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Changes in L-isoaspartyl Methyltransferase, Storage Components and Anti-oxidant Enzymes Activities During Accelerated Ageing in Cucumber (*Cucumis sativus* L.) Seeds*

¹Vasudevan Ayyappan, ²Ganapathi Andy, ³Selvaraj Natesan,

¹Chang Won Choi and ²Manickavasagam Markandan

¹Department of Biology and Medicinal Science, Pai Chai University,
Daejeon 302 735, Republic of Korea

²Department of Biotechnology, Bharathidasan University,
Tiruchirappalli 620 024, Tamilnadu, India

³Department of Botany, Periyar E.V.R. College (Autonomous),
Tiruchirappalli 620 023, Tamilnadu, India

Abstract: Cucumber seeds were assayed to determine whether the onset of tolerance to drying condition was related to changes in some biochemical and physiological properties associated with accelerated ageing. In the present study, accelerated ageing of seeds at 42±1°C and 100% relative humidity reduced the moisture content from 36.2 to 6.4%, with initial mean dry weight of 146.4 mg g⁻¹ decreased to 30.6 mg g⁻¹. The initial seed germination of 84.6% reduced to 50.6% and the percentage of electrolytes leakage significantly increased by ten times of initial activity accompanied by a correlative decrease in L-isoaspartyl methyltransferase (L-IAMT) activity. A reduction in the total content of storage components such as proteins, sugars, starch and lipids, except an increase in amino acids, was recorded during the accelerated ageing. The decrease in the activities of anti-oxidant related enzyme, peroxidase (POX), was also noticed. Exceptionally, catalase (CAT) showed different pattern by early increase in its activity, a peak activity at 4th day and later decrease under the same condition. The present study suggested that seed deterioration and loss of viability were resulted from the decrease in L-IAMT activity and the changes in storage components as well as anti-oxidant related enzymes. Under the condition the degradation of stored reserves in *Cucumis sativus* was enhanced.

Key words: Accelerated ageing, catalase, *Cucumis sativus*, L-IAMT, peroxidase

Introduction

Cucumber (*Cucumis sativus* L.) is one of the important horticultural crops belonging to the family *Cucurbitaceae*, mainly propagated by seeds. Even though huge quantities of seeds are produced, most of them cannot utilize because of vigorous loss in viability. In seed banks, seeds are stored under optimal storage conditions (low temperature and low seed moisture content/relative humidity) to prolong the seed viability. When a large number of seed samples are preserved in seed bank for longer periods, it is recommended to monitor its vigour and viability at regular intervals (normally after every 5 years). The deterioration of the stored seed is a natural phenomenon and the seeds tend to loose viability even under ideal storage conditions (Bhatti and Sato, 1997). The rate of

Corresponding Author: Dr. Vasudevan Ayyappan, Department of Biology and Medicinal, Science,
Pai Chai University, Daejeon 302 735, Republic of Korea Fax: 82-042-5205380

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seed deterioration varies greatly from one species to another and even among varieties of the same species. The seeds with low viability are rejuvenated/multiplied. Frequent multiplication results in genetic drift due to which genetic integrity is impaired. It also involves high risk of out crossing and mechanical mixture during multiplication. So it is very important to prolong the seed longevity.

There are number of factors that affect seed longevity in storage (Mc Donald, 1999; Powell, 1998). Among these factors, temperature and seed moisture content/relative humidity are well studied. There are some other factors which can decrease seed longevity. Varietal differences in seed viability may also be one of the limiting factors. Therefore, it is important to assess seed vigour and viability during storage. Seed aging is an important parameter to assess/estimate the seed viability and vigour. Accelerated aging is a good vigour test for various crop seeds (Anonymous, 1983) could be used to predict storability of seed lots (Tyagi, 1992). Seeds subjected to accelerated ageing lost vigour sooner than viability (Gorecki *et al.*, 1992).

