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Characterization of groundnut seeds through SDS-PAGE profiling during release of fresh seed dormancy via ethrel treatment

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Abstract
In India, groundnut is cultivated in the rabi-summer period, rainy and post-rainy seasons. A short period of 10-15 days fresh seed dormancy is required in the Spanish type of groundnut to prevent in-situ seed germination in the field due to unseasonal rains at the time of crop maturity. Therefore the main aim of present study was to found different proteins which are involved in germination process during release of fresh seed dormancy. For that uniform size mature seeds were harvested and treated freshly in laboratory with 0.05% ethrel solution under sterile condition and sampled at 6 HAI (Hours after Imbibition), 12 HAI and 24 HAI, while seeds without treatment were taken as control and stored immediately after harvesting. Groundnut seed protein SDS-PAGE profiling of NRCG-14380 was carried out at four different stages. The result indicated variation in banding profiling at all four stages. These bands were extracted from gel and digested with trypsin and subjected to UPLC-MS/MS for protein identification. The result revealed identification of 64 different proteins, majority of them was identified as seed storage protein. Hence it can be concluded that seed storage proteins play immense role during dormancy release to prove energy to the developing seed during germination.

Keywords: SDS-PAGE, protein profiling, groundnut, dormancy, germination, ethrel

Introduction
Groundnut (*Arachis hypogaea*) is an annual warm-season plant of the legume family, cultivated since ancient times for various food, feed and fodder purpose ((Pardee, 2002) [10]. It is originated in South America. The seeds contain 40-50% oil, 20-30% proteins and are an excellent source of B vitamins (Javaid *et al*., 2004) [7]. The seeds are a great source of vitamins B and include 40– 50% oil, 20–30% proteins, and other nutrients (Gore *et al*., 2022) [5]. Seed dormancy and germination are major events to protect life cycle of next generation (Chaudhari *et al*., 2023) [3]. Groundnut species *fastigiata* possesses short period of primary dormancy that means upon high moisture condition during seed maturation stage, it may leads to dormancy loss results in pre-harvest sprouting (PHS) when pods are still attached to its mother plant. PHS reduces grain quality and yield of groundnut by up to 20–40% (Reddy *et al*., 1985) [12]. Ethylene is one of the main causes of germination in case of groundnut.
Upon imbibition, the seed rapidly resumes metabolic activities like respiration, enzymatic activity, RNA and protein synthesis. Hydrolytic enzymes are mainly secreted and catalyze the depolymerisation of starch and protein reserves in the starchy endosperm. Degradation products are absorbed and translocated to the seedling for further growth and development (Bentsink *et al*., 2008; Potokina *et al*., 2002) [1, 11]. Most enzymes which are involved in major reserve mobilization are apparently synthesized de novo during seed germination. However, this activation of different enzymes and reserve mobilization varies from crop to crop and hence seed germination study in groundnut became evident to understand these important events at protein level.
Seed dormancy is important in plant ecology and agriculture because it allows seeds to overcome periods unfavourable to seedling formation. Some studies have been conducted to better understand how germination is controlled by various environmental factors and chemicals used in groundnut (Shelar *et al*., 2014) [14]. However, little is known about the process by which embryos emerge from seeds and complete germination and how embryo emergence is blocked in dormant seeds (Bewley, 1997) [2]. These physiological processes are influenced by both genetic and environmental factors like light, temperature, water, time of seed dry storage.

Hence, present study was conducted to understand the role of some major proteins playing crucial role during different stages of seed germination.

Materials and Methods

Seeds sowing and sample collection

The experiment material comprised of seeds of NRCG 14380 groundnut genotype used for the present study were obtained from Indian Council of Agricultural Research (ICAR)-Directorate of Groundnut Research (DGR), Ivnagar Road, Junagadh, Gujarat, India. After attaining maturity, pods were harvested and shelled properly without damaging testae immediately after harvest. The seeds were considered as mature which showed browning of inner part of pods. These mature seeds are selected and treated with 0.05% (v/v) ethrel solution to induce germination and break dormancy. Germination pattern was observed for the sampling and total four stages were selected for current study. Different pre- and post- germination stages were selected for the sampling. Samples were collected at different time point *viz.* 0 hours after incubation (HAI), 6 HAI, 12 HAI and 24 HAI of the treatment and stored immediately at -80 °C for further downstream analysis.

