Seed desiccation tolerance and dormancy of three endangered New Zealand species: *Carmichaelia williamsii*, *Clianthus puniceus* and *Hibiscus diversifolius*

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Abstract
At least one third of New Zealand's indigenous plant species are threatened with extinction and strategies for conserving endangered flora are urgently required. One strategy is to use *ex situ* seed storage as a complement to *in situ* conservation. Successful *ex situ* storage of seed requires knowledge of the seed storage behaviour, optimal storage conditions and germination requirements of the species being stored. For many threatened species, however, this information is either incomplete or unavailable. In this study, preliminary experiments were conducted with three threatened species, *Carmichaelia williamsii, Clianthus puniceus* and *Hibiscus diversifolius,* to determine their desiccation tolerance and dormancy status. Seeds were tested for germination following desiccation and dormancy-breaking treatment. Seeds of all three species could be dried to moisture contents of 2.9-3.7% without losing viability. All three species became predominantly hardseeded at approximately 10% moisture content. However, *C. puniceus* became permeable to water again at moisture contents below 6%. In all species, manual scarification of seeds improved germination to 96-100% compared with 5-20% in untreated seeds. Dormancy in these species is a function of the seed coat preventing water uptake by the dry seed. While seeds of these species are most likely desiccation tolerant and thus can potentially be stored for long periods under conventional conditions, the loss of dormancy of *C. puniceus* at very low moisture contents is of concern. More work is needed to confirm the long-term storage behaviour of these species.
Keywords: *ex situ* conservation, seed storage behaviour, New Zealand flora

Introduction

New Zealand possesses a unique and diverse flora of 2,300-2,470 taxa, with most species (80%) being endemic (Dopson *et al*. 1999). Currently 34% of these taxa are classified as threatened or naturally uncommon, with 11 presumed to be extinct (Warmington *et al*. 1996; de Lange *et al*. 2004). In order to combat further species loss, a range of conservation solutions including both *in situ* protection and restoration and *ex situ* conservation is required.

In situ conservation aims to conserve species within their natural habitat by managing that habitat in a sustainable way. *Ex situ* conservation, in contrast, conserves species outside their natural habitat (Cochrane *et al*. 2007). This approach is most appropriate where populations and/or plant numbers are small or threatened by disease and/or human activities or habitat change. *Ex situ* storage provides a resource that can be used for re-vegetation if *in situ* storage fails. The two approaches should, therefore, be regarded as complementary, with *ex situ* storage providing insurance against sudden loss of species in *in situ* storage (Cochrane *et al*. 2007).

Seed preservation is one of the most commonly used methods of *ex situ* conservation (Theilade & Petri 2003). *Ex situ* storage usually requires drying of seed to low moisture content (3-7% fresh weight basis), depending on the species, and storage at low temperature, preferably at –18°C or cooler. Temperature and moisture content of the seed are major factors in determining whether viability is retained in storage (Hong *et al*. 2001; Theilade & Petri 2003). For many New Zealand threatened species, however, the information on seed biology, seed storage requirements and seed dormancy release mechanisms is either incomplete or unavailable.

Carmichaelia williamsii Kirk (Fabaceae) has a conservation assessment of 'nationally endangered' based on its restricted distribution and small population size (Hitchmough *et al*. 2007). It is restricted to the northern offshore islands (particularly the Poor Knights Islands and the Aldermen Islands) and to two small remnant populations near East Cape. Some populations are at risk from coastal erosion. The species is principally pollinated by New Zealand honeyeaters. Loss of these pollinators will reduce seed production (Heenan 1996; Brandon *et al*. 2004; New Zealand Conservation Network 2005a; Hitchmough *et al*. 2007).

Clianthus puniceus (G. Don) Sol. ex Lindl. (Fabaceae) is cultivated widely, but wild populations are classified as 'nationally critical'. In 2005 only one naturally occurring plant population was known in the wild at a site near the Kaipara Harbour. At this site *C. puniceus* is threatened by summer droughts, browsing animals and competition from weeds (Shaw & Burns 1997; New Zealand Conservation Network 2005b; Hitchmough *et al*. 2007).