Drying tolerance allows seeds to shift a developmental process to a germinative mode (Kermode, 1995), to reduce the concentration of biochemical components and to synthesize certain metabolic products in responsive to dehydration. L-isoaspartyl methyltransferase (L-IAMT) (EC 2.1.1.77) is an enzyme hypothesized to play a role in limiting and repairing age-induced damage to proteins. This enzyme catalyzes the methyl esterification of L-isoaspartyl residues using S-adenosyl-L-methionine as the methyl group donor and this reaction can initiate its conversion back into the normal L-aspartyl forms (Lowenson and Clarke, 1991). In tomato, this enzyme was developmentally regulated and inactivated or degraded during accelerated ageing from one to four days at 45°C and 100% relative humidity (Kester *et al.*, 1997). In addition, various biochemical changes were also observed during accelerated ageing viz., proteins (Nautiyal *et al.*, 1985; Basavarajappa *et al.*, 1991; Ravikumar *et al.*, 1998; 2002), amino acids (Coolbear *et al.*, 1984; Basavarajappa *et al.*, 1991; Ravikumar *et al.*, 1998; 2002), sugar (Bernal-Lugo and Leopold, 1992; Ravikumar *et al.*, 1998; 2002) starch (Basavarajappa *et al.*, 1991; Bernal-Lugo and Leopold, 1992; Ravikumar *et al.*, 1998; 2002), lipid (Harman and Mattick, 1976; Powell and Matthews, 1981) and anti-oxidant enzymes such as catalase (Streb and Feierabend, 1996; Scandalios *et al.*, 1997) and peroxidase (Nkang, 1988; Basavarajappa *et al.*, 1991; Ravikumar *et al.*, 1998; 2002).

In this study cucumber seeds were treated under accelerated aging to analyze germination, moisture content, electrolytes leakage, L-IAMT activity, storage components and anti-oxidant enzymes activities with a view to evolve seed storage strategies. Specially, we focused on L-IAMT catalyzed reaction and analyzed its activity by Michaelis-Menten kinetics.

Materials and Methods

Plant Material and Ageing Conditions

Seeds of cucumber (*Cucumis sativus* L.) cv. Poinsett 76 were procured from Indo-American hybrid seeds Pvt. Ltd., Bangalore, India. They were subjected to accelerated ageing at 42±1°C and 100% relative humidity (RH) for 8 days in a closed water bath in a controlled incubator to a final moisture content of 20% (fresh weight basis). Seeds were kept in such a way that they never got direct contact with water during the experimental period. The seeds were subjected to biochemical analyses in the 0-8 days. One hundred and twenty seeds were used for each experiment.

Germination Tests and Seed Moisture Content Determination

Germination tests were performed by placing the seeds (125 seeds in 3 replicates) in boxes containing sand moistened with water to 9% (w/w) at an alternate temperature of 20/30°C (16/8 h) with a photoperiod of 8 h. After 8 days, seedlings were characterized according to ISTA (1993). A seed was tolerant to ageing when it germinated and gave a normal seedling, or intolerant when it did not germinate

or did not give a normal seedling. The germination percentage for control and aged seeds were determined. Seed moisture content was determined by the ISTA method (1993). Fifty seeds per sample were ground and dry matter was evaluated by oven drying at 130°C for 1 h. Results were expressed as percentage of fresh weight. Results corresponded to the means of the moisture content values obtained with three measurements±SD.

Electrolytes Leakage Measurements

Electrolytes leakage measurements were carried out using an automated seed conductivity meter (Forma Scientific Instruments, USA) with 50 seeds placed individually in 5 mL deionized water at 20°C for 24 h. The leakage was expressed as a percentage of total electrolytes in the tissue (fresh weight basis). The changes in total electrolytes leakage between control and aged seeds were evaluated.

Substrates for L-IAMT

Peptide substrates such as VYP-L-isoAsp-HA or KASA-L/isoAsp/-LAKY were synthesized and purified by HPLC in Indian Institute of Science, Bangalore, India. Ovalbumin (chicken egg, Grade VII) was purchased from Sigma (St Louis, Mo).

Preparation of Seed Extracts for L-IAMT

Cucumber seeds were ground to a fine powder in liquid nitrogen in a mortar with a pestle. The powder of the seed extract was suspended in 5 mL of chilled extraction buffer containing 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, pH 7.5, 10 mM beta-mercaptoethanol, 1 μM leupeptin, 1 mM phenylmethanesulfonyl fluoride, 10 mM sodium hydrosulfite, 10 mM sodium metabisulfite per gram of seeds and the contents stored at 4°C for 10 min. The suspension was spun at 1200×g for 3 min at 4°C. The supernatants were collected and centrifuged at 172,000×g for 1 h at 4°C. The supernatants of this spun was then filtered through one layer of Mira cloth (Sigma, St Louis, Mo) and stored at -20°C. These soluble extracts were used as the enzyme source of the L-IAMT.

