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The role of fractures and lipids in the seed coat in the loss of hardseededness of six Mediterranean legume species

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SUMMARY

Changes in the seed coat morphology of 12 annual legumes were studied using environmental scanning electron microscopy (ESEM). The seeds of *Biserrula pelecinus* L. cv. Casbah, *Ornithopus sativus* cv. Cadiz, *Trifolium clypeatum* L., *T. spumosum* L., *T. subterraneum* L. cv. Bacchus Marsh, *Trigonella balansae* Boiss. & Reuter., *Trigonella monspeliaca* L. and *Vicia sativa* subsp. *amphicarpa* Dorthes (morthes.) were examined by ESEM after exposure to field conditions for 6 months, while those of *Medicago polymorpha* L. cv. Circle Valley, *Trifolium clypeatum* L., *T. glanduliferum* Boiss., *T. lappaceum* L., *T. spumosum* L., and *T. subterraneum* L. cv. Dalkeith, were examined after 2 years' exposure. The entry of water into seeds was followed by covering various parts of the seed coat with petroleum jelly and soaking the treated seeds in dyes.

As the seeds softened over time, more and larger fractures appeared on the seed coat. Water entered the seed either through fractures, over the seed coat as a whole or through the lens. It is hypothesized that the formation of fractures occurs after physicochemical changes in the seed coat, probably associated with changes in the amount and nature of seed coat lipids.

The newly matured whole seeds of *M. polymorpha* cv. Circle Valley, *T. clypeatum*, *T. glanduliferum*, *T. lappaceum*, *T. spumosum*, and *T. subterraneum* cv. Dalkeith were analysed for lipid content in 1997. The seed coats of *T. subterraneum* cv. Dalkeith and *T. spumosum* were separated from the cotyledons and examined in detail for lipid content.

The lipid content of whole seeds ranged from 48 (*T. lappaceum*) to 167 mg/g (*T. subterraneum* cv. Dalkeith). Total lipid of the whole seeds of *T. subterraneum* cv. Dalkeith and *T. glanduliferum* declined by about 9 mg/g over 2 years, while in *T. spumosum* it declined by about 17 mg/g.

In contrast, the major fatty acids in the seed coat declined by 0·67 mg/g over the 2 years. Change in seed coat lipids showed a marked similarity to changes in hardseededness for both *T. subterraneum* cv. Dalkeith and *T. spumosum*. The results strongly suggest that seed softening is associated with loss of lipids in the seed coat, because lipids have physical characteristics that are altered at temperatures experienced in the field.

INTRODUCTION

The morphology and anatomy of the seed coat in legume seeds have been described by many authors (Corner 1951; Esau 1977; Fahn 1990). The hilum, micropyle and lens in seeds of legume species differ not only in structure, but also in function (Hamly 1932; Hyde 1954; Tran & Cavanagh 1984). There are two schools of thought on the role of the seed coat. The first is that the seed coat has specialized regions for water loss and uptake such as the hilum, micropyle and lens. The second is that the whole seed coat is involved in water exchange.

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Hyde (1954) first postulated that the hilum of ripe seeds in *Trifolium repens* L., *T. pratense* L. and *Lupinus arboreus* Sims acts as a hygroscopic valve. He found that the testa of hard seeds is impermeable to water except at the hilum, which opens and allows the seed to lose moisture when the external relative humidity is low (10–15%). McDonald *et al.* (1988) reported that water penetrated the seed coat around the hilum area; but also that water can penetrate the seed coat at other sites. Lush & Evans (1980) found that the micropyle, sometimes regarded as a breathing hole or pore, plays a significant role in water uptake.

The lens is a region of structural weakness in the seed coat of some legumes (Hamly 1932). Water entry appears to be through the lens initially, after artificial treatments are applied such as percussion, hot water, high temperature and microwave (Tran 1979; Cavanagh & Tran 1980; Gopinathan & Babu 1985; Manning & van Staden 1987). However, recent results suggest that although the lens region is the primary site of water entry, other sites allow water entry later (Serrato-Valenti *et al.* 1995). On the other hand, Quinlivan (1968) observed that water penetrates at random locations on the seed coat but not at the hilum or lens. Hagon & Ballard (1970) and Quinlivan (1971) further demonstrated that the permeability of the whole testa, or of random sites on the testa, is conditional on the seed moisture content being in the range of 90–140 mg/g. Egley (1979), Abdullah *et al.* (1991) and Morrison *et al.* (1992) also found that water enters at sites other than the lens.

Since lipids are known to be affected by temperatures in the range experienced in Australian summers (Vertucci 1992; Grindstaff *et al.* 1996; Gunstone 1996), it seems likely that there are changes in the lipid content and structure over this period.

Surprisingly little is known about changes in the chemical composition of the seed coat of legumes during the seed softening process. The surface of the seed is covered by a hydrophobic layer, which consists of an insoluble, polymeric and structural component and a complex mixture of lipids (Hamilton & Hamilton 1972; Kolattukudy *et al.* 1981; Graven *et al.* 1996). The components of this layer are primarily cutin, suberin and waxes, all of which are lipids with different fatty acid compositions. Other substances such as callose, phenolics, silica and pectin in the seed coat are also likely to be involved in seed coat impermeability (Serrato-Valenti *et al.* 1993, 1994; Morrison *et al.* 1995; Graven *et al.* 1996). While there is some evidence suggesting that callose and phenolics do not play a role in seed coat impermeability (Serrato-Valenti *et al.* 1989; Beresniewicz *et al.* 1995), most evidence strongly suggests that lipids do (Espelie *et al.* 1980; Riggio-Bevilacqua *et al.* 1985; Serrato-Valenti *et al.* 1994). Whether and how these lipids change during seed softening is not known.

The hypothesis underlying the current study on the role of seed coat lipids is that the thermal degradation of the lipid component of seed coats is of primary importance in the softening of hard seeds. Furthermore, the effects of high temperatures could induce an alteration in the quality and quantity of the lipid component of the seed coat, eventually resulting in the fracturing of the seed coat.

Some important questions about changes in the seed coat during the softening of hard seeds remain unanswered. Firstly, what changes occur to the seed coat during the softening process? Secondly, where does water penetrate the seed coat after the seeds become permeable? Finally, what is the relationship between changes in seed coat morphology and imbibition after exposure to field conditions? The present study attempts to answer these questions.

