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Tocopherol and Organic Free Radical Levels in Soybean Seeds during Natural and Accelerated Aging[1]

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ABSTRACT

Soybean seeds which had aged in long-term storage ("natural aging") or by exposure to high temperature and humidity ("accelerated aging") were analyzed for their tocopherol and organic free radical contents. Tocopherol levels remained unchanged during both types of aging. Three principal tocopherol homologues (α, γ, δ) were present in fairly constant proportions throughout. Organic free radical levels were also remarkably stable, presumably due to the relatively immobile environment of the dry seed. These results, taken in conjunction with previous data on the stability of unsaturated fatty acids in soybean seeds, indicate that it is improbable that lipid peroxidation need play a significant role during natural or accelerated aging in this species.

Deterioration of membranes (22) and damage to nucleic acids (3) have both been identified as major factors in the declining viability of stored seed. Deleterious changes in membranes have been invoked to explain the enhanced leakage of solutes which accompanies seed imbibition in aged soybean seeds (16). One widely promoted hypothesis to explain this deterioration is that aging of seeds leads to lipid peroxidation, which is in turn responsible for membrane perturbation consequent to its alterations of unsaturated fatty acids. Kaloyereas (9) was probably the first to suggest that peroxidation of polyunsaturated fatty acids (of pine seed) could be correlated with, and might be a primary cause of, loss of viability. An essentially similar hypothesis was presented by Malysheva (12). More recently, Villiers (22) has specifically suggested that peroxidative changes in the unsaturated fatty acids of the seed membranes are intrinsic to the loss of germinability and vigor, and Harman and Mattick (8) have found that the decrease in germination rate (during several weeks of accelerated aging) was paralleled by a significant reduction of dienoic and trienoic fatty acids in the total lipid fraction from pea seeds. In a previous study (18), we were unable to find comparable trends in the fatty acids of soybeans undergoing accelerated aging. Such changes are apparently also lacking in aging corn seed (unpublished work of N. W. Pammenter, cited in ref. 1). The absence of a demonstrable alteration of the unsaturated fatty acids indicates that membrane-related or other effects of aging may not be mediated through lipid peroxidation, at least in soybean and corn.

Because of our previous inability to find evidence of decreased fatty acid unsaturation in artificially aged soybeans (18), we have directed our attention to possible changes in tocopherols since tocopherols act as natural antioxidants and, hence, oxidative changes should be more readily apparent in this fraction. In soybeans (and presumably other seeds) tocopherols are associated with both membrane and storage lipids (25).

Parallel to the tocopherol study reported in this paper, we have also pursued a more direct analysis of organic free radical status in seeds using ESR.[4] Roubal (19) used ESR on dry biological systems to indicate that lipid peroxidation gave rise to dramatic changes in the free radical signal. Conger and Randolph (4) reported remarkably stable levels of organic free radicals during natural seed aging. We have pursued similar studies with both naturally and artificially aged soybean seeds.

MATERIALS AND METHODS

Soybean seeds (*Glycine max* (L.) Merr.) were obtained from the following sources: cv. 'Wayne' from Maumee Valley Seeds, Woodburn, Ind.; cv. 'Custer' and cv. 'Magna' from Dr. J. E. Specht, University of Nebraska, Lincoln; cv. 'Amsoy 71,' cv. 'Calland' and cv. 'Wells' from Dr. J. R. Wilcox, Purdue University, Lafayette, Ind. Within any one variety, the seedlots that were produced in different years were all grown at the same location. All seed samples had been maintained cool and dry during long term storage, following accepted practice. Seeds designated in this paper as "unaged" were harvested 18 to 24 months prior to the experiments.

Accelerated aging of Wayne seeds was achieved by incubation in closed plastic boxes at 40 C and close to 100% RH (5). Following treatment, the seeds were allowed to air-dry until their original weight had been restored. For germination assays, 50 seeds were rolled in germination papers moistened by capillary action. Radicle length was measured after 3 days of growth in darkness at 24 C. Seeds with radicles longer than 10 mm were considered to have germinated. Growth was measured as the mean length of all emerged radicles.

