# An investigation into the effects of ultraviolet radiation on *Leucadendron laureolum* (Lam.) Fourc. seeds.

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## Abstract

The effects of four different doses of biologically effective radiation: visible (> 400 nm; 0 kJ $m^{-2} d^{-1}$); UV-A (320 - 400 nm; 0.45 kJ $m^{-2} d^{-1}$); UV-B ambient (280 - 320 nm; 8.89 kJ $m^{-2} d^{-1}$) and UV-B enhanced (280 - 320 nm; 11.43 kJ $m^{-2} d^{-1}$), over 1, 2, 4, 8 and 16 weeks, were investigated in serotinous *Leucadendron laureolum* (Lam.) Fourc. seeds. Germination studies revealed a depression in seed germination vigour and totality after one week of irradiation in the UV-B irradiated seeds. This depression disappeared in seeds irradiated for longer time periods, which suggests that cellular changes following UV-B irradiation might be an initial 'adjustment' response. Evidence of increased free radical scavenging activity of glutathione reductase was observed with both increased irradiation dosage and increased exposure period. Protective mechanisms in the seed are therefore playing a more important role in the prevention of cellular damage at higher irradiation levels and longer exposure times. Increases in rates of electrolyte leakage in seeds after longer exposures to ultraviolet irradiation, coupled with ultrastructural evidence of initial stages of seed damage (such as lipid coagulation), indicated that ultraviolet radiation may accelerate seed deterioration. These changes in glutathione reductase activity, electrolyte leakage and cellular ultrastructure, which all indicate potential slight damage in seeds of higher irradiation doses and longer exposure periods, did not depress seed germinability. Thus the irradiation levels and exposure times tested in this study suggest that ultraviolet radiation does not pose a real threat to the species in an ecological context.

## Introduction

Depletion of stratospheric ozone, first reported two decades ago (Johnston, 1971 and Cutchis, 1974), has been attributed to anthropogenic pollutants released into the atmosphere (Gleason *et al.*, 1993). This has resulted in increased levels of solar ultraviolet-B (UV-B, 280 - 320 nm) radiation at the earth's surface (Johanson *et al.*, 1995 and Madronich *et al.*, 1995). Concerns for biological ecosystems have been heightened (Teramura & Sullivan, 1994), because of various adverse effects that have been observed on different organisms, following UV-B exposure (Tevini, 1993b). UV-B radiation effects on plants have been studied at molecular, tissue, whole plant, population and ecosystem levels (Tevini & Teramura, 1989; Biggs & Joyner, 1994; Strid *et al.*, 1994 and Caldwell *et* al., 1995). Direct UV-B-induced damage to the plant photosynthetic apparatus (Teramura & Sullivan, 1994) and genome (Strid *et al.*, 1994) have been demonstrated, as well as indirect photomorphogenic responses to UV-B, including reductions in plant biomass (Singh, 1996), altered plant height, leaf area and thickness (Dillenburg *et al.*, 1995), stem elongation (Caldwell *et al.*, 1989), flowering times (Caldwell *et al.*, 1995) and seed and pollen production and viability (Tevini & Teramura, 1989 and Musil, 1994). Most studies on the effects of enhanced UV-B on plants have focused on vegetative rather than reproductive phases. This scarcity of reproductive phase studies, especially on seeds, may be attributed to protection from excessive UV-B radiation by the surrounding fruit or seed coat and when buried in the soil. Yet in arid and disturbed environments, seeds may be exposed to extended periods of sunlight prior to germination.

In mediterranean-type ecosystems of South Africa, Australia, California and the Mediterranean basin, many plant species are killed by fire and regenerate from seeds stored in the soil, and in condensed inflorescences (cones) in the plant canopy, termed serotiny (Gill & Groves, 1980 and Kruger & Bigalke, 1984). Several important South African plant families from the Cape fynbos have serotinous members, including the Proteaceae (*Protea*, *Leucadendron* and *Aulax*), Ericaceae (*Erica sessiliflora*), Asteraceae (*Phaenovoma* and *Helipterum*), Cupressaceae (*Widdringtonia*) and Bruniaceae (*Berzelia* and *Nebelia*) (Bond, 1985).

Massed fruit (hereafter loosely referred to as seed) release by serotinous taxa following fire is considered a means of reducing post-dispersal predation through satiation (O'Dowd & Gill, 1984). This is because seeds of serotinous taxa contain a high incidence of structures (wings, plumed perianths, dense villose hairs) adapted for wind dispersal and are not concealed (buried) from predators by ants like myrmecochorous seed of non-serotinous taxa (Bond, 1985 and Rebelo, 1995). Serotinous species typically exhibit a physiologically imposed embryo dormancy, released on exposure to low temperature, which contrasts with predominantly seed coat-imposed dormancy in the myrmecochorus taxa (Bond, 1984 and Brits, 1986). Seed dormancy in serotinous taxa is essential for seed survival over the dry summer, following untimely release of seeds in late winter or spring by aseasonal fires (Bond, 1984).

Although physiologically imposed dormancy of serotinous seeds promotes seed survival over summer, regeneration failure of serotinous Proteaceae stands in the first winter following aseasonal fires is common (Bond, 1985). It was proposed that the critical factor explaining this is the longer period of time seed lies exposed to granivores (Bond, 1985), and not a paucity of mature seeds in the pre-fire canopy (Jordaan, 1981). However, evidence of ultraviolet-induced photodegradation of plant structural compounds, such as cellulose and lignin in wood (Hon, 1991), suggests that seeds may also be significantly impacted by the sun's emissions where exposed for long periods on largely barren soil surfaces in arid or disturbed areas.

Ultraviolet radiation-induced photodegradation of biomolecules resulting in seed coat and embryo damage may be mediated by free radical formation (Larson, 1988) and seed aging (Sung, 1996). Free radicals enhance molecular damage through chain reactions involving electron transfer between molecules (Foyer *et al.*, 1994). Efficient antioxidant scavenging systems have evolved in plants (Foyer *et al.*, 1994) and seeds (Sung, 1996), which function to reduce free radicals to a minimum. Ascorbate peroxidase, glutathione reductase, superoxide dismutase and peroxidase form part of this protective system in seeds (Sung, 1996 & Wang *et al.*, 1991).

A balance between rates of free radical generation and antioxidant scavenging is necessary in dormant seeds to maintain seed viability and integrity (Sung, 1996). This balance in a seed, following exposure to light, may be upset by an increase in free radical formation due to photooxidative stress (Foyer *et al.*, 1994). Photodegradation of seeds and free radical formation may be further exacerbated by fluctuating chemical characteristics of the environment (Hon, 1994) and high or fluctuating environmental temperature (Bewley & Black, 1994).

Light however is not exclusively damaging to plants, but functions also as an environmental cue. Alteration of light absorbing biomolecules (pigments) in plants, on exposure to specific wavelengths of light, play an important ecological role in acquiring information about the environment, and mediating appropriate plant responses (Aphalo & Ballaré, 1995). The classic example in seeds of light inhibition or promotion of germination via photoreceptor dormancy imposition or release is that of the dimorphic (Pr, Pfr) pigment phytochrome, located in the seed coat. The effects of even short exposure of seeds to red light (dormancy release via Pr → Pfr conversion) and far red light (dormancy enforcement via Pfr → Pr conversion) have been well documented (Gutterman, 1993 and Bewley & Black, 1994). Ultraviolet light also interacts with photoreceptors (Ballaré *et al.*, 1995), and has been implicated in phytochrome photoconversion by UV-B (Pratt & Butler, 1970 in Ballaré *et al.*, 1995). Yet the functioning of ultraviolet light as an environmental information cue has been largely unexplored.

The potential consequences for dispersed seeds exposed for long periods to the sun's emissions on barren soil surfaces are alterations to the structure, permeability and chemical composition of their outer integuments; leading to increased sensitivity to attack by fungal pathogens, untimely germination due to disrupted mechanically or chemically enforced dormancy mechanisms, and cellular (enzyme and DNA) damage induced by UV absorption and free radical formation during the photodegradation process.

In the desert annual *Dimorphotheca pluvialis*, it was found that UV-B irradiation of polymorphic seeds resulted in altered germination accompanied by decreased photochemical efficiency, reduced seed production and increased sensitivity to photoinhibition in plants cultivated from the irradiated seeds (Musil, 1994). Preliminary investigations by Musil (pers. comm.) in several serotinous species in the Proteaceae suggested that prolonged exposure (two to four months) of these seeds to ultraviolet light may have induced a secondary dormancy.

In view of these findings, the objectives of the study were to investigate the role of specific wavelengths of ultraviolet radiation in possible seed damage and the observed secondary dormancy effect in a serotinous member of the Proteaceae. Seed germination studies following different ultraviolet radiation dosages and exposure periods were conducted to determine the requirements for secondary dormancy induction. A red light treatment of half of the seeds prior to germination was included to see whether secondary dormancy, if induced by the ultraviolet modification of a photoreceptor (possibly phytochrome), could be reversed by red light. Damage effects of different ultraviolet wavelengths over time were examined by studies of electrolyte leakage (indicator of cell membrane integrity) , free-radical-scavenging enzyme activity (indicator of damage prevention) and seed tissue ultrastructure (for visible changes) in the seeds. Serotinous *Leucadendron laureolum* (Lam.) Fourc. seeds were chosen for the study.

