Do heat and smoke affect the permeability of the *Grevillea* seed coat to large molecular weight compounds?

Flowers and ripening fruits of *Grevillea linearifolia*

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Abstract

Seeds of east Australian *Grevillea* species germinate in response to fire-related cues such as heat and smoke. The seed coat is responsible for dormancy in *G. linearifolia*: a possible dormancy mechanism is the existence of barriers to diffusion of large molecular weight compounds in the seed coat. Such internal barriers are known to be involved in the dormancy of fire-responsive seeds overseas eg *Emmenanthe pendulioflora*, and the permeability of these barriers is altered by smoke. This model was tested for *G. linearifolia* by investigating the permeability of the seed coat to diffusion of large molecular weight compounds, and whether this changed after exposure to fire cues. The germination characteristics of seeds to heat, smoke, and combined exposure was tested. The penetration of the dye Lucifer Yellow into intact seeds was examined after 24 and 48 hours of exposure, and the penetration of the dye from the inside of the seed coat outwards was examined after 24 hours. Histochemical staining with Nile Red and Acridine Orange was used to locate cuticles, suberin and lignin.

About one-fifth of untreated seeds germinated; both heat by itself, and smoke by itself, increased germination; greatest germination (up to 80%) was observed after treatment with both fire cues. The seed coat of untreated seeds was impermeable to Lucifer Yellow, irregardless of whether the dye was diffusing inwards or outwards, and three barriers to diffusion were identified. Treatment with heat or smoke slightly increased penetration of the dye, but did not completely remove the barriers. Suberin was identified in secondary walls of exotestal and mesotestal cells, and was absent from primary cell walls. Movement of Lucifer Yellow occurred through the middle lamella and primary cell wall of suberized cells; movement of the dye was impeded where suberin was absent.

The *Emmananthe* model was not supported: fire cues did not significantly decrease barriers to diffusion of large molecular weight compounds in the seed coat of *Grevillea*, and must be breaking dormancy by another mechanism.

Introduction

Seeds of many species in fire-prone environments limit germination to the post-fire period by being dormant when shed from the parent plant; dormancy is broken by a fire, allowing germination to proceed. Seeds of east Australian *Grevillea* species show this pattern. Seeds respond to fire-related germination cues such as smoke or heat; the greatest response is to the combination of the two (Kenny, 2000; Morris, 2000). Dormancy in *G. linearifolia* is controlled by the seed coat, as its removal resulted in full germination of all dissected embryos (Morris *et al*., 2000).

A number of mechanisms of seed coat dormancy are known (1) a mechanical restriction of germination, (2) preventing the exit of inhibitors from the embryo, (3) the presence of chemical inhibitors in the seed coat, (4) the restriction of water uptake and (5) the restriction of oxygen uptake (Bewley and Black, 1994). Some of these have been ruled out for *Grevillea*. The seed coat of *Grevillea* is not impermeable to water: seeds imbibe freely when exposed to water (Morris, 2000; Morris *et al.*, 2000). Furthermore, the seed coat inhibitor model was not supported by experiments of Morris *et al.* (2000); dissected embryos still germinated if re-inserted into the seed coat.

A version of the 'prevention of inhibitor escape' model is known for some smoke-responsive chaparral species, which have a sub-dermal barrier that is semi-permeable, allowing water to enter but excluding large molecular weight solutes eg. *Emmenanthe penduliflora, Phacelia grandiflora, Romneya coulteri* and *Dicentra chrysantha*. Exposure to smoke removed the barrier to diffusion of large molecular weight compounds (Keeley and Fotheringham 1998). This was confirmed in some detail for *Emmenanthe penduliflora* by Egerton-Warburton (1998), who showed that the barrier was an internal cuticle in which pores opened up after smoke treatment. It was hypothesized that the embryo of unsmoked seeds contained a large molecular weight germination inhibitor; smoke, by altering the permeability of the internal cuticle, would allow this inhibitor to diffuse out of the seed, and germination to occur (Keeley and Fotheringham 1998; Egerton-Warburon 1998). This mechanism was confirmed, and the inhibitor isolated and identified (Egerton-Warburton and Ghisalberti, 2001). Whether a similar mechanism applies to *Grevillea* dormancy is unknown.

An earlier study detailed the anatomy and histochemistry of the seed coat of untreated seeds from three species (*G. linearifolia*, *G. buxifolia* and *G. sericea*; Briggs *et al.*, 2005). These three species produce the oat-type seed which has an oil-filled eliosome at the chalazal end (Olde and Marriott, 1995; Auld and Denham, 1999). The seed coat had two layers and was similar in all three species. The outer integument or testa was three-layered, with an exotesta (suberized), mesotesta and lignified endotesta. The inner integument or tegmen consisted of two or three layers of non-lignified yet sclerenchymatous elongated cells. A crushed cell layer was found between the tegmen and the embryo. Between the eliosome and the embryo was a multilayered hypostase. Two internal cuticles were identified by histochemical staining with Nile Red and Sudan Black B (the thickest between the endotesta and the tegmen and a thinner cuticle over the inner surface of the endotegmal layer). Suberised secondary walls were found in the exotesta and hypostase.

The hypothesis of seed coat impermeability to large molecular weight compounds was tested for a limited number of unheated, unsmoked seeds of *G. linearifolia* using a tracer dye (Briggs *et al*. 2005). Lucifer Yellow CH (FW 457.2) is a double negatively charged molecule that has frequently been used as an apoplastic tracer and had already been used in the study of seed coat dormancy in *Emmenanthe penduliflora.* The inwards movement of Lucifer Yellow was halted in the seed coat, so that Lucifer Yellow did not penetrate into the embryo. These results were consistent with the hypothesis of internal barriers to diffusion of large molecular weight compounds in the seed coat of unheated, unsmoked *Grevillea* seeds.