MATERIALS AND METHODS

Species used, the production of seeds and exposure of the seeds to field conditions

A total of 12 species were used in the study. Eight species (*Biserrula pelecinus* L. cv. Casbah, *Ornithopus sativus* cv. Cadiz, *Trifolium clypeatum*., *T. spumosum*., *T. subterraneum* L. cv. Bacchus Marsh, *Trigonella balansae* Boiss. & Reuter., *Trigonella monspeliaca* L. and *Vicia sativa* subsp. *amphicarpa* Dorthes.), were sown in May 1998 at the Shenton Park Field Station. Seeds were harvested in November 1998 and the pods were returned to the field at the Shenton Park Field Station in December 1998. These seeds were sampled in June 1999. The experimental area was covered with clear polycarbonate roof sheeting supported on a steel frame 1 m above the soil surface to protect the experiment from rainfall. Previous experience has shown that temperatures under the polycarbonate sheeting are very close to outside temperatures.

A further six species (*Medicago polymorpha* L. cv. Circle Valley, *Trifolium clypeatum* L., *T. glanduliferum* Boiss., *T. lappaceum* L., *T. spumosum* L., and *T. subterraneum* L. cv. Dalkeith) were sown in May 1996 at the University of Western Australia Field Station (Shenton Park, 31° 57′ south and 115° 51′ east). Seeds of each species were scarified by machine (to soften the impermeable seed coat) and inoculated with appropriate rhizobia before sowing. All species were sown in rows 4 m long and 2 m apart, at a rate of 1 g/m. Species were randomly allocated into one block at four replicates. Fertilizer (superphosphate and potash, 3:1) was applied at the rate of 10 g/m^2 before sowing. The plots were hand-weeded and watered as necessary. The seeds of all species were harvested in November 1996.

The pods were placed in separate mesh envelopes (20 × 15 cm) and carefully located in three replicates on the soil surface at Shenton Park in January 1997

for 2 years. Pods were included in each envelope for counts of hardseededness to be taken at 4-weekly intervals until June, with final measurements in December 1997 and 1998. The three replicates of pods were pooled and stored at a constant temperature of 4 °C.

Seed coat images

Six to eight seeds of each of the species were recovered after 0, 0·5, and where appropriate for 1, 1·5 and 2 years in the field and the surface imaged using an 'Electro' Scan model E3 environmental scanning electron microscope (ESEM). Images were collected at 15 kV and with a chamber gas pressure of 2·0 torr. Water vapour was used as the imaging gas. Images from each seed were taken at random sites on the seed coat surface. The width of fractures in the seed coat was measured using the NIH image program (version 1.16).

Sites of penetration of water through the seed coat

Experiment 1

Various parts of the seed coat of *O. sativus* cv. Cadiz, *T. subterraneum* cv. Bacchus Marsh and *V. sativa* ssp. *amphicarpa* exposed for 6 months and *T. subterraneum* cv. Dalkeith, *T. spumosum* and *T. clypeatum* exposed for 2 years were covered with petroleum jelly using a stereo microscope at ×10 magnification. The petroleum jelly was applied such that the seed could only imbibe through either the hilum or the lens, or through the remainder of the seed coat. Additionally the whole seed coat was covered with petroleum jelly, which prevented the seed from imbibing water completely. Five seeds were selected for each species and treatment. After the petroleum jelly was applied at the different sites, the seeds were immersed in Petri dishes in two dyes (0·01 mg/g sulfur rhodam and 10 mg/g fuchsin acid). All species were soaked in the dyes for 6 h except *O. sativus* cv. Cadiz, which was soaked for 30 min. Each stained seed was examined externally, and internally after bisecting the seed through the lens and hilum with a razor blade under stereo microscopy at ×10 magnification. Where the seed remained firm and no dye had penetrated the interior, the seed was defined as hard. Where the seed was soft or swollen and the interior was dyed and wet the pathway of water entry was followed. The number of seeds penetrated by the dyes for each treatment was counted at the end of the test.

Experiment 2

To further clarify the point of entry of water, seeds of *T. clypeatum* were sampled at seven times in the first year of field exposure and the same four treatments as in Expt 1 applied. Six seeds were used for each test. In this case the only dye used was 10 mg/g fuchsin acid solution. The seeds were immersed for 6 h and the experiment was replicated three times. The method was same as Expt 1 described above.

Seeds used for lipid study

The species used in the lipid study were six Mediterranean clovers and medics (*M. polymorpha* cv. Circle Valley, *T. clypeatum*, *T. glanduliferum*, *T. lappaceum*, *T. spumosum* and *T. subterraneum* cv. Dalkeith). Bulked samples of the mature, whole seeds of six species were sampled in 1997 and analysed in triplicate for lipid content. *T. glanduliferum*, *T. spumosum* and *T. subterraneum* cv. Dalkeith, were further tested in 1999 to determine whether the lipid content of whole seeds changed after exposure to field conditions for 2 years. The seed coats of *T. spumosum* and *T. subterraneum* cv. Dalkeith were examined in detail for lipid content and hardseededness at 4-weekly intervals during the summer and autumn of 1997 and 1998 and in December 1997 and 1998. Seed samples were tested for hardseededness immediately after sampling, while samples to be tested for lipids were stored at 4 °C until analysis during 1999.

Hardseededness

Pods of the newly matured seeds of *T. spumosum* and *T. subterraneum* cv. Dalkeith were gently rubbed on a corrugated rubber mat to remove seeds. Three replicates of 100 clean seeds each were placed on moist filter paper in Petri dishes and incubated at 15 °C. The numbers of imbibed and germinated seeds were counted on days 7 and 14. The percentage of hard seed was calculated from the proportion of seeds remaining not imbibed and germinated at day 14.

Separation of the seed coat

Seeds of *T. spumosum* and *T. subterraneum* cv. Dalkeith that had not been exposed to field conditions (harvested November 1996 and stored at 4 °C), and seeds that had been exposed for 2 years (collected December 1998) were ground in a domestic coffee grinder for 2 min. This was sufficient time for the seeds to shatter into several fragments: seed coat, cotyledons and seed coats with fragments of cotyledon attached. Seed coat pieces were separated from the cotyledons using different sizes of sieve (0·5, 0·71, 1 and 2 mm) and a sorting tray. Seed coats could easily be identified by texture and colour under a stereo microscope at ×10 magnification. Any small cotyledon fragments were removed in sorted seed coats using forceps under a stereoscopic microscope. The seed coat pieces with attached cotyledons were then further ground in the coffee grinder for 1 s, breaking them into tiny pieces for easy separation.

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Residual cotyledon pieces were removed with forceps under a stereoscopic microscope leaving a pure seed coat preparation.

The procedure was repeated until 4 g of seed coat were obtained for each legume sample. Whole seeds were ground to a powder with the domestic coffee grinder.

Lipid extraction

For each sample, lipids were extracted from the whole seed or the seed coat using the Soxhlet method (Gunstone 1996; Hemming & Hawthorne 1996). A sample (1 g) of the powdered seed or seed coat was put into a Soxhlet thimble. Samples were extracted for 6 h with 20 ml of hexane (995 mg/g). This solvent was evaporated to constant weight using nitrogen gas and the extracted lipids were weighed. Each sample was extracted in triplicate.

