Ageing-induced changes in nutritional and anti-nutritional factors in cowpea (*Vigna unguiculata* L.)

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Abstract This study examined the role of seed ageing in the control of anti-nutritional factors in cowpea (*Vigna unguiculata* L.). In differently aged seeds of three cultivars of *V. unguiculata* (V240, V78 and V585), germination ability and vigour were studied. Effort was also made to assay trypsin inhibitor, phenol and phytic acid, perform protein profiling in these seeds. High vigour lots (V240 and V585) registered maximum increases in germination of aged seeds. The contents of nutritional factors such as total protein and carbohydrate declined with decrease in seed vigour lots. Anti-nutritional factors such as phytic acid, phenolic content and trypsin inhibitor activity decreased and varied in low and high vigour seed lots. Polypeptide banding pattern significantly varied in the high, medium and low vigour seeds. Notably, proteins with the highest relative mobility of 0.98 and lowest molecular weight of 11.5 kDa and lowest relative mobility of 0.17 and highest molecular weight of 102.0 kDa were observed in all the vigour lots. Results implied the decline in vigour of *V. unguiculata* seeds under conditions of controlled ageing can be related to the decline in content of

major nutritional factors (total carbohydrates and proteins) required for the growing embryo during seed germination. Additionally, decreases in the contents of anti-nutritional factors phytic acid and phenols, and the activity of trypsin inhibitor in particular are connected with the decrease in seed vigour irrespective of *V. unguiculata* cultivars. The use of short duration controlled ageing technique can, at least partially, reduce the negative effects of anti-nutritional factors, and eventually improve the nutritional quality of *V. unguiculata* seeds.

Keywords Seed ageing · Anti-nutritional factors · Cowpea · Trypsin inhibitor

Introduction

As a major source of globally preferred and affordable plant-based proteins and minerals, legumes (grain legumes/pulses) are contributing to healthy and balanced human diet for many centuries (Bohra et al. 2014; Anjum 2016; FAO 2016). Cowpea (*Vigna unguiculata* L.), an important food legume is grown in semi arid regions including Africa, Brazil and India, and is generally grown for its high protein (25%) and low fat (1.9%) level (Maia et al. 2000). Considering nutritional value of legume crops for human health, the significance of pulses as nutritious seeds for a sustainable future has been emphasized (FAO 2016). Notably, the presence of several anti-nutritional factors (such as trypsin inhibitors, phenols, tannins) and mineral chelating agents (such as phytic acid and oxalic acid) in *V. unguiculata* are significantly limiting its nutritional value and the consumption. In fact, the occurrence of these anti-nutritional factors hinders the nutrient's digestibility and absorption in human/animal (Maia et al. 2000;

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Ramakrishna et al. 2008; Ghumman et al. 2016; Parmar et al. 2017). Successful removal or minimization of the highlighted anti-nutritional factors in the food legumes (including *V. unguiculata*) can help in realizing their full potential (Gonçalves et al. 2016). The commonly used strategy of cooking, boiling, dehulling, sprouting and germination may not be significant in the minimization of the effect of anti-nutritional factors in *V. unguiculata* (Cestelle et al. 2012; Muzquiz et al. 2012).

Given above, this study aimed to explore the role of ageing in the control of anti-nutritional factors in *V. unguiculata*. In *V. unguiculata* seeds varying in age and vigour, efforts were made to examine the changes occurring in the major nutritional (proteins and carbohydrates) and anti-nutritional (trypsin inhibitor, phenol and phytic acid) factors.

Materials and methods

Materials

All chemicals used in the present study were of analytical or molecular grade, and purchased from Sigma-Aldrich (USA) unless specified otherwise. *V. unguiculata* seeds of V240, V578 and V585 cultivars, used in the present study were procured from the Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi (India).

Seed ageing-related experiments

The seed moisture content was determined using the high constant temperature oven method (ISTA 2007). In order to prepare samples for artificially induced seed-ageing assays, seeds were conditioned to 20% moisture content, sealed in tri-layered laminated aluminum foil packets and placed in an oven set at $45\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for periods ranging from 24 to 168 h (7 days). Aged seeds were dried on laboratory bench for 48 h to 72 h to original moisture content (8 to 9%). All seed lots were tested for their germination and seedling vigour as detailed below. Based on the performance of the seeds, three vigour categories were identified: high vigour lot (24 h aged seeds), medium vigour lot (72 h aged seeds) and low vigour lot (120 h aged seeds). Seeds from all three categories were used for the analysis of nutritional and anti-nutritional factors. Untreated seeds served as control.

