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**Desiccation response of seed of *Clianthus* spp., *Carmichaelia muritai*, *Pittosporum crassifolium* and *Pittosporum eugenioides***

**A thesis presented in partial fulfilment of the requirements  
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## ABSTRACT

New Zealand has a rich, diverse and unique of plant life. However, the conservation status of the New Zealand indigenous vascular flora is deteriorating, with 7.6% of this flora regarded as threatened with extinction. A series of conservation approaches are required to protect species against further loss. Developing *ex-situ* conservation of these species requires basic information such as seed storage behaviour and seed germination requirements to be determined. However, for many species this information is missing or incomplete.

The objective of this study was to determine seed storage behaviour (response to desiccation), and/or seed coat characteristics in selected New Zealand native species. Five native tree and shrub species were studied: *Carmichaelia muritai*, *Clianthus puniceus*, *Clianthus maximus*, *Pittosporum eugenioides*, and *Pittosporum crassifolium*.

Seeds of *Clianthus maximus*, *Clianthus puniceus*, and *Carmichaelia muritai* were found desiccation tolerant at low moisture content (down to ~2.5%), suggesting the storage behaviour is orthodox; storage trials need to be conducted to confirm this. In contrast, the storage behaviour of *Pittosporum eugenioides* and *Pittosporum crassifolium* appears to be non-orthodox since there was some loss of viability upon drying to low moisture contents. Moisture sorption isotherms were determined for *Pittosporum eugenioides* and *Pittosporum crassifolium*.

This research also demonstrated that loss of water impermeability occurred with desiccation of *Clianthus maximus*, *Clianthus puniceus*, and *Carmichaelia muritai* seeds, to ultradry moisture as a result of cracking to the seed coat surface, in particular the extrahilar and lens area.

The findings of this study provide further seed coat morphology, seed biology (seed storage behaviour and germination) information that will contribute to the development of *ex-situ* conservation strategies for New Zealand's indigenous flora.

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# 1 INTRODUCTION

The plant life of New Zealand is rich, diverse and unique. New Zealand is one of the world's 34 biodiversity hotspots (Phoenix et al., 2006). However, the conservation status of the New Zealand indigenous vascular flora is deteriorating, with 7.6% of this flora regarded as threatened with extinction (de Lange et al., 2009). About 38% (897 taxa) of New Zealand's total indigenous vascular flora was classified as extinct, threatened, or at risk taxa (de Lange et al., 2009). There are a number of reasons for this deteriorating situation for New Zealand's indigenous vascular flora, including human activity, for instance the extensive pastoralism impacting on indigenous grassland, with more frequent fire and heavy grazing especially by sheep or rabbits. In the future wide ranging climate change is likely to be significant (Halloy & Mark, 2003). A series of conservation approaches are required to protect seed species against further loss.

Both *in-situ* conservation and *ex-situ* conservation are considered as conservation solutions for plant genetic diversity. *In-situ* conservation maintains recovering populations of species in the surrounding where they have developed their particular features. This approach aims to protect the species and enable evolution and propagation within their natural habitats. However, *in-situ* conservation is difficult to achieve with some or all the population compared with *ex-situ* conservation, because *in-situ* conservation requires large areas, and it is costly to maintain the natural habitats such as national parks, biosphere reserves, and wildlife sanctuaries (Convention on Biological Diversity, 2014). *Ex-situ* conservation strategies range from seed to whole plant, pollen or vegetative tissue (Maunder et al., 2004). One *ex-situ* conservation approach is seed banking. In the seed bank, seed will be banked under conditions that will maintain seed viability for decades. A standard approach of seed storage within genebanks (seed banks) was proposed by FAO/IPGRI (1994) with the aim of storing as many accessions as possible under suitable and sustainable conditions for long term conservation. However, seed storage duration may vary amongst species. It is essential therefore to understand the storage behaviour of any particular species.

The standard approach for seed storage is storage at sub-zero temperatures at 3-7% seed moisture content (FAO/IPGRI, 1994). However, not all species' seeds can survive at such low moisture contents. Seed storage behaviour is partly defined by the capacity of seed to survive desiccation. Roberts (1972) classified two categories of seed storage behaviour:

orthodox and recalcitrant. Subsequently, Ellis et al. (1990) introduced a third category of intermediate behaviour between orthodox and recalcitrant. For seed of many species in the New Zealand flora information on storage behaviour is either unknown or incompletely known. This includes *Pittosporum eugenioides* for which the storage behaviour is uncertain (Royal Botanic Gardens Kew Seed Information Database (SID), 2015).

Seed germination is the most efficient approach to test seed viability before and after storage. However, for many species, seeds are unable to germinate because of either environmental constraints or innate factors within the seed. The result is the seed being dormant. For example, seed of some Fabaceae species develop a water-impermeable seed coat (“hard seed”), which imposes seed dormancy. Some threatened species within the New Zealand flora have been identified as having this characteristic (Park, 2012; Conner & Conner, 1988). Park et al. (2010) reported that at ultra-dry moisture water impermeability in *Clianthus* spp. was lost. However, the mechanism by which this impermeability is lost is unclear.

The aims of this study are to determine:

1. Seed storage behaviour in two *Pittosporum* spp. (*P. eugenioides* and *P. crassifolium*).
2. The mechanism by which water impermeability in *Clianthus puniceus* and *Clianthus maximus*, is lost.
3. Whether *Carmichaelia muritai*, another New Zealand Fabaceae, loses water impermeability at ultra-dry moisture.

Specifically, the objectives of this research were to:

1. Determine whether the seed coat of *Carmichaelia muritai* becomes water permeable at low moisture contents.
2. Identify changes in seed coat characteristics for *Carmichaelia muritai*, *Clianthus puniceus* and *Clianthus maximus* associated with loss of water.
3. Characterise the desiccation sensitivity of *Pittosporum eugenioides*, and *Pittosporum crassifolium*.
4. Understand the relationship between seed moisture content and relative humidity in *Pittosporum eugenioides*, and *Pittosporum crassifolium*.

## 2 LITERATURE REVIEW

### 2.1 Indigenous flora in New Zealand: threats and conservation strategies

The indigenous flora of New Zealand is unique, having evolved in isolation for millions of years. About 80% of the plants are endemic (Department of Conservation, 2014). The flora of New Zealand is described as one of the world's biodiversity hotspots by Conservation International (2004), and about 1-15% of the total New Zealand land area remains covered with indigenous plants (Department of Conservation, 2014). However, New Zealand plant species diversity is decreasing and more species are becoming threatened (for example, *Gentianella* spp., *Carex* spp., *Hypericum* spp., and *Olearia adenocarpa* Molly et Heenan) or even extinct (such as *Myosotis cinerascens* Petrie, *Myosotis laingii* Cheeseman) (de Lange et al., 2009; de Lange et al., 2013). The major reasons are habitat loss as a result of both natural and human activities, invasion of introduced plants species, and the expansion of human activities, for instance the extensive pastoralism into the grassland, with more frequent fire and heavy grazing especially by sheep or rabbits (Department of Conservation, 2014; Hobbs et al., 2006). Also, climate change, particularly global warming could cause the extinction of a number of species in their natural habitat. For example, about 200-300 indigenous alpine plant species of New Zealand could be lost with a rise temperature of 3 °C over the next 100 years (Halloy & Mark, 2003). New Zealand has more introduced plant species than indigenous species, by number of species the indigenous species are only 7.8% and the rest 92.2% are introduced plants (including naturalised plants) (Department of Conservation, 2000). About 8% of introduced plants have established self-sustaining populations (New Zealand Plant Conservation Network, 2012), and only a small number of exotic species (about 300) have become invasive weeds (Howell, 2008). The majority of plant introductions into New Zealand were undertaken intentionally. As reported by Department of Conservation (2014), 12% of introduced invasive species were brought into the country for the purpose of agricultural, horticultural or forestry, while over 70% were brought in as ornamental plants, with accidental introductions accounting for a further 11%, and the remaining 7% are indigenous species (Williams & Cameron, 2006). The unpredictability of accidental introductions increases the difficulty of management and control.

The long term survival of indigenous plants as well as the genetic diversity within native species is threatened by the introduced plants in New Zealand (Department of Conservation, 2014). A number of invasive weeds, for example, threaten native species by taking over their

habitat or competing for nutrients with indigenous species. Invasive tree species such as *Pinus contorta* compete for forest space with native trees and plants, and pine needles form a carpet which discourages regeneration of native forest floor species. The Department of Conservation (2000) have reported the introductions have potential to replace the native plants. New Zealand has more introduced conifers and flowering plants than native plant species, with the estimated number of introduced species with established wild populations being 240 (in the year 2000) and increasing every year. Thus, a series of conservation projects have been proposed with the aim of protecting against the loss of native biodiversity.

A national seed bank project, the New Zealand Indigenous Flora Seed Bank, was established in 2013. It aims to collect the seeds of New Zealand native flora as part of an *ex-situ* conservation strategy to conserve native plant biodiversity within New Zealand, and both threatened and common species will be collected. There are two approaches to the conservation of plant genetic diversity: *in-situ* and *ex-situ* conservation. *In-situ* conservation is defined as where wild or cultivated species are retained within natural ecosystems and habitats, whereas in *ex-situ* conservation is where germplasm is retained outside its original habitat(s). *Ex-situ* conservation can be achieved using whole plants in field genebanks or botanical gardens, seeds in a seed bank, or specific parts of the plant for example the roots, dormant buds or pollen (Jarvis et al., 2000). An advantage of using seed banking as an *ex-situ* conservation strategy is that it allows a wide range of species to be conserved together in one place (New Zealand Plant Conservation Network, 2014).

Seed banks are facilities where seeds are stored under cold and dry conditions. This prolongs the viability of seed and conserves them for future use. However, seed banking faces a number of issues. The key issues include identification of seeds, the quality of banked seeds, assessment of storage needs, characteristics that affect long term storage, incomplete information of storage behaviour, germination and dormancy factors, and insufficient knowledge of the physiology of the seeds. Some of these issues will be discussed in the rest of this review.

## **2.2 Managing quality during seed storage: germination**

One of the key techniques is the use of germination tests over time; assessment of seed viability prior to and during storage is an essential management tool for long term storage (Rice et al., 2006). The storage life of a seed is inevitably limited, all seed, as with any living organism will eventually die. Storage of poor quality seed along with improper storage

practices or management may result in a decrease in storage life. There are a number of approaches for the assessment of seed viability including the cut test, x-ray analysis and/or staining with tetrazolium chloride (Tz) (Terry et al., 2003), but the most direct measure of viability is the germination test. A seed has the capacity to germinate over a wide range of environment parameters (Baskin & Baskin, 2004). In addition to the basic requirement for adequate moisture, a suitable temperature and a supply of oxygen, seed germination may also be affected by other factors such as light and scarification (Finch-Savage & Gerhard, 2006). However, as a result of seed dormancy, an intact viable seed may not be able to complete germination even under favourable growing conditions (Hilhorst, 1995; Bewley, 1997; Li & Foley, 1997). For seed germination to be assessed as part of managing the seed in long term storage, for many species, particularly wild species, an understanding of dormancy; the different types of dormancy; and the mechanisms by which dormancy is imposed is essential.

### **2.2.1 Definition of dormancy**

As simply defined above, seed dormancy is when an intact viable seed does not have the capacity to complete germination under favourable conditions (Hilhorst, 1995; Bewley, 1997; Li & Foley, 1997). Baskin and Baskin (2004) have a more detailed definition of a dormant seed, as being one that does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors (such as light and temperature) that otherwise are favourable for its germination. Dormancy can be considered as a block to the completion of germination. This block to germination occurs differently amongst species and enables different species to adapt to a wide range of environments. Dormancy also enables a seed to delay germination until conditions are most suitable for the resultant seedling to develop into a plant (Hilhorst, 1995; Bewley, 1997; Li & Foley, 1997; Baskin & Baskin, 2004; Fenner & Thompson, 2005). However, dormancy is difficult to define because it is measured only when germination is absent (Finch-Savage & Gerhard, 2006).

Dormancy is not only associated with the absence of germination, but it is also used to determine the conditions required for germination (Fenner & Thompson, 2005). As already discussed when a seed requires specific environmental factors in addition to adequate moisture, a suitable temperature and oxygen for stimulating germination, it is dormant. The specific conditions can be wide temperature fluctuations (Probert, 2000), light/darkness (Baskin & Baskin, 2004), nitrate (Batak et al., 2002), and chemical signals (such as abscisic acid) (Krock et al., 2002). Temperature has been widely accepted to regulate both dormancy

and germination, and light has also been considered to both stimulate germination and release dormancy (Baskin & Baskin, 2004; Fenner & Thompson, 2005). Non-dormant, seed on the other hand, no longer requires additional environmental factors for germination to proceed. Dormancy, as mentioned above, is a seed feature that determines the conditions which suitable for germination, hence, dormancy can be released by any factor that extends the requirements for germination.

Some researchers have defined dormancy based on stages. Hilhorst (1995) and Bewley (1997) said that a freshly matured dormant seed, where the dormancy has developed during seed maturation on the mother plant, has primary dormancy. Technically, a non-dormant seed has the ability to germinate over the widest range of environmental conditions (Baskin & Baskin, 2004). When the seed is non-dormant but will not germinate because one or more of adequate moisture, a suitable temperature and oxygen is absent then it is said to be quiescent (Harper, 1957; Harper, 1977). If the missing environmental factors are not provided the seed may enter secondary dormancy. Secondary dormancy occurs in seeds after seed dispersal. However, dormancy is not an all (complete dormancy) or nothing (non-dormancy) stage through the seed's life cycle (Baskin & Baskin, 2004; Finch-Savage & Gerhard, 2006). Baskin and Baskin (2004) proposed a dormancy cycle explaining the cycle between dormancy and non-dormancy of seeds with non-deep physiology dormancy. Baskin and Baskin (1985) suggested that seed dormancy cannot be simply classified as primary dormancy, conditional dormancy and secondary dormancy, and that the dormancy cycle is just a series of dormancy states of non-deep level of the class physiology dormancy (PD). This classification is defined according to the timing of dormancy. However, dormancy has been classified using other systems.

### **2.2.2 A classification system for seed dormancy**

A number of schemes for classifying seed dormancy have been published. Of those Harper's scheme (Harper, 1957; 1977) has been the most frequently utilized for seed ecology and whole-seed physiology studies (Baskin & Baskin, 2004). Harper (1957, 1977) classified dormancy as innate (primary dormancy), enforced (quiescence) and induced (secondary dormancy). However, some researchers (Baskin & Baskin, 1985; Vleeshouwers et al., 1995; Thompson et al., 2003) suggested that Harper's scheme is inadequate to describe the wide diversity of seed dormancy types. Lang's 'universal' system of endodormancy, paradormancy and ecodormancy, is supposed to be used across all types of plant dormancy, not just seed (Lang et al., 1985; Lang et al., 1987; Lang, 1987). Baskin and Baskin (2004) concluded the

problem with this approach is: (1) it is too cumbersome to apply to a sample of extant seed plants; (2) as a physiologically based approach, it does not give proper recognition to the importance of a water-impermeable seed coat or underdeveloped embryos; and (3) it does not include the levels of dormancy or types of dormancy-break. A comprehensive classification system of seed dormancy was proposed by Nikolaeva (1969). This system reflects the fact that seed dormancy is determined by both seed morphological and physiological characteristics (Finch-Savage & Gerhard, 2006). Baskin and Baskin (1998, 2004) also proposed a dormancy classification system based on Nikolaeva (1969), which comprehensively classified seed dormancy into classes, levels and types. Five classes of dormancy were identified: physiological dormancy (PD), morphological dormancy (MD), morph-physiological dormancy (MPD), physical dormancy (PY) and combinational dormancy (PY+PD).

#### **2.2.2.1 Physiological dormancy**

Physiological dormancy (PD) is the most prevalent dormancy form in most seed species, particularly those from temperate regions (Finch-Savage & Gerhard, 2006). Physiological dormancy prevents embryo growth and germination until chemical changes occur in the seed (Fenner & Thompson, 2005). Seeds with embryo-based dormancy may either be morphologically immature (or have an undifferentiated embryo) (see 2.2.2.2 Morphological Dormancy), or the presence of germination inhibitors such as abscisic acid (ABA) and/or the absence of or deficiency in growth hormones such as gibberellic acid ( $GA_3$ ) that may prevent embryo growth (Morris et al., 1989; Bewley & Black, 1994; Baskin & Baskin, 1998; Baskin & Baskin, 2004). The growth of the embryo is reduced to such an extent that it is not strong enough to break through the testa (seed coat) and surrounding tissue. PD is broken when the inhibitors are reduced or no longer produced by the seed. An application of a gibberellic acid such as  $GA_3$  or cold/warm stratification helps alleviate PD. Following Nikolaeva (1977), Baskin and Baskin (2004) differentiate PD into three levels: deep, intermediate and non-deep. Non-deep PD is further differentiated into five classes based on the physiological responses to temperature during the dormancy-break.

#### **2.2.2.2 Morphological Dormancy**

Morphological dormancy (MD) occurs in seeds with underdeveloped or undifferentiated embryos (Baskin & Baskin, 2004). In such seeds, the mother plant releases them when the embryos have not fully differentiated; therefore, the seeds need time to ripen under

appropriate conditions, meaning germination can be delayed from a few weeks to a few months.

In seeds with morph-physiological dormancy (MPD) dormancy, the seeds have an underdeveloped embryo combined with a physiological component to the dormancy. Eight levels of MPD are recognised. For MPD a combination of dormancy-breaking treatments is usually needed for seed to germinate, e.g. warm or/and cold stratification combined with application of gibberellic acid (GA<sub>3</sub>). In seeds with MPD compared with MD, embryos need a longer period of time for growth (Baskin & Baskin, 2004).

### **2.2.2.3 Physical or Coat-imposed Dormancy**

The embryo development allows the radicle to grow and protrude through the tissues (seed or fruit coat and endosperm) that cover the embryo. The restraint imposed by the covering tissues must be weakened sufficiently to allow germination (Bewley & Black, 1994).

Physical or coat-imposed dormancy is a dormancy imposed on the embryo by the seed coat or other surrounding tissues such as endosperm, pericarp or extra-floral organs. Seeds with this type of dormancy will germinate readily in the presence of water and oxygen once the seed coat and other surrounding tissues are removed or scarified. Five mechanisms of coat-imposed dormancy have been proposed by Morris et al. (2000). These are (1) interference of water uptake because of the seed coat impermeability to water; (2) interference with gas exchange, reducing oxygen supply to the embryo or hindering escaping of carbon dioxide; (3) mechanical constraint, where tissues surrounding the embryo impose a mechanical constraint to expansion of the embryo; (4) retention of inhibitors, the seed coat may prevent the escape of a range of chemical inhibitors from the seed; and (5) prevention of the exit of inhibitors from the embryo.

