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Proteomic analysis of ageing in black gram (*Vigna mungo* L.) seeds and its relation to seed viability

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Abstract

The Indian minimum seed certification standards recommend that black gram seeds should have a minimum germination of at least 75% in order to pass the germination standard. The aim of this study is to investigate, using 2D-PAGE analysis, the possible proteome changes in black gram seeds when their germination rate falls below 75% and thereby, identify proteins that correlate with loss of seed viability during seed ageing. Firstly, in order to obtain seed samples with germination rate less than 75%, for 2D-PAGE analysis, we analyzed the proteomic (using SDS-PAGE) and physiological changes in seeds, which were artificially aged for 1 to 10 days at 98 ± 2% relative humidity and 40 ± 1° C temperature. The results revealed the major physiological and protein changes occurs from sixth day onwards and germination rate falls below 75% at sixth day, suggesting that 2D-PAGE seed samples can be obtained from 6-day-artificially aged seeds. Secondly, we carried out 2D-PAGE analysis using fresh and 6-day-artificially aged seeds and characterized 16 differentially expressed proteins in aged seeds. These proteins were identified using MALDI-TOF-MS and classified into 8 functional groups. Out of 16 proteins, 4 were up-regulated and 12 were down-regulated, indicating that these proteins play an important role in seed viability. Seven down-regulated proteins were functionally related to cell structure, transporters, metabolism and transcription, and one up-regulated protein was related to defense. In summary, this study has identified proteins that correlate with seed deterioration and loss of viability during ageing in black gram seeds.

Keywords: Actin, Cell structure, Black gram, Proteomics, PaO, Seed ageing, Seed viability, Vigour, VARS.
Abbreviations: 2D-PAGE_ two dimensional – polyacrylamide gel electrophoresis; CD_critical differences; CHAPS_3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; DPPH_1,1-diphenyl-2-picrylhydrazyl; DTT_dithiothreitol; EDTA_ Ethylene diamine tetraacetic acid; EF-Tu_elongation factor Tu; HCCA_α-cyano- 4-hydroxycinnamic acid; HEPES_2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; MALDI-TOF/MS_Matrix-assisted laser desorption ionization – time of flight / Mass spectrometry; PaO_pheophorbide a oxygenase; RNAP II_RNA polymerase II; SDS-PAGE_sodium dodecyl sulfate – polyacrylamide gel electrophoresis; SNARE_soluble N-ethylmaleimide-sensitive factor adaptor protein receptors; TCA_tricarboxylic acid; TEMED_N,N,N',N'-tetramethylethylenediamine; VARS_Valyl-tRNA synthetase

Introduction

Seed is the prime source of crop production and the successful field establishment and crop performance are mainly attributed to the quality of seed used for sowing. In any crop, seeds have to be invariably stored as carryover seed for sowing in next season. During the storage period, seed deterioration is inevitable and it results in loss of seed vigour and viability (McDonald 1999). Reduction in vigour and viability of seeds may affect field performance and productivity of the subsequent crops (TeKrony and Egli 1991). Till date, there is no concrete evidence that explains the molecular mechanism of seed deterioration. However, mounting research data pinpoints that the production of free radicals during storage is a major cause for disruption of cellular membranes and damage to protein and nucleic acid, which ultimately results in deterioration of cell organelles and seed ageing (Bailly 2004; Kibinza et al., 2006). It has been proved that proteins are the major targets of free radicals due to their abundance in biological systems (Davies 2005). But information on the proteome changes in dry seeds during storage is only scanty. Recently in *Arabidopsis thaliana*, 18 differentially expressed proteins were identified in dry mature seeds after controlled deterioration treatment (85% relative humidity, 20°C) (Rajjou et al., 2008). While Xin et al. (2011) identified that 40 spots were differentially

expressed in dry mature seed after artificial ageing treatment in maize seeds and demonstrated that the proteome change could take place even in the dry state of ageing seeds. Many earlier researchers also proved the suitability of artificial ageing to study the pattern of seed deterioration in various crops (Kibinza et al., 2006, Jatoi et al., 2001; Scialabba et al., 2010). Black gram (*Vigna mungo* L.) is a protein rich food, containing about 26% protein, which is almost three times that of cereals. It ranks fourth among the major pulses cultivated in India. Black gram supplies a major share of protein requirement of vegetarian population of the country. It is consumed in the form of split pulse as well as whole pulse, which is an essential supplement of cereal-based diet. However, in the past decade, due to unavailability of high quality black gram seeds, there was a reduction in area of black gram production (ASSOCHAM 2012). This necessitates the understanding of molecular mechanism underlying seed deterioration. In order to provide seeds of high quality, Indian minimum seed certification standards have fixed '75% germination' as minimum requirement for selling the black gram seeds (Tunwar and Singh 1988). Therefore, we are interested to investigate, using 2D-PAGE analysis, the possible proteome changes in black gram seeds when their germination rate falls below 75% and thereby, identify proteins that correlate with loss of seed viability during seed ageing. Understanding the changes in the proteome associated with ageing could help us dissect the molecular basis of seed longevity and hence prevent or slowdown the progressive loss of germination vigour.

Results

Reduction in germinability and vigour of black gram seed during artificial ageing

Germinability and other vigour parameters (i.e., shoot and root length, dry matter production, and vigour index) declined gradually during artificial ageing. Fresh seeds showed higher germination ability (99%), however, during ageing, germinability declined and reached 14% at 10^{th} day. Importantly, at the 6^{th} day, the germinability of the seeds deteriorated to 58%, which is lesser than the minimum germination limit (75% germination) required by seed certification standard. In addition, a concomitant reduction in vigour index was also observed (Fig. 1). The shoot and root length and dry matter production declined due to artificial ageing. The decline in shoot and root length was significant only after 9-days of ageing. However, the reduction was not significant for dry matter production (Fig. 2).

