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STEROLS IN RELATION TO AGEING OF SEEDS OF *HELIANTHUS ANNUUS* AND *CICER ARIETINUM*

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Key Word Index—*Helianthus annuus*; Compositae; *Cicer arietinum*; Leguminosae; seed viability; accelerated ageing; sterols; steryl esters; steryl glycosides.

Abstract—Under accelerated ageing at high relative humidity and high temperature for 4 days germination and membrane permeability remained unaffected both in sunflower and chick pea seeds. However, the steryl glycoside concentration in the pooled leachate increased progressively with ageing. Total sterols, as well as steryl glycosides and free sterols of the seeds, increased with a concomitant decline in steryl esters under accelerated ageing. Pretreatment with the sterol biosynthesis inhibitor SK & F 7997A_3 prevented the increase of total sterols under accelerated ageing conditions but there were increases in the amounts of steryl glycosides and free sterols and a decrease in steryl ester after such treatment, therefore, indicating interconversions of the various sterol types. Accelerated ageing also caused increases in free amino acids and soluble carbohydrate. Low relative humidity–high temperature and high relative humidity–low temperature failed to produce such effects.

INTRODUCTION

Accelerated age induced changes in seeds have been studied in detail [1, 2], but no studies have yet been made on changes in the sterol concentration. Free sterols are effective plant membrane stabilizers [3], and steryl glycosides and steryl esters are abundant in intracellular organelles [4]. If the cell membrane is the site of ageing damage [5], sterols are likely to be liberated. On the other hand, recent work [1] has shown that accelerated ageing did not cause membrane deterioration in viable seeds but some internal biochemical changes took place which ultimately reduce seed vigour.

An attempt has been made in this study to observe the changes in sterol concentrations and their interconversions in seed undergoing deterioration through accelerated ageing. For assessing age induced damage some other biochemical changes were also considered in addition to germination and the leaching of electrolytes from the seeds.

RESULTS AND DISCUSSION

The germination ability of chick pea and sunflower seeds were found to be significantly high, up to 4 days of accelerated ageing under a high relative humidity of 100% both at 40° and 20°. Under a low relative humidity of 30% and at a temperature of 40° germination was also significantly high. On the other hand after 6 days of accelerated ageing at 100% relative humidity and 40°, germination of the seeds of both the cultivers declined significantly, whereas in the other two cases no such fall was evident (Table 1). Experiments have also shown that 4 days of accelerated ageing caused some intrinsic biochemical changes without any physical manifestation and these were evident from increases in soluble carbohydrate and amino acid concentrations in sunflower seeds (Table 2). No such changes have been noted in seeds kept under low relative humidity–high temperature and high relative humidity–low temperature. Such effects of accelerated ageing by high relative humidity–high temperature are reported in different seed types [1].

Table 1. The effect of accelerated ageing on the germination of gram (*Cicer arietinum*) and sunflower (*Helianthus annuus*) seeds.

Ageing (days)	Treatment*	Germination (%)†	
		Sunflower	Gram
0		90 (b)	90 (b)
2	100% RH–40°	98 (b)	95 (b)
	100% RH–20°	92 (b)	92 (b)
	30% RH–40°		
4	100% RH–40°	95 (b)	85 (b)
	100% RH–20°	97 (b)	91 (b)
	30% RH–40°	97 (b)	87(b)
6	100% RH–40°	65 (a)	72 (a)
	100% RH–20°	92 (b)	86 (b)
	30% RH–40°	87 (b)	98 (b)

*RH, Relative humidity.

†Significance based on Duncan's Multiple Range Tests at 5% level. Values with a common letter within columns are not significantly different.

In the present study, an increase in electrolyte leaching did not occur at least up to 4 days of accelerated ageing. However, during this period steryl glycosides in the leachate increased significantly. The results, therefore, indicated that accelerated ageing might not affect membrane permeability but metabolically the tissues might have suffered from some sort of alteration in their biochemical make-up, confirming the observations of Perl *et al.* [1]. It is known that free sterols are an effective plant

Table 2. Changes of protein, free amino acid and soluble and insoluble carbohydrate in sunflower seeds at various stages of accelerated ageing at 100% relative humidity and 40°.

Ageing (days)	Protein in percentage of initial dry wt	Free amino acid in percentage of initial dry wt	Soluble carbohydrate in percentage of initial dry wt	Insoluble carbohydrate in percentage of initial dry wt
0	18 (a)	0.01 (a)	9.0 (a)	6.9 (a)
2	19 (a)	0.04 (b)	11.7 (b)	7.6 (b)
4	15 (a)	0.14 (c)	16.8 (c)	7.6 (a)

Significance based on Duncan's Multiple Range Tests at 5% level. Values with a common letter within columns are not significantly different.

membrane stabilizer [3] and sterols are reported to prevent leakage through the membrane [6]. Plant sterols occur mainly in the membranes [4]. There are reports of age induced changes in sterol levels in plants [7, 8]. In tubers [7] and cotyledons [8] steryl esters increased the most during ageing, whilst in leaves free sterols accounted for the largest increase [9]. A possible explanation to account for an increase in free sterols during ageing is that, in mature leaves, the free sterols are not only a structural component of membranes but are also part of a non-metabolic pool [10]. In the present analyses gradual increases of total sterols, free sterols and steryl glycosides were noted progressively with ageing at high relative humidity–high temperature only (Table 4). While the increase in total sterol indicated the enhanced synthesis of sterol under the accelerated ageing environment, the increase in glycosides and free sterols might be accounted for either by synthesis *de novo* or by interconversion from steryl esters. In any case the increase in the content of sterol glycosides in seed under accelerated ageing conditions (high relative humidity–high temperature), appears to explain the origin of the increased amount of steryl glycosides in the pooled leachate (Table 3) under imbibed conditions, though signs of membrane damage were not evident from electrolyte leaching (Table 3).