Baskin et al. (2000) use the term physical dormancy (PY) in relation to dormancy caused by one or more water-impermeable layers of palisade cells in the seed coat or fruit coat, which block water reaching the embryo. Mechanical or chemical scarification breaks dormancy/promotes germination in seeds with PY. In some taxa of Fabaceae (such as Mimosoideae, Faboideae), dormancy break may also be achieved by heating (fire or soaking in boiling water) to disrupt the seed coat.

Combinational dormancy (PY+PD), occurs where a water impermeable seed or fruit coat is combined with physiological embryo dormancy (Baskin & Baskin, 2004). The physiological

component is at the non-deep level (Baskin & Baskin, 1998). In most seed species with combinational dormancy, PY will release first in dry storage or in the field within a few weeks as they after-ripen (Baskin & Baskin, 1998). And in some genera such as *Cercis* (Fabaceae) and *Ceanothus* (Rhamnaceae) that are more deeply dormant, they require a period of low temperature stratification to release from PD.

In long term storage, the knowledge of dormancy breaking and germination conditions is essential for testing seed viability. Germination results can be inaccurate if there is failure to break dormancy. For successfully establishing *ex-situ* conservation, the determination of optimum germination factors and treatments to alleviate dormancy is critical.

### **2.3 Seed storage**

The purpose of seed storage is to retain the initial seed quality for a period of time. Seed quality factors that need to be considered during storage include: germination, vigour, viability, and physical integrity (Hanson & Chin, 1999). Seed storage has been practiced since prehistoric times and people have long developed techniques to store small amounts of seed for future use. With the development of agricultural practice, people have used increasingly sophisticated technology to meet the demand for the maintenance of seed quality during seed storage to ensure that viability and vigour are retained to maximise the emergence of the seed when sown. The purpose of seed storage can range from short term storage, such as from one sowing season to another, through to long term germplasm storage. Germplasm storage of seed is used to conserve biodiversity. However, the storage longevity of seed can be affected by a number of factors ranging from internal genetic factors within the seed, the environment the seed is exposed to during seed development and the post-development storage environment the seed is exposed to, in particular temperature and relative humidity.

#### **2.3.1 Seed storage behaviour**

To successfully achieve *ex-situ* conservation using seed banks, knowledge of seed storage behaviour is essential. Firstly there is the need to determine whether storage in a seed bank is possible for the species to be stored, and secondly, there is the need to determine suitable seed handling techniques for that species. For conventional seed bank storage the ability to survive desiccation to low moisture, generally that in equilibrium with around 15% relative humidity, is essential. However not all seeds have this capacity and several types of seed storage behaviour are recognised.

### 2.3.1.1 Types of seed storage behaviour

Roberts (1972) divided seed storage behaviour into two categories: orthodox and recalcitrant. Subsequently, Ellis et al. (1990) defined a third category between orthodox and recalcitrant: intermediate. The categories are based on the response to desiccation, longevity once desiccated and tolerance of low temperature.

Orthodox seeds, can be desiccated to low moisture contents about 5% and below without loss in viability, and will retain viability at that moisture. For orthodox seeds there is a direct relationship between moisture and temperature and storage life. The lower the moisture and temperature the longer the storage life. Seeds of this type are mostly shrub and tree genera in the temperate, the tropics and subtropics, for example, *Pinus* L., *Pseudotsuga* Carr., and *Acacia* L. (Bonner, 2008).

Intermediate seeds, can be desiccated to 10-12.5% moisture content, however seeds lose viability when desiccated to lower moisture content. Seeds of this category can also be chilling or freezing sensitive during dry storage at low temperature (10 to 12 °C or 0 °C to -20 °C) (Hong & Ellis, 1996). Intermediate seeds native to temperate regions are generally tolerant to sub-zero temperatures, the storage temperature of such seeds is below 5 °C for example, *Champereia manillana* and *Schefflera octophylla* (Chen et al., 2007). In contrast, intermediate seeds from tropical areas are sensitive to low temperature and supposed to be stored above 10 °C, for example, *Cordeauxia edulis* (Andersson et al., 2007).

Recalcitrant seeds cannot survive desiccation to moisture contents below 15-20% and are also chilling-sensitive (Hong & Ellis, 1996; Hong et al., 1996). Seeds of this type, such as *Quercus* and *Theobroma*, are mostly from shrub or tree species and about 47% of species are from tropical moist forests (Li & Pritchard, 2009). A number of New Zealand native species have recalcitrant seeds (for example, *Dysoxylum spectabile*) (Park, 2012), or other native species show decreased viability on desiccation (for example, *Ripogonum scandens*) (Bannister, Bibby, & Jameson, 1996). As such, seed collectors face problems with collecting recalcitrant seeds germplasm and handling, and there are great risks that seeds either germinate or deteriorate before they arrive back at the seed bank.

Although, a number of protocols have been published to determine seed storage behaviour of a particular species (Hong & Ellis, 1996; Vertucci & Roos, 1990; 1993; Vertucci, Roos, & Crane, 1994), it is not always easy to determine the storage behaviour category of all seed species. Classification of seed storage behaviour is still uncertain in a number of species. For

example, seeds of neem (*Azadirachta indica*) have been characterized by complex storage behaviour, such as orthodox (Tompsett & Kemp, 1996; Nayal et al., 2000), and intermediate (Hong & Ellis, 1998; Sacandé et al., 2000). Storage handling, chilling temperatures, and imbibition damage have all been suggested as contributing to their complex storage behaviour (Sacandé, Buitink, & Hoekstra, 2000).

### **2.3.1.2 Acquisition of desiccation tolerance**

Although desiccation tolerance is critical to seed storage, there is no easy method to identify when a seed is desiccation tolerant. Seed tolerance to desiccation depends on the developmental status of the seed, and requires the completion of morphological development or completion of seed reserve accumulation (Kermode & Finch-Savage, 2002; Hay & Smith, 2003). Seed development and maturation can be divided into different stages. In the early stage of development seeds are not desiccation tolerant. Seeds develop desiccation tolerance during later stages of development and maturation, however, the capability and degree of desiccation tolerance varies among species (Galau et al., 1991).

Seed moisture content declines during seed development and maturation. As moisture declines, potential damage may occur from the following causes including: (1) mechanical damage caused by a decline in cell volume (Farrant & Sherwin, 1998; Oliver & Bewley, 1998); (2) metabolism-induced damage, caused by an unbalanced metabolism at intermediate moisture content (Pammenter & Berjak, 1999); and (3) desiccation damage *sensu stricto*, this is denaturation caused by the loss of moisture associated with macromolecular surfaces (Vertucci & Roos, 1993). In response, seed has evolved a number of mechanisms of desiccation tolerance to protect against this damage (Kermode & Finch-Savage, 2002). These mechanisms or processes include: (1) intracellular physical characteristics, such as a reduction in the volume of vacuoles and accumulation of seed food reserves (protein, lipids); (2) intracellular de-differentiation, which minimizes membrane surface area; (3) accumulation of proteins, particularly of late embryogenic abundant proteins (LEAs) that are associated with the development and maturation of orthodox seeds (Pammenter & Berjak, 1999; Kermode & Finch-Savage, 2002); (4) existence and operation of an antioxidant system; and (5) the metabolism switches off (Pammenter & Berjak, 1999). Species that are not tolerant of desiccation (recalcitrant seeds) either lack or have an incomplete expression of this suite of mechanisms or processes.

### **2.3.2 Factors affecting longevity in storage**

The storage potential of an individual seed can be influenced by more than one factor. The combination of factors that can influence storage life can include genetic, pre-harvest and environment elements.

#### **2.3.2.1 Genetic factors**

Genetic factors affect seed longevity in storage. Different species can have considerable variation in storage potential and longevity. It has long been understood that seeds of some species, for example in the Fabaceae family, can survive storage for long periods and that many of these long-lived species have hard water-impermeable seed coats (Daws et al., 2007). Harrington (1972) lists a number of species with longevity records of ten or more years. These include species within the genera *Albizia*, *Cassia*, *Goodia* and *Trifolium* at 147, 158, 105 and 100 years respectively. All these genera are members of the Fabaceae family. Seed longevity also shows a wide range of variation within species. For example, weed seeds of *Chenopodium album* L. persisted in the soil for 10 years with 28% to 41% viability, while other experiments reported viable seeds after 6 years and 39 years in cultivated soils (Lewis, 1973). Inheritance of seed longevity is not limited to species level, it can be seed at the cultivar level as well. Shands et al. (1967) found seeds of the cultivar Oderbrucker barley has greater resistance to germination loss in storage than other cultivars. Similarly, Ellis et al. (1982) found differences of longevity in the storage of various cultivars of chickpea, cowpea, and soybean. Not all the seed species, cultivars or even an individual seed within a genetic group are destined to have same longevity in storage. Seed longevity depends on the storage approaches and conditions for specific seed species.

Seed shape, size, and organization of seed structures affect seed longevity in storage. Small seeds are normally less readily damaged during harvest and processing compared with large seeds. Roberts (1972) evaluated the protection from mechanical damage during harvesting, handling, and processing provided to seed by their shape and concluded that spherical seed was more protected than other flat or irregularly shaped seeds. However, the extent of damage is also influenced by other factors, such as coat thickness and strength, and brittleness of the seed .

#### **2.3.2.2 Pre-harvest factors**

Pre-harvest factors can affect seed longevity in storage. Ideally, seeds when harvested in the case of commercial crops or collected in the case of wild species, for storage are mature, of

normal size and relatively free of mechanical injuries and micro-organisms. Immature seed or mechanical-injured seed will have a reduced storage life, however, the latter is less of a problem for wild species, because wild species are generally collected and subsequently handled by hand. Also, seed may deteriorate before harvesting if produced in warm and humid climates. Weather, especially seasonal changes, is the most obvious pre-harvest factor affecting seed viability and storability (Harrington, 1972; Schmidt, 2000). Woltz et al. (2006) evaluated the effect of freezing rate, freezing temperature and duration on seed germination and vigour during seed development and maturation of six corn hybrids. Pre-harvest sprouting is the major reason for significant economic loss in the grain industry around the world. Under wet weather conditions, seeds lack of adequate dormancy can cause pre-harvest sprouting damage in barley (Li et al., 2004). However, this is less of a problem in wild species where the inherent dormancy mechanisms have not been removed as part of a selection process as has occurred in many of the cultivated species.

### **2.3.2.3 Environmental factors**

Temperature and seed moisture content are the two factors that have the greatest effect on seed longevity in storage. The lower the temperature and seed moisture content the greater the longevity in storage of orthodox seeds. Harrington (1972) proposed a relationship to demonstrate the effect of storage temperature and seed moisture content on longevity. That for each 1% reduction in seed moisture content below 14% and down to 5% moisture content the storage life of the seed was approximately doubled, and that between 0 °C and 50 °C a decline of 5 °C doubled the longevity. However, this guide is only an estimate and the relationships do not hold under extreme storage atmosphere.

In seed storage, the relationship between the relative humidity of the seed storage environment and seed moisture is essential; assuming a water permeable seed coat, the seed moisture will equilibrate to the relative humidity of that of the surrounding air. This is termed the equilibrium moisture content of the seed. This means the seed moisture content can be effectively changed to an optimum by manipulating the ambient relative humidity before and/or during storage (FAO, 1985). The partition of moisture in seeds versus the surrounding air is described by a moisture isotherm, this is a curve describing the specific relationship between seed moisture content, relative humidity and temperature when a certain system is at equilibrium (Vertucci & Roos, 1993). The isotherm will show what equilibrium moisture content will be at a certain relative humidity level. Isotherms are specific to individual seed lots, although usually approximated to individual species, and are due to factors at a given

relative humidity and temperature, such as seed composition, whether seed is gaining moisture or losing moisture, and temperature (Gold & Hay, 2014). Also, previous researchers have constructed moisture sorption isotherms for a variety of species at various temperatures, for example, Chen (2003) constructed sorption isotherms of pea seeds with three different drying treatments at five temperatures (5, 15, 25, 35, and 50 °C); Menkov (2000) determined equilibrium moisture contents in lentil seeds by using the gravimetric static approach at 5, 20, 40, and 60 °C over a range of relative humidity from 11 to 87%. Moisture isotherms can be used to help manage the seed drying process for seed, particularly seed being dried for long-term storage in a seed bank, and for studies of comparative longevity of seed lots and for testing desiccation tolerance of seed prior to seed bank storage (Gold & Hay, 2014).

#### **2.4 The role of a hard seed coat in seed storage life**

The seed coat is the natural defence against various environmental conditions. In storage a hard seed coat protects the seed from external stress such as hydration stress, mechanical stress, microorganism invasion, and humidity fluctuations (Mohamed-Yasseen et al., 1994).

Mechanical stress can reduce the storability of seeds. A hard seed coat protects seed from mechanical injury and results in longer seed viability. Bass (1980) reported that mechanical injury is a significant factor in seed storage life, particular in small seeds (Corner, 1976). Lignin content of the seed coat is a characteristic which has been suggested as being associated with resistance to mechanical injury in soybean (Alvarez et al., 1997). However, whether lignin content in the seed coat is associated with seed coat thickness or not is still a matter of controversy (Agrawal & Menon, 1974; Capeleti et al., 2005).

A hard seed coat acts as a physical defence against external threats. It provides protection against fluctuations in humidity, which could encourage growth of microorganisms (which initially infect through handling of the seed surface) (Mohamed-Yasseen et al., 1994) which can reduce seed storage life. Microorganisms damage the seeds by producing exocellular enzymes and toxins (Halloin, 1986). These can enter the seeds through the natural opening, such as the micropyle or cracks on surface, or may even obtain entry through thin seed coats (Neergard, 1977; Herman, 1983).

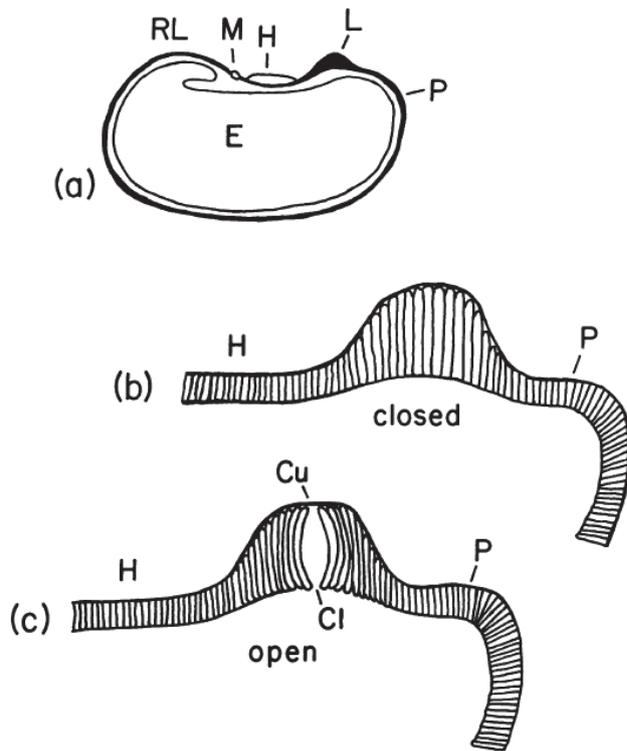
Seed coat colour can also be associated with seed storage life (Mohamed-Yasseen et al., 1994). A number of researchers (Van tier Maesen, 1984; Starzinger et al., 1982) have reported a link between seed coat colour and coat impermeability, particularly in Fabaceae

family with dark coloured seeds being more impermeable than light coloured seeds. Phenolic compounds which give the seed coat a dark colour have been suggested as contributing to hardseededness. Phenolic compounds also have another role of inhibiting microorganism growth (Gillikin & Graham, 1991; Halloin, 1986; Vaquero et al., 2007).

The Fabaceae, also known as Leguminosae family, is the third largest family of angiosperms. A number of Fabaceae species are used as crops for human and animal consumption as well as oil, fuel, fertilisers, timber, fibre, and horticultural food varieties (Lewis et al., 2005). The Fabaceae can vary from annual and perennial herbs to trees, shrubs and vines as well as a few aquatics (Rundel, 1989). The fruit of Fabaceae is the primary unifying characteristic of the family (Polhill, 1994). The Fabaceae fruits are commonly referred to as pods. The pods often have one chamber, with a few exceptions having two chambers, separated by a septum. Seeds of Fabaceae are produced and stored inside the pods. Many Fabaceae produce seeds with physical dormancy where the seed is unable to imbibe water. A phenomenon that is termed having an impermeable or hard seed. The seed coat remains hard compared with permeable seeds, which soften during imbibition (Rolston, 1978; Angiosperm Phylogeny Group (APG), 1998; Baskin et al., 2000). The impermeability of the coat is caused by the presence of one or more palisade layers of macrosclereids, and these cells have a feature of thickening the cell wall (Rolston, 1978). In the seed coat, an anatomical structure that acts as the “water gap”, has been associated with physical dormancy (Van Assche et al., 2003). The water gap in Fabaceae is in the lens area, it is an opening in the palisade cell layer. Water gaps are closed at seed maturity, and open when an appropriate environmental signal occurs (Figure 2.1). Once open, it cannot close. Thus, the timing of germination of seeds with physical dormancy is controlled in nature to determine the environmental conditions required for the water gaps to open (Baskin, 2003). In addition, in most cases, seed coat constraint of the embryo is mechanically weakened over time. This is achieved either by enzymes produced by the embryo or by external physical factors such as fluctuating temperature, the action of microorganisms, and heat (Adkins et al., 2002), thus water impermeable seed coats can change to permeable by both internal and external factors.

Water-impermeable seeds have a number of ecological and agricultural significances. They: (1) maintain seed quality when ambient relative humidity is high, by preventing deterioration; (2) help delay germination until adequate moisture is able to reach the embryo (Bennett, 1959); (3) enable seed of perennial species to persist in the soil seed bank; and (4) provide a seed source for reseeding in annuals (Rolston, 1978). However, for Fabaceae used as

agricultural and horticultural crops, the crops often establish poorly due to the delayed germination from hard seeds (Harrington, 1916) resulting in delayed harvest, and poor yield in annual crops (Baciu-Miclaus, 1970). For this reason the physical dormancy that may have been present in the wild-type ancestors of today's crop species has been removed or reduced either directly or indirectly during the selection process.



**Figure 2.1** A stylized seed of a species of legume. (a) Whole seed. (b) Portion of the seed coat showing water cap closed. (c) Portion of the seed coat showing water cap open. Cl, cleft; Cu, cuticle; E, embryo; H, hilum; L, lens; M, micropyle; P, impermeable palisade layer of seed coat; RL, radicle lobe (Baskin, 2003).