Changes in biochemical parameters

Disintegration of cell membrane integrity is correlated with ageing ,Therefore, to get a better understanding on the intensity of free radical activity and cell membrane integrity during seed ageing, antioxidant potential through DPPH test and electrical conductivity of seed leachates were analysed. The results showed a gradual decrease in antioxidant potential with the progress of artificial ageing with concomitant increase in electrical conductivity of seed leachates. The decline of antioxidant potential was steep from third day and also the increase in electrical conductivity of seed leachates was pronounced from third day (Fig. 3). This clearly indicates that ageing reduces the free radical scavenging capacity and increases the disintegration of membrane integrity in seeds.

Changes in protein pattern during artificial ageing

There were no major differences in seed protein pattern up to fifth day of artificial ageing suggesting that artificial ageing does not appreciably affected the protein pattern up to 5 days. However, significant differences in protein pattern were observed from sixth days onwards. Most of the changes were observed in proteins of molecular weight ranging between 31.00 and 66.20 kDa. The changes were mostly reduction in band intensity. The disappearance of protein band at 60.21 kDa in 6 to 10-day artificially aged seeds is interesting (Fig. 4). The disappearance of protein bands and significant reduction in germination below the minimum germination standard from sixth day kindled our interest to compare the proteome of fresh and 6-day artificially aged seeds.

Proteome changes in dry seeds during ageing

There were totally 782 spots detected both in fresh and 6-day artificially aged seeds. Among these, we selected 16 spots that were differentially expressed by artificial ageing for our further analysis. Out of 16, 4 were up- regulated spots and 12 are down-regulated spots (Table 1 and Fig.5).

MALDI-TOF/MS identification of proteins affected by ageing

MALDI-TOF/MS analysis of the 16 differentially expressed spots revealed the identity of the proteins (Table 1 and Fig. 6). On the basis of putative protein functions, these proteins were grouped into eight categories as described by Bevan et al. (1998), Cell structure (3 spots, 18.75% of total identified spots), Protein destination and storage (2 spots, 12.50%), Protein synthesis (2 spot, 12.50%), Transporters (2 spot, 12.50%), Transcription (1 spots, 16.25%), Metabolism (1 spot, 6.25%), Disease/defence (1 spots, 6.25%), and unclear or uncharacterized (4 spots, 25.00%). One spots each from protein destination and storage (spot U16), protein synthesis (U15), disease/defence (U12) related proteins and one unclassified proteins were up-regulated, while the others were down-regulated (Fig. 6).

Discussion

Physiological and biochemical basis of seed deterioration

Though seed ageing starts immediately after development and maturation in plants (Anderson and Baker 1983; McDonald 2004), freshly harvested and well-processed black gram seeds usually, have above 90 % germination. But when seeds were subjected to prolonged storage, the rate of deterioration increased with passage of time and the market value of seed lot declines and ceases when the germination rate falls below the prescribed 75% germination standard. To prevent or to slowdown the rate of deterioration, a better understanding on the molecular changes that causes the seeds to lose vigour and viability below the germination standard. is warranted. In the present investigation, physiological and biochemical basis of seed deterioration was studied by subjecting seeds to artificial ageing. As expected, all the physiological parameters *viz*., germination, shoot and root length, dry matter and vigour started to decline with increase in ageing duration (Fig. 1 and 2). Similar reduction in physiological performance concomitant with increase in aging duration was also reported in pea (Jatoi et al., 2001) and rice (Kapoor et al., 2011). Changes in free radical

scavenging enzymes, increase in free radical production, degradation of protein and DNA, increase in amino acid pool were shown as reasons for reduction in vigour and viability during ageing (McDonald 2004). In the present study, biochemical observations revealed a concomitant decrease in antioxidant potential and an increase in solute leakages which is evidenced from elevated electrical conductivity, substantiating the earlier findings that membranes are damaged by increased free radical attack during artificial ageing of seeds (Baily 2004; Bewley 1986; Crowe et al., 1992; Vertucci and Farrant 1995; Leprince et al., 1999; Bailly et al., 2008). The result was in conformity with that of Vasudevan et al. (2012). In a study with transgenic tobacco, Lee et al. (2010) reported that higher levels of ion leakage in aged non-transgenic tobacco seeds leading to decreased germination rate and suggested that higher expression of antioxidants like Cu/Zn-superoxide dismutase and ascorbate peroxidase genes in transgenic tobacco resulted in reduced ion leakage and maintained vigour and seed viability during ageing.

Changes in protein pattern show a reduction in intensity of protein bands in aged seeds and loss of a protein band of molecular weight 60.21 kDa from the 6^{th} day of ageing. Loss of protein bands may be due to post-translational modifications and degradation during ageing (Rajjou et al., 2008). The result was in harmony with that of Machado et al. (2001). Similar loss of bands in 6 and 9-day artificially aged seeds as compared to control was reported by Vasudevan et al. (2012). They also demonstrated that the biochemical changes during accelerated ageing were similar to natural ageing except the rate at which they occur.

These preliminary studies have clearly indicated that prolonged ageing increases the loss of viability with concomitant loss in antioxidant potential, cell membrane integrity and proteins. From the interesting observation that both the reduction in germination below germination standard and loss of protein bands coincided in 6-day artificially aged seeds, we conjectured that the protein damage is pivotal for loss of seed viability. Therefore, further comparative proteome studies with fresh and 6 day artificially aged seeds were carried out.