Assay of L-IAMT Activity

A vapor-diffusion assay (Gilbert *et al.*, 1988) was used to determine the L-IAMT activity. The method involves the transfer of radiolabelled methyl groups by the enzyme from S-adenosyl-L-methyl-¹⁴C Met to a peptide substrate such as VYP-L-IsoAsp-HA or KASA-L/IsoAsp/-LAKY or to the protein substrate ovalbumin. The methyl esters subsequently are hydrolyzed with a base and the resulting ¹⁴C-methanol is quantified. Unless otherwise described, the reaction mixture (total of 40 μL) consists of 500 μM of VYP-L-/iso-Asp/-HA, 10 μM of [¹⁴C] AdoMet (57 mCi mmol⁻¹, Amersham Pharmacia Biotech, NJ), 0.2 M sodium citrate buffer (pH 6.0) and 12 μL of the crude enzyme preparation. As a control, the endogenous activity was measured by incubating the enzyme with buffer alone instead of the peptide. The reaction was allowed to proceed at 45°C for 1 h and stopped by quenching with 40 μL of 0.2 N NaOH/1% (w/v) SDS. The contents were vortexed and 60 μL of this mixture was then spotted onto a 1.5×8 cm pleated filter paper (no. 1650962, Bio-Rad Laboratories, Hercules, CA), which was placed in the neck of a 20 mL scintillation vial containing 5 mL of counting fluor (Safety Solve High Flashpoint Cocktail, Research Products International, Mt. Prospect, IL). The vials were capped and incubated for 2 h at room temperature. During this period ¹⁴C-methanol diffuses into the fluor and the un-reacted [¹⁴C] AdoMet stays on the filter paper. Quantification was done by removal of the paper and counting the vials in a scintillation counter. Peptide-specific activity was calculated by subtracting the endogenous activity from the activity in the presence of the peptide. Reaction for each substrate concentration was

performed in duplicate and the values represent the mean±standard error. K_m and V_{max} values were calculated by fitting the data to the Michaelis-Menten equation using the DeltaGraph program (version 4.0). Three replicates of control and aged seeds under different condition each weighing about 1 g were used to study various biochemical changes using standard methods. The activity of L-IAMT was expressed as unit tissue⁻¹ fresh weight.

Estimation of Storage Components

Total soluble protein was determined by precipitating the protein with 1 mL of 10% (w/v) trichloroacetic acid and then using Lowry *et al.* (1951) method. Bovine serum albumin (Sigma, St Louis, Mo) was used as the standard. Results were expressed as mg g⁻¹ dry weight. For estimation of sugars, amino acids and starch 1 g of the seed sample was homogenized in 10 cm³ of 80% methanol, centrifuged at 8000×g for 10 min at 5°C and the supernatant was used for the analyses of amino acids, starch and sugar. Total free amino acids were estimated by the methods of Troll and Cannan (1953). To 1 cm³ of the sample, 0.1 cm³ of 80% phenol was added, kept in boiling water for 10 min, then 0.2 cm³ of 5% ninhydrin was added in boiling water for further 10 min. The mixture was then cooled and the absorbance read at 575 nm. L-glycine was used as a standard. Total soluble sugars were estimated by the methods of Duboil *et al.* (1956). The absorbance was measured at 490 nm. Glucose was used as standard. Starch was estimated by the methods of McCready *et al.* (1950). After the methanol extraction, 6.5 cm³ of perchloric acid and 5.0 cm³ of distilled-water, were added to the pellet and centrifuged at 6400×g for 5 min. To 0.5 cm³ of the supernatant, 4.5 cm³ of distilled water and 10 cm³ of 0.2% anthrone were added. The absorbance was measured at 630 nm. Glucose was used as the standard. Results were expressed as mg g⁻¹ dry weight. Lipid contents were estimated by the method of Bragdon (1951). One gram of seed powder was soaked in chloroform for 48 h, centrifuged and the chloroform extract was evaporated to dryness. To this 10 cm³ of K₂Cr₂O₇-H₂SO₄ reagent was added and diluted with equal volume of distilled water. The absorbance was read at 580 nm. Stearic acid was used as the standard. Results were expressed as mg g⁻¹ dry weight.

