Mechanical constraint model of seed coat dormancy in *Grevillea*

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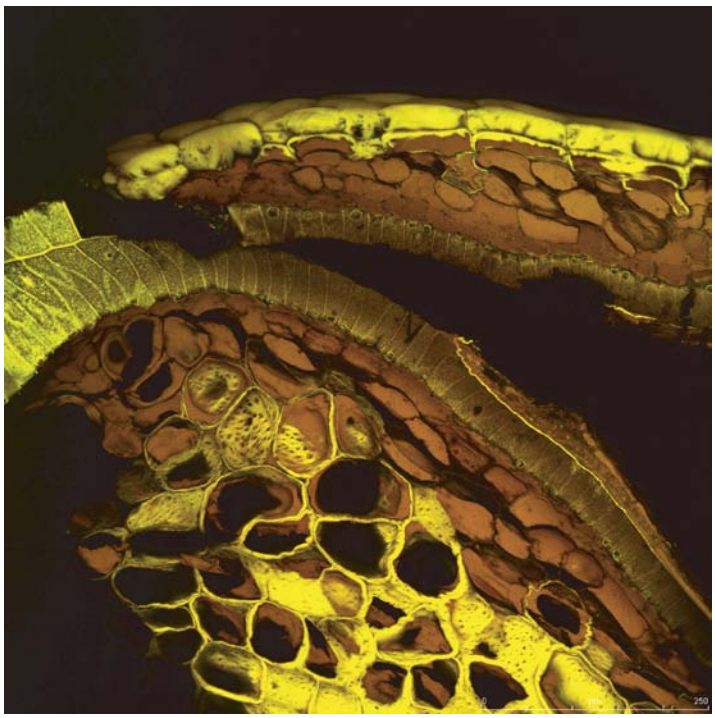

Confocal microscope image through the seed coat of a germinating *Grevillea juniperina* seed, showing the fracturing of the layers of the seed coat. Embryo removed for photograph.

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Abstract

Dormancy of seeds in some East Australian *Grevillea* species is controlled by the seed coat, as excised embryos germinate fully. This project investigated whether the mechanical constraint mechanism of seed coat dormancy applied in *Grevillea juniperina* and *G. linearifolia*. The anatomical basis of breaking of the seed coat by the emerging radicle was investigated using confocal light microscopy. The force required to break through the seed coat was investigated by applying a force from the interior of bisected seed coats (with the embryo removed), in an attempt to simulate the action of the radicle. Compressive forces were applied to seeds from the outside, as a comparison. Both these methods were used on control, and heated and smoked seeds, to determine whether the fire cues affected seed coat strength. The maximum force that the embryos could develop in osmoticum over a range of water potentials was examined for control, and heated and smoked seeds.

The anatomical investigations showed that as the radicle began to grow, it forced apart the inner and outer micropyle, with fractures running between cells, along the cell walls, extending through the layers of the seed coat to the exterior. The tip of the radicle emerged to the outside through the dorsal seed coat near the micropylar tip of the seed, rather than through the tip itself. Estimates of the force required to break through the seed coat from the inside ranged from 0.1 – 0.4 MPa after one day of imbibition; there was no significant difference between the control or treated seeds in the force required. The force required after 14 days of imbibition was slightly less than after one day, but still not significantly different between treatments. The method used to estimate the force required to break through the seed coat from the inside was difficult to implement, and the results must be treated with caution as a result. The compressive force required to break the seed coat after one day of imbibition did not differ with treatment either; however, there was weak evidence of a shift in the shape of the frequency distribution with treatment, which would be consistent with the seed coat of some proportion of heated and smoked seeds being weakened by one or both of the fire cues. More work would be required to substantiate whether this very tentative conclusion is correct. The maximum force that could be exerted by half-excised embryos was at least 0.3 MPa for *G. juniperina*, and at least 0.4 MPa for *G. linearifolia*. The range of water potentials used did not

allow determining what the full maximum force was for either species, and whether the fire cues altered this maximum force. Further work is required to confirm the tentative conclusions that it was possible to reach in this work.

Introduction

Seeds of east Australian *Grevillea* species generally do not germinate when shed from the parent plant (ie they are dormant), but show increased germination after exposure to fire-related cues such as heat shock and smoke (Morris 2000, Kenny 2000). Dormancy is imposed by the seed coat, as removal of the seed coat results in germination of all dissected embryos (Morris, Tieu & Dixon 2000).

However the mechanism by which the seed coat controls dormancy is not fully known. Potential mechanisms of seed coat dormancy include: acting as a mechanical barrier ('mechanical barrier' model); preventing the exit of germination inhibitors ('inhibitor exit' model); presence of germination inhibitors in the seed coat itself ('seed coat inhibitor' model); restricting water uptake (as in wattles and peas, the 'water-impermeable' model); and restricting oxygen uptake ('oxygen diffusion' model; Bewley & Black 1994). Three of these mechanisms have been investigated to date: *Grevillea* seeds take up water freely, whether scarified or not, so the seed coat is not a barrier to water movement (Morris 2000, Morris, Tieu & Dixon 2000). Re-insertion of dissected embryos back into seed coats did not restrict germination either, so the seed coat inhibitor model was not supported (*ibid.*). Treatment of seeds with heat and smoke did not alter the permeability of the seed coat to large molecular weight compounds, so the inhibitor exit model was not supported (Briggs & Morris 2007).

The models of seed coat dormancy still remaining to be tested for *Grevillea* are (1) seed coat acting as a mechanical barrier; and (2) seed coat restricting gas exchange. The first model was the subject of this project (seed coat acting as a mechanical constraint on the embryo.) Briggs *et al.* (2005) described the structure of the seed coat of *Grevillea* (outer testa with three layers; inner tegmen) and identified layers that could potentially act as mechanical barriers to expansion of the embryo (palisade layer of endotesta; sclerenchyma layer of tegmen). Whether these layers act

to mechanically constrain the embryo is completely unknown; until this hypothesis is tested, it cannot be eliminated (or accepted) as a cause of seed coat dormancy for these seeds.

Methods to estimate the mechanical strength of the seed coat are not well developed. The problem that arises in attempting such estimation is that the embryo exerts its thrusting force from inside the intact seed at germination. For experimenters to duplicate this is technically very challenging; any attempt to exert a force from inside the intact seed means that some manipulation of the seed coat is first required to allow the force to be generated internally, and such manipulation by itself may affect the structure of the seed coat, and thus any estimates of its strength. A more common approach has been to use compressive force applied externally; while easier to do and measure, this approach does not simulate well the force the embryo must generate to break through the seed coat from the inside.

We made an attempt to measure the resistance of the seed coat to rupture from a force applied internally, using dissected seed coats. While this straight away introduces an element of artificiality (seed coat is not intact), the question of interest was whether the fire cues affected the force required to rupture the seed coat from the inside; so the comparison of controls *vs.* treated seeds was of interest. If the values measured are relative rather than absolute measures of the force required, the comparison is still valid provided the forces are measured in the same way across treatments. We also applied a compressive force to the seed coat, using an instrument designed to test the resistance to compression of foods, for comparison with the first method.

The other possible effect that one or both of the fire cues could have on the *Grevillea* seed is to stimulate growth of the embryo so that it can exert greater thrust and so break through the seed coat. This effect is called an increase in the 'growth potential' of the embryo; a common way to measure it has been to grow fully or partly excised embryos in osmoticum over a range of water potentials, to determine the lowest water potential at which germination can still occur. The absolute value of this water potential is taken as a measure of the maximum (positive) thrust that the embryo can generate, and for a number of species, dormancy-breaking treatments have been found to increase it (Nabors and Lang 1971; Baskin and Baskin 1998). This approach was tried with partly excised seeds of *Grevillea.*

The aims of this project were to determine:

- the anatomical basis of the rupturing of the seed coat by the emerging radicle;
- the physical forces required to break the seed coat;
- whether the force required to break the seed coat changed after exposure to fire-related cues;
- whether fire-related cues increase the growth potential of the embryo, allowing it to exert greater force to break through the seed coat

Methods

Anatomy of germination

Seeds of *Grevillea juniperina* and *G. linearifolia* were imbibed with distilled water and kept at 20°C in day/night cycle of 12/12. Ungerminated seeds were removed at selected days following imbibition and germinated seeds were removed when the radicle had just broken through the seed coat. Seeds of *G. linearifolia* were fixed for 24 hrs with 2.5% glutaraldehyde-3.0% paraformaldehyde in phosphate buffered saline, tripled rinsed with distilled water, slowly dehydrated with ethanol and stored in 70% ethanol at 4°C until hand-sectioned. Seeds of *G. juniperina* were examined unfixed. Sections were stained with various fluorochromes and examined with a Leica laser scanning confocal microscope (LSCM). Stains used were: Berberine Hemisulphate/ $FeCl_3$, Congo Red, Acridine Orange. Seeds that had been used for the LuciferYellow CH apoplastic tracing study (Briggs and Morris, 2008) were also examined for the structure of the micropyle.

