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Two-Dimensional Gel Electrophoresis of Protein profile from Groundnut (*Arachis hypogaea* sp. Virginia) at Flower Development Stage

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A B S T R A C T

Protein is play critical role in flower development. Profiles of total flower development proteins isolated from different stages (Bud, Flower and Peg) peanut cultivar GJG-22, were studied using two-dimensional gel electrophoresis. Protein is extracted by trichloro acetic acid/acetone precipitation method. Comparative analysis was carried out of spots identified using platinum master software at three different flower stages. Protein revealed that total 9193 protein spots with the pH range 4 - 7 and 23-312 kDa range recorded. Out of 9193 spots, maximum 3413 spots were found in pH range 6 - 7 in groundnut samples. Proteomic analysis reveals the translational products of gene expression of plant during flowering stage.

Introduction

The most precarious step in any proteomics study is sample extraction and preparation. In this regard, proteomic analysis of plant tissues involves a number of practical challenges that are typically more problematic than with other samples. In addition to having relatively low protein concentrations, plant tissues are often higher in proteases enzyme and materials that severely restrict with downstream protein purification and analysis, including cell wall and storage polysaccharides, phenolic mixtures, lipids and a wide-ranging of secondary metabolites (Granier, 1988; Tsugita and Kamo, 1999).

Groundnut or peanut (*Arachis hypogaea* L.) is one of the principal economic crops of the world (Cobb and Johnson, 1973). It is most popular and universal legume crops, cultivated in more than 100 countries. Groundnut plays an important role in the agricultural and industrial economy of the country. *Arachis*

hypogaea has the widest distribution of any *Arachis* species. More than half of the production area that accounts for 70% of the groundnut growing area falls under arid and semi-arid regions (Reddy *et al.,* 2003). It is a rich source of oil (49.24 g/100 g), protein (25.80 g/100 g), carbohydrates (16.13 g/100 g), minerals, fiber and vitamins (USDA National Nutrient database).

In groundnuts, the basic reproductive units constitute the flowers. Flowering and flowers play an important role in all seed crops, yield is dependent largely upon the basic reproductive units available. Proteomic analysis reveals the translational products of gene expression of plant during flowering stage. Analysis of proteins is a direct approach to define the function of their associated genes as it linked to genome sequence information, which is important for functional genomics. There are scanty reports of proteome analysis that focus on the study during flower development of peanut genotype (Kottapalli *et al.,* 2008; Liang *et al.,* 2006).

Production of high-quality protein samples is the critical initial step for proteomic analysis. Although numerous of the sample preparation methods had been reported, TCA/acetone precipitation was still a very useful method for minimizing protein degradation/modification and removing interfering compounds such as polysaccharides, polyphenols, pigments and lipids (Saravanan and Rose, 2004; Manadas *et al.,* 2006). However, the precipitated proteins, especially those from recalcitrant tissues, were difficult to resolubilize (Gorg *et al.,* 2004; Carpentier *et al.,* 2005). Repeated precipitation in TCA/acetone presumably causes aberrant and irreversible protein refolding and aggregation. This always results in the loss of certain groups of proteins such as membrane proteins or other hydrophobic proteins and hard-to-obtain well-resolved 2-DE maps.

To increase the solubility of precipitated proteins, some chemicals such as chaotropes (urea and thiourea), surfactants (CHAPS) (Valcu and Schlink, 2006), 3-(4-heptyl) phenyl 3-hydroxypropyl dimethylammonio propane sulfonate (C7BzO), SDS (Wang *et al.,* 2006) and reductants (b-mercaptoethanol, DTT, tributylphosphane (TBP) (Ruan and wan, 2007; Gomez- Vidal *et al.,* 2008) were added to lysis buffer to break hydrogen bonds, discharge hydrophobic interaction and diminish disulfide bridges. Several physical-aided resolubilization procedures such as sonication and repeated freeze–thaw (Hopkinson *et al.,* 2005) were also reported. However, these methods were time and labor consuming and the rate of resolubilization depended on given plant tissues and skill of researcher (Zhang *et al.,* 2011).

