The seed ecology of

Lebeckia ambigua

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I acknowledge that this research was conducted on the traditional lands of the Whajuk Noongar people. I recognise the strength, resilience and capacity of Aboriginal people in this land and pay respects to all Noongar people and Elders both past and present.

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Abstract

Lebeckia ambigua is a perennial legume native to the heathlands of the Fynbos biome in the Western Cape region of South Africa. The species has been identified as a potential agricultural cultivar for the south west of Western Australia, where the edaphic conditions are similar and equally challenging. The species is very fecund and the seed produced has a very high percentage of hard seed dormancy (88-99%). This seed ecology needs to be understood in the domestication of the species.

Successful integration of *L. ambigua* into any agricultural system will require an understanding of the species-specific nature of its hard seed dormancy, and the environmental and artificially imposed triggers required to break it. This will ensure that enough seed germinates to successfully establish the legume in new sowings. This was the main focus of the research presented here.

A germination level of 68% of the seed was achieved in response to optimisation of a physical scarification method. Further, *L. ambigua* seeds displayed a high tolerance to applications of moist heat, and thus the best overall germination (73%) was encountered in response to simulated fire experiments.

However when *L. ambigua* seeds were left in the field, exposed to normal environmental conditions, less then 10% of seed softened to become germinable in the first winter (24 weeks of exposure), and only an additional 30% had softened by the following winter (57 weeks of exposure). Seed was originally collected from geographically separated and discrete populations of *L. ambigua* in South Africa.

Substantial variation was also detected in the occurrence and breakdown of hard seed between provenances. This provides an opportunity for further selection of desired seed traits during the domestication of the species.

These results are quite unique when set against the most common perennial legumes currently utilised in agriculture. Instead the hard seed dynamics discovered in *L. ambigua* are more comparable to the annual legumes used in ley farming systems. This potentially provides an opportunity to integrate a perennial legume phase in a uniquely flexible way, by exploiting the hard seed traits of the new legume. It may be possible to regenerate stands from hard seed banks in the same way as applied in conventional annual ley farming systems, and this would be revolutionary when applied to a perennial species.

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Section 1 General review of Literature

1.1Introduction

1.1.1 The importance of seed ecology studies

The first recorded studies into seed germination ecology were conducted by the Greek philosopher Theophrastus (372-287 BCE)(Baskin and Baskin, 2001). Attempts to describe the methods by which germination is controlled in many different plant species have followed. Individual species have a distinctive seed phenology as a consequence of their ecological and evolutionary origins. Seeds within a population display different responses to their environments depending on a combination of their genetics, the environment of their maternal plant and the environment the seed has been subjected to (Baskin and Baskin, 2001). In many cases the successful domestication of new agricultural cultivars has been reliant on a good understanding of the physiology and behavior of the seed.

Seed dormancy imposed by a water impermeable hard seed coat is the main mechanism that regulates germination in the Fabaceae family (Rolston, 1978). Almost all agriculturally domesticated legume species are descended from wild ancestors with high levels of hard seed and asynchronous timing of germination (Williams and Elliott, 1960).

High seed costs and poor germination are major constraints to the adoption of new agricultural species (Dear and Ewing, 2008, Boersma *et al.*, 2007). In some agricultural systems a high proportion of dormant seed is undesirable; normally high germination rates are required to rapidly establish a uniform crop (Boersma *et al.*, 2007). However in some cases, high levels of hard seed impermeability is desirable (Chapman and

Asseng, 2001). Ley farming systems, for example, rely on the regeneration of pasture legume species from seed "banks" that persist under a year or more of cropping (Howieson *et al.*, 2000). Producing a bank of hard seed, which is resilient to premature softening, is therefore required to ensure optimal productivity in these systems (Chapman and Asseng, 2001).

When there is a need to develop new agricultural cultivars, understanding the specific dynamics of the reproductive system is essential to best integrate the plant into an agricultural system. This is discussed in further detail in section 1.3.

1.1.2 Legumes, ley farming and soil fertility

The agricultural systems of southern Australia are considered some of the most advanced low input systems in the world (Keating and Carberry, 2010). Their success is built on the productivity and dominance of pasture legumes grown in ley farming systems (Nichols *et al.*, 2007). The majority of the region is characterized by poor soil fertility and low annual rainfall (Howieson *et al.*, 2000). This provided an enormous challenge to early European settlers attempting to achieve adequate production through conventional agricultural practices. Despite intermittent advances in farming technology and machinery through the 18th and 19th century, Puckridge and French (1983) believe that crop yields did not increase from those of the first European settlers until the implementation of ley farming in the 20th century.

The ley farming system involves rotating between crop and pasture phases. The pasture phase is usually a mixed sward comprised of self regenerating annual plants including legumes (Bathgate and Pannell, 2002). The pasture phase increases the soil

fertility and impedes the build up of pests and diseases that occur in the crop phase, increasing subsequent crop yields (Loi *et al.*, 2005). Animals can also graze the pasture, providing meat and wool while simultaneously helping to control weeds through eating them (Loi *et al.*, 2005).

Because of these benefits, when managed appropriately, ley farming systems can also reduce the reliance on agrichemicals such as pesticides and herbicides within a cropping sequence (Hochman *et al.*, 2013). It is widely believed that the use of these chemicals has reached unsustainable levels leading to an increase in frequency of herbicide resistant weeds, which further compromises productivity (Hochman *et al.*, 2013).

Legumes are central to maintaining the productivity of the pasture phase of the ley farming system (Bathgate and Pannell, 2002). Their ability to assimilate atmospheric nitrogen through symbiotic association with rhizobia enables them to replace nitrogen exported through cropping (Nutt, 2012) and provides higher protein, higher quality feed for animal enterprises (Li *et al.*, 2008). The legume-rhizobia symbiosis is a fundamental system for the health and function of global terrestrial ecosystems (Herridge *et al.*, 2008) and is considered second only to photosynthesis as the most important biochemical process on earth (Lindstrom *et al.*, 2010). Currently it is estimated that legumes are present in 93% of improved pastures in southern Australia (Peoples and Baldock, 2001) and produce around 80% of the nitrogen found in Australian grain (Herridge and Howieson, 2005).

It is estimated that Australia saves 3 billion dollars AUD annually on fertiliser expenses by utilizing symbiotic nitrogen fixation (Herridge and Howieson, 2005) and 40-70

million metric tons of nitrogen is provided globally (Sessitsch *et al.*, 2002). Reducing the need for industrially produced nitrogen fertilisers is of paramount importance to address the major issues of green house gas emission, fossil fuel depletion, rising cost of fertilisers and nitrogen leaching which threaten the sustainability of current agriculture (Revell *et al.*, 2013). A reduction is most obviously achieved by expanding the use of legumes and increasing the efficiency of their ability to biologically fix nitrogen that could in turn increase their adoption.

1.1.2.1 The need for new perennial cultivars

Despite the historical success of ley farming in southern Australia there are vulnerabilities that remain and constrain the sustainability of the system (Loi *et al.*, 2005). The major issues that have been highlighted by a number of past reviews are summarised in Table 1.1. Figure 1.1 demonstrates how each of these issues can be considered part of a negative feedback loop leading to a loss of production and profitability of both animal and crop operations. Figure 1.1 also illustrates how all the issues stem from two antecedent problems: a small suite of adapted annual legumes and no suitably adapted perennial legume options. Other factors such as declining terms of trade and changing rainfall patterns are also major contributing factors (Howieson *et al.*, 2000). However these precursory factors are not considered in the scope of this model as they are outside the direct control of researchers.

A sustained research and development effort over the past 20 years has domesticated a number of new annual pasture legume options for southern Australian farming systems and these are having remarkable impact (Nichols *et al.*, 2007).

However the lack of productive perennial legumes adapted to the low rainfall areas of southern Australia is potentially the biggest obstacle to the economic and environmental sustainability of the region's agriculture. Perennial legumes can utilise water over summer through deep roots in a manner that more closely resembles the original flora (Hobbs *et al.*, 1995). Thus they can conceivably restore the hydrological balance of this vast region, aiding in the reduction of dryland salinity (Caccetta *et al.*, 2010). Additionally, because the perennial plants continue to grow over the summer months, forage maybe provided for livestock during the key feed gap, greatly diminishing the input costs to animal enterprises (Byrne *et al.*, 2010).

Perennial species are successful in higher rainfall regions where a number of grasses and legumes are edaphically suited (Bolger *et al.*, 2005). However despite consensus on the importance of perennial legumes in agriculture and a recent considerable research effort, there still remains no widely suitable or economically viable perennial legume cultivar for the low rainfall regions of southern Australia (Monjardino *et al.*, 2010).

Table 1.1. A summary of the major issues identified in the southern Australian agricultural region from recent reviews. (*) Indicates that the corresponding issue was raised by the review.

Major issues	*Bathgate and Pannell (2002)*	*Brock* et al. *(2013)*	*Cocks (2001)*	*Dear and Ewing (2008)*	*Dear* et al. *(2003)*	*Hochman* et al. *(2013)*	*Howieson* et al. *(2000)*	*Loi* et al. *(2005)*	*(Moore* et al.*, 2009)*	*Nichols* et al. *(2007)*	*Robinson* et al. *(2007)*	*Suriyagoda* et al. *(2013)*
1.Feed gap for stock			*		*				*		*	*
2.Low biodiversity					*	*	*	*		*		
3.Increased use of Nitrogen fertilizer		*				*						
4.Over use of Agri-chemicals						*		*		*		
5.Inorganic waste runoff		*				*				*		
6.Herbicide and pesticide resistance						*	*	*		*		
7.Salinity	*		*	*	*		*	*		*	*	*

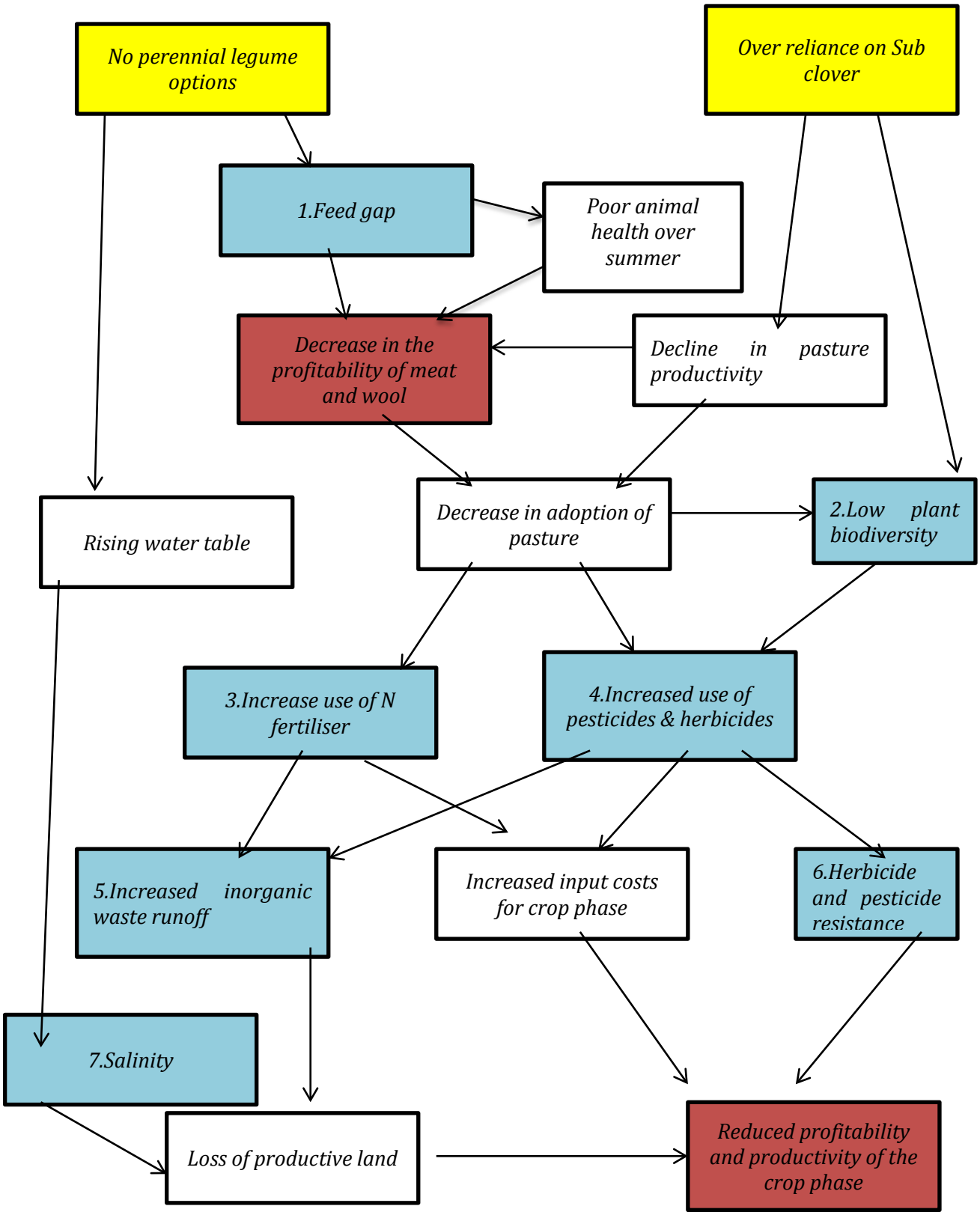

Figure 1.1. A demonstration of how each issue is related: Blue; An issue commonly raised in reviews, Yellow: An issue which can be addressed, Red; An outcome

1.1.3 The Fynbos and the Wheatbelt

The Wheatbelt is an iconic agricultural region of southern Australia (ABARE, 2014). The area has a well defined Mediterranean-type climate with the rain falling mostly in winter, and the summers typically hot and dry with plant growth severely limited by the lack of surface soil moisture in this period (Hobbs *et al.*, 1995). Australian native plants are typically unsuitable for use in agriculture in this region (Cocks, 2001). Because of this, Australian agriculture has a long and successful history of introducing plants from similar Mediterranean-type bioclimatic environments (Howieson *et al.*, 2008). Along with the southwest of Australia there are four other regions of the world with Mediterranean-type climates: The Chilean coast, the Mediterranean basin, the Western Cape of South Africa and the Pacific coast of North America (Reinten and Coetzee, 2002). The Mediterranean basin in the northern hemisphere has been the predominant source of germplasm for agricultural cultivars since antiquity (Howieson *et al.*, 2000). Many northern hemisphere agricultural plants have been intensively selected *in situ* for agriculture over a very long time and subsequently are very well adapted to intensive grazing and cultivation (Dear and Ewing, 2008). The perennial legumes of the Mediterranean basin are more frequently encountered in the wetter areas, and it seems that much of the Wheatbelt is not edaphically suitable for them (Cocks, 2001). Development of a new perennial cultivar requires the discovery of a species naturally adapted to nutrient depleted soils and climate similar to that of the Wheatbelt (Cocks, 2001).