Fatty acid analysis

The fatty acid composition was determined by gas chromatography (GC) on the lipid samples by converting the extracted lipids to fatty acid methyl esters (FAMEs) (Ai 1997; Alonso *et al.* 1997). Each of the lipid samples (15–30 mg) was dissolved in 4 ml hexane in a stoppered tube for the whole seed samples, and 2 ml hexane for the seed coat samples. Then 1·5 ml of methanolic potassium hydroxide (2 M) was added to the lipids of the whole seed and seed coat samples to produce the FAMEs. After mixing for 1 min and standing for 15 min, the hexane layer containing the FAMEs were separated and transferred into glass vials. One μl of the hexane fraction (FAMEs) was used for the gas chromatography.

Gas chromatography analysis was performed on a GC-17A V3 (SHIMADZU, Japan) equipped with flame-ionization detector (FID) and flow splitter. Chromatographic conditions used in analysis of lipid fatty acid methyl esters in the seeds and seed coats of legumes by GC with a BPX-70 (50 m × 0·22 mm) capillary were set at 200 KPa for the column head pressure with 35 of split flow ratio and 250 and 285 °C for injector and detector temperatures respectively (Jimenez De Blas & Del Valle Gonzlezález 1996). In the GC oven, initial temperature of 170 °C with initial time 35 min at 5 °C/min and final temperature of 220 °C with final time 5 min were set. Helium and hydrogen were used for carrier and detector gases respectively.

Figure 1 shows a typical chromatogram of the seed coat of *T. spumosum*. In the chromatogram, major peaks were identified against standards (SupelcoTM 37 component FAME mix) and by mass spectrometry (MS, Hewlett Packard Mass Selective) analysis.

The samples of transesterified lipids from whole seeds of the six species were analysed with a GC-17 A V3 and GC/MS. Each peak and its mass from the mass spectrometer represented molecular fragments and the 'parent peak' of the ionized molecule of the fatty acid. The molecular formula of each fatty acid was then determined according to the mass and specific ions.

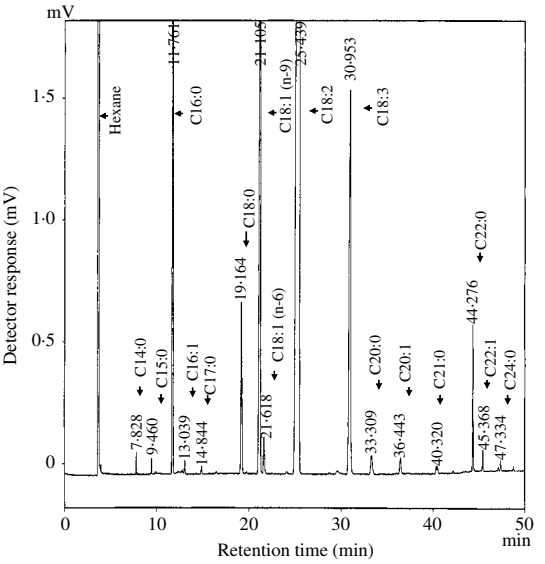

Fig. 1. GC chromatogram of the lipids from whole seeds of *T. spumosum* before exposure in the field.

Statistical analysis

As the seeds were tiny and the operation of covering the various parts of the seed coat was difficult, only five seeds were used for each species and treatment without replication. It was therefore not possible to compare the species in Expt 1. However, using the species as replicates an estimate of the variation between sites of water entry could be calculated. These data were analysed using analysis of variance from the SPSS version 7.5 statistical software system. Tukey's multiple range test and paired-samples *t*-tests were used to compare means. R^2 was obtained from a linear regression analysis.

RESULTS

Change in seed coat morphology during the seed softening

For most species there were no detectable fractures on the seed coat of fresh seeds. However, there were some visible fractures on the seed coats of *O. sativus* cv. Cadiz, *T. spumosum* and *V. sativa* ssp. *amphicarpa* (Table 1). In particular, fresh seeds of *O. sativus* cv. Cadiz had many large fractures (average width

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Table 1. *Seed diameter and fracture width (μm) on the seed coat of 12 species during seed softening. Six species were exposed to field conditions for 2 years and the remainder were exposed for 6 months*

Species	Seed diameter (mm ± S.E.)	Before field exposure (μm ± S.E.)	After field exposure for 6 months (μm ± S.E.)	After field exposure for 1 year (μm ± S.E.)	After field exposure for 2 years (μm ± S.E.)
B. pelecinus Casbah	2·0 (0·05)	N/D	N/D		
M. polymorpha Circle Valley	1·6 (0·02)	N/D	0·2 (0·02)	0·5 (0·04)	0·5 (0·02)
O. sativus Cadiz	1·8 (0·04)	2·9 (0·24)	22·4 (1·80)		
T. clypeatum	2·9 (0·04)	N/D	0·6 (0·03)		
T. clypeatum	2·9 (0·04)	N/D	0·7 (0·04)	0·7 (0·05)	0·9 (0·03)
T. glanduliferum	1·0 (0·01)	N/D	N/D	0·2 (0·02)	0·2 (0·02)
T. lappaceum	1·1 (0·01)	N/D	N/D	N/D	0·4 (0·02)
T. spumosum	1·8 (0·02)	0·4 (0·04)	0·8 (0·12)		
T. spumosum	1·6 (0·02)	0·7 (0·05)	1·1 (0·07)	1·2 (0·10)	2·1 (0·13)
T. subterraneum Bacchus Marsh	2·7 (0·04)	N/D	0·5 (0·05)		
T. subterraneum Dalkeith	2·6 (0·03)	N/D	0·4 (0·02)	0·4 (0·02)	0·5 (0·02)
Trigonella balansae	1·6 (0·02)	N/D	0·4 (0·07)		
Trigonella monspeliaca	1·0 (0·03)	N/D	0·3 (0·07)		
V. sativa amphicarpa	3·8 (0·10)	0·2 (0·06)	0·4 (0·04)		

N/D indicates no detectable fractures on the seed coats examined.

2·9 μm). The number and size of fractures were not associated with seed diameter.

After field exposure for 6 months, fractures appeared on the seed coat of most species. However, there were no detectable fractures on the seed coats of *B. pelecinus* cv. Casbah, *T. lappaceum*, and *T. glanduliferum* at this stage. Of the species with fractures, those of *O. sativus* cv. Cadiz were the widest (22·4 μm), while those of *M. polymorpha* cv. Circle Valley were the narrowest (0·2 μm). There were slight differences in the fracture sizes of *T. spumosum* and *T. clypeatum* in the 2 years. The fractures further widened in those species exposed for 2 years. *T. spumosum* had the widest fractures (2·1 μm) and *T. glanduliferum* (0·2 μm) the narrowest at the end of the experiment.