Here, we describe an effective and all-purpose application protocol for protein extraction andtwo-dimensional gel electrophoresis (2DE) analysis from Groundnut (*Arachis hypogaea ssp.* Virginia) tissues, specifically flower development tissues.

Materials and Methods

Plant materials

Gujarat Junagadh Groundnut-22 (GJG-22) genotype of groundnut (*Arachis hypogaea ssp.* Virginia) was collected for study based on new released variety from "Oil Seed Research Station" of Junagadh Agricultural University, Junagadh, Gujarat, India. Groundnut plants were grown in a net house. A total 12 tissue samples was collected, including nine vegetative tissues and three stages of flower development. Flower bud, open flower and peg are denoted as Stage 1, Stage 2 and Stage 3 respectively. At least three biological replicates were collected for each tissue sample. Each stage of plant included samples were collected; First tetra foliate leaf (UL),

Last tetra foliate leaf (LL) and Main internode bearing primary branch(S). Three stages each from flower bud, flower and peg development were frozen in liquid nitrogen immediately after harvest and stored at -80^0 C prior to protein extraction.

Protein extraction

Protein extracts were prepared in biological triplicates for each developmental stage. For protein extraction, 1 gm of each plant tissues were used. Grind fresh/frozen plant tissues into fine powder in liquid nitrogen, add ice-cold TCA/acetone (containing 0.07% v/v β-mercaptoethanol, 5 ml/g tissue) and transfer this mixture into a centrifuge tube. Place the tube on ice for 5 min. Centrifuge at 10 000 ×g for 5 min at 4^0 C and discard the supernatant. Repeat this step until a clear supernatant is achieved. Wash the pellet with 1.5 ml ice-cold acetone twice. Invert the tube on a clean piece of absorbent paper to drain the access acetone. Then add phenol (containing 0.5% w/v DTT, 2 ml/g tissue) and mix thoroughly, place the tube at RT for 10 min.

Centrifuge at 10 000 × g for 5 min at 4^0 C and transfer the supernatant to a new tube. Repeat this procedure thrice and put the supernatant together. Add five volumes of cold methanol (containing 0.1 M ammonium acetate) to the collected phenol, mix thoroughly and keep at -20^0 C for at least 10 min to precipitate the protein. Centrifuge at 10 000 × g for 10 min at 4^0 C and discard the supernatant carefully. Add 1 ml pre ice-cold methanol to wash the pellet, centrifuge at 10 000 × g for 5 min at 4^0 C and discard the supernatant. Repeat thisprocedure thrice to remove remaining ammonium acetate and phenol.

Freeze-dry the pellet; the product can be stored at -80^0 C or used directly for SDS-PAGE and IEF. Protein concentration was determined using the 2D-Quanti Kit (GE-Healthcare®), with bovine serum albumin (BSA) as standard.

Two-dimensional PAGE electrophoresis

All two dimension (2DE) procedure was followed as GE health care 2D manual and standard method adopted by (Jiang *et al.,* 2008 and Han *et al.,* 2009). The each 12 samples were diluted with a rehydration buffer (8 M urea, 2% CHAPS, 10% glycerol, 1% bromophenol blue, 0.5% IPG-buffer, 0.28% DTT) to 50-100 µg (silver staining) or 300-350 µg protein (CBB staining) per 100 or150µL, respectively, and was applied *via* anodic cup loading. The strips of 24 cm and pH 4–7(GE Healthcare®) were rehydrated for at least 10-20 h in 400 µL rehydration buffer. IEF was carried out on the EttanIPGphor 3 (GE Healthcare®) at 20^0 C with current limit 50mA/strip: 1 h at 200 V, 7 h at 500 V, 1 h at 1000 v, 8 h at 8000 V (gradient), 5 h at 8000 V, and 4 h at 5000 V.