#### 2.4.1 *Clianthus* and *Carmichaelia*

##### 2.4.1.1 *Clianthus* spp.

The *Clianthus* genus comprises two species of shrubs in the Fabaceae family which are endemic to the North Island of New Zealand (Heenan, 2000). The two species are, *Clianthus maximus* Colenso and *Clianthus puniceus* (G.Don) Sol. ex Lindl. (Morris & Ballance, 2008). The common name for both species is kakabeak. This comes from the shape of flowers which are thought to resemble the beak of a New Zealand native bird, the kākā (Dawson & Lucas, 2011). *Clianthus* are recorded as being the first endemic plants which were cultivated as horticultural plantings by Maori in New Zealand (Colenso, 1885; Metcalf, 1987).

*C. maximus* is markedly different from *C. puniceus*. *C. maximus* is a large shrub, and more rarely a small tree, up to 4 m high. The leaves are 15-20 cm long, with 15-30 pairs of dark green leaflets. Leaflets are glossy on the upper surface. The flowers are in dark red or pink colour. In contrast *C. puniceus* is a small shrub, growing only to around 2 m high. The leaves are 15 cm long, with 15-20 pairs of olive-green to grey-green leaflets. The leaflets are dull on the upper surface. The flowers are red, pink or white in colour (Heenan, 2000). Flowers of both species are a typical papilionaceous blossom (flowers with the characteristic irregular and butterfly-like corolla) (Heenan, 1998). Flowering is mostly between August and January, but on rare occasions, flowering may occur throughout the year (NZPCN, 2014b). The reproductive approach of *Clianthus* is thought to be mainly cross-pollination. This is because the stigma of *C. puniceus* and *C. maximus* is covered with a cuticle. This cuticle prevents self-pollination, but when it is ruptured pollination can occur (Heenan, 1998). Plants can also be propagated vegetatively by stem layering and hardwood cuttings (Shaw & Burns, 1997; Metcalf, 1995).

Maori used kakabeak for gifting and trading. Plants are thought to have been transported throughout the country. The complete pre-human distribution of the plant is therefore unknown, but, in the past, it is known to have once grown in Northland, Auckland, Great Barrier Island, Coromandel, Lake Waikaremoana, the East Cape and Hawke's Bay. Currently, *Clianthus maximus* is found at several sites on the East Cape, Te Urewera National Park, around Wairoa, and in Boundary Stream Mainland Island in Hawke's Bay, while *Clianthus puniceus* is restricted to an island in the Kaipara harbour (NZPCN, 2014b). Although in extensive cultivation, both species of *Clianthus* are seriously threatened with extinction in the wild because animals such as goats and rodents eat the plants faster than they can grow. As a result their conservation status was classified as nationally critical in 2012 (NZPCN, 2014b). Only one plant of *Clianthus puniceus* is left in the wild, and *Clianthus maximus*, had dropped from 2000 plants in 1990 to just 153 plants in 2014 (Gibson, 2009; NZPCN, 2014b). *Clianthus* is threatened by other factors. *Clianthus puniceus* is vulnerable to competition from weeds and summer droughts. *Clianthus maximus*, at all sites, is threatened by a range of diseases, natural senescence, fire, weed control operations, and the unstable erosion-prone nature of the habitat (Department of Conservation, 2006).

Efforts to save *Clianthus* from extinction on the East Coast and in Hawke's Bay, have included protecting remaining populations from browsing animals, undertaking replanting into suitable sites and surveying potential sites for replanting (Thorsen, 2003).

Studies on *Clianthus* spp. have provided information on the biology of the species including taxonomy (Heenan, 2000), conservation status (de Lange et al., 2010), pollination system (Heenan, 1998). Also, *Clianthus* spp. have an impermeable seed coat. However, Park (2012) found that seeds of *Clianthus* spp. exhibited a loss of water impermeability when desiccated to low moisture content. Park (2012) suggested this is caused by a disruption of the cells in the lens and extrahilar regions which allowed entry of water and a loss of seed hardseededness and germination. This suggestion was based on the observation of the seed coat surface using scanning electron microscopy but the suggestion has not been unequivocally verified.

#### **2.4.1.2 *Carmichaelia muritai* (A. W. Purdie) Heenan.**

The *Carmichaelia* genus also belongs to Fabaceae family; the genus consists of 24 species, of which 23 species are native and endemic to New Zealand, and one species on Lord Howe Island (Wagstaff et al., 1999). *Carmichaelia muritai*, commonly known as coastal tree broom, is a rare small tree with a number of upright leafless branches inhabiting the coastal Kaikoura area.

*C. muritai* has a single or multiple stems and can grow up to 6 m high, with leafless branches. The plant produces a white flower with purple markings in erect racemes. Flowering is from December to January. The yellow-green seed of around 1 mm in length is produced within a pod, with only a single seed per pod. The species is currently found only naturally at Clifford Bay, Marlborough, but it is in cultivation (Grüner, 2003). *C. muritai* is easily propagated from fresh seed once the dormancy imposed by the hard seed coat is alleviated. This is done by scarifying, for example, by chipping the hard seed coat, to expose the endosperm. Plants can also be propagated from hard wood cuttings (NZPCN, 2014a).

Studies on *C. muritai* have provided information on the biology of the species including wood anatomy (Heenan, 1997), taxonomy (Heenan, 1998), conservation status (de Lange et al., 1999, de Lange et al., 2013), habit-heteroblasty and leaf-heteroblasty (Heenan, 1997). However, little is known about the biology of the seeds. Metcalf (1995) suggests seeds of *Carmichaelia* spp. will germinate in their seed pods. Park (2012) confirmed coat-imposed dormancy in *Carmichaelia williamsii*. Eleven species of *Carmichaelia* including *C. muritai* are reported to retain high seed viability for up to 24 years storage. This has been related to the presence of an impermeable seed coat in the seed (Heenan & Grüner, 2001).

The conservation status of *C. muritai* has been classified as nationally endangered since 2012 (de Lange et al., 2013). There are two small populations in the wild, both are threatened. One population is under threat from summer drought, competition from weeds, coastal erosion, and browsing animals. The other, while currently flourishing, is vulnerable to fire.

#### **2.4.1.3 Pittosporum spp.**

*Pittosporum* belongs to the Pittosporaceae family. The New Zealand flora includes 26 species of *Pittosporum*, all of which are endemic with *Pittosporum crassifolium* Banks et Sol. Ex A. Cunn. and *Pittosporum eugenioides* A. Cunn. being the most common. Both are grown domestically as hedges, because of the ability of their foliage to readily regenerate (Weston, 2004). Currently, neither *P. crassifolium* nor *P. eugenioides* is threatened with extinction. However, *P. crassifolium* rarely regenerates on offshore islands because the fruit are eaten by rats, rodents (NZPCN, 2014c). Although neither of these species is threatened, both represent common structural components of the forest types they come from, and conservation of those structural elements is an important component of an overall conservation approach.

*P. crassifolium* is valued as an ornamental and shelter plant, especially in coastal areas, because it is extremely resistance to the salt winds. The tree can reach up to 9 m high and has white underside on its leaves, and red to purple flowers with a strong scent. Flowering mainly occurs in early spring and fruiting in autumn. In late autumn, the fruits burst open to reveal sticky black seeds (Crowe & O'Flaherty, 1999).

*P. eugenioides* is commonly known as lemonwood. The tree can grow up to 12 m tall and light green shiny and wavy edged leaves the give off a strong lemon scent when crushed. Flowering occurs in late spring when bunches of cream flowers produced also emit a sweet fragrance (Weston, 2004; Crowe & O'Flaherty, 1999). The fruits are two to three-valves with each capsule containing black seeds covered with sticky mucilage (Burrows, 1996).

Seeds of *P. crassifolium* and *P. eugenioides* can be difficult to germinate. For example Moore et al. (1994) found almost no germination when imbibed and a loss of viability when *P. crassifolium* and *P. eugenioides* seeds were moist stored for 3 to 4 months in the light at 21 °C. However, low temperature stratification at 4 °C for 8 weeks or more resulted in almost full germination of seeds of *P. eugenioides*, but not with seeds of *P. crassifolium* (Moore et al., 1994). Burrows (1995) suggested that germination may be being inhibited by chemical inhibitors in the fleshy fruit tissues or in the mucilage of *Pittosporum* seed. Germination of seeds of *Pittosporum* spp. is improved when the surrounding mucilage is removed with

detergent (Burrows, 1996 by pers. comm. with G. C. Platt). In nature, the removal of the mucilage and the inhibitory tissues from the seeds of *Pittosporum spp.* is normally the result of passage through birds (Burrows, 1994). Burrows (1996) also found that some seeds of *Pittosporum* appeared to subsequently die as a result of fungal and bacterial infection and that the removal of the mucilage from the seeds was responsible for the infection from these micro-organisms.

### **3 THE EFFECT OF DESICCATION ON FABACEAE**

#### **3.1 The effect of desiccation on *Clianthus* spp.**

##### **3.1.1 Introduction**

*Clianthus*, a genus of legume in the Fabaceae family, comprises two shrub species native to New Zealand. Legumes are known for having seeds that store for many years (Priestley, 1986). This longevity is due to the seeds having a hard, impermeable seed coat, which enables the seed to remain at low moisture content in storage and be stored long-term at room temperature (Murdoch & Ellis, 1992). Seeds of *Clianthus* spp. have an impermeable seed coat but Park (2012) found they lost water impermeability when desiccated to low moisture content. Park (2012) suggested this was caused by disruption of the cells in the lens and extrahilar regions which allowed entry of water and a loss of seed hardseededness and germination. This was not unequivocally verified but there is evidence to support this suggestion, in that Park (2012) observed cracks occurring in the lens and extrahilar regions, suggesting that these parts are physically the weakest region of the seed coat. The lens region is believed to be the primary site of water entry into the seed in many species due to its relatively thin cell wall (Manning & Van Staden, 1987), and in leguminous seeds the lens region acts as the site of initial water entry and regulates the rate of water uptake. Indeed damaging the lens is more effective in stimulating germination without increasing seedling abnormalities than complete excision of the lens (Manning & Van Staden, 1987).

This chapter investigates whether the loss of hardseededness in *Clianthus* spp. is a result of cracking to the lens and extrahilar regions and clarifies the point of water entry. The objective is to provide greater understanding of the behaviour of *Clianthus* seed, and support development of long term conservation strategies for New Zealand native species.

##### **3.1.2 Materials and methods**

###### **3.1.2.1 Seed material**

Seeds of *Clianthus maximus* and *Clianthus puniceus* were obtained from a range of sources, Massey University (Palmerston North), Otari Botanical Garden (Wellington), and Proseed Company (Dunedin) (Table 3.1). All seeds were transported to Massey University (Turitea Campus) where seeds were stored at 5 °C until needed for experimentation. Seeds of both species were used in all experiments.

Prior to experimentation seeds were split into 4 replicates using the hand halving method (ISTA, 2004). Seeds were placed on a smooth clean surface and thoroughly mixed into a mound with a spatula, then the mound was divided in half and each half was mounded again and halved into quarters. Each quarter was halved again to make 8 portions. The 8 portions were arranged in rows and alternate portions were mixed and retained. The process was repeated until a sample of the required weight is obtained. Seeds of each replicate were then packed into plastic ziplock bags and labelled.

### **3.1.2.2 Seed measurement**

For each seed lot, prior to any other experimental work, the dimensions (length and breadth) of seed (Table 3.1) was measured using a digital vernier caliper and the initial seed weight measured using a five decimal-place digital balance.

### **3.1.2.3 Seed desiccation**

#### ***3.1.2.3.1 Determination of moisture content***

Seed moisture content (SMC) of fresh seeds was determined using the low-constant-temperature oven method as described in the International Rules for Seed Testing (ISTA, 2014). Four replicates of 50 seeds of each species were cut in half and weighed with a digital balance before and after drying for 17 hours in a  $103 \pm 2$  °C forced air oven. SMC was calculated as the percentage of water on a fresh weight basis.

#### ***3.1.2.3.2 Seed desiccation experiment***

Seed lots were dried to a number of target moisture contents (10%, 5%, and 2.5%) chosen according to the IPGRI screening protocol (IPGRI-DFSC, 2004). Four replicates of 50 seeds from each sample were 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 SMC was calculated using the following formula (IPGRI-DFSC, 2004):

$$\text{Weight of seed (g) at target moisture content} = \left( \frac{100 - IMC}{100 - TMC} \right) \times \text{initial seed weight (g)}$$

where IMC = initial seed moisture content

TMC = target seed moisture content

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 room for 24 hours (IPGRI-DFSC, 2004).

Once the seed was reached the target weight, seed moisture and germination were determined.

#### **3.1.2.4 Determination of whether a loss of hardseededness is due to the disruption on seed coat**

##### ***3.1.2.4.1 Determination of the water entry point***

All seeds used in this determination were first checked with a dissecting microscope to identify intact seeds without visible damage. To determine the point of water entry after each desiccation treatment on each species, as suggested by Park (2012), four replicates of 60 seeds (20 seeds per treatment) were treated as follows: (1) Vaseline<sup>®</sup> was applied as a blocking material to the lens and hilum area; (2) Vaseline<sup>®</sup> was applied to the extrahilar and hilum area; and (3) a nil treatment control where no Vaseline<sup>®</sup> was applied.

##### ***3.1.2.4.2 Imbibition of Vaseline<sup>®</sup>-treated seed***

To determine if the Vaseline<sup>®</sup> treatments were preventing water uptake, single seeds were weighed using a five decimal-place balance, immersed in tap water for 24 hours, removed from the water, re-weighed and then returned to the water. Seeds were recorded as imbibed when the amount of water uptake exceeded an additional 50% of the initial weight (Park, 2012).

##### ***3.1.2.4.3 Treatment of methylene blue uptake***

For imbibed seed (i.e. seed that imbibed after immersion in tap water (3.1.2.4.2)), methylene blue was used to track water entry into the seed and determine the point of water entry and whether this correlated with where cracks in the seed coat that may allow water entry were observed. Four replicates of 5 randomly selected Vaseline<sup>®</sup> blocked seeds of each species were immersed in methylene blue (1% in water) for 24 hours; stained seed was examined under an Olympus Provis AX-70 microscope (Orozco-Segovia et al., 2007).

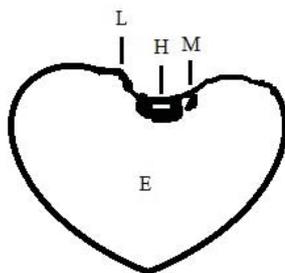
#### **3.1.2.5 Determination of germination**

Seed germination was determined using four replicates of 50 seed of each species for each treatment (initial germination, desiccation to 5% target moisture content, and desiccation to 2.5% target moisture content). Before testing for germination, seeds at less than 10%

moisture content were humidified above water at 20 °C for 24 hours to avoid the possibility of imbibition injury during rehydration (Park, 2012). Seeds were placed between moist folded 38 lb regular weight seed germination paper (Anchor Paper Company, Saint Paul, Minnesota) and held in closed plastic boxes and incubated at 20 ± 2 °C under a 16 h light/8 h dark photoperiod. 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). Germination was scored every 7 days. After 21 days any seeds that had not imbibed were scarified by removing a small piece of the seed coat in the cotyledon area with a scalpel.

### 3.1.2.6 Scanning electron microscopy (SEM) observation

Surface features of seeds at target moisture content of 2.5%, 5% and 10% were examined using scanning electron microscopy (SEM) to determine if the seed surface of *Cliaanthus* had cracked after desiccation. Samples were mounted onto aluminium stubs using double-sided tape and a small amount of silver paint to aid with conductivity. The stubs were then coated with approximately 100 nm of gold using a Balzer SCD 050 sputter coater. Samples were viewed in a FEI QUANTA 200 Scanning electron microscope at an accelerating voltage of 20 kV.



**Figure 3.1** A typical seed of both *Cliaanthus* spp. defining each area of seed coat. **E**, extrahilar; **H**, hilum; **L**, lens; **M**, micropyle.

### **3.1.2.7 Data analysis**

Data were first examined with a goodness of fit test (Shapiro-Wilk tests) to test the normality of data. Data that were normally distributed were analysed using an analysis of variance (ANOVA) followed by Tukey's Studentized Range (HSD) test for multiple comparisons. Where data were not normally distributed, the analysis used was a non-parametric ANOVA followed by Bonferroni (Dunn) t Tests for multiple comparisons. Data were ranked before the non-parametric ANOVA. Data were transformed by an arcsine square-root transformation before ANOVA. A significance level of  $P \leq 0.05$  was used for all tests. Data analyses were all performed using SAS software (SAS 9.3, SAS Institute Inc., NC, USA).

### **3.1.3 Results**

#### **3.1.3.1 Seed morphology and dimensions**

The dimensions of the seeds of *Clianthus maximus* and *Clianthus puniceus* were similar irrespective of location or species (Table 3.1). The seeds were around 3.2-3.3 mm in length, 2.6-2.8 mm in breadth and 0.01 g in weight. Seeds of both species obtained from the commercial supplier were larger in length, breadth, and seed weight than those directly collected. *C. puniceus* seeds are kidney shaped, and coloured olive green with various black stripes or blotches, whereas *C. maximus* seeds are grey or black (Figure 3.2).

**Table 3.1 Characteristic of *Clianthus maximus* and *Clianthus puniceus*. Seed materials were collected from various locations. Fifty seeds per replicate were used for measuring 1000 seeds weight and dimension (length and breadth).**

Species	Locations of collection	Lat/long coordinates	Date of collection	Number of seeds used per replicate	Length (mm)	Breadth (mm)	1000 seeds weight (g)
<i>C. maximus</i>	Otari Botanical Garden, Wellington	-41.265524, 174.751143	02 April 2014	50	3.24 ± 0.013 B	2.66 ± 0.015 B	10.44 ± 0.071 B
<i>C. maximus</i>	Dunedin (purchased from Proseed )	unknown	June 2014 (arrival date)	50	3.28 ± 0.013 A	2.79 ± 0.016 A	10.66 ± 0.073 A
<i>C. puniceus</i>	Dunedin (purchased from Proseed )	unknown	June 2014 (arrival date)	50	3.30 ± 0.017 A	2.76 ± 0.019 A	10.39 ± 0.130 A
<i>C. puniceus</i>	Massey University, Palmerston North	-40.385087, 175.614064	18 February 2014	50	3.17 ± 0.013 B	2.64 ± 0.015 B	9.34 ± 0.108 B

Numbers followed by the same letters within the same species and column are not significant different (LSD,  $P \leq 0.05$ )



**Figure 3.2** A single seed of *Clianthus maximus* (a) and *Clianthus puniceus* (b). Bars indicate 2 mm.

### 3.1.3.2 Initial moisture content and germination

The moisture content of *C. puniceus* (Proseed), and *C. puniceus* (Massey) seeds was similar at around 10%, whereas seeds of *C. maximus* were significantly different in moisture content depending on collection location. Seeds obtained from Proseed (Dunedin) had 11% moisture content, while seeds collected from Otari Botanical Garden (Wellington) had 7.4%. Seed of *C. maximus* (Proseed), *C. puniceus* (Proseed), and *C. puniceus* (Massey) had 97%, 96% and 98% germination, respectively. The germination of *C. maximus* (Otari) was 94%. The remaining non-germinated seeds were dead.

