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Research Article

EFFECT OF SEED AGEING ON PROTEIN QUALITY AND QUANTITY IN MAIZE

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Abstract: Storage of maize seeds under ambient hot and humid conditions is very problematic since these conditions deteriorate seed quality faster. Many biochemical changes are linked to the process of seed deterioration and the changes during accelerated aging were mostly the same as those in natural ageing with only difference being the rate at which they occur. Decreased vigour and viability follows increased ageing. Impairment in the quantity and quality total soluble protein content and protein profiles also showed alteration in their number and intensity of bands in aged seeds. Quantitative and qualitative changes in protein were detected during ageing. This work establishes association between seed viability and age accumulation, due to protein deterioration, in maize seeds.

Keywords: SDS, Maize, Protein, Seed deterioration

INTRODUCTION

Maize (*Zea mays* L.) is the third most important food grain in India after wheat and rice. To meet increasing demand, many hybrids were developed and released by both public and private sectors. The information on storability of newly released maize hybrids under ambient storage conditions is important. Since adequate seed storage is a common agricultural problem as it is very difficult to maintain seed viability and vigour during storage period particularly in tropical regions with a high humidity. Seeds are to be stored from the day of harvest up to the time of next sowing. For seeds men, storability of seeds is of much concern because seeds are to be saved for a longer period. The seeds will undergo deterioration after the physiological maturity and the process of deterioration will be aggravated if the storage conditions are not at their optimum. High temperature, relative humidity, seed moisture content, light exposure and an extended storage period have all been found to adversely affects seed vigour.

In recent years a rapid ageing method where seeds are subjected continuously to high temperature and in saturating humidity has been recognized as an important technique in estimating the rate of seed deterioration in storage. In rapid and slow ageing (natural ageing), the pattern of deterioration preceding the death is the same whether seed survives for few hours or decades. Accelerated ageing is an important procedure for understanding the events that lead to the loss of seed viability (Olga Stan, 1997). Many hypotheses have been proposed regarding causes of seed ageing such as loss of vigour (Syed *et al.,* 2012) and viability in terms of germination (Deshpande *et al.,* 1991; Drochioiu *et al.,* 1993) due to many physiological changes like cell membrane perturbation in deteriorated seeds. Physiological markers (germination, seedling vigour) are universally adopted as classical tools to understand the mechanism of seed deterioration in terms of vigour potential. However the exact biochemical changes involved in seed deterioration were scanty in order to support the above hypothesis. Keeping in view of the above facts an experiment was designed to study the relationship between protein deterioration and seed viability.

MATERIAL AND METHODS

Experimental storage conditions:

These studies were carried out on seeds of newly released maize hybrid Hema. Seeds were surface sterilized with 5% sodium hypochlorite (NaOCl) solution for 5 minutes and rinsed with double distilled autoclaved water (Gholami and Golpayegani, 2011). The seeds were air dried at 28°C±1 for 24 h in the laboratory. Seeds were subjected to accelerated ageing treatment at two temperature levels (40°C & 42°C) and four relative humidity levels (85, 90, 95 & 100 per cent) for 0, 3, 6, 9 and 12 days in a controlled ageing chamber. For germination and vigour tests three replications of hundred seeds were taken out at predetermined intervals. Following the ageing treatments, the moisture content was recorded as described by ISTA (2010). The seeds were air dried at 28°C±1 in the laboratory until their original moisture content of 12% (Kranner *et al.,* 2012) was restored. Then seeds were tested for following seed quality parameters.

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Germination percentage: It was determined as per ISTA rules for seed testing (Anon., 1996). The seeds were placed in rolled paper towels. Hundred seeds of four replications were tested at a constant temperature of 25°C. The germination first and second counts were recorded on fourth and seventh day respectively and per cent germination was expressed on normal seedling basis.

