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Two-hundred-year seed survival of *Leucospermum* and two other woody species from the Cape Floristic region, South Africa

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Abstract

The ability of orthodox seeds to survive long-term dry storage is a key prerequisite for *ex situ* seed conservation in genebanks. However, only a few credible observations of seed survival for ≥200 years have been reported. In this paper, seed survival is shown for three species under suboptimal storage conditions for a documented time of ≥203 years and carbon dated at 218–270 years. Two species that germinated are in the *Fabaceae* (*Liparia* sp. and *Acacia* sp.). A water-impermeable seed coat, and hence an inability to equilibrate with ambient relative humidity, may contribute to long-term survival of these species. The third species that germinated (*Leucospermum* sp.) does not have a water-impermeable seed coat, and long-term survival may be linked to an oxygen-impermeable barrier in the seed. These results for species from the Cape Floristic Region, South Africa, suggest adaptation for extreme longevity in seeds of species of seasonally dry, Mediterranean environments.

Keywords: *Fabaceae*, *Leucospermum*, longevity, *Proteaceae*, seed storage

Introduction

Seed longevity of orthodox seeds (*sensu* Roberts, 1973) increases in a predictable and quantifiable way with decreases in both temperature and relative humidity; this can be modelled using the seed viability equation (Ellis and Roberts, 1980). Using this approach and species-specific parameters, exceptional life-spans can be predicted for a range of species. For example, seeds of *Sorghum bicolor* are predicted to take >10,000 years to fall from 98 to 50% viability at −20°C and 15% relative humidity (RH) (Kuo *et al.*, 1990). Such predictions form the rationale for the long-term *ex situ* storage and conservation of seeds in genebanks.

However, parameters used in the viability equation are usually based on accelerated ageing of seeds under comparatively harsh conditions, particularly with respect to temperature (usually 50–60°C; reviewed in Pritchard and Dickie, 2003). Consequently, although seeds of some species may survive, at least in theory, for hundreds to thousands of years under suitable conditions, there is a need for confirmatory data for extreme longevity under more natural long-term storage conditions.

In this context, there are a number of reports of extreme longevity under 'natural' conditions. However, several of these records are unreliable. For example, viable wheat and pea seeds are alleged to have been recovered from Pharaoh's tombs in Egypt. However, these are now thought to be the result of hoax or modern contamination (Priestley, 1986). Similarly, there are reports of seed longevity on a time scale of >1700 years for *Lupinus arcticus* (Porsild *et al.*, 1967), *Chenopodium album* and *Spergula arvensis* (Odum, 1965) and *Nelumbo nucifera* (Libby, 1955). However, in all these cases, the dating of seeds relied on circumstantial evidence, in particular their association with archaeological artefacts of known age. Consequently, such reports have been viewed sceptically (Godwin, 1968; Bewley and Black, 1982; Priestley, 1986).

None the less, there are reliable reports of extreme longevity in two species. Individually carbon-dated seed coats from germinated seeds of *Nelumbo nucifera*

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have been dated at 200–1288 years old (Shen-Miller, 2002; Shen-Miller *et al.*, 2002). Similarly, a viable seed of *Canna compacta* has been removed from a ceremonial rattle, consisting of a *Juglans australis* nutshell found in Argentina. The nutshell itself was dated at *c.* 600 years old; the *Canna* seed could only have been inserted inside the nutshell while it was still developing (Bewley and Black, 1982).

There are also several reports of germination of *c.* 100-year-old seeds. For example, viable seeds have been reported from herbarium specimens of between 90 and 110 years old (Bewley and Black, 1982; Steiner and Ruckenbauer, 1995). In addition, seeds of three species (*Verbascum blattaria*, *V. thapsus* and *Malva rotundifolia*) from the long-term seed burial experiment set up by Dr Beal in 1879 (Beal, 1905) germinated after 100 and 120 years (Kivilaan and Bandurski, 1981; Telewski and Zeevaart, 2002). In the present study, the evidence base for seed longevity in excess of 200 years is extended by reporting on germination tests of 35 South African species.