Estimations and assays

Germination and seedling vigour

Germination tests were conducted using 50 seeds in three replications. Germination was assessed employing the

‘Between paper method’. Herein, seeds were equidistantly placed over wet paper towels and wrapped in wax paper followed by incubation in a seed germinator maintaining adequate humidity (over 90%) and a temperature of $25\text{ }^{\circ}\text{C}$. Seedling evaluation and germination counts were performed on the 7th day (ISTA 1985). *V. unguiculata* seedling vigour was estimated following the method of Perry (1978). Seeds of *V. unguiculata* were placed on wet paper towel as in the case of germination test, but in a single line. Three replications of 25 seeds were plated for seedling vigour evaluation. Observations were taken on the seventh day. The lengths of roots and shoots of all the germinated seedlings were measured in centimeters and root or shoot vigour of a replicate was calculated as the sum of the root or shoot length of all germinated seedlings divided by 25, the total number of seeds plated. As mentioned also above, three vigour categories namely high vigour lot (24 h aged seeds), medium vigour lot (72 h aged seeds) and low vigour lot (120 h aged seeds) were identified based on the performance of the seeds (Table 1).

Carbohydrate content

Total carbohydrate was estimated by phenol sulphuric acid method (Dubois et al. 1956). *V. unguiculata* seed powder (100 mg) was extracted by boiling with 5.0 ml of 80% ethanol for 15 min. Supernatant was decanted into a fresh tube and the residue was re-extracted with 5.0 ml of ethanol. The two supernatants were combined and made up to 10 ml. From this total volume, 0.1 ml of extract was taken in another to which 0.9 ml of water and 1.0 ml of phenol were added. Further 5.0 ml of 96% sulphuric acid was added and mixed well. After 10 min, the content was incubated in water bath shaker at $25\text{--}30\text{ }^{\circ}\text{C}$ for 20 min. Absorbance was read at 490 nm in a spectrophotometer (Beckman DU 7400). Phenol reagent was used as the blank. The amount of total carbohydrate in the extract was calculated using a standard graph of glucose.

Anti-nutritional factors

The list of anti-nutritional factors in this study included phytic acid, total phenols, and trypsin inhibitory activity. Ground *V. unguiculata* seed samples (0.5 g) were stirred using a magnetic stirrer in 10 ml of 3.5% HCl for 1 h. Contents were centrifuged at $3000\times g$ for 10 min. Phytic acid content was assayed in the supernatant following the method of Vaintraub and Lapteva (1988). A suitable aliquot of the supernatant was diluted with 3.5% HCl to make up to the 3.0 ml. To which, 1.0 ml of Wade reagent (0.03% solution of $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ with 0.3% sulphosalicylic acid) was added and centrifuged. Absorbance was read at 500 nm where pure phytic acid (Sigma-

Table 1 Data representing seed vigour categories of *Vigna unguiculata* cultivars

Vigour test traits	Cultivars								
	V1 (V240)			V2 (V578)			V3 (V585)		
	Vigour categories								
	High	Medium	Low	High	Medium	Low	High	Medium	Low
Root length	12.88 ^a	9.75 ^b	4.38 ^d	8.38 ^a	4.82 ^b	1.73 ^c	14.77 ^a	9.89 ^c	3.20 ^c
Shoot length	15.62 ^a	11.38 ^{bc}	6.93 ^d	9.98 ^a	5.17 ^b	2.03 ^c	18.53 ^a	12.07 ^c	4.75 ^c
Seedling vigour	28.50 ^a	21.13 ^b	11.32 ^d	18.37 ^a	9.98 ^b	3.77 ^c	33.30 ^a	21.95 ^c	7.95 ^c

Data presented are means of three replications. Values are rounded off to nearest integer up to two places of decimal. Values with different letter in same column are significantly different at $P \leq 0.05$

Aldrich, USA) was used as the standard. The amount of phytic acid was calculated from the organic phosphorus by assuming that one molecule of phytic acid contained six molecules of phosphorus. In order to estimate total phenolic content, extraction of phenols was first performed. Seed powder (100 mg) was taken in a test tube with 5.0 ml of 80% ethanol. The tube with sample was boiled for 1 h at 90 °C, cooled and subsequently the clear solution decanted into a clean tube. To the residue, 5.0 ml ethanol was added and the process of boiling and cooling was repeated. Clear solution was added to the earlier collected extract in a tube and the volume was made up to the 10 ml with 80% ethanol. The sample tube was centrifuged at 6000×g for 15 min, and the supernatant was saved and for the estimation of total phenol. Total phenolic content was estimated spectrophotometrically following the Prussian blue method (Butler 1982). To 3.0 ml of the obtained above supernatant 100 µl of 0.008 M K₃Fe (CN)₆, 100 µl of 0.05 FeCl₃ in 0.1 M HCl were added. Absorbance was read after 25 min at 722 nm. The results are expressed as µg of catechol per gram of the sample and calculated from a standard graph prepared from a range of concentration of pure catechol.