Since European settlement sixty four percent of the native vegetation of the Wheatbelt has been cleared for mixed farming enterprises (predominantly wheat and sheep) and the region contributes $4.5 billion a year annually to the agricultural output of

Australia (ABARE, 2014). The remaining native vegetative communities of the south west of Western Australia are unique with a very diverse range of endemic plant species (Beard *et al.*, 2000). The Kwongan heathlands are a vegetative community that consists of sclerophyllous shrubs which grow on the sandy soils with the lowest fertility (Beard *et al.*, 2000). Kwongan heathland remnants are found throughout the Wheatbelt region (Figure 1.2). The Kwongan heathlands have very high levels of species richness and endemism, and are considered a hotspot of floral biodiversity (Beard *et al.*, 2000).

Several ecological reviews (Wisheu *et al.*, 2000, Hobbs *et al.*, 1995, Goldblatt, 1997) have compared the environments of the Mediterranean-type bioclimates and concluded that the biome that most closely resembles the Kwongan heathlands in terms of soils, disturbance regimes and vegetation composition is the Fynbos . The Fynbos biome is a belt within the Western Cape of South Africa (Reinten and Coetzee, 2002). The Fynbos also exhibits rich botanical diversity, and in particular the biome boasts a very large diversity of perennial legumes, offering a valuable resource for investigation into new species to domesticate (Sprent *et al.*, 2010) .

The soils of both regions are very old and come from similar parental material (Hobbs *et al.*, 1995). Processes of weathering have yielded coarse-grained sand soils, with very little clay content and a lack of water holding capacity (Goldblatt, 1997). Both the soils are also relatively poor in nutrients compared to the other Mediterranean-type regions (Wisheu *et al.*, 2000). The low availability of nutrients, particularly phosphorous, is limiting to plant growth and significant in the type of vegetation that can be supported (Goldblatt, 1997).

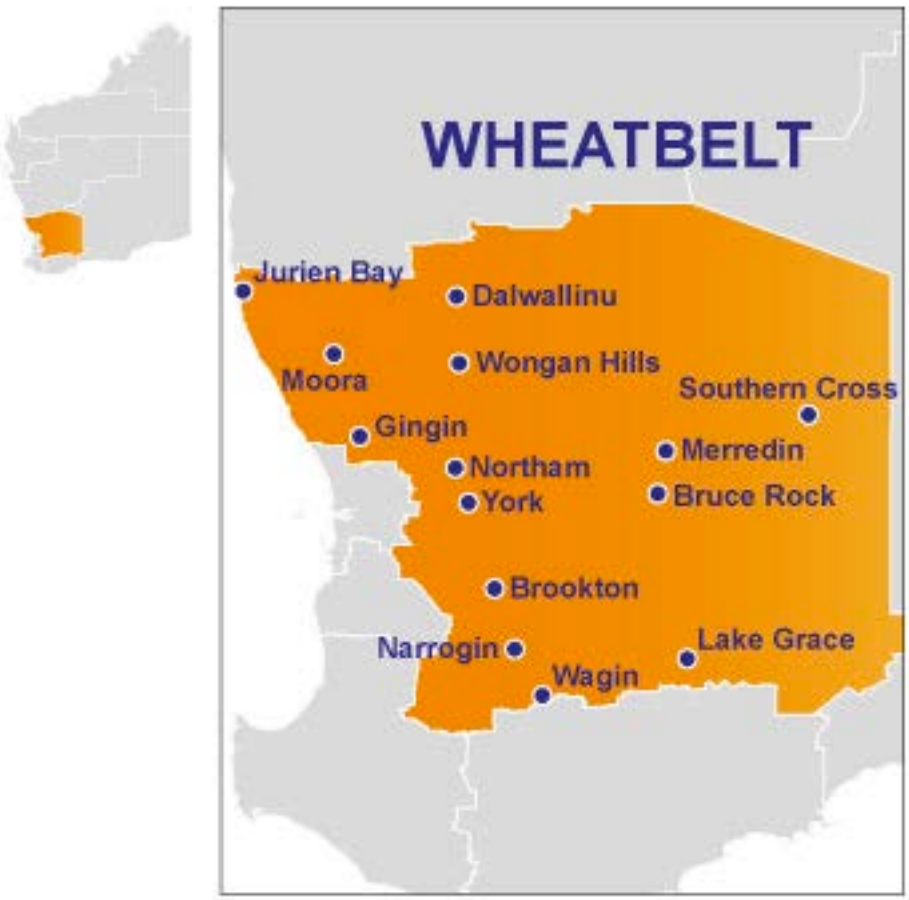

Figure 1.2. The Wheatbelt of the south west of Western Australia, Department of regional developement (2014).

The similar challenge posed by both soils is considered the major reason that the floral communities of the Fynbos and the Kwongan are comparable (Wisheu *et al.*, 2000). The remarkably low nutrient concentration constrains the growth of the vegetation, and sclerophyllous shrubs are the principal life form for both regions (Wisheu *et al.*, 2000). Extensive and deep root systems are also required to access water and nutrients on the freely draining soils, so generally only plants with large tap roots and specialised structures are able to thrive (Hobbs *et al.*, 1995).

1.1.3.1 Fire and grazing disturbance ecology

Fire is a disturbance that is routinely present in all of the Mediterranean-type biomes. The dichotomy of the weather favours sclerophylous and semi deciduous vegetation that can conserve water during the summer months (Moreno and Oechel, 1994). This combination inevitably leads to an accumulation of highly flammable material and eventually a fire will spark during a dry period (Pausas, 1999). The vegetation in these regions has evolved to thrive under this disturbance (Rutherford *et al.*, 2011). The frequency and intensity of the fires has a very big influence on the vegetative composition and life history (Pierce and Cowling, 1991). The Fynbos and Kwongan have much shorter intervals between fire disturbances than the other Mediterranean-type regions (Wisheu *et al.*, 2000). This could be a significant factor in accounting for the differences these biomes have to other regions and the similarity they have to each other (Wisheu *et al.*, 2000).

One manner in which the Kwongan and Fynbos do differ is in their interaction with grazing animals. Many native Australian legumes contain toxins that deter grazing by animals (Dowling and McKenzie, 1993). *Gastrolobium*, *Gomphelobium* and *Acacia* are common genera in WA and species within these genera naturally produce sodium monofluoroacetate (Short *et al.*, 2005). Native animals have co-evolved with these species, developing a resistance to the compound (Short *et al.*, 2005). The compound is lethal to introduced animals, including agricultural species, and therefore plants that produce sodium monofluoroacetate are completely unsuitable for grazing (Cocks, 2001). In contrast a large diversity of ruminants and browsers, both native and introduced, have grazed the Fynbos vegetation (Radloff *et al.*, 2014). Though it has been poorly studied, herbivory has had an important influence on the vegetation

structure and development of the Fynbos through its evolution (Kraaij and Novellie, 2010). Freegrazing by agricultural ruminants introduced to the fynbos in current times provides a high intensity of grazing pressure on the native vegetation without adverse effects to livestock health (Radloff *et al.*, 2014).Therefore the Fynbos may be an ideal place to search for perennial legume species that are suited to the edaphic niche of the Wheatbelt and are amenable to grazing.

1.2 The ecology of *Lebeckia ambigua*

Lebeckia ambigua is a perennial legume endemic to the Cape Floristic Region (CFR)(le Roux and Van Wyk, 2007). *L. ambigua* was identified as a potential candidate for domestication in the agricultural systems of southern Australia during reconnaissance expeditions to the Western Cape of South Africa between 2002-2007 (Howieson *et al.*, 2008). The sites targeted for collection were within the Fynbos biome characterised as having very poor fertility and an annual rainfall of less than 300ml. These challenges are congruent to those encountered in the low rainfall areas of the Western Australian Wheatbelt.

1.2.1 The evolution and diversity of the *Lebeckia* genus

The generic concept of *Lebeckia* as defined by Bentham (1844) and Harvey (1862) described 36 species precinctive to the Cape floristic region (Boatwright *et al.*, 2009). The Genus was not re-examined in any depth until Boatwright et al (2009) 147 years later, partially as a result of the interest from Australian researchers in exploiting the

genus for agriculture. In this study molecular evidence combined with morphological and anatomical data revealed the genus to be polyphyletic. The group was subsequently redefined to contain only 14 species (*Lebeckia* strictus) with other species re-assigned to the reinstated *Calobota* genera and the new genera *Wiborgiella*.

Lebeckia strictus is placed within the Crotalarieae tribe based on the presence of an open androcieal sheath (Boatwright *et al.*, 2009). Crotalarieae is the largest tribe of papilionoids in Africa containing over 1200 species (Boatwright *et al.*, 2008). Crotalarieae contributes over 50% of the diversity of the Genistoid alliance, the major radiation of Fabacea in the southern hemisphere (Boatwright *et al.*, 2008). The Crotalarieae tribe is currently believed to be monophyletic and this is well supported by molecular, morphological and chemical data (Le Roux *et al.*, 2011). Recent molecular studies show the tribe can be divided into 4 major lineages with *Lebeckia* forming part of the cape clade along with the newly defined *Wiborgiela, Calobota and Aspalathus* (Figure 1.3). The *Lebeckia* genus strictus is morphologically distinguished from the rest of the former broader generic concept by their phyllodinus, acicular leaves and their 5+5 anther arrangments (le Roux and Van Wyk, 2009). However it is easy to confuse species within the group and they are commonly misidentified (le Roux and Van Wyk, 2007). *L. ambigua* is most readily differentiated from the other species in the genera by the inflorescence arrangement (Figure 1.4). All species have a terminal multi flowered raceme but *L. ambigua* can be distinguished by the fact that they branch into 2-3 lateral racemes which also have a terminal inflorescence.

A great variety of fruit structure is displayed throughout the Crotalaria tribe (Boatwright *et al.*, 2009).The pods of *L. ambigua* are very slender, comparatively long and are dehiscent with thin walls (le Roux and Van Wyk, 2009). Six or more small

oblong seeds are produced per pod (Boatwright *et al.*, 2009). Large variation is seen between collected individuals for pods and seeds; this is suspected to be an indicator of provincial speciation. This is discussed further in section 2.

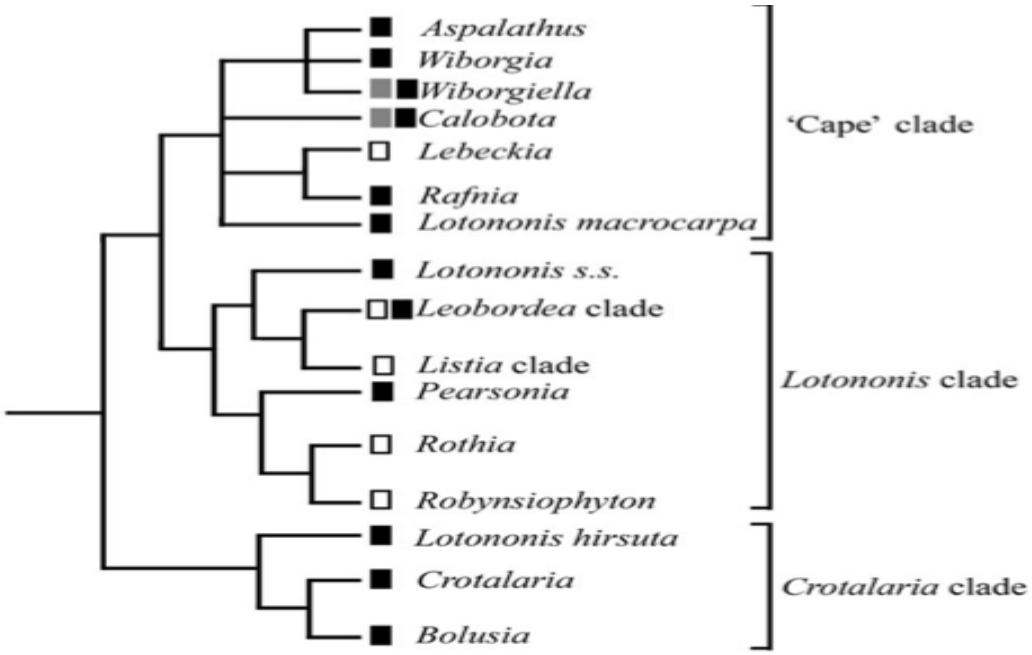

Figure 1.3. The 4 major lineages of the tribe Crotalarieae (Le Roux et al., 2011).

