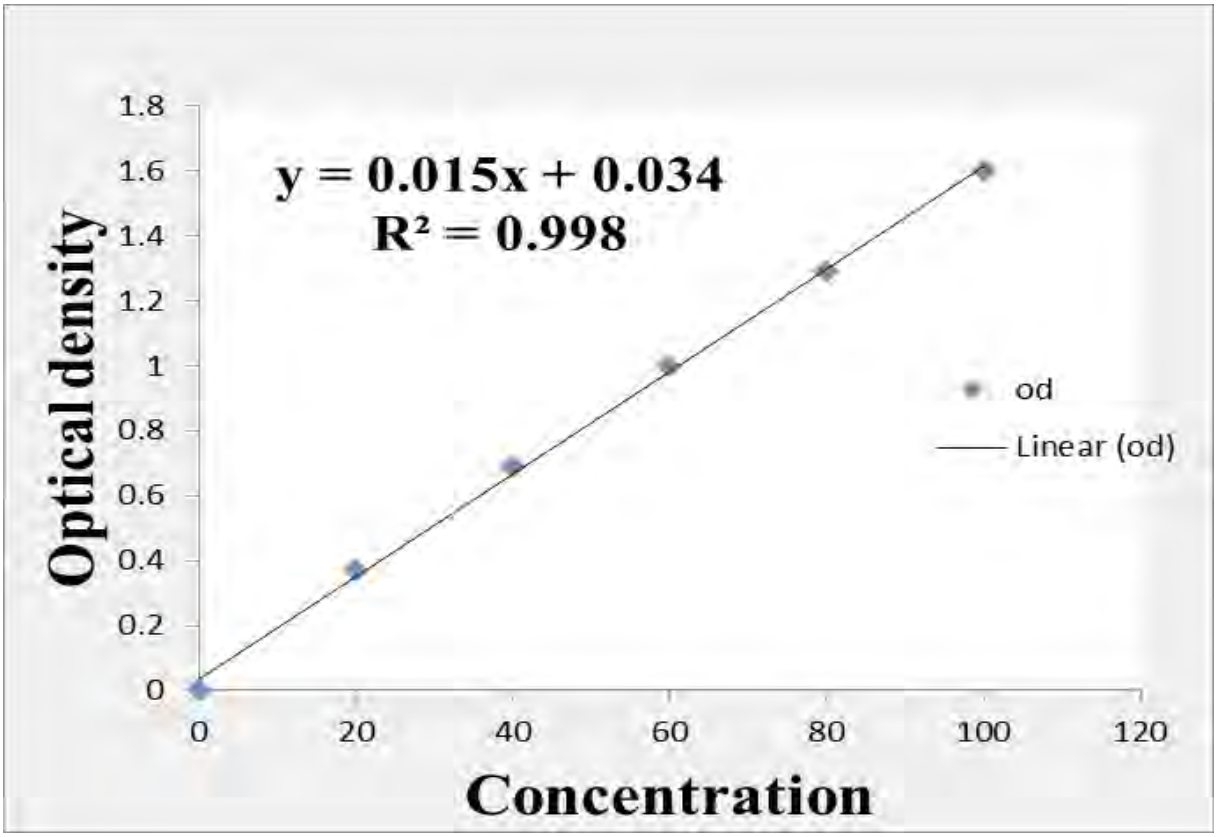


Fig. 3: Standard Curve for Protein Quantification

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1D- analysis (SDS-PAGE): To solubilize proteins, each sample (25 µg) was mixed with 1X Tris-glycine buffer (0.025 M Tris base, 0.912 M glycine and 0.1% SDS (Sodium dodecyl sulfate), left at -80 °C for 2 min, and immediately protein renaturation by placing on ice for 2-3 minutes. Protein samples and molecular ladders were loaded on acrylamide gels (5% stacking gel, pH 6.8 and 10% separating gel, pH 8.8) and finally electrophoresed at 60 V using Tris-glycine tank buffer. The compositions of both gels are shown in table 1.

Gel Image Analysis: Gels were kept in staining solution (50% methanol, 10% GAA (Glacial acetic acid)) and 0.05% Coomassie Brilliant Blue R-250), placed on a rotating platform for 2 h, rinsed in ddH2O for 1 min, and finally destained in destaining solution (30% methanol and 10% glacial acetic acid) to make the background clearer and finally the comparison was made from all the three different protocols (Protocol 1, 2 and 3) used for three different tissues (shoot, root and seed).