Internal force required to break the seed coat –

The following method was adapted from Nabors and Lang (1971) to estimate the thrust needed by the radicle to pierce the abutting tegmen and palisade endotestal layers i.e. the layers believed responsible for mechanically restraining the embryo, using dissected seed coats. The strength of the seed coat was tested at two temporal points during germination in *Grevillea linearifolia*. Seeds (2 x 15 seeds from untreated controls and 2 x 15 seeds from Heat+Smoke treatment) were placed on filter paper (moistened with distilled water) in 9cm sterile Petri dishes for 24 hours (end of

Phase 1 of water uptake) or left for 13 days (end of Phase 2), transversely bisected halfway along their length, then the embryo was carefully removed to avoid damaging the inner layers of the seed coat and the micropyle; the empty shells were then placed (dorsal side down) on moistened filter paper to prevent drying out and to simulate the hydrated condition of the seed coat prior to germination. The micropylar end of each embryo-less shell was inverted over the sharp tip of a map pin that was affixed to a balance. The emptied seed coat was grasped between the thumb and index finger and pulled down onto the pin until the tip emerged through the seed coat. The force of the pull from commencement to breakthrough was recorded by a digital camera in video mode focused on the balance readings, and the force exerted at breakthrough of the pin recorded (Nabors and Lang 1971). The surface area of the map pin tip was calculated as 2.9 mm^2 (conical shape assumed) and the applied forced calculated as units of pressure.

External (compression) force required to break seed coat

We have continued work on testing the mechanical strength of the seed coat but have approached it differently. The TA-XT2 is a machine that can be programmed to compress an object (ie peach, piece of bread) for a predetermined depth and record the force needed to reach that depth. We adapted this process and applied a 2mm diameter metal probe (flat surface) to the dry and fully imbibed seeds of *Grevillea juniperina*. In our initial trial using untreated seeds, we tested the micropylar, middle and chalazal regions of the seed and found that there was a difference in force required to push the probe 0.3-0.6 mm into the seed. Fully imbibed seeds required less force than dry seeds and the least force was needed at the micropylar end in both hydration states. The compressed seeds were then sectioned, stained and examined with confocal microscopy. We found that the seed coat split along the same lines as occurred during germination in *G. linearifolia.* A second small trial tested untreated (16) and heat+smoke (24) treated seeds. Seeds were imbibed for 24hr, blotted and then placed under the probe. Each time the probed was centred 2mm from the micropylar end of the seed and the depth was 0.4mm (the depth from the dorsal surface to the tegmal layer of the seed.

Anatomy of compression

Seeds of *Grevillea juniperina* were placed on Whatman's No 1 filter paper in 9cm sterile Petri dishes and seven ml of Fungarid solution was added to each dish. Plates were kept at 20°C in day/night cycle of 12/12. Seeds were removed after 24 hours and 14 days and subjected to compression by the TA-XT2 machine (see above). Compressed seeds were hand-sectioned (MLS), stained with Congo Red and examined by a Leica LSCM.

Measurement of Embryo Growth Potential using PEG8000 –

All glass ware, distilled water used for rinsing and preparation of solutions (PEG 8000 solution, Fungarid©, bleach etc.), filter paper and forcepts were sterilized for 20 minutes at 120°C. Aseptic techniques were used throughout the surface sterilization procedure, preparation of PEG 8000 solutions and setting up of the experimental Petri dishes.

Preparation of Smoke Water –

Aerosol smoke was generated by a bee-keepers smoker using dried eucalyptus leaves and twigs. The smoke was pulled (by under vacuum) through distilled water until the solution was lightly coloured and smelled of smoke (approximately 20 minutes). Four such smoke solutions were prepared and sterilized.

Preparation of PEG 8000 solutions –

The various water potential solutions were prepared according to the formula below taken from Michel (1983):

$$\psi = 0.129[PEG]^2\,T - 14.0[PEG]^2 - 0.4[PEG]$$

where T = 20°C and $[PEG] = \frac{g\text{PEG 8000}}{g\text{H2O}}$

For example, a solution of PEG8000 with a water potential of -0.6032 MPa was prepared by adding 0.212g PEG 8000 to each gram of distilled water. Zero water potential was sterile distilled water.

For *G. juniperin*a, a stock solution of PEG8000 (-0.6032 MPa) was diluted with distilled water to achieve water potentials of -0.05, -0.10, -0.15, -0.20, -0.25, -0.30

MPa; another stock solution of PEG 8000 (-0.802 MPa) was diluted to achieve water potentials of -0.30, -0.40, -0.50, -0.60, -0.70, -0.80 MPa in the extension of this experiment.
For *G. linearifolia*, individual PEG 8000 solutions (water potentials of -0.25, -0.325, -0.375 and -0.40 MPa) were prepared, using the above formula.

Surface Sterilization of Seeds: - Ten seeds were placed into each glass tube, covered with 10 mL of Fungarid©-0.05% Triton X-100 solution and left for 24 hours. The Fungarid©-Triton X solution was then removed and the seeds washed three times with 10 mL of distilled water. After removal of the third washing solution, 10 mL of 75% ethanol was added to each tube, agitated for 6-8 seconds, decanted, then replaced by 10 mL of bleach (1/11 dilution of Domestos© equalling 0.45% sodium hypochlorite, 0.109% sodium hydroxide) and left for 10 minutes. Following removal of the bleach solution, the seeds were washed four times with distilled water and the final rinsing solution discarded.

Experimental Procedure: Grevillea juniperina
Untreated controls – surface sterilized seeds were aseptically transferred onto filter paper, transversely bisected using a disposable scalpel blade and the micropylar half transferred to a 10 cm glass Petri dish with one Whatman's No 1 filter paper; the chalazal half was discarded. A new scalpel blade was used for each batch of 10 seeds. Twelve mL of one of the water potential solutions was added to each Petri dish. N.B. This part of the experiment was later extended to include water potentials of -0.30, -0.40, -0.50, -0.60, -0.70 and -0.80 MPa in order to find the water potential at which no germination occurred.

Heat Treatment and Smoke-Water Treatment – surface sterilized seeds were transferred to dry filter paper, air-dried for 20 hours, transferred into clean, dry, glass tubes, closed off by a cotton bung then heated (separately for each batch of seeds, Morrison and Morris 2000) for 10 minutes at 80°C. Following heat treatment, each glass tube was air-cooled for 5 minutes then the seeds were tipped into glass vials containing one of four replicates of smoke water (diluted 1/11with distilled water). After one hour, the smoke water was decanted and the seeds tipped onto fresh filter

paper, transversely bisected and the micropylar half transferred onto the experimental plate (as per the controls). Twelve mL of one of the water potential solutions (0.00 to -0.30 MPa) was added to each correspondingly labeled Petri dish.

For the untreated controls and the treated seeds, there were two replicates of each water potential treatment. Each Petri dish was sealed with Parafilm and placed into a ziplock bag to reduce loss of water vapour and thereby alteration to the water potential. The Petri dishes were placed into a Contherm Phytotron Climate Simulator set at Day/Night cycle of 12/12hr, 20°C, RH 47%, Lux 180µ E. Dishes were removed and examined for radicle protrusion every three days. Germinated embryos were aseptically removed from the Petri dishes when the radicle was 1-2 mm in length.