Figure 2 shows the development of fractures on the seed coats of *T. subterraneum* cv. Dalkeith, *T. spumosum* and *O. sativus* cv. Cadiz during the course of the experiment.

Sites of penetration of water through the seed coat

Experiment 1

Water was able to penetrate 0·73 of the seeds through fractures on the seed coat and 0·87 of the seeds through the lens (no significant difference at $P<0·05$ level; Table 2). Water did not penetrate the seed coat if the whole surface was covered with petroleum jelly, nor when the hilum alone was exposed.

Experiment 2

The seeds of *T. clypeatum* did not imbibe when the seed coat surface was fully covered by petroleum jelly (Table 3). Likewise, there were few seeds where dye penetrated through the hilum. However, where either the lens or the fractured seed coat were exposed the proportion of seeds imbibing rose from 0·06 initially to 0·67 in December 1997. There were no significant differences at any sampling time between the treatments where the lens and seed coat remained uncovered, indicating that seeds softened at both sites at roughly the same time. The relationship between the percentage of hard seeds and the number of seeds that imbibed through the lens ($R^2=0·85$, $n=48$, $P<0·05$) and through the fractured seed coat ($R^2=0·71$, $n=48$, $P<0·05$) was highly significant in both cases.

Lipid content and fatty acid composition of the whole seed

The lipid content of whole seeds ranged from 48 (*T. lappaceum*) to 167 mg/g (*T. subterraneum* cv. Dalkeith) (Table 4). Major fatty acid elements of the seed lipids of all six legumes were palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), behenic (C22:0) and lignoceric (C24:0) acids. Some species showed only traces of some of the fatty acids. For example, in *T. clypeatum* heptadecanoic (C17:1), erucic (C22:1) and nervonic (C24:1) acids were below detectable

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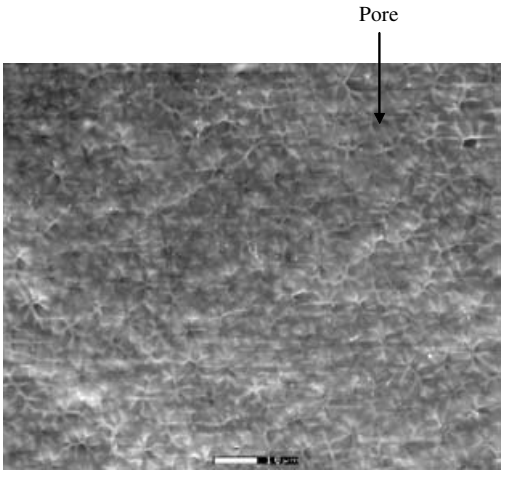
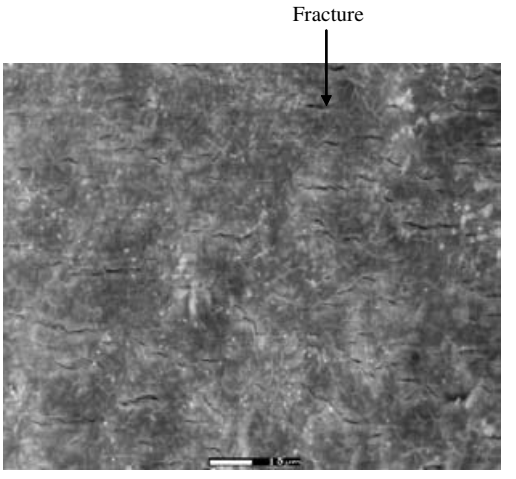
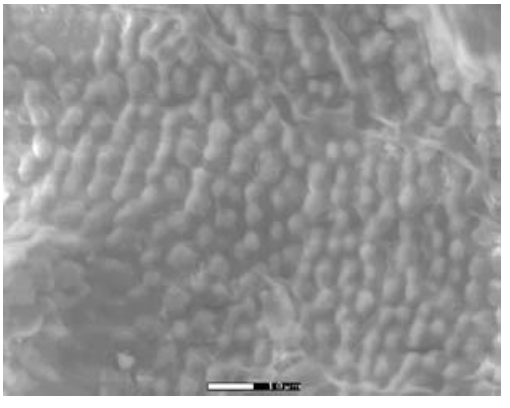


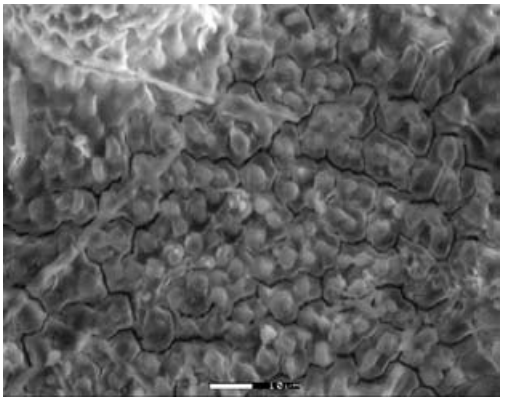
A 1. Initial seed of Dalkeith



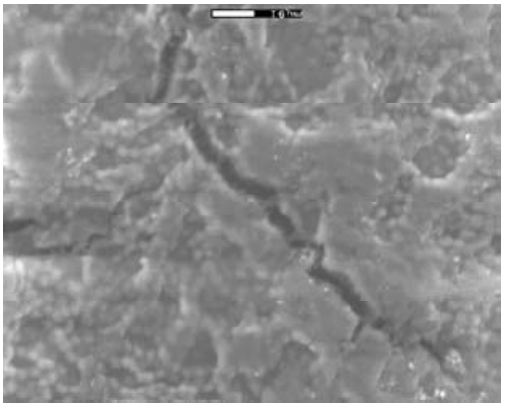
A 2. Dalkeith seed exposed for one year



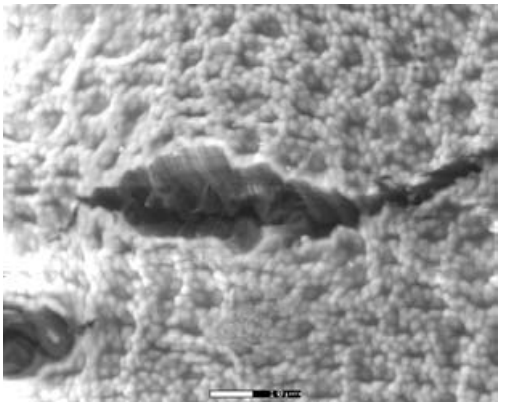
B 1. Initial seed of *T. spumosum*



B 2. *T. spumosum* seed exposed for one year



C 1. Initial seed of Cadiz



C 2. Cadiz seed exposed for six months

Fig. 2. The development of fractures on the seed coat of *T. subterraneum* cv. Dalkeith, *T. spumosum* and *O. sativus* cv. Cadiz during the course of the experiment. Images were taken using ESEM.