For the extraction of trypsin inhibitor, first the grounding of *V. unguiculata* seeds (100 mg) was using a coffee mill to a particle size of 20-mesh sieve. The seed flour was defatted by mixing with 5.0 ml of hexane (w/v) for 10 min (Hines et al. 1992). The mixture was filtered through filter paper (Whatman No. 1) and the sediment was rinsed thrice with hexane in order to remove the residual oil in the seed flour. The defatted flour was air-dried at ambient temperature (28–30 °C) until dry and free of hexane odour. NaCl (0.15 M) was used to extract trypsin inhibitor. The solution was added into the defatted flour with a ratio of 10: 1 (v/w) and shaken at 180 rpm at ambient temperature. The supernatant was recovered by centrifuging the slurry at 5000×g for 30 min. Estimation of trypsin inhibitor activity was done in the supernatant using Benzoyl DL Arginine *p* Nitro Anilide (BAPNA) as substrate (Benjakul et al. 2000). A solution containing: 100 µl of inhibitor solution

(previously diluted to possess 50–70% inhibition), 200 µl (20 µg ml⁻¹) of bovine pancreas trypsin and 100 µl of distilled water was pre-incubated at 37 °C for 10 min. Subsequently, to the above solution, 500 µl (0.4 mg ml⁻¹) of BAPNA (pre-warmed to 37 °C) was added and vortexed immediately to initiate the reaction. After incubating for 10 min, the reaction was terminated by adding 100 µl of 30% acetic acid (v/v). Finally, the reaction mixture was centrifuged at 8000×g for 3 min. Absorbance was read at 410 nm where p-nitroaniline released was estimated. One unit of trypsin-inhibitor activity was defined as 0.01 decrease in absorbance at 410 nm under the assay condition.

Protein extraction, purification, quantification and profiling

Weighed quantity of seeds were soaked overnight, crushed with pinch of quartz by adding 5.0 ml of cold extraction medium (phosphate buffer: pH 7.0; 0.01 M). Phosphate buffer contained mercaptoethanol (10 mM) and phenyl-methane sulphonylfluoride (2.8 mM). The homogenate was centrifuged in a refrigerated centrifuge at 4 °C at 10,000 rpm for 15 min (Chittora and Purohit 2012). The crude protein thus extracted was further purified by gel filtration using Sephadex (G25). Sephadex (1.0 gm) was mixed with 15 ml distilled water and allowed to swell overnight. The gel matrix was poured into a 15 cm column and was allowed to settle. Subsequently, the crude protein solution (1.0 ml) was poured into the column and was eluted with 4.0 ml of extraction buffer (0.05 M phosphate buffer; pH 7.0; 0.05 M). First, 1.0 ml eluted sample was discarded and the rest was used as a source of protein. The quantification of proteins was done as per the Bradford method (1976) using Pro Pure Proteomic grade Protein assay Bradford kit (Code: M173-Kit, Amresco). Purified protein extract (20 µl) was taken and 80 µl 0.15 M NaCl added. One ml of Bradford reagent was added and vortexed. The mixture was allowed to stand at room temperature for 2 min and the absorption read at 595 nm in a UV–visible spectrophotometer (DU Beckman 6400) against the

reagent blank. The amount of protein was calculated by standard graph prepared by using 0.5 mg ml Bovine Serum Albumin as standard. The protein content was expressed as mg protein g⁻¹ fresh weight of the seeds. Finally, the qualitative changes in protein pattern were studied employing protein profiling that was done through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using separating (10%) and stacking (4%) gels (Laemmli 1970).

Statistical analysis

All the data obtained in triplicate were statistically analyzed by two-way ANOVA by Proc GLM for comparison of means using LSD (SAS).