SK & F 7997A_3 [tris-(2-diethylaminoethyl) phosphate] inhibits sterol biogenesis [11] and inhibition occurs during conversion of squalene 2,3-oxide to cycloartenol [12]. In the present experiment total sterols in SK & F pretreated seeds under accelerated ageing did not show a further increase in contrast to water pretreated seeds kept under the same ageing conditions (Table 5). However, increases in the endogenous levels of steryl glycosides and free sterols with ageing at high relative humidity–high temperature occurred in both the cases. The results, therefore, indicated that the increment of steryl glycosides and free sterols during ageing was mostly due to interconversion from steryl esters.

EXPERIMENTAL

Materials. Seeds of *Helianthus annuus* cv Modern and *Cicer arietinum* L. cv B-108 were obtained from Oil Seed and Pulses Research Station, Berhampore, West Bengal, India. Steroids were obtained from Sigma, U.S.A. SK & F 7997A_3 was obtained from Smith Kline & French, Philadelphia, U.S.A.

Methods. Fully viable seed lots of sunflower and chick pea exhibiting *ca* 100% germination were used for the present work.

Table 3. The effect of accelerated ageing of chick pea and sunflower seeds on dry weight loss, leaching of electrolytes, and sterols in leachate*

Ageing (days)	Treatment†	Dry weight (mg/g initial fr. wt)		Conductivity (mMhos/g seeds)		Sterol in leachate (μg/100 mg initial dry wt)	
		Sun-flower	Chick pea	Sun-flower	Chick pea	Sun-flower	Chick pea
0		942 (a)	928 (b)	0.33 (a)	0.32 (a)	24.3 (a)	13.3 (a)
2	100% RH–40°	943 (a)	920 (b)	0.32 (a)	0.31 (a)	26.8 (a)	27.0 (b)
	100% RH–20°	945 (a)	921 (b)	0.33 (a)	0.29 (a)	25.7 (a)	12.5 (a)
	30% RH–40°	952 (a)	920 (b)	0.32 (a)	0.29 (a)	24.5 (a)	17.7 (a)
4	100% RH–40°	941 (a)	975 (a)	0.32 (a)	0.32 (a)	36.5 (b)	27.7 (b)
	100% RH–20°	944 (a)	913 (b)	0.32 (a)	0.30 (a)	21.2 (a)	15.2 (a)
	30% RH–40°	948 (a)	915 (b)	0.31 (a)	0.30 (a)	23.5 (a)	16.1 (a)

*Significance based on Duncan's Multiple Range Tests at 5% level. Values with a common letter within columns are not significantly different.

†RH, Relative humidity.

Table 4. Effect of accelerated ageing of chick pea and sunflower seeds on internal sterol and its derivatives

Ageing (days)	Treatment*	Sunflower† (μg/100 mg initial dry wt) Total sterol	Steryl ester	Free sterol	Steryl glycosides	Chick pea† (μg/100 mg initial dry wt) Total sterol	Steryl ester	Free sterol	Steryl glycosides
0		365 (b)	247 (c)	91 (a)	26 (a)	176 (a)	62 (c)	52 (a)	62 (a)
2	100% RH–40°	355 (a)	194 (b)	101 (b)	60 (b)	192 (b)	55 (b)	62 (b)	75 (b)
	100% RH–20°	361 (b)	249 (c)	82 (a)	30 (a)	181 (a)	61 (c)	55 (a)	65 (a)
	30% RH–40°	359 (b)	245 (c)	87 (a)	27 (a)	172 (a)	59 (c)	50 (a)	63 (a)
4	100% RH–40°	443 (c)	177 (a)	158 (c)	108 (c)	222 (c)	30 (a)	98 (c)	94 (c)
	100% RH–20°	373 (b)	249 (c)	93 (a)	31 (a)	168 (a)	58 (c)	49 (a)	61 (a)
	30% RH–40°	368 (b)	241 (c)	96 (a)	31 (a)	183 (a)	57 (c)	61 (b)	65 (a)

*RH, Relative humidity.

†Significance based on Duncan's Multiple Range Tests at 5% level. Values with a common letter within columns are not significantly different.

