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Accelerated Ageing Mediated Seed Longevity Prediction and Assessment of Seed Deterioration Pattern through 2D-Gel Electrophoresis in Chickpea (*Cicer arietinum* L.)

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ABSTRACT

Background: Accelerated ageing is a method to assess seed storage performance by exposing the seeds to higher temperature and relative humidity to make seeds lose its vigour and viability quickly. The seed physiological parameters are then compared with the natural ageing to derive certain conclusions. But, the molecular pattern of seed deterioration under accelerated ageing varies compared to that of natural ageing.

Methods: Chickpea, seed lots of variety JG-11 and Annigeri-1 were subjected to natural ageing in cloth bag with nine per cent initial seed moisture under ambient conditions. Simultaneously, representative sample of same seed lot were also aged at 41°C and 95±2% humidity up to 120 hours.

Result: Both the ageing methods had significant negative effect on seed physiological and biochemical quality parameters. The germination potential of seeds tested after 48 hours of accelerated ageing (83.5%) was equal 10 months of natural ageing (85%) in JG-11, while also in Annigeri-1, 48 hours of accelerated ageing (78%) was nearly equal to 10 months of natural ageing (80%). The protein expression analyzed through 2D-PAGE at similar germination potential brought out by two independent ageing methods showed higher protein down regulation ratio (3.4) in accelerated ageing than in natural ageing (3.2) unveiling its rapidity in the seed deterioration process. Therefore, accelerated ageing can be used for predicting chickpea seed longevity.

Key words: 2D electrophoresis, Catalase, Germination, Protein expression, Storage prediction.

INTRODUCTION

Deterioration is an irreversible phenomenon in all bio-entities which steadily continues till they are dead. Seed deterioration is a serious inevitable problem when stored under conditions without appropriate control on temperature and humidity. Higher suboptimal temperature and humidity increases the metabolic rate of the seeds leading to loss of vigour quickly. Accelerated seed ageing is a controlled imposition of higher temperature and relative humidity to seed environment which is widely used for testing seed vigour and to correlate the performance of seed in field (Pandey *et al*., 1999). Nowadays, it is recognized as a rapid, simple and inexpensive way to determine the relative seed storability with great scope for understanding mechanism of ageing and associated process of deterioration. The application part of the use of accelerated ageing for seed longevity assessment also depends on the type of seed and inherent ability to withstand the artificial ageing treatments. Some researchers are of the opinion that physiological changes occurring inside the seeds undergoing accelerated ageing are considered to be as same as those occurring during natural ageing with only difference in rate of change (Vijay *et al*., 2010), meanwhile others believe that the accelerated ageing cannot depict actual seed deterioration events of natural ageing. Under these circumstance it is necessary to assess the rate of seed deterioration in chickpea under accelerated and natural ageing conditions. So that, the technique can be used precisely in predicting the relative storability of different seed lots from which seed producers and organizations get benefited in making management decisions regarding which seed lot to be retained and which needs to be marketed immediately adhering to minimum quality standards for seed certification. Therefore, the present investigation was undertaken to test the pattern of seed viability and vigour loss in ambient storage condition and upon subjecting to higher temperature and relative

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humidity with associated physiological and biochemical changes.

The change in metabolic activity of seed is best predicted by change in protein expression and type. Routine laboratory generally uses Two-dimensional gel electrophoresis (2DGE) to study the abundance level of proteins with ability of resolving even complex protein mixtures from seed protein samples. The technique is a basic and core component of the proteomics with a capacity to resolve and separate thousands of proteins in a single gel. In the study of biological system, proteomics is often considered as advanced step and complicated than genomics as the organism's genome is more or less constant, but the protein expression profile alters with slight change in environment. The strength of 2D beside its technical challenge is the ability to profile complex mixture of proteins and its post translational modifications and isoforms which are mostly deposited as spots in vertical/ horizontal axis of 2D-PAGE (Ong and Pandey, 2001). In addition, it's also possible to elute relatively pure protein for further analysis from resolved spots. Therefore, studying seed proteins using 2D-GE to understand seed deterioration pattern can provide reliable data to draw fact full phenomena. In the present experiment, we highlight the marked reduction in seed quality parameters in accelerated ageing and natural ageing and its associated protein expression as validation of rapid seed deterioration in accelerated ageing process.

MATERIALS AND METHODS

The present study was conducted in the Department of Seed Science and Technology and Department of Agricultural Biotechnology, University of Agricultural Sciences, Dharwad, India. Chickpea seeds of varieties JG-11 and Annigeri-1 of certified class procured from Seed Unit were dried to the safe moisture level (9%), packed and stored (25°C) for natural ageing. Simultaneously, the seeds were subjected to accelerated ageing conditions. Bimonthly observations were recorded for various seed quality parameters during natural ageing. The experiment was laid in FCRD (Factorial completely randomized design) involving two varieties (JG-11 and Annigeri-1) as main factor and six accelerated ageing durations as sub factors.

Accelerated ageing

The methodology described by Delouche and Baskin (1973) was followed with little modifications. To petri plates, 40ml of distilled water was added above which a wire mesh was placed in a way avoiding contact with water. On top of mesh, 60 grams of fresh seeds were uniformly spread in a single layer and exposed to accelerated ageing conditions of 41°C temperature and 95±2 per cent RH for 0, 24, 48, 72, 96 and 120 hours and 400 seeds are tested for germination (ISTA, 2013) in four replicates following between paper method and incubated in seed germinator at 25°C temperature and 90% RH up to final count (8 days).