## Materials and Methods

### Seed material and irradiation treatments

One year old seeds (batch nos 631/75) were obtained from the Kirstenbosch Botanical Gardens, Cape Town. Fifty *L. laureolum* seeds were placed in open 90 mm diameter Petri dishes. There were 48 Petri dishes, 12 of which were covered with 1 mm thick polycarbonate film (which excluded transmission of light below 400 nm). Another 12 were covered with 0.12 mm thick polyester (Mylar type D) film (excluding transmission below 320 nm), and the remaining 24 dishes were covered with 0.075 mm thick cellulose acetate film (excluding transmission below 280 nm).

Artificial UV radiation was supplied daily over an 8 hour period, centred on the solar noon, by fluorescent sun lamps (Phillips TL/12 40W UV-B) suspended above the Petri dishes in a polycarbonate cladded greenhouse. Absolute spectral irradiances of lamps beneath the films were measured after sunset with a monochromator spectroradiometer (IL-1700, International Light Inc, Newburyport, USA), calibrated for absolute responsivity and checked for wavelength alignment, that was interfaced with a personal computer. Measured spectral irradiances were weighted with the action spectrum for intact plant DNA (Quaite *et al.*, 1992), normalized at 290 nm, and integrated over wavelength to obtain biologically effective UV irradiances as a function of distance from the lamp source.

There were four irradiation treatments: visible (> 400 nm), UV-A (320 - 400 nm), UV-B ambient and enhanced (280 - 320 nm) radiation. For UV-B treatments, distances between lamps and seeds were adjusted to provide biologically effective doses for seeds under cellulose acetate films of 8.89 kJ $m^{-2}$ $d^{-1}$ (UV-B1) and 11.43 kJ $m^{-2}$ $d^{-1}$ (UV-B2) which approximated the doses that would be received under clear skies during summer at 1000 m elevation in the south-western Cape (33°S) under normal ozone levels and anticipated 20% ozone depletion respectively, as computed from an empirical model (Green, 1983). In the visible and UV-A radiation treatments, seeds were maintained at the same distances from lamps as those in the ambient UV-B (UV-B1) treatment. Biologically effective doses received by seeds under polycarbonate films were 0 kJ $m^{-2}$ $d^{-1}$ (visible), and under polyester films 0.45 kJ $m^{-2}$ $d^{-1}$ (UV-A).

At weekly intervals, positions of Petri dishes under the fluorescent lamps were randomized, and films covering Petri dishes replaced to ensure uniformity of UV-B transmission. Over the irradiation period, photosynthetic flux densities (PFD), as measured with a quantum sensor (LI 189, Li-Cor, Lincoln, NE, USA) above the Petri dishes averaged 1605 ± 210 μmol $m^{-2}$ $s^{-1}$ around the solar noon.

**Electrolyte leakage**

Rates of electrolyte leakage from irradiated seeds provided an index of cell membrane integrity (Simon, 1974), and thus embryo damage (Bewley & Black, 1994). Three Petri dishes were removed at random from each irradiation treatment 1, 2, 4, and 8 weeks after commencement of irradiation. At each sampling interval, 100 seeds from each irradiation treatment were weighed and placed individually into wells of a CM100 Multiple Conductivity Meter (Reid and Associates, Durban) interfaced with a computer, and 1 $cm^3$ of ultra-pure water (deionized distilled water filtered through a Millipore purification system) added to each well. The remaining seeds were frozen in liquid nitrogen and stored for later enzyme analysis.

Prior to conductivity measurements, wells were thoroughly rinsed with ultrapure water and the conductivity meter calibrated at 4 volts for 30 minutes in the ultrapure water. Conductivity measurements were taken in each well at 30 s intervals for a period of 30 minutes and regressions of conductivity increase against time computed for each seed. Regressions with correlation coefficients ($r^2$) below 0.80 were rejected. Rates of electrolyte leakage (μA $cm^{-3}$ $min^{-1}$ $g^{-1}$) for each seed were computed from the slopes of the regressions and corresponding seed masses.

**Enzyme analysis**

Activities of the free-radical scavenging enzymes ascorbate peroxidase and glutathione reductase were used as an index of free radical formation (Foyer *et al.*, 1994). Methods of enzyme extraction and assay preparation of ascorbate peroxidase were a modification of Wang *et al.* (1991). Methods of glutathione reductase enzyme extraction and assay preparation were a modification of Foyer & Halliwell (1976).

Embryos were dissected from approximately 40 frozen seeds sampled from each irradiation treatment after 1 and 8 weeks of exposure. Embryos were weighed, and double the weight of polyclar AT added to the seeds (to minimize the interference of phenolics). For ascorbate peroxidase, enzymes were extracted by grinding the embryos in 4 ml of cold 50 mM Tris buffer (pH 7.7) and 1 ml freshly prepared 2 mM sodium ascorbate. For glutathione reductase, 4 ml of a cold 1 mM $K_2HPO_4$ buffer (pH 7.5) was used for enzyme extraction. The resulting thin pastes were placed in eppindorfs and centrifuged twice for 10 minutes at 4°C to remove particulate matter. The supernatants (which contained the enzyme extracts) were placed on ice to minimize enzyme activity. 300µl samples of each enzyme extract were refrozen for later protein analysis. Sixteen assays were performed for both ascorbate peroxidase and glutathione reductase from two extractions.

<u>Ascorbate peroxidase</u>

100 µl of 2 mM sodium ascorbate and 700 µl of 25 mM $K_2HPO_4$ (pH 6.1) were added to 100 µl samples of the enzyme extract. The resulting mixture was placed in a quartz cuvette to which 100 µl of 0.1 mM $H_2O_2$ added. The change in absorbance (at 290 nm) was measured every 0.3 seconds over a period of 1 minute using a double beam UV/Vis scanning spectrophotometer (CARY 1E, Varian, Australia). The change in optical density per minute (indicating utilization of $H_2O_2$ by ascorbate peroxidase) was used to calculate ascorbate peroxidase activity (described below).

Glutathione reductase

665 µl of 0.3 M $K_2HPO_4$ (pH 7.5), 100 µl of 3 mM $MgCl_2$ and 10 µl of 0.1 mM EDTA and 25 µl of freshly made 0.15 mM NADPH were added to 100 µl samples of the enzyme extract, and left to stabilize for 15 minutes. The resulting mixture was placed in a quartz cuvette, and the change in absorbance (at 340 nm) measured for 1 minute. This was necessary to correct for any GSSG-independent oxidation of NADPH. 100 µl of freshly made 10 mM GSSG was added to initiate the enzyme reaction, and the change in absorbance was again measured for 1 minute. The change in optical density per minute (indicating oxidation of NADPH) was used to calculate glutathione reductase activity (described below) from absorbance measurements every 0.3 seconds at 340 nm over a 1 minute period with the scanning spectrophotometer.

Protein analysis

Total protein content (mg) in the enzyme extracts was determined according to the method of Bradford (1976). 5 ml of reagent (100 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml of 95% ethanol, 100 ml of phosphoric acid and 850 ml of distilled water) was added to 100 µl samples of the enzyme extract. The solution was allowed to stand for 15 minutes to allow for colour development. Absorbances were measured at 595 nm with the scanning spectrophotometer.

Standards comprised 100 µl of bovine serum albumin containing between 0 and 1 mg $ml^{-1}$ of protein in Tris buffer (pH 7.7). Blanks (the buffer and added reagent) were measured in each series, and subtracted from the absorbancy values. Protein assays were performed in triplicate.

Enzyme activity

Enzyme activity was calculated as:

$$(\text{Absorbance change } min^{-1} / \text{constant}) \times (1 / \text{protein content mg})$$

Constants were 0.0028 for ascorbate peroxidase and 0.0063 for glutathione reductase. Enzyme activities are expressed for ascorbate peroxidase as the oxidation of ascorbate $min^{-1}$ $mg^{-1}$ protein and for glutathione reductase as the oxidation of NADPH $min^{-1}$ $mg^{-1}$ protein.

**Germination**

After completion of each batch of conductivity measurements, surface water was removed from the seeds with absorbant paper. Seeds were placed onto filter paper in sterilized 90 mm diameter Petri dishes (10 seeds per dish). Half of the seeds were exposed to red light for 24 hours and the other half kept in the dark. Petri dishes containing seeds to receive red light exposure were arranged between two white light sources (40 W) screened by red light filters (35 cm X 35 cm). No significant heating effect was measured from the red lights over the 24 hour exposure period.

