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Proteomic Analysis of Hydro primed Seeds of Maize Hybrid and its Parental Lines

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ABSTRACT: Maize (*Zea mays* L.) known as queen of cereals and globally it is among the most widely grown field crops. Hydropriming is an efficient method to improve seed performance under unfavourable arid and semi-arid maize producing regions. Though the hydropriming process is simple and offers huge potential to increase uniform plant stand, yet the molecular mechanism behind it is still unknown. Studies on the changes in expression of proteins due to hydropriming in maize seeds were attempted. In this study, genetically pure seeds of maize hybrid; PEHM 5 and its parents (Female – CM 150, Male - CM 151) were taken and divided in two groups before imbibing them in water for 30 h at 25°C. In first group, hydroprimed seeds were dried to their original moisture content under shade at room temperature at 25 ±2°C while in second group the hydroprimed seeds were dried using the blotting paper and immediately evaluated for germination and differential proteomic analysis with untreated seeds. Increased abundance of proteins belonging to the family of antioxidant enzymes in soaked and primed maize seeds might have resulted in significantly improved seed germination. This also revealed that different proteins are expressed during imbibition and different phases of seed priming. The differential expression of protein spots between the primed and control seeds could possibly be the protein markers for enhanced seed vigour.

Keywords: 2-D electrophoresis, Embryo proteins, Hydropriming, Isoelectric focussing, Maize seed, Proteome profiling

Seed or planting materials of a crop or a plant is a very important component of crop production as good seed interacts with the good environment around it and determines the health of the crop or plant [1]. These seeds must germinate and establish healthy plants to harness the potential harvest of the crops. Germination is a complex process and requires optimum conditions of temperature, oxygen and water. Germination *sensu stricto* refers uptake of water followed by protrusion of radical [2]. However, it is difficult to study biochemical aspects of germination as the process does not occur synchronously in seed lot [3]. Seed priming is usually practiced in crops (including maize) to attain optimal seed performance through hasten germination and seedling emergence especially under adverse climatic conditions of the arid and semi-arid regions of India [4]. It is believed that priming induces an oxidative stress thereby producing reactive oxygen species (ROS). To counteract damaging effect of ROS, the level of many antioxidant enzymes like catalase and increases during priming [5]. The beneficial effects in various crops include increased seed germination with uniform seedling emergence and increased yields [6-7]. In addition, hydropriming is uncomplicated and inexpensive technique which is preferred by farming community of developing countries [4]. Hydropriming has been found proficient to increase germination and seedling establishment specifically in abandoned agricultural areas of Northern India which was reflected by up regulation of ROS scavengers and metallothioneins (MTs) genes the molecular indicators of seed quality [8].

Seed priming is a complex metabolic process as seeds are dried back after completion of phase II of imbibition, so some processes of germination might begun and some might have completed [9]. Under such circumstances, proteomics may be helpful to provide an insight into a large number of processes occurring simultaneously during priming [10]. Comprehensive studies in many crops have been reported on seed priming and its physiological effects. These revealed that the activity of antioxidant enzymes; superoxide dismutase (SOD),

catalase (CAT) and peroxidase (POX) along with the compatible solute contents (proline and soluble sugars) increased substantially during priming and germination [11-12]. Proteome analysis of seed germination during priming especially hydropriming and osmopriming indicated the presence of degraded products of the storage protein 12S-cruciferin β-subunits recognized by MALDI-TOF spectrometry in *Arabidopsis thaliana* [13]. Job *et al.* [14] while working on seed priming of sugar beet reported that the B-subunit of 11-S globulin is degraded by an endoproteolytic attack on the A-subunit resulting in built up of degradation product. Abundance of catalase isoform was also detected by the proteomic analysis during hydropriming. Thus, during seed priming, synthesis or activation of enzymes involved in mobilization of storage proteins occurs.

Successful germination process thus, requires intense DNA repair and improved antioxidant functions and these can considered as markers of seed vigour [15-16]. Proteomics is a useful approach to identify protein and gain information on its biological function and thus is becoming a widely applicable research tool in the field of seed science [12, 17-19].

Maize is amongst the most important food and feed crop world-wide and third most important cereal crop in India. Numerous works done previously point towards the beneficial effects on seed vigour linked with pre-sowing enhancement treatments specifically drying followed by soaking. The study was undertaken to know if there were differences in expression of proteins in surface dried seeds and in seeds that were dried back to original moisture content after imbibition. Additionally, it was for the first time that such an experiment was conducted in the maize hybrid and its parental lines, to the best of our knowledge.

MATERIALS AND METHODS

Treatment details

To evaluate the effect of hydropriming for 30 h at 25°C [20] on expression of protein genetically pure and fresh seeds of maize hybrid, PEHM-5 and its parental lines (Female, CM-150 and Male, CM-151) were obtained. Before starting the experiment, the moisture content of all the genotypes was assessed using standard hot air oven method [21]. The initial moisture content observed was 10.4, 10.2 and 10.2 per cent in seeds of CM-150, CM-151 and PEHM-5, respectively. The seed lots of maize hybrid and its parental lines were subjected to the following treatments *viz*.; Control seed (Unprimed), hydropriming in distilled water for 30 h at 25°C and removed from the water followed by only surface drying with blotting papers and used immediately (Soaked) and hydropriming with distilled water for 30 h at 25°C and dried back to the initial moisture content by spreading surface dried seeds under the shade at room temperature at 25 ±2°C (Primed).