Electrical conductivity

As per ISTA (2013) guidelines, the seeds were surface sterilized in 1% sodium hypochlorite for 30 seconds and rinsed with distilled water twice. Sterilized seeds were soaked in 25ml distilled water and incubated at 25°C for 12h. The clear leachate was collected and the volume was made up to 25ml with distilled water. ELICO conductivity bridge was used to measure electrical conductance at 1.0 cell constant and expressed in desi Simons per meter (dSm^{-1}).

Seedling vigour index

The seedling vigour index is the product of per cent seed germination and total seedling length in centimetres (Abdul-Baki and Anderson, 1973).

Seedling Vigour Index = [Germination % × (Root length + Shoot length)]

Catalase activity

Crude extract was prepared by homogenizing 5 seeds in pestle and mortar with 2ml PBS (50mM, pH 7) containing Triton X-100 (1%). The homogenate was centrifuged for 10 minutes at 15000 rpm at 4°C. The supernatant was used for catalase assay as suggested by Aebi (1983), by measuring the disappearance of H_2O_2. A 1.0 ml reaction cocktail containing 100µl enzyme extract and PBS (50mM, pH 7) was made to react with 100µl of 10mM H_2O_2. Change in absorbance at 240nm was read through spectrophotometer (Eppendorf, Biospectrometer-Kinetic) at every 15 seconds time scan. It was expressed in terms of µg/mg protein/min.

Protein differential expression

Protein was isolated from fresh seeds, 48 hours accelerated aged and 10 months naturally aged seeds of JG-11 variety based on the results obtained. The whole analysis was done for three independent technical replicates.

Protein extraction and solubilization

Seeds were fine powdered using liquid nitrogen in sterilized pre-chilled pestle and mortar. Protein was extracted by suspending 150mg of dry powder in 800µl of extraction buffer (500mM Tris-HCl, 50mM EDTA, 700mM sucrose, 100mM KCl, 2% β-mercaptoethanol and 1mM PMSF with pH 8.0) and incubated for 5 mins at 4°C (Hurkman and Tanaka, 1986). Equal volumes of Tris-buffered phenol of pH 8.0 was added and vortexed for 30 seconds. Upper phenol phase with total soluble proteins obtained after centrifugation for 10 minutes at 12000 rpm was precipitated using 4 volumes of 0.1M ammonium acetate in ice cold methanol. Pellets were washed thrice with methanol to remove any pigments and then vacuum dried. Pellets were dissolved in 300µl of solubilisation solution (2M thiourea, 7M urea, 20mM DTT and 4% CHAPS) to every 150mg of initial sample (Amalraj *et al.,* 2010). The isolated protein was kept in -80°C until further use. The protein was quantified by following Bradford (1976) method using BSA as standards.

Isoelectric focusing (IEF)

IEF was performed using 180µg of protein sample diluted in 250µl of two-dimensional rehydration buffer [8M urea, 2M thiourea, 2% CHAPS, 20mM DTT, 0.5% pharmalyte (pH 4-7) and 0.05% bromophenol blue] to rehydrate immobilized pH gradient strips (11cm; pH 4–7). IEF strips were passively rehydrated with proteins and focusing was performed for 30,000 Vh in PROTEAN i12 IEF (Bio-Rad) at 20°C. The focused IPG strips were then reduced with 1% DTT in 3ml of equilibration buffer [6M urea, 50mM Tris-HCl pH 8.8, 30% glycerol and 2% SDS], followed by alkylating through 2.5% iodoacetamide in same buffer. The equilibrated strips were then placed on 12% polyacrylamide gel as described by Laemmli (1970) and sealed using 0.5% agarose containing 0.002% bromophenol blue. Second dimension electrophoresis was carried out using Bio-Rad electrophoresis unit at 60 mA/gel at 25°C. The current was stopped once the tracking dye reached bottom of the gel. Later, the gels were stained with 1.2% coomassie brilliant blue R-250 in staining solution (50:40:10 of ddH_20 : methanol : Acetic acid v/v) for 5 hours and then destained (50:40:10 of ddH_20 : methanol : Acetic acid v/v) until the background was clear.

Protein spot analysis

Gel image acquisition in TIFF format was done using Bio-Rad densitometer (GS-800) and subjected to Bio-Rad's PD-Quest 2D analysis tool. Manual alignment of gel images was done to superimpose the landmark protein spots to prepare master gel image and the fold change was fixed to log 1.8 where, $P \leq 0.05$ is considered significant.

The experimental data thus obtained were statically analyzed by the procedure prescribed by Sundararaj *et al.* (1972).

RESULTS AND DISCUSSION

Effect of accelerated and natural ageing on the chickpea seeds is clearly illustrated in the present study. The accelerated aged seeds of varieties JG-11 and Annigeri-1 had a significant effect on seed physiological and biochemical parameters (Fig 1). The variety JG-11, recorded significantly higher seed germination (60.3%), seedling vigour index (1219), catalase (11.3µg/mg protein/min) and lower electrical conductivity (0.427 dSm^{-1}) compared to Annigeri-1 (56.1%, 1088, 9.9µg/mg protein/min and 0.446dSm^{-1}, respectively).

Seed germination and seedling vigour index drastically reduced with advancement in ageing durations. Significantly higher seed germination and seedling vigour was seen in fresh seeds (97.8% and 2591, respectively), while, 120 hours of accelerated aged seeds did not germinate and as a consequence there was zero seedling vigour index (Fig 1A and 1B). Similarly, with the progress in natural ageing, germination per cent in both the varieties (Fig 2A and 2B) declined by recording an average of 60.5 per cent at 18 months after storage with significant lower seedling vigour index (1006).