Hibiscus diversifolius Jacq. (Malvaceae) appears restricted to the northern most extremity of the North Island (from about Reef Point and Doubtless Bay north) (New Zealand Conservation Network, 2005c). The largest populations are known to occur on the eastern side of the Te Paki dune wetland area. This species is classified as 'nationally vulnerable', and is under severe threat from browsing animals, particularly wild cattle and horses. Some populations at Tokerau Beach have been destroyed by coastal housing development (de Lange *et al*. 2004; New Zealand Conservation Network 2005c; Hitchmough *et al*. 2007).

In this study, experiments were conducted to determine the desiccation tolerance, dormancy status and germination of *C. williamsii*, *C. puniceus* and *H. diversifolius* seed.

Methods

Seed material

C. williamsii, *C. puniceus* and *H. diversifolius* seed pods were collected from the Auckland Botanic Gardens in 2007. The collected pods were couriered to Massey University (Manawatu Campus, Turitea Site) in paper bags within 12 days of collection (Table 1). The seeds were extracted from the pods by hand and the initial moisture content (MC) and germination determined.

Determination of moisture content and germination

The moisture content of the whole seed was determined using the low-constant-temperature oven method described in the International Rules for Seed Testing (ISTA, 2007). Four replicates of 10 (*C. williamsii*) or 25 seeds (*C. puniceus* and *H. diversifolius*) were cut in half and weighed before and after drying in a 103°C ± 2°C oven for 17 h. MC was calculated as the percentage of water on a fresh weight basis.

Seed germination was determined using four replicates of 20 (*C. williamsii*), 25 (*H. diversifolius*) or 50 (*C. puniceus*) seeds. Seeds were placed between moist folded 38 lb regular weight seed germination paper (Anchor Paper Company, St. Paul, Minnesota) held in closed boxes and

incubated at 20°C. A seed was considered to have germinated when a normal seedling had developed; a seedling was classified as normal when it had a well developed primary root and intact hypocotyl and cotyledons (ISTA, 2007).

Seed desiccation experiment

Seed samples were dried to target MCs of 10%, 5% and 2.5%. Seed from each sample was mixed with an equal weight of silica gel in polythene bags and placed in a desiccator and held at 20°C. Seed samples were monitored daily for target weights. The target weight that corresponded to each target MC was calculated using the following formula (adapted from ISTA, 2007):

Weight of seed (g) at target moisture content =

$$\left(\frac{100 - \text{IMC}}{100 - \text{TMC}}\right) \times \text{initial seed weight (g)}$$

Where IMC = initial seed moisture content
TMC = target seed moisture content

Once the target weight was reached seed MC and germination were determined as described above. To avoid imbibition damage of seed at 10% moisture content and below, seeds were humidified before germination by placing them above water in a closed container at 20°C for 24 hours (IPGRI-DFSC, 2004).

Seed dormancy experiment

Seeds that had not germinated after 3 weeks were counted and manually scarified with a scalpel by cutting the seed coat in the cotyledon area. Scarified seeds were returned to 20°C to continue the germination process for another 6 days after which ungerminated seeds were classified as viable when they showed no sign of infection and were firm when pressed. Normal seedlings were assessed as previously described.

Data analysis

Analysis of Variance (ANOVA) was conducted on sample means of each treatment. Where significant effects were detected in the ANOVA (P=0.05), means were compared using the LSD (Least Significant Difference) test. Prior to analysis, data were checked for normality. No transformations were necessary. SAS® for Windows (Release 9.13, SAS Institute, Cary, North Carolina) was used for analysis of all data.

Results

Initial characteristics

The initial characteristics of the seeds of the three species used in the experiments are shown in Table 1. The moisture content of *C. williamsii* and *C. puniceus* seeds on receipt was around 20% and germination over 95%. In contrast, seeds of *H. diversifolius* had 12.4% MC and low germination (46%).

Table 1 Initial characteristics of *Carmichaelia williamsii*, *Clianthus puniceus* and *Hibiscus diversifolius*.