Tocopherols were isolated and analyzed by a modification of the method of Walker and Slinger (23). Whole seeds (5 g) were ground for 30 s in a Waring coffee mill and then extracted for 30 min at room temperature with 25 ml chloroform/methanol (2:1, v/v) under an argon atmosphere. The solution was filtered through Whatman GF/A glass microfiber paper, made up to 30 ml volume, and partitioned against 6 ml 0.9% (w/v) NaCl. An internal standard of 5,7-dimethyltocol (Supelco, Inc.) was added at this stage. The organic phase was dried in a rotary evaporator and the lipid subsequently re-dissolved in 55 ml ethanol. A 5-ml sample was removed and dried for gravimetric determination of total lipid yield. To the remaining 50 ml lipid solution, 1 ml 10% (w/v) ethanolic pyrogallol was added as antioxidant. The lipids were saponified under argon at 90 C, using a saturated KOH solution

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[4] Abbreviations: ESR: electron spin resonance; g: spectroscopic splitting constant.

(2.5 ml). After 5 min, the solution was cooled on ice, partitioned with 50 ml *n*-hexane and 50 ml of H_2O, washed three times against 50 ml H_2O containing 2 ml 10% (w/v) ethanolic pyrogallol, evaporated, redissolved in a few ml of benzene, and stored in the dark under argon at −20 C.

The tocopherol fraction was purified by one-dimensional TLC on precoated 250-μm Silica Gel HR plates (Analtech, Inc.) using benzene as solvent. The plates were sprayed with 0.01% (w/v) rhodamine 6G, and the sectors containing tocopherols (visible under UV light) were scraped off, eluted with peroxide-free diethyl ether, and derivatized using pyridine/*bis*(trimethylsilyl)trifluoroacetamide (4:1, v/v) for 1 h at room temperature. The trimethylsilyl derivatives were analyzed using a Hewlett-Packard 5730A gas chromatograph equipped with a flame ionization detector and coupled to an electronic integrator. Separations were performed on a 365-cm glass column (internal diameter, 4 mm) packed with 0.5% (w/w) Apiezon L on 100/120 mesh Gas-Chrom Q (Applied Science Labs) at 240 C with N_2 (37.5 ml min^{-1}) as carrier. The identification of tocopherols was based on co-chromatography with authentic standards (Eastman Organic Chemicals and Sigma Chemical Co.).

Organic free radical analysis was performed on the cotyledons alone, which were dried over $CaSO_4$ until their water content was close to 3.5% (wet weight basis). Twenty cotyledons were ground briefly in a porcelain mortar and sieved through two layers of nylon mesh (pore size, approximately 400 μm), and the fines were returned to a $CaSO_4$ desiccator prior to analysis within a few hours. The ground seed was analyzed in quartz tubes using a Varian E-104 (x-band) ESR spectrometer. Radical concentration in the tissue was determined by the amplitude of the first derivative signal. The absolute organic free radical concentration was estimated by reference to Mn^{2+} signals naturally present within the seed material, acting as an internal standard. To determine the Mn content, samples (1 g) were wet-ashed for 3 h using H_2SO_4/HNO_3 (1:1, v/v) containing 1% (w/v) La_2O_3 to sequester Pi (14). After ashing to dryness, the samples were boiled for 10 min in 20 ml 1 M HCl and then made up to a volume of 7.5 ml in 100 mM HCl. Mn content was determined using a Perkin-Elmer 305A atomic absorption spectrophotometer.

RESULTS

The six cultivars used in the present study showed good germinability (average 90%) and vigorous growth in the unaged state. A decline was evident with age for all cultivars. In two cultivars (cv. Custer and cv. Magna), the oldest seed used in our experiments was completely dead (Table I).

