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Free radical accumulation and lipid peroxidation in testas of rapidly aged soybean seeds: a light-promoted process

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Abstract

The role of free radical-induced damage as a cause of loss of vigour in seeds is by no means resolved. In this contribution, the effects of environmental treatments known to reduce viability rapidly were compared with the effects of long-term, low-temperature storage on germination, hypocotyl growth and free radical accumulation and lipid peroxidation in soybean seeds. Accelerated aging was achieved by incubating seeds at 35°C and 1% relative humidity over H_2SO_4 for up to 69 days in the light and in darkness. In contrast, seeds under long-term storage were maintained at 5°C and 6% moisture content in darkness for up to 6 years. At 35°C there were rapid and significant reductions in rates of seed germination and hypocotyl extension. Loss of viability and declining vigour were associated with increases in lipid peroxidation and free radical build-up but the latter, surprisingly, was largely confined to the testa rather than the cotyledon. Exposure to light greatly enhanced lipid peroxidation and increased organic free radical accumulation in the translucent testas of seeds, but not in the cotyledons. Similar responses to light were recorded in testas detached from seeds. These results show that in soybean the testa is a significant locus of free radical degenerative events induced by high temperature combined with low moisture.

Keywords: Free radicals, *Glycine max*, lipid peroxidation, oxidative stress, seed viability, testa.

Introduction

Seeds deteriorate during prolonged storage, but the rate of deterioration varies greatly among species (Priestley, 1986; Roberts, 1986). While the exact cause of loss of seed viability is still not well-defined, many studies have implicated damage to membranes as one causative factor (e.g. Parrish and Leopold, 1978; Pearce and Abdel Samad, 1980; Bewley, 1986). In the presence of oxygen, aging of seeds is often, though not always, associated with peroxidation of polyunsaturated fatty acids (Harrington, 1973; Stewart and Bewley, 1980; Wilson and McDonald, 1986; Senaratna *et al.*, 1987; Hendry *et al.*, 1992; Hendry, 1993). Lipid peroxidation has considerable potential to damage membranes and may be important in the deterioration of stored seeds and reduced longevity of seeds under natural conditions. Other organic molecules subject to oxidative attack include proteins (Sun and Leopold, 1995) and nucleic acids (Osborne, 1980) though the literature on these is less extensive. Increased free radical damage and decline in activity of free radical-processing systems during accelerated aging has been linked to loss of viability under high moisture regimes, for example in sunflower (e.g. Bailly *et al.*, 1996).

Soybean seeds can be stored at low moisture content and low temperature for many years, while aging can be accelerated during exposure to high temperatures, a technique widely used in the study of seed storability and deterioration. In this present study, we have investigated the germination, hypocotyl expansion, accumulation of stable free radicals and lipid peroxidation of soybean seeds stored for up to 6 years and, by contrast, in seeds rapidly aged over 10 weeks. Our aim was to assess the significance of free radical generation and lipid peroxidation during declining vigour of dry-stored soybean seeds.

Materials and methods

Plant material

Seeds of soybean (*Glycine max* L. Merr. cv. Williams 82) were obtained from the collection of the USDA-ARS, National Seed Storage Laboratory, Fort Collins, Colorado, USA.