Estimation of Anti-oxidant Enzymes

Seeds were ground in liquid nitrogen and then homogenized in 20 mL of potassium phosphate buffer (0.1 M, pH 7.8) containing 2 mM-dithiothreitol, 0.1 mM EDTA and 1.25 mM polyethylene glycol 4000 and mixed for 15 min. The homogenate was centrifuged at 16,000×g for 15 min and the supernatant was filtered through Miracloth and desalted on a PD 10 column (Pharmacia). Catalase (CAT) (EC 1.11.7.6) activity was determined by according to Bailly *et al.* (1996). Results were expressed as units nmol H₂O₂ mg⁻¹. (protein) min⁻¹. Peroxidase (POX) (EC 1.11.1.7) activity was estimated by the method of Malik and Singh (1980). One gram of seed was homogenized in 0.1 M phosphate buffer of pH 6.5 and centrifuged at 2000×g for 10 min. To the supernatant 0.5 cm³ of orthodianisidine solution (1 mg in 1 cm³ of methanol) and 0.2 cm³ of 0.2 M H₂O₂ were added. Increase in the absorbance was recorded every 30s up to 3 min at 430 nm. Results were expressed as units mg⁻¹ (protein) min⁻¹.

Results and Discussion

Changes in Moisture Content and in Dry Weight

The moisture content of seeds was decreased with extended ageing conditions (Fig. 1). The initial moisture content of 36.2% (fresh weight basis) was decreased to 6.4% at 8th day of ageing. However, the dry weight of seeds was sharply decreased with accelerated ageing. The initial mean dry weight of seeds was 146.4 mg g⁻¹. At the end of ageing the dry weight was decreased about 30.6 mg g⁻¹ (Fig. 1).

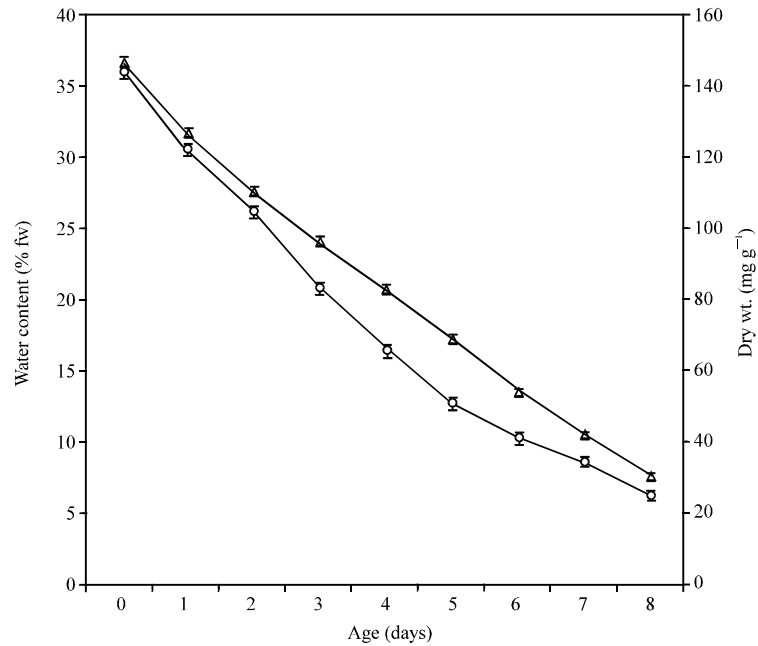


Fig. 1: Changes of moisture content (○) and dry weight (Δ) of cucumber seeds before and during accelerated ageing. Mean of three measurements±SE