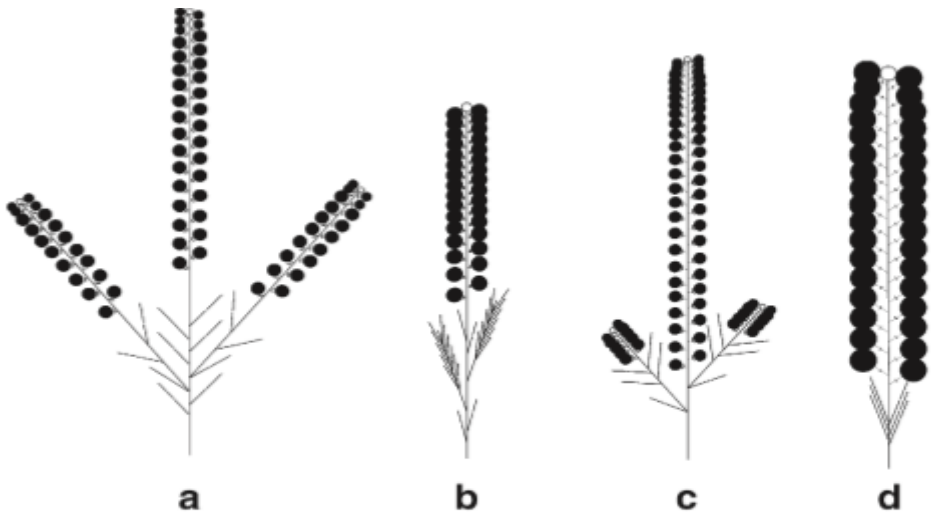

Figure 1.4. Inflorescence structure of Lebeckia species a) L. ambigua b) L. brevicarpa c) L gracilis d) L. sepiaria (le Roux and Van Wyk, 2007).

1.2.2 The distribution and edaphic adaptations of *Lebeckia ambigua*

L. ambigua is distributed throughout the coastal regions of the Western Cape province (Figure 1.5) and demonstrates a ruderal habit frequently colonising disturbed areas such as roadsides and on deep sandy soils (le Roux and Van Wyk, 2007). *L. ambigua* has been encountered at an altitude as high as 900m but is more typically found in low-lying regions (le Roux and Van Wyk, 2007). The soil pH of collection sites in the expeditions of 2002-2007 ranged from 5.5-6.5.

Survival on deep sandy soils is likely to be dependent on the ability to produce a long woody taproot. This enables the plant to reach the water table, which commonly accumulates at depths beneath the freely draining sands (Lefroy *et al.*, 2001). The physiology and architecture of the root system of *L. ambigua* has yet to be studied in any great detail but as water is a key limiting resource and the soils hold very little water, access to the water table is required to thrive in these conditions (Hobbs *et al.*, 1995).

Along with access to water, the conservation of water within the plant is of equal importance to success in a region with very high solar radiation (Hobbs *et al.*, 1995). *L. ambigua* has phyllodinus acicular leaves (needle shaped) (le Roux and Van Wyk, 2009), which have a very low surface area to volume ratio.

This adaptation strongly limits the amount of water lost through transpiration. Mucilage cells are also present in the leaves of *L. ambigua* (Boatwright *et al.*, 2009). These cells are often associated with plants from Mediterranean-type climates and are thought to increase water storage and reduce transpiration (Boatwright *et al.*, 2009). Curiously the leaves also lack layers of parenchyma cells instead forming complete

circles of palisade cells (Boatwright *et al.*, 2009). Parenchyma cells aid transport of water and gases (Raven *et al.*, 2013); whether their exclusion is an adaptation to drought is not known.

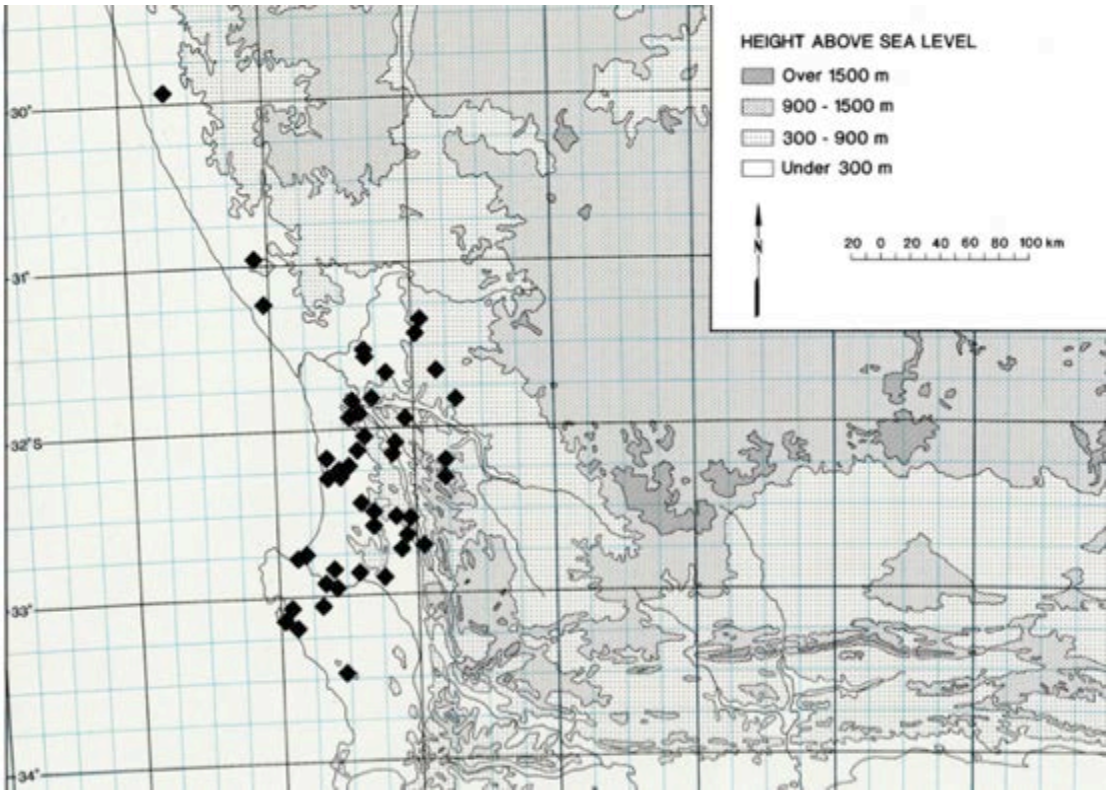

Figure 1.5. The known distribution of L. ambigua in the western cape region of South Africa. Locations from which L. ambigua has been sampled are indicated by (•) (le Roux and Van Wyk, 2007)

L. ambigua develops cluster roots (Figure 1.6) which arise when a portion of the lateral root becomes highly branched, forming many closely spaced, hairy roots (Raven *et al.*, 2013). These specialised root zones are able to release exudates that solubilise immobile nutrients and increased surface area, both of which improve the plant's efficiency of nutrient acquisition (Lambers *et al.*, 2006). Phosphorous appears to be the most influential nutrient on initiation and regulation of cluster roots, though the genetic and environmental controls are still poorly understood (Lambers *et al.*, 2006).

Figure 1.6: Cluster root formation on L. ambigua, excavated from field experiment in Tincurrin Western Australia. Photo of courtesy of Miss Sofie De Meyer, Centre for Rhizobium Studies Murdoch University.

Phosphorous is a very limited resource within the soils of both the Fynbos and Kwongan heathlands and this adaptation is prevalent within the native plant communities of these regions (Raven *et al.*, 2013). In particular, the Proteaceae family is a very prominent member of both plant communities and the cluster root adaptation is almost ubiquitous for all of its 1600 species (Lambers *et al.*, 2006). Cluster roots have previously been discovered in a number of other plant families including Fabaceae (Hocking and Jeffery, 2004).

Cluster roots are thought to occur most commonly on species that do not form arbuscular mycorrhizal associations (Lambers *et al.*, 2006). However the majority of information on this evolutionary novelty has been compiled from studies on a very narrow suite of Proteaceae species and the Fabaceae species *Lupinus albus* (Hocking

and Jeffery, 2004). As the knowledge of cluster roots increases, more species which form these specialised roots are identified and the understanding of the diversity of physiology and function is augmented (Lambers *et al.*, 2006). Although no mycorrhizal symbiotic associations have been detected on *L. ambigua* to date, this does not necessarily mean that the species is precluded from forming these symbioses. The specific physiology and root architecture of *L. ambigua* needs to be investigated further to improve the understanding of the requirements and ecological contributions of the species.

1.2.3 Rhizobial symbiosis

Nitrogen is an essential macronutrient for plant growth and in many environments it is the most limiting (Bengtsson *et al.*, 2011). The major contribution of accessible nitrogen to terrestrial ecosystems is through the legume-rhizobia symbiosis (Bontempts, 2009). A suitable strain of rhizobia is able to infect the root tissue of a legume and form specialised symbiotic organs termed nodules (Allen and Allen, 1981). Once the nodule is formed, the host plant provides the rhizobia with carbon and other elements required for metabolism; in exchange the plant receives nitrogen (N) in the form of ammonia (Udvardi and Poole, 2013). The direct access to plant available nitrogen through symbiosis confers an advantage on legumes in low nitrogen environments and infertile soils (Thrall *et al.*, 2007). In this way legumes can exist as pioneer species, colonizing the poorer soils then improving fertility and subsequently their biodiversity (Ampomah *et al.*, 2012).

The ability to form symbiosis with rhizobia is almost ubiquitously displayed within the

Fabacea family and is considered one their most distinguising traits (Gyaneshwar *et al.*, 2011). A wide diversity is displayed across nodulating plants for how the symbiosis is formed, with which bacteria it can partner, and the conditions required for the symbiosis to be effective. Information on the nature of the symbiotic relationships of a legume is very important to increasing our understanding of their ecology (Gyaneshwar *et al.*, 2011) and is integral to developing and managing the species in an agricultural context (Howieson and Ballard, 2004).

During the plant collecting expeditions in South Africa nodules found on *L. ambigua* were also collected and isolated (Howieson *et al.*, 2013). Howieson *et al* (2013) provides a comprehensive report on the phylogeny, morphology and nitrogen fixation effectiveness for all the strains that were isolated and authenticated.

Nodule phenotypes appear to be a synamorphic characteristic and therefore an important source of information for increasing our understanding on the evolution and ecology of legume species (Gyaneshwar *et al.*, 2011). *L. ambigua* forms crotaloid indeterminate nodules (Figure 1.7) (Howieson *et al.*, 2013). The mode of infection is not yet identified but nodules are able to form on both the lateral and tap roots (Howieson *et al.*, 2013). Field observations from both Australia and the native Fynbos show that *L. ambigua* appears to be a "reluctant" nodulator, forming very few nodules in the field and often low down on the root systems. This is an interesting observation that could reflect the ecology and life-strategy of the species. It is also important to consider this in agricultural systems, to ensure abundant ecosystem N is able to be provided by the plant.

The legume symbiosis exercises specificity in relation to which rhizobial strains are

able to form effective nodulation (Perret *et al.*, 2000). The specific recognition pathways and molecular exchange in symbiosis is complex and thoroughly reviewed by Oldroyd (2013).It has recently been demonstrated that some legume hosts are able to "select" their preferred symbiont even when their numbers are significantly fewer (Yates *et al.*, 2011). Understanding the host specificity in symbiosis is crucial to optimise N fixation, promote legume growth and therefore increase the benefit of the legume to agriculture (Sessitsch *et al.*, 2002).

L. ambigua forms effective symbiotic associations with rhizobia from the Burkholderia genus (Howieson *et al.*, 2013). So far, five distinct phylogenetic groups within Burkholderia have been identified from Lebeckia nodules collected in the native range (Howieson *et al.*, 2013).

1.2.3.1 Burkholderia

The Burkholderia genus was first described by Yabuuchi *et al* (1992) based on 16s rRNa sequencing, DNA-DNA homologous values and the composition of lipids and fatty acids. The genus is part of the beta class of the proteobacteria (Gyaneshwar *et al.*, 2011). Though several species in the genus were known to be free living nitrogen fixers (diazotrophs) (Elliott *et al.*, 2007a) it was not purported that any species could form symbiotic associations with legumes until Moulin *et al* (2001) demonstrated a strain of *Burkholderia tuberum* could form nodules. Prior to this discovery the ability to nodulate legumes was thought to be confined to the alpha proteobacteria (Parker *et al.*, 2007).

Burkholderia strains have since become recognized as the predominant N fixing nodule occupants of native Brazillian Mimosa species (Bontempts, 2009). The symbiosis

between these two genera appears to be ancient, stable and highly effective suggesting that they may have co-evolved together (Bontempts, 2009) (Gyaneshwar *et al.*, 2011).

Figure 1.7. L. ambigua nodulated by Burkholderia sprentiae (WSM4184). Photo courtesy of Miss Sofie De Meyer Centre for Rhizobium Studies Murdoch University

The Fynbos biome seems also to be a major reservoir of Burkholderia capable of forming symbiotic nodules (nodulating Burkholderia)(Gyaneshwar *et al.*, 2011). Along with Lebeckia, Burkholderia has been reported to effectively nodulate members of the *Rhynchosia* (Garau *et al.*, 2009), *Cyclopia* (Elliott *et al.*, 2007a) and *Asphalathus* (Vandamme *et al.*, 2002) genera, which are papilionoid legumes endemic to the Fynbos region (Elliott *et al.*, 2007b). Exploration of rhizobial Burkholderia endemic to the Fynbos has only recently begun and undoubtedly only a small fraction of the diversity have so far been uncovered (Garau *et al.*, 2009, Elliott *et al.*, 2007a).

Significant differences have been noted between the Brazilian and Fynbos nodulating Burkholderia populations in terms of their core genomes, compatible hosts and host range (Gyaneshwar *et al.*, 2011, Elliott *et al.*, 2007b). The two appeared to be

phylogentically separate and distinct when sequences of their nod genes were analysed (Garau *et al.*, 2009). Therefore it is suggested that there are at least two distinct lineages of rhizobial Burkholderia, having arisen separately from the different genetic centers of Brazil and South Africa (Gyaneshwar *et al.*, 2011).