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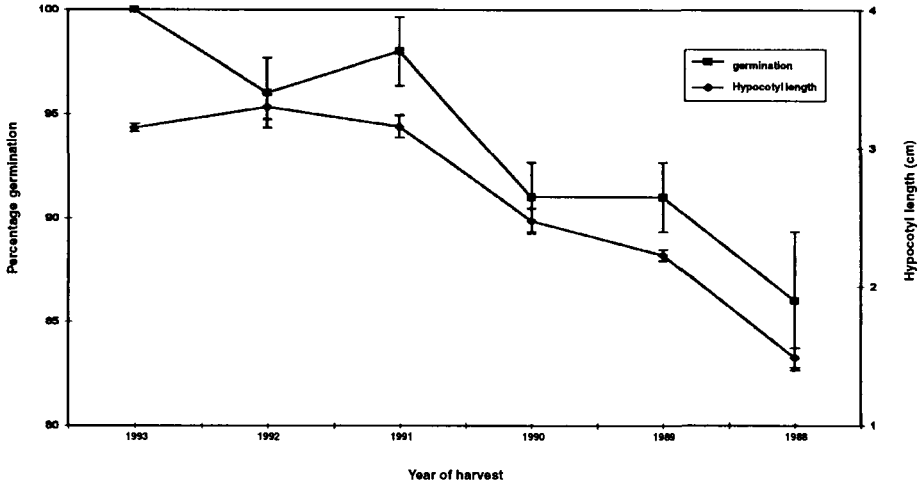

Figure 1. Germination (%) and hypocotyl expansion in soybean seeds held under long-term cold,dry (5°C, 6% moisture content) storage conditions.

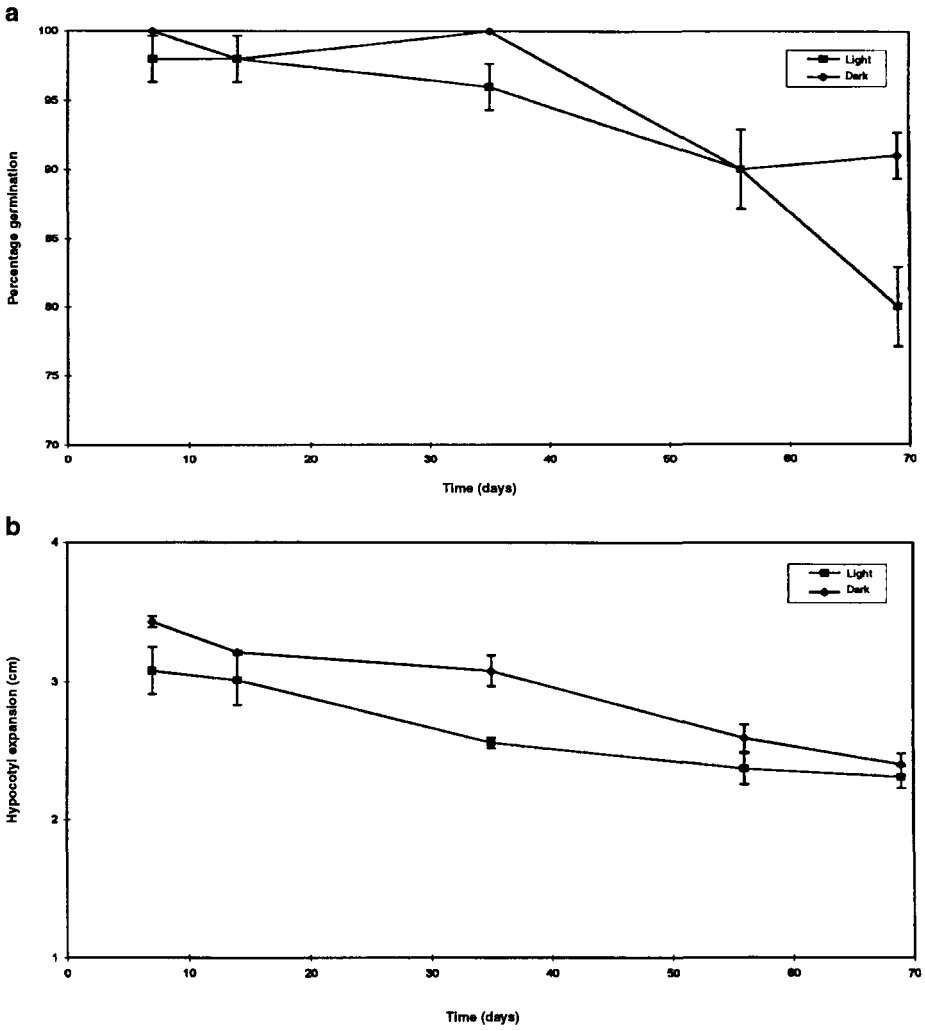

Figure 2. **a**) Germination and **b**) hypocotyl expansion in seeds aged at 35°C and 1% relative humidity (high temperature-low moisture) under light (■) or dark (◆).