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Table 2. *The number of seeds* (*out of 5*) *imbibing water using petroleum jelly to cover various parts of the seed coat. The seeds of* T. subterraneum *cv. Dalkeith,* T. spumosum *and* T. clypeatum *were exposed in the field for 2 years while the other species were exposed for 6 months.* O. sativus *cv. Cadiz was soaked in the dyes for 30 min and the others for 6 h*

Stains	0·1 mg/g sulphur rhodam				10 mg/g fuchsin acid			
Species	Covered surface	Exposed hilum	Exposed lens	Exposed fracture	Covered surface	Exposed hilum	Exposed lens	Exposed fractures
O. sativus Cadiz	0	0	4	5	0	0	4	5
T. clypeatum	0	0	5	3	0	0	5	3
T. spumosum	0	1	4	5	0	2	5	4
T. subterraneum Dalkeith	0	0	5	2	0	0	5	3
T. subterraneum Bacchus Marsh	0	1	5	5	0	1	5	4
V. sativa amphicarpa	0	1	3	3	0	1	2	3
Means* (±S.E.) D.F. (4)	0·00 (<0·001)	0·10 (0·004)	0·87 (0·061)	0·77 (0·099)	0·00 (<0·001)	0·13 (0·061)	0·87 (0·090)	0·73 (0·061)

* Means expressed as a proportion of seeds imbibing for the treatment meaned over the six species.

Table 3. *Effect of covering various parts of the seed coat with petroleum jelly on the imbibition of* T. clypeatum. *Measurements of the softened seeds, expressed as proportions, were taken from December 1996 to December 1997*

Various parts	Initial (±S.E.)	Jan (±S.E.)	Feb (±S.E.)	Mar (±S.E.)	Apr (±S.E.)	May (±S.E.)	Jun (±S.E.)	Dec (±S.E.)
Covered surface	0	0	0	0	0	0	0	0
Exposed hilum	0	0·06	0·06	0	0	0·06	0·06	0·06
Exposed lens	0·1 (0·06)	0·2 (0·06)	0·3 (0·10)	0·3 (0·10)	0·4 (0·15)	0·6 (0·06)	0·7 (0·06)	0·7 (0·00)
Exposed fracture	0·1 (0·06)	0·2 (0·00)	0·2 (0·06)	0·4 (0·15)	0·5 (0·10)	0·6 (0·06)	0·6 (0·06)	0·7 (0·00)
Softseededness	0·3 (0·01)	0·3 (0·01)	0·4 (0·02)	0·4 (0·05)	0·4 (0·06)	0·6 (0·02)	0·8 (0·03)	0·8 (0·01)

levels. However, there was great variation in the proportion of individual fatty acid to total lipid content between species (Table 4).

The proportion of saturated acids in the fatty acids was 0·18–0·25. The ratio of saturated to unsaturated ranged from 0·2–0·3. These observations illustrate that although the ratio of fatty acid composition varied greatly among the six legumes, those of saturated to unsaturated acid exhibited little variation (Table 4).

Total lipids in the whole seeds of *T. subterraneum* cv. Dalkeith and *T. glanduliferum* declined by about 9 mg/g over 2 years, while in *T. spumosum* they declined by about 17 mg/g (Table 5).

Changes in lipid content of the seed coat

The lipid contents of the seed coats were much less than those of the seeds as a whole. For example, mature seeds of *T. subterraneum* cv. Dalkeith contained 167 mg/g lipids compared with a content of only 21 mg/g in the seed coat (Tables 5 and 6). Similarly, whole seeds of *T. spumosum* contained 58 mg/g lipids compared with a content of only 11 mg/g in the seed coat. After exposure of the pods in the field for 2 years, the lipid contents of seed coats decreased further to 9 mg/g for *T. subterraneum* cv. Dalkeith and 6 mg/g for *T. spumosum* (Table 6).

Change in the total lipids in the seed coat shows a marked similarity to changes in hardseededness (Fig. 3). In the case of *T. subterraneum* cv. Dalkeith the seed coat lost 0·6 of their lipid content over the 2 years, almost all of which occurred in the first year. About 0·8 of the hardseededness was also lost in the first year. In the case of *T. spumosum* the similarity between lipid loss and seed softening was even more marked. The amount of lipids fell during the summer and autumn of each of the 2 years, almost exactly reflecting the loss in hardseededness. About 0·4 of the lipids were lost over the 2 years, of which approximately equal amounts were lost in the first and second years. Thus, the seed population lost almost half of its hard seeds in the first year and most of the remainder in the second year.

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Table 4. *Proportion of individual fatty acids* (*mean and* S.E. *both* $\times 10^{-4}$) *in lipids and total lipid content* (*mg/g dry matter*) *from mature whole seeds of six annual legumes. The proportion of fatty acids was calculated on the basis of peak areas relative to total peak area* (*mV* × *s; millivoltage* × *second*) *in the chromatogram*