Seedling vigour index-I: Ten normal seedlings were selected from the standard germination test at random in each replication on final count. The shoot length was measured from collar region to the point of attachment of cotyledons and root length from the collar region to the tip of the primary root, sum of shoot and root length constitute the seedling length and mean was calculated and expressed in centimeters. It was computed by adopting the formula as suggested by Abdul-Baki and Anderson (1973) and expressed in whole number.

$$\text{SVI-I} = \text{Germination (\%)} \times \text{Mean seedling length (cm)}$$

Seedling vigour index-II: The seedlings used for seedling length measurement was used for estimating dry weight. They were dried in a hot air oven maintained at 80 ± 2°C for 48 hours. After drying, the weight of dry seedlings was recorded and the mean seedling dry weight was calculated and expressed in milligrams. It was computed by adopting the formula as suggested by Abdul-Baki and Anderson (1973) and expressed in whole number.

$$\text{SVI-II} = \text{Germination (\%)} \times \text{Mean seedling dry weight (mg)}$$

Total soluble seed protein (µg g^{-1}): The total soluble seed protein was estimated as per the method ascribed by Lowry *et al.,* (1951). The procedure followed is as follows:

a. Preparation of seed powder

Forty eight hours primed seeds as per the treatments were thoroughly washed and excess moisture was blotted with tissue paper and seeds were immediately frozen with liquid nitrogen. Seeds were ground by using pestle and mortar and the powder was transferred to eppendorf tubes and stored in sealed containers at 4°C until analysis.

b. Preparation of reagents

- Solution A: 20g of anhydrous sodium carbonate + 4 g of sodium hydroxide dissolved in 1000 ml of water.
- Sodium potassium tartarate solution (1.35 %): 1.35 g of sodium potassium tartarate dissolved in 30ml water and volume was made up to 100 ml.
- Copper sulphate solution (5.5 %): 5.5 g of Copper sulphate was dissolved in 30ml water and volume made up to 100 ml.
- Solution B: 1.0 ml of (1.35 %) Sodium potassium tartarate + 0.1 ml of (5.5 %) Copper sulphate solutions were mixed together.
- Solution C: 50 ml of solution 'A' was mixed with 1 ml of solution 'B', just before use.
- Folin Ciocalteu Reagent (FCR): Commercial grade reagent was diluted 1:1 (V/V) using distilled water before use.
- Standard Bovine Serum Albumin (BSA) solution: 50 mg of BSA was dissolved in water and volume made up to 50 ml to get a stock standard. A standard curve was prepared to get 500 µg ml^{-1}.

c. Sample extraction

One hundred milligram of oven-dried seed sample was extracted in 10ml of 0.1M Sodium phosphate buffer, pH 7.0 for one hour on a magnetic stirrer at room temperature. The extract was centrifuged at 10000rpm for 15 min. The supernatant was used for the estimation of total soluble protein content.

d. Estimation of total soluble seed protein

The required quantity (0.1 ml) of supernatant was diluted to 1ml with double distilled water. Five ml of solution 'C' was added and mixed thoroughly. After 10 minutes, 0.5 ml of FCR was added and mixed immediately. The mixture was incubated for 30 minutes under dark and then the absorbance of the solution was recorded at 660nm against a reagent blank. A standard curve was constructed with BSA, with the concentration of 100 to 500 µg. The standard curve was used to estimate the actual protein content of the unknown sample and expressed in µg g^{-1} of seed.

Electrophoretic characterization of total soluble seed protein by SDS- PAGE

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of total soluble seed protein of accelerated and natural aged seeds was carried out by using 12.5 per cent polyacrylamide gel according to the method prescribed by Laemeli (l970) with slight modifications.

i. Sample preparation

Accelerated and natural aged seeds were used for preparation of sample. Aged seeds (0.5 g) were ground with liquid nitrogen. To this slurry of seed material, 600 µl of extraction buffer (Tris glycine-0.125 M, pH 6.8) was added and agitated thoroughly and kept at 40°C overnight for protein extraction. Then the suspension was centrifuged under refrigeration at 12,000 rpm for 15 min and the clear supernatant was collected. Take 100 µl supernatant, for this add 300 µl of chilled acetone and kept at room temperature for one hour. After incubation spin at 10,000 rpm for 15 minuts.

Because of spinning pellet was formed then through the supernatant and dry the pellet till acetone smell over. For this add 25µl of tris glycine buffer. This protein extract (20µl) was dissolved in an equal amount of working buffer and kept in boiling water at 90°C for 5 minutes, cooled and again centrifuged at 10,000 rpm for 5 minutes and the supernatant was collected and used for loading on to the gel.

ii. Electrophoresis

The upper and lower reservoirs of electrophoretic unit were filled with electrode buffer (pH 8.3- with SDS). Then 30 µl of protein extract was loaded into the wells of stacking gel by layering them under electrode buffer using micropipette. A current of 1.5 mA per well with a voltage of 100 was applied until the tracking dye crossed the stacking gel. Later the current was increased to 2 mA per well and voltage up to 150. The electrophoresis was stopped when the tracking dye reached the bottom of the resolving gel, which took six to eight hours.

iii. Silver staining of protein gel

Silver staining was done for gels to get protein profile. The reagents used for staining are given below table.