Significant changes in biochemical parameters were also observed due to accelerated and natural ageing (Fig 1). The accelerated ageing treatments resulted in membrane damage reflected in terms of higher solute or ion leakage (Fig 1C) from seeds aged up to 120 hours (0.490 dSm^{-1}) as compared to control (0.378 dSm^{-1}). Similar rate of loss of membrane integrity was also recorded in natural ageing (Fig 2) up to 18 months (0.495 dSm^{-1}). Nevertheless, JG-11 was comparatively tolerant to its membrane damage as depicted with significant lower value of EC (0.425dSm^{-1}) than that of Annigeri-1 (0.435 dSm^{-1}).

Similarly, both the methods of seed ageing also had significant impact on catalase enzyme (Fig 1D and 2D). Between the varieties, JG-11 maintained significant higher catalase activity (10.9µg/mg protein/min) compared to Annigeri-1 (10.1µg/mg protein/min) in natural ageing. While, in accelerated ageing also JG-11 had higher average catalase (11.3µg/mg protein/min) than Annigeri-1 (9.9µg/mg protein/min). Eloquent higher catalase was observed in fresh seeds (13.2µg/mg protein/min) and did not change statistically until 2nd month of natural ageing and up to 24 hours of accelerated ageing period. Later on, it reduced drastically with ageing period, reporting 5.1 and 8.1µg/mg protein/min, respectively in 120 hours of accelerated ageing and 18 months of natural ageing.

Prediction of storability of seeds

The seed germination potential decreased significantly irrespective of type of ageing and duration of ageing, but pattern of reduction was not uniform for most of the seed quality parameters tested. Accelerated ageing caused the decline in seed germination of chickpea varieties at higher rate of reduction compared to that of natural ageing (Table 1).

Present study demonstrates that, in case of JG-11, 48 hours of accelerated ageing with 83.5 per cent germination was equal to 10 months of natural ageing with 85 per cent of germination. In similar way, 48 hours of accelerated ageing in Annigeri-1 seeds recorded 78 per cent of germination which was nearly equivalent to 10 months of natural ageing with 80 per cent seed germination.

Protein profile in fresh seed, 10 months naturally aged and 48 hours accelerated aged seeds

The average of three technical replicates of protein sample from JG-11 variety of fresh seeds, 10 months naturally aged and 48 hours of accelerated aged seeds resolved 230, 183 and 138 protein spots, respectively, with corresponding match rate of 87, 71 and 46 per cent to master gel (Table 2). Upon using fresh seeds protein profile in comparative background to form a master gel (Fig 3), it revealed that, among the relatively analysed 122 spots between fresh and 48 hours of accelerated aged seed protein sample, 68 were differentially expressed (1.8 fold change; $p \leq 0.05$) out of which 28 protein spots significantly up regulated and 40 were down regulated (Fig 4, Supplementary graph). Correspondingly, in between fresh and 10 months naturally aged seed sample, out of 138 common spot count, 96 showed differential expression of which 39 protein spots observed statistically as up regulated and 57 as down regulated (Fig 5, Supplementary graph).