Germination experiments

Seed germination was carried out on four replications each of fifty seeds. Seeds were incubated by placing amid two sheets of moist paper towel and positioned in the walk-in-germinator set at 25°C. First and final counts were taken on 4th day and 7th day, respectively [21]. A seed was viewed as germinated when radicle obtruded through seed coat. On day of final count, the germination percentage was considered based on number of normal seedlings.

Preparation of total soluble protein extracts

Total soluble proteins extracts were readied from unsoaked dry seeds, soaked (30 h) seeds and hydroprimed (30 h) seeds of all genotypes in quadruplicate. Total proteins were mined in the phosphate buffer (pH 7.0) as illustrated by Harder *et al.* [22] including the protease inhibitor cocktail (Sigma); 5,300 units DNase I, 1,600 units RNase A, and 0.2% (v/v) Triton X-100. After 10 min at 4°C, 14 mm dithiothreitol was supplemented and the total protein extracts were stirred for 20 min at 4°C. Soluble proteins were taken out in chilled-distilled water. 2-D Electrophoresis was used for separation of maize seed protein extract.

2-D Electrophoresis

Proteins were first separated by electrophoresis in consistent with charge. Isoelectrofocusing (IEF) was done with 250 µg of proteins of the different extracts using gel strips forming an immobilized linear pH gradient from 3 to 10. Strips were rehydrated for 18 h at 22°C with the thiourea/ urea rehydration buffer as illustrated by Harder *et al.* [22] containing 2% (v/v) Triton X-100, 20 mm dithiothreitol, and the protein extracts. IEF was performed at 22°C in the IPGphor III electrophoresis system (GE Healthcare Amersham Pharmacia Biotech) for 1 h at 300 V and for 7 h at 3,500 V. Proteins were then separated according to size. Equilibrated gel strips were placed on top of vertical 10% (w/v) polyacrylamide gels [23]. A denaturing solution (1% [w/v] low-melting agarose, 0.4%

[w/v] SDS, 0.15 m bis-Tris, and 0.1 m HCl) was loaded on gel strips. After agarose solidification, electrophoresis was performed at 10°C in a buffer (pH 8.0) containing 25 mm Trizma base, 200 mm taurine, and 0.1% (w/v) SDS for 1 h at 35 V and 110 V overnight. For each condition analyzed, 2-D gels were made from independent protein extractions.

Protein staining and analysis of 2-D gels

Gels were stained with 0.1% (w/v) Coomassie Blue R-350 (CBR350) in 10% acetic acid and destained with 10% acetic acid. Stained gels were examined with the Sharp JX-330 scanner equipped with the Labscan version 3.00 from Amersham Pharmacia Biotech. Image analysis was performed with the ImageMaster 2-D Elite version 4.01 software (GE Healthcare Amersham Pharmacia Biotech). After spot finding and background subtraction (mode: average on boundary), 2-D gels were arranged, matched, and quantitative determination of the spot volumes was carried out (mode: total spot volume normalization). For each analysis, statistical data showed a high level of reproducibility between normalized spot volumes of gels formed from the independent protein extractions. Specific spots were expressed as showing variations during germination and priming when their volumes were appreciably different (at least 1-fold in relative abundance) in the three analyzed stained gels from each extraction.

Mass spectrometry and protein identification

Differentially expressed protein spots from control (untreated/unprimed), soaked (imbibed) and hydroprimed (redried) seeds were excised from the gels and digested using trypsin. Proteins were reduced (10 mM DTT), alkylated (50 mM iodoacetic acid) and then digested with 10 mg/ml trypsin for 16 h at 37°C in 50 mM ammonium bicarbonate. The supernatants were vacuum-dried and dissolved in 10 µL 0.1% trifluoroacetic acid and 0.5 µL added onto a matrix consisting of 0.5 µL of 5 mg/ml 2, 5-dihydroxybenzoic acid in water: acetonitrile (2:1) [24]. The outsourced vender employed various types of search parameters in MALDI-TOF/TOF analyzer for scrutiny of digested protein fragments. Only significant scores ($P<0.05$, score>60) of positive protein were used to envisage the proteins.