Fatty acid and lipids	*T. subterraneum* Dalkeith (±S.E.)	*T. spumosum* (±S.E.)	*T. lappaceum* (±S.E.)	*T. glanduliferum* (±S.E.)	*T. clypeatum* (±S.E.)	*M. polymorpha* Circle Valley (±S.E.)
Myristic (C14:0)	5 (<0·1)	11 (<0·1)	17 (<0·1)	10 (<0·1)	8 (<0·1)	31 (<0·1)
Pentadecanoic (C15:0)	6 (<0·1)	10 (<0·1)	11 (<0·1)	11 (<0·1)	5 (<0·1)	10 (<0·1)
Palmitic (C16:0)	1744 (5·0)	1438 (10·0)	1406 (20·0)	1443 (7·0)	1776 (8·0)	1368 (11·0)
Palmitoleic (C16:1)	16 (<0·1)	11 (<0·1)	4 (2·0)	5 (<0·1)	44 (<0·1)	7 (<0·1)
Heptadecanoic (C17:0)	11 (<0·1)	8 (<0·1)	5 (1·0)	15 (<0·1)	8 (<0·1)	8 (<0·1)
cis-10-Heptadecanoic (C17:1)	2 (<0·1)	N/D	N/D	5 (<0·1)	N/D	N/D
Stearic (C18:0)	486 (1·0)	240 (1·0)	180 (2·0)	192 (<0·1)	351 (2·0)	130 (<0·1)
Oleic (C18:1)	1739 (1·0)	1186 (3·0)	840 (5·0)	1411 (1·0)	1400 (2·0)	847 (1·0)
Linoleic (C18:2)	5767 (5·0)	6017 (7·0)	4936 (12·0)	2482 (2·0)	5038 (4·0)	1360 (2·0)
Linolenic (C18:3)	111 (<0·1)	824 (2·0)	2380 (18·0)	3963 (7·0)	1030 (2·0)	5210 (16·0)
Arachidic (C20:0)	52 (<0·1)	45 (<0·1)	43 (<0·1)	55 (<0·1)	92 (<0·1)	165 (<0·1)
Eicosenic (C20:1)	15 (<0·1)	29 (<0·1)	12 (<0·1)	50 (<0·1)	19 (<0·1)	36 (<0·1)
Heneicosanoic (C21:0)	5 (<0·1)	19 (<0·1)	13 (<0·1)	27 (<0·1)	14 (<0·1)	21 (<0·1)
Behenic (C22:0)	35 (<0·1)	130 (2·0)	122 (2·0)	273 (1·0)	195 (3·0)	244 (1·0)
Erucic (C22:1)	5 (<0·1)	18 (<0·1)	2 (1·0)	18 (<0·1)	N/D	8 (<0·1)
Lignoceric (C24:0)	4 (<0·1)	12 (1·0)	18 (<0·1)	30 (1·0)	15 (1·0)	510 (3·0)
Nervonic (C24:1)	N/D	6 (3·0)	9 (<0·1)	N/D	N/D	9 (1·0)
Total saturated*	235 (5·0)	191 (8·0)	181 (2·4)	204 (8·0)	247 (7·0)	249 (8·0)
Total unsaturated†	762 (5·0)	809 (9·0)	819 (24)	793 (8·0)	753 (6·0)	748 (17)
Saturated/unsaturated	0·31 (<0·1)	0·24 (<0·1)	0·22 (<0·1)	0·26 (<0·1)	0·33 (<0·1)	0·33 (<0·1)
Lipids (mg/g dry matter)	167 (1·4)	58 (0·6)	48 (1·1)	68 (0·9)	64 (1·6)	63 (0·5)

* Total saturated includes Myristic (C14:0), Pentadecanoic (C15:0), Palmitic (C16:0), Heptadecanoic (C17:0), Stearic (C18:0), Arachidic (C20:0), Heneicosanoic (C21:0), Behenic (C22:0) and Lignoceric (C24:0).
† Total unsaturated includes Palmitoleic (C16:1), cis-10-Heptadecanoic (C17:1), Oleic (C18:1), Linoleic (C18:2), Linolenic (C18:3), Eicosenic (C20:1), Erucic (C22:1) and Nervonic (C24:1).
N/D: Not detected.

Table 5. *Total lipid content* (*mg/g dry matter*) *of the whole seed at different exposure times in the field*

Species	Before exposure (±S.E.)	Exposed 1 year (±S.E.)	Exposed 2 years (±S.E.)
T. glanduliferum	68 (0·9)	63 (1·4)	59 (1·8)
T. spumosum	58 (0·6)	50 (2·0)	41 (0·2)
T. subterraneum Dalkeith	167 (1·4)	164 (3·7)	158 (6·6)

Change in fatty acid profile in the seed coat over the 2 years of field exposure

For the freshly harvested seeds, the major fatty acids in the seed coats of *T. subterraneum* cv. Dalkeith and *T. spumosum* consisted of palmitic, stearic, oleic, linoleic and linolenic acids (Table 6). The proportion of individual fatty acids in the total showed some differences between the species. For example, in *T. subterraneum* cv. Dalkeith the proportion of stearic was 0·043 of the total while in *T. spumosum* it was 0·039. *T. subterraneum* cv. Dalkeith had a proportion of 0·168 oleic acid compared with 0·081 in *T. spumosum*. Finally, the proportion of linolenic in *T. subterraneum* cv. Dalkeith (0·02) was less than in *T. spumosum* (0·10).

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Table 6. *Proportion of individual fatty acids* (*mean and* S.E. *both* $\times 10^{-4}$) *and total lipid content* (*mg/g dry matter*) *of the seed coats of* T. subterraneum *cv. Dalkeith and* T. spumosum *before exposure to field conditions and after 2 years' exposure. The proportion of fatty acids was calculated on the basis of the peaks of each fatty acid relative to the total peak area* (*mV* × *s; millivoltage* × *second*) *in the chromatogram*

Lipids and fatty acid	*T. subterraneum* Dalkeith (before exposure) (± S.E.)	*T. subterraneum* Dalkeith (exposed 2 years) (± S.E.)	*T. spumosum* (before exposure) (± S.E.)	*T. spumosum* (exposed 2 years) (± S.E.)
Lauric (C12:0)	6 (4·0)	28 (13·0)	N/D	148 (50·0)
Myristic (C14:0)	13 (<0·1)	10 (6·0)	N/D	N/D
Pentadecanoic (C15:0)	16 (<0·1)	34 (7·0)	N/D	N/D
Palmitic (C16:0)	1887 (5·0)	1914 (8·0)	1956 (14·0)	2158 (10·0)
Palmitoleic (C16:1)	11 (5·0)	N/D	N/D	N/D
Heptadecanoic (C17:0)	14 (<0·1)	N/D	N/D	N/D
Stearic (C18:0)	430 (1·0)	387 (1·0)	265 (1·0)	291 (2·0)
Oleic (C18:1)	1681 (2·0)	1693 (5·0)	813 (5·0)	862 (15·0)
Linoleic (C18:2)	5558 (6·0)	5446 (25·0)	5825 (12·0)	5625 (32·0)
Linolenic (C18:3)	201 (1·0)	184 (3·0)	996 (1·0)	801 (13·0)
Arachidic (C20:0)	73 (1·0)	87 (7·0)	N/D	N/D
Eicosenic (C20:1)	14 (3·0)	N/D	N/D	N/D
Behenic (C22:0)	68 (1·0)	125 (3·0)	97 (2·0)	117 (4·0)
Erucic (C22:1)	16 (<0·1)	64 (1·0)	49 (1·0)	N/D
Lignoceric (C24:0)	12 (<0·1)	29 (6·0)	N/D	N/D
Total saturated*	252 (5·0)	261 (26·0)	232 (14·0)	271 (54·0)
Total unsaturated†	748 (5·0)	739 (27·0)	768 (14·0)	729 (54·0)
Saturated/unsaturated	0·34 (<0·1)	0·35 (<0·1)	0·30 (<0·1)	0·37 (<0·1)
Lipids (mg/g dry matter)	21 (2·5)	9 (1·3)	11 (0·3)	6 (0·3)

* Total saturated includes Lauric (C12:0), Myristic (C14:0), Pentadecanoic (C15:0), Palmitic (C16:0), Heptadecanoic (C17:0), Stearic (C18:0), Arachidic (C20:0), Behenic (C22:0) and Lignoceric (C24:0).
† Total unsaturated includes Palmitoleic (C16:1), Oleic (C18:1), Linoleic (C18:2), Linolenic (C18:3), Eicosenic (C20:1) and Erucic (C22:1).
N/D: Not detected.