Materials and methods

Seedlot details

Seeds of the study species were collected from the Cape Region of South Africa around 1803.The likely identity of species, seed numbers available and mean seed mass are presented in Table 1.

These seeds were found among the papers of Jan Teerlink, who was on a Dutch East Indiaman, the *Henrietta*, captured by British privateers on its return journey from the Cape of Good Hope. Letters accompanying the seeds include some dated in China in November 1802 and at the Cape in March 1803. However, based on the species annotations on the packets, the seeds are from the Cape where the Dutch East India Company had its own botanic gardens.

The wallet containing the seeds was (re-)discovered in 2005 in the High Court of Admiralty collections stored in the National Archives at Kew, UK. The collections include documents and other items removed from captured ships. The wallet also contained several samples of Chinese silk that appeared to be in good condition: there was no evidence of mould or damp damage to the swatches of silk. Prior to arriving at the National Archives in 1996, the wallet was stored at the Tower of London (*c.* 1803–1857) and then at Chancery Lane (1857–1996). Consequently, the seeds were only stored under temperature- and humidity-controlled conditions (15°C and 50% RH) for the past 10 years. However, in previous storage the temperature was likely to have been comparatively cool, consistent with a cellar (*c.* 15–18°C).

Germination tests

Due to the often low seed numbers (in many cases <10, see Table 1), a limited range of germination conditions was used. All seeds were sown on the surface of 1% (w/v) agar in water in 90 mm diameter Petri dishes. For those species likely to display physical dormancy (*Fabaceae*), seeds were chipped using a scalpel and sown at 20/10°C (8 h light). Since the majority of the remaining species occur in families known to exhibit a positive germination response to smoke (e.g. *Ericaceae* and *Proteaceae*; Brown *et al.*, 2003; Brown and Botha, 2004), all remaining seedlots were treated using an aqueous smoke solution, according to recommended guidelines (Brown and Botha, 2004; Brown and Duncan, 2006). In addition, since many Cape species germinate at comparatively low temperatures, ensuring that germination occurs only during winter rains (Brown and Botha, 2004), seeds were sown at 20/10°C, with the 8 h elevated temperature period coinciding with the light period. Seeds were scored daily for germination, with radicle emergence of more than 1 mm classified as germinated.

Those seeds that germinated were subsequently potted into a sterile sandy loam soil and grown in a glasshouse at *c.* 20°C.

Radiocarbon dating

The seed coat was removed from four individual seeds of *Protea argentea* and four replicates of two seeds each of *Liparia villosa*; the embryos were used for radiocarbon dating. Embryos were placed in 0.5 M HCl and heated to 80°C for 2 h, then filtered, rinsed in reverse osmosis water and re-filtered through pre-weighed, small lengths (*c.* 15 mm) of 8 mm outer diameter quartz, containing quartz wool to trap the seed material.

Samples were subsequently sealed, under vacuum, into quartz combustion tubes. Sample tubes were then heated to 850°C for 8 h, and allowed to cool before cracking open, followed by CO_2 recovery and purification. CO_2 was cryogenically trapped using liquid nitrogen (−196°C), and then expanded back to a gas by removal of the liquid nitrogen followed by gentle heating. Subsamples of CO_2 were removed for graphitization (1 or 2 ml depending on the yield) and subsequent atomic mass spectrometry ^{14}C analysis and ^{13}C analysis.

CO_2 was converted to graphite using the method of Slota *et al.* (1987), and then pressed into aluminium sample holders for subsequent radiocarbon (^{14}C) analysis. ^{13}C analysis (for correcting the sample activity for fractionation) was undertaken on the second subsample of CO_2, using an isotope ratio mass spectrometer.