Statistical analysis

Genotypes and treatments were taken as factors for analysis of variance (ANOVA) means and compared with least significant difference (LSD) using SPSS 10.0. The

Table 1. Effect of various hydropriming treatments on germination (%), mean germination time (days) and protein content (%) of maize hybrid PEHM 5 and its parental lines

Genotypes	Germination (%)				Mean germination time (days)				Protein content (%)			
	Unprimed (Control)	Soaked (surface dried)	Primed (redried)	Mean	Unprimed (Control)	Soaked (surface dried)	Primed (redried)	Mean	Unprimed (Control)	Soaked (surface dried)	Primed (redried)	Mean
CM 150	85.0 (67.6)*	95.0 (77.1)	93.0 (75.2)	91.0 (73.3^{a})	4.67	2.50	3.07	3.41^{a}	9.24	9.72	9.49	9.48^{a}
CM 151	80.0 (63.4)	86.0 (68.3)	87.0 (68.6)	84.0 (66.8^{b})	5.43	3.70	4.27	4.47^{b}	8.69	9.22	8.97	8.96^{b}
PEHM 5	92.0 (73.9)	96.0 (78.5)	95.0 (77.1)	94.0 (76.4^{c})	4.70	2.33	2.60	3.21^{c}	9.35	9.82	9.59	9.59^{c}
Mean	86.0 (68.3^{a})	93.0 (74.2^{b})	92.0 (73.9^{b})		4.93^{a}	2.84^{b}	3.31^{c}		9.09^{a}	9.59^{b}	9.35^{c}	
CD (p=0.05)												
	Genotype (A)		2.7				0.19				0.054	
	Treatment (B)		2.5				0.23				0.053	
	Interaction (A X B)		NS				0.34				NS	

*Figure in parentheses are arcsine transformed values

arcsine square root transformation of germination percentage data was using formula =+ASIN (SQRT ((value)/100))*180/3.14 in MS excel [25].

RESULTS AND DISCUSSION

Plant life cycle starts with seed germination. Mean germination time (MGT) is critical for plant stand under stressful growing conditions, as lower MGT is an indicator of higher seed vigour. Upon imbibition, the seed switches from metabolically inactive stage to dynamic stage. The results of proteomic analysis obtained for soaked and primed seeds of maize hybrid and its parents, which could characterize germination, vigour and identify seed quality enhancement (SQE) treatment are elaborated below.

The data on effect of diverse hydropriming treatments on germination (%), mean germination time (days) and protein content (%) in seeds of hybrid PEHM 5 and its parents of maize has been given in table 1. Under optimal conditions (25°C), dry mature seeds of hybrid PEHM 5 and its parents (CM 150 and CM 151) germinate at 36 h of imbibition and 50 per cent of the seeds germinated at 52 h of imbibition. Dry mature seeds were hydroprimed (30 h) and divided into two groups. In first group, hydroprimed seeds were dried under shade at room temperature at 25 ±2° to their original moisture content while in second group the hydroprimed seeds were dried by blotting paper and immediately evaluated. Both the groups showed considerable head- start in germination and substantially reduced T_{50} *i.e.* time to attain 50% germination, indicating remarkable differences in the effectiveness of soaked and primed strategies. Both soaked and primed treatments, however, significantly influenced the seed germination capacity of hybrid PEHM-5 and its parents (Female, CM-150; Male, CM-151). The influence of hydropriming on germination percentages among the genotypes was also significant. Increase in percentage of germination for non-dried and redried seeds of CM 150 by 3.9 and 9.4 per cent; that of CM 151 by 5.6 and 8.7 per cent whereas in PEHM 5 it led by 3.7% and 2.3% respectively, as compared to control unprimed seeds. The hydropriming; soaked (only surface dried) and primed (soaked and redried) treatments also significantly reduced the MGT as compared with untreated seeds. Primed seed resulted in significantly higher protein content (9.59%) in non dried and 9.35% in dried seeds over control (9.09%). Whereas among the genotypes, the hybrid PEHM 5 showed heterosis and resulted in 9.59% which was significantly higher than its parental lines.

For all the genotypes, the highest reduction of the MGT was observed only after surface drying (soaked) strategy. Being retained largely after non-drying of seeds such physiological advancement of primed seeds resulted in faster germination upon rehydration [26]. The faster germination of hydroprimed seeds for soaked (only surface dried) and primed (soaked and redried) strategies appeared to be associated with faster and uniform germination preceding vigorous seedlings growth. Bray *et al.* [27] also reported the advantages of hydropriming treatment by eliminating the variation in germination performance of different vigour seed lots.

Jisha and Puthur [28] reported beneficial effect of halopriming in three *V. radiata* (L.) varieties with better growth performance of the seedlings due to the metabolic changes associated with seed priming. Thus, improved germination performance of primed seed might be ascribed to the onset of early metabolic events during hydration leading the seed physiological state to the brink of radicle protrusion. Differences in percent germination and MGT could be accredited to de-novo protein synthesis and repair of nucleic acids [8], as noticed in hydropriming treatment strategies that also resulted in significant differences in protein content (%) of maize hybrid PEHM 5 and its parents. Seed priming is an easy and economical technique which could improve seed quality parameters, especially under stress conditions [12]. We also noticed that hydroprimed maize seeds (CM-150, CM-151, and PEHM-5) showed faster germination compared to control and this would also be imitated in proteome stage of the hydroprimed maize embryo. Similar results were observed in pyrethrum [29] where it was reported that hydropriming shortened the delay of MGT at all osmotic potentials, and improved the germination percentage in distilled water (from 52% to 59%) and Ibrahim [30] elaborated that the seed priming stimulates the pre-germination metabolic processes and provides faster and synchronized germination.