Storage and aging treatment

For short-term treatments, seeds from the 1993 harvest were aged at Fort Collins by storing over H_2SO_4 at 35°C (1% RH) in cool-white, fluorescent light (90 µmol m^{-2} s^{-1}) or in the dark. Seed parts were aged in a similar manner. Samples were removed from the aging treatment after 7, 14, 35, 56 or 69 days and, in separate experiments, after 14 months. For long-term treatments, seeds from the harvests of 1988–93 were stored at 5°C in the dark at 6% moisture content. After treatment samples were express air-freighted to the UK for analysis. Mean values (± 1 SE) are shown in the figures with Spearman's correlation coefficients given in the text.

Germination treatment

Seeds were hydrated for 16 h in plastic boxes (18 × 26 cm) with tight fitting covers, the bottom of the boxes being lined with wet paper towels and the seeds placed in Petri dishes on top of the damp paper. Following hydration, seeds were rolled in Whatman 1 filter paper in contact with a reservoir of water sufficient to keep the seeds moist. After 96 h at 25°C in the dark, percentage germination and hypocotyl expansion were recorded.

Lipid peroxidation product estimation

Lipid peroxidation was determined as the concentration of thiobarbituric acid-reactive substances, equated with malonylaldehyde (MDA), as originally described by Heath and Packer (1986) but modified as in Hendry *et al.* (1993), where the products were quantified from the second derivative spectrum against standards prepared from 1,1,3,3- tetra-ethoxypropane. All determinations were of a minimum of 5 replications, each of one intact seed or about 50 mg of testa tissue.

Electron paramagnetic resonance (EPR) response

Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ER 200 D spectrometer, as described by Leprince *et al.* (1990), care being taken to position the sample reproducibly in the spectrometer cavity. Other parameters were adjusted as necessary to obtain the most resolved spectra. Free radical concentrations were estimated by the height (in cm) of the first derivative spectrum corrected for instrument gain and expressed on an unimbibed seed weight basis. Minimum replication for EPR analysis was 3 samples.

Fatty acid analysis

Polyunsaturated fatty acid content was determined as described by Hendry and Thorpe (1993) where 50 mg of ground tissue was extracted with borate buffer pH 9.0, 3 ml of KOH was added to 1 ml of extract and incubated in sealed tubes for 6 hours at 80°C. Following centrifugation, the saponified extract was incubated with lipoxidase enzyme (60 000 U/ml), (Sigma Chemicals) for 20 minutes at 25°C. Absorbance was recorded with active and boiled enzyme at 234 nm and estimated against a linoleic acid (Sigma Chemicals) standard. Replication was 5 samples.

Results

Seed aging and viability

When stored for up to 6 years (5°C, 6% moisture), seed lots showing 100% germination shortly after harvest declined in germination from 100% (1993 harvest) to about 86% (1988 harvest) while the rate of hypocotyl expansion decreased to about one half over the 6 years (Fig.1). Seeds from the 1993 harvest subjected to high temperature, low moisture treatment in the dark or light for 69 days showed decreases in germination to 91% (dark-treated) and 80% (light-treated) (Fig. 2a). There was a 2-fold decline in hypocotyl length (Fig. 2b) with treatment.

Lipid peroxidation in cotyledon and testa

In the long term-stored seeds lipid peroxidation increased by about 15% over 6 years in the embyros (which here included the cotyledons and embryonic axes) (Fig. 3). However, no significant change in lipid peroxidation was observed in the testas of these long term-stored seeds (data not shown) nor was there any correlation between lipid peroxidation and hypocotyl length or percentage germination. In contrast, in seeds subjected to high temperature and low moisture for 69 days, there was a highly significant increase (>60%) with time in lipid peroxidation in the testas of seeds subjected to illumination (Fig. 4a), strongly correlated with duration of aging treatment (r^2 = 0.968). In the cotyledons (which sample also included the axes), much smaller (10–15%) increases in lipid peroxidation were noted (Fig. 4b).