The aim of the present study was to confirm the existence of putative barriers to diffusion of large molecular weight molecules for a larger number of unsmoked, unheated seeds, and to determine whether smoke, heat, or the combination of the two, affected this permeability.

Methods

Plant material and treatments

Seeds of *Grevillea linearifolia* were collected during November, 2006 from Killarney Heights and Manly Dam, Sydney, NSW Australia and stored in paper bags at room temperature (RT). Three treatments were used: (i) heat (ii) smoke (iii) heat and smoke. To apply heat shock, seeds in open glass Petri dishes were heated at 80^0 C for

ten minutes in a fan-forced oven, and this process was repeated separately for each replicate container. Seeds were smoked in a glass chamber, each replicate container being smoked individually (Morrison and Morris, 2000). Smoke was produced by burning dry fine fuel litter from a eucalypt forest in a bee keeper's burner forced out by an electric air-pump through a condensing tube, which both cooled and dried the smoke. For seeds that received both treatments, seeds were smoked within one to two minutes after heating. Treated seeds were stored in paper envelopes at RT until examined. Treatments were applied independently four times, with seven – ten seeds in each application.

Test for germinability of seeds

In March-April 2007, a sub-set of the seed lot was treated with the fire cues to determine the germination response of the seeds. For each treatment, six replicates of five seeds each were separately treated with heat, smoke, or heat and smoke (see above). Controls were unheated and unsmoked. Treated and control seeds were transferred to plastic 9 cm Petri dishes on one layer of Whatman No. 1 filter paper, moistened with aqueous fungicide (0.1% Fongarid®) and incubated at a constant 20°C under a 12/12 hour light/darkness cycle. Seeds were regularly checked (approximately weekly) for germination. A seed with a 1-mm long radicle was scored as a germinant and removed. The experiment was ended after eight weeks when the rate of germination was very low eg. no germination had been recorded for 14 days. Germination was calculated as a percentage of initial seeds per Petri dish. Data were analysed as a Two-Way Factorial ANOVA, with smoke and heat as fixed effects. Tests of assumptions indicated transformation of percentage germination data was not required.

Apoplastic tracing with Lucifer Yellow-CH

Inward Diffusion through whole seeds*:* Whole untreated (ie control) and treated seeds were placed into1.5 ml micro-Eppendorf tubes containing 200 μL of 0.1% Lucifer Yellow-CH dilithium salt (ProSciTech C122, CAS #67769-47-5) and placed in the dark for 24 hours at RT. Examination of a small number of replicates indicated that after 24 hours the dye had not reached the embryo, therefore the

diffusion experiment was repeated with the diffusion time doubled to 48 hours. Lucifer Yellow (LY) was replaced with fixative and seeds fixed (see below).

Outward Diffusion through halved seeds: Control and treated seeds were imbibed on moistened filter paper (one hour), blotted, stuck onto microscope slides with double-sided sticky tape and the top part of the seed coat was dissected off.. The exposed embryo was carefully prised out avoiding damage to the tegmen. Microscope slides with attached seed coat shells were placed into Petri dishes on top of moistened filter paper. Lucifer Yellow CH (0.1%) was applied to the inner surface of the seed coat, the dish was covered and placed in the dark at RT for 24 hours. After 24 hours LY was removed and the seed coats were fixed (see below). This experiment was carried out twice: firstly using seeds three months old (three seeds from each treatment) and again when seeds were12 months old (10 seeds from each treatment). Germination of untreated seeds was greater in the older seed lot than in the original experiment (unpublished data).

Fixation of tissue

All seed material was fixed for 24 hours at 4°C in 1 ml of 2.5% glutaraldehyde/3% paraformaldehyde in PBS. Whole seeds traced with LY were rinsed with fixative, cut under fresh fixative into four segments (2-3 mm in length) and transferred to clean vials; empty shells were rinsed with fixative then placed into fresh fixative without further cutting. To determine aldehyde-induced autofluorescence, control and treatment seeds were imbibed for 20 minutes, sectioned under fixative into four segments, then fixed as above. Following fixation, seeds were rinsed with three changes of distilled water, gradually dehydrated to 40 or 50% ethanol, then stored at 4°C until examined.

Histochemical staining

Hand-cut transverse and longitudinal sections were taken from imbibed, unfixed seeds or fixed LY-traced seeds (control and treated) using one half of a double-sided razor blade. LY-traced sections were first examined without counter staining, then the same section was stained with Nile Red (see below).

For *cuticles and neutral lipids,* sections were covered by freshly diluted Nile Red (40 μg/ml) (Oparka and Read, 1994), stained for 40 to 60 minutes in the dark, rinsed with distilled water then mounted in glycerol or distilled water.

For *cutin, lignin, negatively charged walls,* sections were covered with freshly made Acridine Orange (0.1% aq) stained for five minutes in the dark (Oparka and Read, 1994), rinsed and mounted in distilled water.

Autofluorescence

To determine any contribution to emission due to autofluorescence, unfixed and fixed control and treatment seeds were also examined at the various wavelengths and gain settings used in this study. Dry seeds were imbibed for 20 minutes with distilled water then hand-sectioned (see above), fixed seed segments were re-hydrated before sectioning. Sections were mounted in water or glycerol for examination.