**Table 3.2** Initial seed moisture content (SMC) and germination of the seeds of *Clianthus maximus* and *Clianthus puniceus*.

Species	Number of seed per replicate used for		Initial SMC (%)	Initial germination (%)
	Initial SMC	Initial germination		
<i>C. maximus</i> (Otari)	50	50	7.4 ± 0.06 B	94 ± 1.7 A
<i>C. maximus</i> (Proseed)	50	50	11.0 ± 0.05 A	97 ± 1.5 A
<i>C. puniceus</i> (Proseed)	50	50	9.9 ± 0.06 A	96 ± 0.8 A
<i>C. puniceus</i> (Massey)	50	50	9.8 ± 0.03 A	98 ± 1.4 A

Numbers followed by the same letters within the same column are not significant different (LSD,  $P \leq 0.05$ )

### 3.1.3.3 Desiccation experiment

Both species of *Clianthus* tolerated desiccation over silica gel to 5.5-5.9% and 2.3-2.5% moisture without loss of viability (Table 3.3). Seeds took 15-19 days to reach around 5.5% moisture and a further 75-77 days to reach around 2.4% moisture. There was no change in germination of either species from any location as a result of moisture declining from 7.4 to 2.5%. Scarification was required for both species to achieve germination. The percentage of germination increased to 94-98% with scarification compared with 2-20% in untreated seeds.

**Table 3.3 Desiccation days, SMC and germination after desiccation of each *Clianthus* seed species.**

Species	Target Moisture (%)	Drying days	Moisture reached (%)	Germination (%)	
				Without scarification	With scarification
<b>C. maximus (Otari)</b>	initial	0	7.4 ± 0.06 A	20 ± 2.6 B	94 ± 1.7 A
	5	15	5.9 ± 0.25 B	94 ± 2.4 A	95 ± 2.1 A
	2.5	92	2.5 ± 0.18 C	99 ± 0.6 A	99 ± 0.6 A
<b>C. maximus (Proseed)</b>	initial	0	11.0 ± 0.05 A	2 ± 1.0 C	97 ± 1.5 A
	5	17	5.6 ± 0.14 B	88 ± 2.9 B	97 ± 1.00 A
	2.5	92	2.5 ± 0.03 C	97 ± 1.5 A	97 ± 1.5 A
<b>C. puniceus (Proseed)</b>	initial	0	9.9 ± 0.06 A	5 ± 1.3 B	96 ± 0.8 A
	5	17	5.6 ± 0.09 B	93 ± 1.0 A	94 ± 0.8 A
	2.5	94	2.4 ± 0.10 C	95 ± 1.7 A	95 ± 1.7 A
<b>C. puniceus (Massey)</b>	initial	0	9.8 ± 0.03 A	7 ± 3.1 B	98 ± 1.4 A
	5	19	5.5 ± 0.13 B	97 ± 1.3 A	99 ± 1.0 A
	2.5	94	2.3 ± 0.08 C	100 ± 0.0 A	100 ± 0.0 A

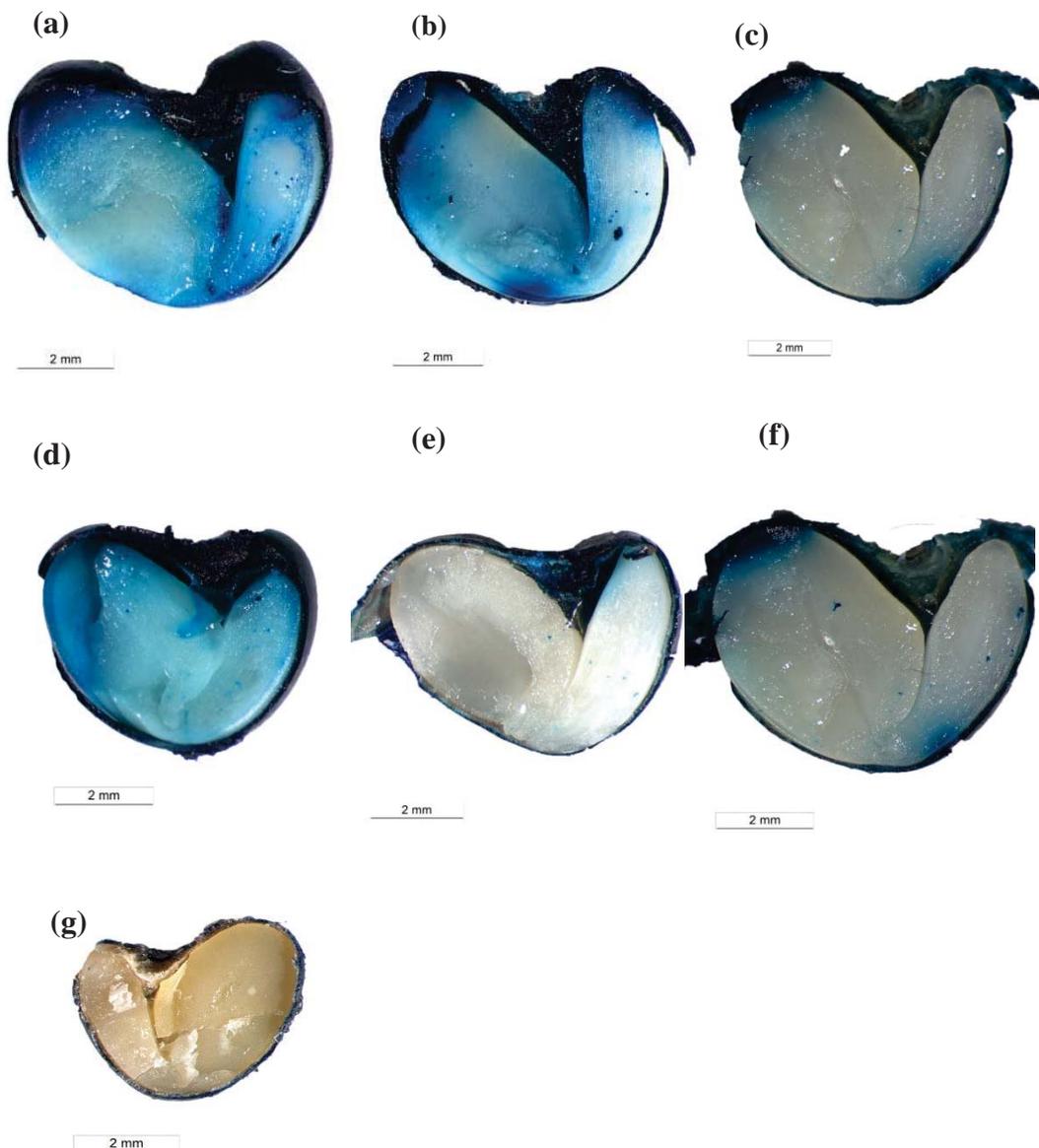
Numbers followed by the same letters within each seed lot and within each column are not significant different (LSD,  $P \leq 0.05$ )

### 3.1.3.4 Changes in seed coat morphology with desiccation

#### 3.1.3.4.1 Structures associated with water permeability

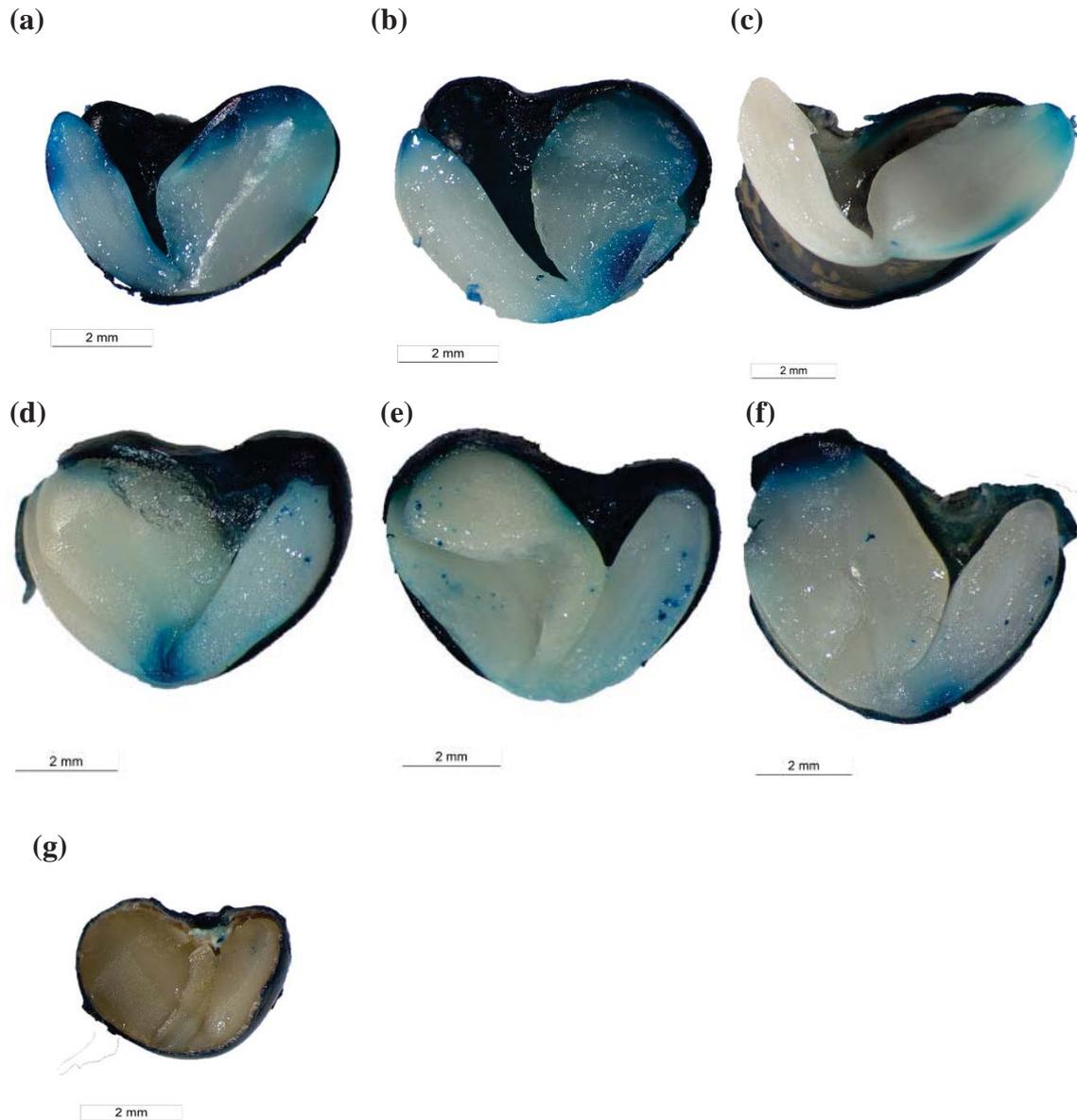
After 24 hours immersed in water, seeds of *C. maximus* and *C. puniceus* at the 2.5% and 5% target moisture contents swelled indicating that the seed had imbibed. The lens, hilum, and extrahilar areas of both *C. maximus* and *C. puniceus* seeds at both 2.5% and 5% target moisture content were fully stained when blockage material (Vaseline<sup>®</sup>) was not applied (Figure 3.3 a, d and Figure 3.4 a, d). This indicates water was able to enter the seed through one or more of the lens, hilum or extrahilar areas. When only the extrahilar and hilum area of seeds were blocked with Vaseline<sup>®</sup>, seeds also imbibed and the lens area was stained, suggesting water moved into the seed through the lens area (Figure 3.3 b, e and Figure 3.4 b,

e). When the lens and hilum areas of seeds were blocked with Vaseline<sup>®</sup>, seeds were imbibed as before and the extrahilar area stained, suggesting water was also able to enter the seed through extrahilar area (Figure 3.3 c, f and Figure 3.4 c, f). After 24 hours immersed in water both *C. maximus* and *C. puniceus* seeds of 5% target moisture content had only a small portion of the population (3-12%) non-imbibed. After scarification germination increased by 1 to 9% (Table 3.3).



**Figure 3.3** Longitudinal sections of *C. maximus* seeds immersed in water for 24 hours followed by uptake of 1% solution of methylene blue for 24 hours: (a) Seed was desiccated to 2.5% target moisture content (TMC) and without blockage material (Vaseline<sup>®</sup>) applied; (b) Vaseline<sup>®</sup> applied to extrahilar and hilum area; (c) blockage material (Vaseline<sup>®</sup>) applied; (d) Seed was desiccated to 5% TMC and without blockage material (Vaseline<sup>®</sup>) applied; (e) Vaseline<sup>®</sup> applied to extrahilar and hilum area; (f)

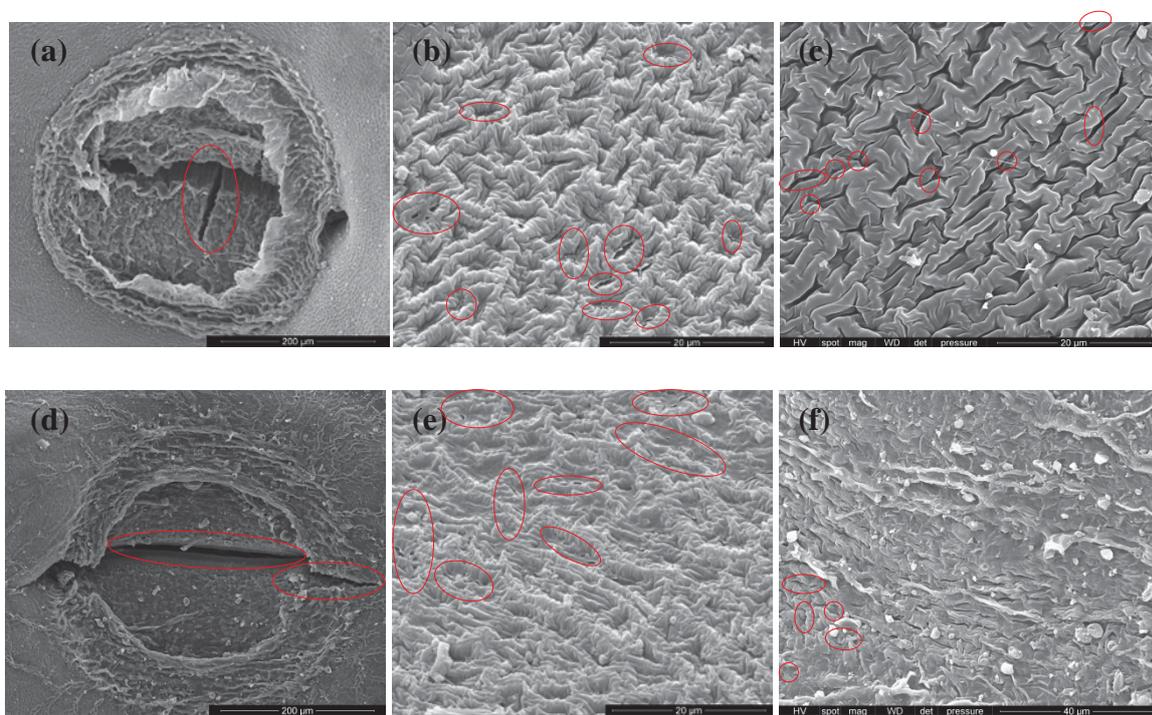
Vaseline<sup>®</sup> applied to lens and hilum area; (g) Seed non-imbibed at 5% TMC when Vaseline<sup>®</sup> applied to lens and hilum area. Bar indicates 2 mm.



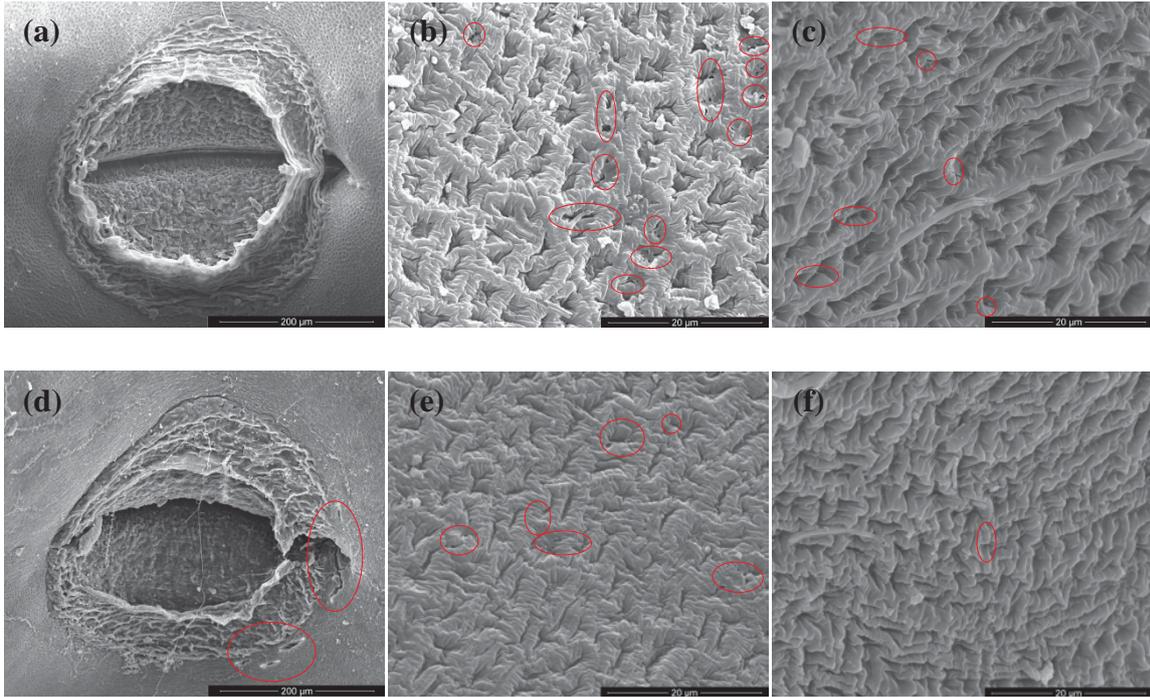
**Figure 3.4** Longitudinal sections of *C. puniceus* seeds immersed in water for 24 hours followed by uptake of 1% solution of methylene blue for 24 hours: (a) Seed was desiccated to 2.5% target moisture content (TMC) and without blockage material (Vaseline<sup>®</sup>) applied; (b) Vaseline<sup>®</sup> applied to extrahilar and hilum area; (c) Vaseline<sup>®</sup> applied to lens and hilum area; (d) Seed was desiccated to 5% TMC and without blockage material (Vaseline<sup>®</sup>) applied; (e) Vaseline<sup>®</sup> applied to extrahilar and hilum area; (f) Vaseline<sup>®</sup> applied to lens and hilum area; (g) Seed non-imbibed at 5% TMC when Vaseline<sup>®</sup> applied to extrahilar and hilum area. Bar indicates 2 mm.