Free radical processes in cotyledon and testa

In seeds under long-term storage conditions there was no correlation between the build-up of a stable free radical (EPR response) and age, neither in the cotyledons nor in the testas. In contrast, in the seeds subjected to high temperature-low moisture treatment there was a highly significant increase in free radical accumulation strongly correlated with duration of treatment (r^2 = 0.910 dark-treated and r^2 = 0.672 light-treated). In absolute terms, the amplitude of the EPR signal was 2-fold greater in testas from light-treated seeds than in testas from dark-treated material (Fig. 5). There was also a strong correlation between the increase in EPR response in the testas and the decline in germination in both light-treated (r^2 = 0.944) and dark-treated tissues (r^2 = 0.869) (Fig. 6).

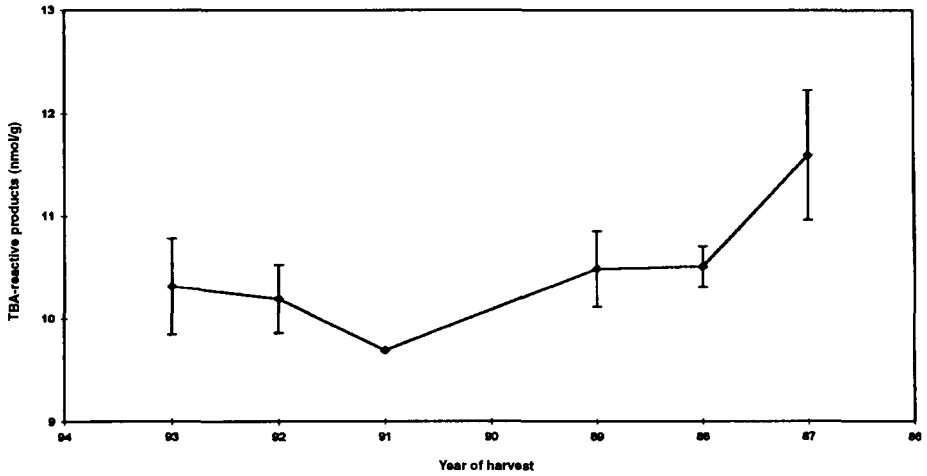

Figure 3. Lipid peroxidation (thiobarbituric acid-reactive products) in soybean seeds held under long-term (cold/dry) storage conditions.