Results and discussion

Germination of aged seeds

The initial percent germination in three cultivars ranged between 89.33 and 94.67, where the high vigour lots (V240 and V585) registered maximum increases. However, in medium vigour and low vigour lots, significant decreases were recorded in all the three cultivars namely V240, V585 and V578 (Table 2). An increased germination in V240 and V585 over the control could have been due to an effect similar to priming during the first 24 h when the metabolic events had commenced within a few hours of exposure to high temperature and proceeded till the seeds were removed next day and the deterioration had not set it yet. Meanwhile, no increase in the germination percentage in V578 is an indication of the late onset of metabolic events and earlier deterioration after priming during the similar period (24 h). Therefore, V240 and V585 seeds can be expected to show an enhanced seed quality in terms of germination and vigour due to repair processes that might have occurred during the initial stages of ageing. Though progressive loss in the seed quality-attributes occurred with

ageing in all cultivars the inter-cultivar differences were highly significant. This kind of variability in response to natural and accelerated ageing can be attributed to genetic constitution and the inherent capacity of a cultivar to withstand stresses. An increase in the percent germination after 24 h of ageing implies that vigorous metabolic repair leading to invigoration of seeds taken place due to high moisture content of the seeds as well as the higher temperature to which the seeds were exposed during ageing (Table 2). Similar observations were reported earlier in several other crops including pea (Jatoi et al. 2001), wheat (Sterlec et al. 2010), pearl millet (Sundareswaran et al. 2009) and rice (Ali et al. 2003).

Nutritional factors

Total protein content declined with decrease in vigour except in the cultivar V240, where a significant increase was recorded in high vigour lot as (vs. control). The protein content was reduced further in medium vigour and low vigour lots, where the maximum reduction was exhibited by V585 (Table 2). A reduction in the total content of storage components such as proteins was also reported earlier in cereals (Agnieszka et al. 2010) and watermelon (Rashed et al. 2010). A decrease in the rate of in vivo protein synthesis was observed in the accelerated aging conditions, in pea and soybean seeds (Gidrol et al. 1998). Reduction in protein content accompanied a rise in the level of amino acids that is indicative of the protein degradation during the process of seed ageing (Mukhopadhyay and Ghosh 1986; Kalpana and Madhava Rao 1997). The loss in seed viability and associated decrease in the protein content may also be due to ageing-induced disorganization of proteins and lipid-phase transition influencing the membrane structure and integrity (Mc Donald 1999).

The decreases in the content of total carbohydrate corroborated with the decreases in the vigour of seed lots. Among the control seed lots, V240 (50.10) and V578 (30.22) exhibited respectively maximum and minimum

Table 2 Carbohydrate and protein in seed vigour categories of *Vigna unguiculata* cultivars

Vigour test traits	Varieties											
	V1 (V240)				V2 (V578)				V3 (V585)			
	Vigour categories											
	C	High	Medium	Low	C	High	Medium	Low	C	High	Medium	Low
Carbohydrate	50.10 ^a	38.64 ^b	32.95 ^c	24.58 ^d	30.22 ^a	25.62 ^b	22.11 ^c	20.86 ^c	36.27 ^a	33.50 ^b	26.26 ^c	25.15 ^d
Protein	34.04 ^a	34.73 ^a	31.28 ^b	26.57 ^c	20.76 ^a	15.52 ^b	11.58 ^b	9.47 ^d	30.61 ^a	29.95 ^a	17.75 ^b	16.55 ^d

Data presented are means of three replications. Values are rounded off to nearest integer up to two places of decimal. Values with different letter in same column are significantly different at $P \leq 0.05$

contents of total carbohydrate (Table 2). Although there was germination and vigour enhancements in the high vigour seed lots of all the cultivars, a conspicuous decrease in the carbohydrate content was noted even in the high vigour lot. The findings of the present study coincide with the earlier observation of Basavarajappa et al. (1991) where also recorded a decrease in the content of carbohydrates during an accelerated ageing. Additionally, reductions in the contents of starch and soluble sugar were also reported earlier in pigeon pea seeds during accelerated ageing (Madhava Rao and Kalpana 1994). These authors attributed the observed reductions to the action of hydrolytic enzymes and respiration. In contrast, increases in the levels of total carbohydrates in seeds with increasing age were also evidenced (Verma et al. 2003). Nevertheless, no simple relationship exists between seed vigour and seed carbohydrate contents. However, the pattern of these changes strongly depended on plant species/cultivar and ageing conditions. Thus, no simple universally valid hypothesis is available to explain the changes in carbohydrates related to seed-quality loss.

Anti-nutritional factors

The control seed lots registered a higher phytic acid content than any other vigour category. In the high vigour seed lot, cultivars V240 and V585 registered a non-significant decline in phytic acid content by 2.80 and 2.22, respectively. In contrast, the decrease was significant in V578. Additionally, a significant decline in the phytic acid content in the medium and low vigour seed categories was observed in all the three cultivars tested herein. These findings coincide with the results of previous works of Chang et al. (1977) and Chitra (1994). In warm, moist environment, there would be increased metabolic activity, phytase activation, and membrane degradation. Hydrolysis by phytase can convert phytate into inorganic phosphorus. Earlier, in white bean, enzymatic hydrolysis was reported to cause increase in the conversion of phytate to inorganic phosphorus during storage at high temperatures and high humidity (Chang et al. 1977). As observed in the current study, the reduction in phytic acid during storage at high temperatures could be the result of similar hydrolysis of phytate by phytase.