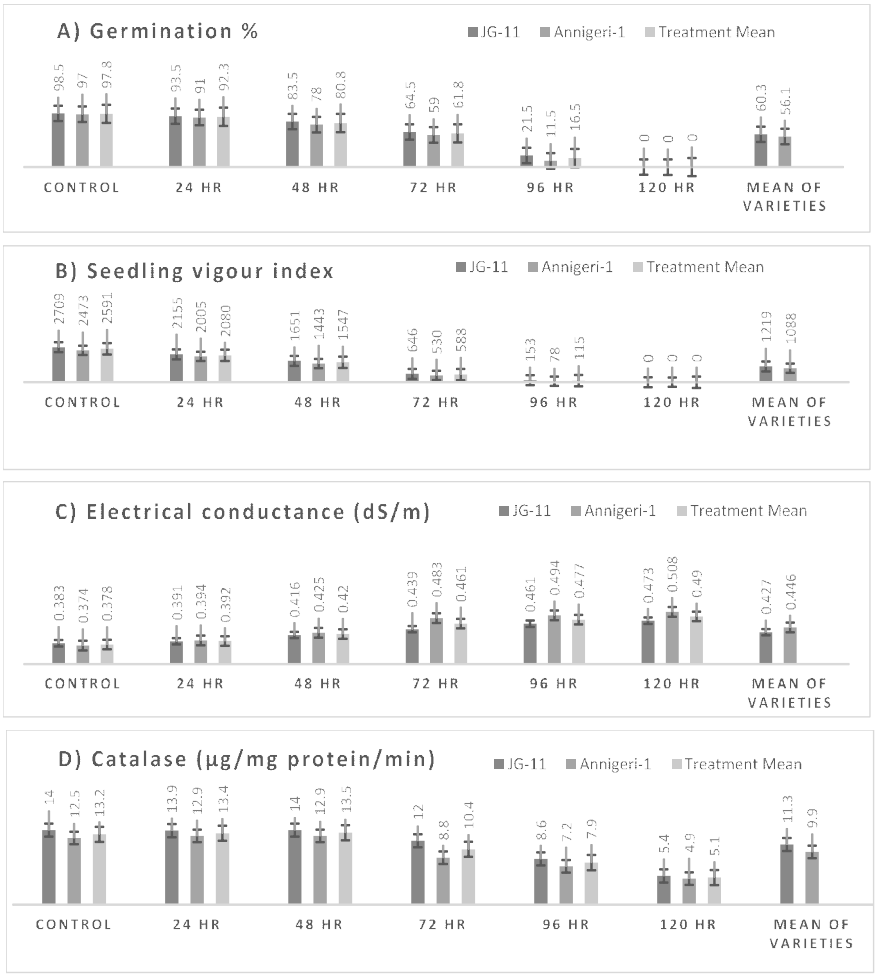

Fig 1: Influence of accelerated ageing conditions on [A] Seed germination; {(Varieties; S.Em=0.6, CD=1.7) (Treatments; S.Em=1.1, CD=3.0)} [B] Seedling vigour index; {(Varieties; S.Em=24, CD=68) (Treatments; S.Em=41, CD=117)} [C] Electrical conductance; {(Varieties; S.Em=0.002, CD=0.008) (Treatments; S.Em=0.004, CD=0.013)} [D] Catalase{(Varieties; S.Em=0.1, CD=0.3) (Treatments; S.Em=0.2, CD=0.6)}. The values corresponding individual bars indicate the average of four replications.

Table 1: Simulation of seed storability through accelerated and natural ageing based on seed germination.

Accelerated	Seed germination (%)			Natural ageing	Seed germination (%)		
ageing duration	V_1	V_2	Mean A	duration	V_1	V_2	Mean N
A_0: Control	98.5	97.0	97.8	N_0: Fresh seed	98.5	97.0	97.8
A_1: 24 hr	93.5	91.0	92.3	N_1: 2nd month	98.0	95.0	96.5
A_2: 48 hr	83.5	78.0	80.8	N_2: 4th month	93.5	89.0	91.3
A_3: 72 hr	64.5	59.0	61.8	N_3: 6th month	89.0	87.0	88.0
A_4: 96 hr	21.5	11.5	16.5	N_4: 8th month	87.0	84.0	85.5
A_5: 120 hr	0.0	0.0	0.0	N_5: 10th month	85.0	80.0	82.5
Mean V	**60.3**	**56.1**		N_6: 12th month	80.0	73.0	76.5
Factors	V	A	V × A	N_7: 14th month	71.0	69.0	70.0
S.Em±	0.6	1.1	1.5	N_8: 16th month	66.0	63.0	64.5
C.D. at 5%	1.7	3.0	4.3	N_9: 18th month	61.0	60.0	60.5
				Mean V	**82.9**	**79.7**	
				Factors	V	A	V × N
				S.Em±	0.3	0.8	1.1
				C.D. at 5%	1.0	2.2	3.5

V_1: JG-11 variety. V_2: Annigeri-1 variety.

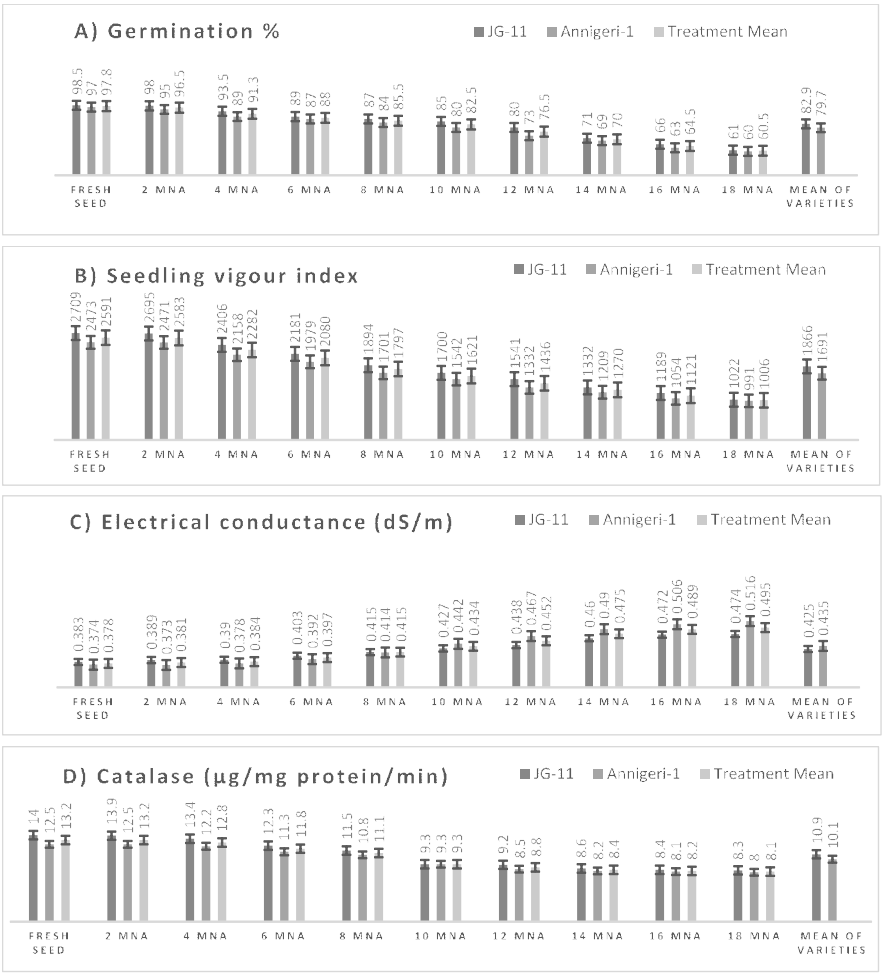

Fig 2: Influence of natural ageing on [A] Seed germination; {(Varieties; S.Em=0.3, CD=1.0) (Treatments; S.Em=0.8, CD=2.2)} [B] Seedling vigour index; {(Varieties; S.Em=18, CD=50) (Treatments; S.Em=39, CD=112)} [C] Electrical conductance; {(Varieties; S.Em=0.002, CD=0.005) (Treatments; S.Em=0.003, CD=0.01)} [D] Catalase {(Varieties; S.Em=0.1, CD=0.2) (Treatments; S.Em=0.2, CD=0.5)}. The values corresponding individual bars indicate the average of four replications.