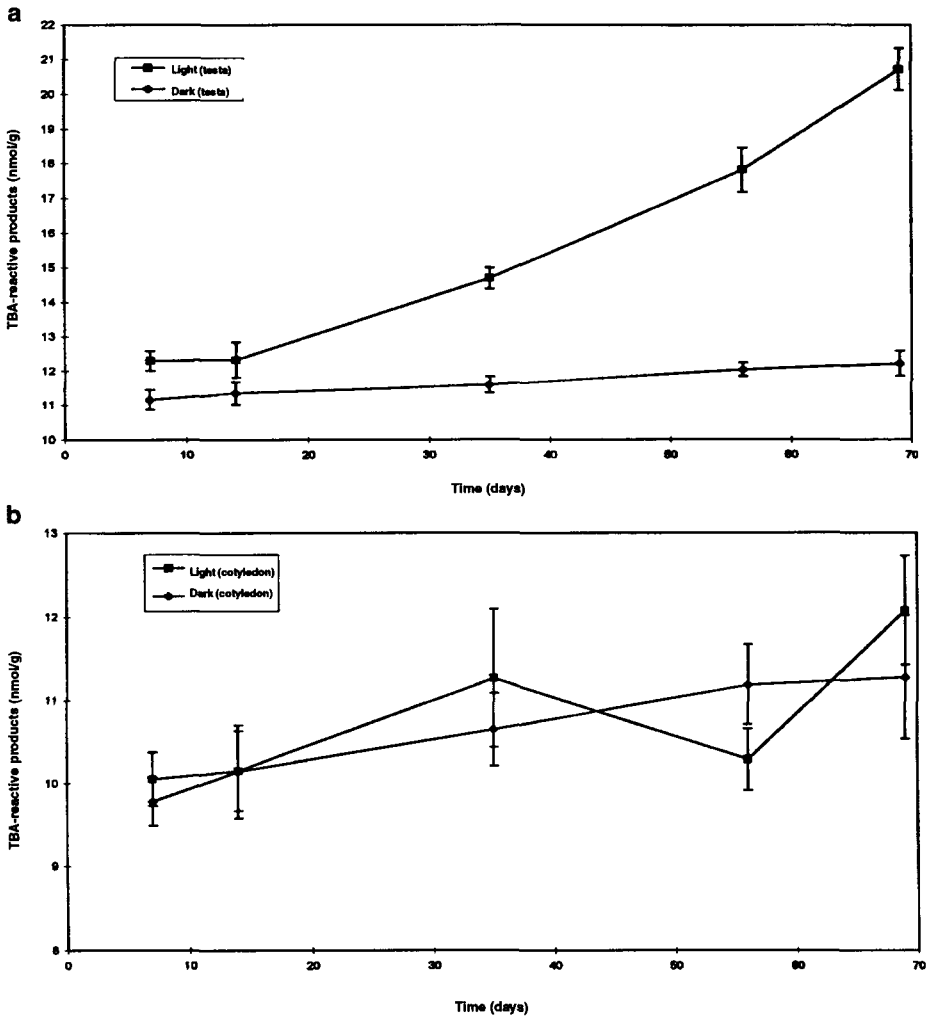

Figure 4. Lipid peroxidation (thiobarbituric acid-reactive products) in **a**) testas and **b**) cotyledons plus axes of soybean seeds aged at 35°C and 1% relative humidity (high temperature-low moisture) under light (■) or dark (◆).

There was, however, no significant difference in EPR response in the cotyledons and axes of seeds aged rapidly under light or dark.

Polyunsaturated fatty acid content

No significant difference in unsaturated fatty acid content of the testas was observed over the course of long-term storage or short-term treatment.

Effect of extending the high temperature-low moisture treatment to 14 months

Germination percentages declined to low values (10% or less) and hypocotyl expansion was reduced 4-fold or more over 14 months (data not shown). Compared with controls (seeds at 5°C, 6% moisture content) the EPR response in the testas of seeds subjected to 14 months of treatment increased 1.2-fold in the dark and 2.7-fold in the light. As in the 69-day treatment, a much greater build-up of free radicals occurred in the testas than in the cotyledons plus axes. Lipid peroxidation was also enhanced in the 14-month treatment compared with controls, though the values were not significantly different from those recorded at 69 days.

Effect of removing the testa before the high temperature-low moisture treatment

Testas isolated from the seeds and then subjected to high temperature treatment for 14 months showed broadly similar responses to testas from intact seeds, including