Protein profiling

Extraction of protein

The extraction was carried out by using protocol of Zhao *et al*., (2015) [19] with minor modification. Also GE health care guidelines were applied wherever needed. The protocol summarized briefly as:

Seed samples of NRCG 14380 were weighed 500 mg and ground to fine powder in liquid nitrogen, and suspended in extraction solution containing 7 M urea, 4% CHAPS, 40 mM Tris-HCl (pH 8.5), 1 mM PMSF, 2 mM EDTA and 10 mM DTT. After 5 minutes, the above suspension was sonicated at 200 W for 5 min. and then centrifuged at maximum speed (13,000 x g) for 15 min. at 4 °C. The supernatant was carefully collected and 5x volume of chilled acetone containing 10% (v/v) TCA was added, and the samples were mixed well and incubated overnight at -20 °C. Samples were centrifuged at 13,000 x g for 15 min. at 4 °C and supernatant was discarded and the precipitates were washed three times with chilled acetone. After air-drying, precipitates were dissolved in lysis buffer containing 7M urea, 4% CHAPS and 20 M Tris-HCl (pH 8.0-8.5). The suspension was sonicated at 200 W for 5 min. and centrifuged at 13,000 x g for 15 min. at 4 °C. The supernatant was collected and transferred to a new tube for further analysis.

Determination of protein content through colorimetric analysis

Protein concentration was determined through Pierce™ BCA protein Assay Kit (Thermo Scientific) and is used to perform highly sensitive and selective colorimetric detection of cuprous ions Cu^{1+} (Smith *et al*., 1985) [15].

Protein profiling through SDS-PAGE

Total protein samples from seeds o NRCG 14380 were analyzed using SDS-PAGE (12% separating gel with 5% stacking gel). Electrophoresis was conducted on vertical slab gel PAGE unit (Bio-Rad) at 25 mA for 2 hrs and then 50 mA for next 1.5 hrs according to Laemmli system (Laemmli U. K., 1970) [9]. Each lane was loaded with 4 µl of protein extract and 2 µl protein molecular weight marker. After the electrophoresis gels were washed to remove excess of SDS and stained with 0.1% commassie brilliant blue-G250 in a mixture of methanol: acetic acid: distilled water in the ratio 40: 10: 50. The gels were destained by using a mixture of methanol: acetic acid: distilled water in the ratio 40: 10: 50 without dye (Sadashivam and Manickam, 1996) [13]. The image of obtained bands in gel was visualized and scanned by image scanner and taken further for analysis of banding pattern.

Protein identification

Protein identification was performed for the different bands collected from dormant genotype which were differentially expressed at respective four stages. For the identification proteins were digested with trypsin by using In-Gel Tryptic Digestion Kit (Thermo Scientific) through a given protocol.

Band preparation and destaining

Scalpel was used to excise protein band of interest from SDS-PAGE gel and bands were cut into 1 x 1 to 2 x 2mm pieces. Destaining solution (200 µl) was added to gel pieces. Samples were incubated at 37 °C for 30 minutes with shaking. Destaining solution was removed and discarded from the tube. The procedure from destaining is repeated once.

Reduction and alkylation

Reducing buffer was prepared and 30 µl was added to each tube containing the sample and incubated at 60°C for 10 min. Samples were allowed to cool and then reducing buffer was removed and discarded from the tube. Alkylation buffer was prepared and 30 µl alkylation buffer was added into each tube. Samples were incubated in the dark at room temperature for 1 hour. Alkylation buffer was removed and discarded from the tubes and samples were washed by adding 200 µl destaining buffer to each tube. Samples were incubated at 37°C for 15 min. with shaking. Distaining buffer was removed and discarded from the tubes.