Table 2: Two dimensional gel analysis report of fresh, 10 months naturally aged and 48 hours accelerated aged.

Protein sample	Spots detected	Match rate to master gel	Significant common spots (p<0.05)	DEP's	Up regulated	Down regulated	Up regulation ratio	Down regulation ratio
*Fresh seeds	230	87%	230	-	-	-	-	-
48 hours acelerated aged seeds	138	46%	122	68	28	40	4.9	3.4
10 months natural aged seeds	183	71%	138	96	39	57	4.6	3.2

*Used as master gel/reference gel.

Seed deterioration is the loss of viability and vigour steadily either due to adverse storage conditions or due to ageing itself. The deterioration pattern is well understood with the test of seed storage and accelerated ageing. Under accelerated ageing seeds which rapidly deteriorate usually show a marked reduction in their ability to germinate (McDonald, 1999) due to alteration in enzymatic function as consequence of higher temperature and humidity with corresponding changes in protein conformation by partial folding or unfolding, monomers or subunits dissociation and condensation to polymers. This influences the potentiality of seed germination and also because of various changes in hydrolytic enzymes, including phospholipase and lipase, phosphatise, DNAase, amylase and even protease which

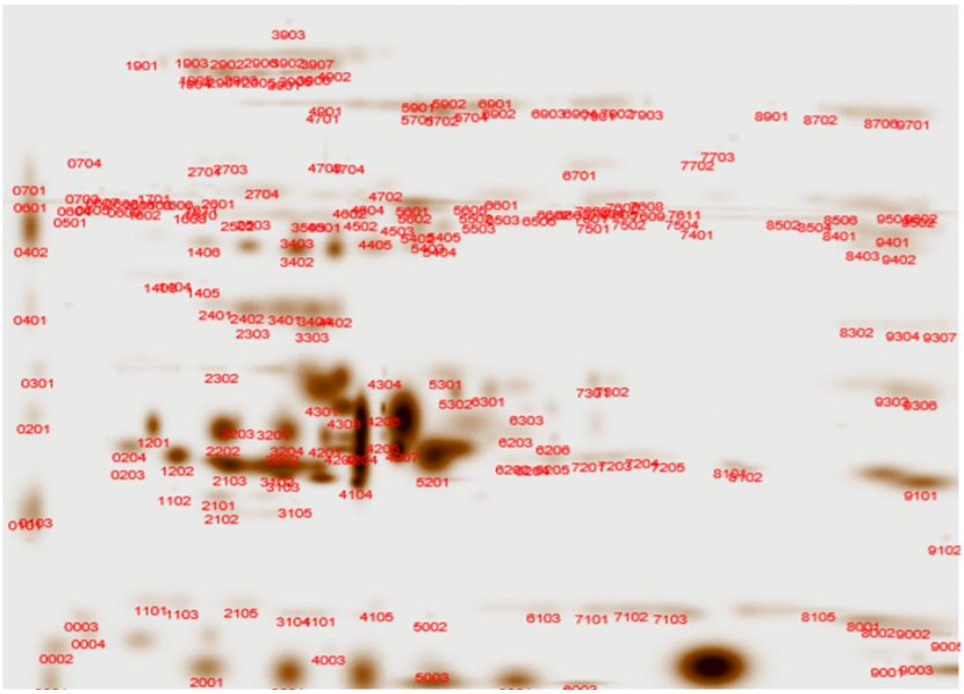

Fig 3: Protein spots with unique spot assignment numbers in master gel obtained from fresh seeds, 48hr accelerated aged seeds and 10 months naturally aged seed protein sample using PD-Quest (Bio-Rad) 2D gel analysis software.