Seeds were then moistened by the addition of 6 ml of a solution benomyl (Benlate, Du Pont) at a concentration of 0.5% active ingredient, to prevent fungal contamination. Seeds were incubated in an Electrocool controlled incubator with a diurnal temperature amplitude of 20°C / 10°C for 16 hr / 8 hr in the dark for 40 days, which approximated the cold winter soil temperatures required for germination. Seeds were considered to have germinated when the radical had protruded to 5 mm. Numbers of germinants were counted for each Petri dish daily.

According to Czabator (1961), seed germination can be expressed in terms of the speed of seed germination (an index of seed vigour) and the completeness of germination (an indicator of seed viability or dormancy). Both seed vigour (represented by the peak value) and completeness of germination (represented by the mean daily germination) were calculated for each Petri dish. The germination value (here referred to as the germination index), which is the product of the peak value and mean daily germination, was calculated, as it is considered a good seed germination index (Czabator, 1961). The total numbers of germinants in each Petri dish, at the termination of the experiment, were recorded and expressed as a percentage.

**Seed ultrastructure**

Seed ultrastructure was examined in seeds sampled from each irradiation treatment after 16 weeks of exposure. Outer integuments (pericarp and testa) were removed and a portion of the embryo excised at the position of radical emergence. Excised portions from five seeds were fixed in 2.5% gluteraldehyde (buffered in 0.1 M $NaPO_4$, pH 7.4, containing 0.5% caffeine) for 18 hours. Fixed samples were washed three times in 0.1 M $NaPO_4$ (phosphate) buffer (pH 7.4), postfixed in 1% osmium tetroxide for 1 hour, and rewashed three times in the phosphate buffer. The samples were then dehydrated in a graded ethanol (30, 50, 70, 80, 90, 95 and 100%) series for 80 minutes, and placed in 100% acetone for 20 minutes. Thereafter they were placed into a solution of 50% acetone and 50% Spurr resin (Spurr, 1969) for 4 hours, and then into solutions comprising increasing proportions of resin : acetone over two days, with a final placement into 100% resin twice. Excised portions were then embedded in resin blocks and allowed to polymerize for 16 hrs at 60°C, and then sectioned with an ultramicrotome (Reichert Ultracut-S, Leica Instruments). Tissue sections were stained according to Reynolds (1963) in uranyl acetate for 10 minutes, washed in distilled water, restained in a solution of lead citrate for 10 minutes, and rewashed in distilled water. A transmission electron microscope (JEOL200CX) was used to view the cellular ultrastructure of the maternal reserve tissue of the seed.

**Statistical analyses**

A single factor analysis of variance was used to test for significant differences in measured parameters between irradiation treatments and exposure periods. Percentage values were arc-sine transformed to correct for non-normality in proportions prior to statistical analysis. Significantly different means were separated using the Student Newman-Keuls multiple range test at $P < 0.05$ (Zar, 1996).

## Results

Differences in effects of different wavelengths (visible, UV-A, UV-B1 and UV-B2) of light after equal exposure times, and then the effects of different exposure times (1, 2, 4, 8 weeks) to the same radiation were tested using a single factor analysis of variance. Means, standard deviations, variance ratios and significance levels of single factor ANOVA's are tabulated in Appendix 1.

**Effects of different ultraviolet radiation doses**

Electrolyte leakage

After 1 week of exposure, there were no significant differences ($p > 0.05$) in rates of electrolyte leakage (Figure 1A). Rates of electrolyte leakage after 2 and 4 weeks exposure were significantly higher ($p < 0.05$) in the UV-A irradiated seeds, than in seeds exposed to other irradiation treatments. Significantly increased electrolyte leakage rates ($p < 0.001$) were evident in seeds irradiated with ultraviolet light after 8 weeks of exposure.

Enzyme analysis

No ascorbate peroxidase activity was detected in any of the *L. laureolum* seeds, despite varying the substrate concentration in the assays (results not shown). Activity of glutathione reductase was detected (Figure 1B). Significantly increased glutathione reductase activities ($p < 0.001$) were measured in seeds exposed to ambient and enhanced levels of UV-B for 1 week. After 8 weeks of exposure, significantly increased glutathione reductase activity ($p < 0.001$) was observed in the UV-B2 irradiated seeds.

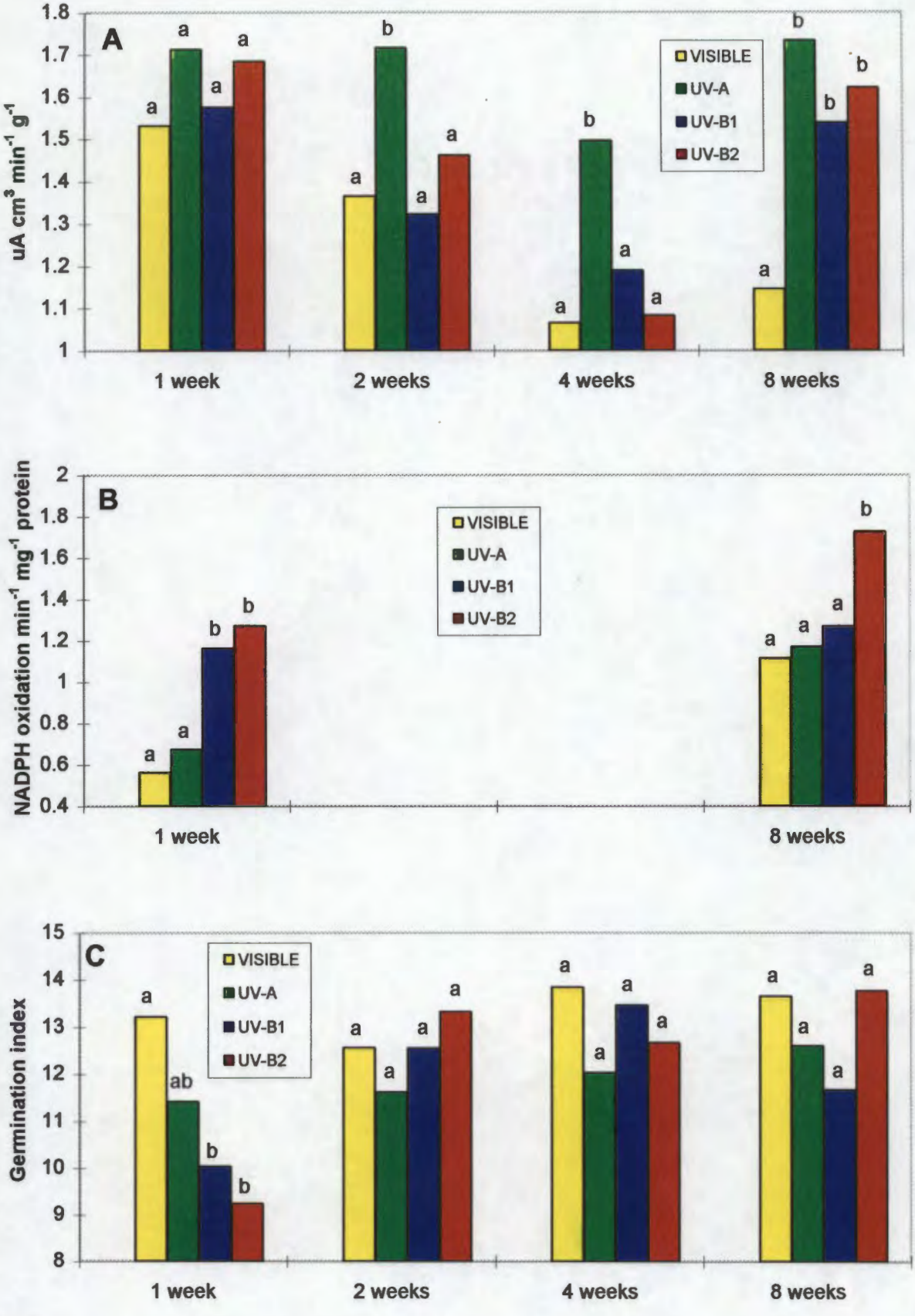


**Figure 1** Effects of different irradiation doses on: **A.** electrolyte leakage, **B.** glutathione reductase activity and **C.** germination index of *L. laureolum* seed. Values with the same letter not significantly different (within treatments) at $P < 0.05$.

Germination

Germination indices in seeds that did not receive red light treatment were compared among irradiation doses (Figure 1C). After 1 week of irradiation, germination indices declined significantly with increasing irradiation dose ($p < 0.01$). No significant differences ($p > 0.05$) in germination values between irradiation treatments were observed with longer exposure periods.

As the germination index incorporates both germination rate and totality, the transformed final germination % and peak value for each treatment were calculated. This was done to determine whether the changes reflected in the germination index (Figure 1C) were due mainly to a change in germination totality (Figure 2A), germination rate (peak value) (Figure 2B), or both. Germination index decline in UV-B treated seeds after 1 week of exposure was found to be the combination of a decrease in germination rate (peak value) and germination totality (transformed germination percentage).