#### 3.1.3.4.2 Scanning electron microscopy observation

The morphology of the seed coat surface of seeds of *Clianthus* spp., altered with seed moisture content (Figures 3.5-3.7). At 2.5% target moisture content, the seed coat cells tended to appear drier and were more bunched together, compared with cells at 5% and the initial moisture content. More significantly, seeds at 2.5% moisture showed cracks in the hilum and extrahilar area in both species (Figure 3.5). Also, at target moisture content of 5% there were cracks in the extrahilar area in both species (Figure 3.6). However, the hilum and extrahilar area remained intact in seed at the initial moisture contents (Figure 3.7).



**Figure 3.5** Scanning electron micrographs of seed coat surfaces of *Clianthus* spp. at 2.5% target moisture content after desiccation. (a-c) hilum, extrahilar, and lens area of seed of *C. maximus*, (d-f) hilum, extrahilar, and lens area of seed of *C. puniceus*. Red circles indicate the details of cracks in the seed coat.



**Figure 3.6** Scanning electron micrographs of seed coat surfaces of *Cliaanthus* spp. at 5% target moisture content after desiccation. (a-c) hilum, extrahilar, and lens area of seed of *C. maximus*, (d-f) hilum, extrahilar, and lens area of seed of *C. puniceus*. Red circles indicate the details of cracks in the seed coat.

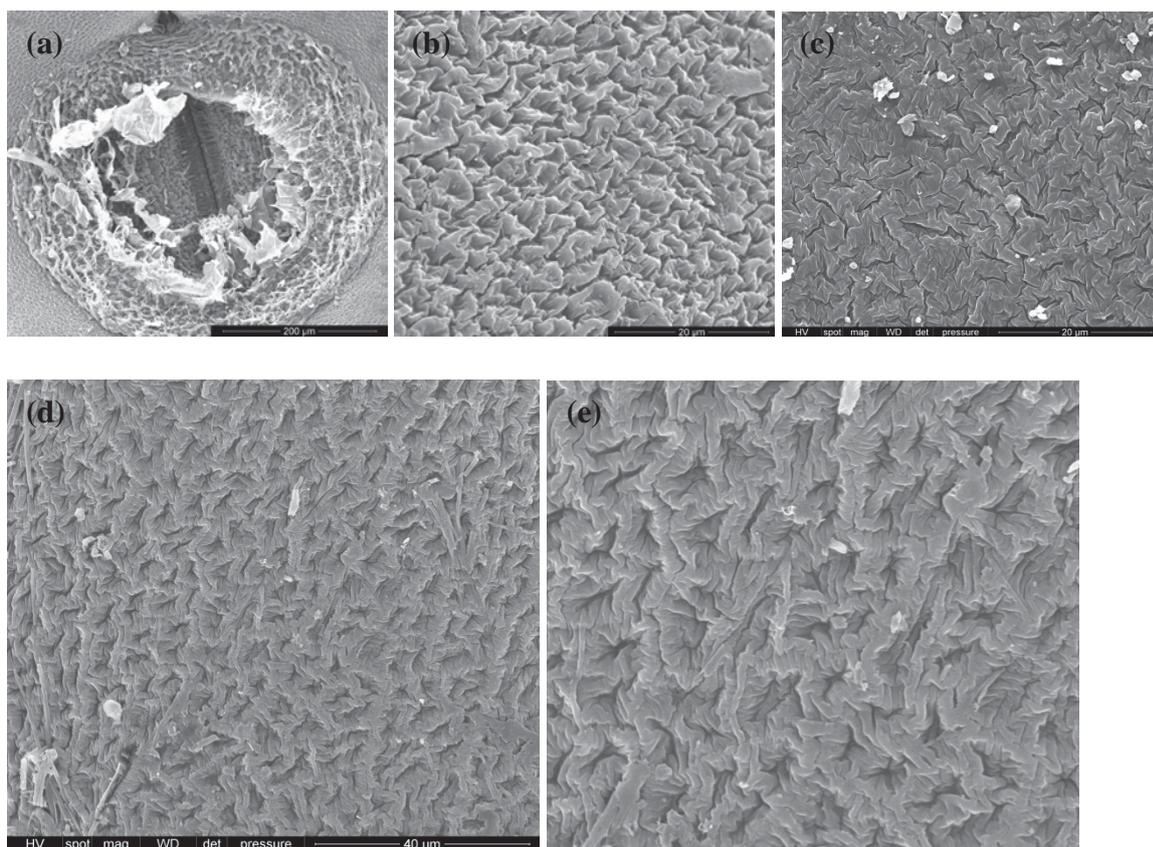


Figure 3.7 Scanning electron micrographs of seed coat surfaces of *Cilianthus* spp. at initial moisture contents. (a-c) hilum, extrahilar, and lens area of seed of *C. maximus*, (d-e) extrahilar, and lens area of seed of *C. puniceus*.

Table 3.4 Summary of the methylene blue penetration and observation of seed coat cracking by SEM in *Cilianthus*.

Species	Moisture Content (%)	Region of seed coat blocked by Vaseline®	Region of seed coat penetrated by Methylene Blue	Evidence of Cracking region by SEM
<i>C. maximus</i>	5	Extrahilar, hilum	Lens	Extrahilar, lens
		Lens, hilum	Extrahilar	
	2.5	Extrahilar, hilum	Lens	Hilum, extrahilar, lens
		Lens, hilum	Extrahilar	
<i>C. puniceus</i>	5	Extrahilar, hilum	Lens	Extrahilar, lens
		Lens, hilum	Extrahilar	
	2.5	Extrahilar, hilum	Lens	Hilum, extrahilar, lens
		Lens, hilum	Extrahilar	

### 3.1.4 Discussion

Park (2012) showed seeds of *Clianthus* species have impermeable seed coats. Scarification is essential to enable these seeds to imbibe, after which germination occurs rapidly. Water impermeability is a result of physical dormancy where one or more water-impermeable layers of palisade cells in the seed coat prevent water reaching the embryo (Baskin et al., 2000). However when desiccated to 3.7% moisture content, both *C. puniceus* and *C. maximus* seeds lost water impermeability (Park, 2010). Similar results have also been found with seeds of some species of Geraniaceae, which also have water-impermeable seed coats. Seeds of *G. columbinum*, *G. dissectum*, *G. lucidum*, *G. molle*, and *G. pusillum* that were stored over silica gel at 20 °C for periods of 3 months and 1 year, lost water-impermeability during the dry storage (Van Assche & Vandeloock, 2006). A similar period of time was taken for impermeability to be lost in *Clianthus*; 92 days for *C. maximus* and 94 days for *C. puniceus*.

This work confirmed the observations of Park et al. (2010) where seed coat permeability increased in both *Clianthus* species with a reduction of seed moisture content to 5% and 2.7%. Park (2012) investigated changes in seed coat morphology of *C. maximus* desiccated to 2.7% moisture and found cracks to the lens and extrahilar regions, whereas the hilum remained intact at 2.7%. This research has shown for the first time that in *C. puniceus* drying to low moisture also results in cracking to the lens and extrahilar areas and was confirmed in a second seedlot of *C. maximus* where cracking occurred to the lens and extrahilar areas at 2.5% and 5% target moisture. In contrast to Park (2012) cracking was also observed to the hilum area but only at 2.5% target moisture content. Similarly, the SEM results for *Clianthus* spp. indicated that seeds at target moisture content of 5% had cracks in the lens, extrahilar and 2.5% had cracks in the lens, extrahilar, and hilum regions, and methylene blue confirmed that both extrahilar and lens regions enabled water entry (Table 3.4). Methylene blue was used to track water entry into the seed and determine the point of water entry and whether this correlated with the areas where cracks in the seed coat that may allow water entry were observed. Methylene blue was chosen because the size of the molecules means the dye will pass through even fine fissures in the seed coat, but will not pass through the intact seed coat. As suggested by Park (2012), seeds were blocked with Vaseline<sup>®</sup> as follows: (1) the lens and hilum area; (2) the extrahilar and hilum area; and (3) a nil treatment control where no Vaseline was applied. The extrahilar and lens regions on seed coat were penetrated, which correlates with cracking being observed in these regions. The hilum area was not evaluated with methylene blue as Park (2012) did not find evidence of cracking to the hilum area.

Cracking to the hilum was found in this study but not by Park (2012). However, Park (2012) did not desiccate *Clianthus* seeds to the extremely low moisture contents of 2.3% and 2.5% for *C. puniceus* and *C. maximus* respectively as in this study. This suggests that the location of the cracking may be a function of the drying moisture but drying of additional seed lots to around 2.5% moisture is needed to confirm this. The hilum may be more resistant to cracking than the other parts of the coat. The hilum area is structurally different to the other areas of the seed coat and is said to act like a hygroscopic valve. There is a fissure along the groove of the hilum. This fissure closes when the seed is surrounded by moist air and opens when the surrounding air is dry (Esau, 1960). This suggests that the hilum area may be more flexible than the extrahilar and lens areas and therefore it is able to tolerate more desiccation. The cracks in the hilum, lens and extrahilar regions may be the result of cracks in the cuticle of an impermeable seed coat as a result of mechanically weakening during drying. The cuticle is about 0.2  $\mu\text{m}$  thick overlaying the palisade layer; it covers all the areas on the seed coat. Ma et al. (2004) found that in another water impermeable species of Fabaceae, soybean (*Glycine max*), cracking to the cuticle allowed the seed coat to become permeable but that the seed coat would remain impermeable when the few cracks are superficial, and would be permeable when they extend through the seed surface.

Seeds exhibiting a water impermeable seed coat have been suggested to survive for long term storage, because the hard seed coat of Fabaceae seeds enables a low degree of metabolism by blocking water and oxygen (Bonner, 2008); however, the loss of water impermeability may reduce the storage life. Storage under low relative humidity helps the transformation of seeds from permeable to impermeable, while storage under high relative humidity has an opposite effect. However, under hermetic, low relative humidity and low temperature storage the Fabaceae seeds may transform from impermeable to permeable (Souza et al., 2001). Loss of water impermeability could be a problem in long term storage, the ability to survive may be affected. Park (2012) reported that the hardseededness of *C. maximus* seeds reduced after 7 month's hermetic storage at seed moisture content under 5% and at low temperature of -20 °C but there was no reduction in viability. However, 7 months is a relatively short storage period – storage for longer periods needs to be assessed.

Fabaceae produce seeds with orthodox storage behaviour (Weber et al., 2005). Also, seeds of this family show remarkable longevity (Baskin & Baskin, 1998). Desiccation to 2.3-2.5% moisture content had no significant effect on germination of *Clianthus* seed with over 95% of seeds surviving desiccation to low moisture content environment. Park (2012) has suggested

that *C. maximus* and *C. puniceus* seeds are orthodox, as most seeds also survived at -20 °C storage for 7 months. Ultra-dry storage has been suggested to be a practical approach for seed banks conserving germplasm. Ultradry storage was first suggested by the IPGRI in 1992 (Hong et al., 2005). The suggestion was that seed could survive for long term storage at moisture contents under 5% even at an ambient temperature of 20 °C. Studies indicated that ultra-dried seeds of carrot, groundnut, lettuce, oilseed rape and onion retain their survival and vigour for 10 years of hermetic storage (Hong et al., 2005), and ultra-dried tomato seeds retained relatively high seed viability and vigour after 13 years storage at 0 °C (Zhang et al., 2001). However, in *Clianthus* spp. drying to seed moistures suitable for ultra-dry storage affects seed coat integrity. Very low seed moisture results in an impermeable seed coat becoming permeable as a result of cracking to the seed coat (Park, 2012). In hermetic storage where moisture content cannot increase this may not be a problem although the effect of damage to the seed coat on storage life is yet to be determined. In an environment where the seed may be exposed to higher relative humidity this is likely to significantly reduce storage longevity. There are some possible impacts that loss of seed coat integrity may have on long term storage: (1) seeds without intact seed coats are more likely to be infected with microflora, which makes seeds deteriorate faster during storage; (2) seeds without intact seed coats are losing the primary defence against mechanical injuries (Mohamed-Yasseen et al., 1994).

### **3.2 The effect of desiccation on *Carmichaelia muritai***

#### **3.2.1 Introduction**

*Carmichaelia muritai*, another indigenous legume in the Fabaceae family, is a tree broom endemic to New Zealand. Tan et al. (2013) reported that *Carmichaelia* is related to the genus *Clianthus* and that *Carmichaelia* and *Clianthus puniceus* are likely to be dependent on the same symbiosis genes (Wagstaff et al., 1999); as the two are closely related they might have similar seed behaviour. There are 24 *Carmichaelia* species in New Zealand and 17 of them, including *C. muritai*, were judged either “At Risk” or “Threatened” in a 2013 conservation assessment (de Lange et al., 2013) meaning these species are key candidates for seed bank storage. There is limited prior research on storage behaviour of *Carmichaelia* spp. One study showed *C. glabrescens*, *C. muritai*, *C. stevensonii*, and *C. torulosa* had hard seed coats (Gruner and Heenan, 2001). Park (2012) found that *C. williamsii* had a hard seed coat and that this was maintained even at 3.7% moisture. On the other hand Connor and Connor (1998) found that, although *C. stevensonii* was water impermeable, viability was markedly reduced after 6.5 years storage when legumes would normally be expected to retain viability

for much longer. One possible explanation is some form of disruption has allowed moisture to enter. There is no evidence presented by Connor and Connor (1998) to suggest that the seed coat is disrupted but it remains a possibility that *Carmichaelia* species could show the same loss of hardseededness as is seen in *Clianthus*? The aim of this section of the research was to determine whether *Carmichaelia* loses hardseededness at low moisture in the same way as *Clianthus*.

### **3.2.2 Materials and methods**

#### **3.2.2.1 Seed material**

Seeds of a single *Carmichaelia* species, *C. muritai*, were used for this experiment. Seed was only available from one location (Otari Botanical Garden, Wellington). Seed was collected on 2 April 2014. Immediately on receipt (Table 3.5) seed moisture content, weight and dimensions were determined.

#### **3.2.2.2 Seed measurement**

The dimension (length and breadth) of seed was measured using a vernier caliper and the initial seed weight was measured using a digital balance.

#### **3.2.2.3 Seed Desiccation**

##### ***3.2.2.3.1 Determination of moisture content***

Seed moisture content (SMC) of fresh seeds was determined as described in 3.1.2.3.1.

##### ***3.2.2.3.2 Seed desiccation experiment***

Seed lots were dried to a range of target moisture contents (10%, 5%, and 2.5%) chosen based on the IPGRI screening protocol (IPGRI-DFSC, 2004). The desiccation experiment was as described in 3.1.2.3.2. Seeds were placed in a desiccator and held at 20 °C until the seed reached the target moisture content.

#### **3.2.2.4 Determination of germination capacity**

Seeds were set to germinate as described in 3.1.2.5. Seeds that had not germinated after 21 days were counted and mechanically scarified by nicking the seed coats with a scalpel.

This test was to determine whether there was loss of hardseededness after desiccation to low moisture content.

### **3.2.2.5 Determination of whether loss of hardseededness is due to the disruption of the seed coat**

#### ***3.2.2.5.1 Determination of the water entry point***

The point of water entry was determined as described in 3.1.2.4.1.

#### ***3.2.2.5.2 Treatment of water uptake***

Seed water uptake was measured as described in 3.1.2.4.2.

#### ***3.2.2.5.3 Treatment of methylene blue uptake***

To determine the point of water entry and whether this correlates with where cracks in the seed coat that may have allowed water entry were observed, methylene blue was again used as a dye for the staining procedure. The measurement was as described in 3.1.2.4.3, except that seed at the initial moisture was also stained.

### **3.2.2.6 Data analysis**

A goodness of fit test (Shapiro-Wilk tests) was applied to test the normality of data. Data that were normally distributed were analysed using analysis of variance (ANOVA) following by Tukey's Studentized Range (HSD) test for multiple comparisons. Where data were not normally distributed and thus analysed using a non-parametric ANOVA followed by Bonferroni (Dunn) t Tests for multiple comparisons were used. Data were ranked before the non-parametric ANOVA. Data was transformed by an arcsine square-root transformation before ANOVA. A significance level of  $P \leq 0.05$  was used for all tests. Data analyses were all performed using SAS software (SAS 9.3, SAS Institute Inc., NC, USA).

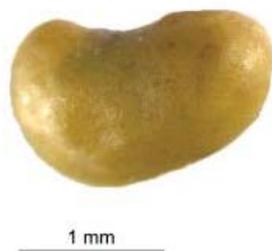
## **3.2.3 Results**

### **3.2.3.1 Seed morphology and dimensions**

The seeds of *Carmichaelia muritai* collected from Otari Botanical Garden, Wellington were dimensionally around one-third to one half the size of the *Clianthus* seed length at 1.76 mm in length and 1.2 mm in breath, but at just 0.0011 g in weight was only around 10% of their weight (Table 3.5). *Carmichaelia muritai* seeds are kidney shaped and yellow-green in colour.

**Table 3.5 Characteristics of *Carmichaelia muritai* seed. Fifty seeds per replicate were used for measuring 1000 seed weight and dimension (length and breadth)**

Location of collection	Lat/long coordinates	Date of collection	Number of seeds used per replicate used	Length (mm)	Breadth (mm)	1000 seed weight (g)
Otari Botanical Garden, Wellington	-41.265524, 174.751143	02 April 2014	50	$1.76 \pm 0.013$	$1.20 \pm 0.008$	$1.10 \pm 0.013$



**Figure 3.8 A single seed of *Carmichaelia muritai*. Bar indicates 1 mm.**

### 3.2.3.2 Initial moisture content and germination

The initial seed moisture content of the seeds of *Carmichaelia muritai* was 7.8%, the germination 86%, and the other 14% of seeds was either abnormal seedlings or dead seeds (Table 3.6).

**Table 3.6 Initial seed moisture content (SMC) and germination of *Carmichaelia muritai* seeds.**

Number of seed per replicate used for		Initial SMC (%)	Initial germination (%)
Initial SMC	Initial germination		
50	50	$7.8 \pm 0.14$	$86 \pm 1.0$

### 3.2.3.3 Desiccation experiment

The hard seed percentage, and as a consequence, germination percentage of *Carmichaelia muritai* changed with desiccation (Table 3.7). *C. muritai* seed had an initial germination of 50% with 41% of the remaining seed not germinating because of a water impermeable (hard) seed coat. However, with desiccation to moisture contents of 5.9% and 2.8% the percentage of

germination increased to 67% and 82% respectively and that of hard seed decreased to 24% and 17% (Table 3.7).