Microscopy

All sections were examined on a Leica TCS SP5 Laser Scanning Confocal Inverted Microscope using 10x or 20x (water/glycerol) objectives. Confocal imaging was through the Leica Application Suite Advanced Fluorescence Software (1.5.1 Build 869). Sections were first examined using epifluorescence microscopy to determine the presence and gross distribution of LY then confocally with an Argon laser and the following excitation wavelengths and PMT combinations. *Nile Red and LY* - 488 nm laser line, PMT1 (500-585 nm) and PMT2 (590-730 nm) or 488 nm and 543 laser lines, PMT1 (500-560 nm) and PMT2 (610-740 nm). *LY and Acridine Orange* - 458nm and 514 nm laser lines, PMT1 (450-510 nm) and PMT2 (580-650 nm). *LY alone* - 458nm laser line, PMT1 (460-570 nm) and PMT2 (572-670 nm). *Acridine Orange alone* - 514nm laser line, PMT1 (520-555 nm) and PMT2 (558-630 nm). Images were collected in 1024x1024 pixels format with eight or 16 times line averaging. Gains were adjusted to give maximum definition, clarity and trueness of colour (ie Lucifer Yellow to appear yellow, cuticles to appear orange-gold as seen under epifluorescence). Images were exported in .tiff format.

Replication

Overall 102 seeds were examined by Confocal microscopy; 22 seeds for the 24 hr LY inward diffusion experiment (four each for controls, heat-treated, smoke-treated and ten heat+smoke treated); 24 seeds for the 48 hr LY inward diffusion experiment (six seeds from each of the treatments and six from the controls); 52 seeds for the 24hr LY outward diffusion (13 from each treatment and controls); the remaining seeds were unfixed or fixed for autofluorescence.

Results

Germination response to treatments

About one-fifth of untreated seeds germinated (Fig. 1). The fire cues increased germination independently and additively, heat increasing germination to 50%, and smoke to 67% (Fig. 1; heat main effect, $F_{1,20} = 11.27$, $P = 0.003$; smoke main effect, $F_{1,20} = 35.27$, $P < 0.001$). Seeds that received both smoke and heat showed *c*. 80% germination (Fig. 1).

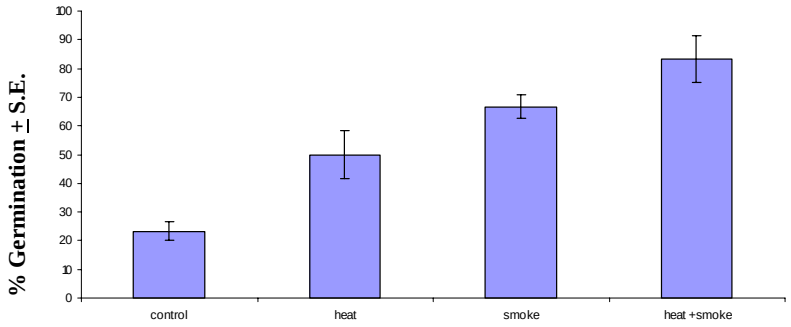


Fig. 1. Mean germination of *G. linearifolia* seeds which were untreated (control), or exposed to heat shock, smoke, or heat and smoke combined.

Zonation of the seed coat

During the initial LY tracing experiments, characteristic differences in depth of diffusion of the dye into the seed coat were detected. This occurred both longitudinally along the seed (termed zones, Fig. 2a) and transversely in the ventral groove (termed regions, Fig. 2b). In TS view, region G1 in zones one, two and four, had two layers of columnar cells in the exotesta increasing to three or four layers in zone three, while regions G2 and G3 usually only had one; exotestal cells of G3 were curved not straight (Fig. 3a). The cells forming the innermost layer of the mesotesta were distinct from those forming the other mesotestal layers. They were small with thin, tightly appressed radial walls and were filled with a brown pigment.

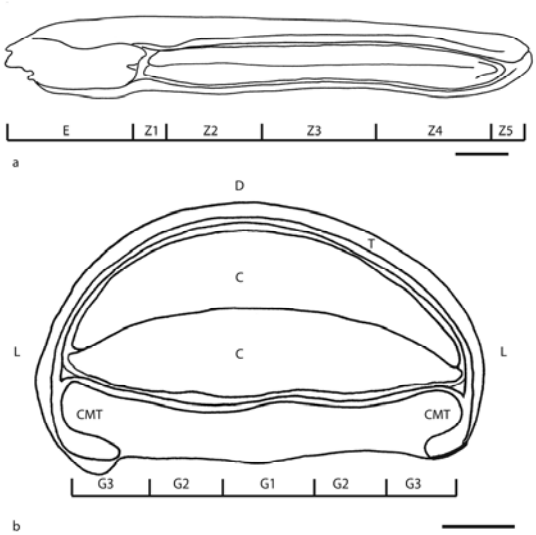


Fig. 2a. Map diagram (LS) showing the longitudinal zonation of the seed. Scale bar = 1 mm; Fig. 2b. Map diagram (TS) showing the regions of the seed in transverse section. Scale bar = 0.5 mm

Autofluorescence

The intensity of autofluorescence depended upon the laser power and gains selected, but when reduced to the setting used to collect the images, autofluorescence was negligible and/or clearly different from the emission colour induced by the dye. There was no difference in autofluorescence colours between fixed and unfixed material.

Distribution of Lucifer Yellow CH following 24 and 48 hours inwards diffusion into intact seeds

After 24 hours immersion in LY, the dye had not passed beyond the middle layer of the mesotesta in any of the control or treated seeds. At the eliosome end, the dye was found in the walls of the oil-filled cells occupying the centre of the eliosome, and in the walls of the adjacent inner tegmal cells. The dye did not penetrate beyond the hypostase. In the dorsal and lateral parts of the seed, it fluoresced intensely in the walls of the squat-shaped exotestal cells; where this layer was broken it was also present in the walls of a few adjacent mesotestal cells. In the ventral groove, the walls of the columnar-shaped exotestal cells exhibited intense LY-induced fluorescence and in the treated (Fig. 3a) and control seeds (Fig. 3b), walls in the outer one to three layers of mesotestal cells also fluoresced. The depth of diffusion by LY was not consistent between the treatments nor between seeds from the same treatment, nor along the length of the same seed nor along the ventral groove (Fig. 3a). The greatest penetration into the mesotesta usually occurred in zones two and three (Fig. 3c).