Seeds exposed to red light treatment after 1 week of irradiation did not exhibit the same decline in germination that was observed in UV-B treated seeds in the non-red light treatment (Figure 3). However, differences in germination indices between red light and no red light treatment for all irradiation doses and exposure periods were not significant ($p > 0.05$).

Seed ultrastructure

Plates 1 and 2 illustrate the ultrastructure typical of cells from embryonic storage tissue from seeds subjected to (A) visible, (B) UV-A, (C), UV-B1 and (D) UV-B2 irradiation treatments in *L. laureolum*. The cells of all seeds had irregular nuclei, typical of seeds in a dry state (Farrant, pers. comm.). However clumping of the nuclear material, which often is an indication of cellular damage (Smith & Berjak, 1995), was not observed in any of the cells.

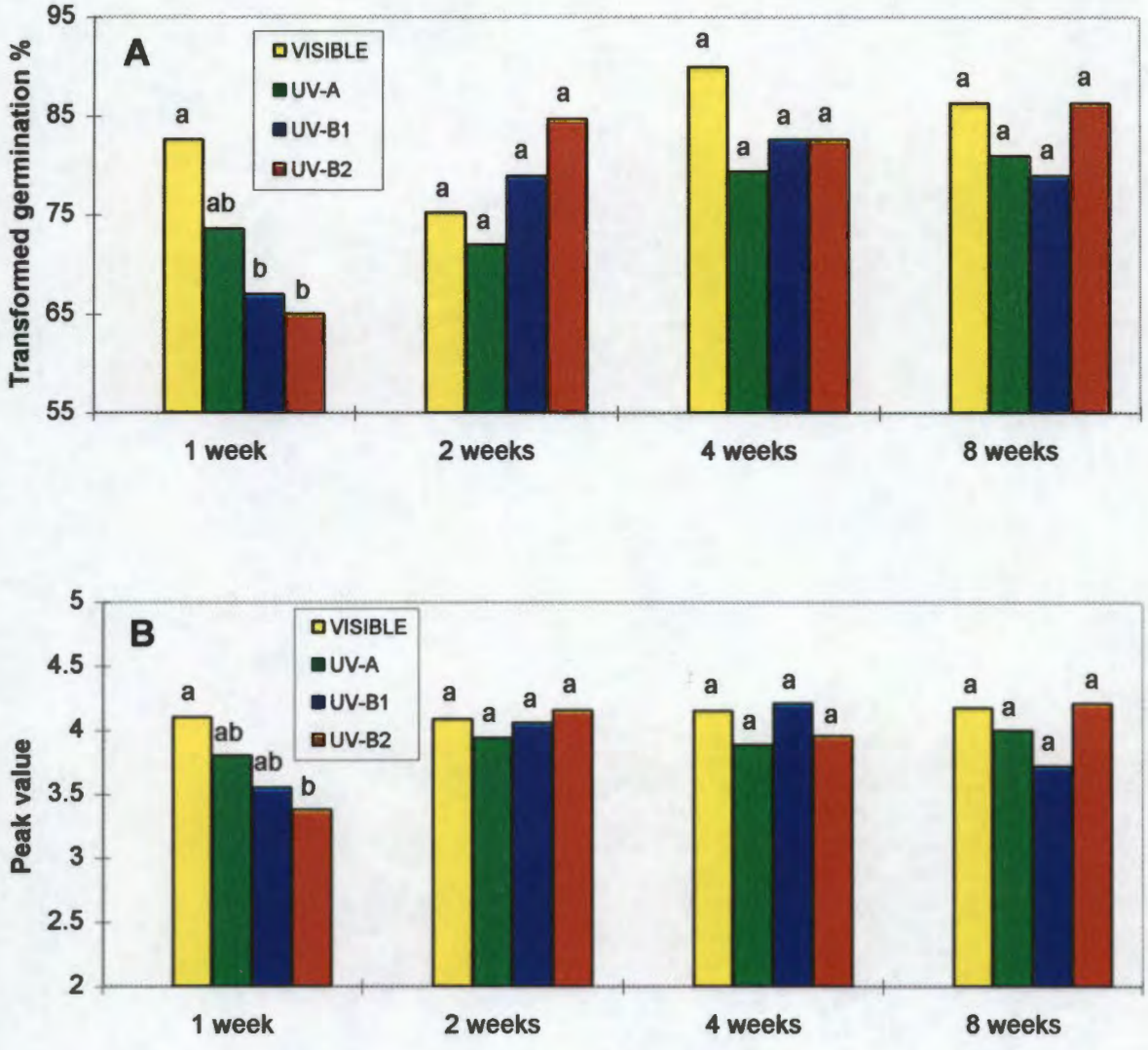


**Figure 2** Effects of different irradiation doses on: **A.** transformed germination percentage and **B.** germination index of *L. laureolum* seed. Values with the same letter not significantly different (within treatments) at $P < 0.05$.

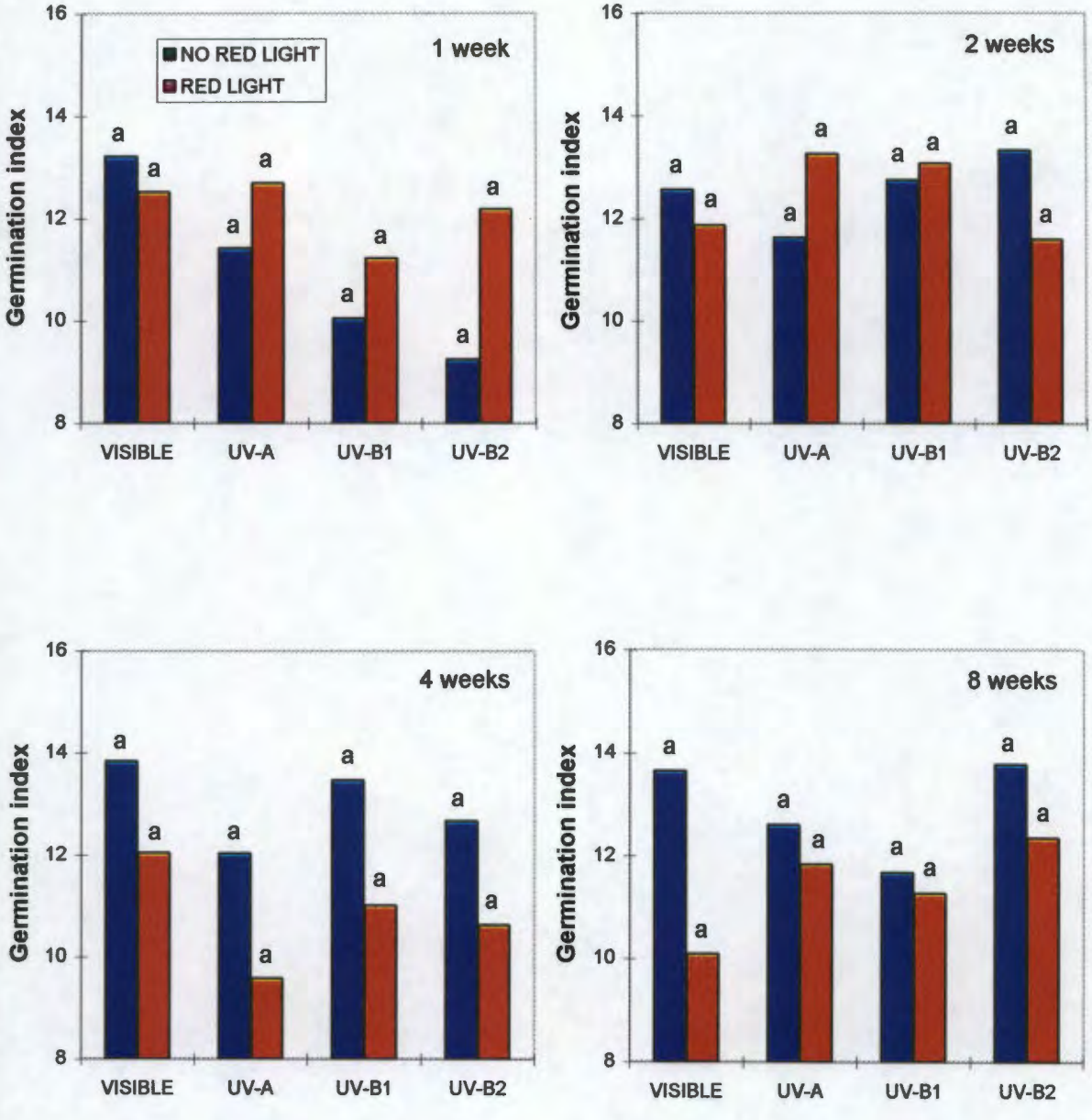


**Figure 3** Effect of 24-hour exposure to red light on germination index of *L. laureolum* seed following differential periods of irradiation with visible and ultraviolet light. Values with the same letter not significantly different (within treatments) at $P < 0.05$.