Experimental Procedure: Grevillea linearifolia

Untreated controls – surface sterilized seeds were aseptically transferred onto filter paper; from each batch of 10 seeds, 5 were left whole and transferred to a 9cm sterile plastic Petri dish and 5 were bisected (as before) and transferred to a separate 9cm Petri dish. A new scalpel blade was used for each batch of 10 seeds. Each 9cm Petri dish had one 9 cm Whatman's No 1 filter paper folded to form two ridges at right angles to each other. Ten mL of one water potential solution (0.00, -0.25, -0.325, -0.375, -0.40 MPa) was added to correspondingly labeled experimental dish.

Heat Treatment and Smoke-Water Treatment – surface sterilized seeds were transferred to dry filter paper, air-dried for 20 hours and treated as above for *G. juniperina* except only one smoke water replicate was used. After one hour, the smoke water was decanted and the seeds tipped onto fresh filter paper to briefly drain. Each batch of seeds was then processed as per the controls.

Heat Treatment – surface sterilized seeds were transferred to dry filter paper, air-dried for 20 hours, transferred into clean, dry, glass tubes, closed off by a cotton bung then heated (separately) for 10 minutes at 80°C. Following heat treatment, each glass tube was air-cooled for 5 minutes then the seeds were tipped into glass vials containing distilled water to imbibe; this was to permit sectioning of the seed without inducing fracture planes. After one hour, the seeds were briefly drained on dry filter paper, before being processed as per the controls.

Smoke Treatment – after surface sterilization, the final rinsing water was replaced with 10mL of diluted smoke water. After one hour, the smoke water was removed and the seeds placed onto dry filter paper to briefly drain. They were then processed as per the controls.

For each water potential (control and treatments), there were two replicates for the bisected seeds (10 seeds in total) and two replicates for whole seed (10 seeds in total).

Each Petri dish was sealed with Parafilm and placed into a ziplock bag then placed into snaplock boxes. The Petri dishes were placed into a Contherm Phytotron Climate Simulator set at Day/Night cycle of 12/12hr, 20°C, RH 47%, Lux 180µ E. Dishes were removed and examined for radicle protrusion every three days. Germinated embryos were aseptically removed from the Petri dishes when the radicle was 1-2 mm in length. All Petri dishes were opened at least once a week for refreshment of oxygen and the water potential solutions were replaced with freshly made up solution at day and day.

Results

Structure of the Micropylar Region and changes induced by germination

Longitudinal sectioning through the micropylar region of the seed revealed an inner micropyle formed by two layers of short, very-thick-walled, unlignified tegmal cells (Fig. 1), and an outer micropyle formed by a single layer of reticulately-lignified palisade-shaped endotestal cells (Fig. 1) that curve "downward" towards the ventral side of the seed (Fig. 2). The micropylar tegmel cells were continuous with the endo- and exotegmal layers of the rest of the seed coat; the abutting mesotegmal cells were similarly continuous with the inner and outer mesotegmal layers (Fig. 1). The cell layers forming both the inner and outer micropyles abut closely "glued together' by the compressed remains of the pollen tube. Overlying the entry into the inner (embryonic) end of the micropyle were the crushed remains of the nucellus (the crushed cell zone from Briggs and Morris 2008 or (2005??)).

The ventral side of the micropylar region is characterised by small to medium-sized, thin-walled, inner mesotestal cells and larger, thicker-walled outer mesotestal cells. The walls of these later cells stain positively with Berberine Hemisulphate (Fig. 2). The dorsal side of the micropylar region is characterized by a few layers of large, thin-walled mesotestal cells overlain by thicker-walled exotestal cells (Fig. 2).

As the elongating radicle pushed into the entry of the micropyle, the inner micropyle opened, the 'glue' fractured and the inner micropylar cell layers were forced apart and a line of separation extended ahead a short distance into the outer micropyle. Continued elongation resulted in the 'shunting aside' of the inner micropylar layers, further widening the first part of the outer micropyle and the generation of a fracture plane that extended between the palisade endotestal cells (Fig. 3) before it crossed into the dorsi-lateral meso- and exotestal layers (Fig. 2). The fracture plane followed the middle lamellae which resulted in a stepped fracture surface (Fig. 4).

Fracture planes also extended laterally away from the micropyle. They were initiated within the mesotegmal layer of the tegmal wedge (Fig. 5). This wedge-shape region is formed from the pinching together of the tegmal layers such that the mid-line is

formed from the abutting together of the endotegmal layer of cells and hence there is no middle lamella "cementing" these cells together. As the wedge started to separate down the mid-line (Fig. 6) the ventral inner mesotestal cells became distorted (Fig. 7), then collapsed; the outer part of the wedge then fractured along the middle lamellae of the exotegmal layer before the abutting dorsal palisade endotestal and mesotestal layers fractured. Again, all the fracture planes followed the middle lamellae instead of breaking across the cells. The radicle emerged through the side of the seed (rather than through the tip of the micropylar end) and continued elongation resulted in the splitting of the seed coat up the side (Fig. 8) then along the dorsal mid-line.

Internal break-through pressure for G. linearifolia: 1 day of imbibition

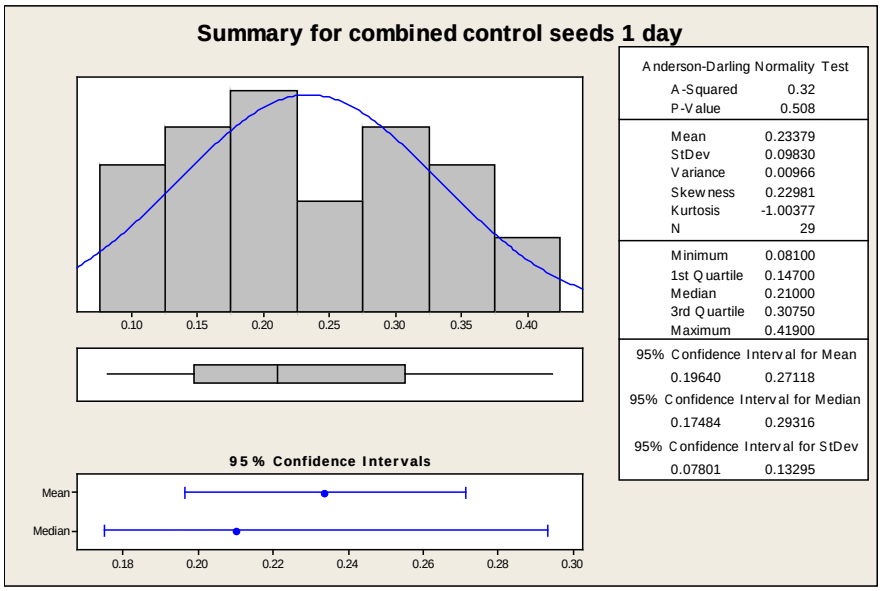
The pressure required to break outwards through the micropylar end of bisected seed coats in the untreated (control) seeds differed little between seeds that were fully intact prior to cutting, and seeds that showed some sign of piercing or external damage (Table 1; comparison of undamaged and damaged means, $F_{1,27} = 0.18$; $P = 0.67$). Consequently both control seed lots were pooled to obtain an estimate of the pressure required to break through the micropylar end with a larger sample size. A small number of the heated and smoked seeds also showed some seed coat damage, but exclusion of these seeds affected the estimate of pressure required to break through very little (Table 1); thus undamaged and damaged seeds were pooled for the treated seeds as well.

The estimate of the internal break-through pressure at the micropylar end for seeds imbibed for 1 day was almost identical for both control (0.234 MPa), and heated and smoked seeds (0.235 MPa; Table 1). The frequency distribution of break-through pressures was approximately normal in both cases (Fig 1; Anderson-Darling test of normality NS for both), though both distributions were slightly flat (negative kurtosis, Fig. 1). Break-through pressures ranged from <0.1MPa up to 0.4 MPa in both control and treated seeds (Fig. 1).

Table 1. Comparison of strength of bisected and imbibed seed coat of control, and heated and smoked seeds, to force applied internally at the micropylar end after **1 day** of imbibition. Data shown for undamaged seeds, damaged seeds (controls only), and for pooled damaged and undamaged seeds. 10 June data.