All the soybean cultivars possessed three principal homologues of tocopherol (α, γ, δ). In all six cultivars examined, the most abundant homologue was γ-tocopherol (ranging in mean value from 77.1% in cv. Amsoy 71 to 61.1% in cv. Wells) accompanied by smaller amounts of δ-tocopherol (31.9% in cv. Wells to 10.6% in cv. Custer) and α-tocopherol (20.2% in cv. Magna to 6.9% in cv. Wells). This distribution of tocopherol homologues is compatible with the results of previous studies (*e.g.* ref. 6). The levels of total tocopherols in naturally aged seeds are shown in Figure 1. None of the correlation coefficients calculated between tocopherol content and age (or tocopherol content and percentage germination) for any of the individual cultivars were significant. Although the overall trend in tocopherol content with age (or percentage germination) is difficult to define from these data, it is evident that a disappearance of tocopherol is not a prerequisite for loss of germination in any of the cultivars studied. Within each cultivar, the proportions of tocopherol homologues demonstrated little evidence of change with age (data not shown). Data from seeds subjected to accelerated aging (cv. Wayne) gave little sign of a change either in total tocopherol present or in the relative proportions of the homologues (Fig. 2).

A characteristic first derivative ESR spectrum for powdered soybean cotyledonary material is shown in Figure 3. The g value of the organic free radical signal (in the center of the spectrum shown) is slightly higher than that of a free electron (g = 2.0023) and close to reported values (g = 2.004-2.0055) given by Conger and Randolph (4). The peak-to-peak line width was in the order of 9 to 10 G. There was no evidence of hyperfine structure in the organic radical signal, nor was it possible to separate it into several components with significantly different g values. No indication was found of a "lipid signal" peak slightly downfield of the central resonance (of the kind recorded by Roubal (19) in dry fish flesh). It is possible, however, that free radical signals derived from peroxidizing lipids might, in our case, have been impossible to resolve from the "general" organic signal. The two extreme signals in the spectra of Figure 3 are contributed by the two center-field transitions of the Mn^{2+} ion. The Mn^{2+} signals were not saturated at all power levels, but the organic radical signal began to saturate at powers exceeding several mw. For this reason spectra were run at 3 mw. The saturation curve obtained by plotting signal intensity against the square root of microwave power (not shown) implies heterogeneous saturation of the organic free radical signal. This suggests that the signal arises from a mixture of molecular species (2). The Mn^{2+} content of the tissues (determined by atomic absorption spectrophotometry) was in the order of 44 μg/g dry weight. Comparison of the organic radical signal with that of Mn^{2+} (making the assumption that all of the Mn^{2+} was observable by ESR) suggests that the cotyledonary material contained approximately 2×10^{17} free radicals/g, within the range of values reported by Conger and Randolph (4) for whole and ground seeds of various species.

The organic free radicals present in the cotyledons of naturally aged seeds showed little change with age (Fig. 4). None of the correlation coefficients calculated between free radical content and age (or free radical content and germination) for any of the individual cultivars were significant. ESR analysis of seeds of cv. Wayne subjected to accelerated aging similarly showed negligible change in organic free radicals (data not shown).

DISCUSSION

We have previously reported (18) that the unsaturated fatty acids of the total (primarily storage) and polar (membrane) lipid fractions of soybean were unaffected by accelerated aging. Despite the fact that linoleic acid constitutes the major fatty acid present (approximately 55–60% in both fractions) and is highly susceptible to free radical induced peroxidation *in vitro*, no evidence for a change during accelerated aging was found (18). The data reported here demonstrate that the tocopherol fraction in soybean seeds is likewise unaffected during aging (natural or accelerated) and that the organic free radicals in the seeds are apparently unchanging under either of these conditions.

The ability of low concentrations of tocopherols to prevent lipid peroxidation is well-documented (*e.g.* ref. 24). It has been shown that 1 molecule tocopherol may afford antioxidant protection to several thousand unsaturated fatty acid molecules, even in relatively immobile systems adsorbed on silica gel (24). Characteristically, the presence of tocopherols in a lipid mixture suppresses autoxidation of unsaturated fatty acids for an interval of time (the "induction period") during which the tocopherol itself is broken down. After the tocopherol has largely disappeared, the rate of autoxidation of unsaturated fatty acids greatly increases.