**Table 3.7 Changes in germination and hard seed percentage of seed of *Carmichaelia muritai* with desiccation over silica gel. Seed was scarified after 21 days to confirm hard seed was viable.**

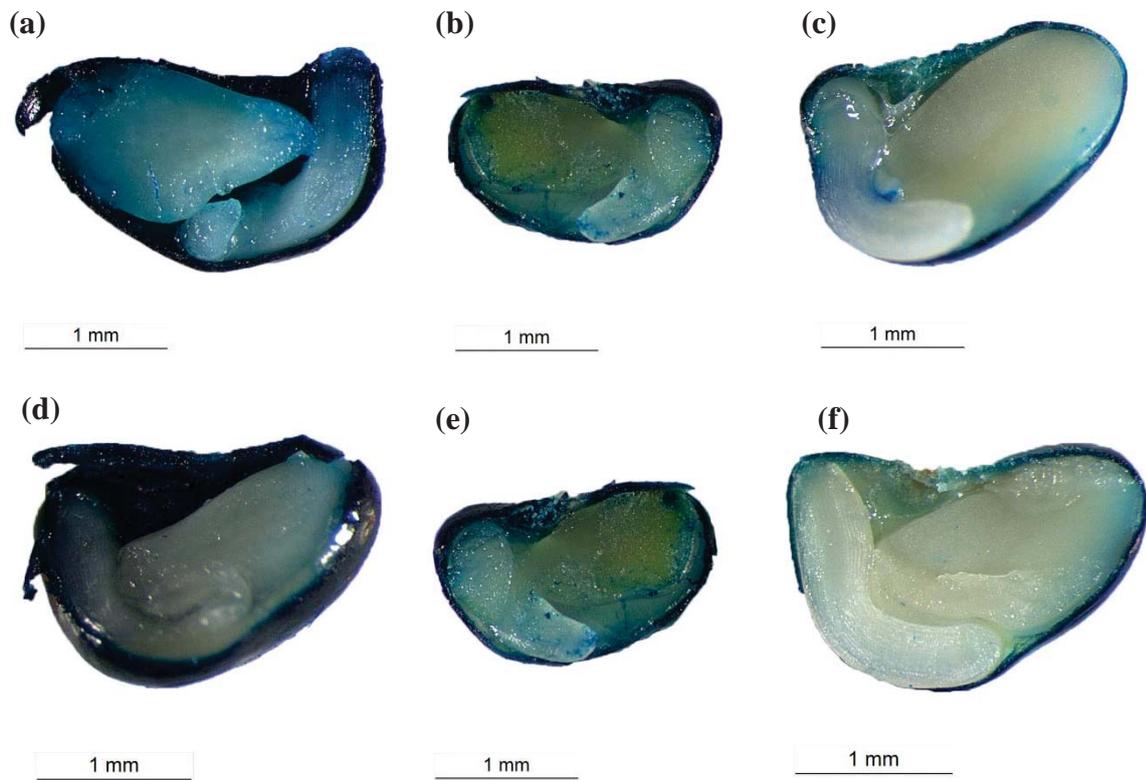
Target Moisture (%)	Drying days	Actual Moisture (%)	Germination (%)		Hard seed (%)
			Without scarification	With scarification	
initial	0	7.8 ± 0.14 A	50 ± 1.0 C	91 ± 1.0 A	41 ± 1.3 A
5	20	5.9 ± 0.02 B	67 ± 2.7 B	91 ± 3.9 A	24 ± 3.5 B
2.5	48	2.8 ± 0.05 C	82 ± 2.7 A	99 ± 1.0 A	17 ± 2.4 B

Numbers followed by the same letters are not significant different (LSD,  $P \leq 0.05$ )

### 3.2.3.4 Changes in seed coat morphology with desiccation

#### 3.2.3.4.1 Structures associated with water permeability

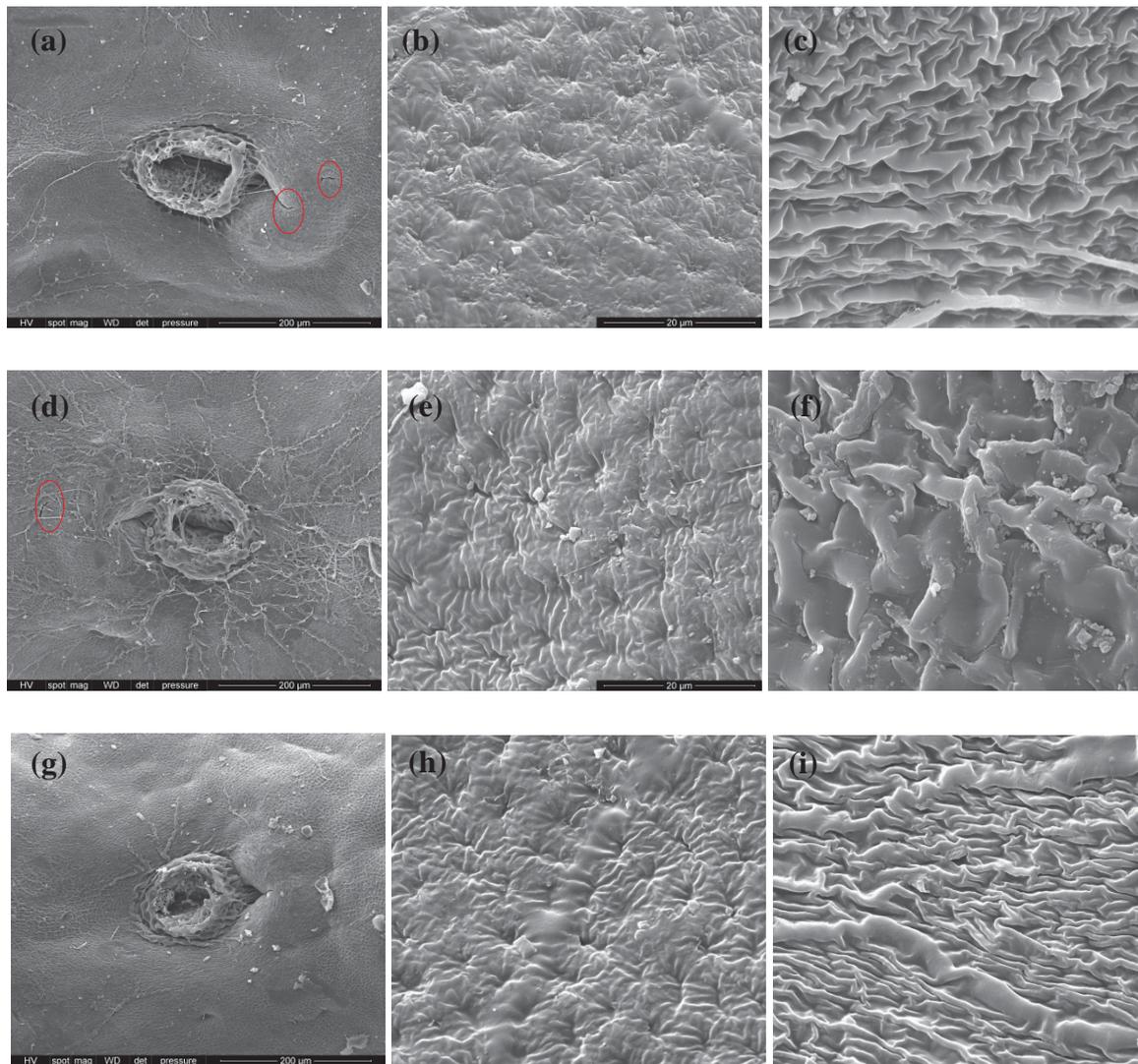
The seed coat of *C. muritai* at both 2.5% and 5% target moisture content were fully stained when blockage material (Vaseline<sup>®</sup>) was not applied (Figure 3.9 a, d), which indicates the water can potentially enter from one or more of the lens, hilum, or extrahilar areas. When the extrahilar and hilum area of the seeds were blocked with Vaseline<sup>®</sup>, seed imbibed and the lens area was stained, indicating water penetrated the seed through the lens area (Figure 3.9 b, e). When the lens and hilum areas were blocked with Vaseline<sup>®</sup>, seeds imbibed and staining to the extrahilar area was observed, suggesting water can also penetrate the seed through the extrahilar area (Figure 3.9 c, f). In seed at the initial moisture content methylene blue staining was observed in the lens, hilum and extrahilar areas.



**Figure 3.9** Longitudinal sections of *Carmichaelia muritai* (a-f) seeds immersed in water for 24 hours followed by uptake of 1% solution of methylene blue for 24 hours. (a) Seed was desiccated to 2.5% target moisture content (TMC) and without blockage material (Vaseline<sup>®</sup>) applied; (b) Vaseline<sup>®</sup> applied to extrahilar and hilum area; (c) Vaseline<sup>®</sup> applied to lens and hilum area; (f) Seed was desiccated to 5% target moisture content and without blockage material (Vaseline<sup>®</sup>) applied; (e) Vaseline<sup>®</sup> applied to extrahilar and hilum area; (f) Vaseline applied to lens and hilum area. Bar indicates 1 mm.

#### 3.2.3.4.2 Ultra-surface seed coat morphology

Changes in the morphology of the seed coat surface of *Carmichaelia muritai*, were observed at different moisture contents (Figure 3.10). Seeds at target moisture content of 2.5% and 5% had cracks in the hilum/micropyle area (Figure 3.10 a, d). However, no cracking was observed in the seed coat at the initial moisture content (Figure 3.10 g-i).



**Figure 3.10** Scanning electron micrographs of seed coat surfaces of *Carmichaelia muritai* at initial moisture content and following desiccation to 5% and 2.5%. (a-c) hilum/micropyle, extrahilar, and lens area of seed at 2.5% moisture content, (d-f) hilum/micropyle, extrahilar, and lens area of seed at 5% moisture content, (g-i) hilum, extrahilar, and lens area of seed at initial moisture content. Red circles indicate the details of cracks in the seed coat.

### 3.2.4 Discussion

Scarification also made a significant difference to germination of *Carmichaelia muritai* seed. Seeds without scarification did not imbibe, while scarification enabled germination to occur rapidly. Only 41% of seeds exhibited a hard seed coat at the initial moisture content of 7.8%. This indicates that at this moisture only about half of this one population of *C. muritai* had a water impermeable seed coat. In contrast, *C. williamsii* at 8.4% and 3.7% seed moisture hardseededness was much higher at 82-84% (Park, 2012). There may be variation in the

extent to which an impermeable seed coat develops in different *Carmichaelia* species or the same species produced in different years and/or locations. Grüner and Heenan (2001) found differences in viability amongst different *Carmichaelia* species stored for a similar number of years and that viability was correlated with seed coat permeability. Seed coat permeability was not assessed at the beginning of the storage period but it may be that the poorer storage at ambient temperatures in some species is a function of lower initial coat impermeability as well as the loss of hardseededness over time. The implication of the seed coat permeability in a significant portion of the population for long term storage at ambient temperatures of *C. muritai* could be: (1) the reduction of seed longevity as seeds are more likely to deteriorate as a result of fluctuating temperatures and exposure to high relative humidity and oxygen; and (2) more likely to be infected with micro-flora, which accelerates the deterioration of seeds (Mohamed-Yasseen et al., 1994).

This presence of hard seed is consistent with another species in the *Carmichaelia* genus. *C. williamsii* was reported to exhibit hard seed by Park (2012). The percentage of *C. williamsii* seeds having an impermeable seed coat increased from 3% to 82% with reduction of moisture content from 19.8% to 3.7%. In contrast, seeds of *C. muritai* with an impermeable seed coat decreased to 17% with reduction of moisture content to 2.8%. The possible reason for this difference may be the initial moisture content and maturation of the seeds. The initial moisture content of *C. muritai* was 7.8% in this study, and *C. williamsii* was 19.2% in the Park (2012) study. Michael et al. (2007) reported that the seed coat usually develops hardseededness at moisture contents between 15% and 54% during maturation drying in *Malva parviflora*. El Balla et al. (2011) reported that seeds of wild okra (*Abelmoschus esculentus*), also in the Malvaceae, changed from permeable seed coat to impermeable with the maturation of the seeds at the moisture content of 29%, and the percentage of hard seed reduced with increasing moisture content. Similar results were also reported by Marbach and Mayer (1974) in wild pea seeds. The lower final moisture content in *C. muritai* may result in cracking to the seed coat.

In this study, in that proportion of the population with a water impermeable seed coat desiccation to lower moisture contents (5.9% and 2.8%) also alleviated coat impermeability in *C. muritai*, in a similar way to the *Clanthus* spp. In contrast at 3.7% moisture in *C. williamsii* 84% of seeds retained a water impermeable seed coat, suggesting species differences in coat permeability. As was observed in *Clanthus* cracks in the seed coat of *C. muritai* appear to allow moisture to enter the previously hard seed. However, a limitation of this experiment is

that only 40% of the *Carmichaelia muritai* seed population had a water impermeable seed coat, and in selecting seed for methylene blue staining after desiccation it was not possible to determine if seed selected had a water permeable coat at the initial moisture content (50% of the population) or if permeability developed as a result of desiccation (17% and 32% of the population at 5% and 2.5% moisture respectively). In seed at the initial moisture content methylene blue staining was observed in the lens, hilum and extrahilar areas indicating that in water permeable seed water was able to enter through all areas of the seed coat. However, in seed at 2.5% and 5% moisture SEM only showed cracks in the hilum/micropyle regions and not in all seeds. No cracks were observed in seed at the initial moisture content. This suggests that desiccation is also increasing water permeability in *C. muritai* seeds as a result of cracking of seed coat structures. This is the first report that seeds of *C. muritai* develop cracks in their seed coat allowing moisture to enter. To confirm this, the same experiment could be undertaken with the addition of soaking seeds as a preliminary screening and only desiccating those seeds that do not imbibe. Cracking have also been found in *Clanthus maximus* (Park, 2012) and other species of Fabaceae, such as soybean (Ma et al., 2004). This and the loss of hardseededness observed by Van Assche & Vandeloos (2006) in species of Geraniaceae suggests that determining if cracking is occurring in other Fabaceae and Geraniaceae would be worthwhile .

An understanding of the storage behaviour in seeds of *C. muritai* is essential for long term storage. The viability of *C. muritai* was not lost when seeds were desiccated to 5.6% moisture content. Viability remained high (99%) when seeds were further desiccated to 2.8%. Similar results were reported by Park (2010) who concluded that *C. williamsii* seeds are of the typical orthodox type, which had no reduction in viability when desiccated to a moisture content of 3.7%. Also, the storage behaviour of *Clanthus* spp. seeds and about 99% of other Fabaceae seeds species has been verified as orthodox (Dickie & Pritchard, 2002). In order to confirm the seed storage behaviour of *C. muritai*, storage at -20 °C for 3 months is still necessary as suggested in the protocol (Hong & Ellis, 1996).

## 4 THE EFFECT OF DESICCATION ON PITTOSPORACEAE

### 4.1 Desiccation sensitivity of *Pittosporum crassifolium* and *Pittosporum eugenioides*

#### 4.1.1 Introduction

Determination of seed storage behaviour for a particular plant species is the first stage in developing an *ex-situ* conservation strategy. For some species, especially trees and shrubs from wet tropical habitats, seed desiccation sensitivity may limit their capacity for conservation in a seed bank. Seed storage behaviour refers to the capacity of seeds to survive desiccation (Gold & Hay, 2008) and seeds are classified as desiccation tolerant (orthodox), desiccation sensitive (recalcitrant), and intermediate (Roberts & Ellis, 1989; Pritchard, 1991). The standard procedures for seed bank storage recommended by FAO/IPGRI (1994) are seed storage at 3-7% moisture content and temperature at -20 °C. Only seeds with orthodox storage behaviour can survive under such conditions therefore it is essential to understand if seeds are tolerant to desiccation to low moisture content before storage. For those species identified as having non-orthodox storage behaviour, *in-situ* conservation may be the best conservation strategy.

For many New Zealand native species, information on seed storage behaviour is either incomplete or not available. The Royal Botanic Gardens Kew Seed Information Database (SID) (2015) indicates that information on the seed storage behaviour of *Pittosporum eugenioides* is incomplete. No information for *Pittosporum crassifolium* is available. Therefore an investigation needs to be conducted for both these species. This chapter investigates seed storage behaviour in these two *Pittosporum*. The objective is to provide details for developing long term conservation strategies for these New Zealand native species.

#### 4.1.2 Materials and methods

##### 4.1.2.1 Seed materials

Seeds of *Pittosporum crassifolium* and *P. eugenioides* at two maturity stages (mature with black fruits and seeds and immature with green fruits and seeds) were collected from Palmerston North (Massey University Turitea Campus) and Wellington (Otari Botanical Garden and Island Bay). Following collection, seed material were then split into 4 replicates using the hand halving method (ISTA, 2004) at Massey University (Turitea Campus).

Immediately upon receipt (Table 4.2) the seeds were extracted from the reproductive structures by hand and the initial moisture content and germination determined.

#### **4.1.2.2 Determination of initial moisture content**

Seed moisture content (SMC) of fresh seeds was determined as described in 3.1.2.3.1.

#### **4.1.2.3 Determination of initial germination capacity**

Both mature and immature seeds were mixed in sieved wood ash to prevent them from clumping, which would limit the seeds ability to absorb water evenly. Seed was placed on blue germination blotters and held in sealed plastic boxes (220×120×40 mm) then chilled at 5 °C for 21 days (Moore et al., 1994). After pre-chilling, seeds were set to germinate at 20 °C with light. Germination was assessed at 35 days (*P. eugenioides*) and 42 days (*P. crassifolium*) excluding pre-chilling time to determine if any normal seedlings had developed. A seed is considered to have germinated and a normal seedling developed when it had a well-developed primary root and intact hypocotyl and cotyledons (ISTA, 2014). At the end of 35 days the viability of any seed that had not germinated was assessed using the tetrazolium test.

#### **4.1.2.4 Tetrazolium testing**

On receipt viability was also assessed using the tetrazolium test. Both mature and immature seeds were soaked in water for 18 hours at 20 °C. A ¼ of the distal end of the seeds was cut off and the remaining seed immersed in 1% tetrazolium solution for 72 hours at 30 °C. After staining, seeds were cut longitudinally and the embryo assessed for viability (Figure 4.3) under a microscope following the tetrazolium testing working sheets for *Pittosporum* species (ISTA, 2003).

#### **4.1.2.5 Seed desiccation experiment**

Both mature and immature seeds were dried to a range of target moisture contents (10%, 5%, and 2.5%) following the IPGRI screening protocol (IPGRI-DFSC, 2004). The desiccation experiment was as described in 3.1.2.3.2.