Based on the range of Fynbos legumes associated with nodulating Burkholderia it seems to be a genus well adapted to the sandy, acid, infertile soils of the Fynbos (Howieson *et al.*, 2013). The ability to persist in the soil is termed saprophytic competence and is a particularly important consideration when introducing legumes to new environments (Howieson *et al.*, 2011). Even though years of co-evolution ensures the legume–rhizobia symbiosis is successful in the native range this may not be the case for the target soils (Lindstrom *et al.*, 2010). In the domestication program for *L. ambigua* an evaluation of the robustness of the Lebeckia–Burkholderia symbiosis in the soils of the Western Australian Wheatbelt, is required, and is subsequently underway.

1.3 Seed dormancy

Seed dormancy is the obstruction to the germination of viable seed under favorable conditions (Leubner, 2014). A number of dormancy mechanisms have been observed across the diversity of angiosperms (Van Staden *et al.*, 1989). These can be broadly described as being either exogenous, endogenous or combined dormancy if methods of both exogenous and endogenous are detected on the same seed (Bewley and Black, 1982).

Exogenous dormancy comprises tissues enclosing the embryo that restrict germination (Nutt, 2012). Alternatively, endogenous dormancy describes situations where

dormancy of the embryo is opposed by the embryo itself (Nutt, 2012). Each type of dormancy can be further divided based on the cause of the dormant state. Exogenous dormancy can be imposed by chemical inhibitors, mechanical structures such as woody growths or though physical means with the presence of a water impermeable seed coat (Baskin and Baskin, 2004). Endogenous dormancy can be classified as physiological, when there is a physiological inhibiting mechanism obstructing germination, morphological when the embryo is underdeveloped, or morphophysiological when both of those methods are simultaneously operating (Baskin and Baskin, 2001).

Physical and physiological dormancy have both been described within the legume family (Baskin and Baskin, 2004). However endogenous physiological dormancy is considered short-lived when it has it has been observed (Rossiter, 1966). A high proportion of seeds from legume species display exogenous physical dormancy (Morrison *et al.*, 1998). This is considered a more persistent characteristic and the main method of germination regulation in the legume family *(Fabacea)* (Morrison *et al.*, 1998, Nutt, 2012).

1.3.1 Hard seed dormancy

An impermeable seed coat is often referred to as "hard seed", which is a type of physical dormancy that regulates germination (Baskin and Baskin, 2001). Fifteen families of Angiosperms have species that display this trait but differences are seen between the families, in the seed coat characteristics, formation and break down (Baskin and Baskin, 2001). In the *Fabacea* family, the seed coat is comprised of a single palisade layer (Baskin and Baskin, 2001). The palisade contains macrosclereid cells and high concentrations of the plant polysaccharide callose. Macrosclereid's are

impermeable to water and callose actively repels water, which in combination render the seed coat impermeable to water (Taylor, 2005, Baskin and Baskin, 2001). To germinate, the seed first needs to imbibe water. Therefore the impermeable seed coat must be breached before germination can proceed (Taylor, 2005).

Hard seeded legumes have a specialized region on the seed coat termed a strophiole (Baskin and Baskin, 2001). A strophiole is adjacent to the hilum and acts like a water valve (Gama-Arachchige *et al.*, 2013). To break the physical dormancy of these legumes, the strophiole must fissure and allow water to travel through the palisade layer and reach the nutrient layer(Baskin and Baskin, 2001).

In natural ecosystems, physical dormancy is environmentally controlled requiring specific environmental cues to make the strophiole fracture, allowing the imbibition of water (Baskin and Baskin, 2001). Heat, moisture levels and scarification from passing through the gut of ruminants are all environmental occurrences that have been recorded as impacting physical dormancy (Cook *et al.*, 2008, Kimura and Islam, 2012, Staden *et al.*, 2000). The environmental signal or combination of signals required appears to be different between species, and reflective of their environment and ecological niche (Leubner, 2014).

Hard seed dormancy can provide a range of ecological advantages and in many natural systems is an important aspect of species fitness (Nutt, 2012). This feature can allow survival through the digestive tract of grazing animals, which in turn can aid in distributing the seed over a greater spatial range (Nutt, 2012). Temporal dispersal can also be aided as the seeds can become viable in different years and competition is reduced between offspring and siblings (Nutt, 2012).

Hard seed dormancy is a common feature in legumes that occur naturally in areas around the world that have a Mediterranean-type climate (Perry *et al.*, 2009). In these systems physical dormancy is most commonly broken by a well ordered, temperature and moisture-dependent physiological process known as "seed softening" (Taylor and Revell, 2002). Taylor (1988) described this process as a 2-stage model, requiring a pre-conditioning stage of a high temperature, then diurnal fluctuations of temperature combined with autumnal moisture to break the seed dormancy. Bare soil surfaces in a Mediterranean-type climate are exposed to very high temperatures (approx. 60°c) and wide fluctuations over summer, providing the first stage of the environmental trigger to break the hard seed dormancy (Taylor and Revell, 2002). Because there is a requirement for a number of fluctuations cycles to transpire and possibly related to an increase in moisture fluctuation substantial softening is generally detected in late autumn (Hanbury *et al.*, 1999). This requirement prevents hard seed from germinating as a result of sporadic rain events in summer. This is important because summer rainfall events are not commonly followed by sufficient timely rainfall to support growth (Loi *et al.*, 1999).

In addition to this, not all hard seeds will germinate in the same year; some will remain dormant but viable for future years (Loi *et al.*, 1999). This provides a reserve seed bank, which can be crucial if there is a poor winter growing season or a major disturbance to growth, and parent plants are unable to set seed that year (Smith *et al.*, 1998, Taylor, 2005).

1.3.2 Inheritance of hard seed and the sources of variation

Hard seed dormancy is shown to be a heritable trait for a number of species (Baskin

and Baskin, 2001). Under identical germination conditions seeds of the same species can display a different germination response and breakdown pattern (Baskin and Baskin, 2001). There is natural variation in the initial presence of a hard seed coat and the pattern of breakdown for any given seed population (Nutt, 2012). This variation is controlled by a combination of genetics and the environment (Rolston, 1978). The factors that influence hard seed are summarised in Figure 1.8. These factors do not operate independently and the relative importance of each factor is difficult to separate (Baskin and Baskin, 2001). Capturing the role inheritance plays in the determination of hard seed requires researchers to have a measure of the environmental influence on the population distribution.

Preconditioning is a phenomenon that was first observed by grain breeders, who noticed that the environment of the parent plant directly influenced the growth of the progeny (Baskin and Baskin, 2001). Nelson *et al* (1970) established that occurrence of hard seed dormancy was particularly influenced by environmental preconditioning.

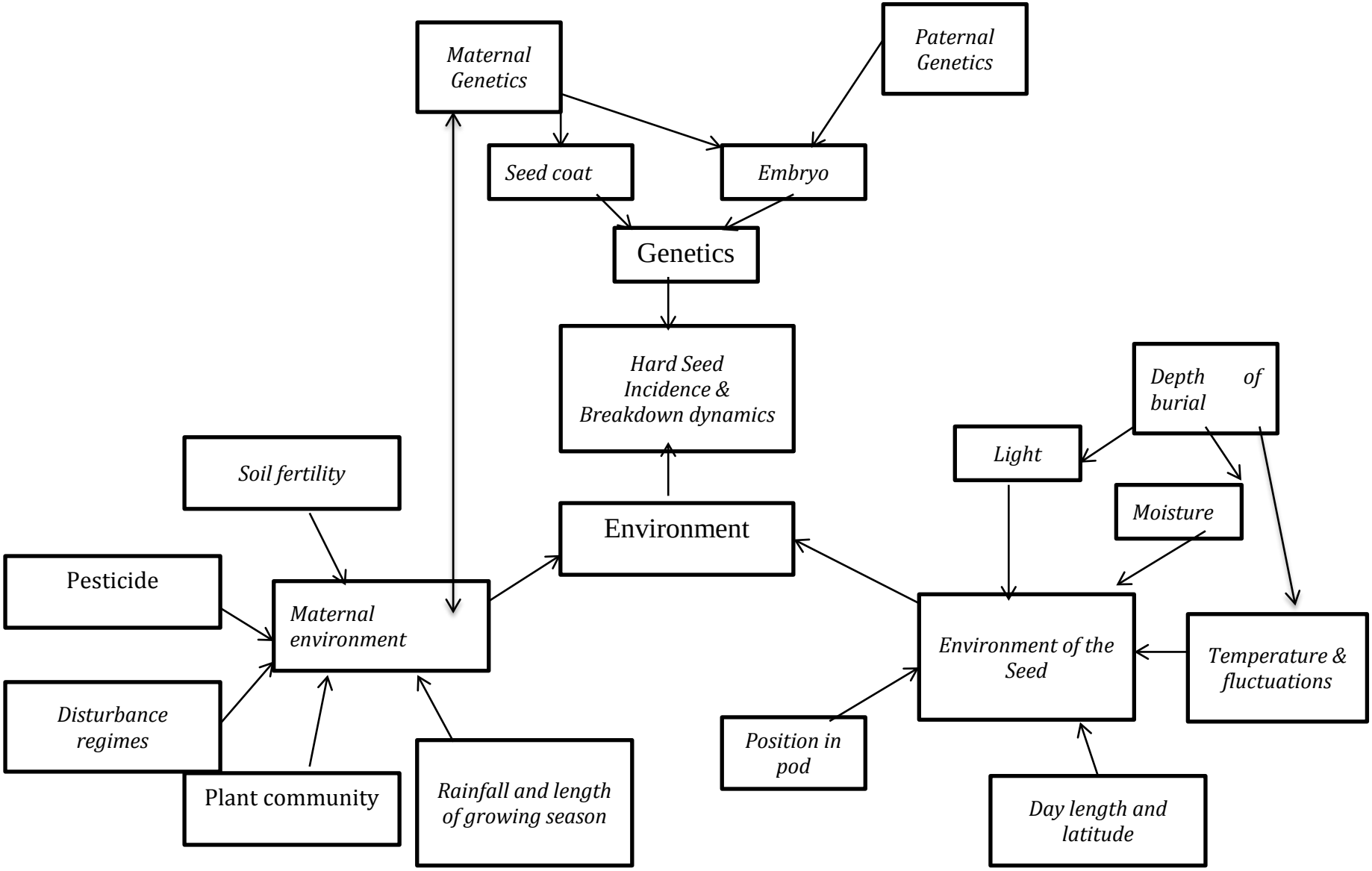

Figure 1.8. Flow chart of the factors that may influence the incidence and breakdown of hard seed and how they interact

It has since been shown that the latitude, elevation, day length, presence of herbicide, length of growing season soil moisture and soil nutrients of the parent plant directly influence the germination dynamics of a number of species (Baskin and Baskin, 2001). The nature of the response and sensitivity to particular factors varies greatly and is specific to the plant species concerned (Morrison *et al.*, 1998).

While it is logical that factors which influence the growth of the plant and the resources available for seed maturation will impact the formation of a hard seed coat, other ways in which the parent plant has been shown to effect this trait are less intuitive. Quinby *et al* (1962) demonstrated that differences in the seed yield of *Triticum aestivum* was influenced by the environmental conditions of the grandparent plants. Therefore an environmental effect beyond the first generation may also influence the incidence of hard seed.

The percentage of hard seed in a population can also be influenced by the ecological interactions of the parent plant. The density and composition of the plant community as well as the presence of fungi and microbes can influence seed characteristics (Baskin and Baskin, 2001). Pierce (1991) believes the intensity and frequency of disturbance regimes such as fire can have a very strong influence on the dynamics of seed populations. The physiological age of the plant can be a factor in determining the levels of hard seededness in the seeds produced (Baskin and Baskin, 2001). Both the number of years a plant has grown and the age at which flowering is induced has been seen to have an effect (Kigel *et al.*, 1979).

Each individual seed may be subjected to an environment that is different prior to collection. Seeds that develop at different positions on the mother plant may also show

differences in their germination requirements (Baskin and Baskin, 2001). Resources are not necessarily allocated to seeds uniformly and individual flowers can mature at different rates as well as on different locations on the plant with slightly different microenvironments (Baskin and Baskin, 2001). Species that develop multi-seeded fruits are commonly identified to have variation as are those which produce multiple inflorescence (Sahai, 1994).

Once the seeds have left the plant, the environment where they gestate can be different and important. In studies this can be controlled by collecting the seeds before they leave the plant but understanding this process can be important in researching seed bank dynamics and regeneration in agricultural systems. Cropping and standard agricultural activity inevitably results in some form of soil disturbance in which seeds can become buried (Taylor and Ewing, 1988). Similarly in a natural ecosystem, wind erosion, trampling and temporary ingestion from browsers can lead to different rates of burial across a seed population (Wisheu *et al.*, 2000). This is particularly true if the natural environment has deep sandy soils (Goldblatt, 1997). The depth of burial can markedly impact the soil temperatures and the fluctuation of the temperatures that the seed are subjected to (Taylor and Ewing, 1988). Loi *et al* (1999) found that across a range of agricultural pasture species, in general, the deeper the burial, the lower the rate of softening and the higher the percentage of hard seeds. However, the degree of response to burial seems to be species specific, somewhat related to how reliant the seed is on the fluctuating temperatures to break dormancy (Taylor and Ewing, 1988).