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Table 1. Details of the seedlots used, including their likely modern botanical name

Description on seed packet	Likely name[a]	Family (*–aceae*)	Mean seed mass (mg)	No. of seeds[b]
'Seeds from a tree with crooked thorns'		*Fab–*?	74.3	5
'Seeds from an unknown *Mimosa*'	*Acacia* sp.	*Fab–*	122.9	2 (1)
'Seeds of the wild water lemons whose fruits are eaten by the savages along the Orange river'	*Citrullus lanatus*	*Cucurbit–*	86.5	5
Arctotis paradoxa	*Ursinia* sp.	*Aster–*	0.47	50
Borbonia lanceolata	*Aspalathus angustifolia*	*Fab–*	6.4	7
Brunia glutinosa	*Staavia glutinosa*	*Bruni–*	1.8	19
Cineraria amelloides	*Felicia amelloides*	*Aster–*	0.78	5
Cliffortia ericoides	*Cliffortia* sp.	*Ros–*	0.48	16
Colutea annua	*Colutea* sp.	*Fab–*	13.8	23
Erica cerinthoides major	*Erica* sp.	*Eric–*	0.11	100
E. cerinthoides minor	*Erica* sp.	*Eric–*	0.15	16
E. flexuosa	*E. imbricata*	*Eric–*	0.09	50
E. mucosa	*E. ferrea*	*Eric–*	0.18	300
Gnaphalium foetidum	*H. foetidum*	*Aster–*	0.2	50
Gnidia oppositifolia	*Gnidia* sp.	*Thymel–*	3.5	13
Heranthumicum stachalina	Unknown	Unknown	9.9	7
Lachnaea conglomerata	*L. capitata* or *Passerina ericoides*	*Thymel–*	11.6	14
Liparia villosa	*Liparia* sp.	*Fab–*	6.0	25 (16)
Lobelia secunda	*Wimmerella secunda*	*Campanul–*	0.02	105
Mimosa giraffe	*Acacia* sp.	*Fab–*	255	3
M. nilotica	*Acacia nilotica*	*Fab–*	243	1
Passerina capitata	*Lachnaea capitata*	*Thymel–*	2.7	11
Protea acaulis	*P. acaulis*	*Prote–*	18.3	9
P. argentea	*Leucodendron argenteum*	*Prote–*	239	9
P. conocarpa	*Leucospermum* sp.	*Prote–*	111	8 (1)
P. cynaroides	*P. cynaroides*	*Prote–*	31.0	5
P. glomerata	*Serruria* sp.	*Prote–*	12.6	8
P. lagopus	*Paranomus* sp.	*Prote–*	7.7	5
P. linearis	*Leucadendron* sp. or *Leucospermum* sp.	*Prote–*	54.6	4
P. pulchella	*P. burchellii*	*Prote–*	16.0	6
P. serraria	*Serruria fasciflora*	*Prote–*	6.3	18
P. speciosa nigra	*P. speciosa*	*Prote–*	28.4	5
P. strobilina	*Leucadendron* sp.	*Prote–*	214.5	3
P. tomentosa	*Leucospermum* sp.	*Prote–*	69.0	6
Zygophyllum morgsana	*Z. morgsana*	*Zygophyll–*	11.1	5

[a] From the International Plant Names Index (http://www.ipni.org).
[b] Values in parentheses refer to the number of seeds that germinated, otherwise it was zero.

The graphite samples from the seed samples, together with graphite prepared from modern reference standard material (SRM-4990C – oxalic acid II), background standards and known age standards were analysed at the Scottish Universities Environmental Research Centre AMS facility (East Kilbride, Scotland). The instrument used was a National Electrostatics Corporation 5 Million Volt terminal instrument, operated at 4.5 million volts for radiocarbon analysis. The data acquired were used to calculate radiocarbon ages according to the standard age equation.

Finally, once the radiocarbon ages had been determined, they were calibrated to the calendar time scale using the University of Oxford calibration software (OxCal 3.10).

Modelling of Sorghum bicolor *longevity*

The seed viability equation (Ellis and Roberts, 1980) was used, with species-specific constants for *Sorghum bicolor* (from Kuo *et al.*, 1990), to predict seed viability after a range of storage periods. Initial seedlot viability was assumed to be 98%. Seed storage temperature was assumed to be 15°C, which reflects conditions in the National Archives where the seeds have been held

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since 1996, and is probably close to conditions in the Tower of London. Two storage RH values were used: 50% to reflect ambient storage (RH in the National Archives is *c.* 50%) and 25% as an approximation of the RH in the two legume seeds. A value of 25% was used as an estimate to gauge the relative magnitude of the effect on storage life-span of having impermeable, as opposed to permeable, seed coats (i.e. those that equilibrate with ambient RH).