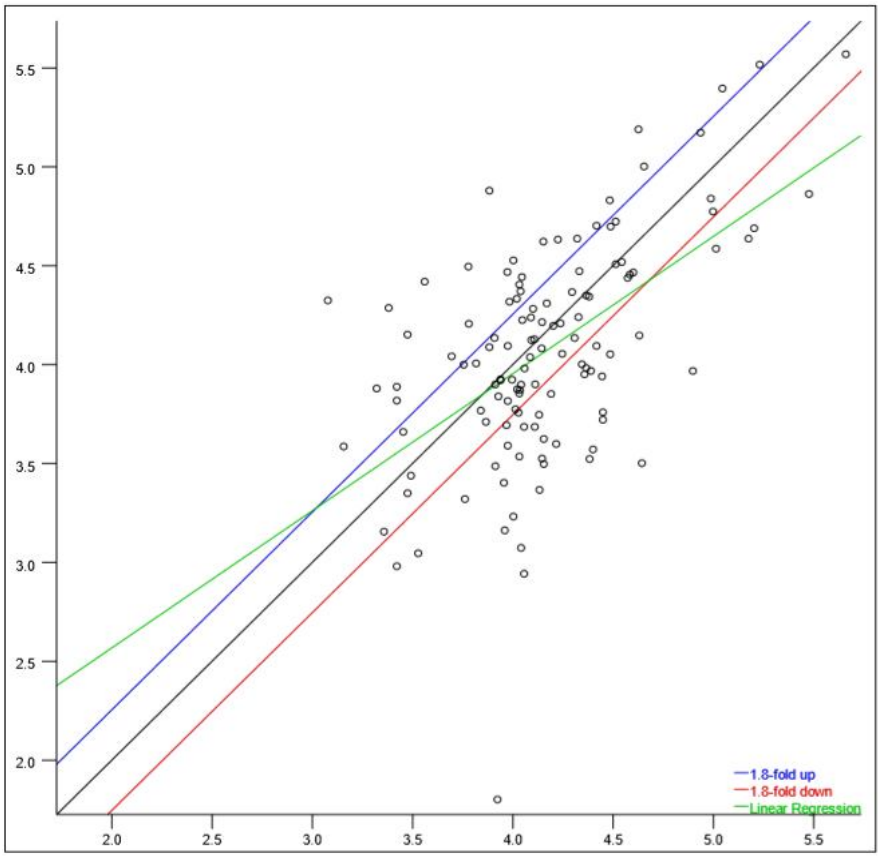

Fig 4: Scatter plot diagram between X = 48 hours accelerated aged and Y = Fresh seeds showing the distribution of 122 spots of which 28 are up regulated (above blue line) and 40 as down regulated (below the red line) with a correlation coefficient of 0.565 and slope 0.693.

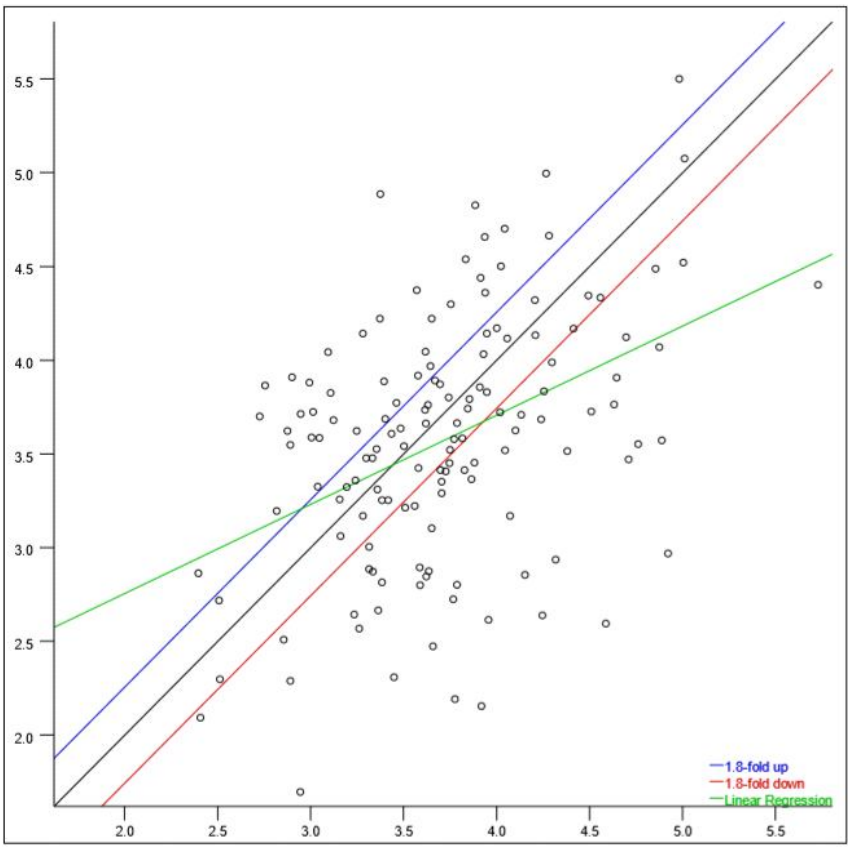

Fig 5: Scatter plot diagram between X = 10 months naturally aged and Y = Fresh seed showing the distribution of 138 spots of which 39 are up regulated (above blue line) and 57 as down regulated (below the red line) with a correlation coefficient of 0.425 and slope 0.475.

were essential for successful germination (Basavarajappa *et al.,* 1991). In many seed species, mechanism of seed deterioration in accelerated and natural ageing is still to be revealed.

Some researchers consider that accelerated ageing induced deterioration is similar to natural ageing, with only difference in speed at which they occur (McDonald, 1999; Galleschi *et al.,* 2002). The present study compares the germination response with associated enzymatic and protein expression under accelerated ageing and natural ageing duration to know the dissimilarity between two ageing methods. It is observed that, Annigeri-1 performance was inferior with respect to its seed physiological and biochemical parameters as compared to JG-11. The poor seed quality of Annigeri-1 might be due to faster rate of seed deterioration resulting in excessive leaching of electrolytes. Whereas, the JG-11 variety as evident from the average electrical conductivity values had better withholding capacity to seed deterioration process than that of Annigeri-1, which made it to survive longer under seed ageing process. Similar varietal differences with respect to maintenance of seed quality parameters were earlier reported by Geeta *et al.* (2007) in pea upon subjecting to accelerated ageing conditions for 21 days. Faster reduction in germination and other parameters were also reported in one-year-old seeds of bitter gourd compared to fresh seeds during accelerated ageing test (Shantappa *et al.,* 2006).

With the advancing natural ageing duration, there was gradual decline in seed germination and seedling vigour index due to inhibition in cell expansion more profusely than cell division rendering root and shoot elongation (Holmfridur *et al.,* 2009). As ageing is an irreversible process, seed germination potential of different varieties of pea in earlier study also gradually declined with age during storage (Geeta *et al*., 2007). Our results were also in concordance with the findings of Vijay *et al*. (2010) in soybean wherein, fresh seeds (90%) gradually declined its germinability below 70 per cent after 10 months of natural ageing. Rapid decline in germination and seedling vigour index might be due to the impairment in mitochondrial membrane paving to reduced energy supply necessary for metabolism to initiate germination (Gidrol *et al.,* 1998) and increased sub-cellular membrane structure and organization changes (Shantappa *et al.,*2006). Similar reduction in vigour index was also reported in onion (Bhanuprakash *et al.* 2010), green gram, red gram and cowpea (Ananthi *et al*., 2015) due to seed ageing.