Treatment	*n*	**Mean (MPa)**	**Standard Deviation**	
Control	13	0.225	0.085	undamaged
	14	0.241	0.111	damaged
	29	**0.234**	**0.098**	**combined**
Heat + smoke	17	0.239	0.101	undamaged
	20	**0.235**	**0.092**	**combined**

(a)



(b)

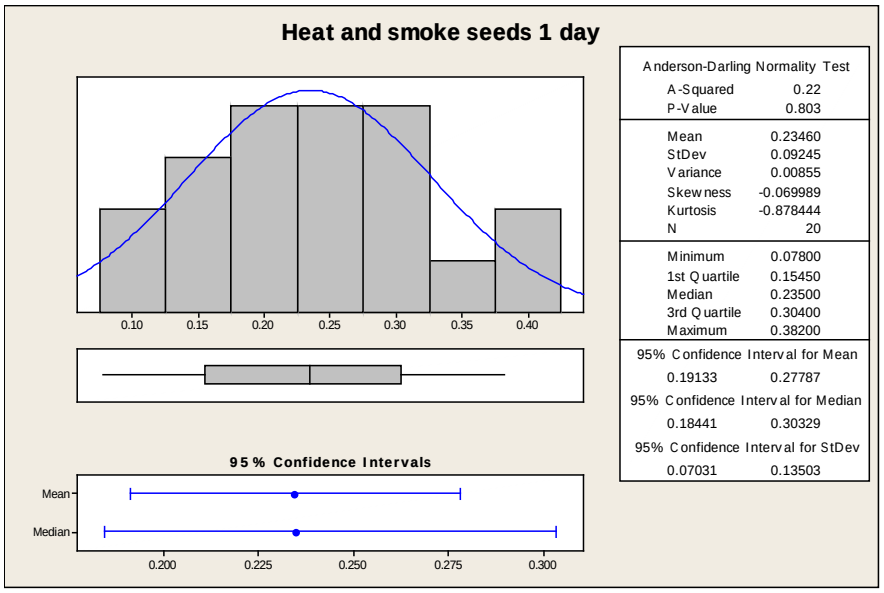


Fig. 1. Frequency distribution of internal break-through pressure at the micropylar end of (a) control, and (b) heated and smoked bisected seeds of *G. linearifolia*; 1 day of imbibition.

Internal break-through pressure: 14 days of imbibition

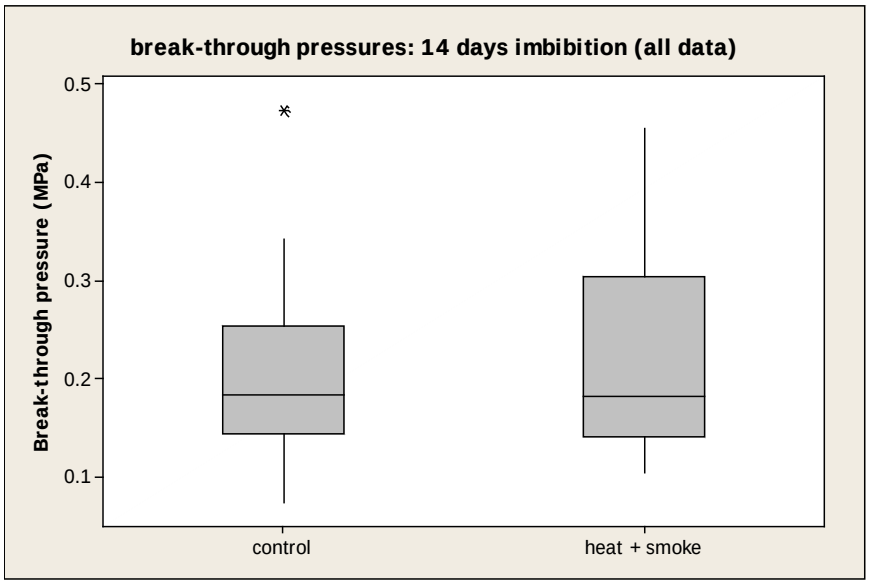
In both the control and treated seed lots, single data points >0.4 MPa were detected as outliers ($P < 0.05$; Fig. 2(a)). While removal of the outliers reduced the estimated mean break-through pressures, the qualitative conclusions drawn remained the same. Firstly, break-through pressures were less after 14 days of imbibition than after 1 day (Tables 1, 2). Secondly, break-through pressures did not differ significantly between control and treated seeds (comparison of means with outliers excluded; $F_{1,27} = 0.12$; $P = 0.73$; Fig. 2(b)).

Whilst the mean break-through pressure did not differ between the two groups of seeds, the *shape* of the frequency distribution did. The frequency distribution of pressures for the control seeds remained approximately normal, as observed for the 1-day seeds (Anderson-Darling test of normality NS; Fig. 3(a)). However, the frequency distribution of pressures for the heated and smoked seeds did not remain normal (Anderson-Darling test of normality, $P = 0.04$; Fig. 3(b)). The change in shape was most apparent in comparison of the mode of the distribution for each treatment (controls = 0.20 MPa; heated and smoked = 0.15MPa; Fig. 3).

Table 2. Comparison of strength of bisected and imbibed seed coat of control, and heated and smoked seeds, to force applied internally at the micropylar end after **14 days** of imbibition. 10 June data.

Treatment	*n*	**Mean (MPa)**	**Standard Deviation**	
Control	14	0.203	0.104	all seeds
	13	**0.185**	**0.075**	outlier excluded
Heat + smoke	15	0.212	0.094	all seeds
	14	**0.194**	0.069	outlier excluded

(a)



(b)

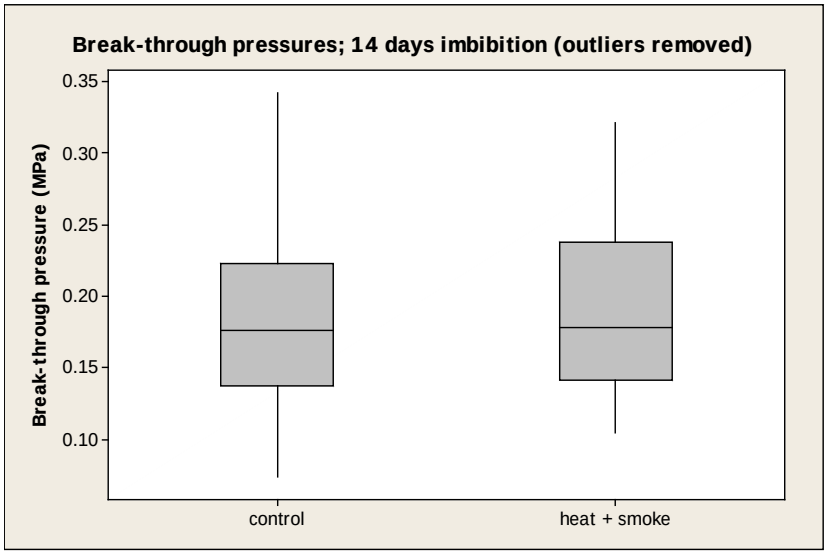
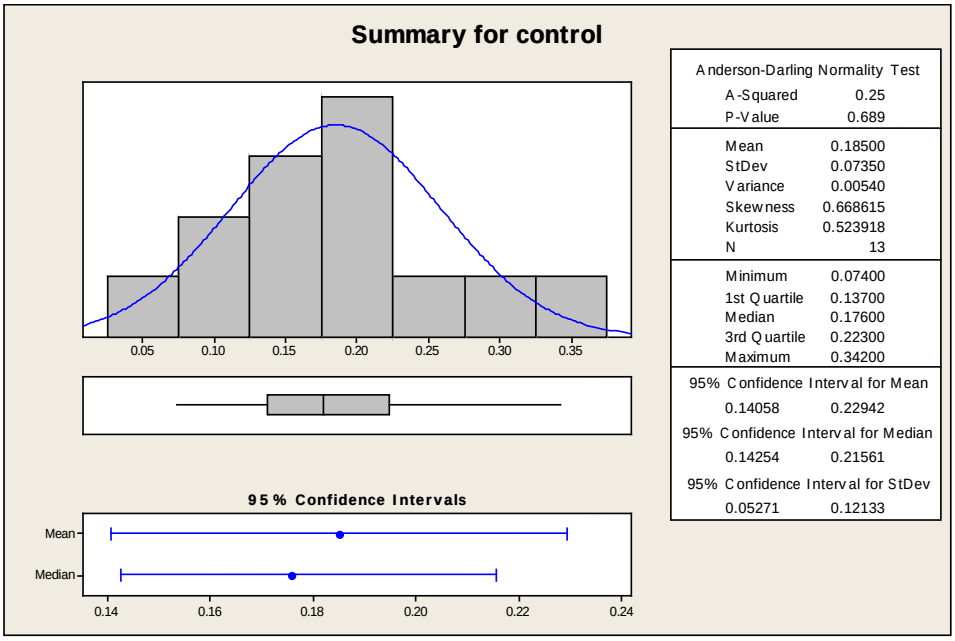


Fig. 2. Box plot of break-through pressures for control and heated and smoked seeds after 14 days of imbibition: (a) all data included; (b) outliers removed. In (a), the values > 0.4MPa are shown as an outlier for the controls, and as an elongated range for the heated and smoked seeds.