Proteome changes due to seed ageing

Down regulation of proteins involved in cell structure maintenance

Proteins involved in maintenance of cell structure are responsible for the intactness of the cell membranes. Many previous studies had positively correlated the loss of cell membrane integrity to loss of seed viability (Sacande et al., 2001; Khan et al., 2004; Ratajczak and Pukacka 2005; Eliud et al., 2010). In the present investigation, we have identified three spots, (D03, D06 and D11) related to cell structure, that were down-regulated when compared to fresh seeds (Table 1). D03 and D11 are actin and actin-101 like protein respectively, in which actin is a cytoplasmic protein that is capable of self-assembly into dynamic filamentous structures. In plants, actin filaments are presumed to play essential roles in many important processes including cell division, cell elongation, establishment of cytoplasmic organization, cytoplasmic streaming (Hussey et al., 2006), and changes in response to bacterial signaling molecules arising from pathogens (Dantan-Gonzalez et al., 2001). Several researchers have reported importance of actin in successful seed germination. In mung bean, actin like protein takes part directly or indirectly in cell division during seedling development (Ghosh et al., 1989). It has been also reported an association to mitochondrial membrane and mtDNA for its structural maintenance and suggested progressive import of actin into cotyledon mitochondria in concert with the conversion of quiescent mitochondria into active forms during seed germination is necessary for the persistent function of mitochondria until the very end of the orderly cell death process that occurs in mung bean seedling cotyledons during germination (Lo et al., 2011). A study in common bean (Villanueva et al., 1999) and maize (Diaz-Camino et al., 2005) confirmed that concentration of actin in embryonic axis is higher than in other part of seeds and concluded that its expression during post-imbibition both at the protein and mRNA levels is essential for morphological changes in developing seedling. This ascertains the role of actin in successful germination. Hence, the down-regulation of actin in aged seeds could result in severe damage to cell membrane leading to its disruption and further substantiate the eminent concept of increased solute leakage in aged seeds.

Apart from actin, another spot D06 related to structural maintenance was also down regulated. It was identified as LIM domain proteins, which act as the core of multiple protein complexes and function in various cellular processes. Cytoplasmic LIM-domain proteins take part in cytoskeletal organization through interaction with cytoskeleton and extracellular matrix (ECM) proteins. LIM Kinases (LIMKs) play an important role in regulating actin cytoskeleton organization in response to various extracellular stimuli (Zheng and Zhao 2007). The majority of the identified cytoplasmic LIM-domain proteins are found to be ABLIM (actin-binding LIM protein) that binds with F-actin and actin-based cytoskeletons and mediate interactions between actin filaments and cytoplasmic targets, and consists of a C-terminal cytoskeletal domain and four N-terminal LIM motifs (Roof et al., 1997, Barrientos et al., 2007). This down-regulation of LIM domain proteins could add to further loss of actin and disruption of cell structure.

Protein storage and destination

Storage proteins are accumulated in high amounts during mid-maturation stage of seed development and will break down during imbibition to supply amino acids for seed germination and seedling development (Li et al., 2007). Among the storage proteins, globulin comprises major composition in pulses (Bassuner et al., 1983; Mandal and Mandal 2000). It was well documented that the content of seed storage protein would decrease upon seed ageing (Kapoor et al., 2010). Further loss in seed vigour and viability in aged seeds and inability of low-vigour seeds to display a normal proteome during germination can be ascribed to protein changes in dry seeds during ageing (Rajjou et al., 2008). In our investigation, the storage protein, 8S globulin alpha isoform precursor (D10) was down-regulated in aged seeds compared to fresh seeds (Table 1). This result is in line with that of Xin *et al.* (2011) and Wu *et al.* (2011) who reported down-regulation of globulin in artificially aged maize seeds. The breakdown of the storage proteins prior to germination might lead to inefficient amino acids supply for synthesis of new proteins essential for seed germination and seedling establishment. During ageing, proteins might be degraded due to free radical attack, which results in improper functioning of proteins. The proteolytic spot U16, identified as 26S proteasome subunit, was up-

Table 1. Functional classification of differentially expressed proteins associated with artificial seed ageing.

Spot No.	NCBI accession no.	Protein name	Theoretical MW (kDa) / pI	Experimental MW (kDa) / pI	MASCOT score	Matched peptides	Sequence coverage (%)	Relative abundance*
Metabolism								
D02	297802676	5' nucleotidase, deoxy (Pyrimidine), cytosolic type C protein	40.4/9.05	40/5.2	81	9	30	0.78
Transcription								
D01	168010051	Largest subunit of RNA polymerase II	99.1/9.34	47/5.5	80	24	29	0.79
Protein synthesis								
D05	255077874	Valyl-tRNA synthetase	16.5/5.23	47/5.4	82	32	24	0.47
U15	336285879	Elongation factor Tu	25.4/4.74	25/5.1	74	6	36	1.46
Protein destination and storage								
D10	108743974	8S globulin alpha' isoform precursor	51.6/5.58	43/5.5	77	7	20	0.44
U16	308800992	Putative 26S proteasome regulatory subunit	41.6/8.51	14/5.4	71	7	20	2.85
Transporters								
D08	242062694	hypothetical protein (contains Sec14p-like lipid-binding domain)	70.8/7.31	44/5.7	51	5	10	0.52
D09	219362943	SNARE domain containing protein	26.1/8.73	14/5.5	40	4	27	0.92
Cell structure								
D03	357503463	Actin	40.2/5.56	43/5.4	69	8	36	0.38
D11	356558578	Actin-101-like protein	41.8/5.31	36/6.8	98	12	47	0.59
D06	167997611	Predicted protein (Contains LIM domain)	20.0/6.09	44/5.8	40	3	29	0.93
Disease / defence								
U12	1935914	Pheophorbide a oxygenase	62.0/8.81	41/5.1	78	7	15	2.91
Unclear / uncharacterized								
D04	77554262	Retrotransposon protein, putative	12.8/8.87	44/5.6	40	5	5	0.67
D13	108710077	Retrotransposon protein, putative, Ty3-gypsy subclass	15.0/8.87	57/5.2	82	29	28	0.62
D07	303279362	Predicted protein	89.2/8.15	42/5.4	75	12	19	0.51
U14	168058176	Predicted protein	46.2/9.18	28/5.1	77	15	42	1.47