The normal functioning of any physiological process is dependent on enzymes, metabolites and membrane structural maintenance. The study demonstrated significant

damage in seed coat membrane stability, leaching more electrolytes due to accelerated and natural ageing as evident from the marked increase in electrical conductance and reduced catalase activity which is a proof for lipid peroxidation or auto-oxidation induced membrane disruption. Earlier studies reported changes in enzymes, tissue permeability, protein stability, ATP synthesis due to ageing (McDonald, 1999; Ananthi *et al.,* 2015), Mali *et al.* (2014) and Vijay *et al.* (2010). Estimation of electrical conductance is a promising method in seed quality studies (Bewley and Black, 1994). The increase in electrical conductivity may be due to auto-oxidation of polyunsaturated fatty acids in the membrane lipids compound involving free radical reactions; and loss of viability and vigour may be linked to changes in membrane properties (Priestley, 1986). Membrane degradation occurs from both hydrolysis of phospholipids by phospholipase and phospholipids oxidation. Therefore, in the simple sense, imbalance in membrane stability might be considered as a result of ageing rather than a cause (Bardel *et al.,* 2002). Nevertheless, the variety JG-11 was better in maintaining its seed membrane integrity evident from the lower electrical conductance compared to Annigeri-1. This might be due to rigid seed coat of JG-11 making the seed to leach less electrolytes.

In seed physiology, Reactive oxygen species (ROS) have a key role. But, their accumulation in seeds during seed desiccation, ageing and germination may induce oxidative stress resulting in membrane damage mediated seed deterioration. Prolonged sequestration of free fatty acids leads to lowering the cellular pH which is detrimental to cellular metabolism sustenance (Copeland and McDonald, 1995). However, to scavenge ROS, cells are endowed with detoxifying antioxidant enzymes. Catalase (CAT) is one such antioxidant entangled to preserve seed viability through normalizing cellular activity and necessary element for seed germination and subsequent growth (Bernal Lugo *et al.*, 2000; Milosevic *et al.*, 2010). As a consequence of higher temperature and higher relative humidity, the activity of catalase in the present study reduced drastically in accelerated ageing and gradually in ambient natural ageing conditions. Bailly *et al.* (1996) reported a possible decrease in antioxidant enzymes is linked to increased lipid peroxidation. Similar reduction in enzyme catalase due to ageing effect was reported in the studies conducted by Khajeh *et al*. (2015) in safflower and Parmoon *et al*. (2013) in milk thistle seeds.

Prediction of seed storability

There are studies showing the suitability of artificial/ accelerated ageing in studying the pattern of seed deterioration and storage assessment in various crops (Kibinza *et al*., 2006; Jatoi *et al.,* 2001; Scialabba *et al.,* 2010). The most powerful tool to predict the storability involves seed germination responses after the accelerated