Results

Germination

In germination tests, seeds of three species, '*Liparia villosa*', '*Protea conocarpa*' and an 'unknown *Mimosa*', germinated. For *L. villosa*, 16 of 25 seeds germinated (64%), for *P. conocarpa*, 1 of 8 (12.5%), while for the '*Mimosa*', 1 of 2 seeds germinated. However, upon examination of the non-germinated '*Mimosa*' seed, it was evident that it had been insect infested at some period in the past 200 years, although both seeds appeared superficially to be intact. The seedlings of all three species appeared normal and healthy, and were successfully transferred to, and grown in, pots (Fig. 1). For the remaining 32 species spanning 10 families, no germination was observed, and seeds rapidly became infested with fungi and failed a subsequent 'cut' test for viability.

Based on changes to plant names tracked in the Royal Botanic Gardens, Kew, International Plant Names Index (2004; web page http://www.ipni.org), it was possible to evaluate the possible modern name(s) of the species in the collection. '*L. villosa*' is likely to be one of three possible *Liparia* species (*L. angustifolia*, *L. laevigata* or *L. vestita*). Similarly, *P. conocarpa* is likely to be one of three *Leucospermum* species (*L. conocarpodendron* subsp. *conocarpodendron*, *L. cuneiforme* or *L. ellipticum*), which are also in the *Proteaceae*. Based on seedling morphology, the 'unknown *Mimosa*' has been classified as an *Acacia*, although it has not been possible to assign a species (G. Lewis, Royal Botanic Gardens, Kew, personal communication).

Modelling of viability loss in S. bicolor *seeds*

Based on the seed viability equation, seeds of *S. bicolor*, held at the approximate seed storage conditions of the South African seeds (15°C and 50% RH), would be predicted to fall from 98 to 0% viability within less than 20 years (Fig. 2). In addition, even if seed RH was assumed to be 10% lower (i.e. 40%), seed viability would still be predicted to have fallen to 0% within 60 years. In contrast, seeds held under conditions more likely to represent the impermeable physically dormant legume seeds (15°C and 25% RH) are predicted to retain a little viability (*c.* 5%) after storage for up to 200 years (Fig. 2).

Carbon dating

^{14}C dating yielded average ages for the two seedlots tested of 270 ± 82 and 218 ± 53 (± SD) years before the present time for *Liparia villosa* and *Protea argentea*, respectively. These values are in broad agreement with an assumed collection date of *c.* 1802, based on the associated, dated letters.

Discussion

It is well known that species live for differing lengths of time both in genebank (dry storage) and soil seed-bank conditions (Pritchard and Dickie, 2003; Walters *et al.*, 2005). However, the mechanistic basis of these intrinsic differences in longevity is unknown, although patterns in longevity are emerging based on species ecology and environment (Pritchard and Dickie, 2003; Walters *et al.*, 2005).

In general, species from semi-arid or seasonal Mediterranean climates may have evolved strategies to ensure long-term seed persistence in the dry state in either the soil or aerial seed bank before conditions are suitable for germination, either in terms of water availability or suitable germination micro-site availability (e.g. after fire). As a result, such seeds are likely to be longer lived in the dry state than those from moister, temperate conditions (Walters *et al.*, 2005). Furthermore, in moist temperate regions, seeds in the soil seed-bank are likely to be hydrated, at least for a large portion of time, which provides an opportunity for DNA repair (Villiers, 1974; Boubriak *et al.*, 1997). Consequently, in such climates there may be limited selection for extreme longevity in the dry state (Thompson, 2000).