Highly resolved 2-D gel patterns shows that the proteins were well separated in the pH range of 3 to 10 and the size range of 10 to 175 KDa. The differential 2-DE maps of the seeds of maize hybrid, PEHM 5 and its parents were done for the treatments. In general, the proteome profiles were different in all the three genotypes (Figure 1). 2-D gel profiles of total proteins from male line (CM 151) in (A) dry (unprimed) seeds detected 268 proteins, while in 30 h soaked (B) seeds 156 proteins were detected and in 30 h hydroprimed (C) seeds 194 proteins

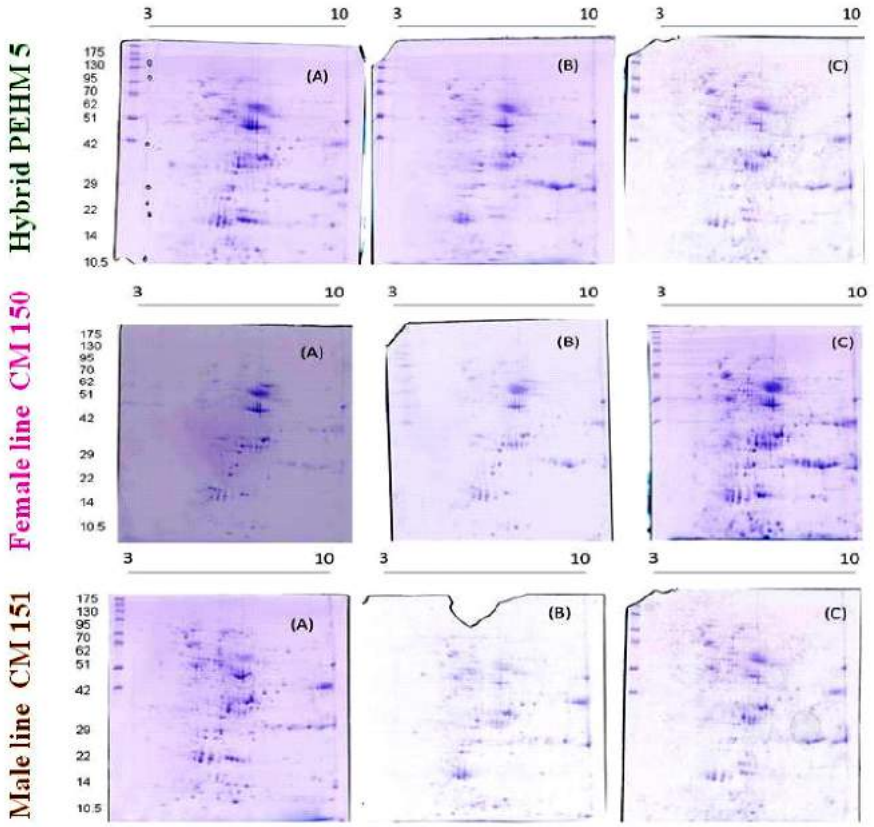

Figure 1. Differentially accumulated proteins shown by 2-D gel profiles from (A) dry (unprimed), (B) 30 h soaked and (C) 30 h hydroprimed seeds of male line, female line and PEHM 5 hybrid of maize

were detected. However, 2-D gel profiles from female line (CM 150) in (A) dry (unprimed) seeds detected 169 proteins, whereas in 30 h soaked (B) seeds 125 proteins were detected and in 30 h hydroprimed (C) seeds 149 proteins were detected. But, the maize hybrid PEHM 5 (A) dry (unprimed), 30 h soaked (B) and 30 h hydroprimed (C) seeds detected 180, 125 and 139 proteins, respectively. Also the spot pattern was observed highly reproducible for independent extractions for two different batches of seeds.

After spot detection, a scatter plot analysis was carried out to check the reproducibility of the 2D gels in different experiments. The 2D gel of control dry seed was considered as reference gel and was matched with gels of the other two treatments with the Image Master 2D Platinum software. Scatter plot analysis between two 2DE maps of control (dry) and 30 h soaked seeds and control (dry) and 30 h hydroprimed seeds of genotype CM 150 (female line) revealed the 125 and 149 matching protein spots, respectively. However, 156 and 194 in CM 151 (male line) whereas 125 and 139 matching protein spots in PEHM 5 (hybrid) were observed with analysis of respective scatter plots. The average coefficients between the reference gel with the gels of 30 h soaked and 30 h hydroprimed seeds were 0.766 and 0.706 (female line); 0.690 and 0.657 (male line); 0.580 and 0.480 (hybrid), respectively (Figure 2).