Digestion

Gel pieces were shrinked by adding 50 µl of acetonitrile and samples were incubated for 15 min. at room temperature. Acetonitrile was carefully removed and gel pieces were allowed to air dry for 5-10 min. Activated trypsin was taken and working solution was prepared freshly as per description given in the kit. Gel pieces were swelled by addition of 10 µl of activated trypsin solution to the tubes. Samples were incubated at room temperature for 15 min. Then 25 µl of digestion buffer was added to each tube and samples were incubated at 37 °C for 4 hours or at 30 °C overnight with shaking. Digestion mixture was removed and placed in clean tubes. To further extract peptides, 10 µl of 1% formic acid solution was added to gel pieces and kept for 5 min. incubation. Extraction solution was removed and added to digestion mixture.

Protein Identification by ultra-performance liquid chromatography electrospray-ionization mass spectrometry (UPLC- ESI-MS/MS)

The bands showing alteration in its content and polymorphic bands in response to different germination time period were cut and in-gel proteolysed with trypsin. The resulting peptides were analysed by UPLC-ESI-MS/MS. The obtained spectra

were used for the identification of the proteins. Protein identification was performed through ProteinPilot^{TM} software (SCIEX, MA, USA) by using *Arachis hypogaea* as standard reference taken from Uniprot and NCBI.

Results and Discussion

Sample collection

Seeds of NRCG 14380 were treated with 0.05% ethrel solution to effect germination (Upadhyay and Nigam, 1999) [16]. The result showed uniformity in different germination stages in both. The seeds were germinated within 24 hour of treatment, so four different pre- and post- germination stages were selected between 0 to 24 hours. Different stages of germination are presented in Figure 1. All four stages seeds possess different morphological characteristics. For example 6 HAI stage seeds get swelling due to imbibition as compared to control, 12 HAI stage seeds get radicle protrusion and 24 HAI stage seeds showed radicle development stage.

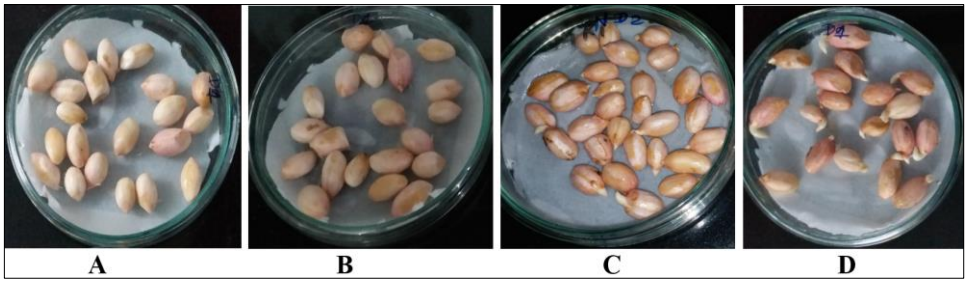

Fig 1: Different stages of seeds showing different morphological characteristics after ethrel treatment. A: Control, B: 6 HAI, C: 12 HAI, D: 24 HAI

Protein profiling and identification

Proteins were extracted, quantified and subjected to SDS-PAGE for profiling of low and high molecular weight proteins. The total soluble seed proteins isolated from dormant and non-dormant genotype were fractioned into 16 different bands of low and high molecular weight which showed variation in interplay among different seed developmental stages. Figure 2 showed typical banding pattern of four stages obtained through staining of gel. The result showed almost similar banding pattern among all the stages except 60, 35 and 15 kD band. The intensity of these bands was reduced subsequently from imbibition to radicle protrusion stage. The identification of protein bands showing difference in band intensity and bands of control stage was carried out and result obtained was presented in Table 1.