*Relative abundance is expressed as the ratio of normalized spot volume in the deteriorated seeds (6 days artificially aged) to the normalized spot volume in the non-deteriorated control seeds (Fresh seeds).

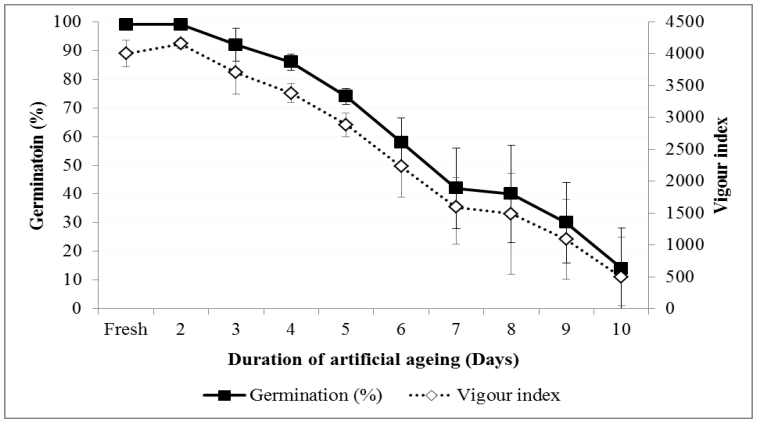

Fig 1. Effect of artificial ageing on seed germination and vigour index. Figure shows that seed germination and vigour index decline as the duration of ageing increases. It can be seen that germination rate has fallen below 75% at sixth day.

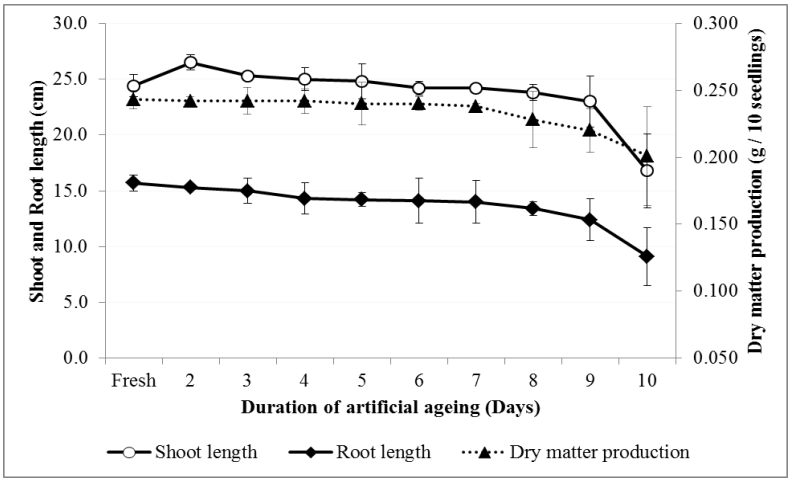

Fig 2. Effect of accelerated ageing on shoot length, root length and dry matter production.

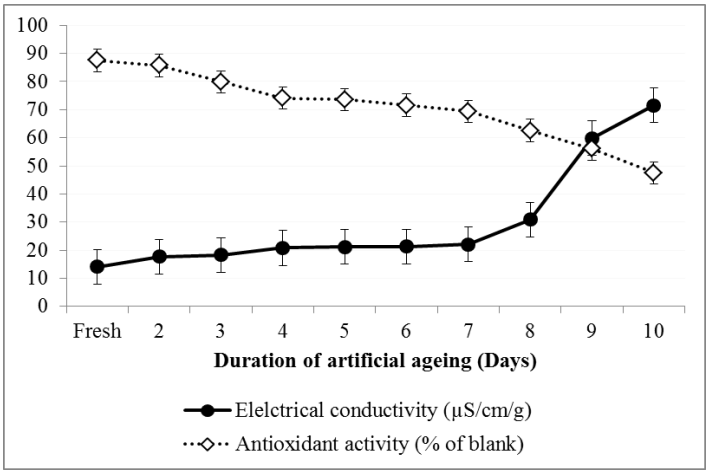

Fig 3. Effect of artificial ageing on antioxidant activity and electrical conductivity of seed leachates.

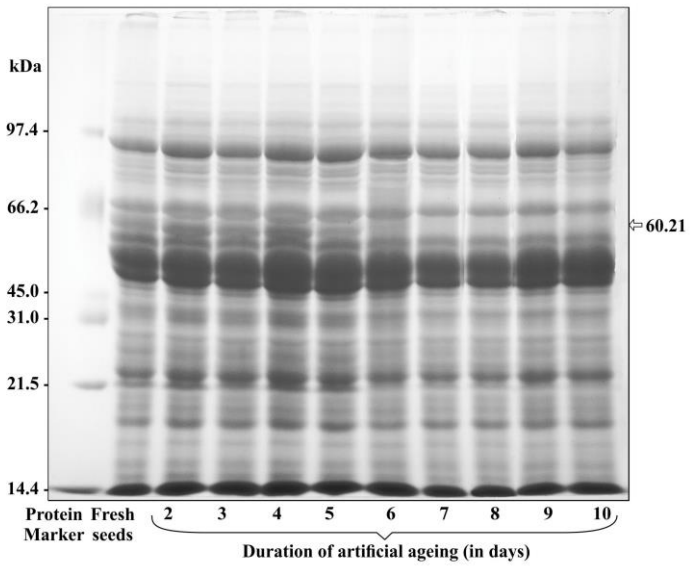

Fig 4. Changes in protein pattern during ageing of seeds. There were no appreciable changes in protein pattern up to five days of ageing but changes were observed from sixth day, for example 60.21 kDa protein band disappeared from sixth day.