After 48 hours inward diffusion, LY still had not reached the embryo or tegmen layers in either the control or treated seeds. In the control seeds, there had been further inward movement of LY through the dorsal and lateral surfaces (Fig. 3d), but again it was variable along the length of the seed and between seeds. In the ventral groove, the depth of penetration by the dye was variable along the groove. In only one seed were traces of the dye found in the endotestal layer and then only in the outer third of the cell (Fig. 3e).

In seeds from the three treatments, LY had also penetrated further into the testa, but again, there was variation between seeds from the same treatment and, furthermore, there was no clear difference between the three treatments. Of all of the seeds examined, in only one heat-treated and one smoke-treated seed (20 min smoke treatment) was LY present in the ventral endotesta overlying regions G1 and G2 (Fig. 3f), but traces were found in the radial walls of the innermost layer of the mesotesta (ie layer five) in one smoke-treated (Fig. 3g) and two heat+smoke (20min smoke) treated seeds. In one heat-treated seed, LY had diffused throughout the curved part of G3 region and into the abutting endotestal cells; it had also diffused through the adjacent lateral mesotestal cells and entered the abutting endotesta cells (not shown). The longitudinal zonation pattern of LY diffusion found in the control seeds was repeated in each of the three treatments.

Distribution of Lucifer Yellow CH following 24 hours outwards diffusion from the embryo cavity

Outward diffusion by Lucifer Yellow was stopped either in the crushed cell zone (Fig. 3h) or in the tegmal layer (Figs 3i,j); no dye was found outside of the outer of the two inner cuticles nor within the abutting endotestal cells. There was a fairly consistent pattern exhibited by all seeds examined from each of the three treatments and the control. In the control seeds LY was mostly confined to the crushed cell layer lying along the ventral innermost inner cuticle (Fig. 3i). Transverse sections from Zones three and four of treated seeds, revealed that the dye was located solely within the crushed cell zone in regions G1 and G2 (Fig. 3h), but in region G3, it was also found within the mesotegmal part of the wedge tissue; it was not present in the exotegmen or abutting endotesta (Fig. 3j). In these seeds, the dye had crossed the innermost cuticle either where it was broken or through cuticular pores (Fig. 3j) Longitudinal sections incorporating Zones three to five revealed that the dye gradually penetrated further outward into the exotegmal layer (Zone four) until, in Zone five, it was all through the tegmen. Medial LS through the micropyle also revealed no trace of LY outside the cuticle, nor within the micropyle, nor within the abutting endotestal cells (not shown).

Staining with Nile Red for cuticles, suberin and neutral lipids

Nile Red stained the outer cuticle overlying the ventral groove (Fig. 3k) and the two inner cuticles (Figs 3h,j) an intense gold, orange or red, depending on the excitation wavelengths used. The external cuticle overlying the lateral and dorsal surfaces was minimal, fragmentary or absent in the controls (Fig. 4a) and treated seeds (Fig. 4b). The thickened suberized walls of the squat (Figs 4a,b) and columnar (Fig. 3k) exotestal cells fluoresced red. As in the earlier study (Briggs *et al*. 2006), one inner cuticle was located between the testa and tegmen, the other on the inner surface of the tegmen (Fig. 3h).

In some control and treated seeds, small groups of mesotestal cells in the dorsal (Figs 4a,b) or lateral regions had thickened, red-fluorescent walls; in the ventral groove, mesotestal cells in the outer layers often had thickened, red-fluorescent walls

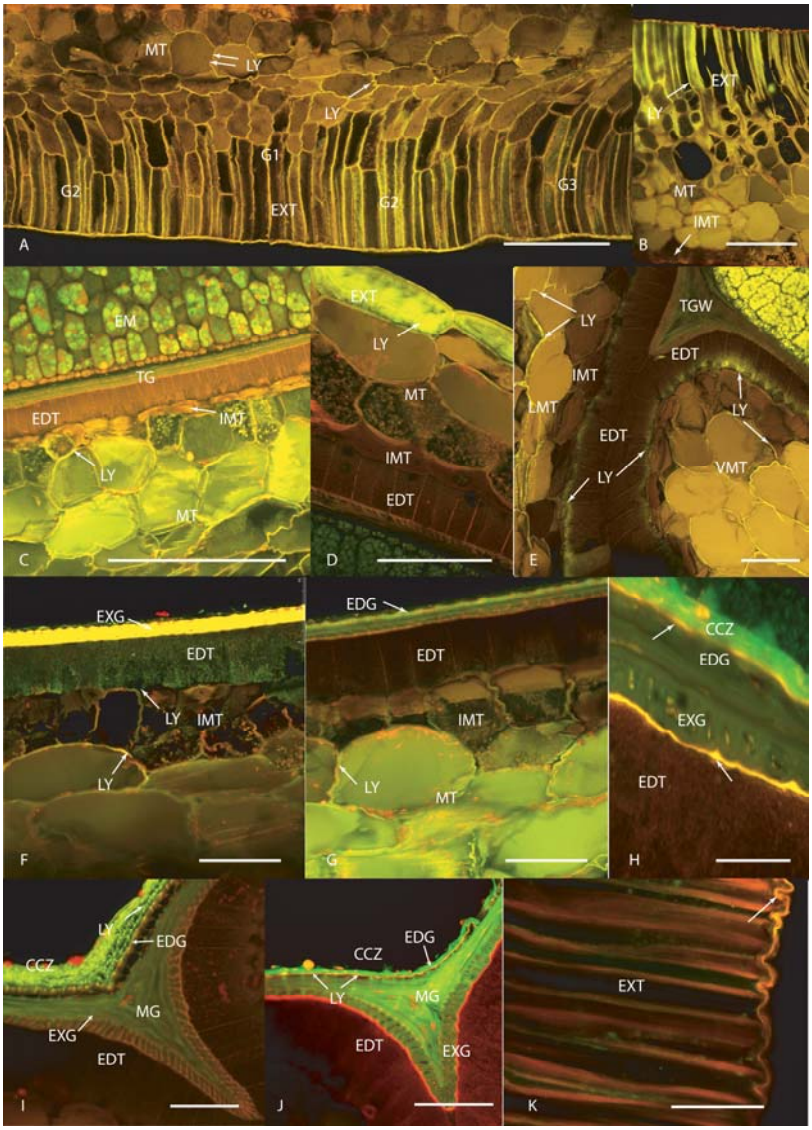
(Fig. 4c). However, the extent of thick-walled mesotestal cells in the ventral groove varied between seeds and even within the various zones of the same seed.