Protein identification can be applied to various conditions of seed biology mainly in model organism or elite agricultural crops. The mature seed is compact well-defined object, easy to access and simple to study under number of given conditions. Numbers of biological processes get initiated after transition from mature developed seed towards dormancy release. In plant science proteins identification through various methods for the study of germination affected by range of biological, chemical and physical condition is in vogue (Wang *et al*., 2015) [17] and dormancy and germination mechanism varies from species to species. In groundnut no such study is defined so understanding seed germination was carried out from seeds of dormant genotype NRCG 14380 at four stages following dormancy release by ethrel solution.

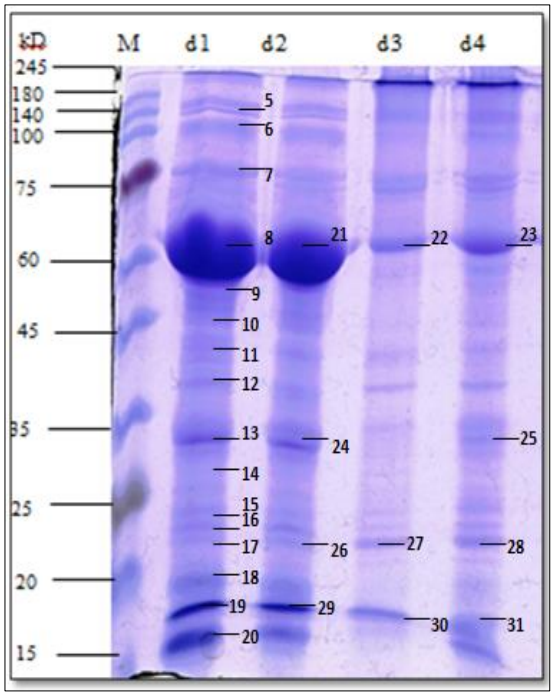

Fig 2: SDS-PAGE profiling (12% gel) of groundnut seeds during different stages of seed germination showing variation in protein bands. Here M represents protein marker and d1, d2, d3, and d4 represents four stages namely control, 6 HAI, 12 HAI and 24 HAI respectively.

The results *in silico* protein identification in present study showed number of proteins majority of them were related to seed storage related proteins. For example in control (0 HAI), AAI domain containing protein, VQ domain containing protein, Arachin, oleosin, different group of LEA proteins and other proteins like ribonucleoproteins, disease resistance protein, seed linoleate 9S-lipoxygenase, sucrose transport protein was identified. While during germination serine hydroxymethyl transferase, NPC intracellular cholesterol transporter 1 isoform, Fatty acyl CoA reductase 4, histone methyltransferase, UDP-glucuronate:xylan alpha-glucuronosyltransferase 2-like isoform, U-box domain-containing protein 3, glucose-6-phosphate 1-epimerase isoform necessary for seedling growth were identified. Also during radicle development stage proteins photosystem I reaction centre subunit N and FAR-RED IMPAIRED RESPONSE were identified indicating onset of photosynthesis.

Table 1: List of proteins identified from SDS-PAGE profiling during control and different stages of seed germination in NRCG 14380 genotype