The resolution of proteins was further improved when the IEF gels were subjected to electrophoresis in the second dimension in presence of SDS. Prior to second dimension analysis, individual strips were equilibrated for 15 min in 3 mL of an SDS equilibration buffer (75mMTris-HCl, pH 8.8, 6 M urea, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) containing DTT (100 mg/10 ml), followed by 15 min in a 3 mL equilibration buffer containing iodoacetamide (250 mg/10 ml).

The second dimension was performed on 12% SDSPAGE gels (1 mm x 18 cm x 20 cm) using a Protean II system (Bio-Rad®) coupled to a Multi Temp III Thermostatic Circulator (GE-Healthcare®) cooling bath. Running was set at 80 v for 20 20 minutes until the sample travels through the IPG strip into separating gel and 150 V until the dye reached the bottom of the gel.

Protein visualization, image analysis and quantification

The gels were then stained with Bio-Safe Coomassie brilliant blue Stain G-250 (Bio-Rad), according to Neuhoff *et al.,* (1988).The gels were kept in staining solution (0.1% Coomassie brilliant blue G 250, 40% methanol,10% Acetic acid and 50% distilled water) for overnight. Gels were repetitive destained with destaining solution (40% methanol and 10% Acetic acid and 50% Distilled water) for until the clear protein visualization. Stained gels were scanned and calibrated with Typhoon FLA Scanner. Image analysis was performed with Platinum Master software (GE healthcare). Spot detection was realized without spot editing. The spots were quantified using the %volume criterion.

Results and Discussion

2-DE analysis of the different stages at flower development in Groundnut (*Arachishypogaea* ssp. Virginia)permitted characterizing protein dynamics involved in flower development, disclosing differential metabolism with specific protein, differential expression at each stage, and those in common during the developmental process to achieve a better protein profile of each developmental stages.

The result of study showed that total 9193 spots were detected in software analysis**.** Out of 9193 spots, 3342 spots in stage 1 (bud stage) and 3864 spots in stage 2 (flower stage) and 1987 spots in stage 3 (peg stage) were reproducibly detected. 75 spots, 194 spots and 170 spots were matched in different stages 1, 2 and 3 respectively. The molecular masses of matched spots were identified with the range of 23 KDa to 312 KDa with pH from 4.00 to 6.76 (Table 1). Some protein spots indicated on the comparative 3-D graphs were showed significantly differentiated the relative expression level between samples (Figure 1). Many spots were shown the significant at different level of expression (Up regulation and down regulation) in all different samples.

Out of 9193 spots, total 3030 spots found between pH 4 to pH 5. 240 spots in UL1, 354 spots in bud, 184 spots in LL1 and 232 spots in S1 and M.W from 24 KDa to 302 KDa in stage 1, 459 spots in UL2, 292 spots in flower, 296 spots in LL2 and 306 spots in S2 and M.W from 24 KDa to 310 KDa in stage 2 and 175 spots in UL3, 45 spots in peg, 173 spots in LL3 and 274 spots in S3 and M.W from 28 KDa to 265 KDa in stage 3 (Table 1).

Total 2750 spots found between pH 5 to pH 6. There were 301 spots in bud, 222 spots in LL1 and 225 spots in S1 and M.W from 24 KDa to 304 KDa in stage 1, 379 spots in UL2, 249 spots in flower, 218 spots in LL2 and 288 spots in S2 and M.W from 23 KDa to 312 KDa in stage 2 and 163 spots in UL3, 68 spots in peg, 195 spots in LL3 and 217 spots in S3 and M.W from 28 KDa to 275 KDa in stage 3; were found between pH 5 to pH 6. Out of 9193 spots, total 2750 spots found between pH 5 to pH 6 (Table 1).