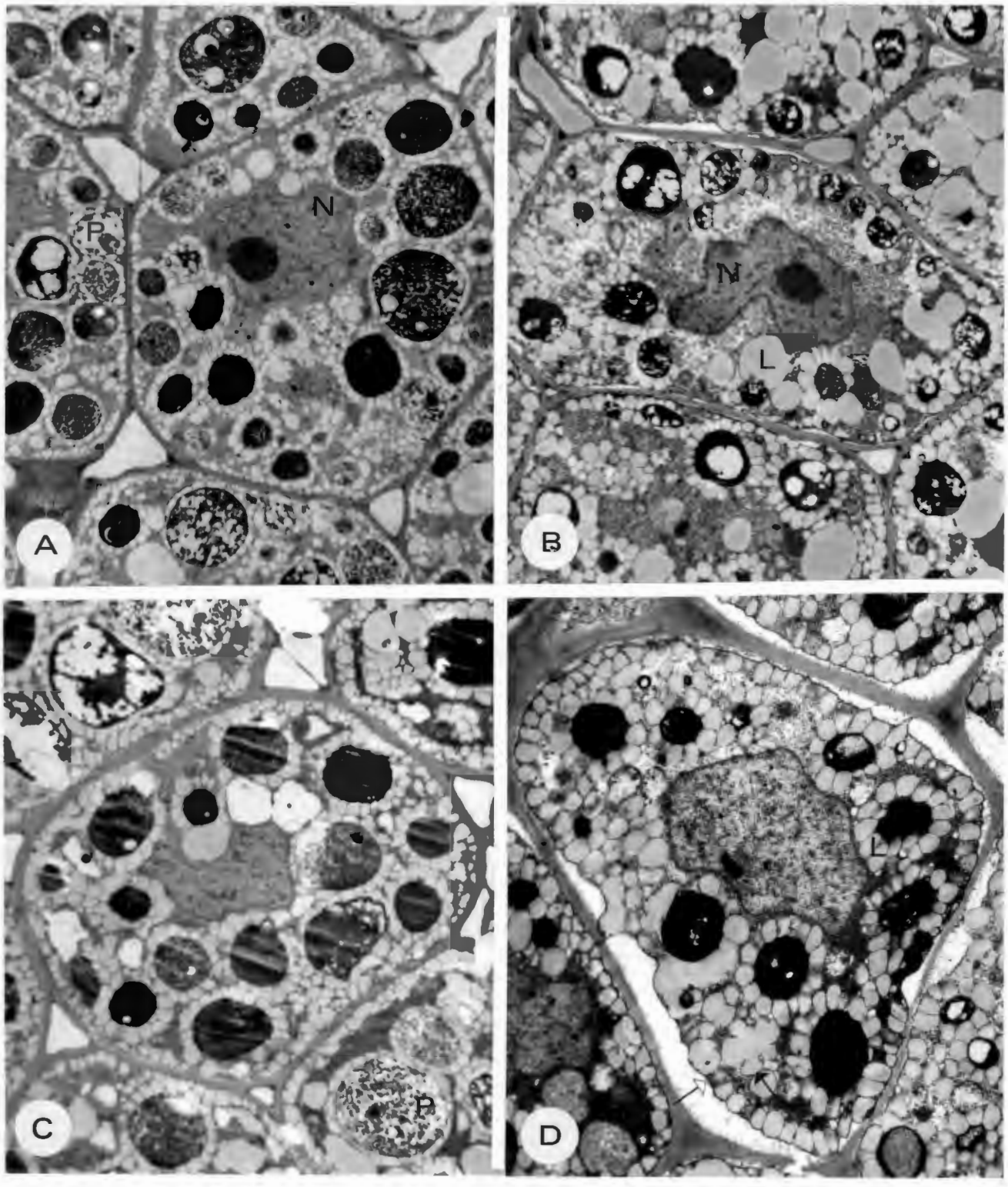


**Plate 1** Cells of storage tissue from embryos of *L. laureolum* exposed to 16 weeks of [A] visible (X 4875), [B] UV-A (X 4380), [C] UV-B1 (X 4250) and [D] UV-B2 (X 8425) irradiation. The solid arrow [D] shows lipid coagulation; the hollow arrow [D] indicates plasmalemma withdrawal. N = nucleus, P = protein body; L = lipid body.

The majority of cellular space was occupied by reserve substances, primarily protein and lipid bodies. Starch grains were mostly absent. Protein bodies were very variable, both within and between cells and treatments. Protein bodies consisting of many or large electron transparent areas contained little protein, whereas electron dense protein bodies contained much protein (Farrant, pers. comm.). Variation in the amount of protein in protein bodies occurred in all seeds. Observations suggested that no real difference existed between protein bodies of seeds of different treatments - yet counts of protein bodies would be necessary to confirm this.

Lipid bodies in seeds exposed to visible light were small, and tightly lined protein bodies and cell walls (Plate 1A). Cells of seeds irradiated with UV-A were quite different (Plate 1B), as coagulation of lipid bodies had taken place, leaving both protein bodies and the cell wall partially exposed to the cytoplasm. Signs of lipid coagulation (solid arrow) in UV-B treated seeds were also visible (Plates 1C and 1D), but there was no lipid movement away from protein bodies and cell walls.

Slight movement of the plasmalemma away from the cell wall (hollow arrow) was observed (for example Plates 1B and 1D) in some cells in all irradiation treatments. However all observations of plasmalemma detachment from the cell wall did not reveal disruption of or damage to the membrane.

Mitochondria from all four irradiation treatments are shown in Plate 2. Generally speaking, few mitochondria and plastids were evident in cells of all treatments, as cells were mostly filled with storage bodies. Mitochondria that were observed were not very active in appearance, and did not possess well defined cristae. According to Bewley and Black (1994), this is typical of dry seeds. Plastids were also present in all cells, but no real differences between treatments were observed. Endomembranes and condensed polysomes were also present, but scarce.

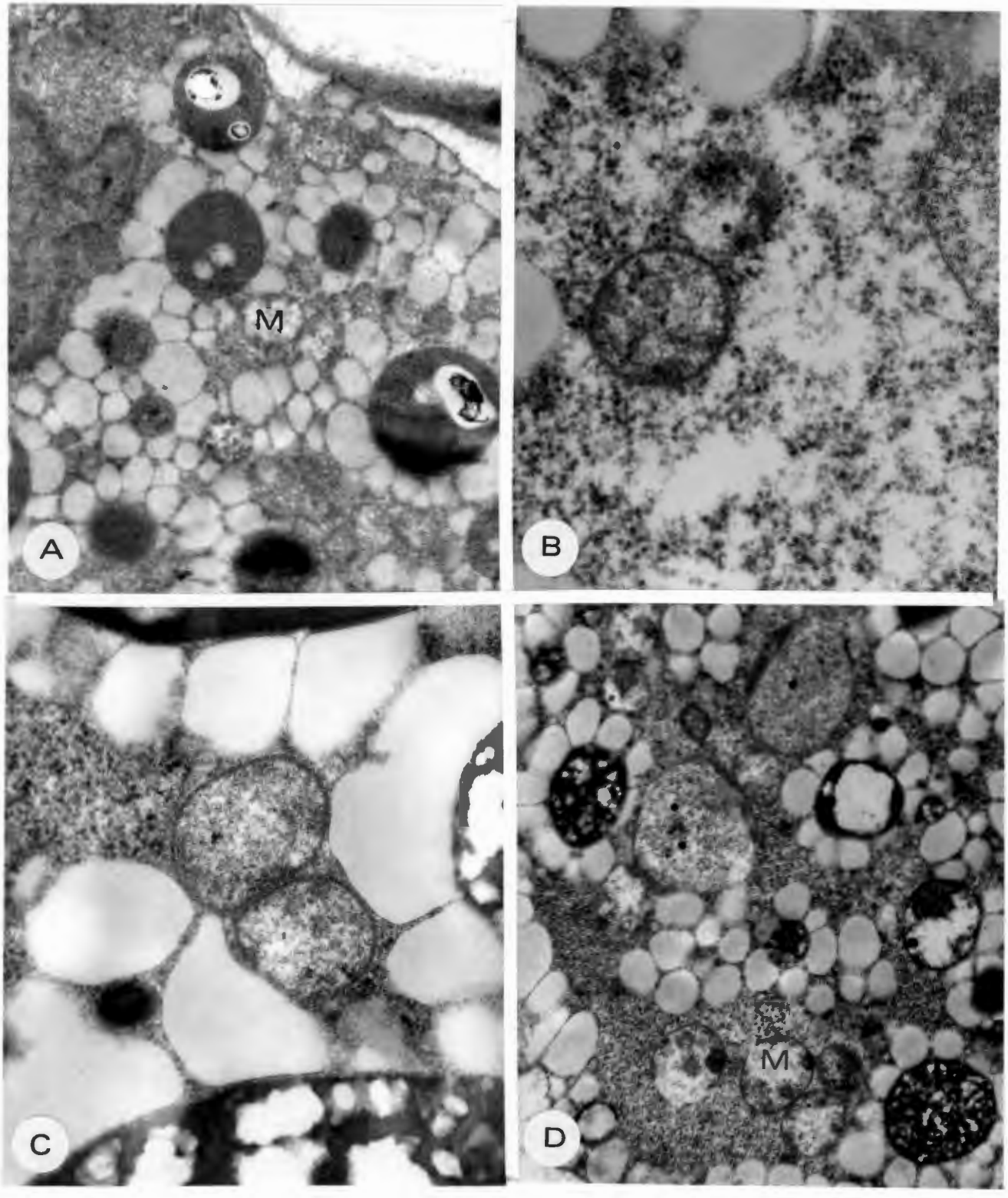


**Plate 2** Mitochondria from storage tissue cells in embryos of *L. laureolum* exposed to 16 weeks of [A] visible (X15750), [B] UV-A (X50700), [C] UV-B1 (X48600) and [D] UV-B2 (X18600) irradiation. There were no obvious differences in numbers or activity of mitochondria between treatments. M = mitochondrion.