It appears that relatively few genes are involved in the inheritance of hard seed (Rolston, 1978). Forbes and Wells (1968) discovered that a single dominant gene controls seed coat impermeability in *Lupinus angustifolius.* Three major genes control

the hard seed dormancy of *Glycine max* (Kilen and Hartwig, 1978). The rate at which dormancy is broken down can be separately genetically controlled by genes unrelated to the occurrence of the dormancy (Baskin and Baskin, 2001). Cross breeding experiments can be used to determine the Mendelian inheritance of the hard seed coat, if pure lines homologous for the traits are able to be obtained and the life cycle of the plant is compatible (Rolston, 1978)

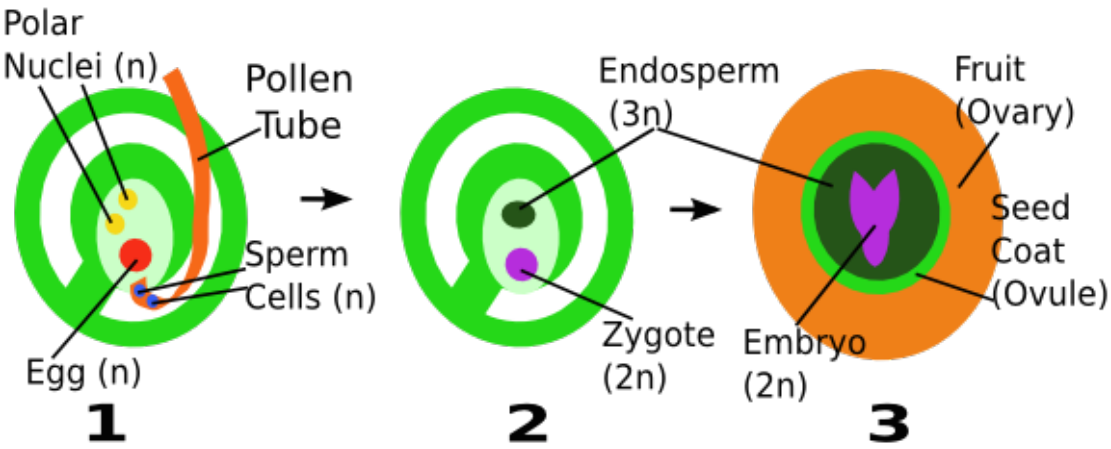

Figure 1.9. Double fertilisation and early seed development. Adapted from Raven et al (2013)

However some genetic inheritance follows a non-Mendelian pattern. The process of double fertilisation means that there are three different combinations of genetic material in the seed (Dumas and Rogowsky, 2008). The endosperm (3n) and embryo (2n) are new combinations of haploids from both the parent plants, while the seed coat is the same unchanged genetic composition of the maternal plant (Figure 1.9)(Raven *et al.*, 2013). Both the embryo and the seed coat can potentially influence the expression of hard seed dormancy and the pattern of breakdown. Hybridization studies can determine whether the maternal genotype of the seed coat is alone in controlling seed dormancy, or if the new genetic combination of the embryo also influences the trait (Baskin and Baskin, 2001).

Pressure for more readily germinable seed will select against dormancy, and this trait is often seen to reduce over the course of domestication (Small, 2011). For example *Pisium sativum* is a legume species for which the wild ancestors commonly display hard seed dormancy and the genes that control the trait have been identified (Weeden, 2007). However over the course of 5000 years of human cultivation none of the genes for hard seed dormancy occur across the genetic diversity of the agricultural cultivars of *P. sativum* (Weeden, 2007). Although this selection process has often been unconscious (Heiser, 1988), research into the genetic controls of the trait has permitted more efficient selection in contemporary programs (Nutt, 2012).

Understanding the inheritance of hard seed has allowed more efficient selection of cultivars with suitable levels of dormancy and predictable breakdown patterns (Nutt, 2012). However most studies have been conducted on species with a long cultivation history. They have had very different selection pressures compared to wild plants and this could bias our understanding (Nutt, 2012, Morrison *et al.*, 1998).

1.3.3 Methods to rupture hard seed dormancy artificially

To ensure uniform plant-densities during establishment, post-harvest seed processing is generally required if hard seed levels are high (Loi *et al.*, 1999). Each species of legume has its own unique seed and pod physiognomy providing different challenges to optimal post harvest processing (Yates *et al.*, 2006). Therefore research and development into the best methods of rupturing the seed coat with minimal seed mortality is essential.

Hughes (1915) studied the best methods for mechanically breaking hard seed dormancy. That work developed scarification techniques that greatly improved germination rates of *Trifolium subteraneum* by damaging the hard seed coats with abrasive surfaces allowing the water to be imbibed. This research discipline has been constantly updated with new technologies and knowledge but scarification still remains the most common method used to mechanically break dormancy (Rolston, 1978).

High intensities of scarification are required to generate germinable seed however this can damage the seed embryo and lead to a loss of viability. Therefore to increase confidence in the process the optimal treatment has been found ideally a point where the process has to be determined at point where damage is minimal but germination is maximised.

Exposing seeds to heat is also a common and popular method to increase germination because it is effective for many species and easy to implement (Kimura and Islam, 2012). Heat treatments can either be wet (water bath) or dry (oven) (Patane and Gresta, 2006). The heat causes the palisade layer to soften and separate from the mesophyll layer underneath, and consequently cracks in the seed coat appear (Baskin and Baskin, 2001). Both the temperature and the time of exposure are thought to be factors in determining the effectiveness of a heat treatment (Baskin and Baskin, 2001). High levels of temperature and exposure will result in increased seed death, so (as for scarification) determining the optimum requires a balance to be found (Patane and Gresta, 2006).

Some studies have suggested that it is the changes in temperature that have more influence on breaking seed dormancy then exposure to extreme temperatures *per se* (Tiryaki and Topu, 2014). Therefore, even when it is unlikely that within the natural environment seed would experience extreme cold, the mechanical action of freezing and thawing the seed can still rupture the seed coat. The shrinking and swelling the seed experiences in the freeze-thaw method makes the seed coat become brittle and eventually crack (Baskin and Baskin, 2001). Repeating the cycles has proven to be more effective in studies reviewed by Kimura and Islam (2012). Extreme cold seems to be more effective at rupturing the seed coat than using liquid nitrogen or other coolants to snap freeze (Kimura and Islam, 2012). The latter method is not effective for many species and appears to be very dependent on the size, shape and particularly the water content of the seeds.

Ecological studies have demonstrated that seeds of many species from environments with frequent fires have adapted to germinate following the physical signals provided by fire (Van Staden *et al.*, 1989). Fire is a common occurrence in the fynbos, the native range of *L. ambigua* (Rutherford *et al.*, 2011). The Mediterranean-type heathlands of the fynbos are dominated by sclerophyllous shrubs that readily burn during the dry seasons, leading to regular fire disturbances, as often as every ten years (Wisheu *et al.*, 2000). The intense heat created can fracture seed coats but some studies suggest that the chemicals within the smoke are responsible for the enhanced germination (Crosti *et al.*, 2006). Some studies have indicated that smoke applied without the associated heat of fire can improve germination of a number of species (Dixon *et al.*, 1995). The responsible chemical compound within smoke has since been isolated and described by Flematti *et al* (2004). Enright and Kintrup (2001) increased germination by exposing

seeds to smoke extract granules dissolved in aqueous solutions. In an environmental setting along with the heat and smoke fire may promote germination in a number of secondary ways. Fertility can be increased by the deposit of nutrients burned in the fire, the chemicals in smoke can suppress the germination of other species that would be competitors and importantly the fire will remove the above ground vegetation to create bare soils so that the seeds are exposed to wider diurnal temperature fluctuations which can further promote hard seed breakdown (Van Staden *et al.*, 1989).

1.3.4 How hard seed banks are exploited in ley farming systems

Post- harvest seed processing is difficult and time intensive, significantly increasing the price of seed for many cultivars of legumes. However, if the hard seed can be exploited in the farming systems by allowing regeneration from a persistent seed bank, the cost of establishment is incurred only once, and is recovered over 10-20 years. This would enable the legume phase to be available "on demand" any time the farmer required.

Hardseeded annual legumes provide great resilience to the low input farming systems of southern Australia (Taylor, 2005). The physical dormancy means that not all of the viable seed is germinated after one winter season, but can persist in the soil, forming a seed bank (Smith *et al.*, 1998). When the paddock is rotated to a crop species the seed bank remains in the soil; those that germinate in the crop are lost, but others germinate and grow when the paddock is left to fallow in subsequent seasons (Loi *et al.*, 1999). Farmers commonly rely on hard seeded legumes to regenerate, to provide pastures after several years of cropping, without the need for re-sowing (Taylor and Revell, 2002). This strategy reduces the financial outlay required for pasture establishment, as

expenditure on seed is only required for the initial introduction of the legume species (Nutt, 2012). Loi *et al* (2005) states that the generation and maintenance of the seed bank is one of the "most vital" components of any modern ley farming system.

Reducing inputs and costs to agriculture is not the only benefit gained by regenerating pastures from hard seed banks. It can also provide protection against year-to-year environmental variation, which is particularly crucial when the environment is least reliable (Smith *et al.*, 1998). Isolated and unseasonably early rains (before the winter growing season) can germinate seeds, with the following dry period leading to their death (Smith *et al.*, 1998). In agriculture this is termed a "false break" (Loi *et al.*, 2005). In southern Australia false breaks are predicted to happen approximately 2 out of every 3 years (Chapman and Asseng, 2001). Regulating germination so that seeds are not ready to germinate until after autumn, when more reliable rains are available, is therefore a wise strategy for maintaining productive pastures.

The farmer also has more operational flexibility if he has a persistent seed bank. The choice between crop and pasture for individual paddocks can be delayed into early winter, allowing for adaptation to changes in economic or environmental conditions.

In recent times a new set of management techniques have been investigated to utilise the unique advantages presented by hard seeded legumes. Summer sowing of harvested but un-scarified seed has been developed (Hackney *et al.*, 2012). If successful, this approach will allow the land manager to make use of the natural break down of hard seed. A particular advantage is that un-scarified hard seed is sown during summer (February 5-15) when there are fewer demands on farm labour (DAFWA, 2014). These seeds will break down over the summer months and germinate for the

break of the winter growing season (DAFWA, 2014). Therefore farmers costs spent on scarifying seeds and hiring labour are reduced.

Twin sowing is another management strategy that helps to reduce the labour and resource costs required to establish a pasture. In this strategy, the farmer sows un-scarified seeds and rhizobium innoculants with the crop in the final year of cropping, before a pasture phase. A proportion of the hard seeds break down underneath the crop and then the following year are ready to germinate in the autumn to begin a pasture phase (DAFWA, 2014).

1.4 Aims of the thesis

L. ambigua is well-adapted to the low rainfall, acid infertile sandy soils of the fynbos biome. For this reason, it may be suitable for development as a perennial forage legume in the edaphically similar regions of southern Australia. To develop a cultivar of *L. ambigua* so that it can be integrated into an agricultural system in southern Australia we need to understand how to propagate the species effectively. This thesis aims to investigate and describe the seed ecology of *L. ambigua* to further this understanding.

Each individual plant species has distinctive reproductive traits as a consequence of evolution and environmental conditions. Plants have evolved strategies and mechanisms to effectively secure their genetic future. Seed ecology is a product of these reproductive dynamics. Therefore understanding the seed ecology of any particular species first requires an understanding of how the breeding system operates in the context of its environment. This is discussed in section 2.

For *L. ambigua* successful propagation requires a high percentage of seed to germinate so that new sowings can be reliably established. For many species this requires post harvest processing to artificially break physical seed dormancy and ensure maximum germination is achieved. Experimentation is required to identify the optimal method to artificially break dormancy, in *L. ambigua* as each species has its own unique seed physiognomy. These experiments are reported in section 3.

Environmental triggers can also break the physical dormancy of seed. Measuring the seed response to field exposure in section 4 allowed us to determine, what percentage of the population germinated, what triggered germination events and over what time frame this occured. This data will inform researchers as to whether *L. ambigua* can regenerate without the need for resowing and if it can persist in a rotation with cropping phases. As the seed in the experiment was produced under identical conditions and was exposed to an identical environment it was also possible to measure the genetic control of this trait. This will be very useful information in a future domestication program to increase the efficiency of selection of *L. ambgiua*.

Section 2: The Breeding system and fecundity of *Lebeckia ambigua*

The reproductive traits of *Lebeckia ambigua* have never previously been reviewed. However during field experiments in Australia a number of observations have been made regarding these traits and these will be provided in this chapter as background.

In general the Australian field studies reveal *L. ambigua* to be a short-lived perennial species living for 3-4 years before senescence. After late winter sowings, *L. ambigua* flowers in the late spring of the following year and then every year at this time for the remainder of its lifespan. During flowering, many (20-50) small yellow flowers are produced that are dispersed along each of the many (5-30) terminal racemes. After fertilization, each flower produces a long slender pod carrying 6-10 seeds. The seeds are less then 2mm in diameter but are very dense, weighing between 1.5 and 2.2 mg. However considerable variation has been observed between individual plants in terms of their reproductive cycle, fecundity and the characteristics of the seed captured.

2.1 Variation in breeding system and seed characteristics

The original *L. ambigua* seed collected for this research came from a number of discrete populations geographically separated by hundreds of kilometres. Genetic isolation as a result of geographic separation can result in large phenotypic variation based on the provenance of the seed (Linhart and Grant, 1996). This is particularly true for breeding and life cycle traits as they are related to fitness within discrete environments (Bischoff *et al.*, 2006). Therefore there may be a very wide range of reproductive behaviours occurring within the collected germplasm of *L. ambigua*.

Figure 2.1. A bee on the flower of L. ambigua in the Murdoch plots. Courtesy of Mrs Regina Carr, Centre for Rhizobium Studies Murdoch University

Local populations of bees have been seen foraging on the flowers of *L. ambigua* in the field (Figure 2.1) and therefore potentially enabling cross-fertilisation (Arceo-Gómez and Ashman, 2014). However, when grown in a glass house isolated from wind, insects or any possible animal vectors, full fertilisation has still been recorded, strongly suggesting that the species is self-compatible. Self compatibility does not preclude cross pollination when flowers are actively worked by bees, so potentially a combination of the two methods occur simultaneously (Nutt, 2012). It is not known, however, the extent to which any of the above fertilisation methods occurs in the natural setting. Cross-fertilisation could potentially increase the genetic variation in the Australian grown population of *L. ambigua* and lead to novel genetic compositions previously restricted by geographically imposed isolation in South Africa. The degree of

outcrossing is also an important consideration for agricultural researchers, as breeding programs and seed production are reliant on a good understanding of the fertilisation dynamics (Devaux *et al.*, 2014).