Differences in phenolic content to various extents were observed in all the cultivars from high vigour seed lots. In V240 and V578, a significant decrease in phenolic content was observed (versus control seed lots). However, a significant increase was recorded in phenolic content of V585 seeds. Additionally, there was an increase in total phenols in the medium and low vigour seed lots of V240 and V578. The observed increase in the content of total phenolics may be due to cellular deterioration during ageing leading to its

greater permeability for the cellular and subcellular content (Hamman et al. 2001). The cellular-membrane damage can also lead to the synthesis of phenolic compounds as a part of the mechanism of curing that have significant association with germination (Ramamurthy et al. 1992). Seed has the capacity to maintain its germination unchanged for some time under conditions of high humidity and temperatures. However, after a certain period of time, a sigmoid loss of germination occurred, accompanied by cell membrane damage. As a result, the seed synthesizes increased amounts of phenolic compounds. Our observation on the phenol content of the *V. unguiculata* cultivars corroborates with a hypothesis, where decreases in the observed seed germination can be due to two opposite effects. First, as a part of the curing mechanism the synthesis of phenolics could have increased; and secondly, the number of seeds having phenolics-synthesizing capacity decreased (Simic et al. 2004).

Regarding the response of another anti-nutritional factor i.e. trypsin inhibitor activity, its activity was observed higher in the control seed lots of all the cultivars. In the high vigour lots of all the cultivars, significant decrease in trypsin inhibitor activity was observed. The medium as well as low vigour seed lots exhibited significantly lower activity as compared to the high vigour lots (Table 3). Report is scanty on the changes in trypsin inhibitor activity due to changes in seed quality. In the present study, various vigour categories of *V. unguiculata* were created by increasing the moisture content of the seeds and subjecting them to controlled ageing (Table 1). It is possible that during this process of ageing, metabolic changes were triggered. However, germination itself was prevented due to high temperature. This could have probably affected the trypsin inhibitor activity and resulted into its significant decrease. Trypsin inhibitor activity of *V. unguiculata* has been reported to decrease with increase in germination period (Malomo et al. 2011). Moreover, the trypsin inhibitor is a heat-labile protein, and the decrease in the activity of trypsin inhibitor is also expected due to exposure of the seeds to various periods to high temperature and high moisture content. This observation was corroborated well with the exhibition of very low trypsin inhibitor activity when compared to seeds exposed to high temperature for longer duration (low vigour seeds), and also to the control seeds (Table 3).

Protein profile

Seed protein extracts of the high, medium and low vigour seeds subjected to SDS-PAGE analysis revealed significant variations in polypeptide banding pattern. V240, V578 and V585 exhibited 23, 21 and 25 total number of protein bands

Table 3 Effect of seed ageing on anti-nutritional factors (phytic acid, phenol and trypsin inhibitor) in the seeds of *Vigna unguiculata* cultivars

V × T	Germination (%)	Phytic acid (mg g ⁻¹)	Phenol (μg of catechol g ⁻¹)	Trypsin inhibitor (mg g ⁻¹)
V _{1C}	89.33 ^{cd} ± 1.33	3.02 ^a ± 0.10	27.77 ^a ± 0.24	25.19 ^a ± 0.43
V _{1HV}	92 ^{bc} ± 0.00	2.79 ^a ± 0.23	24.59 ^c ± 0.24	23.52 ^b ± 0.10
V _{1MV}	81.33 ^c ± 1.33	2.04 ^{bc} ± 0.01	27.30 ^b ± 0.19	21.31 ^c ± 0.83
V _{1LV}	38.67 ^g ± 0.67	1.39 ^d ± 0.08	28.40 ^a ± 0.40	10.67 ^h ± 0.50
V _{2C}	94.67 ^{ab} ± 2.67	2.99 ^a ± 0.18	14.96 ^e ± 0.15	17.49 ^e ± 0.13
V _{2HV}	88.67 ^{cd} ± 1.76	2.05 ^{bc} ± 0.03	12.72 ^g ± 0.29	15.36 ^f ± 0.04
V _{2MV}	56.67 ^f ± 1.76	1.7 ^{cd} ± 0.13	13.93 ^f ± 0.13	15.00 ^f ± 0.22
V _{2LV}	30.67 ^h ± 1.33	1.69 ^{cd} ± 0.19	16.72 ^d ± 0.12	11.45 ^h ± 0.06
V _{3C}	93.33 ^{bc} ± 1.33	2.36 ^b ± 0.08	13.40 ^f ± 0.16	19.31 ^d ± 0.12
V _{3HV}	98.67 ^a ± 1.33	2.22 ^b ± 0.04	15.35 ^e ± 0.07	18.21 ^e ± 0.07
V _{3MV}	85.33 ^{de} ± 2.67	1.73 ^{cd} ± 0.10	17.26 ^d ± 0.10	17.50 ^e ± 0.15
V _{3LV}	37.33 ^g ± 1.33	1.71 ^{cd} ± 0.13	12.69 ^g ± 0.03	13.23 ^g ± 0.25
LSD (5%)	4.88	0.38	0.61	0.99