Following desiccation, the moisture content was determined and seeds were germinated as described in 4.1.2.3, rehydrating the seeds when necessary by placing them above water in a closed container at 20 °C for 24 hours (IPGRI-DFSC, 2004). Tetrazolium testing was applied to non-germinated seeds after 35 days to assess viability.

#### 4.1.2.6 Producing seed sorption isotherms

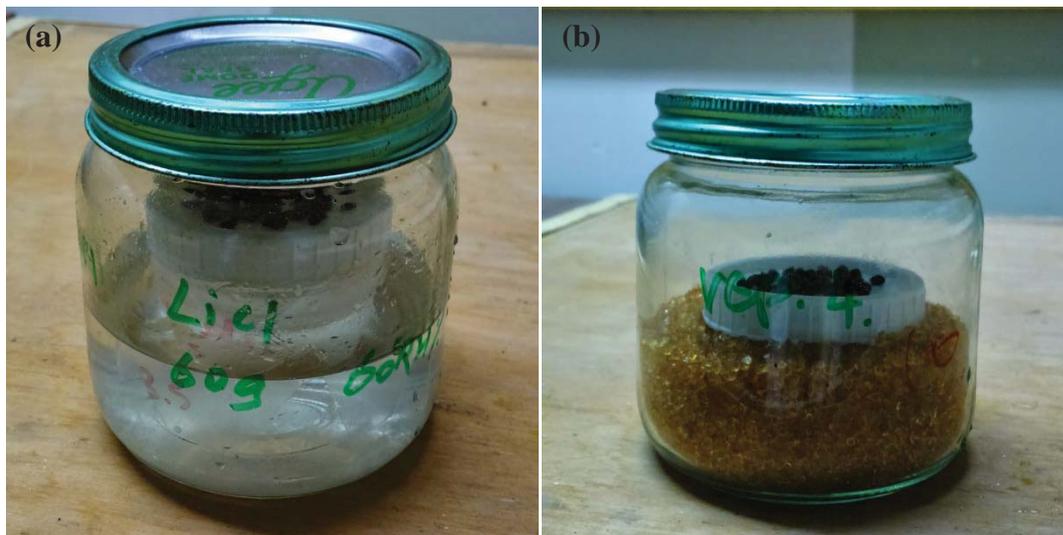
For the isotherm study, seeds were equilibrated at 20 °C over saturated lithium chloride salt solutions for up to 1 to 2 weeks depending on the size of the seeds. To create a series of relative humidity environments (RH) different solutions of lithium chloride were prepared by adding different weights of lithium chloride to 200 ml of reverse osmosis water (Table 4.1) (Gold & Hay, 2014). Solutions were mixed using a magnetic stirrer. Once prepared, four replicates of each RH of 12 suitably-sized air-tight containers were filled with solution, and labelled with the lithium chloride concentration and RH level. The solutions were allowed to equilibrate in sealed containers for at least 24 hours, at a constant temperature of  $20 \pm 0.5$  °C. Seed samples of 5 g (*Pittosporum crassifolium*) and 1 g (*Pittosporum eugenioides*) per replicate were held by a plastic mesh support above each LiCl solution in each jar (Figure 4.1 a).

To provide a point below 10% RH on the curve, a sample of seeds from each treatment replicate was equilibrated with silica gel (5% RH) (Figure 4.1 b). Seed equilibrium relative humidity (eRH) was determined when the equilibrium point had been reached which was monitored by measuring the loss/gain in weight of seeds until there was no further change. Seed moisture content was determined as described and the EMC plotted against measured eRH (Gold & Hay, 2008). eRH in each RH chamber were measured by a Hygrochron Temperature and Humidity iButton (DS 1923-F5) data logger (temperature accuracy:  $\pm 0.5$ °C from -10°C to +65°C).

After 1 to 2 weeks seeds were germinated as described in 4.1.2.3, rehydrating the seeds when necessary by placing them above water in a closed container at 20 °C for 24 hours (IPGRI-DFSC, 2004).

**Table 4.1 Weight of LiCl (g) with predicted equivalent RH% generated at 20 °C compared with direct measurement using a Hygrochron Temperature and Humidity iButton (DS 1923-F5) data logger. (Gold & Hay, 2014).**

Weight of LiCl (g)	Target RH (%) at 20 °C	RH (%) measured at 20 °C	
		Palmerston North Collection	Wellington Collection
174	11	28.0 ± 1.04	26.7 ± 0.64
147	15	29.2 ± 0.76	28.4 ± 0.95
128	20	30.0 ± 0.76	32.1 ± 0.76
104	30	35.7 ± 1.24	38.1 ± 0.25
88	40	41.7 ± 0.70	47.4 ± 0.37
74	50	51.3 ± 0.52	58.4 ± 1.06
60	60	56.1 ± 1.89	66.0 ± 1.02
50	70	60.6 ± 2.63	72.0 ± 0.75
34	80	69.1 ± 1.31	78.1 ± 0.89
26	85	74.5 ± 1.06	82.7 ± 0.34
20	90	78.2 ± 1.01	85.7 ± 0.44
8	95	80.9 ± 1.13	87.6 ± 0.28



**Figure 4.1 Air tight jars with lithium chloride solution, showing the plastic lid support used to hold *Pittosporum* seeds above the solution (a) and above the silica gel (b)**

#### 4.1.2.7 Data analysis

A goodness of fit test (Shapiro-Wilk tests) were applied to test the normality of data. Data that were normally distributed were analysed using an analysis of variance (ANOVA) following by Tukey's Studentized Range (HSD) test for multiple comparisons. Data that were not normally distributed were analysed using non-parametric ANOVA followed by Bonferroni (Dunn) t Tests for multiple comparisons. Data were ranked before non-parametric ANOVA. The data were analyzed by using linear regression. Linear regression was applied to analyze the relationship between seed moisture content and relative humidity. A

significance level of  $P \leq 0.05$  was used for all tests. Data analyses were all performed using SAS software (SAS 9.3, SAS Institute Inc., NC, USA).

### **4.1.3 Results**

#### **4.1.3.1 Seed material morphology and dimensions**

The dimensions of the *Pittosporum eugenioides* and *Pittosporum crassifolium* seed differed between harvest locations (Table 4.2). The dimensions and 1000 seed weight of the *P. eugenioides* seeds was, around 2.8-3.1 mm in length, 2.2-2.5 mm in breadth with an average seed weight of 0.005-0.007 g. In contrast seeds of *P. crassifolium* were both larger and heavier at 4.4-4.6 mm in length, 3.3-3.6 mm in breadth, and 0.03 g in weight. Mature *P. eugenioides* capsules have 2 to 3 valves in a black, ovoid to elliptic shaped fruit, and black seeds with yellow mucilage (Figure 4.2 a), whereas immature *P. eugenioides* capsules have 2 to 3 valves in green, and ovoid to elliptic shaped fruit, seeds are yellow-green with mucilage (Figure 4.2 b). *P. crassifolium* has woody capsules, with 2 to 4 valves, and grow black, ovoid or elliptic shaped seeds that are covered with yellow mucilage (Figure 4.2 c).

**Table 4.2 Characteristic of *Pitosporum eugenioides* and *Pitosporum crassifolium*.**

Species	Locations of collection	Lat/long coordinates	Date of collection and arrival at Massey University	Number of seeds used per replicate	Length (mm)	Breadth (mm)	1000 seed weight (g)
<b>P. eugenioides (mature)</b>	Massey University, Palmerston North	-40.385087, 175.614064	09 September 2014	50	2.86 ± 0.029	2.38 ± 0.027	4.94 ± 0.560
<b>P. eugenioides (immature)</b>	Massey University, Palmerston North	-40.385087, 175.614064	09 September 2014	50	2.82 ± 0.021	2.35 ± 0.021	4.81 ± 0.042
<b>P. crassifolium</b>	Massey University, Palmerston North	-40.385087, 175.614064	09 September 2014	50	4.38 ± 0.031	3.33 ± 0.036	26.46 ± 1.205
<b>P. eugenioides (mature)</b>	Otari Botanical Garden, Wellington	-41.265524, 174.751143	20 October 2014	50	2.87 ± 0.022	2.21 ± 0.027	5.56 ± 0.401
<b>P. eugenioides (immature)</b>	Otari Botanical Garden, Wellington	-41.265524, 174.751143	20 October 2014	50	3.09 ± 0.027	2.48 ± 0.027	6.88 ± 0.758
<b>P. crassifolium</b>	Island Bay, Wellington	-41.335729, 174.773226	20 October 2014	50	4.59 ± 0.035	3.61 ± 0.032	31.99 ± 3.034



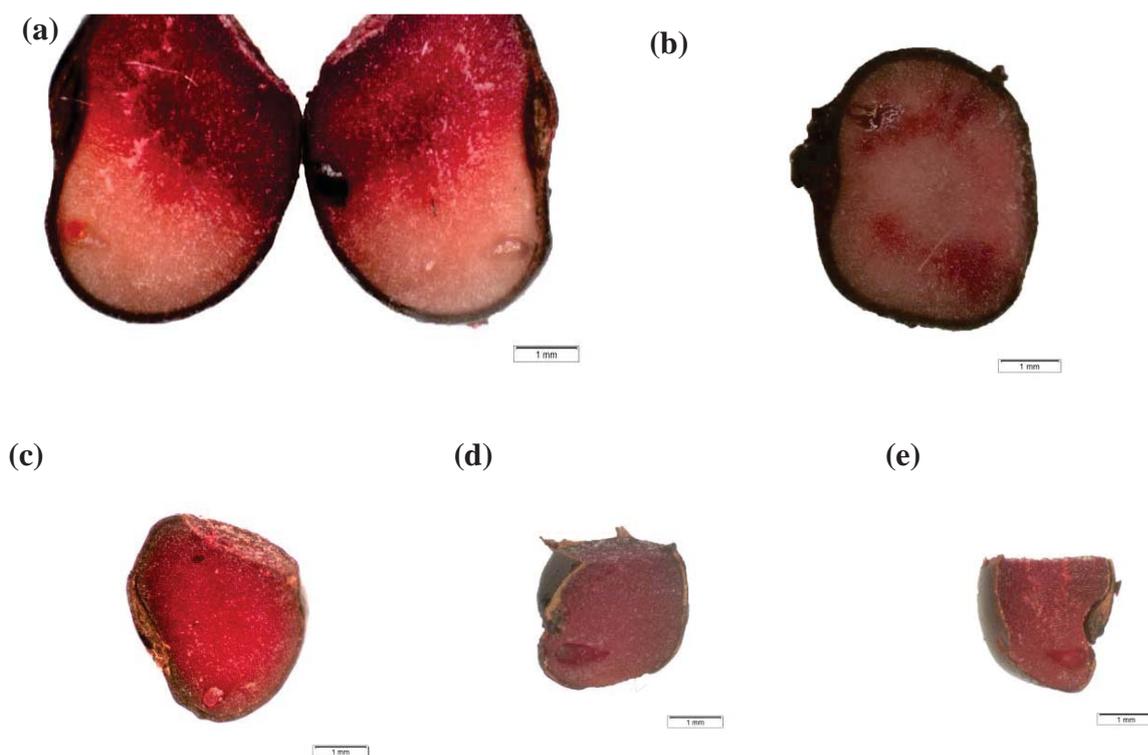
**Figure 4.2** Capsules of (a) mature *Pittosporum eugenioides*, (b) immature *Pittosporum eugenioides*, (c) *Pittosporum crassifolium* (collected at Massey University, Palmerston North).

#### 4.1.3.2 Initial moisture content and viability

The initial seed moisture content and viability of the seeds of *Pittosporum eugenioides* and *Pittosporum crassifolium* are shown in Table 4.3. The moisture content of mature *P. eugenioides* collected at Massey University, Palmerston North was 10.8% compared with 19.9% moisture for that collected in Island Bay, Wellington. For immature from both locations moisture was nearly 70%. In contrast the moisture content of *Pittosporum crassifolium* was around 16% for both locations. The seed viability differed depending on species and collection location but viability was over 80% for all seed lots (Table 4.3). Germination of immature seed and mature seed of *P. eugenioides* was poor despite the high viability of the seed. In contrast viability at the end of the germination test differed between locations. It was higher for *P. crassifolium* than *P. eugenioides* for seed collected at Palmerston North and lower for seed collected at Wellington. Viability for fresh seed at both locations was the similar for species respectively (Table 4.3).

**Table 4.3 Seed moisture content, germination and viability of immature and mature *Pittosporum eugenioides* and mature *Pittosporum crassifolium* seeds collected at Massey University, Palmerston North and Island Bay Wellington.**

Species	Number of seed per replicate used for			Initial SMC (%)	Germination test (%)	Viability of fresh seed TZ testing (%)
	Initial SMC	Initial germination	Initial viability (TZ testing)			
<b>P. eugenioides (mature, Palmerston North)</b>	50	50	25	10.8 ± 0.14	16 ± 2.6	89 ± 3.4
<b>P. eugenioides (immature, Palmerston North)</b>	50	50	25	67.9 ± 0.73	0.0 ± 0.00	94 ± 2.6
<b>P.crassifolium (Palmerston North)</b>	50	50	25	15.8 ± 0.64	75 ± 2.9	82 ± 4.2
<b>P. eugenioides (mature, Wellington)</b>	50	50	25	19.9 ± 0.27	93 ± 1.3	99 ± 1.0
<b>P. eugenioides (immature, Wellington)</b>	50	50	25	68.5 ± 1.15	1 ± 0.5	90 ± 2.0
<b>P.crassifolium (Wellington)</b>	50	50	25	16.8 ± 0.35	53 ± 6.7	82 ± 6.6



**Figure 4.3** Examples tetrazolium viability testing of *Pittosporum* spp. Non-viable seeds with incomplete staining to the endosperm tissue of *Pittosporum crassifolium* (a-b). Viable seeds with complete staining to the endosperm tissue of *Pittosporum crassifolium* (c) and *Pittosporum eugenioides* (d-e). Bar indicates 1 mm.

#### 4.1.3.3 Desiccation experiment

The desiccation experiment for seeds collected at Massey was conducted on September 30, 2014, and for seeds collected in Wellington on October 22, 2014, seeds were stored in sealed plastic bags to keep moist. Mature *Pittosporum eugenioides* collected from Massey University, Palmerston North had low initial germination with 15.5%, however germination was 68.5% for seed stored in a moist environment (vermiculite), but it reduced to 29% when seeds were desiccated to 3% target moisture content. Mature *Pittosporum eugenioides* from Wellington had high initial germination with 93%, and it was maintained in a moist environment at 93%. Desiccation reduced viability to 8.5%. *Pittosporum crassifolium* from both locations had similar results: moist environment maintained the viability at around 70% or above, however, desiccation reduced viability.

**Table 4.4 Desiccation days, SMC and viability assessed by the tetrazolium test after desiccation of *Pittosporum eugenioides* and *Pittosporum crassifolium* seeds.**

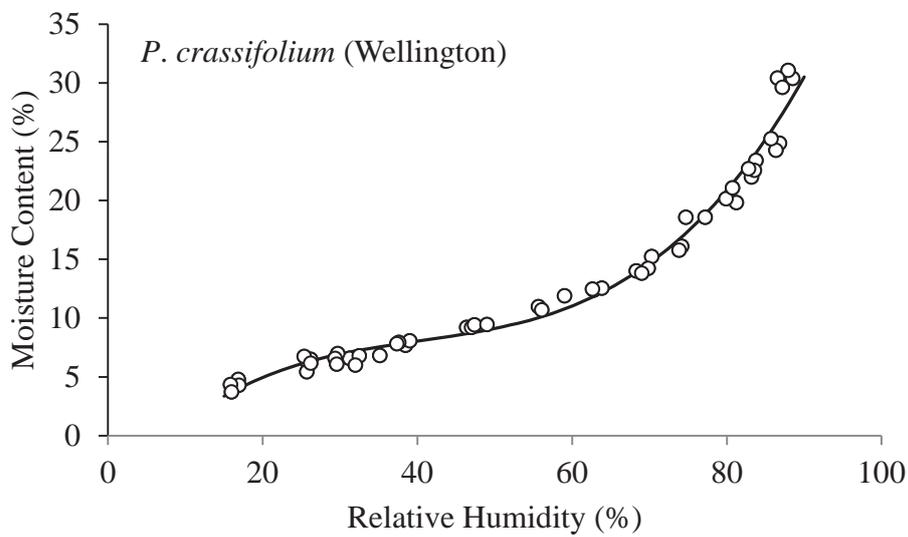
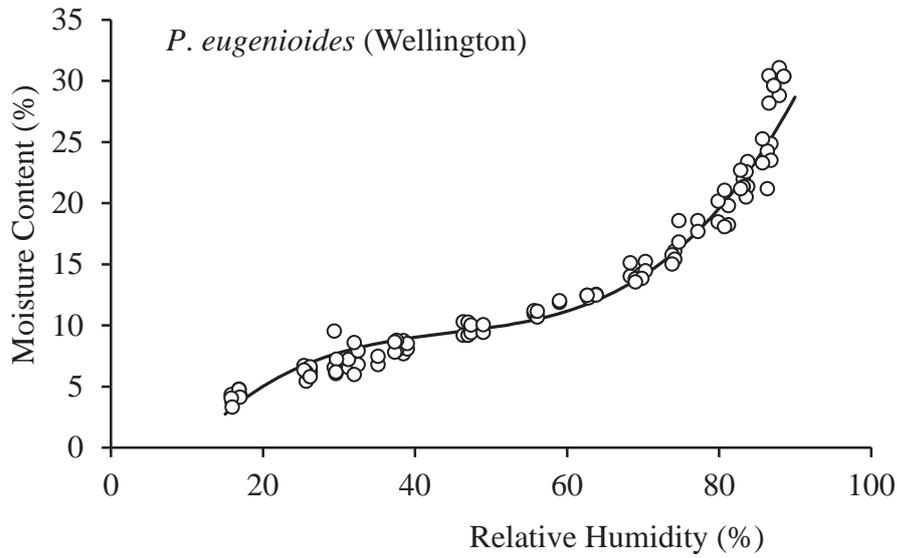
Species	Target SMC (%)	Drying days	Actual SMC (%)	Viability (%)
<b>P. eugenioides</b> (mature, Palmerston North)	initial	0	10.8 ± 0.14B	16 ± 2.6 C
	5	17	5.3 ± 0.19 C	32 ± 4.4 B
	3	23	3.5 ± 0.01 C	29 ± 4.0 BC
	Control*	23	41.5 ± 0.98A	69 ± 1.7 A
<b>P. eugenioides</b> (immature, Palmerston North)	initial	0	67.9 ± 0.73B	0 ± 0.0 A
	10	11	10.2 ± 0.22C	1 ± 0.5 A
	5	14	5.9 ± 0.06 D	0 ± 0.0 A
	3	18	3.6 ± 0.21 E	0 ± 0.0 A
	Control	18	74.5 ± 0.54A	0 ± 0.0 A
<b>P. crassifolium</b> (Palmerston North)	initial	0	15.8 ± 0.64B	75 ± 2.9 A
	10	8	9.8 ± 0.06 C	59 ± 2.1 AB
	5	11	5.4 ± 0.17 CD	56 ± 1.8 AB
	3	14	3.9 ± 0.07 D	40 ± 1.4 B
	Control	14	35.8 ± 2.50A	68 ± 8.8 A
<b>P. eugenioides</b> (mature, Wellington)	initial	0	19.9 ± 0.27B	93 ± 1.3 A
	10	9	10.1 ± 0.26C	87 ± 1.3 A
	5	32	4.6 ± 0.14 D	56 ± 4.1 B
	3	40	3.9 ± 0.31 D	9 ± 2.2 C
	Control	40	41.8 ± 1.38A	93 ± 2.4 A
<b>P. eugenioides</b> (immature, Wellington)	initial	0	68.5 ± 1.15B	1 ± 0.5 A
	10	9	10.0 ± 0.14C	0 ± 0.0 A
	5	32	5.5 ± 0.11 D	0 ± 0.0 A
	3	40	3.0 ± 0.25 D	0 ± 0.0 A
	Control	40	74.5 ± 0.91A	0 ± 0.0 A
<b>P. crassifolium</b> (Wellington)	initial	0	16.8 ± 0.35B	53 ± 6.7 AB
	10	7	9.8 ± 0.32 C	50 ± 3.4 B
	5	16	5.3 ± 0.14 D	44 ± 7.5 B
	3	34	3.3 ± 0.20 D	36 ± 3.2 B
	Control	34	41.2 ± 1.60A	73 ± 3.6 A