Staining with Acridine Orange for cutin, lignin and negatively charged material

The fluorescence emission spectra induced by Acridine Orange depended upon the excitation wavelength and PMT ranges used. In unfixed control material excited with 514 nm laser line, the suberized walls of the exotesta fluoresced yellow- red and the lignified walls of the wing endotesta fluoresced yellow; the unsuberized walls of the mesotestal, exotegmen and embryo also fluoresced yellow. However, when excited with 488nm laser line, the lignified walls fluoresced emerald-green. When LY-traced sections were counter-stained with 0.1% AO and excited at 488nm, the LY-induced yellow fluorescence could be clearly seen amongst the AO-induced orange-red suberised walls of the exotesta (Fig. 4d) and the emerald-green fluorescent lignified walls and interior lacework of the wing endotesta (Fig. 4e). Where LY was present in the non-suberised walls of the mesotesta, the yellow fluorescence was often masked and the walls fluoresced orange-brown (Fig. 4f).

Location of Lucifer Yellow in the apoplast and relationship to the suberised wall layer

LY-induced fluorescence was only found in the primary wall layer of the columnar exotestal cells ie outside the suberised secondary wall (Figs 4g,h). In the ventral mesotestal cells, LY-induced fluorescence occurred as a solid continuous line along the outer surface (or primary cell wall) of cells with a suberised secondary wall layer (Fig. 4i), but in non-suberised cells, it occupied the entire wall (Fig. 4j). In the lateral and dorsal mesotesta LY-induced fluorescence was again in the primary wall of non-suberized cells (Fig. 4k). In the squat-shaped exotestal cells, LY-induced fluorescence was in the thin outer primary wall layer and also within the non-suberized parts of the lamellated, suberized secondary wall (Figs 4k,l).



Figs 3 a-c. Movement of Lucifer Yellow (LY) into the seed following 24 hours immersion. XS.

Fig. 3a. Heat and smoke-treated seed, Zone two, ventral. LY had penetrated up to the innermost layer of the mesotesta (double arrows) in G1 and G2 regions. There are two-three layers of columnar-shaped cells in the G1 region. These cells are probably exotestal in origin. The intensity of LY-induced fluorescence is greatest in the columnar exotestal cells, decreasing in the outer squat-shaped mesotestal cells (single arrow) and is weakest further inwards (double arrows). Scale bar = 250 μm

Fig. 3b. Control seed, ventral. LY-induced fluorescence was found halfway into the mesotestal layer of the testa. Scale Bar = 180 μm

Fig.. 3c. Heat and smoke-treated seed, Zone three, ventral, merged image. LY had penetrated through the mesotesta reaching but not entering the innermost mesotestal layer (IMT). Embryo (EM), endotesta (EDT), tegmen (TG), mesotesta (MT).

Scale bar = 250 μm

Figures 3d - 3g. XS through whole, intact seeds that were immersed in Lucifer Yellow for 48 hours.

Fig. 3d. Control seed Zone three, dorsal. LY has diffused beyond the exotesta into the outer mesotestal layer. Endotesta (EDT), exotesta (EXT), innermost mesotestal layer (IMT), mesotesta (MT). Scale bar = 50 μm

Fig. 3e. Control seed, Zone two, ventral G3. LY penetrated through the lateral mesotesta (LMT) into the outer part of the endotesta cells forming the neck region of the endotesta wing (WEDT). LY has also diffused through the curved area of the ventral groove into the endotesta. Endotesta (EDT), innermost mesotestal layer (IMT), tegmal wedge (TGW). Scale bar = 100 μm

Fig. 3f. Smoke-treated seed, Zone three, ventral, G1. LY penetrated through the mesotesta and into the endotesta (EDT). Innermost mesotestal layer (IMT), exotegmen (EXG). Scale bar = 50 μm

Fig.3g. Smoke-treated seed, Zone three, ventral, G1. LY has diffused through the mesotesta (MT) and entered the radial walls of the innermost layer of the mesotesta (IMT), but has not progressed into the abutting endotesta (EDT). Scale bar = 50 μm

Figures 3h-3k. XS of seeds that were halved, the embryo carefully removed and Lucifer Yellow applied to the cavity for 24 hours outward diffusion.