Data presented are means of three replications. Values are rounded off to nearest integer up to two places of decimal. Values with different letter in same column are significantly different at $P \leq 0.05$ (V₁, V₂ and V₃ indicate V240, V578 and V585 varieties, respectively; C, HV, MV and LV indicate control, high vigour, medium vigour and low vigour seed lots, respectively)

respectively. Total numbers of protein bands observed were less in the low vigour seed lots than in control seeds of cultivar V240, while no differences were observed in the number of bands between various vigour categories of V578 and V585. However, the differences in the intensity and thickness of some bands could be observed in all the cultivars (Tables 4, 5, and 6; Fig. 1).

In V240 and V585, decrease in seed vigour resulted into decrease in the intensity as well as the number of bands; whereas, V578 did not exhibit difference in the number of bands among the various vigour categories (Fig. 1). In V240, protein with the maximum relative mobility of 0.93 and minimum molecular weight (mol. wt.) of 16 kDa was observed in all the vigour categories. The proteins with relative mobility of 0.64 of mol. wt. of 50.0 kDa were absent in the low vigour category (Table 4). The protein-banding pattern in the control and various vigour categories of V585 indicated the maximum number of 25 bands in control seeds (Table 5). Proteins with the highest relative mobility of 0.98 and lowest mol. wt. of 11.5 kDa and lowest relative mobility of 0.17 and highest mol. wt. of 102.0 kDa were observed in all the vigour lots. In the cultivar V578, proteins with maximum relative mobility of 0.97 and lowest mol. wt. of 10.5 kDa and that with minimum relative mobility of 0.21 and the highest molecular weight of 98.5 were recorded in all the vigour lots. The proteins with relative mobility of 0.60 of mol. wt. of 54.0 kDa were absent in the low vigour category. Our results corroborate well with earlier studies on the seeds of amaranth (Kehinde et al. 2013), peanut (Vasudevan et al. 2012), rice (Das et al. 2010) and French bean (Machado

Table 4 Relative mobility of protein in seed vigour categories of *Vigna unguiculata* cultivar V240

S. no.	Control	HV	MV	LV
1	0.21 (101)	0.21 (101)	0.21 (101)	0.21 (101)
2	0.22 (99)	0.22 (99)	0.22 (99)	0.22 (99)
3	0.26 (94)	0.26 (94)	0.26 (94)	0.26 (94)
4	0.27 (92.5)	0.27 (92.5)	0.27 (92.5)	0.27 (92.5)
5	0.28 (92.0)	0.28 (92.0)	0.28 (92.0)	0.28 (92.0)
6	0.31 (90.0)	0.31 (90.0)	0.31 (90.0)	0.31 (90.0)
7	0.37 (80.5)	0.37 (80.5)	0.37 (80.5)	0.37 (80.5)
8	0.39 (78.0)	0.39 (78.0)	0.39 (78.0)	0.39 (78.0)
9	0.45 (72.0)	0.45 (72.0)	0.45 (72.0)	0.45 (72.0)
10	0.52 (64.0)	0.52 (64.0)	0.52 (64.0)	0.52 (64.0)
11	0.55 (60.0)	0.55 (60.0)	0.55 (60.0)	0.55 (60.0)
12	0.58 (57.0)	0.58 (57.0)	0.58 (57.0)	0.58 (57.0)
13	0.59 (55.0)	0.59 (55.0)	0.59 (55.0)	0.59 (55.0)
14	0.64 (50.0)	0.64 (50.0)	0.64 (50.0)	–
15	0.7 (43.0)	0.7 (43.0)	0.7 (43.0)	0.7 (43.0)
16	0.74 (38.0)	0.74 (38.0)	0.74 (38.0)	0.74 (38.0)
17	0.8 (31.0)	0.8 (31.0)	0.8 (31.0)	0.8 (31.0)
18	0.81 (29.5)	0.81 (29.5)	0.81 (29.5)	0.81 (29.5)
19	0.82 (29.0)	0.82 (29.0)	0.82 (29.0)	0.82 (29.0)
20	0.84 (27.0)	0.84 (27.0)	0.84 (27.0)	0.84 (27.0)
21	0.86 (24.0)	0.86 (24.0)	0.86 (24.0)	0.86 (24.0)
22	0.89 (22.0)	0.89 (22.0)	0.89 (22.0)	0.89 (22.0)
23	0.93 (16.0)	0.93 (16.0)	0.93 (16.0)	0.93 (16.0)