Solution A	Solution B	Solution C	Solution D
Methanol - 100ml	Sodium thiosulfate- 0.04g per 200ml	Silver nitrate - 0.4g per 200ml	Sodium carbonate-12g per 200ml
Acetic acid - 25ml		Formaldehyde- 150µl	Solution B-1ml
Formaldehyde – 100ml			
Distilled water - 200 ml			

200 ml of solution A was added to the gel after electrophoresis. Keep on shaking for about 1hr, Wash the gel with 50% methanol thrice for 10 min, Then pour solution B, Keep on shaking for about 5 min, Wash with DDW thrice for 20 seconds, Then pour solution C, Keep on shaking for about 20 min, Wash with DDW thrice for 20 seconds, Then pour solution D, Keep shaking till the bands develop since sodium carbonate is used as a developer, Wash the gel with DDW to intensify the bands, After staining fix the gel in 7% acetic acid

iv. Evaluation and Documentation

The electrophoregrams of the gel were prepared by measuring the distance of each band from the point of loading. The Rm value was calculated as given below:

$$\text{Relative mobility (Rm)} = \frac{\text{Distance travelled by the protein}}{\text{Distance travelled by the tracking dye}}$$

Bands were numbered on the basis of increasing Rm values; the relative intensities and mobilization of protein bands and presence or absence of specific bands or combination of different bands were analyzed. The presence or absence of specific band or group of bands as well as band intensity was taken as the criteria to characterize the seed proteins extracted from aged and unaged seeds of maize.

Statistical analysis:

The experimental data was statistically analyzed as per the methods outlined by Sundararaj *et al.*, (1972) adopting "Fisher's Analysis of Variance Techniques". Critical difference (CD) values were computed at 5 per cent level wherever 'F' test was significant.

RESULTS

Table 1: Seed germination and seedling vigoure index-I as influenced by temperature, relative humidity and ageing in maize.

Treatments		Germination (%)			Seedling vigour index-I		
		T_1	T_2	Mean	T_1	T_2	Mean
	Unaged	100	100	**100**	4073	4073	**4073**
	A_3	92	88	**90**	3189	2553	**2871**
R_1	A_6	74	68	**71**	2067	1515	**1791**
	A_9	50	34	**42**	832	330	**581**
	A_{12}	02	02	**02**	11	07	**09**
	A_3	94	90	**92**	3401	2948	**3174**
R_2	A_6	78	71	**75**	2207	1766	**1987**
	A_9	55	52	**54**	1021	947	**984**
	A_{12}	03	02	**03**	21	09	**15**
	A_3	96	91	**94**	3700	3063	**3382**
R_3	A_6	81	78	**80**	2342	2251	**2297**
	A_9	60	51	**56**	1201	882	**1041**
	A_{12}	34	18	**26**	508	122	**315**
	A_3	97	94	**96**	3916	3511	**3714**
R_4	A_6	89	89	**89**	2893	2764	**2828**
	A_9	73	69	**71**	1856	1640	**1748**
	A_{12}	38	31	**34**	584	302	**443**
		T×R					
	R_1	64	58	**61**	2034	1696	**1865**
	R_2	66	63	**65**	2145	1949	**2047**
	R_3	74	68	**71**	2365	2078	**2222**
	R_4	79	77	**78**	2664	2458	**2561**
		T×A					
	A_3	95	91	**93**	3552	3019	**3285**
	A_6	81	77	**79**	2377	2074	**2226**
	A_9	60	52	**56**	1227	950	**1089**
	A_{12}	19	13	**16**	281	110	**195**
	Mean	**66**	**61**		**1998**	**1695**	
		S.Em±	**CD (P=0.01)**	**CV (%)**	**S.Em±**	**CD (P=0.01)**	**CV (%)**
	T	0.150	0.561		8.54	31.86	
	R	0.213	0.794		12.07	45.06	
	A	0.238	0.888		13.50	50.37	
	TR	0.301	1.123	1.70	17.07	63.72	3.04
	TA	0.336	1.255		19.09	71.24	
	RA	0.476	1.776		27.00	100.75	
	TRA	0.673	2.511		38.18	142.48	