Band No.	Protein identified	Sequence	Modifications	Observed mass	Conf. %	Genotype
5	Retrotrans_gag domain-containing protein	EVFPLEVCATDGK	Carboxymethyl(C)@8	1464.6	87.6	tr|A0A444XSZ0
5	REVERSED Uncharacterized protein	ELSGVKKEAASASGK	-	1460.7	70.7	RRRRRtr|A0A445CZZ5
5	CLP protease regulatory subunit CLPX1, mitochondrial	TSWGGSNLGKDLPSPK	-	1640.2	98.9	XP_025700427.1
5	putative H/ACA ribo-nucleoprotein complex subunit 1-like protein 1 isoform X2	MRPPRGGGRGGGFR	-	1456.3	94.9	XP_029149935.1
5	REVERSED WPP domain-interacting tail-anchored protein 2 isoform X3	ELSGVKKEAASASGK	-	1460.7	78.8	RRRRRXP_025701871.1
6	PHD-type domain-containing protein	ARRPGEAGLQGVIR	-	1476.1	71.9	tr|A0A445BW55
7	Uncharacterized protein	GLSILVPAERR	-	1211.5	92.5	tr|A0A445CJ57
7	Lipoxygenase	NLRGDGKGER		1098.2	92.5	tr|Q4JME7
7	REVERSED L18 ribosomal protein	VDIGNDILSQVVAR	-	1492.9	92.5	RRRRRtr|A0A0I9QPT2
8	AAI domain-containing protein	ATPAAPSSSQAPPLSSTPK	-	1795.2	90	tr|A0A444ZBQ5
8	REVERSED VQ domain- containing protein	MSQPKPPNNPPPR; INKGIKNVGLSDNI	Deamidated(N)@9	1460.5	82.3	RRRRRtr|A0A444WVQ0
8	small nuclear ribonucleoprotein SmD3b	GASLGVGRGRAVAMR	-	1456.4	96.5	XP_025653046.1
8	protein ENHANCED DISEASE RESISTANCE 4	SGVNFSSDDYSGYEFHSVDRVAGPSSNSNK	-	3210.8	74	XP_025603421.1
8	protein HEADING DATE 3A-like	RENSPTGSSRR	-	1247.7	89.1	XP_025692301.2
9	Uncharacterized protein	EEISVDNGEVRRGVEEK	Deamidated(N)@7	1944.1	99	tr|A0A445EPC4
9	Protein kinase domain-containing protein	HFDYDAGSSSSSFDDYSTSSSSGGGVSVLYTRSMEFYDHK	Oxidation(M)@34	4378.1	99	tr|A0A444X409
9	Uncharacterized protein	TYQSFVAAAGVLR	-	1383.2	98.6	tr|A0A445BKA2
9	uncharacterized protein LOC112785465	DVHIVTDNAANYVAAKR	-	1855.0	99	XP_025684712.1
9	CRM-domain containing factor CFM3, chloroplastic/mitochondrial	EEISVDNGEVRRGVEEK	Deamidated(N)@7	1944.1	99	XP_025630862.1
9	type I inositol polyphosphate 5-phosphatase 5-like	DQLNIEREAGRVFNGFQEGR	Deamidated(N)@14	2334.8	92.6	XP_025648148.1
10	AAI domain-containing protein	TPSPSPSAEGSSQNGK	Deamidated(N)@14	1528.2	90	tr|A0A444XUA1
12	ANK_REP_REGION domain-containing protein	LTKLNHLVKLMLMVSR	-	1895.1	82.6	tr|A0A445ATE2
13	Protein FAR1-RELATED SEQUENCE	TYQSFVAAAGGHRK	-	1490.6	97.2	tr|A0A445CL22
13	Uncharacterized protein	VTFPIALLNNFKMGFTYGLFIDAFK	-	2868.4	89.3	tr|A0A445AN62
13	probable 2-oxoglutarate-dep. dioxygenase At3g111800	TYEGYGSRLGVEK	-	1459.4	89.4	XP_025683956.1
13	xylose isomerase-like	YTDVGLYAYTSVK	-	1479.2	81.8	XP_025640442.1
16	Oleosin	RGYDVSGGGVK	-	1093.36	96.6	tr|A0A444YZD4
16	AAI domain-containing protein	FEGSGGDGDQGSNSTLSFCK	Deamidated(N)@13, Carboxymethyl (C)@19	2051.7	99	tr|A0A445C040
17	TPR_REGION domain-containing protein	QLILGDHFPVGGDMGSSER	-	2015.1	97	tr|A0A445EMH8
17	Trehalose 6-phosphate phosphatase	ITPNNNPSWIVGHPSALKMFDEMIWNSK	Deamidated(N)@26	3226.7	90.8	tr|A0A445CE97
17	Late embryogenesis abundant protein group 1 protein	TLEAQEHLAEGR	-	1351.7	99	tr|E5FHY1