Total 3413 spots found between pH 6 to pH 7. 292 spots in UL1, 496 spots in bud, 301 spots in LL1 and 270 spots in S1 and M.W from 25 KDa to 304 KDa in stage 1, 435 spots in UL2, 319 spots in flower, 261 spots in LL2 and 362 spots in S2 and M.W from 24 KDa to 312 KDa in stage 2 and 138 spots in UL3, 75 spots in peg, 220 spots in LL3 and 244 spots in S3 and M.W from 28 KDa to 276 KDa in stage 3 (Table 1).

In protein profiling, maximum (3413) spots were found between pH 6 to 7 and minimum (2750) spots were found between pH 5 to 6. But highest number of up regulated spots was found between pH 6 to 7. So, it appears that maximum growth and development responsive protein spots were lies near 6-7 pH range.

Fig.1 2DE analysis of 12% PAGE with pH 4-7 gradient strips were stained using CBB R 250 of groundnut samples. A1: Bud, A2: Lower leaf-1, A3: Stem-1, A4: Upper leaf-1, B1: Flower, B2: Lower leaf-2, B3: Stem-2, B4: Upper leaf-2, C1: Peg, C2: Lower leaf-3, C3: Stem-3, C4: Upper leaf-3

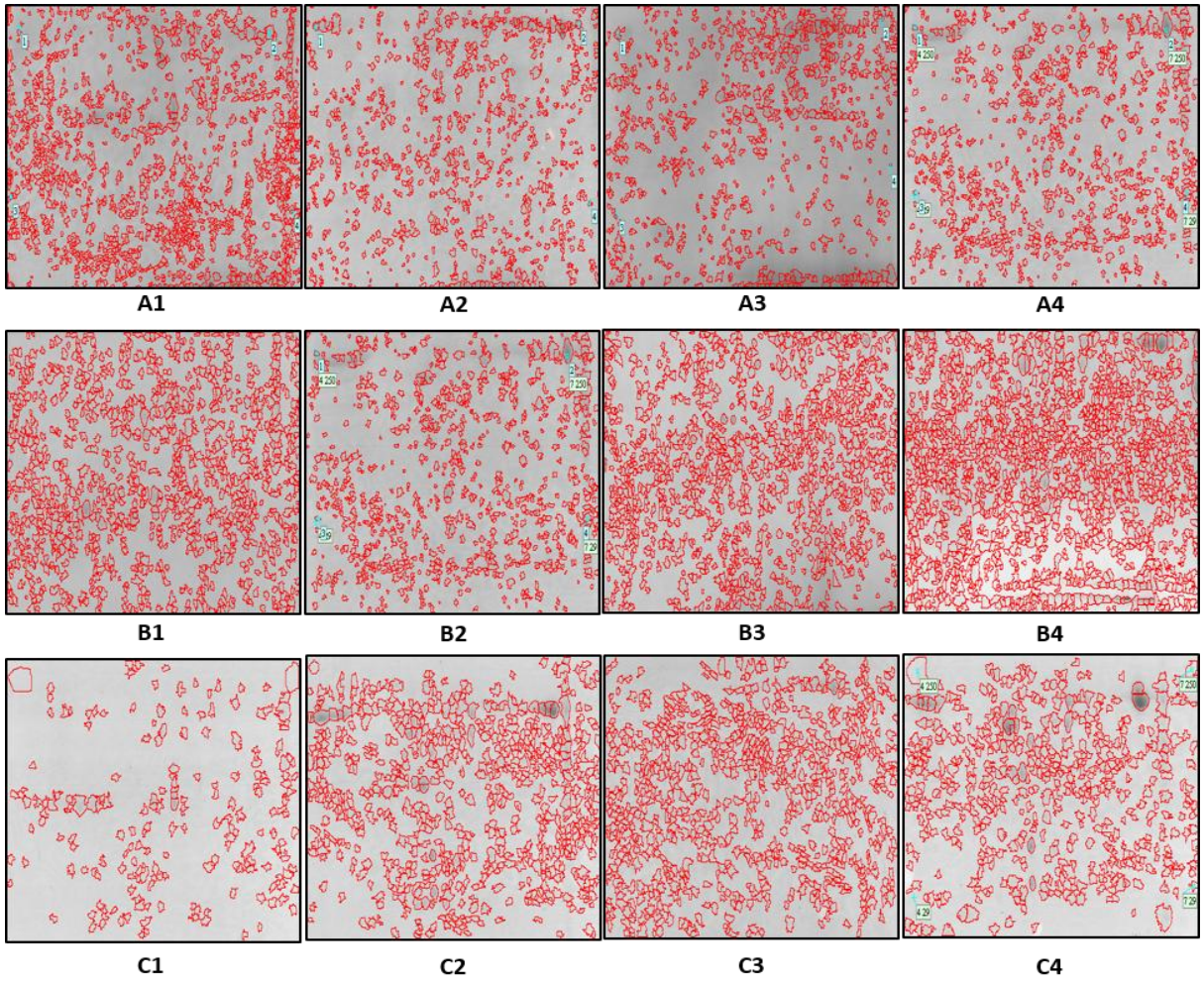

Table.1 Protein spots with PI group and molecular weight ranges of different samples

PI Range	No. of spots												Total no. of Spots
	UL1	BUD	LL1	S1	UL2	FLOWER	LL2	S2	UL3	PEG	LL3	S3	
PI (4-5)	240	354	184	232	459	292	296	306	175	45	173	274	3030
MW	24-301	24-302	24-290	24-298	23-308	23-295	24-310	24-312	28-253	28-240	28-257	27-265	
PI (5-6)	225	301	222	225	379	249	218	288	163	68	195	217	2750
MW	24-285	24-304	24-302	24-301	23-312	23-310	23-312	23-310	27-265	28-275	27-243	27-272	
PI (6-7)	292	496	301	270	435	319	261	362	138	75	220	244	3413
MW	24-304	24-294	24-288	25-285	24-312	24-293	24-310	23-312	28-250	27-245	28-276	28-266	
Total Spots	757	1151	707	727	1273	860	775	956	476	188	588	735	9193