Moreover, 2-DE maps of female and male lines and maize hybrid PEHM 5 showed differential seed proteins abundance between soaking (imbibition) and priming (soaking and re-drying) treatments. MALDI-TOF analysis of these differentially expressed proteins (indicated by enclosed box in figures 3, 4 and 5) revealed more than one fold difference between control (unprimed) and soaked (only surface dried) and primed (soaked and redried) seeds. The numbers of protein spots have also been depicted in these figures. Those selected specific up-regulated and down-regulated protein spots in control (unprimed), 30 h soaked and 30 h primed (soaked and redried) seeds are e.g., protein spot no. 21, 47, 91, 92,

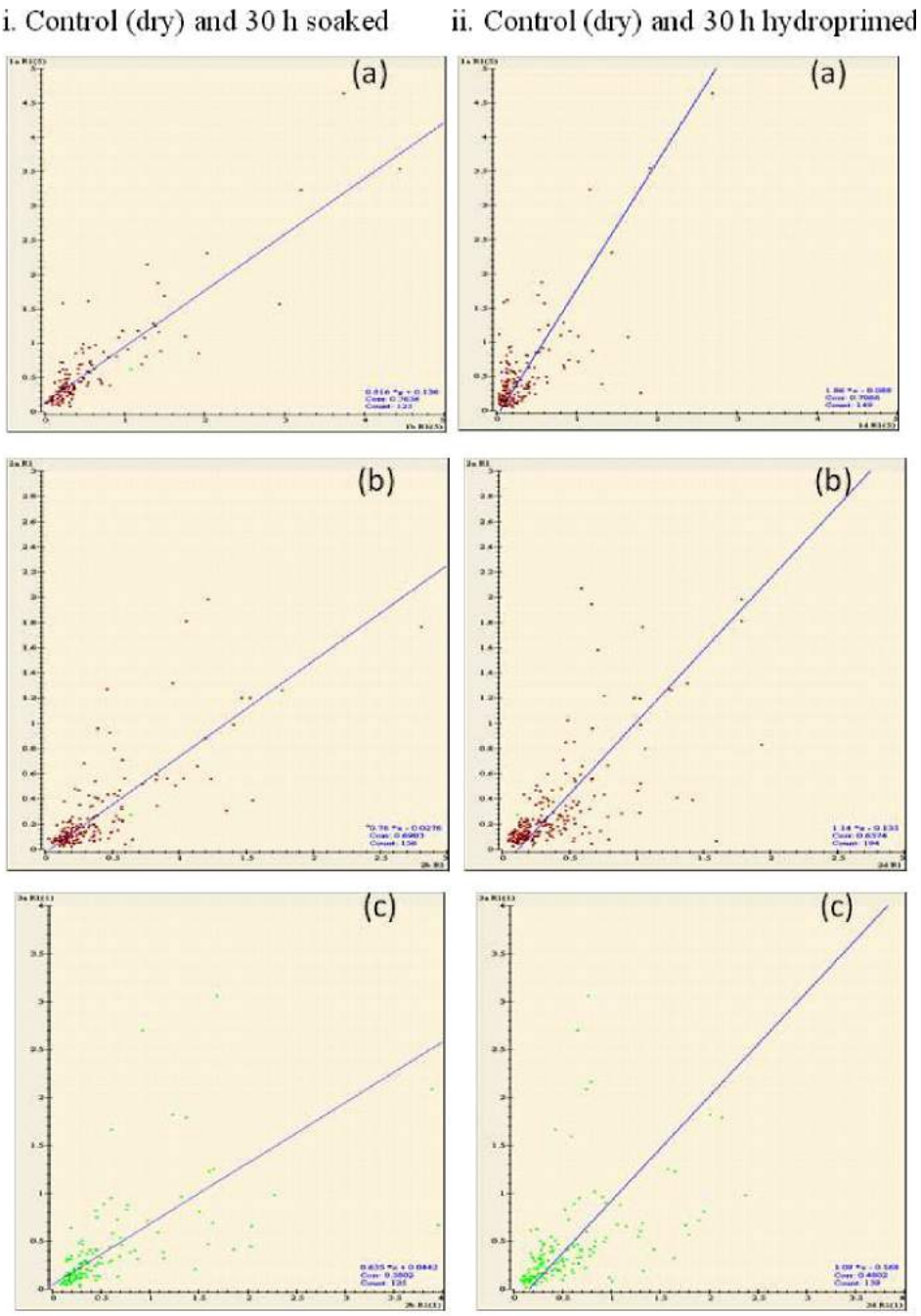

Figure 2. Scatter plot analysis between 2DE maps of control (dry) with 30 h soaked (i) and 30 h hydroprimed (ii) seeds of female line (a), male line (b) and PEHM 5 hybrid (c) of maize

93, 94, 97, 102, 117, 122, 149, 166 in case of maize female line CM 150; protein spot no. 2, 4, 26, 28, 31, 32, 38, 54, 69, 125, 204, 215 in case of maize male line CM 151; and, protein spot no. 0, 14, 23, 25, 26, 28, 29, 36, 41, 44, 45, 46, 59, 77, 97, 98, 99, 100, 129 in case of maize hybrid PEHM 5. These specific spots vary in abundance in control (unsoaked), 30 h soaked and 30 h primed (soaked and redried) seeds respectively in all the three genotypes (Figures 3, 4 and 5).