**Effects of different ultraviolet radiation exposure periods**

Electrolyte leakage

Rates of electrolyte leakage (Figure 4A) from seeds in all irradiation treatments declined from 1 week to 4 weeks of irradiation exposure, significantly in visible light and UV-B treatments ($p < 0.05$). For UV-B irradiation treatments, the rate of electrolyte leakage after 8 weeks of irradiation was significantly higher ($p < 0.05$) than seeds exposed to 4 weeks of irradiation, but not significantly different ($p > 0.05$) to the shorter exposure periods.

Enzyme analysis

In all irradiation treatments excepting UV-B1, a significant increase ($p < 0.01$) in glutathione reductase activity was observed between 1 week and 8 weeks of exposure (Figure 4B). The percentage increase in enzyme activity between the 1st and 8th weeks was greatest in seeds exposed only to visible radiation, and declined with increasing irradiation dose (Table 1).

**Table 1** Percentage increase of glutathione reductase activity in *L. laureolum* seeds from week 1 to week 8 for the different irradiation doses.

| Irradiation dose | Glutathione reductase activity increase (%) |
|---|---|
| Visible | 97.7% ** |
| UV-A | 73.4% ** |
| UV-B1 | 9.1% |
| UV-B2 | 35.8% * |

Significance: * $p < 0.01$; ** $p < 0.001$.

Germination

Germination indices within treatments generally increased over time. This increase was significant ($p < 0.05$) in the UV-B irradiated seeds between week 1 and the longer exposure periods. The percentage increase in the germination index between the 1st and 8th weeks was greatest in seeds exposed only to visible radiation, and increased with increasing irradiation dose (Table 2).

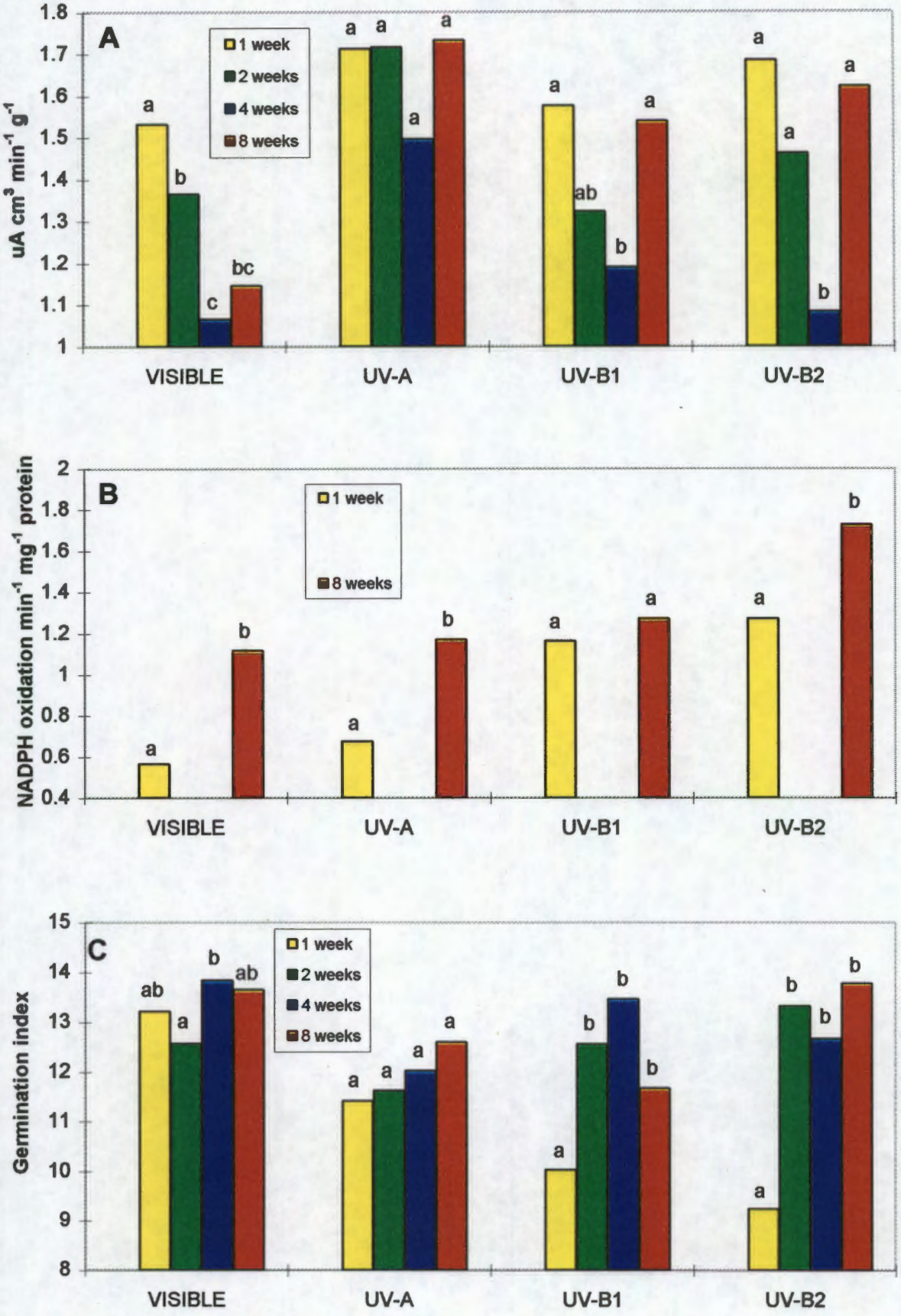


**Figure 4** Effects of different exposure periods on: **A.** electrolyte leakage, **B.** glutathione reductase activity and **C.** germination index of *L. laureolum* seed. Values with the same letter not significantly different (within treatments) at $P < 0.05$.

**Table 2** Percentage increase of the germination index of *L. laureolum* seeds from week 1 to week 8 for the different irradiation doses.

| Irradiation dose | Germination index increase (%) |
|---|---|
| Visible | 3.3% |
| UV-A | 10.3% |
| UV-B1 | 16.2% * |
| UV-B2 | 48.9% ** |

Significance: * $p < 0.05$; ** $p < 0.01$.

## Discussion

The decline in germination of *L. laureolum* seeds with increasing irradiation dose after 1 week of exposure reflected both a reduction in seed vigour and totality of germination. Diminished seed vigour is known to be indicative of seed damage or deterioration (Smith & Berjak, 1995), whereas decreased germination totality may reflect seed damage and viability loss, or alternatively enforced dormancy, in which the seed is viable, but germination is prevented from taking place (Bewley & Black, 1994). However as temperature requirements for dormancy breaking / seed germination in this species were met (Brits, 1986 and Mustart & Cowling, 1991), this germination depression must consequently be attributed to some other factor.

Rates of electrolyte leakage did not increase with decreasing germination indices in the first week, which would be expected if cellular (especially membrane) damage was occurring (Mohamed-Yasseen *et al.*, 1994 and Smith & Berjak, 1995). It seems that the observed decline in germination may be due to inhibition, rather than seed damage. This germination inhibition may have been mediated by a photoreceptor, since this pattern of decline was not evident in the differently irradiated seeds exposed to red light. UV-B radiation has been implicated in phytochrome photoconversion (Pratt & Butler, 1970 in Ballaré *et al.*, 1995), and it is possible that UV-B might be mediating a response in phytochrome, or another photoreceptor, that is also sensitive to red light.

An increase in glutathione reductase activity was observed in UV-B irradiated seeds after 1 week of exposure. The increase in glutathione reductase activity, which prevents free radical damage (Smith *et al.*,1989), was indicative of increased free radical formation. Increased activity of this free radical scavenging enzyme with increasing irradiation exposure, suggests that the increased potential damage to the seed by free radicals is being ameliorated by greater activity of these enzymes in the seed tissue. The absence of ascorbate peroxidase activity in these seeds suggest that this enzyme is not used in *L. laureolum* seeds to prevent free radical damage.