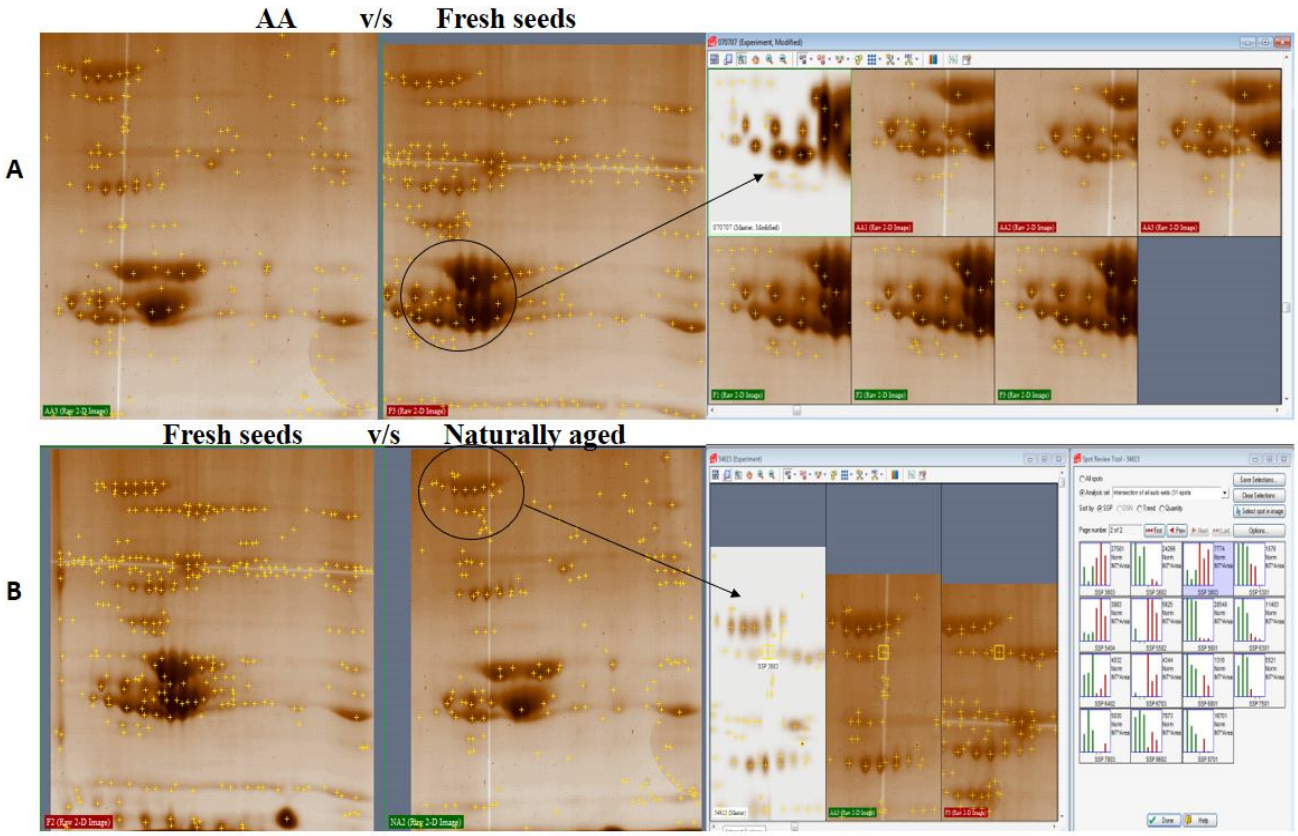

Fig 6: Differential protein expression analysis between **(A)** 48 hours accelerated aged seeds and fresh seeds; **(B)** fresh seeds and 10 months naturally aged seeds using PD-Quest (Bio-Rad) 2D gel analysis software.

ageing (Delouche and Baskin, 1973). Routinely, the seed physiological quality evaluated by the standard germination test is assumed as most practicable and reproducible parameter (Torres *et al.,*2004) and can be better correlated with the physiological status of seed from both the ageing methods. In the present experiment, seed germination after 48 hours of accelerated ageing in JG-11 and Annigeri-1 was closely equal to 10 months of natural ageing. Ananthi *et al.* (2015) also predicted storage potential of seeds in green gram by accelerated ageing technique based on seed germination criteria wherein, six days of accelerated ageing was equal to nine months of natural ageing by registering nearly same germination of 75 and 73 per cent, respectively under accelerated and natural ageing. Similarly, Shantappa *et al*. (2006) predicted the storability of bitter gourd seeds through accelerated ageing technique and reported that 72 hours of accelerated ageing was equal to 12 months of natural ageing by registering nearly same germination of 87.2 and 87.0 per cent, respectively under accelerated and natural ageing conditions. Mali *et al.* (2014) reported 79.7 per cent of seed germination under four days of accelerated ageing of soybean seeds which was equal to 10 months under natural ageing in gunny bag (73.3%) and 12 months in polylined gunny bag (73.0%).

Protein differential expression

Various research conclusions points towards the free radical production during seed ageing causing proteins and nucleic acids degradation apart from membrane disruption. Hence, monitoring the changes of protein gives better understanding of the deterioration mechanism with addition to physiological tests. The differential regulation of proteins analyzed by 2D gel electrophoresis implicates about the ongoing metabolic process inside seeds even at similar germination level brought out by two independent ageing methods are different from each other (Fig 6). Specifically, higher protein spots match rate in naturally aged seed sample may suggest slow or steady degradation of proteins possibly than compared to accelerated aged seeds sample. The higher spot match rate also indicates that the resolved proteins from 10 months old seeds belongs to more of fresh seeds. Whereas, lower spot match rate of 48 hours accelerated aged seed protein sample signified that there was drastic deviation in the type, isoforms and amount of proteins as found in fresh seeds. From this variation in protein profile, it could be inferenced that, accelerated ageing involves seed deterioration process at faster level than the normal ageing process. Higher protein down regulation ratio due to accelerated ageing also indicates loss of higher number of proteins and associated function leading to rapid loss of vigour and viability in accelerated aged seeds. Neto *et al*. (2001) also reported decrease in the seed germination, vigour and characteristic protein banding pattern of *Phaseolus vulgaris* under natural and accelerated ageing conditions and opined that the degradation pattern of proteins was different in natural and accelerated ageing suggesting different driving factors on seed's physiological and molecular deterioration mechanisms. Study on artificial ageing in black gram for six days performed by Sekar *et al.* (2015) also reports decrease in germination potential below 75 per cent with corresponding differential expression of 16 proteins of which 4 and 12 were up and down regulated, respectively. Likewise, 81 proteins in control deterioration were differentially accumulated as revealed by 2D electrophoresis in *Brassica napus* seeds (Yin *et al.,* 2015). Lv *et al.* (2016) identified a total of 162 DEPs categorized in metabolism, energy supply and defence/stress responses, in artificially aged wheat seeds compared to un-aged seeds revealing the inability of seed to protect from ageing induced degradation of the stored food, impairment in energy supply due to imbalance in metabolic activity and ultimately resulting in seed deterioration.

CONCLUSION

Seed deterioration process evaluated through germination response and other physio-biochemical changes under accelerated ageing conditions was faster than that occurred in natural ageing. This difference is mainly because of higher temperature and relative humidity, the main detrimental factors of seed deterioration process. Protein expression at similar germination level under accelerated and natural ageing also depicted the faster degradation process under accelerated ageing method as it had comparatively lower number of resolved spots as well as lower match rate to the fresh seed protein sample and hence higher protein down regulation ratio. Therefore, rapid deterioration process under accelerated ageing technique can be used creditably in predicting the storage life of seeds.

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Conflicts of interest/Competing interests

Authors declare no conflict of interest.

Authors' contributions

SSP performed the experiment and analyzed the data. ASS, BKN and KPU conceptualized and designed the experiment. SSP and ASS drafted and finalized the manuscript.

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