Many species in the *Fabaceae* have water-impermeable seed coats. During the last phase of seed development (maturation drying), the hilum acts as a one-way valve, permitting the loss, but not uptake, of water from the seed (Hyde, 1954). Consequently, such seeds will equilibrate with the lowest relative humidity experienced (Murdoch and Ellis, 2000). As a result, such seeds are effectively 'isolated' from potentially high ambient relative humidity conditions. Thus, such seeds might be expected to survive longer under suboptimal storage conditions (high RH) than seeds that are permeable, i.e. that can equilibrate with ambient relative humidity. Interestingly, two of the three observed species that germinated are in the *Fabaceae* ('unknown *Mimosa*' and *L. villosa*), and many

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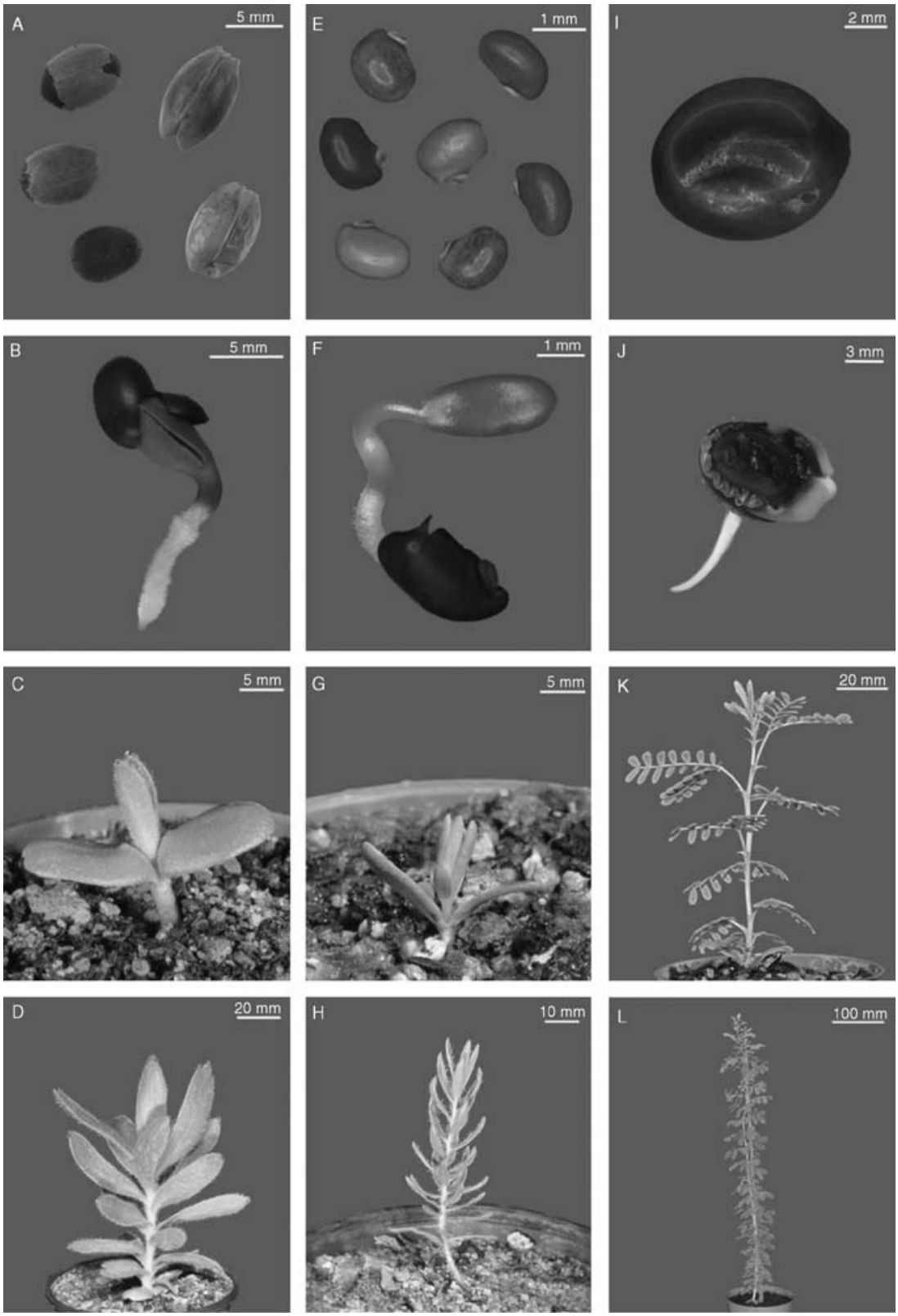

Figure 1. Seeds and seedlings of *Protea conocarpa* (A–D), *Liparia villosa* (E–H) and 'unknown *Mimosa*' (I–L).