regulated during ageing. The 26S proteasome is an ATP dependent proteolytic complex that regulates protein turnover in all phases of plant development by degrading ubiquintylated protein (Sullivan et al., 2003). It plays a myriad of roles during seed development (Ferreira et al., 1995) and germination (Miyawaki et al., 1997; Soos et al., 2010). 26S proteasome is made up of 31 different subunits arranged into two complexes, 20S core protease and two 19S regulatory particles. Later is responsible for identifying ubiquintylated proteins and the former exert catabolic reaction on it and releases amino acids (Moon et al., 2004; Smalle and Vierstra 2004). Up-regulation of 26S proteasome during ageing indicates the production of damaged proteins and substantiate breakdown of storage proteins. This result is in harmony with that of Delaplace *et al*. (2009) who reported up-regulation of proteasome complexes in aged potato tuber.

Transcription and protein synthesis

Neo-synthesis of protein from long-lived mRNA during the initial phase of imbibition is essential for successful germination rather than *de novo* transcription. But, *de novo* transcription is essential for subsequent seedling growth and establishment in *Arabidopsis* (Rajjou et al., 2004) and rice (Sano et al., 2012). In our study, down-regulated spot D01 was identified as larger subunit of RNAP II enzyme which plays major role in DNA mediated transcription to synthesis mRNA and other micro-RNAs. Down-regulation of RNAP II could result in unfavorable modifications in the transcription process (Osborne et al., 1974). The abnormality in the transcription results in the decline and loss of RNA synthesizing activity or the synthesized RNA molecules could be defective in the protein synthesizing system (Sen and Osborne 1977). Zalewski (1989) related rye seed deterioration to lower ability of RNA synthesis during germination. The lower ability of RNA synthesis was due to low polymerase activity of aged seeds (Grilli et al., 1995). Recently, Rajjou et al. (2004) correlated the decline in germination rate and subsequent seedling growth of aged *Arabidopsis* seeds to blockage in transcription during later phase of germination. It is well established in chick pea that cotyledons of dry seed contains preformed mRNA (Matila et al., 1980) and translation of these long-lived mRNA is essential for radicle protrusion (Rajjou et al., 2004). VARS is a member of aminoacyl- tRNA synthetases family, which catalyzes the reaction of charging valine with its cognate tRNA during translational process (Tamura et al., 1994). Substantial amount of valine was reportedly found in pulses seeds (Mandal and Mandal 2000). In the present study, spot D05, which corresponds to VARS, was down-regulated in aged seeds. This down-regulation of VARS during ageing could impair translational addition of valine during imbibition and results in malformed protein. Zhang and Somerville (1997) reported loss of seed viability in transgenic *Arabidopsis*, due to alteration in the expression of VARS. Hence down-regulation of both RNAP II and VARS could be one of the causes for poor germination and reduced growth of seedlings in aged seeds. Though translating enzyme VARS was down-regulated in aged seeds, intriguingly, another protein (spot U15) namely EF-Tu, that participate in elongation of protein during translation (Slobin 1980) was up-regulated in aged seeds. Dell Aquila et al. (1976) reported presence of different forms of EF in wheat embryos and related its presence to viability. Many studies had reported up-regulation of EF-Tu during germination which correlated to increase in protein synthesis especially, proteolytic enzymes (Sano et al., 2012; Potokina et al., 2002; Mak et al., 2009). Our conjecture is that the up-regulation of EF-Tu even in dry seeds might be responsible for up-regulation of other proteins such as proteolytic complex 26S proteasome, defense related protein, PaO and other uncharacterized protein in aged seeds.

Transporters and signal transduction

The two spots D08 and D09 belong to the transportation group. D08 is a Sec14p-like lipid-binding domain, which is found in phosphatidyl inositol transfer family protein and is down-regulated in aged seeds of black gram. It is a ubiquitous cytosolic domain involved in transport of phospholipids from their site of synthesis in the endoplasmic reticulum and golgi to other cell membranes. They are highly conserved proteins (Kader 1996). It polarizes membrane growth of *Arabidopsis* root hairs (Vincent et al., 2005). Another spot, D09 is SNARE domain protein and it was also down-regulated in aged seeds. The SNARE complex is a key regulator of vesicular traffic, executing membrane fusion between transport vesicles or organelles and target membranes (Ebine et al., 2008). It appears that reduction in Sec14p-like lipid-binding domain and SNARE during ageing could impair the transport of cargos' from cotyledons to proliferative embryo during germination and hence cause malformed seedlings.