	C. williamsii	*C. puniceus*	*H. diversifolius*
Collection date	20/12/2007	17/12/02007	31/08/2007
Arrival date	27/12/2007	27/12/2007	12/09/2007
Number of seeds prepared	270	1920	660
Seed weight (mg ± S.E.)	19.8 ± 0.48	22.4 ± 0.32	11.3 ± 0.12
Initial moisture content (%)	19.2	22.8	12.4
Germination (%)	98	94	46

Desiccation tolerance

Seeds of *C. williamsii*, *C. puniceus* and *H. diversifolius* tolerated desiccation over silica gel to 2.9-3.7% MC while maintaining their initial germination rate. *C. williamsii* seed germination of 96-98% was maintained as MC declined from 19.8% to 3.7% (Figure 1a). *C. puniceus* seeds tolerated desiccation to about 3.7% MC without loss of initial germination (Figure 1b). *H. diversifolius* seed had poor initial germination (46%), but germination did not decline further with desiccation to 2.9% (Figure 1c). For *H. diversifolius* the dead seed percentage at 12.4, 10.8 and 2.9% MC was 50%, 37% and 41%, respectively.

Seed dormancy

The proportion of seeds of *C. williamsii* and *C. puniceus* having an impermeable seed coat increased as moisture content declined to 3.7% and 10.3%, respectively. However, hardseedness in *C. puniceus* decreased markedly when seeds were then further dried to 3.7%. Seeds with an impermeable seed coat required scarification for germination to proceed. After scarification, the germination percentage increased to 96-100% for all three species compared to 5-20% in control seeds (Table 2).

Table 2 Hardseededness percentages of fresh and after drying on silica gel and changes of germination of without scarification and after scarification of viable seeds of *Carmichaelia williamsii*, *Clianthus puniceus* and *Hibiscus diversifolius*.

Drying time (days)	Moisture content (%)	Hardseededness (%)	Germination (% of viable seeds)	
			Without scarification	After scarification
C. williamsii				
0	19.8	3 B	95 Aa	98 Aa
5	8.4	86 A	14 Bb	96 Aa
35	3.7	82 A	13 Bb	97 Aa
C. puniceus				
0	22.8	25 B	73 Bb	96 Aa
2	10.3	92 A	5 Cb	96 Aa
10	5.3	12 C	85 Ab	96 Aa
35	3.7	9 C	84 Ab	96 Aa
H. diversifolius				
0	12.4	95 A	5 Ab	100 Aa
1	10.8	89 A	11 Ab	97 Aa
19	2.9	80 A	20 Ab	98 Aa

Numbers following the same uppercase letters are not significantly different within each species; numbers within rows sharing the same lowercase letters are not significantly different (LSD, $P<0.05$)