17	AAI domain-containing protein	IHEMMTEEEKQR	Oxidation(M)@4 and 5	1574.7	99	tr|A0A445CQP8
17	AAI domain-containing protein	MESSRSSPSK	-	1093.4	99	tr|A0A445AW03
17	Arachin 25 kDa protein	VDEPMTIRVAFHSTCM	Carboxymethyl(C)@15	1895.5	99	sp|P04149
17	REV. Mannose/glucose-binding lectin (Fragment)	VQTSHLVRGVTSK	-	1409.7	99	RRRRRtr|Q43377
18	CDT1-like protein b	QFMSPEDNSSTTTNK	Oxidation(M)@3	1704.2	99	XP_025644343.1
18	uncharacterized protein LOC112726510	VQEIQEMARQAR	-	1456.2	99	XP_025631705.1
18	trihelix transcription factor GT-2 isoform X1	LGDSALLGGGGSSGGASDDAVAAAAAVAASGGGGGSNNSGDDER	-	3702.4	99	XP_025621109.1
18	REVERSED peptidyl-prolyl cis-trans isomerase FKBP62	VLVEDLDKPNEWK	-	1583.3	98.1	RRRRRXP_025691721.1
19	AAI domain-containing protein	ITLAPCLSYLRR	Carboxymethyl(C)@ 6	1464.6	98.9	tr|A0A445AL67
19	Uncharacterized protein	LHLKPWGFALPHYGDVSK	-	2063.1	96.4	tr|A0A445DN78
19	Seed linoleate 9S-lipoxygenase	MSFLFNK	-	885.9	83	tr|A0A6B9V640
22	C2 domain-containing protein	ITERVENSSMATR	-	1491.8	98.2	tr|A0A444YD63
22	Serine hydroxymethyltransferase	MEKAKLLNYNNYAEISMATK	-	2330.1	92.1	tr|A0A444WW04
22	Uncharacterized protein	AQGVHVSGCVVVVR	Carboxymethyl(C)@9	1464.1	74.9	tr|A0A445CM73
22	uncharacterized protein LOC112742843	VTLAKGQWAK	-	1099.2	97.2	XP_025647867.1
22	protein SRC2 homolog	ITERVENSSMATR	-	1491.7	88	XP_025665931.1
23	DUF223 domain-containing protein	VLVNVEDATGSAR	-	1328.8	67.8	tr|A0A445B550
23	uncharacterized protein LOC112715909 isoform X3	VLVNVEDATGSAR	-	1328.8	99	XP_029145543.1
23	NPC intracellular cholesterol transporter 1 isoform X2	IEPGLEQQIALPR	-	1461.2	98.7	XP_029147694.1
23	60S ribosomal protein L24	RPYSRSIVGATLEVIQK	-	1915.6	90.2	XP_025663704.1
23	probable fatty acyl-CoA reductase 4	ATDTESVAHR	-	1085.5	87.1	XP_025669768.1
25	REVERSED histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH1	TTDFVYENADGEK	-	1488.3	85.7	RRRRRXP_025624760.1
25	UDP-glucuronate:xylan alpha-glucuronosyltransferase 2-like isoform X2	IEKPSFFDQIMGK	-	1536.5	87.8	XP_025660387.1
25	FAR-RED IMPAIRED RESPONSE 1-like	DVRNYISRYLR	Deamidated(N)@4	1456.2	86.4	XP_025638601.1
27	Elongation factor G, mitochondrial	NIGISAHIDSGK	-	1210.1	96.5	tr|A0A445CV40
27	Uncharacterized protein	MAEVAVTIVVEK	-	1286.9	86.6	tr|A0A444WRG
27	disease resistance RPP8-like protein 3	MAEVAVTIVVEK	-	1286.9	91.6	XP_025698930.1
27	REVERSED U-box domain-containing protein 3	CGNYEESSR	Carboxymethyl(C)@1	1099.3	87.2	RRRRRXP_025644732.1
29	ferredoxin, root R-B2	SSSSLSSVKNASK	-	1281.3	81.3	XP_025675667.1
30	putative glucose-6-phosphate 1-epimerase isoform X2	NQQGGESDGRGGVEVSK	-	1704.1	99	XP_025699937.1
30	photosystem I reaction center subunit N, chloroplastic	APKAIKESELGTR	-	1399.9	99	XP_025683666.1
30	probable inactive nicotinamidase At3g16190 isoform X2	QPNWSKPSLVRSKSGLGLGLGPVDPLEMAAGAWNR	-	3689.5	99	XP_025676629.1
30	uncharacterized protein LOC112750216	NNSDFSNSHPDSDGAEDVAITPNCCVHK	Carboxymethyl(C)@24 & 25	3090.8	99	XP_025654604.1