Figures 1 and 2 show that the tocopherol levels in aging soybeans do not show a pronounced decline. Additional evidence for this lack of tocopherol oxidation arises from the observation that the proportions of the three tocopherol homologues remain fairly constant throughout aging. Different homologues scavenge free radicals with markedly different efficiencies, so the proportions of the homologues present in a mixture undergoing oxidation

Table I. *Effects of Natural and Accelerated Aging on Subsequent Performance of Seeds during Germination*

Results are expressed as per cent germination with mean axis length (±SD) in mm given in parentheses.

Natural Aging	Years of Aging							
	0	1	2	3	4	5	8	10
Magna	88% (45.8 ± 21.9)	46% (32.9 ± 14.4)			0			
Custer	86% (48.2 ± 25.2)	60% (44.9 ± 26.3)			0			
Amsoy 71	93% (45.3 ± 16.7)	88% (41.5 ± 18.6)	92% (44.5 ± 19.2)	88% (47.3 ± 19.1)		74% (41.2 ± 16.4)	66% (24.2 ± 12.4)	
Wells	84% (42.9 ± 18.8)	88% (44.3 ± 21.0)	96% (47.0 ± 18.4)	86% (50.5 ± 2.37)			74% (32.7 ± 14.5)	
Calland	92% (45.7 ± 21.9)	76% (48.5 ± 23.6)	66% (33.6 ± 17.3)	74% (54.4 ± 22.7)				30% (20.7 ± 8.6)

Accelerated Aging	Days of Aging				
	0	1	2	3	4
Wayne	94% (57.4 ± 16.3)	92% (48.5 ± 14.8)	70% (38.7 ± 16.5)	54% (33.9 ± 14.2)	28% (20.4 ± 7.2)

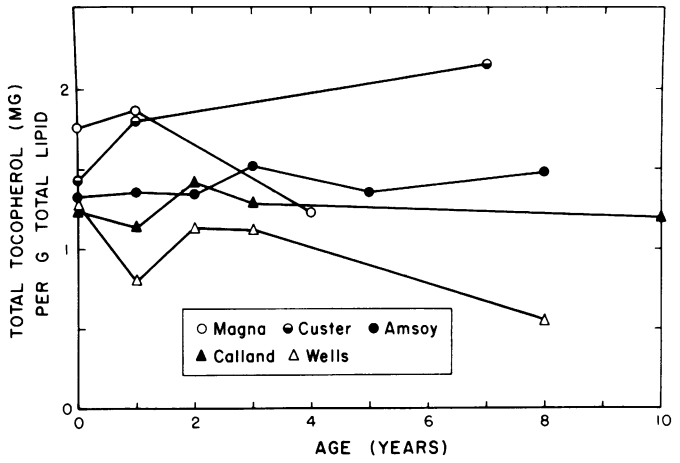

FIG. 1. The total tocopherols present in five cultivars of soybeans undergoing "natural" aging in long-stem storage. Each point represents a single observation.