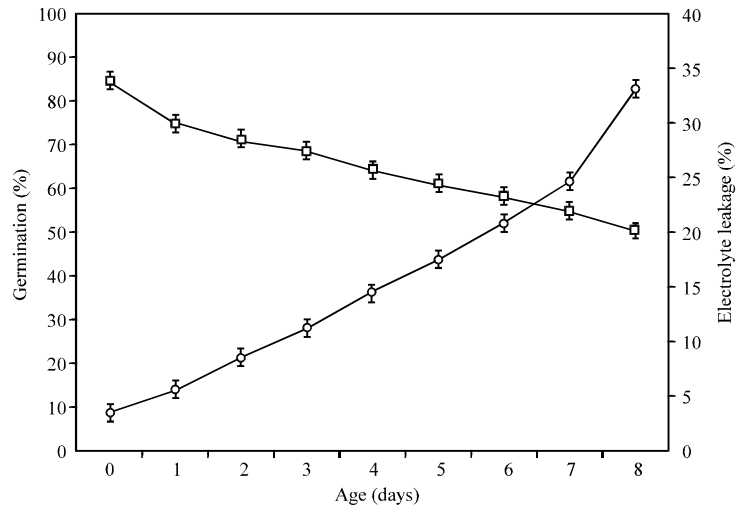


Fig. 2: Germination ability (□) and percentage of electrolyte leakage (○) of cucumber seeds before and during accelerated ageing at 42±1°C and 100% RH. Seed germination ability and vigour are expressed as percentage of normal seedlings obtained after 8 days at 25°C. Seed vigour is characterized by electrolyte leakage measured at 20°C after ageing of seeds. Mean of three measurements±SE for seed germination and means of 50 measurements±SE for seed vigour

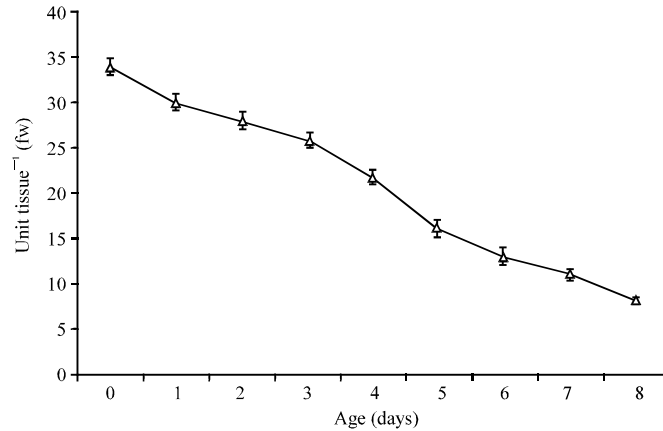


Fig. 3: Activity of L-IAMT (\blacktriangle) in cucumber seeds before and during accelerated ageing at $42\pm 1^\circ\text{C}$ and 100% RH. Activity was recorded by unit per tissue fresh weight in each cucumber seed. Mean of three measurements \pm SE

It was suggested that the drying tolerance of cucumber seeds increased with loss of water content during accelerated ageing, as previously shown in leguminous species (Blackman *et al.*, 1992; Sanhewe and Ellis, 1997; Corbineau *et al.*, 2000).

Changes in Seed Germination Ability and Vigour During Accelerated Ageing

On the other hand, extended treatment in accelerated ageing reduced seed germination ability. This result was in collaborative with Bailly *et al.* (2001) who reported that ageing of bean seeds for 11 days at 40°C markedly altered their germination. The initial germination ability was 84.6% which decreased to 50.6% at the end of ageing (8th day) (Fig. 2). To assess seed vigour, electrolytes leakage was measured. Electrolytes leakage tended to increase with seeds that treated with more time extension in accelerated ageing, corresponding to an increase in seed sensitivity to heat treatment (Fig. 2). Therefore, the accelerated ageing at controlled conditions and the electrolytes leakage have been considered as good indicators of seed vigour in various species (Mc Donald, 1999).

Expression of L-IAMT Activity in Cucumber Seeds

To determine a role of L-IAMT in accelerated ageing conditions, its enzymatic activity was measured both in dry and imbibed cucumber seeds. The activity of enzyme was correlated with the amount of protein. In the present study, we found that the L-IAMT activity was abundant in the non-dried seeds on per gram protein but decreased sharply as the seed undergoes dehydration (Fig. 3). Mudgett *et al.* (1997) suggested that seeds aged pre-maturely by heating do not make lower the enzyme level but still accumulate damage and have a reduced viability, indicating that the level of L-IAMT in seeds may not be sufficient to repair all of the damage. Like in previous findings (Mudgett and Clarke, 1993; Kester *et al.*, 1997; Mudgett *et al.*, 1997), the accelerated ageing of cucumber seeds at 42°C decreased germination as well as L-IAMT activity. In ageing seeds, a role for this enzyme has been speculated that naturally aged barley seeds have reduced L-IAMT levels and higher accumulation of unrepaired L-isoaspartyl residues coupled with lower germination of seeds (Kester *et al.*, 1997).