Among the changed spots, protein spot 0,25,46,45 were more abundant in soaked (only surface dried) seeds and were less abundant in the primed (soaked and redried) seeds. However, protein spots 21, 97, 47, 92 were more

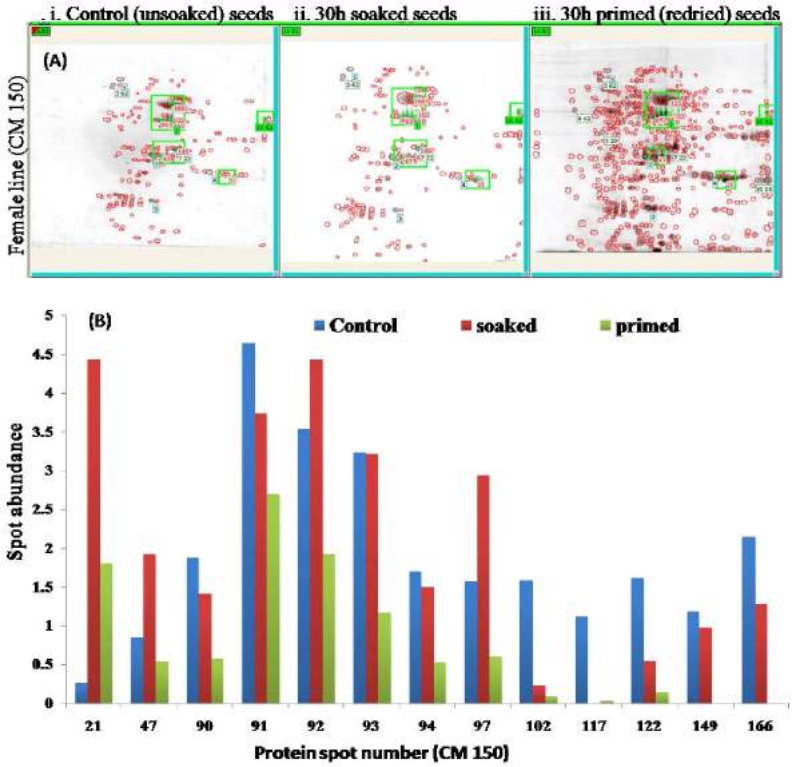

Figure 3. A. 2-DE maps of some seed proteins spots of maize female line (CM 150) and B. abundance of specific up-regulated and down-regulated protein in control, 30 h soaked and 30 h primed (redried) seeds

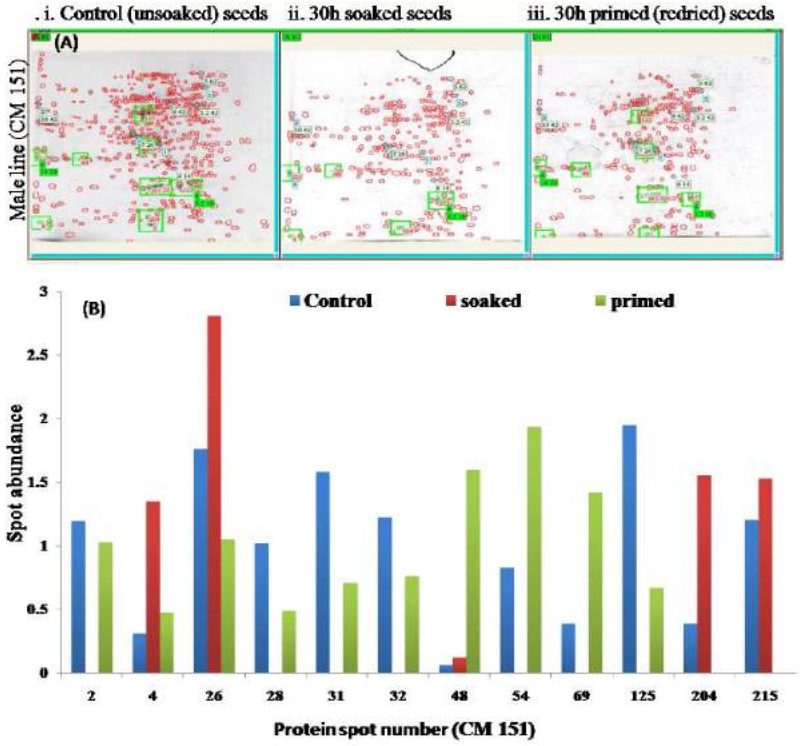

Figure 4. A. 2-DE maps of some seed proteins spots (above) of maize male line (CM 151) and B. abundance of specific up-regulated and down-regulated protein in control, 30 h soaked and 30 h primed (redried) seeds