In addition, to a lesser extent, lauric, myristic, pentadecanoic, palmitoleic, arachidic, eicosenic and lignoceric acids were found in the seed coat of fresh seeds for *T. subterraneum* cv. Dalkeith, but were rarely and/or not detected for *T. spumosum*. This result indicates that composition of the seed coats differed between the species. However, the ratio of saturated to unsaturated fatty acids was similar: 0·30 and 0·34 for *T. subterraneum* cv. Dalkeith and *T. spumosum*, respectively.

During the period of exposure to field conditions the proportions of the various fatty acids in the lipids remained approximately constant. Thus, in *T. subterraneum* cv. Dalkeith, linoleic acid was 0·556 of total lipids at seed maturity and 0·545 after 2 years' exposure. Similarly, in *T. spumosum*, the same acid was 0·583 at seed maturity and 0·563 after 2 years' exposure. None of these figures were significantly different (Table 6). There was some increase in some of the shorter chain acids (e.g. pentadecanoic in *T. subterraneum* cv. Dalkeith) but these are associated with extremely low proportions. In general, there was no change in the relative proportions of the fatty acids over the 2 years of exposure.

Change in the quantity of fatty acids during seed softening

With only two exceptions (lauric and erucic acids in *T. subterraneum* cv. Dalkeith) there were smaller amounts of the individual fatty acids after 2 years of field exposure than in the mature, unexposed seed. By and large these decreased at a common rate, about 0·67 of the amount present in the mature seed, equivalent to the total loss in lipids (Table 7). The proportions of unsaturated and saturated acids remained the same.

DISCUSSION

Many authors have observed that fractures develop on the seed coat with 'artificial' softening caused by percussion and boiling water (Hagon 1971; Dell 1980; Baker *et al.* 1987; Cavanagh 1987; Russi *et al.* 1992; Read *et al.* 2000). These fractures have not necessarily been associated with seed softening. For example Russi *et al.* (1992) believed that the 'cracks' were not deep enough to penetrate through the outer layers of the seed coat. Nevertheless, it seems clear in

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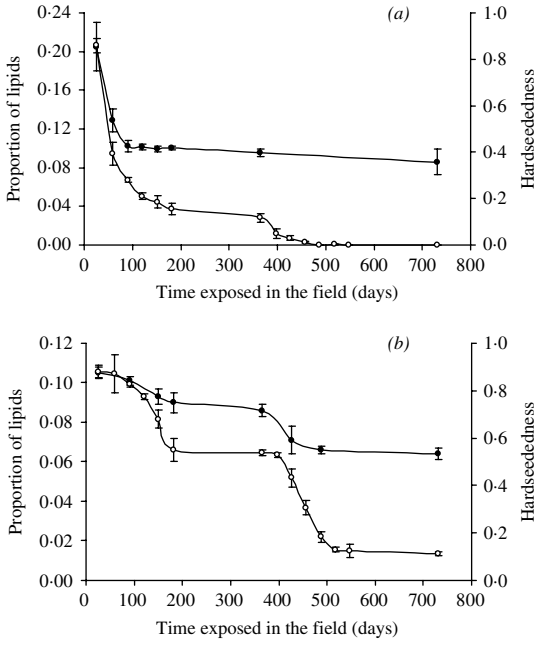

Fig. 3. Change in lipid content (●) of the seed coat and hardseededness (○) over 2 years for (*a*) *T. subterraneum* cv. Dalkeith subterranean clover and (*b*) *T. spumosum* exposed to field conditions. The changes in lipids of the seed coat were reflected in the patterns of seed softening for both legumes.

the present study that one of the main changes that takes place on the seed coat during the softening process is the development of fractures with 'natural' softening.

In general, the development of fractures has been attributed to the alternating temperatures that Mediterranean legumes experience in summer. The argument is that as the temperature rises and falls the seed coat expands and contracts, gradually weakening the structure of the seed coat. This can be seen as a physical explanation of seed softening and has been put forward by Quinlivan (1961) amongst others. For example, at Shenton Park the average minimum and maximum surface temperatures between 1 December 1996 and 28 February 1997 were, respectively, 17 and 61 °C. There were a number of occasions when the daily maximum temperature reached 70 °C on the soil surface. It is argued that the repeated contraction and expansion of the seed coat creates a mechanical force from the cumulative heating energy of high and fluctuating temperatures, which results in the fracturing of the seed coat.

High temperatures and alternatively fluctuating temperatures are key factors which are responsible for the hard seed softening process (Quinlivan 1961). The development of fractures might have three stages. Firstly, the seeds contract and expand repeatedly under high and fluctuating temperatures. For this process to be completed, a critical amount of cumulative thermal energy would be needed. Secondly, shallow fractures appear on the seed coat, caused by changes in the seed coat morphology. This is likely to depend on the mechanical properties of the seed coat and vary between species. Finally, as the seeds are exposed to longer periods of temperature fluctuation, the fractures would deepen and widen.

Hamly (1932), Quinlivan (1968) and Hanna (1984) reported that fractures occurred in the lens region and other sites on the seed coat. The initial cracking for the tissues occurred around the hilum and the most common separation (fracture) is perpendicular to the raphe (along the axis of the micropyle, hilum and lens) (Yaklich & Barla-Szabo 1993).

The results in Fig. 3 strongly suggest that seed softening is associated with the loss of lipids in the seed coat. The similarity between the change in hardseededness and the lipid content over time is most striking, especially in *T. spumosum*. However, even in *T. subterraneum* cv. Dalkeith the loss in hardseededness and the reduction in lipid concentration is almost exactly the same. It is difficult to resist the idea that there is a causal relationship, although this cannot be inferred with certainty.

Both seed softening and lipid loss occur at a time of high temperatures. There is ample evidence that high temperatures are necessary for the hard seeds of legumes to soften (Quinlivan 1971; Taylor 1981). However, lipids are also affected by high temperatures. Each fatty acid component has its own specific melting point. Lipids, which are a mixture of fatty acids, may be wholly solid, wholly liquid or frequently a mixture of solid and liquid phases at any given temperature (Gunstone 1996). The fatty acids detected in the seed coats of *T. subterraneum* cv. Dalkeith and *T. spumosum* have melting points that range from 22–85 °C (Gunstone 1996), similar to the range of temperatures experienced during summer in Western Australia. If the proportion of solid and liquid phases and the change of this proportion depend on the seed coat structure and morphology interacting with environmental temperatures over time, it can be expected that the nature and composition of the lipids will determine the level of hardseededness and its resistance to field conditions in each species. Thus the daily alternating temperature cycles experienced in summer and autumn could cause the two phases (solid and liquid) of lipids to also alternate. If so, the effect of temperature on the seed coat could be explained in terms of its effect on the amount and composition of the lipids in the seed coat.