Enzymatic Property of L-IAMT in Aged Cucumber Seeds

Soluble extract of cucumber seeds was assayed for initial velocity towards peptide substrate (9VYP/L-iso-Asp/HA0) as well as protein substrate (ovalbumin) to determine the K_m and V_{max} values.

Table 1: Kinetic constants of L-IAMT (L-Isoaspartyl methyl transferase) activity in soluble extracts of various aged cucumber seeds to a peptide substrate (Vyp-L-isoAsp-HA) and protein substrate (ovalbumin). Values are mean of three replicates±SE

Ageing condition (d)	Vyp-L-isoAsp-HA		Ovalbumin	
	K_m (mM)	V_{max} (p mol min ⁻¹)	K_m (mM)	V_{max} (p mol min ⁻¹)
0	0.38±0.24	3.2±0.86	1.26±0.98	11.6±0.76
1	0.32±0.36	6.4±0.74	1.84±0.94	10.2±0.84
2	0.26±0.24	8.6±0.76	2.06±0.78	8.4±0.78
3	0.21±0.22	10.3±0.78	3.2±0.86	7.3±0.68
4	0.19±0.06	12.4±0.86	4.3±0.78	6.1±0.76
5	0.15±0.09	16.6±0.78	6.2±0.86	5.4±0.74
6	0.13±0.09	21.2±0.75	8.6±0.84	3.8±0.78
7	0.08±0.09	24.8±0.86	10.4±0.98	2.6±0.86
8	0.05±0.09	36.4±0.78	13.8±0.84	2.8±0.78

In the present study, the K_m values for the peptide and ovalbumin substrates varied by ageing condition. For peptide substrate the K_m values decreased with extended ageing condition (Table 1). In contrast, the K_m value for ovalbumin substrate increased with accelerated ageing. It is interesting that, the relative V_{max} value for the peptide substrate was increased more than ovalbumin substrate during accelerated ageing. Kester *et al.* (1997) reported that the K_m values for peptide and ovalbumin substrates of various plant extracts differed when these extracts were subjected to heat at 45°C and 100% RH. Present study was in collaborative with Kester *et al.* (1997) who reported that V_{max} value for the peptide substrate was increased more than ovalbumin substrate during accelerated ageing.

Estimation of Storage Components

The amount of storage components within seeds showed high variability and related with seed sensitivity during accelerated ageing. Even though the increase of specific proteins may modulate water loss in tissues during dehydration (Chandler and Robertson, 1994; Wang *et al.*, 2002), a large population of proteins is present in dry seeds, which needs to be maintained in the normal form in the absence of active protein synthesis. In the present study, total proteins were decreased to half of the initial content (Fig. 4A) at 8th day of ageing as previously reported (Nautiyal *et al.*, 1985; Basavarajappa *et al.*, 1991; Ravikumar *et al.*, 1998, 2002), while the amount of amino acids increased gradually as previously reported (Coolbear *et al.*, 1984; Basavarajappa *et al.*, 1991; Ravikumar *et al.*, 1998, 2002) (Fig. 4A). The increase in the amount of amino acids was by hydrolysis of proteins during ageing.

Sucrose found to be a good protectant against dehydration stress in drying-tolerant plant tissues (Pelah *et al.*, 1997; Sun and Leopold, 1997; Sun and Gouk, 1999). Total soluble sugars reduced from initial content, even though the rate of decrease was very low (Fig. 4B). The reduction of sugar content may be due to hydrolysis, proved by Amadori and Maillard reactions (Feeney and Whitaker, 1982). In addition, starch (Fig. 4B) and lipid (Fig. 4C) content also significantly decreased at 6-8th day of ageing. The decrease in lipid content also observed in some plants viz., cucumber (Koostra and Harrington, 1969), peanut (Harman and Mattick, 1976; Pearce and Abedel Samad, 1980; Powell and Matthews, 1981), soybean (Stewart and Bewley, 1980), sunflower (Gidrol *et al.*, 1989), tomato (Francis and Coolbear, 1984) and bamboo (Ravikumar *et al.*, 1998; Ravikumar *et al.*, 2002). The content of soluble sugars, starch and lipid decreased during accelerated ageing, suggesting that seeds are vulnerable to desiccation.