Seed storage proteins are synthesized in precursor form of size around 60 kDa during maturation stage of seed on mother plant and are widely distributed among higher plant species (Gallardo *et al*., 2001) [4]. Our SDS-PAGE study clearly demonstrated accumulation of 60 kDa proteins belongs to AAI domain containing proteins, VQ domain containing protein, REVERSED sucrose transport protein SUC 1, protein HEADING DATE 3A-like, small nuclear ribonuclear protein smD3b, protein enhanced disease resistance 4. AAI domain containing protein largely belongs to trypsin/alpha-amylase inhibitors which get degraded during radicle protrusion stage suggesting augmentation of amylase and other proteolytic activities eventually. VQ domain containing proteins are a class of plant-specific proteins with conserved single short amino acid motif and involved in diverse developmental processes including seed development. These are transcriptional regulators also involved protein-protein interactions in signaling pathways (Jing *et al*., 2015) [8]. Protein smD3b belongs to core component of spliceosomal U1, U2, U4 and U5 and snRNP carry out splicing of cellular pre-mRNA involved in number of developmental processes of plant. Disease enhanced protein related transcripts accumulated during seed maturation in our transcriptome study (unpublished). Pathogen resistance protein enhances desiccation tolerance (Huang *et al*., 2012; Wang *et al*., 2012) [6, 18] during after-ripening stage, hence accumulated at maturation stage.

Our study also found intensity reduction of 35 kD proteins

identified as protein FAR-1 related, uncharacterized protein, probable 2-oxoglutarate-dependent dioxygenase and xylose isomerase-like at seed maturation stage. REVERSED histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH1 and protein FAR-RED IMPAIRED RESPONSE 1-like was found at imbibition stage and elongation factor G, mitochondrial, uncharacterized protein, disease resistance RPP8-like protein 3, REVERSED U-box domain-containing protein 3 were identified at 24 HAI stage. Similarly 15 kD protein identified in mature seeds was seed storage protein while during germination putative glucose-6-phosphate 1-epimerase isoform X2, photosystem I reaction center subunit N (chloroplastic), probable inactive nicotinamidase At3g16190 isoform X2 and uncharacterized protein LOC112750216 was identified at imbibition stage. The other identified proteins from SDS-PAGE are mentioned in Table 1.

Total 64 proteins were identified which revealed majority of proteins were related to energy metabolism. Lipids were degraded through lipoxygenase and proteins were through protease and 26S proteasome system. Also lipoxygenase augmented removal of ROS during rapid mobilization of reserve material during seed germination process. These data aid to understand comprehensive physiological status and reserve mobilization processes in crop seed germination.

Conclusion

Dormancy and germination are complex phenomena controlled by multiple factors. Seed germination gets initiated during imbibition stage. During imbibition number of seed storage proteins are degraded which was further reduced during subsequent stages to generate de novo proteins necessary for seed germination. It also produces small amount of energy. Protein kinase activates number of hydrolytic enzymes necessary for proteolysis. Hence it can be concluded that SDS-PAGE profiling followed by protein identification can be one of the important molecular technique for basic understanding of biological processes at protein level.

Conflict of interest: The authors declare no conflict of interest

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