Seeds that were exposed to longer time periods of irradiation interestingly do not show the same decline in seed germination as was observed in seeds irradiated for 1 week; germination in all irradiation treatments was high. Conductivity measurements indicated an increase in electrolyte leakage in seeds irradiated for longer exposures with ultraviolet light. This suggests that slow accumulation of membrane damage in the seed with higher irradiation doses may occur over longer time periods of irradiation.

Ultrastructural studies, although a less sensitive method of detecting seed damage, can reveal signs of seed deterioration by the following ultrastructural changes: (1) chromosome aberrations and chromatin clumping indicating DNA damage; (2) changes in food reserves; (3) changes in mitochondrial status, indicating differences in respiratory activity and ATP production and (5) membrane alterations (Smith & Berjak, 1995). Signs of severe seed deterioration, such as broken membranes or nuclei clumping, were not observed in any of the seeds.

Plasmalemma withdrawal from some cells was observed, but this was consistent throughout the irradiation treatments, and was possibly due to the dry state of the seed and / or the fixation process (Smith & Berjak, 1995). The interesting difference between irradiation treatments was the beginning of lipid coagulation, and movement of lipid bodies away from the plasmalemma and protein bodies, in seeds that had received ultraviolet light.

Lipid coagulation is known as a subcellular event associated with membrane deterioration in the seed (Smith & Berjak, 1995). Movement of lipid bodies away from the plasmamembrane observed in ultraviolet irradiated seeds in the ultrastructural study correspond well with the patterns of electrolyte leakage that were observed seeds exposed to 8 weeks of irradiation. Lipid changes in cells therefore supports the evidence obtained from conductivity studies of initial damage to seed membranes at higher irradiation doses.

Seeds that were exposed to irradiation for longer periods of time exhibited greater glutathione reductase activity. However the percentage increase in glutathione reductase activity between the 1st and 8th weeks declined with increasing irradiation dose. Assuming that the formation of free radicals are linearly related to the length of exposure, it would appear that the UV-B treated seeds do not produce as much glutathione reductase as would be expected. This poor response of UV-B treated seeds compared with the other treatments may indicate that glutathione reductase saturation in the cell could take place, whereafter higher rates of free radical production would begin to cause damage. This is however speculative, as other antioxidant systems (such as superoxide dismutase) which were not measured, could have increased activity to compensate for the relatively lesser amounts of glutathione reductase activity in seeds after 8 weeks of irradiation.

The depression in seed germination with increased irradiation dose after 1 week, with little indication of seed damage, and no subsequent germination depressions in longer irradiation exposures, could possibly be explained by an initial response or adjustment of the seed to the initial supply of high light energy. If the response of seeds to the stimulus is slow, as would be expected in dry seeds that have a slow metabolic rate, then it is plausible that week 1 might still be reflecting those changes. These proposed changes occurring in the few days after seed exposure might be ones that initiate processes of cellular protection or repair, of which glutathione reductase may be one.

Alternately, dependent on the stage of seed afterripening, the depression in germination in ultraviolet treatments after 1 week of irradiation, which disappears over a longer time period, may indicate an initial slowing of seed after-ripening by ultraviolet light. Slowed afterripening may have lead to lowered germination, which over a longer time period disappeared as all seeds became maximally germinable. Seeds were however one year old, and thus it is more likely that depression in germination was due to an adjustment of the seed to the initial supply of high light energy, rather than slowed seed afterripening in higher doses of irradiation.

Slight cellular damage (from ultrastructural and conductivity studies), and increased activity in damage prevention systems (antioxidants) after 8 weeks of irradiation exposure were noticed in seeds that received higher doses of irradiation. This damage was, however, not sufficient to influence seed vigour or viability, indicated by good seed germination in all irradiation treatments at longer exposure times.

Exposure to UV-B may however become important in influencing seed germination or dormancy at longer exposure times. For example, if the graph of glutathione reductase activity were tentatively projected into longer time periods of irradiation, eventually a stage could be reached where the potential for cellular damage to occur may supercede the mechanisms of damage prevention (such as free radical scavenging enzymes) and damage repair. This point would be reached a lot quicker in dry seeds, because of their inherent slow rate of metabolism, compared with plants (Bewley & Black, 1994). Long term irradiation of dry seeds with a greater proportion of high energy light might lead to the formation of free radicals and other subcellular damage faster than what the antioxidants and repair systems of the seed can deal with.

**Future research**

It is unclear whether the difference in the initial depression in germination of seeds exposed to UV-B irradiation after 1 week is real. Studies on processes occurring within the seed during the first week of irradiation would be of value. Processes following the sudden exposure of seeds to light once released onto the ground by fire, would also be of interest.

The potential for damaging effects of ultraviolet light on seeds are clearly obvious in the increase in the free radical scavenging enzyme, glutathione reductase, both over irradiation dose and exposure time. It would be interesting to determine which antioxidant enzymes other than glutathione reductase (for example superoxide dismutase) are activated in the seed, which may assist in protection.

Protective systems other than antioxidants exist in plants which prevent or ameliorate ultraviolet radiation damage. One of these preventative systems are UV-B absorbing molecules, for example phenylpropanoids (Tevini & Teramura, 1989) and flavonoids (Middleton & Teramura, 1993) in leaves and tanniniferous substances in the pericarp of seeds (Beneke *et al.*, 1992). An increase in the number of these molecules in plants following ultraviolet exposure have been shown to decrease or prevent damage (Caldwell, 1983 and Strid *et al.*, 1994). A qualitative and quantitative study of changes in ultraviolet absorbing pigments over different irradiation doses and exposure periods in seed coats of serotinous species, would indicate whether this may be an additional mechanism of protection in the seed.

This study focused on the effect of ultraviolet radiation alone on seed germination behaviour. In nature, it is rarely such a simple cause-and-effect scenario; rather effects are due to a combination of environmental factors, which may enhance or depress ultraviolet effects. Temperature, for example, may result in more pronounced seed damage in combination with ultraviolet light. Irradiation experiments including the variation of another factors (for example summer and winter temperatures) would be useful.

Lastly, a study on vegetative plant performance following several generations of seed irradiation may result in effects that are far more damaging than single generation irradiation studies, as damage may accumulate over several generations. Damage to and changes in DNA, resulting in increased mutation, may result in ecologically significant effects.

## Conclusion

This study has highlighted the importance and necessity of research into the effects of ultraviolet radiation on seeds, especially in terms of seed damage. Results have indicated that higher irradiation doses and longer exposure times of ultraviolet light to seeds are causing initial signs of damage in seeds. Although this study reveals that realistically seed viability and vigour under the tested conditions are not being reduced, the combination of ultraviolet light with changes in other environmental factors, such as temperature may cause more alarming damage. Combination studies associated with increased UV-B radiation (due to ozone reduction), may over successive generations lead to decreases in seed vigour, and possibly even seed viability. Such changes might then be ecologically important, influencing seed germination, seedling establishment and even the competitive ability of the plant. It must be stressed, however, that these results should not be extrapolated to other serotinous species, as species responses to ultraviolet radiation are known to be highly variable.

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## Appendix 1

**Table A** Sample sizes (N), variance ratios (F) and significance levels for single factor ANOVA of electrolyte leakage, enzyme analysis and germination in *L. laureolum* seeds exposed for different periods to irradiation.

| Parameter | N | F | Significance level |
|---|---|---|---|
| **Electrolyte leakage** | | | |
| 1 week exposure | 383 | 0.756 | P = 0.5193 |
| 2 weeks exposure | 376 | 3.663 | P = 0.0126 |
| 4 weeks exposure | 345 | 5.190 | P = 0.0016 |
| 8 weeks exposure | 381 | 5.643 | P = 0.0009 |
| **Glutathione reductase activity** | | | |
| 1 week exposure | 47 | 20.800 | P < 0.0000 |
| 2 weeks exposure | - | - | - |
| 4 weeks exposure | - | - | - |
| 8 weeks exposure | 42 | 10.637 | P < 0.0000 |
| **Germination index** | | | |
| 1 week exposure | 20 | 5.309 | P = 0.0099 |
| 2 weeks exposure | 20 | 0.724 | P = 0.5526 |
| 4 weeks exposure | 20 | 0.892 | P = 0.4665 |
| 8 weeks exposure | 20 | 1.867 | P = 0.1758 |
| **Transformed absolute % germination** | | | |
| 1 week exposure | 20 | 4.915 | P = 0.0132 |
| 2 weeks exposure | 20 | 1.381 | P = 0.2847 |
| 4 weeks exposure | 20 | 0.974 | P = 0.4294 |
| 8 weeks exposure | 20 | 0.710 | P = 0.5603 |
| **Peak germination value** | | | |
| 1 week exposure | 20 | 3.418 | P = 0.0429 |
| 2 weeks exposure | 20 | 0.315 | P = 0.8140 |
| 4 weeks exposure | 20 | 0.631 | P = 0.6058 |
| 8 weeks exposure | 20 | 2.813 | P = 0.0726 |