Values in parenthesis are molecular weight of proteins in kDa

HV high vigour, MV medium vigour, LV low vigour

Table 5 Relative mobility of protein in seed vigour categories of *Vigna unguiculata* cultivar V578

S. no.	Control	HV	MV	LV
1	0.21 (98.5)	0.21 (98.5)	0.21 (98.5)	0.21 (98.5)
2	0.23 (96.0)	0.23 (96.0)	0.23 (96.0)	0.23 (96.0)
3	0.24 (95.0)	0.24 (95.0)	0.24 (95.0)	0.24 (95.0)
4	0.25 (94.0)	0.25 (94.0)	0.25 (94.0)	0.25 (94.0)
5	0.26 (93.5)	0.26 (93.5)	0.26 (93.5)	0.26 (93.5)
6	0.29 (89.0)	0.29 (89.0)	0.29 (89.0)	0.29 (89.0)
7	0.36 (81.0)	0.36 (81.0)	0.36 (81.0)	0.36 (81.0)
8	0.45 (70.5)	0.45 (70.5)	0.45 (70.5)	0.45 (70.5)
9	0.52 (63.0)	0.52 (63.0)	0.52 (63.0)	0.52 (63.0)
10	0.56 (58.5)	0.56 (58.5)	0.56 (58.5)	0.56 (58.5)
11	0.57 (56.5)	0.57 (56.5)	0.57 (56.5)	0.57 (56.5)
12	0.6 (54.0)	0.6 (54.0)	0.6 (54.0)	–
13	0.63 (50.0)	0.63 (50.0)	0.63 (50.0)	0.63 (50.0)
14	0.68 (45.0)	0.68 (45.0)	0.68 (45.0)	0.68 (45.0)
15	0.72 (40.0)	0.72 (40.0)	0.72 (40.0)	0.72 (40.0)
16	0.8 (30.5)	0.8 (30.5)	0.8 (30.5)	0.8 (30.5)
17	0.81(29.5)	0.81(29.5)	0.81(29.5)	0.81(29.5)
18	0.83 (26.5)	0.83 (26.5)	0.83 (26.5)	0.83 (26.5)
19	0.86 (23.5)	0.86 (23.5)	0.86 (23.5)	0.86 (23.5)
20	0.9 (19.0)	0.9 (19.0)	0.9 (19.0)	0.9 (19.0)
21	0.97 (10.5)	0.97 (10.5)	0.97 (10.5)	0.97 (10.5)

Values in parenthesis are molecular weight of proteins in kDa

HV high vigour, MV medium vigour, LV low vigour

et al. 2001). A study based on the size exclusion chromatography revealed the contribution of proteins with varying kDa in making the total proteins in white cowpea protein isolate (WCPI; consisting of five main proteins: 872, 273, 133, 2.6 and 1.3 kDa) and red cowpea protein isolate (RCPI; consisting of five main proteins: 956, 296, 137, 2.6 and 1.3 kDa) (Shevkani et al. 2015). Meanwhile, more aggregated proteins occurred in RCPI when compared to WCPI. The authors suggested that the proteins from red *V. unguiculata* might have denatured to a greater extent than that from white *V. unguiculata*. Additionally, these isolates were also explored for application in rice muffins. Earlier, the proteins of high mol. wt. (872 and 956 kDa) were argued to represent aggregated proteins that were formed as a result of partial protein denaturation during the preparation of protein isolates (Shevkani and Singh 2015). The loss of bands observed in the medium and low vigour categories of V240 and V585 correspond to the decline in the protein content due to protein degradation during moderate or severe ageing as explained above. As observed also earlier, the consistent loss of band intensity indicates losses in protein functions and suggests

Table 6 Relative mobility of protein in seed vigour categories of *Vigna unguiculata* cultivar V585