Table 1: Constituents of 12% separating and 5% stacking gel

12% Separating gel	
Component	**Volume**
M.Q H_20	3.3ml
Bisacrylamide 30%	4.0ml
Tris pH(8.8)	2.5ml
SDS (10%)	100µl
APS (10%)	200µl
TEMED	8µl
5% stacking gel	
M.Q H_20	2.1ml
Bisacrylamide (30%)	0.5ml
Tris(1M)	0.38ml
SDS (10%)	30µl
APS (10%)	30µl
TEMED	5µl

RESULTS AND DISCUSSION

Several techniques have been employed for extraction of proteins from different tissues across various crops , such as , phenol extraction for plasma membrane protein from barley (Hurkman and Tanaka, 1986), TCA-acetone extraction for rice, Arabidopsis, maize, and cucumber leaves (Cho *et al*., 2006). An appraisal of the current methodologies indicates that the protein extraction efficiency is tissue and/or species specific. Even though there are substantial reports of different extraction protocols in several crops, no comprehensive experimental reports are available for effective extraction of high quality proteins from diverse tissues of buckwheat. In the present study, we attempted to standardize a protocol that results in higher extraction efficiency from different buckwheat tissue by evaluating the yield and resolution of the protein bands on 1-D gel produced from these extraction protocols. Three distinct methods for extracting protein from leaves, roots and seeds of field-grown buckwheat were optimized. The possibility of a common protein extraction methodology producing optimal high quality protein yield from all of these ontogenetically varied tissues was believed to be doubtful because the metabolic components were expected to be tissue-specific. As a result, in this investigation, desired tissues were weighed, homogenized, and then exposed to various extraction techniques depending on the buffers utilized (Protocol 1, 2, and 3). Traditionally, proteins are extracted using an aqueous buffer, detergents, or direct precipitation (Michaud and Asselin, 1995). In addition to the commonly used trichloroacetic acid (TCA)/acetone precipitation approach (Cho *et al*., 2006), phenol extraction followed by methanol/ammonium acetate precipitation has been proven to be efficient for proteome research (Hurkman and Tanaka, 1986). The quality and quantity of proteins extracted from diverse tissues using Protocols 1-3 were assessed and compared in this study. Regardless of the tissue utilized for protein extraction, the phenol extraction methodology produced the highest yield in mature buckwheat seeds when compared to the other two protocols (Table 2). Earlier research compared the efficacy of TCA/acetone and phenol-based methods for extracting proteins from refractory tissues, with the latter technique being more effective in eliminating interfering chemicals (Carpentier *et al*., 2005). In this context, addition of phosphate buffer to TCA-Acetone helped in maintaining pH of the solution, which resulted in extraction of high quality protein from leaves and roots.

After extracting proteins from various tissues using Protocols 1, 2, and 3, they were then separated using 1-D gel (SDS PAGE). A comparison was made on the basis of resolution of bands formed. Although Protocols 1 and 3 produced better outcomes than Protocol 2, Protocols 1 and 3 were found to be more suitable for relatively hard (seeds) and soft tissues (leaves and roots) respectively. While as Protocol 2 produced a considerably lighter colored pellet with all of the tissues, making it unsuitable for 1-D PAGE (Wu *et al*., 2014).

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Table 2: Protein yields from buckwheat seeds, leaves and root with Phenol, Phosphate+TCA-acetone (Trichloroacetic acid) and TCA-acetone (Trichloroacetic acid) extraction methods