Fig.. 3h. Heat and smoke-treated seed, Zone three, G 1, counterstained with Nile Red. LY was confined to the crushed cell zone (CCZ). The two inner cuticles (arrows) fluoresced gold. Endotegmen (EDG), exotegmen (EXG), endotesta (EDT). Scale bar = 25 μm

Fig.. 3i. Control seed, Zone three, tegmal wedge. LY is retained in the crushed cell zone (CCZ) with no passage through the inner cuticles. Endotegmen (EDG), exotegmen (EXG), endotesta (EDT), mesotegmen (MG). Scale bar = 50 μm

Fig.. 3j. Heat and smoke-treated seed counterstained with Nile Red, Zone three, tegmal wedge. LY was present in the crushed cell zone (CCZ) and within the tegmal layers. Stronger lines of LY indicated where the dye has moved into the tegmen via cuticular pores (arrows). A break in the CCZ and the inner cuticle also permitted movement of the dye into the tegmen. The thicker inner cuticle (large arrow) between the endotesta and the exotegmen fluoresces red rather than orange. Endotesta (EDT), endotegmen (EDG), mesotegmen (MTG), exotegmen Scale bar = 50 μm

Fig. 3k. Control seed, unfixed, Zone one, ventral, G1, stained with Nile Red. The suberized walls of the exotestal (EXT) cells strongly fluoresce red. The overlying outer cuticle (arrow) fluoresce orange gold. Scale bar = 50 μm

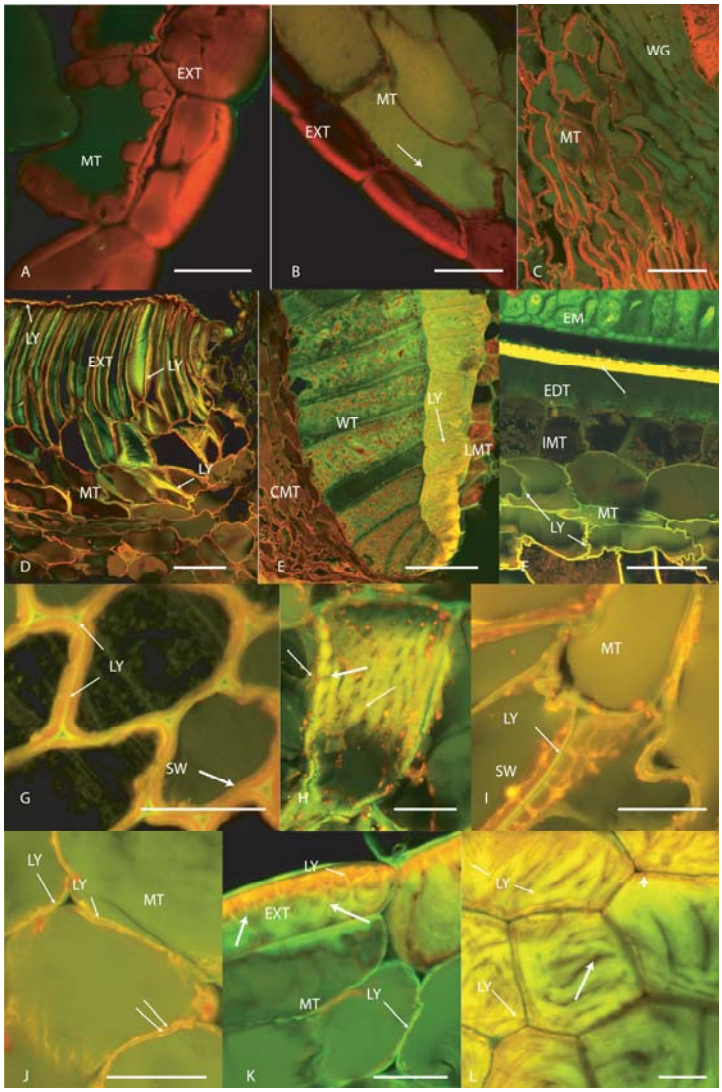


Fig. 4a. Control seed, fixed, hypostase Zone one, dorsal, stained with Nile Red. Strong red fluorescence from the suberized walls of the squat-shaped exotestal cells (EXT) and underlying outer mesotestal cells (MT). Unsuberized walls show no fluorescence. There is no sign of an overlying cuticle. Scale bar = 25 μm

Fig. 4b. Heat and smoke-treated seed, fixed, Zone two, lateral, stained with Nile Red. Strong red fluorescence from the thick walls of the exotestal (EXT) cells, weaker from the thinner suberized walls of the adjacent mesotestal cells (MT). No outer cuticle is present. The unsuberized walls are not fluorescent but there is aldehyde-induced fluorescence from the vacuolar contents (arrow). Scale Bar = 50 μm

Fig. 4c. Control seed, fixed, Zone one hypostase, ventral, G3, stained with Nile Red. Suberized mesotestal (MT) walls stained red, but unsuberized mesotestal walls are unstained. Strong red fluorescence from the wing endotesta cells (WG). Scale bar = 75 μm

Figs 4d-4f. Seeds immersed in Lucifer Yellow for 48 hours, rinsed, fixed and sections counterstained with Acridine Orange.