(a)



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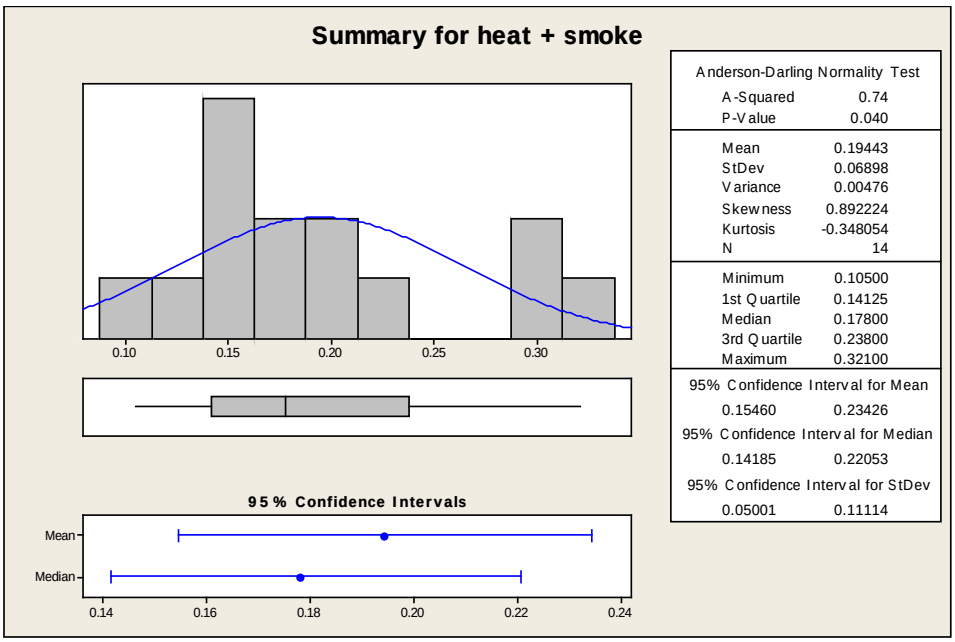


Fig. 3. Frequency distribution of internal break-through pressure at the micropylar end of (a) control, and (b) heated and smoked bisected seeds of *G. linearifolia*; 14 days of imbibition.

Compressive force to break seed coat of G. juniperina: 1 day of imbibition

The mean compressive force required to break through the seed coat at the micropylar end of *G. juniperina* did not differ significantly between the control, and the heated and smoked seeds ($F_{1,43}$ = 0.21; P = 0.648; Figs 4, 5). However, there was the possibility of a change in the *shape* of the frequency distribution of compressive force required to break the seed coat between the two treatments, with a shift to lower compressive force apparent for some of the heated and smoked seeds. This was evident in the relative positions of the mean and median; for the controls, the median compressive force of 366 g exceeded the mean of 355 g, while for the smoked and heated seeds, the reverse applied (median of 347 g < mean of 373 g; Fig. 5). This pattern is visually apparent in the box plot shown in Fig. 4 below. Skewness differed between the two distributions, being -0.40 for the controls, but 0.156 for the heated and smoked seeds (Fig. 5). The mode of the distribution centred on 300 g for the heated and smoked seeds, but on a higher value (367 g) for the control seeds. These changes in the frequency distribution of compressive force required for breaking of heated and smoked seeds would be consistent with a hypothesis of a lessening of this force for some fraction of the seed population, presumably by one or both of the fire-related cues.

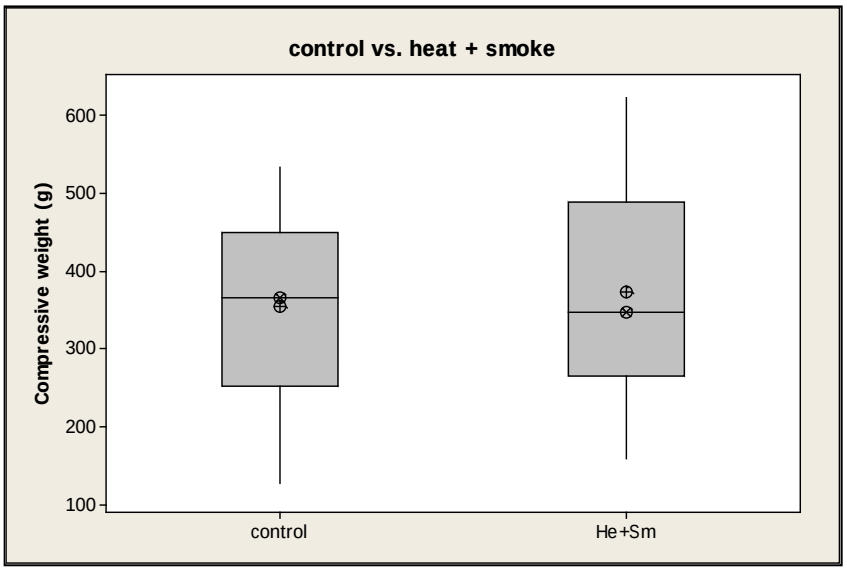
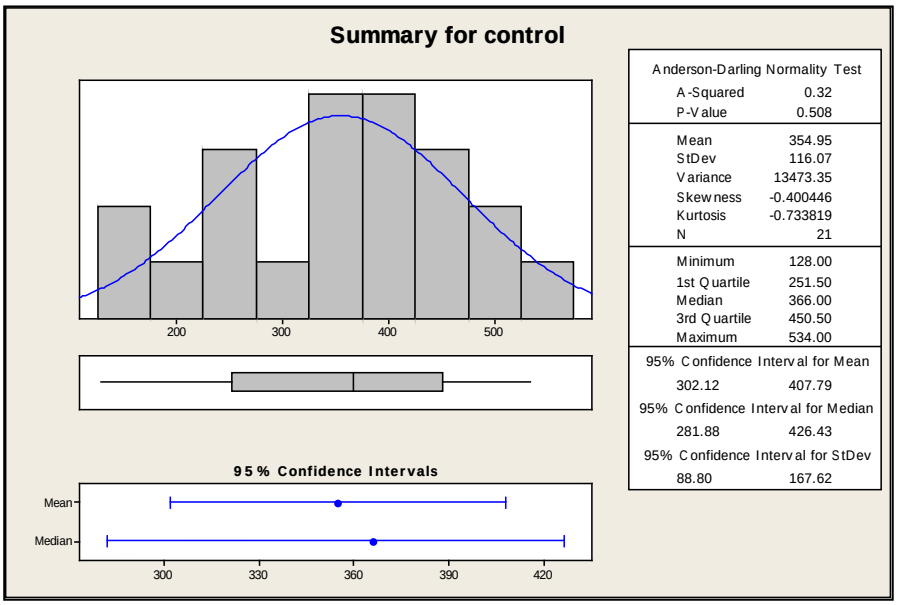


Fig. 4. Box plot of compressive force required to break the seed coat of *G. juniperina* for control and heated and smoked seeds after 1 day of imbibitions.