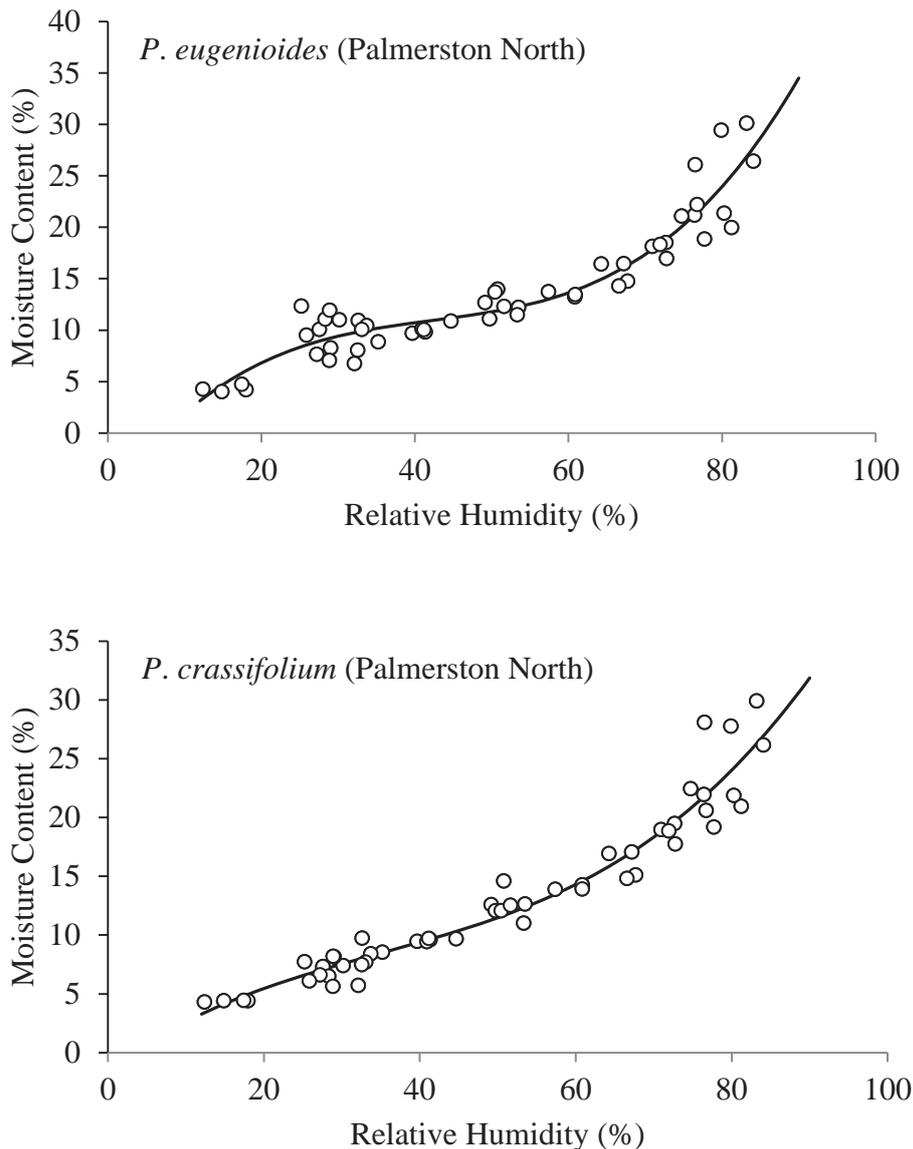
Numbers followed by the same letters within each seed lot and within each column are not significant different (LSD,  $P \leq 0.05$ ) \*Control: seeds stored with moist vermiculite

#### 4.1.3.4 Sorption isotherms for *Pittosporum eugenioides* and *Pittosporum crassifolium*

Sorption isotherms for mature *Pittosporum* seeds collected in Palmerston North were determined on September 17, 2014, seeds collected in Wellington were determined on October 29, 2014; seeds were stored in sealed plastic bags to keep moist. The mean RH in the LiCl containers for both *Pittosporum* species collected from Wellington were at 26.7% to 87.6%; and 28.0% to 80.9% for both *Pittosporum* species collected from Massey University,

Palmerston North. In this relative humidity range the seed moisture content that *P. eugenioides* (collected from Wellington) reached ranged from 4% to 29.2% and 4.3% to 30.3% for *P. crassifolium* seeds. For seeds collected from Palmerston North, moisture contents reached for *P. eugenioides* were 4.3% to 28% and for *P. crassifolium* seeds 4.4% to 28% of (Figure 4.3).





**Figure 4.4** Moisture sorption isotherms for *Pittosporum eugenioides* and *Pittosporum crassifolium* seeds collected from Massey University, Palmerston North; Otari Wilton’s Botanical Garden Wellington and Island Bay Wellington and equilibrated with a range of relative humidities using a range of LiCl solutions at 20 °C.

The percentage viability of *Pittosporum eugenioides* and *Pittosporum crassifolium* seeds from two locations after equilibrating at various RH is presented in Table 4.5. The survival of *Pittosporum* spp. from Palmerston North was maintained, even at low RH of 5% over the silica gel. However, seeds of *Pittosporum* spp. collected from Wellington gradually lost viability with the reduction of RH%, with 59.5% of *P. eugenioides* and 49.0% of *P. crassifolium* with equilibrating over 5% RH, and 91% of *P. eugenioides* and 86.0% of *P. crassifolium* with equilibrating over 95% RH.

**Table 4.5 Changes in viability of *Pitosporum eugenioides* and *Pitosporum crassifolium* seeds *Pitosporum eugenioides* and *Pitosporum crassifolium* at various RH environments.**

Equilibrating with silica gel and weight of LiCl (g)	Target RH (%)at 20 °C	SMC (%) of each species				Germination (%)			
		<i>P. eugenioides</i> (mature, Palmerston North)	<i>P. crassifolium</i> (Palmerston North)	<i>P. eugenioides</i> (mature, Wellington)	<i>P. crassifolium</i> (Wellington)	<i>P. eugenioides</i> (Palmerston North)	<i>P. crassifolium</i> (Palmerston North)	<i>P. eugenioides</i> (Wellington)	<i>P. crassifolium</i> (Wellington)
<b>Silica gel</b>	5	4.3±0.15 H	4.4±0.03 I	4.0±0.29 J	4.3±0.22 I	17±4.9 A	62±6.7 A	60±4.9 B	49±5.3 B
<b>174</b>	11	8.9±1.30 G	6.3±0.48 HI	6.1±0.11 IJ	6.1±0.26 HI	18±3.6 A	72±2.9 A	85±4.9 A	49±4.4 B
<b>147</b>	15	9.7±1.07 FG	7.2±0.40 GH	7.8±0.76 HI	6.3±0.13 H	25±3.5 A	65±5.0 A	91±2.4 A	64±5.9 AB
<b>128</b>	20	9.8±0.57 FG	7.6±0.18 FGH	7.4±0.16 HI	6.8±0.09 H	23±4.7 A	68±6.1 A	88±0.8 A	66±1.2 AB
<b>104</b>	30	10.1±0.27 FG	9.1±0.36 FG	8.7±0.06 GH	7.9±0.06 GH	19±3.5 A	73±5.2 A	91±1.9 A	74±3.5 AB
<b>88</b>	40	10.1±0.27 FG	9.5±0.05 F	10.1±0.06 FG	9.3±0.06 G	22±5.2 A	78±2.2 A	90±2.1 A	77±6.6 A
<b>74</b>	50	12.1±0.58 EFG	11.9±0.32 E	11.7±0.32 EF	11.5±0.41 F	25±4.9 A	82±3.8 A	89±2.2 A	77±4.1 A
<b>60</b>	60	12.9±0.28 EF	13.3±0.43 DE	13.3±0.65 DE	13.2±0.42 F	21±1.7 A	79±5.2 A	94±1.5 A	81±5.3 A
<b>50</b>	70	14.2±0.22 DE	14.6±0.26 D	14.7±0.34 D	15.3±0.41 E	24±6.4 A	80±2.5 A	94±0.5 A	77±5.5 A
<b>34</b>	80	17.0±0.44 CD	17.6±0.44 C	17.7±0.35 C	19.6±0.62 D	24±5.7 A	79±1.9 A	94±1.5 A	78±3.5 A
<b>26</b>	85	19.4±0.93 BC	19.5±0.36 C	20.3±0.72 B	21.7±0.67 C	25±4.0 A	74±2.6 A	96±1.2 A	78±6.0 A
<b>20</b>	90	20.9±0.32 B	21.8±0.31 B	22.3±0.61 B	24.4±0.40 B	22±3.3 A	70±4.2 A	90±2.8 A	79±3.8 A
<b>8</b>	95	28.0±1.03 A	28.0±0.77 A	29.2±0.48 A	30.3±0.29 A	26±3.8 A	73±4.5 A	91±1.3 A	86±3.5 A

Numbers followed by the same letter within each column are not significantly different (LSD,  $P \leq 0.05$ )

#### 4.1.4 Discussion

Seeds of *Pittosporum* spp. from Wellington appear to show some desiccation sensitivity, as the viability reduced when seeds were desiccated to the target moisture content of 3% over the silica gel, but not all viability was lost. This suggests the seed storage behaviour of the two *Pittosporum* spp. evaluated is non-orthodox. The Royal Botanic Gardens Kew Seed Information Database (SID), (2015) suggests that *P. eugenioides* seed is possibly recalcitrant, whereas no data is reported for *P. crassifolium*. The seed storage behaviour of 11 taxa of *Pittosporum* is listed in the seed information database. Of these 8 are suggested as displaying orthodox storage behaviour and 3 recalcitrant. For both *Pittosporum* spp. species evaluated, mature seeds maintained high viability in moist vermiculite at pre-chilling and 20 °C for 14 to 40 days. Seed moisture rose in seed stored in vermiculite suggesting that when the seed was harvested maturation drying had begun. Hong and Ellis (1996) concluded that seed of species from moist areas typically display desiccation sensitivity, especially those from rainforests. The geographic range of *Pittosporum* in New Zealand is in forest and scrub from the coast to subalpine habitats, where relative humidity is high at 70-80 % in coastal habitats. One collecting location in this study was coastal (Wellington) and one inland (Palmerston North). The desiccation sensitivity of the seed lots from the coastal locations appeared to have greater desiccation sensitivity than those from inland suggesting locations may affect the desiccation sensitivity. However, further work needs to be done to confirm this by collecting seeds from a greater number of different locations/different relative humidity environments. No immature *P. eugenioides* seeds, survived desiccation over the silica gel or in moist vermiculite suggesting that the seed when harvested was not sufficiently mature to survive independently of the parent plant. A characteristic of immature seed stored in vermiculite was fungal infection but this was also a problem, in mature seed. Fungi and bacteria generally parasitize newly harvested non-orthodox seeds, which results in a decline in viability during storage (Park, 2012). Other researches also reported the phenomenon of fungal infection reducing the seed viability during the storage trials (Mycock & Berjak, 1990).

Seeds of *Pittosporum* spp. showed delayed germination. The mechanisms for overcoming these delays are ecologically significant as they enable the seeds, interacting with those factors that overcome these delays, to exert some control over the time of germination. Burrows (1989) investigated several New Zealand native and introduced flora species to illustrate the patterns of delayed germination, four main classes of delay were proposed: (1) physical inhibition (thick seed coat); (2) environmental inhibition (particular environmental

requirements for germination); (3) immature embryos; and (4) seed dormancy (biochemical blocking). Seeds of *P. eugenioides* as well as *P. tenuifolium* do not germinate on the mother plant, and remain on the plant becoming fully mature. Seeds are produced within a fruit. The fruit wall may prolong the delay of germination. Burrows (1996) successfully germinated *P. eugenioides* seeds with 65-90% germination achieved in a series of dark treatments and on soil, but for seeds still in the fruit only 0-20% of seed germinated. Seeds of both species in this study were pre-treated by cold stratification at 5 °C for 2 weeks before germination at 20°C but germination did not occur at 35 days. After that, the tetrazolium test was applied to assess viability of un-germinated seed. The most likely reason that stratification treatments did not alleviate dormancy in *Pittosporum* seeds is the limited time of the pre-chill treatment. Moore et al. (1994) reported that 8 weeks and 12 weeks of the cold stratification treatments resulted in almost complete germination seeds of *P. eugenioides* with 93% and 100%, but neither stratified nor scarified seeds of *P. crassifolium* germinated (Moore, 1991); tetrazolium tests were also conducted on un-germinated seeds to assess viability. However, there no information in the literature to indicate the causes of the delay in germination in *P. crassifolium* seeds.

Equilibrating or maintaining seeds at specific moisture levels is always used for comparative longevity studies, desiccation sensitivity testing, and seed/fruit ripening in seed banks (Gold & Hay, 2014). In this study, equilibrating *Pittosporum* seeds at several specific moisture levels was used to produce seed sorption isotherms to describe the relationship between moisture content in the seed and ambient relative humidity. Atkins (1982) suggested that the initial moisture content and temperature of seeds affect the subsequent shape of sorption isotherm. The moisture content of *Pittosporum* seeds had a direct response to ambient RH, which gradually increased with exposure to an increasing RH environment (Figure 4.4). Similar shaped curves of water sorption isotherm were reported for tomato seeds (Sogi et al., 2003) and lentil seeds (Menkov, 2000), but sorption isotherms were obtained at various temperatures.

In the sorption isotherm study, there is also some loss of viability in both *P. eugenioides* and *P. crassifolium* from Wellington between 4-10% seed moisture content, but again not all viability is lost. However, the very low initial viability in the *P. eugenioides* from Palmerston North, seems to be an outlier compared with all the other results (including the dessication for this seed lot). The reason for this is unknown.

Germination still did not occur after cold stratification treatments (21 days) in the sorption isotherm study; the tetrazolium test was also again used to assess viability of un-germinated seeds. The limited time of the pre-chilling treatment before germination is again likely to be the reason for the low germination.

## 5 CONCLUSIONS

### 5.1 Overview of main outcomes

The objective of this research was to determine seed storage behaviour and seed coat characteristics in selected New Zealand indigenous species. The outcomes of this research makes a contribution to the development of seed conservation strategies for seed of species in the New Zealand flora. The threatened species of *Clianthus* spp. and *Carmichaelia muritai* as well as the non-threatened *Pittosporum eugenioides* and *Pittosporum crassifolium* were selected for this seed conservation study, because of the potential of all species for extinction, incomplete seed conservation information and the availability of seed.

The main outcomes of this research were:

- Seeds of *Clianthus maximus*, *Clianthus puniceus*, and *Carmichaelia muritai* were desiccation tolerant to low moisture content of 2.5%, 2.3%, and 2.8%, respectively suggesting the storage behaviour of these seeds is orthodox. Therefore, these species could survive conventional seed storage approaches as suggested by Park et al. (2010).
- Seeds of *Clianthus maximus*, *Clianthus puniceus*, and around 50% seeds of *Carmichaelia muritai* did not imbibe water without scarification; however, manual scarification allowed seed imbibition to occur rapidly, and germination to follow.
- Seeds of *Clianthus maximus*, *Clianthus puniceus*, and *Carmichaelia muritai* lost water impermeability when dried to moisture contents of 5.6%, 5.5%, 5.9% and 2.5%, 2.3%, 2.8%, respectively.
- The loss of water impermeability in *Clianthus maximus* and *Clianthus puniceus* seeds, was a result of cracking to the seed coat surface, in particular in the extrahilar and lens area of seeds at 5% SMC, in the extrahilar, hilum and lens area of seeds at 2.5% SMC. SMC appears to be affecting the location of the cracking with cracking to the hilum only observed at very low (2.5%) seed moisture.
- The loss of some viability during the desiccation of *Pittosporum eugenioides* and *Pittosporum crassifolium* seeds suggests that these species are non-orthodox in their seed storage behaviour.
- The moisture sorption isotherm for *Pittosporum eugenioides* and *Pittosporum crassifolium* seeds in specific relative humidity level were developed.

## 5.2 Future research studies

These outcomes suggest a number of areas for future research:

- (1) Further investigations of the seed coat features of other *Carmichaelia* spp. is required to improve the understanding of the conservation strategy needed and if damage to the seed coat is occurring at low seed moisture environments.
- (2) Long term storage of *Carmichaelia* spp. seeds at low moisture content (2.5%, 5% and 10%) and various temperatures (-20 °C, -5 °C, 5 °C, 20 °C and 40 °C) environments for timespans such as 6 months, 9 months, 12 months, 2 years, 5 years and 10 years are needed to determine if loss of seed coat impermeability is reducing storage life.
- (3) For *Pittosporum eugenioides* and *Pittosporum crassifolium* seeds, further research on seeds from different locations at different RH levels, and determination of the storage behaviour in other *Pittosporum* species is needed.
- (4) The mechanisms for the delay in germination of *Pittosporum eugenioides* and *Pittosporum crassifolium* seeds needs to be determined, and protocols for alleviating dormancy developed.

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