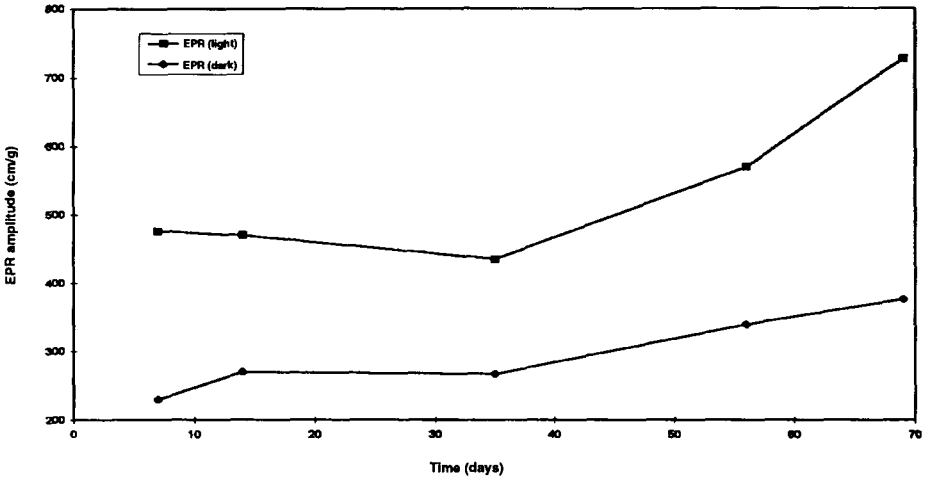

Figure 5. EPR response as the amplitude of the signal at constant settings, per g of testas from seeds aged under light (■) or dark (♦).

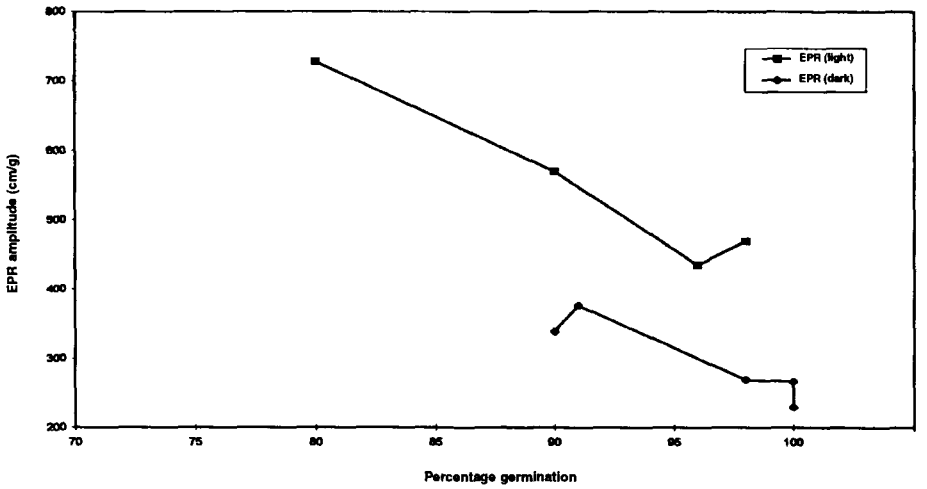

Figure 6. Relationship between EPR response in testas and percentage germination in seeds aged under light (■) or dark (♦).

Table 1. Effect of removing the testas from seeds prior to 14 months of high temperature-low moisture treatment in light or darkness. Values in brackets are those of tissues derived from intact seeds maintained under identical conditions for 14 months

Tissue	Illumination	EPR response (cm/g tissue)*	Lipid peroxidation (nmol/g tissue)*
testa	dark	210 ± 4.45 (237 ± 20.6)	10.4 ± 0.21 (12.2 ± 0.89)
	light	488 ± 23.3 (541 ± 42.7)	13.0 ± 0.32 (15.6 ± 0.39)
cotyledon	dark	5.80 ± 0.68 (9.06 ± 0.39)	14.3 ± 0.62 (14.3 ± 0.40)
	light	30.8 ± 5.75 (21.7 ± 3.16)	16.7 ± 0.73 (13.8 ± 0.20)

* ± SD.

significant light-enhanced increases in free radical accumulation and in lipid peroxidation (Table 1). In absolute terms there was a small but barely significant (P=0.05) decline in both EPR response and in lipid peroxidation. In the cotyledons free of testas for 14 months (and exposed to high temperature-low moisture), a broad pattern of light-enhanced increases in free radical accumulation and in lipid peroxidation was recorded, though the absolute values were not (or barely) statistically significant from those recorded in cotyledons from intact seeds.