Metabolism

The spot D02, identified as 5' nucleotidase, deoxy (Pyrimidine), cytosolic type C protein, was down-regulated in aged black gram seeds. Cytosolic 5' nucleotidase catalyzes hydrolysis of phosphate esterified at carbon 5' of the pyrimidine and release nucleoside and phosphate (Zimmermann 1992). It was also found to play a major role in cytokinin metabolism (Tomaz and Marina 2010). Pyrimidine nucleotides are the building blocks for the direct synthesis of DNA and RNA and they also participate in the metabolism of a large number of other cellular components from sugar interconversion to cellular polysaccharides to glycoproteins and phospholipids (Kafer et al., 2004). In a study with *Arabidopsis*, it was found that elevated pyrimidine degradation increased germination, seedling growth and also higher seed production (Cornelius 2011). Therefore, down-regulation of 5' nucleotidase in aged seeds could alter the intracellular pool sizes of pyrimidine nucleotide and affect normal cellular metabolism during seed germination.

Defence / disease

Seeds are prone to microbial infection during ageing. Harman (1983) reviewed the mechanism of seed infection and pathogenesis during seed storage. Seeds usually develop their own natural defense mechanism against pathogen attack (Dalling et al., 2011). One of the up- regulated spot U12 was identified as PaO. In seeds, studies on role of PaO is scanty except its role in chlorophyll degradation during maturation of canola seeds (Chung et al., 2006). However, in plants, apart from chlorophyll catabolism, PaO has been reported to be a cell death suppressor in *Arabidopsis* (Pruzinska et al., 2003) and maize (Gray et al., 1997). Recently its role in disease tolerance was also reported in wheat (Tang et al., 2013). This is the first report that PaO is up-regulated in aged seeds, suggesting PaO might play an important role in defence against pathogenic attack during ageing.

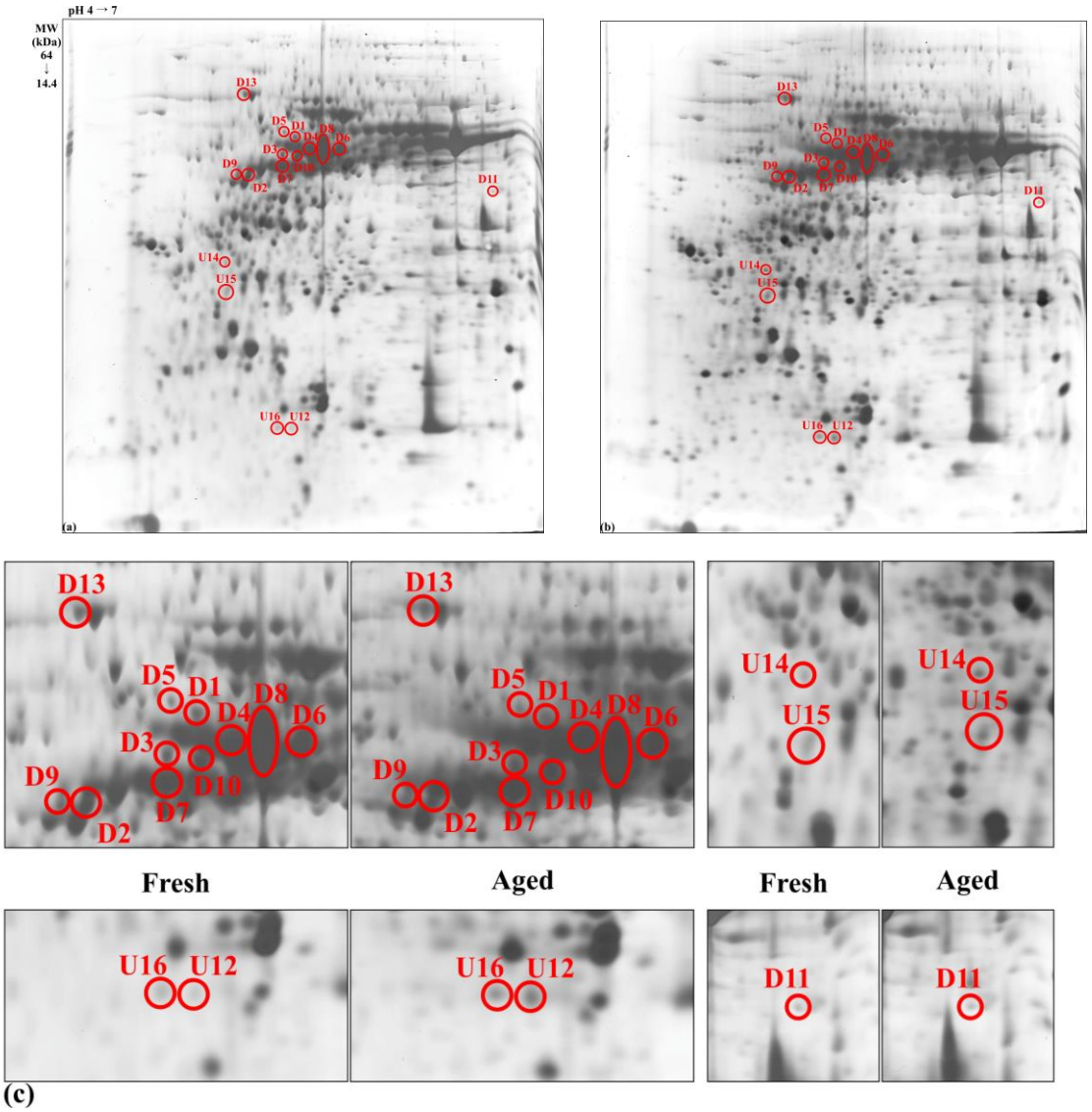

Fig 5. Differential expression of proteins due to artificial ageing of black gram seeds. (a) 2D- PAGE map of control seeds. (b) 2D-PAGE map of 6-day-artificially aged seeds. (c) Shows the up and down-regulated spots.