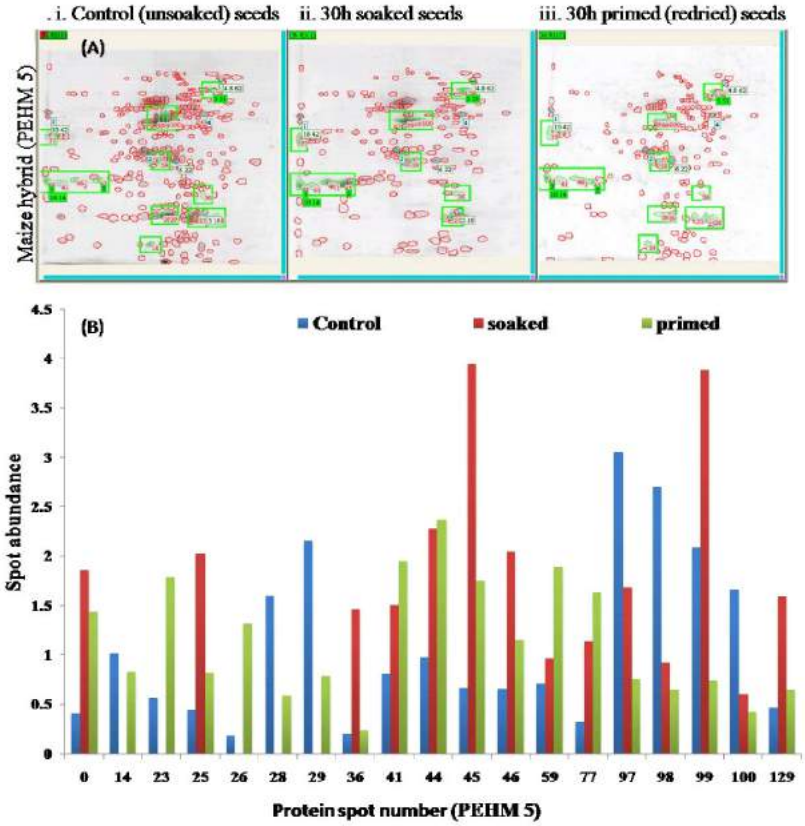

Figure 5. A. 2-DE maps of some seed proteins spots (above) of maize hybrid (PEHM 5) and B. abundance of specific up-regulated and down-regulated protein in control, 30 h soaked and 30 h primed (redried) seeds

abundant in primed seeds and were less abundant in the soaked seeds. Similarly, certain spots which were down-regulated included spot 90, 91, 94, and 122 were more abundant in control (unprimed) seeds and were less abundant in the soaked and primed seeds respectively. Certain down-regulated spots which were hardly detected in soaked seeds are spots 2, 28, 125, 31 and those which were absent in primed seeds, are spot 204, 125, respectively (Figure 6).

Protein spots with increase and decrease in profusion between both the priming strategies (soaked and surface dried; soaked and redried) and unprimed seeds were compared. Overall, 169, 268 and 180 protein spots showed more than one fold increase in abundance in genotypes CM-150, CM-151 and PEHM-5 respectively. Among them 125, 156 and 125 protein spots changed during imbibition for 30 h. Moreover, 149, 194 and 139 protein spots showed more than one folds changed in abundance during hydropriming in genotypes, CM-150, CM-151 and PEHM-5, respectively. Thus, it is observed that hydropriming could have brought about very subtle change in protein profile of maize seeds. Between both the priming strategies and unprimed maize seeds, protein abundance of around 98% spots were almost the same except for few specific embryo protein spots showed higher abundance. This could be attributed to lesser moisture content attained (35.6%) during priming for 30 h compared to requirement of 39.8% M.C. for maize seed germination leading to decreased metabolic activities [31]. Moreover, Coomassie brilliant blue (CBB) staining used is less sensitive to detection of low-abundance proteins. In our present study, computation of heat maps of all the proteins detected in 2DE in the priming strategies and unprimed seeds of all the three genotypes further elaborated quantitative and visualised patterns of proteins across treatments and genotypes in a matrix form (Figure 7).

Up or down regulation of proteins in among different genotypes and treatments were assessed based upon their spot volume. There were cases of more abundance of protein spots in primed (soaked and redried) seeds and less in the soaked (only surface dried) seeds. Certain