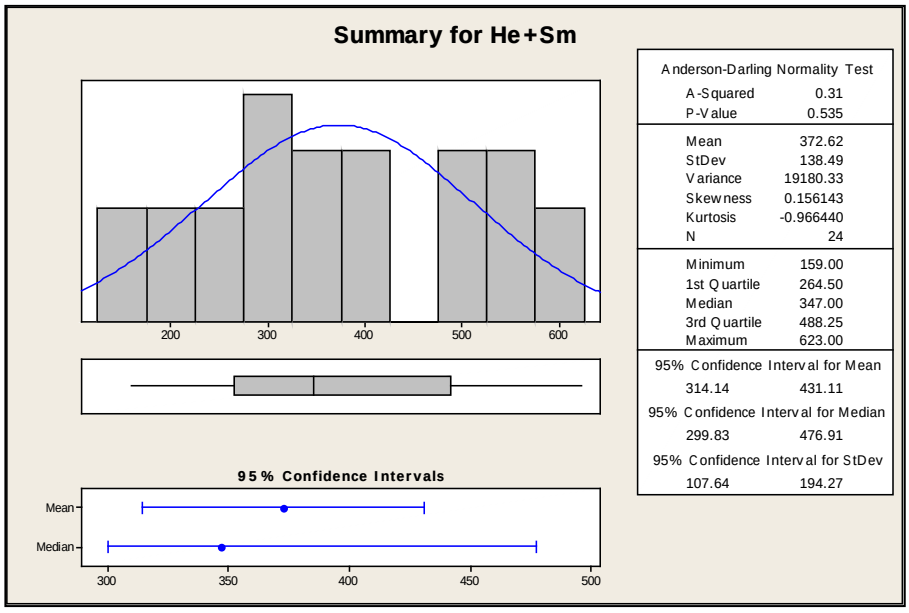


Fig. 5. Frequency distribution of external compressive force to break the seed coat of (a) control, and (b) heated and smoked bisected seeds of *G. juniperina*; 1 day of imbibition.

Determination of embryonic growth potential

For *G. juniperina,* germination (elongation of the embryo > 1 mm) occurred in the majority of half-seeds of over the range of water potentials used; up to 70 – 80% of embryos were still able to extract sufficient water at -0.30 MPa to elongate (Fig. 6). When the range of water potentials was extended to lower values in a subsequent experiment, to determine the water potential at which no germination would occur in untreated half-seeds, limited germination (of 10% of embryos) occurred only at water potentials of -0.60 MPa and -0.80 MPa respectively (data not shown). The implication from the two experiments was that germination was still possible at -0.3 MPa for the majority of embryos, but not at lower water potentials.

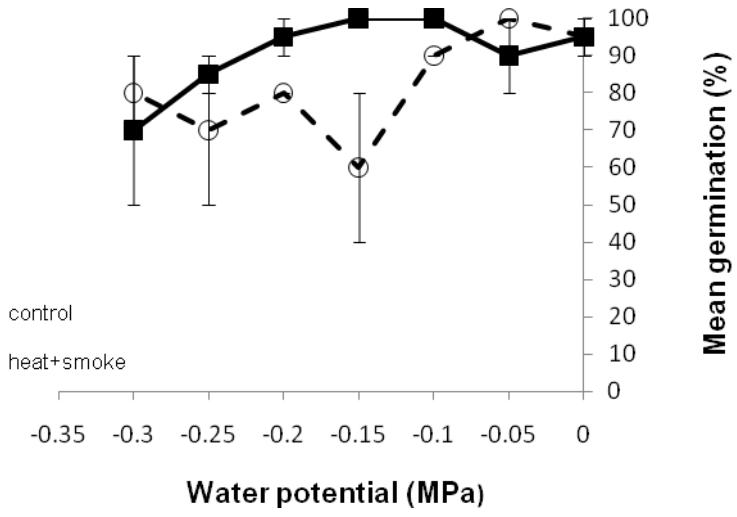


Fig. 6. Germination of half-seeds of *G. juniperina* over a range of water potentials. Open circles, dashed line = controls; solid squares, solid line = heated and smoked seeds. Bars = SE.

For *G. linearifolia*, a substantial proportion of the embryos in half-seeds were also able to germinate in water potentials down to -0.4 MPa (Fig. 2). Only untreated seeds were kept at a water potential of 0 MPa, and all of these germinated; the proportion that germinated at lower water potentials was variable, whether untreated or treated with fire cues, but in most cases was 50 – 100% (Fig. 2). The maximum growth potential that the embryo of both these species can generate would appear to be greater than 0.40 MPa.

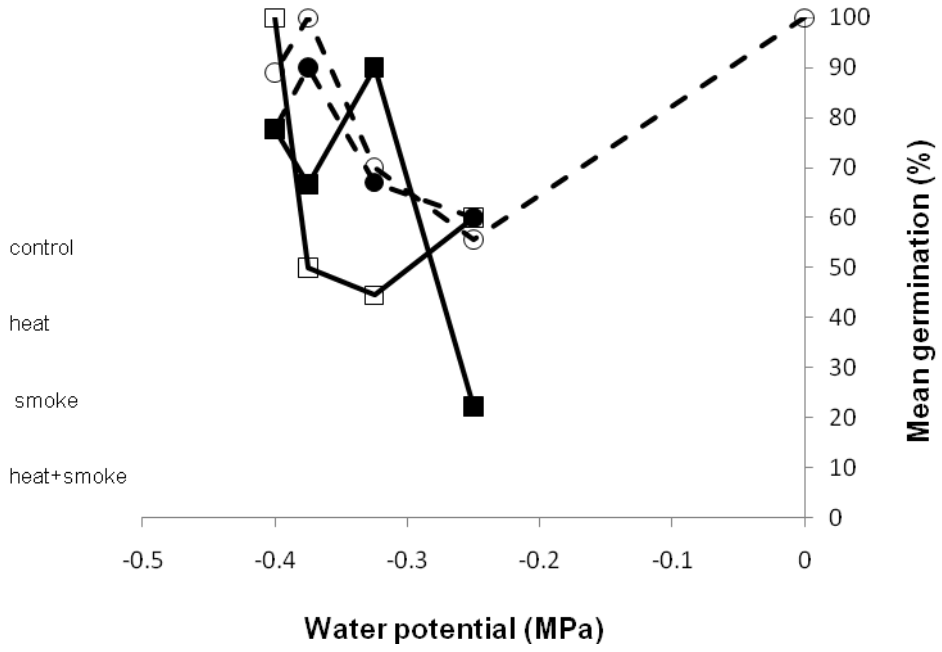


Fig. 6. Germination of half-seeds of *G. linearifolia* over a range of water potentials. Open circles, dashed line = controls; open squares, solid line = heated; closed circles, dashed line = smoked; solid squares, solid line = heated and smoked seeds. Bars = SE.

Discussion

Anatomical basis for and forces involved in breaking the seed coat

During germination, the elongating radicle applies an outward-directed force as it pushes against, then apart, the tegmal cells that formed the inner micropyle: this results in compressive stress forces being applied to these cells (N.B. this is around the entire 360° circumference). However, these tegmal cells do not deform or fracture. As the radicle extends into the inner part of the outer micropyle, compressive stress forces are again applied. However, fracture planes develop shortly thereafter, breaking across or between the dorsal, lignified palisade endotestal cells of the micropylar end as well as along the tegmal wedge and up through the lateral-dorsal side, thereby allowing the radicle to break through the seed coat. This direction of breakage may be due to (i) the dorsal and lateral sides being thinner then the ventral side of the seed coat and (ii) the downward curvature of the palisade endotestal cells which results in a possibly weaker part of the endotesta that lies directly ahead (i.e. horizontal plane) of the elongating radicle coupled with the potentially "bracing" nature of the slightly thicker-walled ventral mesotesta.

In fracture mechanics theory, the application of an imposed force *P* can occur in three modes:

> *In mode I, P produces tensile stress acting normal to the plane of the crack and tends to open the incipient crack. In mode II, P produces shear stress acting parallel to the plane of the crack and perpendicular to its front and tends to slide the two crack faces relative to one another. In mode III, P produces shear stress acting parallel to the plane of the crack and parallel to its front and tends to tear the crack front open*. (Farquhar and Zhao 2006)

Stress fields close to a crack are considered, in theory, to have the "same shape regardless of the larger geometry of the body". However, the heterogeneity of the material, directionality and absolute size of the crack (when compared to the size of the body), results in spatial variation of the stress (Farquhar and Zhao 2006). In *Grevillea juniperina* and *G. linearifolia*, the initial elongation of the radicle could be considered to mimic "mode I" whereas the development of the fracture planes along the tegmal wedge mimics the "tearing" mode III.