S. no.	Control	HV	MV	LV
1	0.17 (102.0)	0.17 (102.0)	0.17 (102.0)	0.17 (102.0)
2	0.2 (99.5)	0.2 (99.5)	0.2 (99.5)	0.2 (99.5)
3	0.21 (97.5)	0.21 (97.5)	0.21 (97.5)	0.21 (97.5)
4	0.22 (97.0)	0.22 (97.0)	0.22 (97.0)	0.22 (97.0)
5	0.23 (95.5)	0.23 (95.5)	0.23 (95.5)	0.23 (95.5)
6	0.24 (95.0)	0.24 (95.0)	0.24 (95.0)	0.24 (95.0)
7	0.32 (85.5)	0.32 (85.5)	0.32 (85.5)	0.32 (85.5)
8	0.39 (78.0)	0.39 (78.0)	0.39 (78.0)	0.39 (78.0)
9	0.47 (69.0)	0.47 (69.0)	0.47 (69.0)	0.47 (69.0)
10	0.5 (65.5)	0.5 (65.5)	0.5 (65.5)	0.5 (65.5)
11	0.52 (63.5)	0.52 (63.5)	0.52 (63.5)	0.52 (63.5)
12	0.53 (62.0)	0.53 (62.0)	0.53 (62.0)	0.53 (62.0)
13	0.59 (55.0)	0.59 (55.0)	0.59 (55.0)	0.59 (55.0)
14	0.63 (51.0)	0.63 (51.0)	0.63 (51.0)	0.63 (51.0)
15	0.67 (46.0)	0.67 (46.0)	0.67 (46.0)	0.67 (46.0)
16	0.7 (43.0)	0.7 (43.0)	0.7 (43.0)	0.7 (43.0)
17	0.73 (39.9)	0.73 (39.9)	0.73 (39.9)	0.73 (39.9)
18	0.79 (33.0)	0.79 (33.0)	0.79 (33.0)	0.79 (33.0)
19	0.8 (32.0)	0.8 (32.0)	0.8 (32.0)	0.8 (32.0)
20	0.84 (27.5)	0.84 (27.5)	0.84 (27.5)	0.84 (27.5)
21	0.89 (21.5)	0.89 (21.5)	0.89 (21.5)	0.89 (21.5)
22	0.9 (20.5)	0.9 (20.5)	0.9 (20.5)	0.9 (20.5)
23	0.94 (16.0)	0.94 (16.0)	0.94 (16.0)	0.94 (16.0)
24	0.97 (12.5)	0.97 (12.5)	0.97 (12.5)	0.97 (12.5)
25	0.98 (11.5)	0.98 (11.5)	0.98 (11.5)	0.98 (11.5)

Values in parenthesis are molecular weight of proteins in kDa

HV high vigour, MV medium vigour, LV low vigour

that the seeds lost genetic integrity at this point of seed ageing (Kehinde et al. 2013). On the contrary, higher number of bands in artificially aged seeds was reported in maize (Basavarajappa et al. 1991) and cotton (Varier and Dadlani 1992). However, Krishnaswamy and Seshu (1989) did not notice any difference in the protein profile of aged and non-aged seeds of maize. Their findings indicated that different species could be distinguished on the basis of the total number of protein bands. Notably, ageing-induced deterioration was reported to increase the extent of protein oxidation, thus inducing the loss of function of all the properties of proteins and enzymes (Loic and Dominique 2008). Additionally, electrophoretic profiles were more efficient in artificially aged seeds compared to naturally aged bean seeds where the physiological quality tests were more sensitive (Freitas et al. 2006). Together, these results suggest that variation in protein degradation patterns

Fig. 1 Protein profiles of cowpea (*Vigna unguiculata* L.) representing different vigour categories (Abbreviations used: V1: V240, V2: V578, V3: V585 cultivars; C: control; PM: protein marker; HV: high vigour; MV: medium vigour; LV: low vigour)

occurs during the artificial and natural ageing, and are driven by different mechanisms (Machado et al. 2001).

Conclusion

A decline in vigour of *V. unguiculata* seeds under conditions of controlled ageing can be related to the decline of content of major nutritional factors (total carbohydrate and proteins) required for the growing embryo during seed germination. *V. unguiculata* cultivars can differ in the rate of loss of viability and vigour for the same ageing period. Decreases in the contents of anti-nutritional factors phytic acid and trypsin inhibitor in particular are connected with the decrease in seed vigour irrespective of *V. unguiculata* cultivars namely V240, V578 and V585. Phenol content can be inconsistent in both medium and low vigour lots of V240, V578 and V585 cultivars. Additionally, the negative effects of anti-nutritional factors can, at least partially, be overcome by using short duration accelerated ageing or controlled ageing technique, that in turn, eventually would improve the nutritional quality of *V. unguiculata* seeds.

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