Fig.. 4d. Control seed, Zone four/five, ventral, G3. [Laser line 488 nm, PMT1 500-598 nm, PMT 2 590-740nm). Lucifer Yellow (LY) is trapped between the outer cuticle and the suberized outer tangential walls (red/brown/orange) of the exotestal cells (arrow). When LY is seen in face view against the suberized walls, it appears more green than yellow (arrows). Under these settings the unsuberized walls of the mesotesta show a orange-brown colour. Scale bar = 100 μm

Fig.. 4e. Heat-treated seed, Zone four/five, G3. [Laser line 488nm PMT1 500-589nm, PMT2 590-740nm; Z Projection 34 steps, 0.75 μm step width). Wing endotesta (WT) adjacent to the raphe vascular trace. Lucifer Yellow (LY) has diffused through the walls of the lateral mesotesta (LMT) into the radial and tangential walls of the outer layer of cells forming the endotestal wing. No diffusion of Lucifer Yellow occurred beyond these cells into the remaining wing cells. The red-brown fluorescence of the cells in the curved mesotestal (CMT) region indicates lack of penetration by LY beyond the ventral exotesta. Under these settings, where Lucifer Yellow was absent in the wing region, the lignin component of the internal lacework wall system and radial walls fluoresced green; pigment in the wing cells and mesotestal cells fluoresced red-brown. Scale bar = 75 μm

Fig.. 4f. Smoke-treated seed, Zone three, G1. Inner half of the testa and abutting tegmen. Lucifer Yellow (LY) had diffused through the mesotesta reaching, but not entering, the innermost mesotestal layer (IMT). The counter-staining by Acridine Orange of thin-walled, unsuberized mesotestal cells has altered the usual yellow colour of Lucifer Yellow to pale orange-brown in some parts of the wall. Mesotesta (MT), endotesta (EDT), embryo (EM), tegmen (TG). Scale bar = 50 μm

FIGS 4g-4l. 24 hours immersed in Lucifer Yellow, fixed, cross-sectioned and sections counterstained with Nile Red.

Fig. 4g. Smoke-treated seed, Zone three. Tangential view through the columnar exotestal cells. Lucifer Yellow (LY) was only found in the outer wall layer overlying the suberized secondary wall (SW) which fluoresces red following staining with Nile Red. No Lucifer Yellow was preserved in the intercellular spaces by fixation. Scale bar = 25 μm

Fig. 4h. Smoke-treated seed, Zone three, G1. Columnar-shaped mesotestal cell showing the presence of Lucifer Yellow in the primary wall but not in the plasmodesmata (large arrow). The red autofluorescent material (double arrows) present in the peripheral layer of the cytoplasm can be seen associated with some of the plasmodesmata. Scale bar = 25 μm

Fig. 4i. Control seed, Zone two, G2. In suberized mesotestal cells, Lucifer Yellow (LY) is only present in the outer, thin primary wall overlying the thickened secondary wall (SW) which fluoresced red with Nile Red. No Lucifer Yellow occurred in the intercellular spaces (arrow). Scale bar = 25 μm

Discussion

This study confirmed the earlier preliminary evidence of Briggs *et al*. (2005) for the presence of barrier(s) to the diffusion of large molecular weight compounds in the coat of untreated *Grevillea linearifolia* seeds. This makes them similar to dormant seeds of chaparral species such as *Emmenanthe penduliflora*. Other similarities include: the external cuticle does not restrict entry of apoplastic dyes, and the seed coat is water-permeable, for both (Briggs *et al*. 2005; Keeley and Fotheringham 1998).

Differences exist however between seeds of *G. linearifolia*, and those of *E. penduliflora:* in the structural location of the barrier(s) within the seed coat, and whether fire cues removed the barrier(s). The seed coat of *E. penduliflora* has a single integument and the barrier to diffusion was an inner cuticle, located on the inner surface of the seed coat adjacent to the endosperm (Egerton-Warburton 1998). The seed coat of *Grevillea*, on the other hand, had multiple barriers to diffusion of large molecular weight compounds; of particular importance were the two inner cuticles resulting from the two integuments of the seed coat. In *G. linearifolia*, the diffusion of Lucifer Yellow outwards from the crushed cell layer through the seed coat was mostly stopped by the cuticle lining the inner surface of the tegmen; the dye appeared in the tegmen only where there were noticeable breaks in the inner cuticle or through cuticular pores near the tegmen wedge. It did not penetrate beyond the outer cuticle lying between the tegmen and endotesta. When Lucifer Yellow diffused from the outside of the seed inwards, the barrier to diffusion lay further out in the seed coat than either internal cuticle. The innermost layer of the mesotesta was the greatest distance inwards that Lucifer Yellow penetrated in most seeds; occasionally, the dye made it past this layer and into the outermost part of the endotesta. The distances for the dye to traverse were similar in the two species (*c*. 200 μm across the seed coat of *Emmenanthe*; *c.* 200 – 250 μm in the dorsal region of *Grevillea* seeds, 550 – 600 μm in the ventral region). The lack of penetration of the dye into the seed coat of *Grevillea* was due to structural features, rather than differences in the distance of diffusion. The exception to the presence of multiple barriers in *Grevillea* occurs where the top ends of the cotyledons are adjacent to the hypostase, which at this location forms a single barrier to diffusion of Lucifer Yellow to or from the embryo. The two integuments are not continuous around the embryo here (Briggs *et al*., 2005).

Unlike in *Emmenanthe*, the fire cues did not remove the barriers to diffusion of large molecular weight compounds in the seed coat of *Grevillea*. In smoked seeds of *E. penduliflora*, Lucifer Yellow appeared in the embryo after two hours of exposure to the dye, as smoke scarified the external cuticle and opened up pores within the internal cuticle (Egerton-Warburton, 1998), allowing the germination inhibitor in the embryo to escape (Egerton-Warburton and Ghisalberti, 2001). In *G. linearifolia*, even after 48 hours immersion in the dye, no Lucifer Yellow penetrated through the seed coat to reach the embryo in smoked or heated seeds. While the fire cues did increase the penetration of the dye into the seed coat, so that the dye was found in the endotesta of several seeds, the barrier to diffusion remained. Nor did the permeability of the two internal cuticles in *G. linearifolia* change after exposure to smoke, as the outwards diffusion experiment showed. Furthermore, no penetration through the hypostase was detected after any of the three treatments. The germinability test of the *Grevillea* seeds showed that the heat and smoke treatments used increased germination, as observed in earlier experiments (Morris 2000); so if the fire cues were breaking dormancy by a version of the *Emmenanthe* mechanism, this should have been detectable in the dye diffusion experiments. Neither smoke, nor heat, nor their combination, removed any of the barriers to diffusion in the seed coat of *G. linearifolia*.