Since spatial variation of stress will be affected by material heterogeneity, the composition of the secondary walls of the tegmen and the testa may be very important in determining where the fracture starts. In the inner micropyle, the secondary walls are a composite matrix of cellulose and hemi-cellulose. Secondary walls are formed by thin lamellae of ordered cellulosic microfibrils that are laid down in a helical arrangement parallel to the long axis of the cell (Reid 1997). Such an arrangement is considered to couple normal and shear behaviours (Lucas et al 1993 cited Farquhar an Zhao 2006). Furthermore, cellulose is recognised as being very strong with a theoretical strength of 25GPa (Vincent 1990), making it 5-10 times stronger than rolled steel when compared on a strength to weight basis. However, this strength is greatly reduced (down to 0.9GPa) when hydrated to 35-40% (Frey-Wyssling, 1952 cited Vincent 1990). Lignification of the secondary wall confers rigidity and hardness by fixing polysaccharide-polysaccharide interactions (Reid 1997).

The force required to rupture the seed coat would be dependent upon how well the various cell layers (and composition of the cell walls) dissipate the initial compression energy resulting from the elongation of the radicle before sudden catastrophic fracture can occur. At the start of elongation, the ordered cellulose microfibrils of the tegmal cells would absorb some of this energy since cellulose is known to have a great capacity to absorb strain energy (Jeronimidis 1980 cited Farquhar and Zhao 2006). Buckling and separation of cells, such as occurs in wood under tension, generates new surface areas for the dissipation of energy (Farquhar and Zhao 2006). Separation of the tegmal cells forming the micropyle, followed by separation along the mid-line of the tegmal wedge, would provide increased surface area for dissipation of energy. Further dissipation would occur when the dorsal mesotestal cells began to be compressed. While there is no evidence of buckling in the lignified palisade endotestal cells, it is possible that the reticulate deposition of lignified secondary wall throughout the interior of the palisade endotestal cells may not only provide strength but possibly flexibility, and thus contribute to the initial dissipation of energy.

However, as the radical enlarges (in cross-section as well as lengthwise), then exertion of force would increase until the separation along the tegmal wedge reached critical “crack length” thereby forcing a fracture pathway through the dorsal seed

coat. The path taken by the developing crack would be influenced by the anisotropic arrangement of the cells in the various layers, the ratio of cell wall thickness to the diameter of the cell and the degree of adhesion (Vincent 1990). Studies on apples and potatoes have indicated that these features, plus the presence/absence of air spaces, greatly affect the mode of failure. Furthermore, "the degree of adhesion can control whether the fracture path goes between cells" as in apples, or as in the case of potatoes (which have smaller, thicker-walled cells that adhere strongly together), through the cells (Vincent 1990). The thin-walled mesotestal cells may not adhere strongly together and hence the fracture plane follows the middle lamellae, whereas the lignified endotestal cells may adhere more strongly, and thus fractures can occur across the cell as well as between the cells.

Physical forces required to break the seed coat; role of fire cues

Two methods were tried in an attempt to estimate the force required to break the seed coat, and whether this changed after treatment with fire cues i.e. an internally-applied force that sought to simulate the radicle breaking through, and an externally applied compressive force. The results of the internally-applied force suggested no weakening of the seed coat by the fire cues after one day, but a possible shift of this force to lower values in seeds that had been heated and smoked, after a longer period of imbibition. However this conclusion is tentative only; the numbers of seeds that could be sampled from each treatment was low (13 controls, 14 treated); the shift (if real) was detected as a lower mode, and change from normality, in the shape of the frequency distribution for the heated and smoked seeds. Finally, we were not convinced that the method used was sufficiently objective and reliable enough to give good data. While Nabors and Lang (1971) used it successfully with bisected lettuce seed coats, we concluded that after numerous attempts, this method was too difficult, too imprecise for reproducibility and did not permit any confidence in the results for *Grevillea*. This was due to the manual nature of this method, the need to push the point through the exact same place for each seed and determining what was the force exerted at the point of breakthrough of the pin. While bearing these caveats in mind, the limited data obtained suggested that the fire cues did not lead to an immediate weakening of the seed coat, but that such weakening was possibly detected in the heated and smoked seeds closer to germination. Such a weakening as seeds

approached germination could be induced by the embryo activating enzymes to weaken cell walls, rather than any direct effect of the fire cues.

The method using an external compressive force was more objective and reproducible than the earlier approach, but suffered from the limitation of not simulating well the direction and type of force exerted by an emerging radicle. Again, seed numbers were limited, and conclusions must be tentative. Interestingly, the results suggested an immediate weakening of the seed coat for a proportion of the heated and smoked seeds, detected again as a possible change in shape of the frequency distribution for this group. If this conclusion is confirmed by further work to expand the numbers of seeds sampled, it is evidence that at least one of the fire cues used weakens the seed coat, and does so immediately.

The type of dormancy operating in *Grevillea* best fits the model of either non-deep physiological dormancy, or intermediate physiological dormancy, in the catalogue of dormancy mechanisms of Baskin and Baskin (1998). In seeds with either type of dormancy, fresh seeds fail to germinate unless they receive a dormancy-breaking treatment of some kind (eg cold or warm temperatures, chemicals, or light); excised embryos grow into normal seedlings however. This raises questions about whether the structures covering the embryo (the seed coat in *Grevillea*), the embryo itself, or an interaction between them, cause dormancy in intact seeds. Baskin and Baskin (1998) conclude that even though excised embryos grow normally from these seeds, the embryo is likely to be involved in controlling dormancy, because evidence to date indicates that dormancy-breaking treatments by themselves do not seem to weaken the structures covering the seed.

Thus the tentative conclusion (from the compressive force study) that at least one of the fire cues may have immediately weakened the seed coat for some fraction of the heated and smoked seeds is very interesting, and worthy of follow-up. If substantiated, it would be the first evidence for this phenomenon in the literature. If sufficient seed can be obtained so that the two fire cues can be tested separately, this would allow determination of whether only one of the cues weakens the seed coat, or whether both do.

Growth potential of the embryo

In other species, dormancy-breaking treatments have resulted in an increase in the growth potential of the embryo (as judged by the lowest water potential at which

germination can occur; Nabors and Lang 1971; Baskin and Baskin 1998). Results with half-seeds of *G. juniperina* did not detect any such difference in the growth potential of embryos from control, or heated and smoked seeds, at water potentials down to -0.3 MPa. Whether such a difference exists, but over a range of even lower water potentials, is unclear. An attempt to extend the range of water potentials to lower values resulted in very little germination from the limited number of seeds used. If there is a strong effect of the fire cues on the growth potential of the embryo, more germination would have been expected from the half-seeds treated with the fire cues over the lower range of water potential. This experiment requires follow-up, using a wider range of water potentials than used in the current study. The evidence to hand however does not support the hypothesis of an increase in growth potential of the embryo after treatment with fire cues, in *G. juniperina*. The results of the same experiment with *G. linearifolia* showed a lot of variability, but again did not support the hypothesis of an increase in growth potential induced by fire cues, at least over the range of water potentials used (down to -0.4 MPa).

Conclusion

Some limited progress has been made in investigating the mechanical constraint hypothesis of seed coat dormancy in *Grevillea*. The anatomy of the rupturing of the seed coat by the emerging radicle has been characterised. Two methods of estimating seed coat strength have been tried, and only one considered reliable and reproducible. That said, it is interesting that the estimates of the break through force by the (less reliable) pin method (up to 0.4 MPa) matched well the estimates (by the osmoticum method) of the force that the embryos can at least generate (0.3 – 0.4 MPa). The tentative conclusion from the compressive force method, that one or both of the fire cues may weaken the seed coat, is worth further investigation. If substantiated, it will be the first evidence of such a weakening by a dormancy-breaking treatment in any seed. More work is also required with the osmoticum method, to determine both the maximum force that embryos can generate, and whether this is altered by the fire cues, as is the case for dormancy-breaking treatments in other species.

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Figure Legend

Fig. 1. Median longitudinal section of the micropylar region of a un-treated control seed. The embryo had been removed and the cavity filled with Lucifer Yellow for 24 hours prior to fixation. Tegmel cells fluoresce yellow due to retained Lucifer yellow. The inner micropyle is formed by the inner and outer tegmel layers that merge along the micropylar channel. The upper portion of the outer micropyle is formed by the endotestal palisade cell layers.