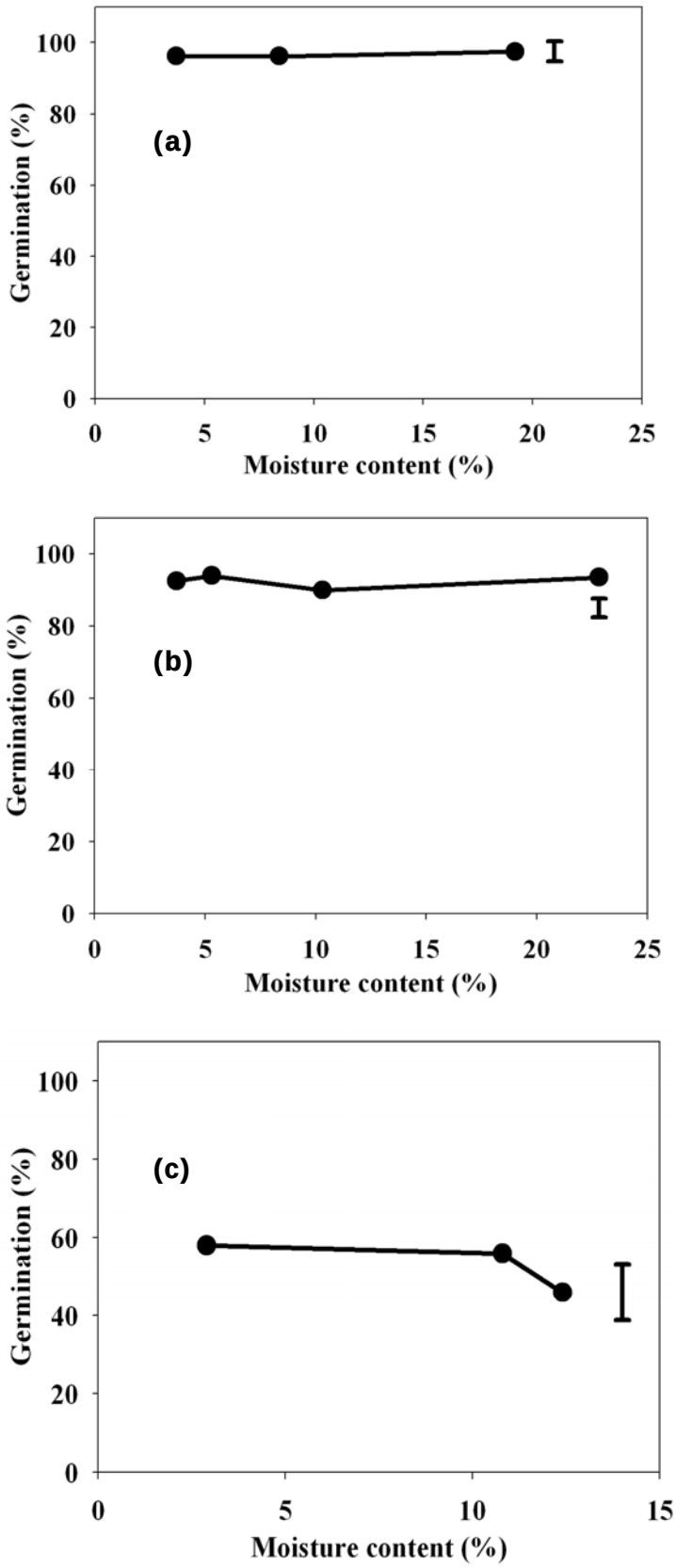

Figure 1 Germination capacity of seeds during desiccation: (a) *C. williamsii*, (b) *C. puniceus*, (c) *H. diversifolius.* Bars indicate LSD (0.05%).

Discussion

Desiccation experiment

Tolerance of seed desiccation is crucial in determining seed storage characteristics. Three categories of seed storage behaviour, orthodox, intermediate and recalcitrant have been identified based on seed desiccation tolerance, longevity and tolerance to low temperatures during storage (Yang *et al*. 2004). Long-term seed preservation for genetic conservation is possible only for species with orthodox seed storage behaviour (Hong *et al*. 2001; Berjak & Pammenter, 2008). In such species seed longevity increases with decrease in storage moisture content (as low as 3-7%) and temperature in a 'quantifiable and predictable way' (Hong & Ellis, 1996; Hong *et al*. 2001; Yang *et al*. 2004).

Desiccation tolerance of *C. williamsii, C. puniceus* and *H. diversifolius* seeds in this investigation is typical of orthodox seed. The desiccation tolerance of *C. williamsii* and *C. puniceus* seeds is similar to that of other Fabaceae of which 97% have been classified as orthodox (Liu *et al*. 2008). For example, seeds of two tree species *Bauhinia purpurea* and *Pongamia pinnata* tolerate drying to moisture contents less than or equal to 5% without loss of viability (Isasi, 2003; Kumar *et al*. 2007). Seeds of other *Hibiscus* species such as *H. cannabinus* have been reported as desiccation tolerant. *H. cannabinus* retained high germination (86%) at 7.2 and 1.1% MC (Hu *et al*. 1998). Also this species retained 66% germination after 39.5 years storage at –18°C indicating orthodox storage behaviour (Walters *et al*. 2005).

In this study, the germination percentage of *H. diversifolius* was low. Angelini *et al.* (1998) commented that low germination percentage (40.8%) of *H. cannabinus* grown in central Italy was due to unfavourable temperature and moisture conditions during seed filling, resulting in rapid seed deterioration and increased seed coat susceptibility to fungal pathogens. This presence of fungi on dead *H. diversifolius* seed observed in this study may suggest a similar problem, but in the absence of data on reserve accumulation in the seed lot, this is speculative.