Estimation of Anti-oxidant Enzymes

Plant tissue dehydration is known to be associated with an increased production of active oxygen species which can react together and trigger numerous deleterious oxidative processes (Leprince *et al.*, 1993; Smimoff, 1993). However, anti-oxidative defense is suspected to play an

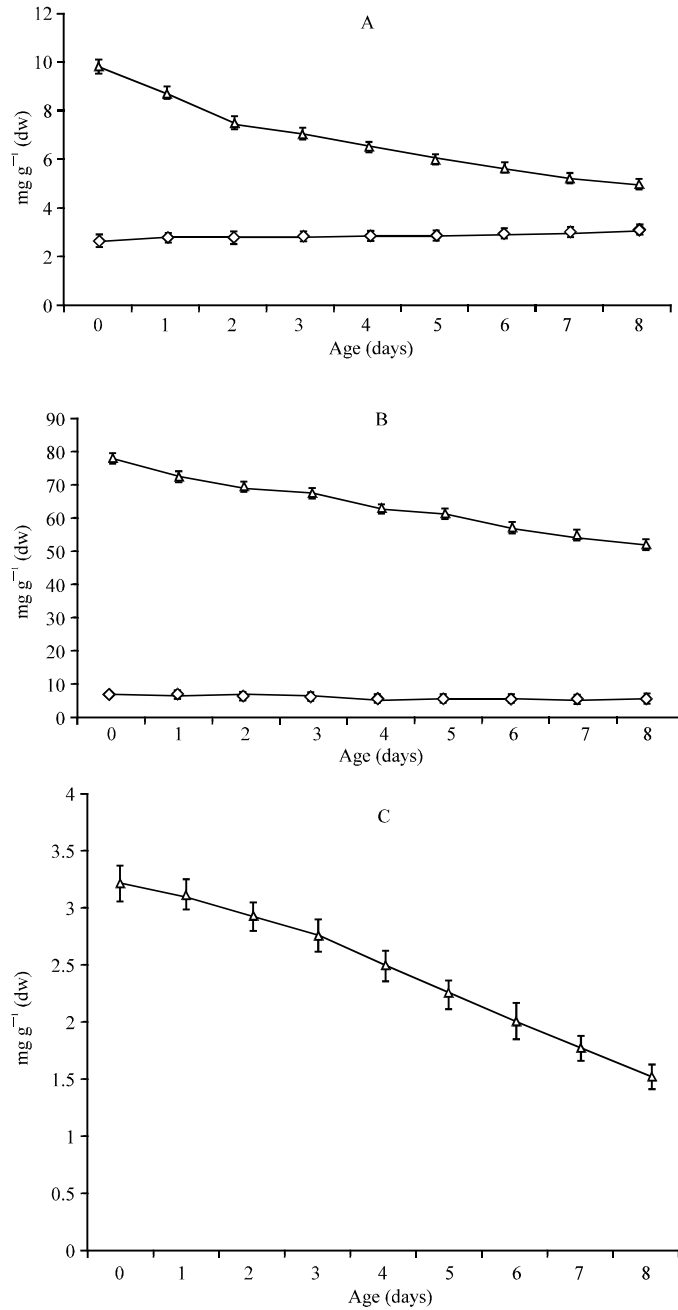


Fig. 4: Changes in protein content (\blacktriangle) and amino acids (\diamond) (A panel), starch (\blacktriangle) and sugar (\diamond) content (B panel) and in lipid content (\blacktriangle) (C panel) before and during accelerated ageing of cucumber seeds at $42\pm 1^\circ\text{C}$ and 100% RH. Mean of three measurements \pm SE

important role in acquisition of drying tolerance (Hendry *et al.*, 1998), even though it is difficult to ascertain their physiological role. Therefore, it was interesting to determine activities of anti-oxidant