Most recent authors believe that water enters softened seed through the lens, which they consider an area of structural weakness (Tran 1979; Cavanagh & Tran 1980; Oliveira *et al.* 1984; Manning & van

Table 7. *Proportion of individual fatty acids* (*mean and* S.E. *both* $\times 10^{-4}$) *and total lipid content* (*mg/g dry matter*) *in the seed coats of* T. subterraneum *cv. Dalkeith and* T. spumosum *before exposure in the field and after 2 years' exposure. Amounts of each fatty acid* (*mg*) *from 1 g powder sample of the seed coat were calculated using external calibration curve* (*Nonadecanoic Acid Methyl Ester, C19:0*)

Fatty acid	*T. subterraneum* Dalkeith (before exposure) (±S.E.)	*T. subterraneum* Dalkeith (exposed 2 years) (±S.E.)	*P**	*T. spumosum* (before exposure) (±S.E.)	*T. spumosum* (exposed 2 years) (±S.E.)	*P*
Lauric (C12:0)	1 (<0·1)	1 (1·0)	−0·26	N/D	3 (<0·1)	
Myristic (C14:0)	2 (<0·1)	1 (1·0)	0·56	N/D	N/D	
Pentadecanoic (C15:0)	2 (<0·1)	2 (<0·1)	0·19	N/D	N/D	
Palmitic (C16:0)	228 (40·0)	78 (4·0)	0·66	90 (40·0)	32 (2·0)	0·65
Palmitoleic (C16:1)	2 (<0·1)	N/D	1·00	N/D	N/D	
Heptadecanoic (C17:0)	2 (<0·1)	N/D	1·00	N/D	N/D	
Stearic (C18:0)	52 (5·0)	16 (1·0)	0·69	13 (5·0)	5 (<0·1)	0·63
Oleic (C18:1)	203 (17·0)	69 (3·0)	0·66	38 (17·0)	13 (1·0)	0·65
Linoleic (C18:2)	670 (119·0)	220 (10·0)	0·67	266 (119·0)	82 (5·0)	0·69
Linolenic (C18:3)	25 (20·0)	8 (<0·1)	0·68	46 (20·0)	12 (1·0)	0·74
Arachidic (C20:0)	9 (<0·1)	4 (<0·1)	0·57	N/D	N/D	
Eicosenic (C20:1)	2 (<0·1)	N/D	1·00	N/D	N/D	
Behenic (C22:0)	9 (2·0)	6 (<0·1)	0·36	5 (2·0)	2 (<0·1)	0·55
Erucic (C22:1)	2 (1·0)	3 (<0·1)	−0·26	3 (1·0)	N/D	1·00
Lignoceric (C24:0)	2 (<0·1)	2 (<0·1)	0·13	N/D	N/D	
Total saturated	304 (47·0)	106 (6·0)	0·65	106 (47·0)	40 (2·0)	0·65
Total unsaturated	901 (157·0)	297 (14·0)	0·67	351 (157·0)	106 (7·0)	0·67
Lipids (mg/g dry matter)	1205 (204·0)	403 (21·0)	0·67	457 (204·0)	146 (9·0)	0·68

* Proportional change of the exposed 2 years over before exposed.
N/D: Not detected.

Staden 1987; Serrato-Valenti *et al.* 1995). The lens contains a small plug (0·3 × 0·25 mm), adjacent to the hilum, which is lost by eruption after heating (Dell 1980). Fairbrother (1991) concluded that fluctuating temperatures cause the cell wall fibres to shrink and swell resulting in the rupture of the lens. It is therefore surprising that in all the species studied in the present work, water enters through fractures distributed over the whole seed coat, including the lens. The results in Table 2 provide no evidence that the lens area fractures first. On the contrary, water appears to enter through the lens and the remainder of the seed coat at the same time throughout the softening period. Lush & Evans (1980) also provide evidence that the whole seed coat, including the lens, has the capacity to control water exchange. Indeed, it appears that the whole seed coat should be regarded as an integrated system in which both water-repellency and mechanical properties of the seed coat are important in the control of water exchange (Ballard 1976).

Ballard (1973) and Egley (1979) stressed that the lens should be studied further to gain more information about seed coat permeability. On the other hand, Morrison *et al.* (1992) believed it likely that, because the whole seed experiences environmental stresses, loss of impermeability will occur at random sites.

The hilum is unlikely to be involved in the response to conditions experienced at Shenton Park. Water entry through the hilum is controlled by a fissure which acts as a hygroscopically activated valve (Hyde 1954). This takes place only when seed moisture is higher than would be experienced at Shenton Park.

The present study strongly supports the idea that water enters the seed at many sites but leaves many questions unanswered. In particular, it does not explain the two stage softening process (Taylor 1981). We need to know more about the chemical composition of the seed coat and how this might influence the development, nature and size of the fractures formed during the softening process.

The role of lipids in seed softening is consistent with Taylor's two stage softening hypothesis (Taylor 1981, 1996) in which a preconditioning period of high temperatures is required before a short period of alternating temperatures (60/15 °C for subterranean clover and 30/10 °C for *M. polymorpha*) finally softens the seeds. According to the results of present study, the two stage process is likely to include physical and chemical changes in seed coat lipids. Physical changes, the result of high temperatures

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in summer, would seem to be confined to the first stage where the polymeric structure of lipids is changed due to a weakening of the hydrophobic bonds. This stage of the process develops slowly with thermal input from the environment, making lipids more vulnerable to degradation, leading to the stage that Taylor has called 'preconditioning'. With alternating temperatures further acting on the thermally degraded seed coat during the second stage, chemical changes to the lipids caused by hydrolysis are possible. Lipid hydrolysis can proceed to form free fatty acids by water in what would be a reaction between the fat and water (Gunstone 1996). The chemical process would require water, which is present either in the seed coat or from the atmosphere, where decreasing temperatures result in increased humidity. This is possibly the reason why low temperatures of 15 or 10 °C are necessary for the second stage in many legume species (Quinlivan 1961; Norman *et al*. 1998); high humidity is more likely at low temperature. The alternating temperatures are therefore involved in the physiochemical changes in the properties of lipids, which occur rapidly over a short period, which is consistent with the rapidity of Taylor's second stage.

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