The results for germination and viability are presented in Table 1. Seed germination diminished noticeably during ageing. Accelerated ageing showed significant ($p<0.05$) reduction in the ability of seeds to germinate whilst the control seeds showed high germination percentages (100%). The changes in viability were less pronounced, but also significantly

reduced. This indicated that the damage occurred in embryonic tissues during ageing did cause seed death but was enough to compromise the germination process. Highest (97%) germination per cent was recorded in three days of ageing whereas least was recorded in twelve days of ageing (2%) irrespective of temperature and relative humidity. Differences in germination percentage were observed among different temperature and relative humidity levels, although those differences were barely significant in viability values (Table 1).

Table 2: Seedling vigoure index-II and total soluble proteins as influenced by temperature, relative humidity and ageing in maize.

Treatments		Seedling Vigour Index-II			Total soluble proteins (µg g^{-1} of seed)		
		T_1	T_2	Mean	T_1	T_2	Mean
	Unaged	809	809	**809**	93	93	**93**
	A_3	606	524	**565**	79	70	**74**
R_1	A_6	404	329	**366**	58	46	**52**
	A_9	196	106	**151**	30	19	**24**
	A_{12}	5	2	**3**	03	02	**03**
	A_3	631	568	**599**	80	75	**77**
R_2	A_6	448	366	**407**	61	50	**55**
	A_9	267	217	**242**	39	36	**38**
	A_{12}	8	3	**5**	04	02	**03**
	A_3	681	581	**631**	87	76	**81**
R_3	A_6	483	458	**470**	66	62	**64**
	A_9	291	210	**251**	41	32	**37**
	A_{12}	115	50	**82**	23	13	**18**
	A_3	712	655	**684**	91	82	**86**
R_4	A_6	549	542	**546**	70	70	**70**
	A_9	385	347	**366**	52	49	**51**
	A_{12}	127	89	**108**	27	18	**22**
				T×R			
	R_1	404	354	**379**	53	46	**49**
	R_2	433	393	**413**	55	51	**53**
	R_3	476	422	**449**	62	55	**58**
	R_4	516	489	**503**	67	62	**64**
				T×A			
	A_3	658	582	**620**	84	75	**80**
	A_6	471	424	**447**	64	57	**60**
	A_9	285	220	**252**	41	34	**37**
	A_{12}	63	36	**50**	14	09	**12**
	Mean	**5.67**	**5.21**		**53**	**47**	
		S.Em±	**CD (P=0.01)**	**CV (%)**	**S.Em±**	**CD (P=0.01)**	**CV (%)**
	T	0.019	0.072		0.216	0.808	
	R	0.027	0.102		0.306	1.142	
	A	0.030	0.114		0.342	1.277	
	TR	0.038	0.144	2.74	0.433	1.616	2.97
	TA	0.043	0.161		0.484	1.806	
	RA	0.061	0.227		0.685	2.555	
	TRA	0.086	0.321		0.968	3.613	

Treatment details for tables:

Temperatures (°C)			Relative humidity			Ageing periods		
T_1	:	40°C	R_1	:	100%	A_3	:	3 days
T_2	:	42°C	R_2	:	95%	A_6	:	6 days
			R_3	:	90%	A_9	:	9 days
			R_4	:	85%	A_{12}	:	12 days

The results pertaining to SVI-I & II as influenced by the different temperatures, relative humidity levels and ageing periods in maize are presented in Table 1 & 2. Seedling vigour index is the one of most important character of quality seed. It's the good measure of field performance of the seed. The value of free fatty acid seedling vigour index decreased with the process of accelerated ageing. Unaged seeds (control) had shown 4073 and 809 of SVI-I & II, however, as accelerated ageing had significant effect SVI-I & II. Highest (3916 & 712) SVI-I & II was recorded in three days of ageing whereas least was recorded in twelve days of ageing (3174 & 655) irrespective of temperature and relative humidity respectively.