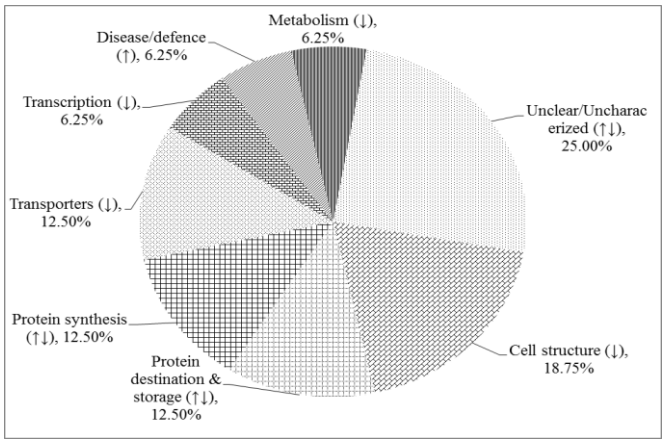

Fig 6. The functional category distribution of the 16 differentially expressed proteins in black gram seeds. The downward arrows (↓) indicate down-regulated proteins and upward arrows (↑) indicate up-regulated proteins.

Unclear and unknown proteins

Two spots, D04 and D13 were identified as retrotransposon protein and Ty3-gypsy subclass retrotransposon protein, respectively. Both were down-regulated in aged seeds. Retrotransposons are ubiquitous in plants and plays a major role in genome duplication (Kumar and Bennetzen 1999). However, their roles in seed are unclear.

Due to the incompleteness of the black gram protein database and the limitation of MALDI-TOF mass spectrometry, two spots, D07 and U14, did not match with any known sequence, and therefore the identity of them could not be verified and hence they represent novel candidate genes. Further studies are required to find the roles of these four proteins in seed ageing.

Materials and Methods

Seed material and artificial aging

Fresh dry seeds of black gram var. TNAU Black gram CO 6 having 98% germination and 8% moisture content were used for the study. Seed deterioration treatment (i.e., artificial accelerated ageing) was performed by placing 20 grams of seeds (which were packed in paper bag with uniform pin head size perforation all over) in an ageing jar maintained at 40 ± 1° C and 98 ± 2% relative humidity, for up to 10 days (Delouche and Baskin 1973). Samples of 2-day to 10-day artificially aged seeds were withdrawn and seed vigour statuses were evaluated.. Fresh seeds, which served as control, were also analyzed.

Seed vigour tests

Fresh and two to 10-day artificially aged seeds were subjected to seed vigour test as outlined by ISTA (2007). Twenty-five seeds in quadruplicate were germinated in roll towel maintained at 25 ± 2°C temperature and 95 ± 3 per cent relative humidity in a germination room. At the end of seven days, number of normal seedlings was counted and the mean was expressed as % of germination. Root length and shoot length of ten normal seedlings, selected at random from each replication, were measured after seven days of germination and the mean was expressed in centimeter and those seedlings used for growth measurement were placed in a paper cover and dried in shade for 24h and subsequently they were dried in an oven maintained at 85 ± 2°C for 48h. The dried seedlings were weighed for estimating the dry matter production and the mean values were expressed in g/10 seedlings. The vigour index was computed as described by Abdul-Baki and Anderson (1973) taking into accounts, the germination % and seedling length in centimeter (sum of root and shoot length).

SDS-PAGE to study protein pattern changes in aged seeds

To study the changes in protein pattern of fresh and 2 to 10-day artificially aged seeds, total protein was extracted from the whole seeds obtained from four replicates using HEPES buffer (50 mM HEPES pH 8.0, 1 mM EDTA and SDS 2% (v/v)). Protein samples (100 µg) were resolved in a 12.5% (w/v) polyacrylamide gel by SDS-PAGE as described by Laemmli (1970). The proteins were visualized by staining with 0.1% Coomassie brilliant blue R 250 dissolved in a 4:1:5 mixture of methanol, glacial acetic acid and distilled water respectively..

Preparation of Total Protein Extracts for 2D-PAGE

Total proteins were extracted from black gram seeds according to Natarajan et al. (2005) using a TCA-acetone buffer. Thirty whole seeds of fresh and 6-day artificially aged seeds were powdered separately in liquid nitrogen using pestle and mortar. A portion (100mg) of the powder was homogenized in 1.5ml 10% (w/v) TCA in acetone with 0.07% (w/v) DTT. Total protein was precipitated for at least 2 h at -20°C. The exaction was centrifuged at 35000 g for 15 min, and the supernatant was discarded. The pellet was washed three times with acetone containing 0.07% (w/v) DTT. Then the pellet was dried under vacuum and stored at -80°C. The dry powder was resuspended in 500µl of lysis buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 1% w/v DTT, 2% v/v Triton X-l00, 0.2% v/v Bio-Lyte pH 3-10) by vortexing at a low speed for 30 min. Insoluble matter was removed by centrifugation at 35000 g for 15 min. Protein concentrations in various extracts were quantified by the Non-Interfering™ protein assay kit (G-Biosciences, St. Louis, MO, USA), in accordance to the manufacturers protocol.