There are other differences between the seeds of *Grevillea* and the chaparral species (Keeley and Fotheringham 1998; Egerton-Warburton 1998). Seeds of *Grevillea* respond to both heat and smoke, whilst the chaparral species respond to smoke only. A palisade-like layer (endotesta) is present in *Grevillea*, but is absent in the chaparral smoke-responders. A further difference between *G. linearifolia* and *E. penduliflora* is that the penetration by the dye through the seed coat was not uniform in the former, either along the seed or from any of the sides. This variability suggested a pattern of "zones" and "regions" in the seed coat structure. These penetration patterns appear to be related to (i) the distribution and location of suberized cells and (ii) to the variation in the number of layers in the ventral groove, particularly in zones two and three where the central corridor of cells (i.e. G1) often consisted of several layers of suberized, columnar-shaped cells. In *E. penduliflora*, such a zonation pattern was lacking and no suberized cells were reported by Egerton-Warburton (1998).

Suberin has been found in many plant organs as part of their normal development (Esau, 1977; Barnabas and Arnott, 1987; Barnabas, 1989; Franke *et al*., 2005) or as a consequence of wounding (Esau, 1977; Lulai and Corsini, 1998). Apart from our earlier findings (Briggs *et al.*, 2005) there are only a few reports of suberized tissues within the seed coat, and in these, suberin was deposited in the chalazal region to seal off the vascular trace (Zee and O'Brien, 1970; Espelie *et al*., 1980; Cochrane, 1983). Suberin is often deposited as lamellae, either throughout the entire wall or concentrated into bands in the radial walls (i.e. Casparian strips). In leaves of the sea grass *Thalassodendron ciliatum,* it is found throughout the cell wall and in the middle lamella between contiguous bundle sheath cells (Barnabas, 1989). In the seed coat of *G. linearifolia*, suberin was only deposited as layers in the secondary wall and not in the primary wall or middle lamella.

Suberized cell walls have three characteristics i.e. they are tissue specific, have a poly(aliphatic) (lipid) domain and a unique lignin-like poly(phenolic) domain (Kolattukudy, 1980; Bernards and Razem, 2001). The term "suberin" has most frequently been used to define the poly(aliphatic) matrix, but most suberized tissues contain small amounts of covalently-linked lignin (monolignols) as well as a significant amount of associated hydroxycinnamic acids. In our present study, the partitioning of Nile Red into the thickened mesotestal and exotestal walls clearly revealed the polyaliphatic lipid domain while similar localization of Acridine Orange revealed the negatively charged lignin-like pholyphenolic domain. The abilities of the confocal LSM were instrumental in separating both components, and together with these two dyes allows a clear, fluorescence-based method for the identification of suberin in plant tissues.

The multi-layered aliphatic polymer, with its associated waxes, is thought to provide suberized tissues with their water impermeability (Kolattukudy, 1980), thus the permeability of tracer dyes through the wall would depend upon the extent of suberization within the wall (Barnabas, 1989, 1994). In *Grevillea*, the suberized secondary cell walls appeared to facilitate the inward movement of Lucifer Yellow as non-suberized mesotestal cells retarded the inward movement during the first 24 hrs. In the ventral groove, the suberized exotestal and adjacent suberized mesotestal cells allowed Lucifer Yellow to move rapidly into the testa but only to the extent of suberization of the tissue. In the lateral and dorsal sides, usually only the squat-shaped exotestal cells were suberized and hence the dye was restricted to this layer.

However, since the squat-shaped exotestal cells had incomplete suberization of the secondary walls, Lucifer Yellow was able to penetrate into the secondary wall layers and accumulate in the non-suberized, pectic portions resulting in the intense fluorescence seen in these cells. Impedance to the dye was only overcome after a further 24 hr immersion (ie 48 hrs in total) that resulted in accumulation of the dye into the apoplast of the non-suberized mesotestal cells.

The slow movement by Lucifer Yellow throughout the seed coat of *Grevillea*, especially in the thin-walled inner mesotestal layers, may be due to strong negative charges in the primary wall and middle lamella, as indicated by Acridine Orange staining and our previous histochemical study (Briggs *et al*., 2005). Acridine Orange is a cationic metachromatic dye that binds to negatively charged groups such as found in DNA, RNA (Hepler and Gunning, 1998), lignin and cutin (Oparka and Read, 1994).

For *Grevillea*, the current study does not support a change in the permeability of the seed coat to large molecular weight compounds after exposure to either fire cue. Whether the embryo of *Grevillea* contains a germination inhibitor (as in *Emmenanthe*) is unknown, and looking for one was beyond the scope of this study. The increased germination following heat, smoke or heat followed by smoke treatments may result from physical effects on the seed coat, or from physiological effects on the embryo (Van Staden *et al*,. 2000). Smoke can substitute for light requirements for germination in Grand Rapids lettuce seed (Drewes *et al*., 1995), and interact with phytohormones to affect germination (van Staden *et al*., 1995; Strydom *et al*., 1996). There is debate about whether smoke acts via hormones, with some evidence against this hypothesis; van Staden *et al*. (2000) argue that the widespread occurrence of the smoke response suggests that it emerged early in the evolution of seed plants, and acts on a trigger for germination at a fundamental stage to provide energy for the process. For *Grevillea*, we are currently investigating whether the fire cues affect the physical properties of the cell walls in the seed coat, and whether such changes are consistent with the ‘mechanical constraint’ model of seed coat dormancy.

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