Large phenotypic variation was recorded for the lifespan and flowering times of individual plants (Figure 2.2). These differences may be responsible for further variation observed in seed characteristics.

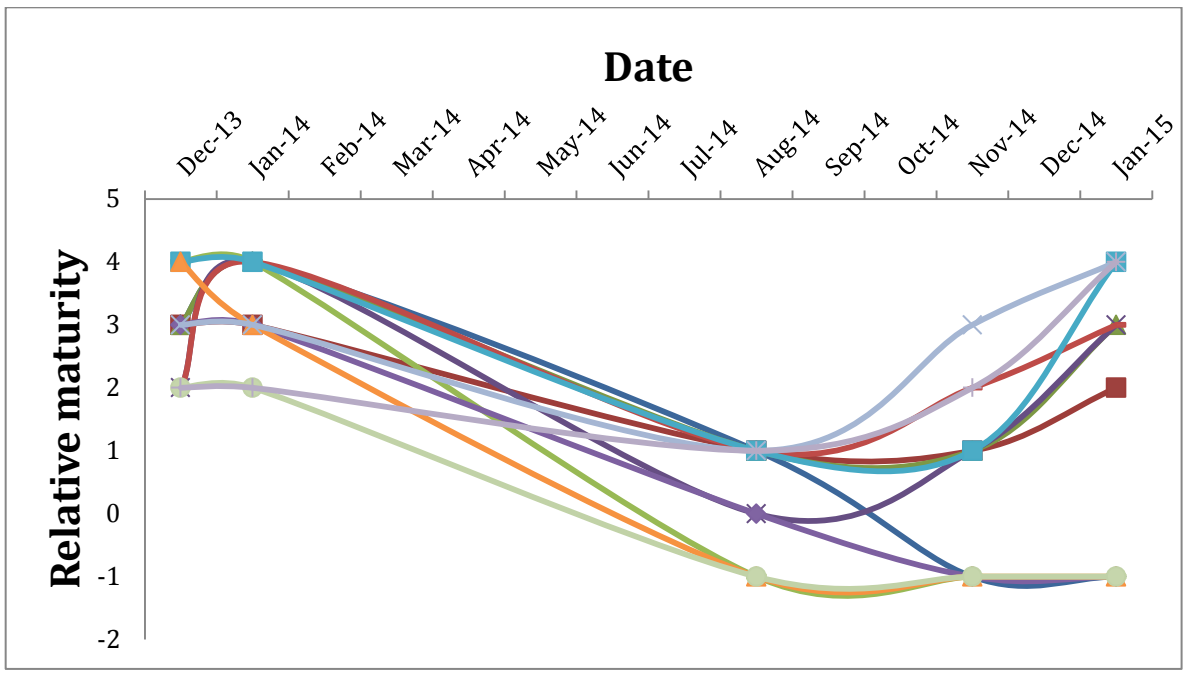

Figure 2.2. The relative maturity (rated 1 immature to 5 very mature, -1 dead) of 12 individual spaced L. ambigua plants growing in the Murdoch plots over two seed production periods.

L. ambigua also displays asynchronous timing in respect to the production and maturation of flowers along the terminal inflorescence of the same plant (le Roux and Van Wyk, 2007). This can influence the uniformity of seed characteristics because at different points of capture, seed can posses different levels of maturity. Immature seed may not have had the opportunity to become fully viable or may behave differently to its genetic disposition (Baskin and Baskin, 2001). During the last stages of development of the hard seed coat, the moisture level is reduced to dehydrate the seed to make the

seed impermeable to water (Hyde, 1954). If the seed has not had a chance to fully dehydrate, the hard seed coat may not have imposed full dormancy and the seed may have been susceptible to imbibition and subsequent germination (Hyde, 1954).

Therefore the timing and method employed to collect seed may also have an influence on the levels of hard seed dormancy detected. The swathing technique was used to harvest the seed in section 3. This method allows seeds to continue to mature post harvesting and should reduce the variation in seed maturity but cannot ensure uniformity (Cenkowski *et al.*, 1989). Further, a percentage of seed may have shattered prior to harvesting, and subsequently lost. Potentially this seed had different hard seed characteristics to the collected population.

In contrast the seed used in section 4 was collected after the seed had dropped to the ground. Potentially this ensured a larger proportion of the seed collected was fully mature. As is turned out the hard seed percentage recorded for this population was 7% higher than that recorded for the population in section 3. However any difference between these populations could also be attributed to the fact that the seed populations were grown under different conditions that could have influenced seed characteristics (Revell *et al.*, 1999).

2.2 Seed size

L. ambigua was found to have a small dense seed (1.6-2.2mg and 0.7-1mm length) compared to many other agricultural legumes and a comparatively large variation in seed size is encountered. The size of seeds can have a large influence on almost all aspects of the germination and establishment of the species (Harper *et al.*, 1970). The

characteristic size of the seed can be considered as a compromise between the energy reserves each seed is afforded and the amount of seed able to be produced (Marshall, 1986). The seed size can also have an important influence on the ability of seed to survive ingestion, with typically smaller hard-coated seeds more likely to remain viable (Ollerton and Lack, 1996).

A number of studies have investigated seed size and how it impacts seedling success across a range of plant species (Ehrman and Cocks, 1996, Beveridge and Wilsie, 1959, Twamley, 1967). While theoretically it is widely supported that lower seed weight leads to reduced seedling vigor, experimental data has provided mixed results, suggesting that the relationship is not straight forward (Marshall, 1986).

Larger seeds with larger energy stores may show increased seedling vigor, allowing them to emerge from greater depths or through competing vegetation (Harper *et al.*, 1970). Beveridge and Wilsie (1959) demonstrated that for seeds of *Medicago sativa,* seedling vigour was correlated with seed size, but that emergence did not display a consistent relationship to seed size.

Certain environmental conditions may favor different seed size predispositions (Bond *et al.*, 1999). Piano and Francis (1992) suggested that wetter clay areas favour larger seeded species while smaller seeds are more suited to drier and sandier conditions. Therefore, this trait displays a high level of adaptive significance and has an immense impact on the fitness of the lineage.

Small seeds tend to mature faster and have a higher surface area to volume ratio and therefore are probably able to germinate faster and with lower water requirements

(Small, 2011). The earlier germination of small seeds along with increased numbers may compensate for a smaller seed size (Marshall, 1986).

2.3 Fecundity

A very high fecundity has been recorded for *L. ambigua* with some mature plants producing more than 20,000 seeds per annum (Figure 2.3). Production of large amounts of harvestable seed is a very desirable agricultural feature but is considered very rare in herbaceous perennial species (DeHaan and Van Tassel, 2014). Inadequate seed production is considered one of the major obstacles to the widespread agricultural adoption of perennial cultivars (DeHaan and Van Tassel, 2014). High fecundity is more associated with an annual life strategy in plants, because they have only one reproductive opportunity before death (this is termed semelparity). Therefore there is evolutionary pressure to allocate more resources into seed production (Pausas and Keeley, 2014). However, the unusual combination of a perennial shrub, with high fecundity, does occupy an ecological niche in some environments.

The Fynbos is a very infertile environment where annual species struggle to persist, and the flora is dominated by perennial shrubs (Rutherford *et al.*, 2011). Charnov and Schaeffer (1973) state that a perennial habit should be favoured in environments with low juvenile survivability. These environments can inhibit adequate recruitment in each annual cycle regardless of how many seeds are produced (Charnov and Schaffer, 1973). In contrast, an established perennial plant can set seed every year it persists, increasing the opportunity that one year will be favorable to more successful

recruitment or a disturbance event will occur to increase the survivability (Pierce and Cowling, 1991).

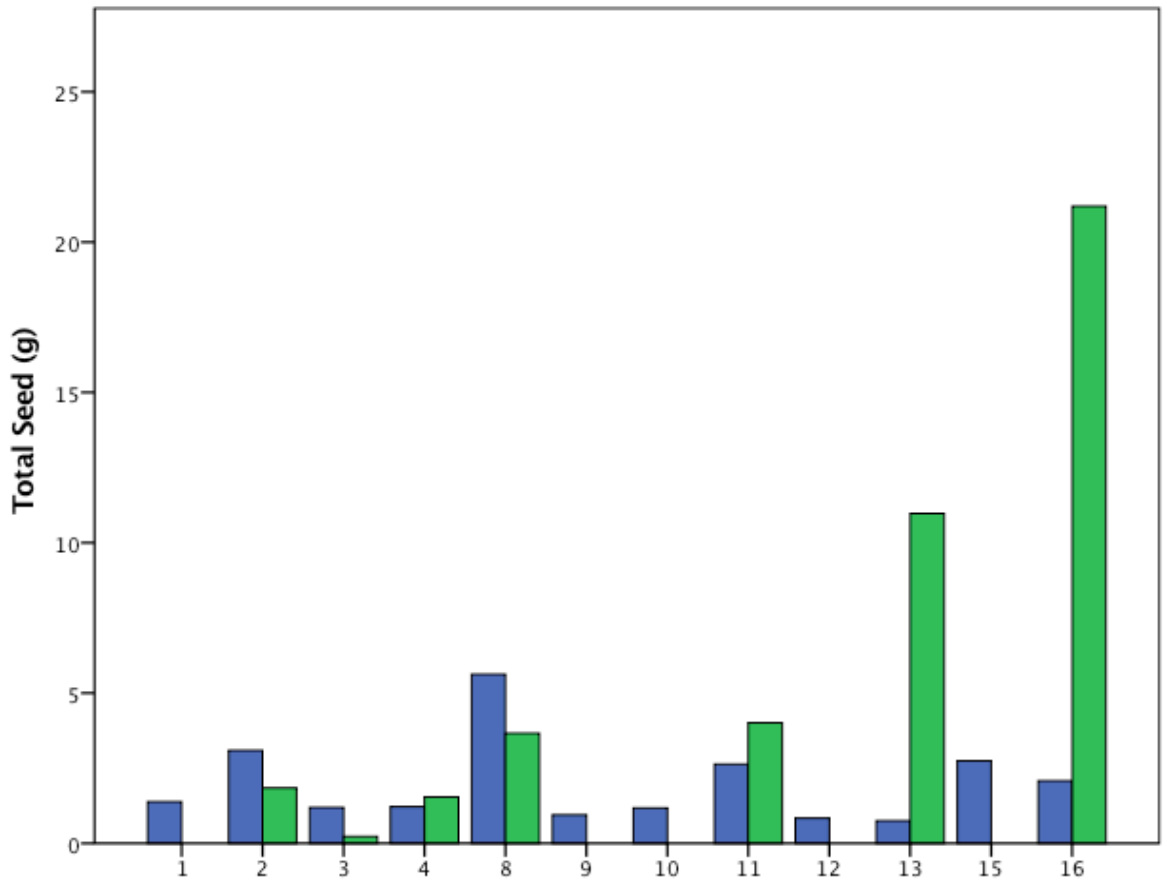

Figure 2.3. Total seed captured (g) from individual spaced L. ambigua plants (1-16) at the Murdoch research plots Blue is the first harvest 10/1/2014 and Green is the second harvest 1/1/2015.

Fire is considered the major disturbance regime of the Fynbos, exerting significant influence on the structure and function of the floral community (Rutherford *et al.*, 2011). Two main strategies are employed by plants to thrive in fire prone environments: Re-sprout from undamaged tissues (re-sprouters) or recruit from seed (seeders) after the fire (Atwell *et al.*, 1999). Species that have the ability to re-sprout have developed the ability to create underground carbohydrate stores, which are protected from the fire. After the fire destroys the vegetation above ground the stores are activated and the plant regrows (Atwell *et al.*, 1999). Seeders either rely solely

(obligate) or partially (facultative) on recruiting from seed post fire (Wisheu *et al.*, 2000).

It is suggested that the resprouting strategy evolved first and certainly is the dominant mechanism encountered across the majority of the known fire prone environments (Pausas and Keeley, 2014). However this dominance is reversed in the Fynbos and Kwongan communities, with species that are able to regenerate from seed more frequently encountered along with the only occurrence of obligate seeder species is encountered (Wisheu *et al.*, 2000). Both of these biomes are characterized by remarkable levels of floral diversity, which is potentially linked to the predominance of seeders (Wisheu *et al.*, 2000). Seeders tend to speciate faster as they sexually recombine their genetic material in each seed (Wisheu *et al.*, 2000). Routine fire disturbance then creates a situation where there is intense competition post fire and isolation from the genetic material of previous generations (Rutherford *et al.*, 2011). Increased speciation results in the very diverse heathlands and subsequent dominance of seeders, but cannot explain what drives the initial occurrence of seeders. It is proposed that the comparatively low fertility and shorter fire intervals that both the Kwongan and the Fynbos experience are also responsible for the predominance of seeders (Wisheu *et al.*, 2000).

Fire disturbance removes all of the above ground vegetation, which had been competing for and accumulating the soil, water and nutrients in the post fire interval (Moreno and Oechel, 1994). Some of the nutrients in the ecosystem are returned to the soil after the fire so the ecosystem has little competition and relatively fertile soil for whichever species is the first to regenerate. Re-sprouting is considered the optimal strategy for regeneration in a fertile environment, where increased access to nutrients

throughout the plant's lifespan allows more carbohydrates to be stored and post fire re-growth to be faster (Wisheu *et al.*, 2000). In contrast, this can be a very slow process in infertile environments. The chance of juvenile survival however is at its highest post fire. Seeds are small and only need a small amount of water to germinate and are able to grow quickly to make the most of the favorable conditions (Pierce and Cowling, 1991). There is also evidence from extant species that obligate seeders have physiological traits which make them more tolerant of drought and water stress (Pausas and Keeley, 2014).