2D-PAGE

For the first dimension, 100µg of proteins in rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.002% Bromophenol blue) were used to rehydrate the 18 cm immobilized linear pH gradient (IPG) strips, pH 4–7. Isoelectric focusing was performed in the Ettan IPGphor 3 system (GE Healthcare) with following steps: 50 V for 1 h, 200 V for 1h, 500 V for 30 min, 4000 V for 1h 30 min, 10,000 V for 14 h, and 50 V for 3 h. Prior to the second dimension, the IPG strips were equilibrated twice for 30 min each in 5 mL/strip of equilibration solution containing 6 M urea, 30% (v/v) glycerol, 2.5% (w/v) SDS, 0.15 M BisTris, and 0.1 M HCl. DTT (50 mM) was added to the first equilibration solution, and iodoacetamide (4% [w/v]) was added to the second. Equilibrated gel strips were placed on top of 12% vertical sodium dodecyl sulphate-polyacrylamide gels (47.5 ml 30% acrylamide / Bisacrylamide, 40.5ml Milli Q water, 30 ml 1.5M Tris HCl, pH 8.8, 1.2ml 10% SDS, 0.6 ml 10% ammonium persulfate, and 60µl TEMED). Agarose sealing solution (0.075 g of low-melting agarose [Gibco BRL], 15 ml of SDS) was loaded onto gel strips. The electrophoresis was performed at 20°C in a 1x electrophoresis SDS buffer at 30 mA/gel constant current. For each condition analyzed, 2D gels were made at least in duplicate and from two independent protein extractions. Data shown in figure 5A and 5B were obtained from fresh and six-day artificially aged seeds, respectively. 2D gels were stained with silver nitrate according to Blum et al. (1987) for densitometry analyses. Image analysis was carried out with Progenesis Samespots software (Nonlinear Dynamics, Newcastle, UK) & Image Master 2D Elite version 4.01 software (Amerschem Biosciences).

Sample preparation for MALDI-TOF

Protein spots were excised from the stained gel and washed first with MilliQ water three times to remove SDS and then with 50% acetonitrile containing 25 mM ammonium bicarbonate to destain three times until the gel is cleansed. The gel plug was dehydrated with 100% acetonitrile for 10 min and dried under vacuum. The gel plug was digested with 10µl of trypsin solution (20µg trypsin/ 1ml 50mM

NH_4HCO_3) for 14 hr at 37°C. After digestion the protein peptides were extracted from the gel plugs using 10µl of extraction buffer (50% acetonitrile, 0.1 % trifluoroacetic acid). The tryptic peptides were collected into siliconized tubes. The extraction procedure was repeated with the same amount of extraction solution. Extracted tryptic peptides were dried by vacuum evaporation and stored at -80°C until analysis or used immediately.

MALDI-TOF- MS Targeting and protein identification by database searching

Tryptic peptides of the extracts were dried and re-dissolved in 1µl of extraction buffer and 1µl of matrix solution (4-HCCA) and targeted onto a MALDI-TOF plate. Matrix-assisted laser desorption/ionization time-of-flight experiments were performed on a Voyager- DE STR mass spectrometer (Applied Biosystems, Franklin Lakes, NJ, USA). For identification of proteins, the peptide mass fingerprinting data were used to search against the database using the Mascot program (http://www.matrixscience.com). The following parameters were used for database searches: taxonomy, Viridiplantae (Green plants); cleavage specificity, trypsin with one missed cleavage allowed; peptide tolerance of 100ppm for the fragment ions; and allowed modifications, Cys Carbamidomethyl (fixed), and oxidation of Met (variable). The protein score was −10*log (P), where P is the probability that the observed match is a random event.

Total antioxidant potential

The total antioxidant potential of the aqueous acetone seed extracts obtained from 30 whole seeds of fresh and two to 10-day artificially aged black gram seeds were assessed based on their scavenging of DPPH free radicals, using a modified DPPH assay (Koleckar et al., 2007). An aliquot of extract (0.5 mL) was mixed with an ethanolic DPPH solution (0.5 mmol, 0.25 mL) and acetate buffer (100 mmol, pH 5.5, 0.5 mL). After 30 min of incubation, the absorbance of the mixture was measured at 517 nm against a blank containing absolute ethanol (0.5 mL) instead of a sample aliquot. DPPH-radical scavenging activity is expressed as % of control

Assessment of membrane integrity

The membrane integrity of fresh and two to 10-day artificially aged seeds were analyzed by assessing the electrical conductivity of seed leachates as described by Hampton and TeKrony (1995). Four replications of fifty seeds from each treatment were drawn, rinsed with distilled water to remove the adhering particles. Then, the surface moisture was removed using blotting paper and seeds were weighed and soaked in 75 ml of distilled water for 8 h at room temperature. After soaking, the seed steep water was decanted to obtain the seed leachate. The electrical conductivity of the seed leachate was measured in a digital conductivity meter with a cell constant of one and expressed as µS cm^{-1} g^{-1}.

Statistical analysis

The data obtained from different experiments were analyzed by the 'F' test of significance following the methods described by Rangaswamy (2010). Wherever necessary, the percent values were transformed to angular (Arc-sine) values before analysis. The CD values were calculated at 5 % probability level. The data were tested for statistical significance.

Conclusion

Our results revealed significant changes in proteome between fresh and six-day artificially aged seeds. Out of 12 down regulated proteins 3 of them belonged to, cell structure related proteins. Understandably, maintainance of cell membrane integrity by cell structure related proteins during storage is more important for the viability of seeds and also for successful cell multiplication during germination. Moreover,, down-regulation of enzymes like RNAP II, VARS, cytosolic 5'- nucleotidase (pyrimidine) during ageing suggests that these proteins play an important role in successful germination of seeds through effective transcription, translation and other metabolic process essential for germination. Down-regulation of storage protein namely 8S globulin and transporters like Sec-14 lipid-binding domain protein and SNARE could desist from supplying adequate nutrients for prolific embryo during germination. These down-regulations might possibly be mediated through up-regulation of proteolytic complex namely 26S proteasome. We believe that the down-regulated proteins play important roles in the transition of seeds from quiescent to active state during germination and hence, the loss of these proteins may be responsible for the loss of vigour and reduction in germination rate below the minimum germination standard. However, to understand the exact function and contribution of these proteins during germination further studies are required.

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