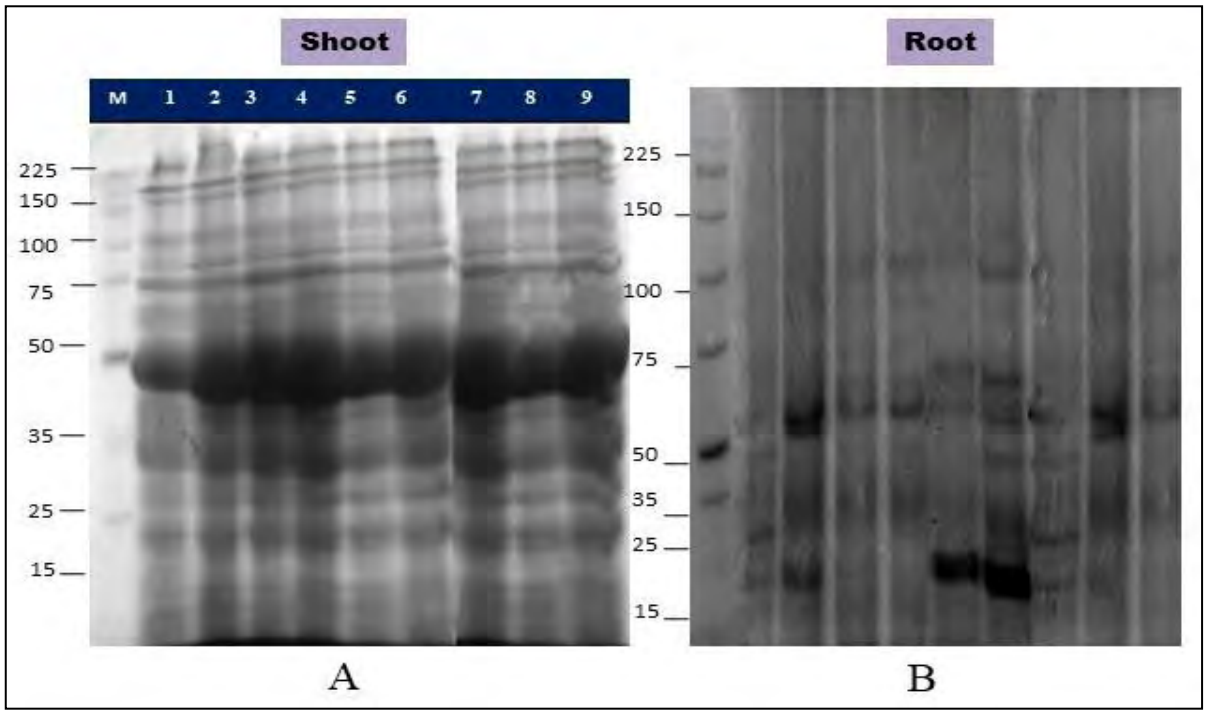
Methods	Genotypes	Root (µg/µl)	Shoots (µg/µl)	Seeds (µg/µl)
Phenol Extraction	BWZ1	1.24±0.02	1.17± 0.07	3.61±0.07
	BWZ2	1.16±0.06	1.23±0.04	3.80±0.05
	BWZ33	1.04±0.03	0.53±0.03	3.75±0.07
	BWZ41	0.37±0.06	0.94±0.03	4.61±0.04
	BWZ69	1.10±0.09	1.00±0.0	3.86±0.06
	BWZ92	1.08±0.07	1.11±0.09	2.93±0.08
	BWM2	0.82±0.05	0.75± 0.03	3.41±0.04
	BWM4	0.75±0.07	0.65±0.07	3.00±0.05
	BWM57	1.09±0.07	1.22±0.06	2.41±0.04
Phosphate + TCA-Acetone	BWZ1	1.18± 0.04	2.01± 0.04	0.82± 0.00
	BWZ2	1.12±0.03	1.92±0.03	1.61±0.06
	BWZ33	1.19±0.03	2.31±0.04	0.22±0.00
	BWZ41	1.82±0.07	2.13±0.03	1.02±0.05
	BWZ69	1.90±0.06	2.10±0.04	1.09±0.00
	BWZ92	1.81±0.04	2.30±0.05	0.75±0.00
	BWM2	1.15± 0.07	2.20± 0.04	0.54± 0.06
	BWM4	1.20±0.04	3.31±0.05	0.34±0.00
	BWM57	1.93±0.06	2.12±0.03	0.51±0.00
TCA-Acetone	BWZ1	0.14± 0.04	0.11± 0.04	0.14± 0.04
	BWZ2	0.09±0.03	0.22±0.04	0.09±0.05
	BWZ33	0.23±0.05	0.47±0.05	0.24±0.05
	BWZ41	0.19±0.06	0.39±0.05	0.16±0.06
	BWZ69	0.02±0.00	0.22±0.05	0.02±0.00
	BWZ92	0.12±0.00	0.13±0.03	0.12±0.00
	BWM2	0.13± 0.00	0.20± 0.05	0.13± 0.00
	BWM4	0.02±0.00	0.06±0.00	0.02±0.00
	BWM57	0.13±0.00	0.22±0.00	0.13±0.00

The light bands observed with Protocol 2 could be attributed to the presence of contaminants in the protein samples, which could have contributed to the low protein yield in these gels. Figure 4 depicts a clear difference in the pattern of protein bands observed using different methods in leaves, roots and seeds. A methodology determined to be suitable for one plant of a species may not be suitable for another. This is demonstrated by studies that found Protocols 1 and 2 to be suitable for different tissues of *Arabidopsis thaliana* and banana leaves (Maldonado *et al*., 2008), however only Protocol 2 was found to be suitable for Brassica seeds (Devouge *et al*., 2007). This work makes it clear that defining a single protein extraction strategy for various tissues which have been provided with developmental-specific metabolites is practically impossible.

The same conclusions were put forth in previous studies (Stalikas, 2007). Overall, this study indicated that Protocol 3 works well for buckwheat leaves and roots and protocol 1 for seeds. As such the protocols for

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higher recovery of protein from different tissues of buckwheat have been established and can be used for high through proteome analysis in future proteome analysis in buckwheat and establishment of buckwheat proteome atlas. The availability of a proteome atlas in the public domain is likely to be a helpful tool in identifying distinct biological features that could eventually be utilized for making abiotic-and/or biotic-stress resistant "smart" buckwheat.



M- Marker; 1- BWM2; 2- BWM1; 3-BWZ92;4-BWZ69; 5-BWZ33; 6-BWM4; 7-BWZ41;8-BWZ2; 9- BWM57

Fig. 4: A. Protein extraction from shoot using Phosphate- TCA acetone Method; B.; Protein extraction from root using Phosphate- TCA acetone Method

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CONCLUSION

In the present study, we established a protocol (Phosphate-TCA-Acetone Buffer) for high recovery of proteins from buckwheat shoots and roots and also established a protocol (Phenol extraction method) for high recovery of proteins from seeds. The extracted proteins from shoots, roots and seeds were further quantified and resolved on SDS-PAGE for validation of the protocol. Our study proved that establishment of proteome extraction is tissues specific as such different methods suited to different tissues. This study have further implications for establishment of a proteome atlas in buckwheat.

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