Proteomic technology is a useful approach for identifying proteins in plants (Yang *et al.*, 2009; Yang *et al.*, 2007; Hossain *et al.*, 2013; Kosova *et al.*, 2011; Li *et al.*, 2013). Recently, a number of proteins that are differentially expressed between peanut aerial gynophores, subterranean gynophores, and early swelling pods, were identified using two-dimensional electrophoresis combined with mass spectrometry (Zhu *et al.*, 2013; Sun *et al.*, 2013). In the current study, we performed 2 DE to provide a comprehensive overview of the proteome profile at flower development stages from peanut. Compared with traditional approaches, fractionation prior to LC−MS/MS analysis significantly increased the number of identified proteins and individual protein coverage (Das *et al.*, 2010; Wang, *et al.*, 2010). We successfully spotted a total of 9193 proteins in peanut at different flower developmental stages. Out of 9193, 3342 at bud developed stage, 3864 at flower development stage and 1987 at peg formation stage is reported. In contrast to other aboveground organs of peanut, the aerial gynophores grow downwards to the ground. Proteins that play positive roles bud to peg formation to final pod formation. Itwas reported that the ABC transporters, heat shock proteins, microtubules, and microtubule-associated proteins play important roles in the plant's development (Collings *et al.*, 1998; Zupanska *et al.*, 2013).

Our analysis of proteome data provided useful information about the mechanism controlling various flower developmental stages. This proteome analysis is first time reported in flower development stage in groundnut. In proteomic analysis, higher protein spots with the different pI range recorded. Out of these spots apart from differentially expressed spots covers large intensity and based on these many spots up and down regulated at bud, flower and peg stages of groundnut. This investigation facilitated the extraction of high-quality protein samples suitable for 2DE-electrophoretic analysis in a wide range of routine plant tissues and recalcitrant plant tissues. And also give the idea for further study on identification of protein on the basis of this proteome analysis.

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