**Table B** Sample sizes (N), variance ratios (F) and significance levels for single factor ANOVA of germination of red light and non-red light treatment of *L. laureolum* seeds exposed to different irradiation doses for different exposure periods.

| Parameter | N | F | Significance level |
|---|---|---|---|
| **Week 1** | | | |
| Visible | 10 | 0.634 | P = 0.4488 |
| UV-A | 10 | 1.336 | P = 0.2811 |
| UV-B1 | 10 | 0.465 | P = 0.5147 |
| UV-B2 | 10 | 3.174 | P = 0.1127 |
| **Week 2** | | | |
| Visible | 10 | 0.206 | P = 0.6621 |
| UV-A | 10 | 1.480 | P = 0.2585 |
| UV-B1 | 10 | 3.120 | P = 0.7667 |
| UV-B2 | 10 | 0.094 | P = 0.1021 |
| **Week 4** | | | |
| Visible | 10 | 1.789 | P = 0.2178 |
| UV-A | 10 | 3.350 | P = 0.1046 |
| UV-B1 | 10 | 5.033 | P = 0.0551 |
| UV-B2 | 10 | 3.642 | P = 0.0927 |
| **Week 8** | | | |
| Visible | 10 | 4.659 | P = 0.0629 |
| UV-A | 10 | 0.363 | P = 0.5635 |
| UV-B1 | 10 | 0.069 | P = 0.7995 |
| UV-B2 | 10 | 0.920 | P = 0.3655 |

**Table C** Sample sizes (N), variance ratios (F) and significance levels for single factor ANOVA of electrolyte leakage, enzyme analysis and germination in *L. laureolum* seeds exposed to different irradiation treatments.

| Parameter | N | F ratio | Significance level |
|---|---|---|---|
| **Electrolyte leakage** | | | |
| Visible | 359 | 6.220 | P = 0.0004 |
| UV-A | 372 | 0.966 | P = 0.4088 |
| UV-B1 | 376 | 3.408 | P = 0.0177 |
| UV-B2 | 378 | 9.075 | P < 0.0000 |
| **Glutathione reductase activity** | | | |
| Visible | 24 | 26.718 | P < 0.0000 |
| UV-A | 23 | 61.179 | P < 0.0000 |
| UV-B1 | 22 | 0.761 | P = 0.3933 |
| UV-B2 | 20 | 9.757 | P = 0.0059 |
| **Germination index** | | | |
| Visible | 20 | 0.706 | P = 0.5621 |
| UV-A | 20 | 0.277 | P = 0.8411 |
| UV-B1 | 20 | 3.885 | P = 0.0292 |
| UV-B2 | 20 | 8.113 | P = 0.0016 |
| **Transformed absolute % germination** | | | |
| Visible | 20 | 3.333 | P = 0.0461 |
| UV-A | 20 | 0.648 | P = 0.5953 |
| UV-B1 | 20 | 2.651 | P = 0.0841 |
| UV-B2 | 20 | 5.994 | P = 0.0061 |
| **Peak germination value** | | | |
| Visible | 20 | 0.058 | P = 0.9809 |
| UV-A | 20 | 0.222 | P = 0.8796 |
| UV-B1 | 20 | 3.120 | P = 0.0554 |
| UV-B2 | 20 | 4.920 | P = 0.0131 |

**Table D** Mean ± standard deviation and sample size (N) for electrolyte leakage, enzyme activity and germination for each week and irradiation treatment in *L. laureolum*.

| Parameter | 1 week exposure | 2 weeks exposure | 4 weeks exposure | 8 weeks exposure |
|---|---|---|---|---|
| **Electrolyte leakage** | | | | |
| Visible | 1.53 ± 0.93 (95) | 1.36 ± 0.78 (94) | 1.07 ± 0.63 (77) | 1.15 ± 0.80 (93) |
| UV-A | 1.71 ± 0.97 (94) | 1.72 ± 1.16 (95) | 1.50 ± 0.90 (88) | 1.73 ± 1.25 (95) |
| UV-B1 | 1.58 ± 1.02 (99) | 1.32 ± 0.72 (92) | 1.19 ± 0.95 (88) | 1.54 ± 1.08 (97) |
| UV-B2 | 1.68 ± 0.90 (96) | 1.46 ± 0.84 (95) | 1.08 ± 0.72 (92) | 1.62 ± 0.97 (96) |
| **Glutathione reductase activity** | | | | |
| Visible | 0.56 ± 0.24 (12) | | | 1.12 ± 0.28 (12) |
| UV-A | 0.68 ± 0.15 (12) | | | 1.17 ± 0.15 (11) |
| UV-B1 | 1.16 ± 0.35 (11) | | | 1.27 ± 0.21 (11) |
| UV-B2 | 1.27 ± 0.28 (12) | | | 1.72 ± 0.37 (8) |
| **Germination index** | | | | |
| Visible | 13.22 ± 1.58 (5) | 12.57 ± 1.56 (5) | 13.84 ± 1.66 (5) | 13.65 ± 1.13 (5) |
| UV-A | 11.42 ± 1.29 (5) | 11.62 ± 2.14 (5) | 12.03 ± 2.88 (5) | 12.60 ± 2.24 (5) |
| UV-B1 | 10.05 ± 2.18 (5) | 12.76 ± 1.71 (5) | 13.46 ± 1.49 (5) | 11.68 ± 1.21 (5) |
| UV-B2 | 9.25 ± 1.56 (5) | 13.32 ± 1.97 (5) | 12.67 ± 1.21 (5) | 13.78 ± 1.62 (5) |
| **Transformed absolute % germination** | | | | |
| Visible | 82.6 ± 10.1 (5) | 75.3 ± 8.24 (5) | 90.0 ± 0.00 (5) | 86.3 ± 8.24 (5) |
| UV-A | 73.6 ± 9.81 (5) | 72.0 ± 10.8 (5) | 79.4 ± 14.6 (5) | 81.0 ± 12.6 (5) |
| UV-B1 | 67.0 ± 6.70 (5) | 78.9 ± 10.1 (5) | 82.6 ± 10.1 (5) | 78.9 ± 10.1 (5) |
| UV-B2 | 65.1 ± 3.64 (5) | 84.7 ± 11.9 (5) | 82.6 ± 10.1 (5) | 86.3 ± 8.24 (5) |
| **Peak germination value** | | | | |
| Visible | 4.12 ± 0.28 (5) | 4.09 ± 0.32 (5) | 4.15 ± 0.50 (5) | 4.17 ± 0.18 (5) |
| UV-A | 3.80 ± 0.16 (5) | 3.94 ± 0.39 (5) | 3.88 ± 0.55 (5) | 4.00 ± 0.37 (5) |
| UV-B1 | 3.55 ± 0.49 (5) | 4.06 ± 0.34 (5) | 4.21 ± 0.43 (5) | 3.72 ± 0.20 (5) |
| UV-B2 | 3.38 ± 0.50 (5) | 4.16 ± 0.40 (5) | 3.95 ± 0.18 (5) | 4.21 ± 0.38 (5) |

**Table E** Mean ± standard deviation and sample size (N) for germination indices of red and no red light treatments of *L. laureolum* seed, for different irradiation doses and exposure periods.

| Germination index | 1 week exposure | 2 weeks exposure | 4 weeks exposure | 8 weeks exposure |
|---|---|---|---|---|
| **Red light** | | | | |
| Visible | 12.52 ± 1.17 (5) | 11.87 ± 3.05 (5) | 12.04 ± 2.51 (5) | 10.10 ± 3.50 (5) |
| UV-A | 12.69 ± 2.08 (5) | 13.26 ± 2.12 (5) | 9.57 ± 0.84 (5) | 11.83 ± 1.80 (5) |
| UV-B1 | 11.22 ± 3.18 (5) | 13.08 ± 1.62 (5) | 11.01 ± 1.94 (5) | 11.27 ± 3.22 (5) |
| UV-B2 | 12.18 ± 3.33 (5) | 11.60 ± 0.71 (5) | 10.62 ± 2.07 (5) | 12.34 ± 2.91 (5) |
| **No red light** | | | | |
| Visible | 13.22 ± 1.58 (5) | 12.57 ± 1.56 (5) | 13.84 ± 1.66 (5) | 13.65 ± 1.13 (5) |
| UV-A | 11.42 ± 1.29 (5) | 11.62 ± 2.14 (5) | 12.03 ± 2.88 (5) | 12.60 ± 2.24 (5) |
| UV-B1 | 10.05 ± 2.18 (5) | 12.76 ± 1.71 (5) | 13.46 ± 1.49 (5) | 11.68 ± 1.21 (5) |
| UV-B2 | 9.25 ± 1.56 (5) | 13.32 ± 1.97 (5) | 12.67 ± 1.21 (5) | 13.78 ± 1.62 (5) |