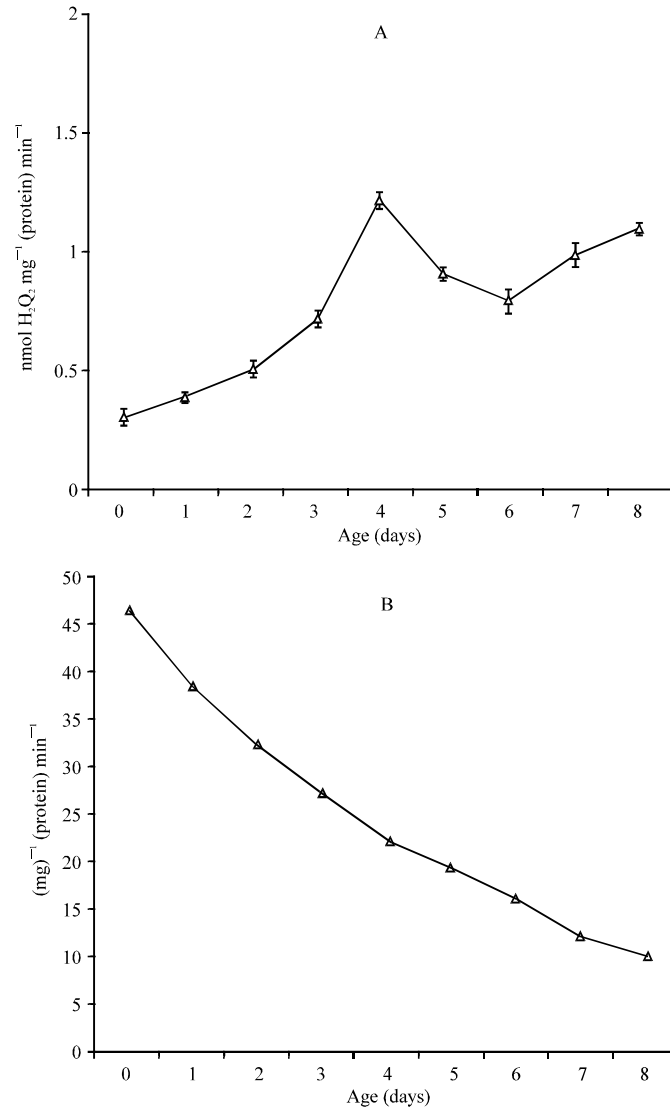


Fig. 5: Changes in catalase activity (Δ) (A panel), peroxidase activity (Δ) (B panel) before and during accelerated ageing of cucumber seeds at $42\pm 1^\circ\text{C}$ and 100% RH. Mean of three measurements \pm SE

enzymes such as CAT and POX. The CAT activity was increased up to 4th day of ageing and it declined at 5th and 6th day (Fig. 5A). In dried mature seeds, acquisition of drying tolerance clearly seems to be associated with high CAT activity (Bailly *et al.*, 2001). It suggests that CAT plays a role during seed desiccation by preventing dehydration-related oxidative damage and that H₂O₂ may play a role in the regulation of CAT gene expression and the transduction pathway of the dehydration signal. In this study the initial increase in CAT activity suggests that seeds induced anti-oxidative defense mechanism in the beginning against accelerated ageing. As CAT has a rapid turnover, conditions inhibiting CAT synthesis will lower the steady-state level of this enzyme (Streb *et al.*, 1993; Streb and Feierabend, 1996; Scandalios *et al.*, 1997). Thus, heat shock and oxidative stress will enhance

inactivation of CAT by preventing synthesis of new enzyme (Hertwig *et al.*, 1992; Feierabend and Dehne, 1996), resulting in a decline in CAT activity. Because the heat shock was applied in the dark, CAT photoinactivation (Feierabend and Engel, 1986; Polle, 1997) was not the cause of the reduction in CAT activity in the present study.

Unlike CAT activity, POX activity significantly decreased by 1/5 of initial activity during accelerated ageing (Fig. 5B). The significant decrease with increasing the storage time in POX activity during accelerated ageing which is similar to previous findings (Nkang, 1988; Basavarajappa *et al.*, 1991; Ravikumar *et al.*, 1998; Ravikumar *et al.*, 2002). Results indicated that the sub-optimal temperature of 20°C decreased germination rate, increased malondialdehyde and total peroxides accumulation and reduced anti-oxidative defense systems of bitter melon seeds, suggesting that sub-optimal temperature effects were associated with lipid peroxidation.

The present study concluded that seed deterioration and loss of viability were due to decrease in L-IAMT activity and changes in storage components, as well as anti-oxidant related enzymes involved in degradation of stored reserves in *Cucumis sativus*.

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