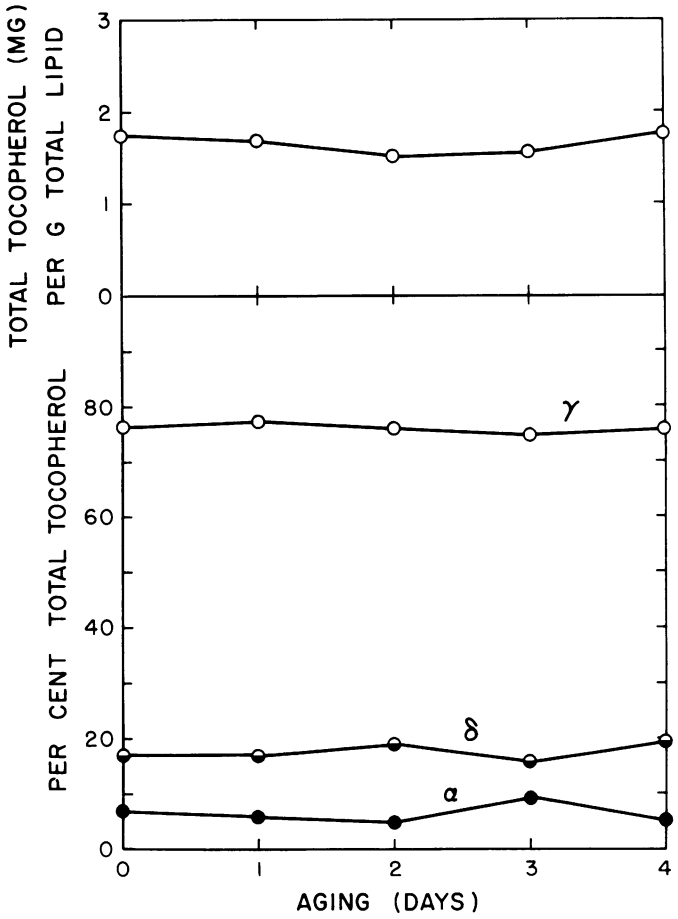

FIG. 2. Effect of accelerated aging on total tocopherol levels (above) and tocopherol homologues (below) in cv. Wayne. Each point represents a single observation.

would be expected to alter quite significantly. Changes of this sort are evident in vegetable oils undergoing oxidation in storage (11). The relative abundance of tocopherols in soybeans at all stages of aging is consonant with the absence of fatty acid oxidation. It is unclear how tocopherols are distributed throughout the seed and whether their presence in all parts of the cell is needed to prevent lipid autoxidation. The data of Yamauchi and Matsushita (25) suggest that in soybean cotyledons the great majority of tocopherols are associated with storage lipid bodies (which constitute well over 90% of the cellular lipid), although a significant proportion is also associated with several membranous fractions.

We are aware of four reports which associate declining tocopherol levels with storage or aging of seed material. Nordfeldt *et al.* (13) found that wheat germs lost 5 to 10% of their tocopherols during 6 months of storage. Malysheva (12) noted that tocopherol levels declined during seed aging but provided no data. Ovcharov (15) claimed that loss of germinability in wheat and corn was associated with declining tocopherol content, but the data he published for the most part show relatively small differences between aged and unaged seed. More recently Sharma (20) has noted a decline in tocopherols of several oil seeds with loss of viability although he was unable to dissociate the effects of extraneous factors such as microbial action from those of aging. Kaloyereas *et al.* (10) reported that briefly dipping seeds in a 1% aqueous emulsion of tocopherol had beneficial effects on their

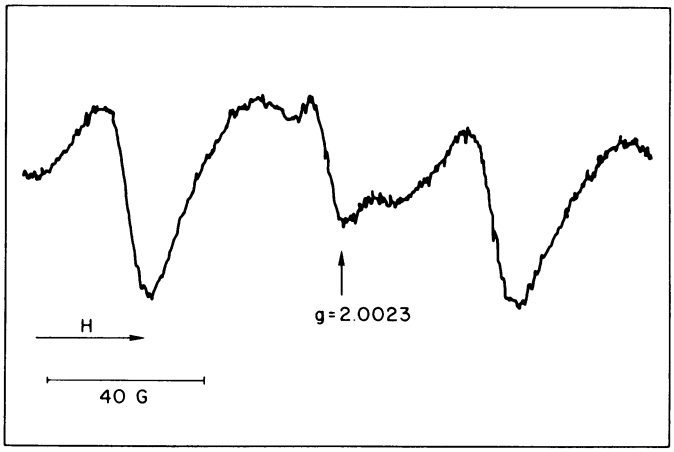

FIG. 3. First derivative ESR spectrum of powdered soybean cotyledons; H: magnetic field strength.