The more seed a seeder species is able to produce during a fire interval, the higher its chances of successful recruitment (Wisheu *et al.*, 2000). For this reason the life cycle of a seeder species must closely correlate to the fire interval (Wisheu *et al.*, 2000). Less predictable regimes and long periods are seen to be detrimental to this regeneration strategy (Rutherford *et al.*, 2011). Pierce (1991) discovered that the longer the interval between fires, the smaller the germinable soil seed bank is, and the less the seed bank represents the above ground vegetation. Therefore the ideal disturbance regime for the obligate seeder species is a predictable, short fire interval. A short interval before fire disturbance causes death can then be seen to be analogous to the kind of pressure that would be put on the semelparous annual species (Pausas and Keeley, 2014).

Therefore it can be hypothesised that *L. ambigua* is faced with the same evolutionary pressures to produce a large amount of seed, as annual species. Further it is required to be a perennial so that it can produce as much seed as possible over different growing seasons during the fire interval to compete in an environment with low juvenile survivability. Additionally, to ensure that a high percentage of seeds germinate at the

ideal time, which would be on the first rains post fire, the species would need to have evolved mechanism to regulate germination (Pausas and Keeley, 2014).

2.4 Hard seed coat

The majority of legumes, including perennial legumes, produce seed with physically imposed (hard seed) dormancy, which restricts and delays their germination (Cocks, 2001). A cross section of *L. ambigua* seed (Figure 2.4) shows there to be a hard seed coat present. The water impermeable palisade layer structure is clearly displayed and the presence of this specialized region is consistent with histological investigations of other species which display hard seed (Rolston, 1978).

The palisade layer is the main structural feature relating to hard seed dormancy (Williams and Elliott, 1960). Additionally the hilum, micropyle and the raphe are also believed to be closely associated with an impermeable seed coat (Ma *et al.*, 2004). The palisade layer of *L. ambigua* is approximately 50μm thick in cross section. Currently it is not well understood whether the thickness of the seed coat is a factor in determining the prevalence of dormancy (Wang and Grusak, 2005). Russi *et al* (1992) investigated 6 legume species collected in Syria and found that differences in seed dormancy between the species could be related to the thickness of the seed coat when the coat thickness was considered relative to the size of the seed. It should be determined whether the persistence of seed coat imposed dormancy in *L. ambigua* is related to variation between seed coat thickness and seed size.

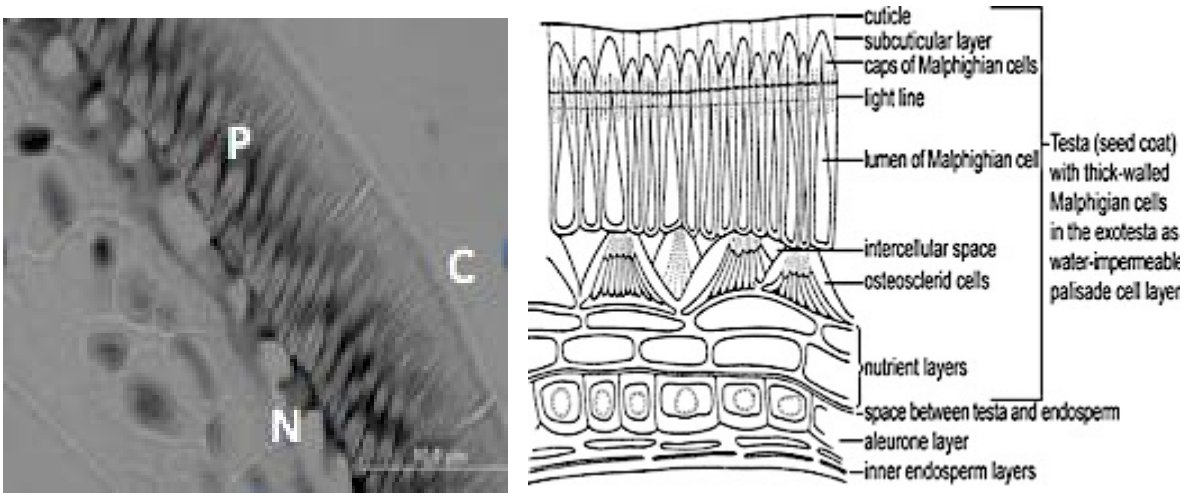

Figure 2.2. Cross section of Legume seed. Left; L. ambigua, with a toluidine blue stain, N: Nutrient layer, P: Pallisade layer (malphigian cells), C: cuticle (methods in appendix A), Right; The stylized seed coat structure of Melilotus alba (Leubner, 2014)

Despite ample empirical data reporting the occurrence of hard seed dormancy, large knowledge gaps in the functioning of the seed coat still exist (Morrison *et al.*, 1998, Cocks, 2001). Although the permeability of a seed coat is related to its structure, it is difficult to be precise in relating observed structures to functions (Ma *et al.*, 2004). Seed coat break down is a dynamic process and analysis through histology is destructive, only providing a snap shot of one point in time (Venier *et al.*, 2012). Therefore experiments which measure the germination response of a seed populations to environmental and artificial elements are required and these are described in section 4 and 3 respectively.

Section 3: Seed germination phenology of *Lebeckia ambigua*

3.1 Introduction

At the time of dehiscence the seeds of *Lebeckia ambigua* are very hard and not readily germinable, methodologies for breaking this dormancy, such that new stands can be readily established, must be developed.

Breaking hard seed dormancy requires the seed coat to be ruptured so that it becomes water permeable (Nutt, 2012). Heating-cooling, freezing then thawing and mechanical scarification are reported as techniques to soften seeds of legumes, with variable results (Kimura and Islam, 2012). Fire and the chemical compounds contained in smoke have also been shown to enhance germination for some species (Pennacchio *et al.*, 2007).

The most common commercial approach to artificially breaking seed coat imposed dormancy is to scratch the seed coat with an abrasive surface (Rolston, 1978). This method termed scarification is favoured for most species because it can reliably produce high germination levels and can also accelerate germination rates (Patane and Gresta, 2006). There is also an additional value in the ease of application, machinery can be reliably up scaled to reproduce the same results on large quantities of seed with minimal effort (Tiryaki and Topu, 2014). The intensity of the scarifying process can be altered by either changing the speed of the abrasive mechanical surface or by changing the time the seed spends in contact with the abrasive surface. However the mechanical abrasion of the seed coat can lead to other parts of the seed becoming damaged, particularly if the intensity of the treatment is high (Ellis and Palmer, 1973). This can lead to an increased loss of seed viability (Tiryaki and Topu, 2014).

Given this information, it is important to conduct research to elucidate the optimal process for rupturing the seed coat to provide germinable seed during the domestication of *L. ambigua*.

3.2 Methods

3.2.1 Nursery plot establishment

To obtain sufficient seed for experimentation, a one hectare plot of pastoral land was sown with 200g of *L. ambigua* seed on the 16/6/11 in Tincurrin, Western Australia (32.9770°S, 117.7750° E). The seed had been sourced from the Murdoch University collection, accession 3.2, originating from provenance 32 (provenance further discussed in section 4). The seed was hand-scarified with glass paper grade P30 and inoculated through new edge microbials 50g peat inoculum bag with nodule bacterial strain WSM4184 just prior to sowing. A CSBP 3-1 mix of superphosphate/potash fertiliser was applied by hand immediately after sowing. Weeds and pests were controlled through the application of 2L of roundup and 200ml of Talstar respectively. Both sprays were applied 5 days before sowing. A further hectare was sown adjacent to the first plot on the 30/8/2012 using the same methods and rhizobial strain.

3.2.2 Harvest and sampling

Seed from the most productive regions of the plots was harvested on the 16/12/2013 using a swathing technique that cut the inflorescences when pods were mature, but not shattered or fallen, and left to dry on the ground (Sheldrick *et al.*, 1985). Pod was collected after one week then threshed and aspirated to yield 3.2 kg of naked seed.

Three sub-samples of 15 g of seed were taken from the top, middle and bottom of the bag that kept the seed stock. These sub samples were then mixed together and divided using a Encott 2-way seed splitter to give a random sample of approximately 40g of seed. Ten grams were further sampled from this for the scarification experiment. The remaining 30g were then divided into groups of fifty seeds using a seed counter until there were 92 samples. These samples were then randomly assigned to either: untreated (4 lots), heat (48 lots), smoke (20 lots) or freeze–thaw experiments (20 lots).

Figure 3.1(left) L. ambigua seeds with soft radicles longer than 2mm, 5 days after placement on wet filter paper to germinate. Figure 3.2 (right) Filter paper with the L.ambigua seed, rolled and stored in a wet tray to preserve moisture before being placed at a constant 15°C to test germintation.

3.2.3 Seed treatments

i) Untreated seed

Four replicates were each placed on separate wet filter paper, then rolled and placed in a tray with excess water (Figure 3.2). The tray was covered with plastic to maintain the moisture and stored in a 15°C room. The seeds were inspected at regular intervals and seeds that were either germinated or had died were recorded and discarded. Seed was defined as germinated when a soft radicle measuring at least 2mm in length was

evident (Figure 3.1). Seed was determined to have died when the seeds became soft and pulpy after water was imbibed. If no water was imbibed the seed was determined to be hard.

ii) Exposure to dry heat

Treatments were placed in a Hitasu t6 experimental oven set to temperatures of 60°C, 80°C, 100°C and 120°C. Seed was removed at time intervals of half an hour, one hour and three hours. Each sample was then allowed to cool for one hour before placing on filter paper to germinate under the same conditions as the untreated treatment. Each treatment was replicated four times.

iii) Exposure to fire and smoke in moist sand

Sixteen lots of *L. ambigua* seed were placed in 25ml petri dishes with oven dried sand and 10mls of room temperature water to regulate the moisture content to 40%. The seed lots were randomly allocated into either treatment 1 (8 lots), treatment 2 or 3 (4 lots). The Petri dishes of treatment 1 were placed in a Jarvis smoker for separate simulated fire treatments. *L. ambigua* chaff, collected from the same nursery plots (3.2.1) in December 2013, dried in a 60°C oven then threshed to be very fine, was placed in a thin layer at the bottom of the smoker. Petri dishes were placed on a metal grill above the chaff and the smoker box was sealed {Figure 3.3}.

A methanol burner was placed underneath the smoker, applying heat and instigating the smoking of the chaff. The temperature was monitored using a Gasmate wireless temperature probe. Heat was only applied to treatment 1 for 7 minutes with the smoker remaining shut for 30 minutes (Figure 3.8). This kept the temperature below 60°C for the experiment.

Treatment 2 underwent the same process except the heat was applied for 27 minutes and the smoker remained sealed for a total of 100 minutes reaching a maximum temperature of 146°c. Treatment 3 was left to sit at room temperature to provide a control.

Figure 3.3. The smoker used for the simulated fire experiments

Prior to attempting either of these methods, seed of both *Kennedia prostrata* and *Hardenbergia comptonia* were subjected to the same regimes. Both species are native Australian legumes with a well-documented response to fire treatments (Auld and O'Connell, 1991). Their improved germination response to the treatment was used to validate the method and also helped to calibrate the temperatures and timings for the *L. ambigua* experiment.

After the conclusion of each experimental regime the replicates were left to sit for 24 hours. Following this the seeds were removed from the petri dishes and placed on filter paper to germinate under the same conditions as those detailed for the untreated replicates.

iv) Exposure to smoke granules and moist heat

The seed lots that had been allocated to this experiment were counted into 16 lots of 30 seeds, by hand. The seeds were placed into a 25ml petri dish and covered with oven-dried sand to fill the dish. Each seed lot was randomly allocated into one of the 4 treatments, so that each treatment had four replicates (Table 3.1).

The soil moisture content of treatment 1 was regulated to 40% by adding 10mls of room temperature water (as in 4.2.3 iii). This treatment was then left to sit. Boiling water (10mls) was applied to treatment 2.

Table 3.1. The different factors imposed on each treatment (+) indicates to which treatment the factor was applied.

Treatment	*Boiling water*	*Smoke granules*
Treatment 1 (control)	-	-
Treatment 2	+	-
Treatment 3	-	+
Treatment 4	+	+

Treatments 3 and 4 had 0.5 grams of Australian wildflower seed starter smoke granules scattered on the surface of the petri dish. Treatment 4 then had 10mls of boiling water added to the petri dish while for treatment 3 10mls of room temperature water was added. The temperature of the sand after the application of boiling water was monitored (Figure 3.8).

After the application of water each treatment was left to sit for 24hours before the seeds were removed from the petri dishes and placed on filter paper to germinate under the same conditions as those detailed for the untreated replicates.

v) Freeze-thaw

Five treatment regimes were developed for the freeze- thaw experiment, each with four replicates. Seed for each treatment was placed in a 25ml Petri dish and covered with oven-dried sand to fill the dish. The soil moisture was regulated to 40% by adding 10mls of water to each as in previous experiments. Treatments 1-4 had their petri dishes placed in the freezer for 1 hour, then removed for one hour in cycles that were: not repeated, repeated once, twice and three times respectively. Treatment 5 was placed in the freezer for an uninterrupted 19 hours. The temperature of the freezer and the petri dishes were recorded every hour. After each respective treatment was finished, plates were allowed to thaw for 2 hours at room temperature before the seeds were sieved out of the sand and set to germinate under the same conditions as the untreated replicates.

vi) Scarification

A sample of ten grams of seed was further separated using the Encott 2-way seed splitter to ensure four groups with at least 2 grams per sub sample. Seeds were slowly hand fed into a Kimseed desktop 1450 rpm scarifying machine. The machine had 4 different speed settings (Table 3.2). Two grams of seed was exposed to each speed setting. After passage the seeds were separated with the Encott 2-way seed splitter until there was a 0.3 g sub-sample (approximately 150 seeds). The remaining seeds were then passed through again, repeating the process until four sub-samples for each speed were obtained. The sub-samples were counted into 3 reps of 50 by a seed-counter, then placed on filter paper to germinate under the same conditions as described for the untreated replicates.