Fig. 2. Median longitudinal section of a germinated Heat and Smoke-treated seed. The fracture plane ran through the medial plane of the tegmel wedge, between the palisade cells that formed the upper portion of the outer micropyle and then through the mesotestal and exotestal cells layers f the dorsal part of the seed coat. Section stained with Berberine hemisulphate and $FeCl_3$.

Fig. 3. Longitudinal section through the outer micropylar region of a germinated Heat and Smoke-treated seed. The fracture line, and separation of the upper and lower parts of the seed coat, followed the cell walls and did not across the cells. Section stained with Congo Red.

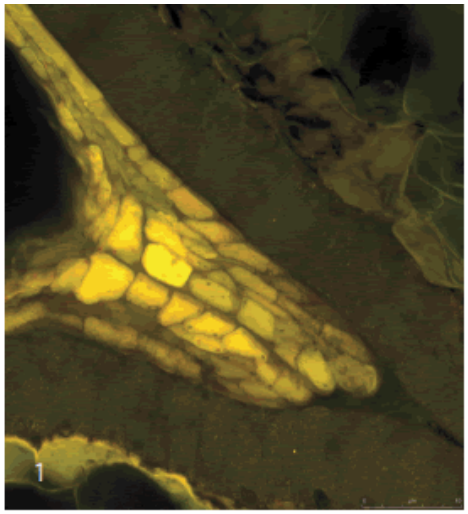
Fig. 4. Longitudinal section through the dorsal part of the seed coat of a Heat and Smoke- treated seed. Again the fracture line followed the cell walls of the mesotestal and exotestal cell layers. Section stained with Congo Red.

Fig. 5. Transverse section through an un-treated control seed just prior to full germination. An incipient fracture plane first occurs in the inner part (i.e. closest to the cotyledons) of the tegmel wedge. Section stained with Congo Red.

Fig. 6. Transverse section through the tegmel wedge of a Heat and Smoke-treated seed just prior to full germination. The fracture line followed the surface of the tegmel cells' walls, and then between the abutting palisade cells that are folded together in this plane of the seed coat. Section stained with Congo Red.

Fig. 7. Transverse section through the tegmel wedge of a Heat and Smoke-treated seed that had fully germinated. The fracture line crossed into the dorsal part of the seed coat via the upper layer of palisade endotestal then ran along the abutting walls of the testal cells. Section Stained with Congo Red and Aniline Blue.

Fig. 8. Macro of a germinated seed. The radicle protruded asymmetrically through the seed coat, forcing apart the dorsal and ventral surfaces. The fracture line first extended along the mid plane of the seed before it ran up the later side to the dorsal region.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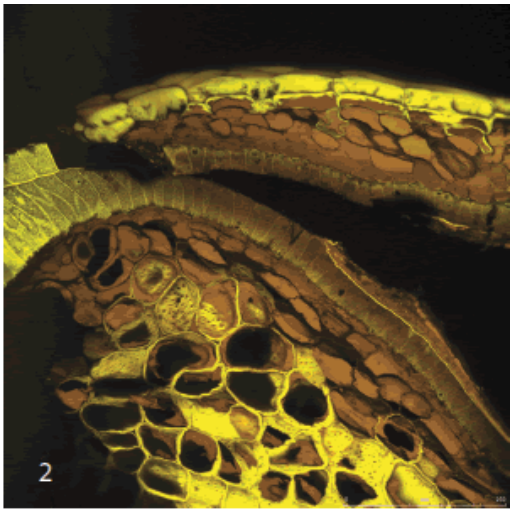
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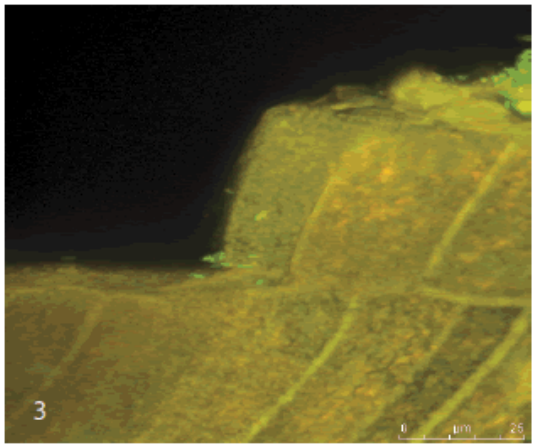
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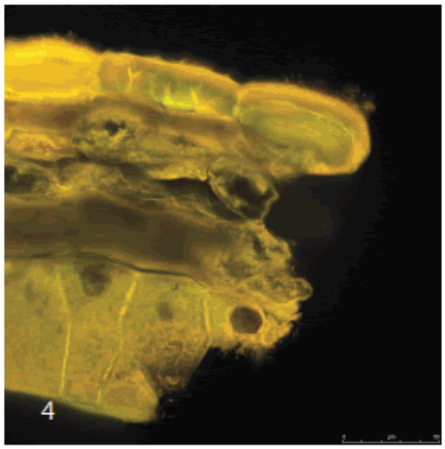
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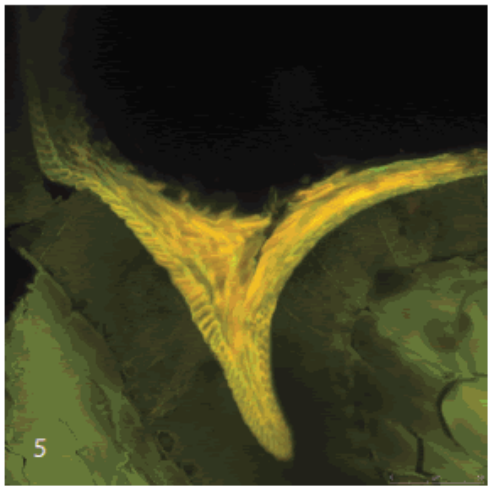
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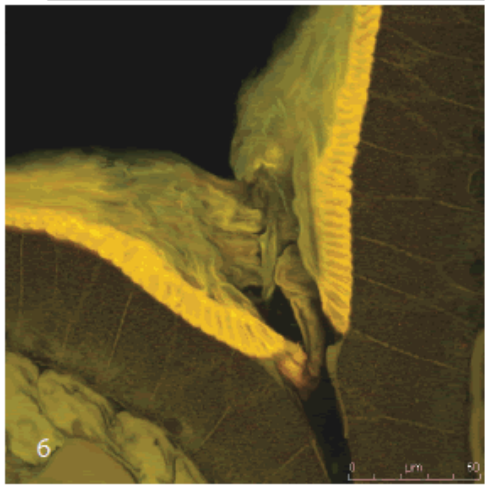
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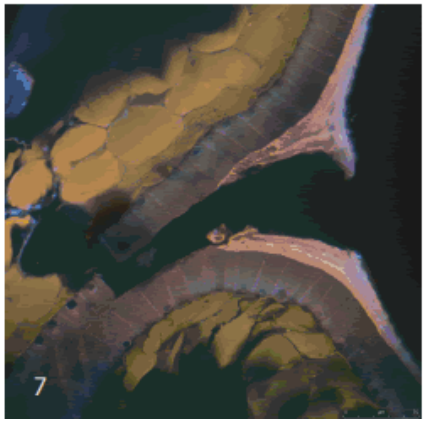
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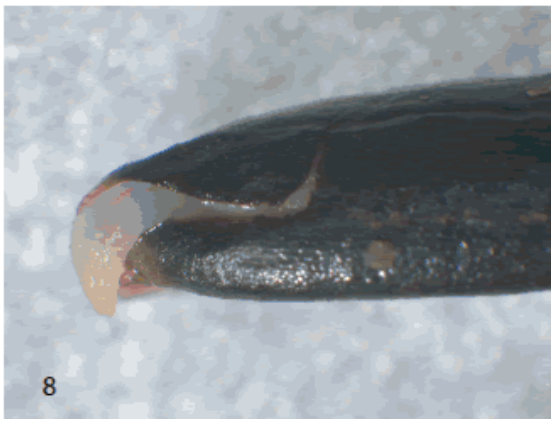
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