Seeds which tolerate desiccation to low moisture content do not necessarily show orthodox storage behaviour. For example, seeds of *Anadenanthera colubrine* (Fabaceae) tolerated desiccation to 4% MC (98% germination), but retained only 20% germination after 23 months of hermetic storage at –18°C and 4% MC (Rojas Espinoza 2005). To confirm that the seed behaviour of the species studied here is orthodox retention of germination in storage at these low moisture contents and low temperature (for example –18°C) will need to be assessed.

Seed dormancy

The results of this study confirm that dormancy in *C. puniceu, C. williamsii* and *H. diversifolius* is a function of the seed coat preventing water uptake by the dry seed. Hardseedness is typical of many other species of Fabaceae and Malvaceae (Mai-Hong *et al*. 2003; Van Assche & Vandelook 2006; Michael *et al*. 2007). For example, Shaw and Burns (1997), Gruner and Heenan (2001) and Westra *et al*. (1996) have reported the scarification is required for germination of *C. puniceus*, *Carmichaelia* spp. and *Hibiscus trionum*, respectively.

In species exhibiting hardseededness, water impermeability of the seed coat usually develops at moisture contents between 15% and 54% during maturation drying of the seed (Michael *et al*. 2007). If seeds are removed from the plant after embryo maturity, but before any drying and impermeability can develop, seeds should be capable of germination (Mai-Hong *et al*. 2003; Usberti *et al.* 2006; Michael *et al*. 2007). This response has been observed in Fabaceae (Mai-Hong *et al.* 2003; Usberti *et al.* 2006) and was observed in the Fabaceae studied here where desiccation also caused an increase of impermeability in comparison to fresh seed.

A water-impermeable seed coat has been suggested as a factor in the ability of seed of some species to survive long-term storage (e.g., *Liparia villosa* is reported to have survived for >200 years) (Daws *et al.* 2007). In many species of Fabaceae, the hilum acts as a one-way valve (Daws *et al.* 2007). This allows loss of water from the seed, but not uptake (Hyde, 1954). Consequently, the seed MC will equilibrate to that of the lowest relative humidity experienced by the seed and remain at that MC irrespective of whether higher ambient relative humidity is subsequently experienced. This ability to remain at low MC under high ambient relative humidity is likely to be a key factor in maximising long-term survival of these species (Daws *et al.* 2007). The effect, if any, of loss of water impermeability below 6% MC in seed of *C. puniceus* on the long-term survival of seed in storage needs to be determined.

The mechanism by which drying to low seed moisture alleviates hardseedness in *C. puniceus* is not known. However, for many Fabaceae, treatments such as acid scarification, hot water, dry heat, and high or fluctuating temperature will alleviate hardseedness by inducing fractures in the lens, hilum, micropyle or seed coat (Zeng *et al.* 2005; Hu *et al.* 2008). Although there is no evidence that drying is involved in loss of impermeability in Fabaceae, dry storage or drying of hard seed can render the seed coat permeable in some species of Geraniaceae. For example, after drying exhumed seeds of *Erodium* and *Geranium* species for 7 days in a desiccator over silica gel, 88-100% of the seeds germinated in 1 or 2 days compared with 0-1% in un-dried seeds (Van Assche & Vandelook, 2006). The current study showed a similar result for *C. puniceus*, where drying for a relatively short period (10 days) in silica gel increased seed coat permeability and, as a consequence, germination increased to 85%.

The present study emphasises the importance of an understanding of the desiccation behaviour and dormancy mechanisms within the seed. At the collection MCs of 19.8% and 22.8% for *C. williamsii* and *C. puniceus* respectively hardseedness had not developed. Desiccation to MCs of 8.4% and 10.3% respectively induced hardseedness and resulted in low germination percentage (5-14%). Failure to recognise the decline in germination with desiccation as being a function of the imposition of dormancy could lead to the erroneous conclusion that viability loss is associated with loss of moisture.

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