Table 5. The effect of accelerated ageing on the concentrations of free sterol, steryl ester and steryl glycoside in sunflower seeds pretreated with SK & F*

Ageing (days)	Pretreatment†	Total sterol (μg/100 mg initial dry wt)	Steryl ester (μg/100 mg initial dry wt)	Free sterol (μg/100 mg initial dry wt)	Steryl glycosides (μg/100 mg initial dry wt)
0	SK&F 500 ppm	367 (a)†	237 (c)	105 (c)	35 (a)
	Water	392 (b)	235 (c)	82 (b)	75 (c)
2	SK&F 500 ppm	362 (a)	186 (b)	130 (d)	46 (a)
	Water	371 (a)	230 (c)	60 (a)	81 (c)
4	SK&F 500 ppm	357 (a)	144 (a)	150 (e)	63 (b)
	Water	416 (c)	181 (b)	130 (d)	185 (d)

*Significance based on Duncan's Multiple Range Tests at 5% level. Values with a common letter within columns are not significantly different.

†Seeds were soaked in SK & F solution for 4 hr, dried to their original weight and then kept at 100% relative humidity–40°. Control seeds were soaked in water for 4 hr, dried to their original weight and aged.

Samples of chick pea containing 7 seeds and of sunflower containing 18 seeds, weighing *ca* 1 g, were taken for estimation of sterols. Samples of chick pea containing 21 seeds and of sunflower containing 54 seeds, weighing *ca* 3 g, were taken for estimation of conductivity and of sterols in the pooled leachate. In each case five replicates were used.

Each sample of seeds was surface sterilized with 0.1% $HgCl_2$ and thoroughly washed with double distilled H_2O. This was followed by treatment with 50 μg/ml of penicillin-G and streptomycin sulphate to avoid growth of micro-organisms. Prior to accelerated ageing, seeds were aseptically dried back to their original weight. For ageing, the seed samples were stored at 100% relative humidity (RH)–40°, 100% RH–20° and 30% RH–40°. Germination ability of seeds kept under accelerated ageing at 100% RH–40°, was found to range between 85 and 98% up to 4 days. Pathological tests during this period did not show any sign of invasion by micro-organisms. However, after 4 days of accelerated ageing there was a significant decline in germination ability along with the appearence of micro-organisms as was evident from pathological tests and expts were, therefore, discontinued after 4 days. Dry wt, internal sterols, conductivity and sterols in the pooled leachate were measured at 0, 2 and 4 days.

The amounts of protein, free amino acid, soluble and insoluble carbohydrate of sunflower seeds were measured from the samples kept at 100% RH–40°.

The dry wt of seeds was determined by the oven drying method (70° for 48 hr). Conductivity was measured by the method of Halder and Gupta [13] and seed leachates were collected after 24 hr of soaking. Protein, soluble carbohydrate and free amino acids were estimated following the methods of Lowry *et al.* [14], McCready *et al.* [15] and Moore and Stein [16], respectively.

Sterol extraction. The sterol extraction procedure was the same as that adopted by Jacobson and Jacobson [17]. Three fractions were separated from the extract, i.e. petrol fraction, $CHCl_3$–MeOH fraction and aq. fraction. The petrol fraction is referred to as the steryl ester fraction and the $CHCl_3$–MeOH (2:1) fraction as the free sterol plus steryl glycosides [17, 18]. The aq. fraction mainly contained steryl glycosides in addition to triterpene glycosides, which were determined by the differences of colour intensity at 430 and 520 nm. Absorption at 430 nm is only due to sterols and absorption at 520 nm is due to sterols and some terpenes. The sterol concn was, therefore, determined from the absorption at 430 nm.

Quantification of sterols. The steryl ester fraction was treated

with 6% KOH in MeOH at 60° for 20 min to liberate free sterols. H_2O (3 ml) was then added to each tube and the sterols were extracted with 3 × 3 ml of Et_2O.

The free sterol plus steryl glycoside fraction in the $CHCl_3$–MeOH phase and the steryl glycoside in the aq. phase were hydrolysed with 2 N HCl in MeOH–H_2O (1:1) at 95° for 1 hr, and the sterols were extracted with 3 × 3 ml of $CHCl_3$–MeOH. 1 N NaOH (3 ml) was added to each extract to neutralize the acid. NaOH was removed and the organic phase was washed × 3 with H_2O. Solvents were removed from each fractions *in vacuo* at 55°. $CHCl_3$ (5 ml) and cold Ac_2O–H_2SO_4 (4:1) (2 ml) were added to each sample, according to the method adopted by Stadtman [19]. The colour intensity was measured at 430 nm.

The absorption spectrum of the reaction product of standard cholesterol showed two peaks, one at 430 nm and another at 520 nm. The absorption spectra of all other sterols showed only one peak at around 420–430 nm. The absorption spectra of triterpenes showed a peak at 520 nm and a low transmission of *ca* 0.01% at 430 nm. Thus, since absorption at 520 nm might be due to interference of terpenes, the colour intensity was measured at 430 nm.

In order to follow the effect of SK & F 7997A_3, sunflower seed samples were soaked in 500 ppm SK & F soln for 4 hr, dried to their original weight and then kept at 100% RH–40° for 4 days. Control seeds were soaked in H_2O for 4 hr, preserved under the same conditions, and the sterols were measured at 0, 2 and 4 days.

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