Protein quantification by Lowry method showed that total soluble protein levels diminished after accelerated ageing at temperature and RH levels (Table 2). Unaged seeds (control) had shown 93 µgg^{-1} of total soluble protein. Highest (91µg g^{-1}) was recorded in three days of ageing whereas least was recorded in twelve days of ageing (2µg g^{-1}) irrespective of temperature and relative humidity respectively.

When protein fractions from accelerated aged seeds were analyzed by SDS–PAGE, the whole lane was stained by silver nitrate, indicating a very wide and non-specific distribution of molecular weights proteins in terms of banding intensity (Figure 1 & 2). Moreover, very high-high intensity bands were observed in the lanes 1, 5, 9 14, 17 (Figure 1) where three day of accelerate samples were loaded. However, as ageing period increases, there was drastic decline in the intensity of the bands. This electrophoretic pattern was consistent with number of bands as well as band intensity. And also here we can clearly observe the effect of effect of temperature and relative humidity on protein deterioration.

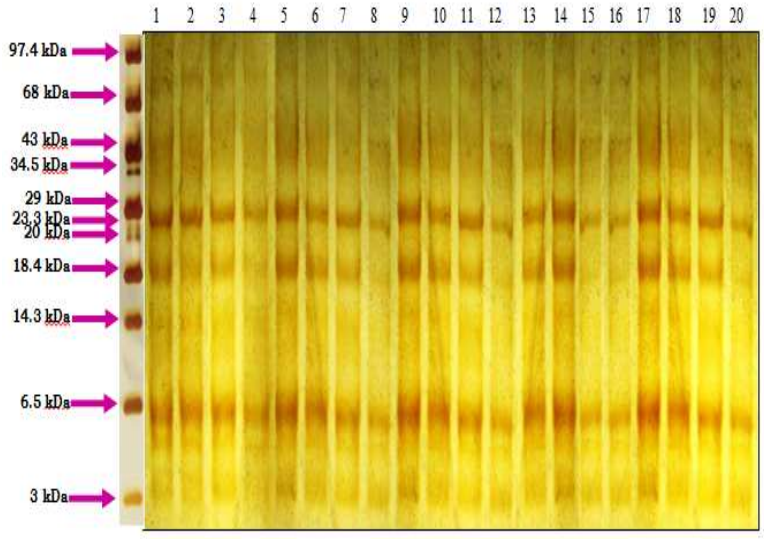

Figure 1: Influence of accelerated seed ageing on total soluble seed protein (SDS-PAGE) profile in maize

Treatment details	Temperature levels (°C)	Relative humidity levels (%)	Accelerated Ageing period
1: $T_1R_1A_3$ 6: $T_1R_2A_6$ 11: $T_1R_3A_9$ 16: $T_1R_4A_{12}$	T_1:40	R_1:100	A_0:Unaged
2: $T_1R_1A_6$ 7: $T_1R_2A_9$ 12: $T_1R_3A_{12}$ 17: $T_2R_1A_3$	T_2:42	R_2:95	A_3:3 day
3: $T_1R_1A_9$ 8: $T_1R_2A_{12}$ 13: $T_1R_4A_3$ 18: $T_2R_1A_6$		R_3:90	A_6:6 day
4: $T_1R_1A_{12}$ 9: $T_1R_3A_3$ 14: $T_1R_4A_6$ 19: $T_2R_1A_9$		R_4:85	A_9:9 day
5: $T_1R_2A_3$ 10: $T_1R_3A_6$ 15: $T_1R_4A_9$ 20: $T_2R_1A_{12}$			A_{12}:12 day

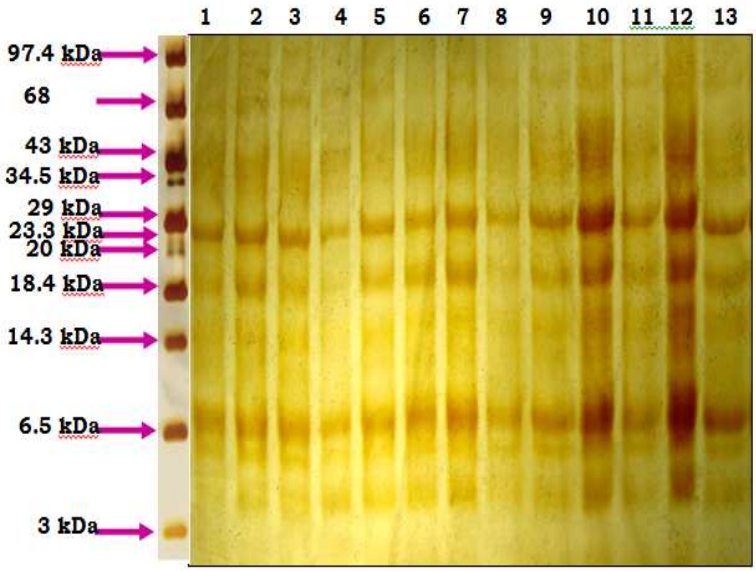

Figure 2: Influence of accelerated seed ageing on total soluble seed protein (SDS-PAGE) profile in maize