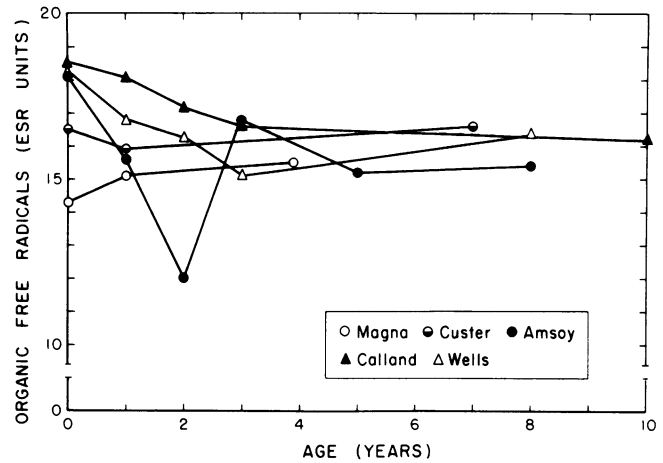

FIG. 4. Effect of natural aging on organic free radical content. One unit of measurement on the ESR spectrum is approximately equivalent to 1.2×10^{16} free radicals/g dry weight of tissue. Each point represents a single observation.

subsequent viability in storage. Whether its action was specifically that of an antioxidant is not evident.

ESR studies by Conger and Randolph (4) on several species of seeds up to 48 years old led them to conclude that organic free radicals in the dry seeds were unusually stable. The change in organic radical signal with age yielded a value for the average half-life of about 200 years. Our data obtained from soybeans also indicate marked stability during both natural and accelerated aging. The decay of radiation-induced organic free radicals in seeds can be correlated with loss of viability, primarily due to injury to nucleic acids (4, 7). Nevertheless, for unirradiated seeds we conclude with Conger and Randolph (4) that it is improbable that changes in endogenous free radicals can account for increased lesions in nucleic acids with age. For similar reasons, it is difficult to implicate free radicals in changes to the lipid fraction. In the dry biological systems studied by Roubal (19), peroxidation of lipids led to increased ESR signals which were noticeably absent or suppressed in the presence of antioxidants. The constant nature of the ESR signal in soybeans coupled with the absence of marked change in the tocopherol fraction, leads to the conclusion that lipid peroxidation need not be a corollary of natural or artificial seed aging.

In a recent communication, Stewart and Bewley (21) have suggested that membrane lipid peroxidation may be associated with accelerated aging of soybean axes (subjected to 45 C at saturating humidity). We have previously presented data (18) showing an absence of change in the total fatty acids of the seed axis aged at 40 C for 5 days. The severity of the aging stress (which killed the seeds within 2 days) employed by Stewart and Bewley (21) prevents a confident assessment of the relationship between lipid peroxidation and loss of viability in their system. Although they were able to demonstrate some loss of linolenic acid in badly deteriorated seed (approximately 30% germination), the most marked changes in susceptible fatty acids were found only in treatments extending beyond the point of death for the seeds. We have previously speculated that lipid oxidation might become significant in soybean seeds *post mortem* (18). Certainly under somewhat milder conditions of accelerated aging (40–41) deficiencies in membrane integrity are evident (16) in the absence of any change to the fatty acids (18). The data given here support this contention and further strengthen the suggestion that lipid peroxidation may be insignificant during natural aging.

In the absence of any convincing evidence in favor of free radical attack as a primary cause of aging in soybean, it is difficult to account for the observed deterioration in membranes (16). In a previous communication (18), we noted small changes in the classes of phospholipids (particularly phosphatidylcholine) present during accelerated aging but no corresponding shift in the proportions of fatty acids. In part the phospholipid decline may have been initiated by phospholipase activity released from sequestration and possibly capable of action even in the severely restricted environment of a semihydrated seed. Whether comparable effects would arise in naturally aged seeds (which do not experience such limited hydration) and whether indeed such changes would have a marked bearing on subsequent cellular viability, remains questionable.