Discussion

Seed germination and hypocotyl expansion of soybean under long-term storage declined over the 6 years. These two measures of declining viability were broadly reproduced by subjecting seeds of the 1993 harvest to 69 days of high temperature-low moisture treatment. However, the significant increases in lipid peroxidation and in EPR responses in the rapidly aged seeds were not (or were barely) apparent in the long term-stored seeds. This apparent anomaly may reflect the presence of repair mechanisms which were functional under the conditions of long-term storage (5°C, 6% moisture content) but where the capacity for repair was greatly exceeded or even repressed in the 69 days of high temperature-low moisture treatment. We have previously shown that free radical accumulation in water-stressed *Zea mays* seedlings is a function of temperature with a Q_{10} of about 2 (Leprince *et al.*, 1990) and it is no surprise to find an apparent temperature-dependent increase in free radical events in soybean seeds maintained at 35° as opposed to 5°C.

The results also demonstrate that both lipid peroxidation and free radical accumulation were significantly correlated with the decline in germinability and hypocotyl extension in rapidly aged soybean seeds. Two features of this phenomenon are particularly noteworthy. Firstly, lipid peroxidation and free radical accumulation were significantly promoted by illumination. To our knowledge light-enhanced free radical generation has not been reported before from dry non-photosynthetic plant tissue and which, in the case of the testa, is probably dead. These light-enhanced events occur whether or not the testa is attached to living cotyledonous tissue. Prolonged treatment for 14 months did not significantly increase these effects beyond those at 69 days. Secondly, the location for lipid peroxidation and free radical build-up was the testa rather than the cotyledon plus axis, an unexpected observation given the high concentration of lipids in the cotyledon. No attempt was made to dissect the axis from the cotyledon; the axis itself would be expected to generate peroxidized lipids (as previously observed by Hendry *et al*, 1992) though **quantitatively** in soybean this would be a minor source compared with the testa.

The apparent correlation between loss of viability and activity of free radical-linked processes in rapidly aged seeds reported here strengthens other evidence (Hendry, 1993) that loss of viability in seeds subjected to high temperature-low moisture treatment is probably closely linked to the ravages of oxidative damage. The intriguing finding that most of the damage (EPR response and lipid peroxidation) was located in the testa raises questions about the possible protective function of the testa in seed storage and the molecular relations, if any, between testa and axis. The effect on the cotyledons of removing the testas prior to the start of the 14-month treatment was not statistically different (or barely so) from the effects recorded on intact seeds in terms of free radical accumulation and lipid peroxidation. It was also apparent that the light-enhanced accumulation of the stable free radical could be reproduced in testas isolated from the rest of the seed. From this evidence, at least in soybean, the testa does not appear to offer protection from (or avoidance of) free radical events in the cotyledon plus axis. More significantly, perhaps, this raises another important issue: there is disagreement in the literature (reviewed in Hendry, 1993) about the correlation between free radical events (usually recorded as lipid peroxidation) and loss of viability in seeds. We suggest that part of this lack of consensus may be due to inherent differences in the organic composition of testas from different seeds and that it would greatly advance the debate if data on lipid peroxidation (and other free radical events and radical processing components) were to be reported not only from the intact seed but from the testa (or testa, pericarp and associated floral structures in cereals) and internal tissues separately. We have previously shown in *Quercus robur* that correlations between whole seed

viability and free radical events can be highly significant when the comparisons are made with the embryonic axis but fail when the cotyledons are included (Hendry *et al.*, 1992).

The build-up of free radicals and the associated peroxidation of lipids is substantially enhanced on illumination, raising further questions on the nature of the light receptor and the possible functional significance of pigmented as opposed to translucent testas in the maintenance of viability. The *in vivo* absorption spectrum of isolated soybean testas between 340 and 740 nm shows only one indistinct broad peak at around 360–365 nm. The functional significance of pigmentation in the testa is a subject we are currently addressing.

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