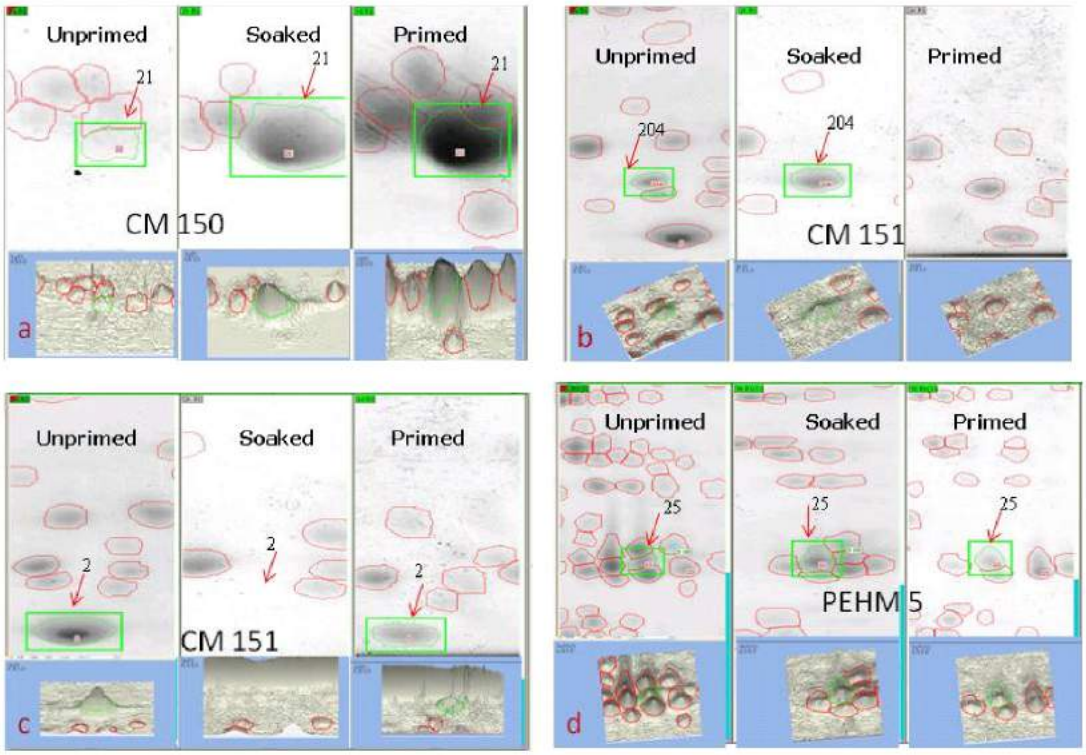

Figure 6. Proteins regulated during treatments as seen by their spot volume; a. up-regulated spot during soaking and priming in CM 150 (female line), b. & c. down-regulated spots during soaking and priming in CM 151 (male line), d. up-regulated spot during soaking and priming in PEHM 5 (hybrid) of maize

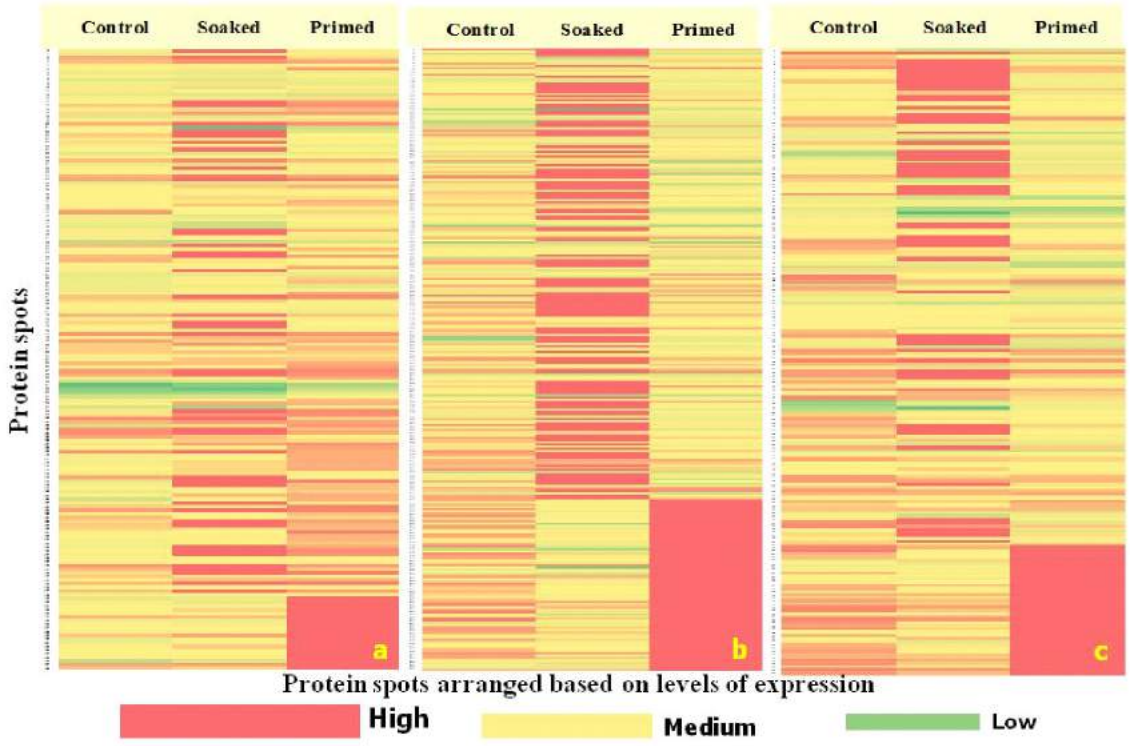

Figure 7. Heat map showing protein order arranged on levels of expression in seeds of; a. female line (CM 150), b. male line (CM 151) and c. Hybrid (PEHM 5) for control, soaked and primed seeds of maize

spots which were down-regulated were more abundant in dry (control) seeds and less abundant in the soaked and or primed seeds. Whereas there were down-regulated spots which were hardly detected in soaked seeds but spots became visible in primed (redried) seeds. The Cupin, Embryonic protein, Globulin, LEA protein, Peroxiredoxin, their isoforms and particularly the abundance of proteins belonging to the family of antioxidant enzymes might have resulted in efficient performance of primed seeds. Catusse *et al.* [32] found that during hydropriming and aging of sugar beet seeds same 18 proteins accumulated. Similarly, Tanou *et al.* [33] established the significance of redox proteomic and processes like protein oxidation, nitrosylation, carbonylation etc, in priming effects.

In conclusion this study revealed that both the hydropriming (30 h at 25°C) strategies hastened germination of maize seeds and lead to changes in the protein profiles. Differentially expressed protein spots involving the primed and unprimed seeds may serve as probable markers for seed priming that can ultimately be used to characterize seed vigour of commercial seed lots. These proteins could help us to understand the various biochemical reactions and metabolisms that occur during seed priming.

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