Treatment details	Temperature levels (°C)	Relative humidity levels (%)	Accelerated Ageing period
1: $T_2R_1A_3$ 6: $T_2R_2A_6$ 11: $T_2R_3A_9$	T_1:40	R_1:100	A_0:Unaged
2: $T_2R_1A_6$ 7: $T_2R_2A_9$ 12: $T_2R_3A_{12}$	T_2:42	R_2:95	A_3:3 day
3: $T_2R_1A_9$ 8: $T_2R_2A_{12}$ 13: $T_2R_4A_3$		R_3:90	A_6:6 day
4: $T_2R_1A_{12}$ 9: $T_2R_3A_3$		R_4:85	A_9:9 day
5: $T_2R_2A_3$ 10: $T_2R_3A_6$			A_{12}:12 day

DISCUSSION

As the ageing period advances, invariable germination and viability values, interestingly showing the high impact of storage conditions on seed stability. Irrespective of temperature and relative humidity in accelerated ageing, 6 days accelerated ageing onwards progressive reduce in germination range from 2.7% to 97% in case of accelerated ageing and 2.3 to 9.5% in case natural ageing was noticed because of parallel increase in number of abnormal seedlings and dead seeds. The findings of the study were in agreement with, Epel (1963) and Akhter *et al.,* (1992). Increase of the age of seeds, percentage of dividing cells decreased. The rate of mitosis is closely related to resultant level of ATP. Hence, it might be that cell division is energy dependent process and thereby the movement of chromosomes mainly depends upon the energy generating system. Toxic materials which are the products of high temperatures and relative humidities in the aged seeds may disturb the respiratory pathways, resulting in the low production of energy containing and other essential compounds-ATP, sugars and protein molecules.

With the decrease in germination percentage in the accelerated aged seeds, vigour index also showed a decline pattern to a tune of 99% in accelerated ageing. The findings of the study were in agreement with Stadtman (2001, 2004). It may be due to a progressive accumulation of oxidative damage of these macromolecules in aged tissues thought to contribute to the decline in biological functions characteristic of the aged phenotype. There is strong evidence that proteins are the most important targets for oxidants (Davies, 2005). Protein metabolisms are altered by carbonylation in deteriorated seeds. Protein metabolism regroups several biological functions, such as protein folding, protein translocation, thermo-tolerance, oligomeric assembly, and switching between active and inactive protein conformations. Impairment of these functions is closely linked with the loss of seed vigor.

The decrease in protein content was observed as ageing advances; the results of the study were in agreement with (Narayanamurthy *et al.,* 2002). Protein deterioration was mainly due to condensation, rearrangement, fragmentation, strecker degradation and polymerization. Condensation is the process of reaction between a reducing sugar and a primaryamino acid. Loss of water from this molecule produces an amine that is able to cyclise, resulting in the formation of an N glycoside (a sugar attached to an NR2 group), in amadori re-arrangement instead of cyclisation of the ammonium ion, an Amadori rearrangement may take place. Alkali catalyzed isomerisation reaction which is followed by fragmentation i.e., glycosylamines and Amadori products are intermediates formed during the course of the Maillard reaction.

The seeds exposed to high relative humidity and high temperature there was change in protein electrophoretic patterns at increased moisture is enough to damage seed or its structures, as this happen in naturally ageing also (Machado *et al.,* 2001). Total 13 bands were scored in accelerated ageing at temperature of 40°C but it was declined to eight in case of accelerated ageing at temperature of 42°C. Thus we can conclude that deleterious of temperature on protein machinery.

CONCLUSION

Results indicated that accelerated aging showed the greatest efficiency for identifying germination and seedling emergence potential, damage of protein machinery which was occurred during storage period.

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