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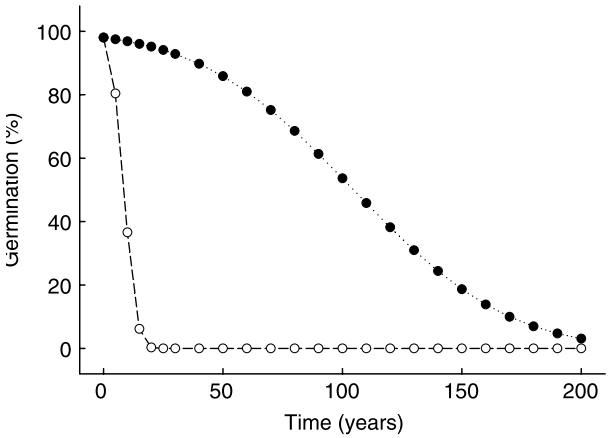

Figure 2. Predicted seed survival of *Sorghum bicolor* at 15°C and two conditions of relative humidity [25% (•) and 50% (o)], based on viability constants from Kuo *et al.* (1990) and the seed viability equation (Ellis and Roberts, 1980).

of the species reported to have survived for >100 years have seeds with impermeable seeds (e.g. *Malva rotundifolia*, *Nelumbo nucifera* and *Canna compacta*; Kivilaan and Bandurski, 1981; Bewley and Black, 1982; Shen-Miller, 2002; Shen-Miller *et al.*, 2002; Telewski and Zeevaart, 2002).

The third species in which there was germination is in the genus *Leucospermum* (labelled as *P. conocarpa*). While seeds of *Leucospermum* are not water impermeable, a number of species have an oxygen-impermeable layer that prevents germination (Brits *et al.*, 1995). Since oxidative stress plays a major role in viability loss during seed ageing (Kranner *et al.*, 2006), it is possible that this layer reduces seed damage from reactive oxygen species.

The remaining 32 seedlots failed to germinate. These included three species in the *Asteraceae* and one in the *Cucurbitaceae*. Both of these families produce seeds that are comparatively short lived at 4–8% moisture content and −18°C (Walters *et al.*, 2005). This, coupled with disparities in long-term survival between the species studied here, all of which originate from the same environment, suggests a phylogenetic component to seed longevity.

Based on seed viability constants estimated from accelerated ageing, *Sorghum bicolor* has been reported to have among the longest-lived seeds. The two legumes in this current study, while long lived, are not necessarily longer lived than *S. bicolor*; at reduced relative humidity (25%) this species is predicted to still retain some viability after 200 years. However, the unclassified *Leucospermum* is exceptionally long lived: under similar storage conditions (50% RH and 15°C), *S. bicolor* would be predicted to have lost all viability after about 10 years. That germination was observed after 203 years suggests that seeds in the genus *Leucospermum* are among some of the longest-lived seeds known.

These data provide further evidence for seeds surviving for prolonged periods of time and still producing healthy seedlings. Furthermore, this appears to be only the third published report of seeds surviving for greater than 200 years for which there is confirmatory data for seed age, both in terms of associated documents and ^{14}C dating of the seeds themselves. Studies on woody species have revealed that, based on seed viability constants, trees/woody species may have considerable variability in longevity and be comparatively short lived (Tompsett, 1994; Medeiros *et al.*, 1998). In this context, 200-year survival of seeds from three woody taxa is the longest validated survival reported for such species, and consequently the suggestion is that seeds from such taxa are not inherently short lived.

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