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LITERATURE CITED

1. BERJAK P 1978 Viability extension and improvement of stored seeds. S Afr J Sci 74: 365–368
2. BORG DC 1976 Applications of electron spin resonance in biology. *In* WA Pryor, ed, Free Radicals in Biology, Vol 1. Academic Press, New York, pp 69–147
3. CHEAH KSE, DJ OBSORNE 1978 DNA lesions occur with loss of viability in embryos of aging rye seeds. Nature 272: 593–599
4. CONGER AD, ML RANDOLPH 1968 Is age-dependent genetic damage in seeds caused by free radicals? Radiat Bot 8: 193–196 (Erratum. Radiat Bot 9: 147)
5. DELOUCHE JC, CC BASKIN 1973 Accelerated aging techniques for predicting the relative storability of seed lots. Seed Sci Technol 1: 427–452
6. GOVIND RAO MK, EG PERKINS 1972 Identification and estimation of tocopherols and tocotrienols in vegetable oils using gas chromatography-mass spectrometry. J Agric Food Chem 20: 240–245
7. HABER AH, ML RANDOLPH 1967 Gamma-ray-induced ESR signals in lettuce. Evidence for seed-hydration-resistant and -sensitive free radicals. Radiat Bot 7: 17–28
8. HARMAN GE, LR MATTICK 1976 Association of lipid oxidation with seed aging and death. Nature 260: 323–324
9. KALOYEREAS SA 1958 Rancidity as a factor in the loss of viability of pine and other seeds. J Am Oil Chem Soc 35: 176–179
10. KALOYEREAS SA, W MANN, JC MILLER 1961 Experiments in preserving and revitalizing pine, onion, and okra seeds. Econ Bot 15: 213–217
11. LEHMANN J, HT SLOVER 1976 Relative autoxidative and photolytic stabilities of tocols and tocotrienols. Lipids 11: 853–857
12. MALYSHEVA AG 1964 Izmenenie biokhimicheskikh svoistv i vskhozhesti semyan maslichnykh kul'tur v protsesse ikh khraneniia. (Changes in the biochemical properties and germination of oil crop seeds in the course of their storage). *In* AL Kursanov, ed, Biologicheskie osnovy povysheniia kachestva semyan sel'skokhozyaistvennykh rastenii. Nauka, Moscow, pp 259–266
13. NORDFELDT S, N OLSSON, G ÅNSTRAND, V HELLSTRÖM 1962 Influence of storage upon total tocopherols in wheat germs. Effect of germination upon total tocopherols in wheat. K Lantbrukshögsk Ann 28: 181–188
14. OELSCHLAGER W, L BESTENLEHNER 1976 Bestimmung von Mangan in Biologischen Materialen, sowie in Mineralfuttermitteln mit Hilfe der Atomsabsorptions-Spektralphotametrie (AAS). Landwirtsch Forsch 29: 224–233
15. OVCHAROV KE 1969 Fiziologicheskie osnovy vskhuzhesti semyan. Nauka, Moscow. (Physiological Basis of Seed Germination) Amerind, New Delhi, 1977
16. PARRISH DJ, AC LEOPOLD 1978 On the mechanism of aging in soybean seeds.

Plant Physiol 61: 365–368
17. PARRISH DJ, AC LEOPOLD 1978 Confounding of alternate respiration by lipoxygenase activity. Plant Physiol 62: 470–472
18. PRIESTLEY DA, AC LEOPOLD 1979 Absence of lipid oxidation during accelerated aging of soybean seeds. Plant Physiol 63: 726–729
19. ROUBAL WT 1970 Trapped radicals in dry lipid-protein systems undergoing oxidation. J Am Oil Chem Soc 47: 141–144
20. SHARMA KD 1977 Biochemical changes in stored oil seeds. Indian J Agric Res 11: 137–141
21. STEWART RRC, JD BEWLEY 1980 Lipid peroxidation associated with accelerated aging of soybean axes. Plant Physiol 65: 245–248
22. VILLIERS TA 1973 Aging and the longevity of seeds. *In* W Heydecker, ed, Seed Ecology. Pennsylvania State University Press, University Park, pp 265–288
23. WALKER BL, SJ SLINGER 1975 Effect of processing on the tocopherol content of rapeseed oils. Can Inst Food Sci Technol J 8: 179–180
24. WU G, RA STEIN, JF MEAD 1979 Autoxidation of fatty acid monolayers adsorbed on silica gel. IV. Effect of antioxidants. Lipids 14: 644–650
25. YAMAUCHI R, S MATSUSHITA 1976 Quantitative changes in tocopherols and their intracellular distribution in cotyledons during soybean germination. Nippon Nogeikagaku Kaishi 50: 525–529