Table 3.2. Speed setting and the relative rpm for the Kimseed desktop 1450 rpm scarifier.

Speed setting	Gear arrangement	RPM
One	1-2	725
Two	1-1.5	966
Three	1.5-1	2175
Four	2-1	2900

3.2.8 Statistical analysis and calculations

Germination (G) and seed death percentage (D) were calculated as cumulative values across all observations (Ranal and Santana, 2006).

$G= (\Sigma A_1 \ldots A_i / P)*100$ and $D=100-G$

A_1 = total germination on day 1, P = Size of initial sample

Germination velocity (GV) was calculated as coefficient of germination rate (Ranal and Santana, 2006).

$GV= 100*(\Sigma A_1..Ai)/(\Sigma A_1 t_1..A_i t_i)$

A_1= total germination on day 1, t_1 = days since wetting

Where there was more then one factor within a treatment, influence was compared with a two-way analysis of variance (IBM, 2013). All other experiments were analysed by applying a one-way analysis of variance to determine any significance in germination between treatments (IBM, 2013). Confidence intervals of 95% are

displayed to indicate the practical importance of differences in seed response to treatments (Kalinowski and Fidler, 2010).

3.3 Results

3.3.1 Effect of exposure to dry heat on seed germination and death

The interaction between temperature and exposure was significant for both the germination percent and seed death (p Value = 0.19, p Value> 0.00 respectively).

No treatment produced more than 25% germination. Seeds exposed to 60°C were unaffected in germination over time, however seeds exposed to 80°C or higher for 1 hour had an increase in germination compared to the untreated seed (Figure 3.4). The highest mean germination was recorded in seed exposed at 100°c for 3 hours.

Seed death increased with exposure to the two highest temperatures. The seed death was maximized with the highest exposure to heat: 120°c over 3 hours (Figure 3.5).

3.3.2 The effect of Fire on seed germination

A preliminary experiment recorded increased germination of *K. prostrata* and *H. comptonia* in response to the simulated fire method (Figure 3.6). The germination percentage for *H. comptonia* increased from 20% to 47% after 30 minutes in the smoker, when the temperature was approximately 60 °C. No increase in seed death was recorded for *H. comptonia* in response to any of the treatments.

These fire treatments were then imposed upon *L. ambigua* and a significant increase in germination was observed *(Figure 3.7).* Treatment 2 reached a temperature of 146 °C and the apparatus was above 100 °C for approximately twelve minutes (Figure 3.8).

Treatment 1 rose to 60 °C after 10 minutes and remained above 50 °C for a further 9 minutes.

The highest average germination percent was for 100 minutes in the smoker with a maximum temperature of 146 °C. No seed death was recorded for the control or treatment 1 in this experiment, however treatment 2 produced seed death of 6.45% (data not shown).

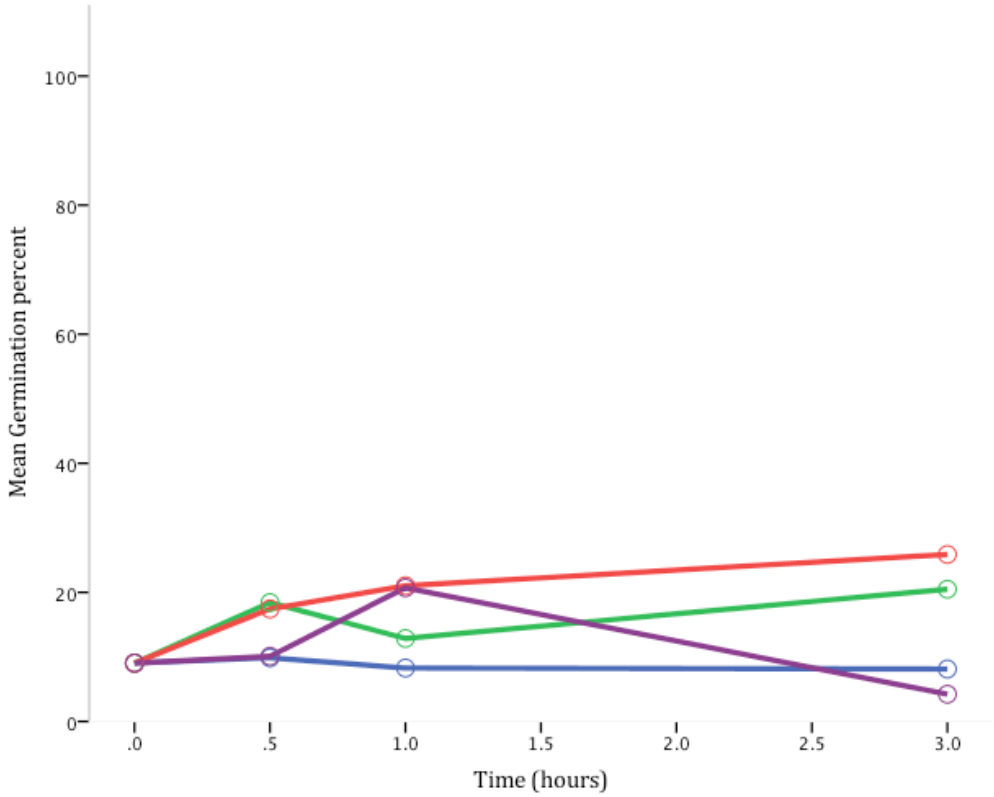

Figure 3.4. L. ambigua seed germination in response temperature illustrated as an interaction plot between three time intervals; Half an hour, one hour and three hours at four temperatures; 60°C (blue), 80°C (green), 100°C (red), 120°C (purple).

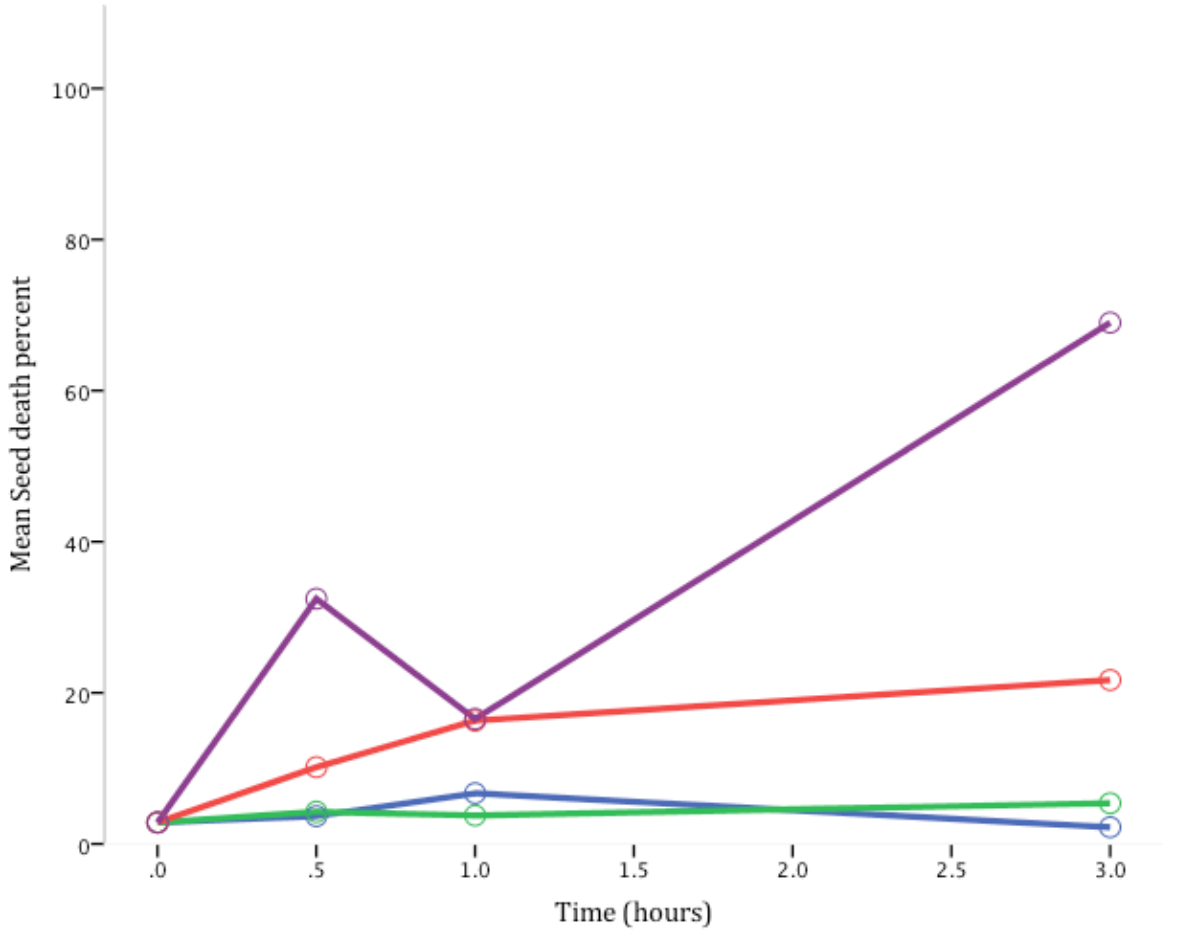

Figure 3.5. L. ambigua seed death response to an interaction plot between three time intervals; Half an hour, one hour and three hours. At four temperatures; 60°C (blue), 80°C (green), 100°C (red), 120°C (purple).

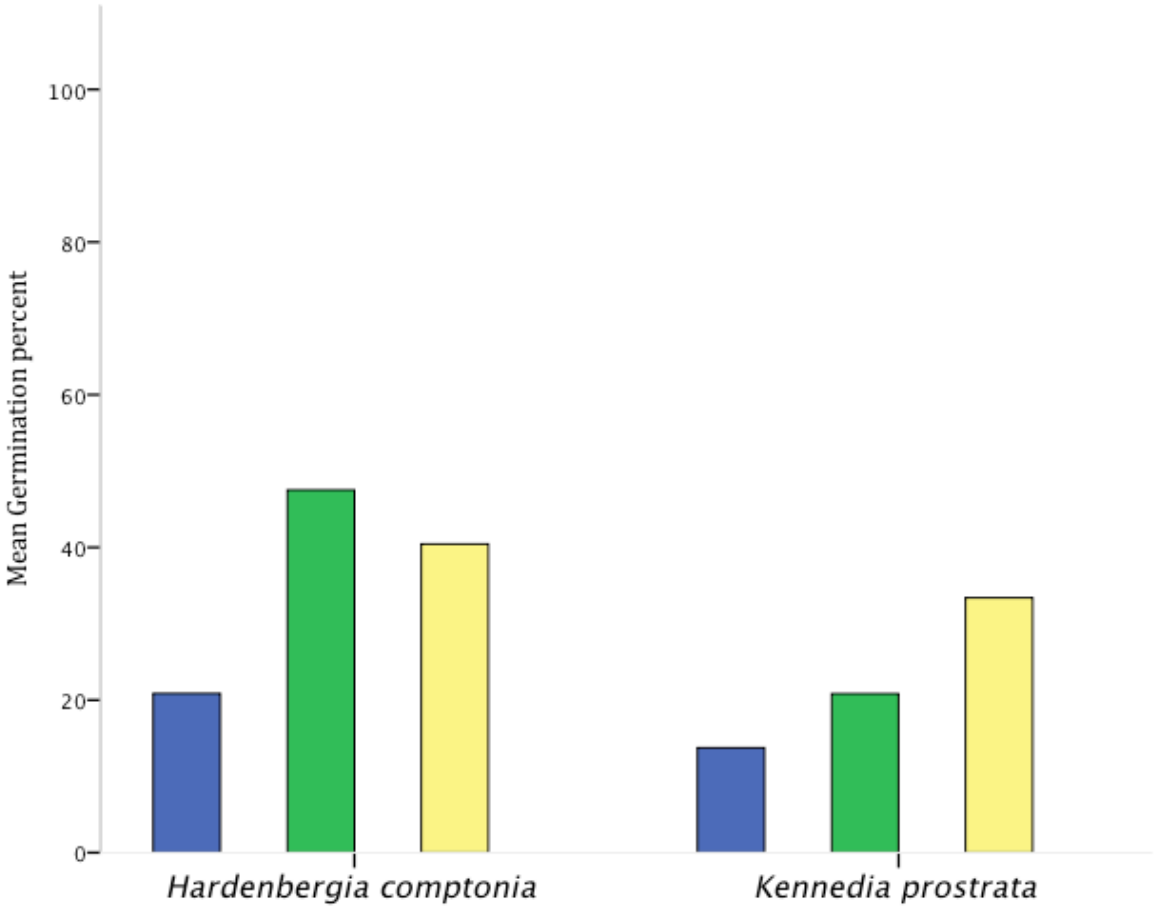

Figure 3.6. The average germination (%) of the seed of Australian native legumes when exposed to; left in petri dishes ■, 30 minutes in smoker ■, 100 minutes in smoker ■

3.3.4 The effect of wet heat and smoke on seed germination

The higher temperature of 100°C and longer exposure time produced the best germination result for *K. prostrata* (14% increased to 34%). High levels of seed death for all treatments applied to *K. prostrata,* (including the control) were recorded.

The presence of smoke did not significantly increase germination of seed of *L. ambigua* in this study (p Value = 0.33)(Figure 3.9). However the application of boiling water did significantly increase germination with 70% of seed becoming germinable (p Value < 0.00). No seed death was recorded for any of the treatments (data not shown). The temperature trend for boiling water added to sand is shown in Figure 3.8.

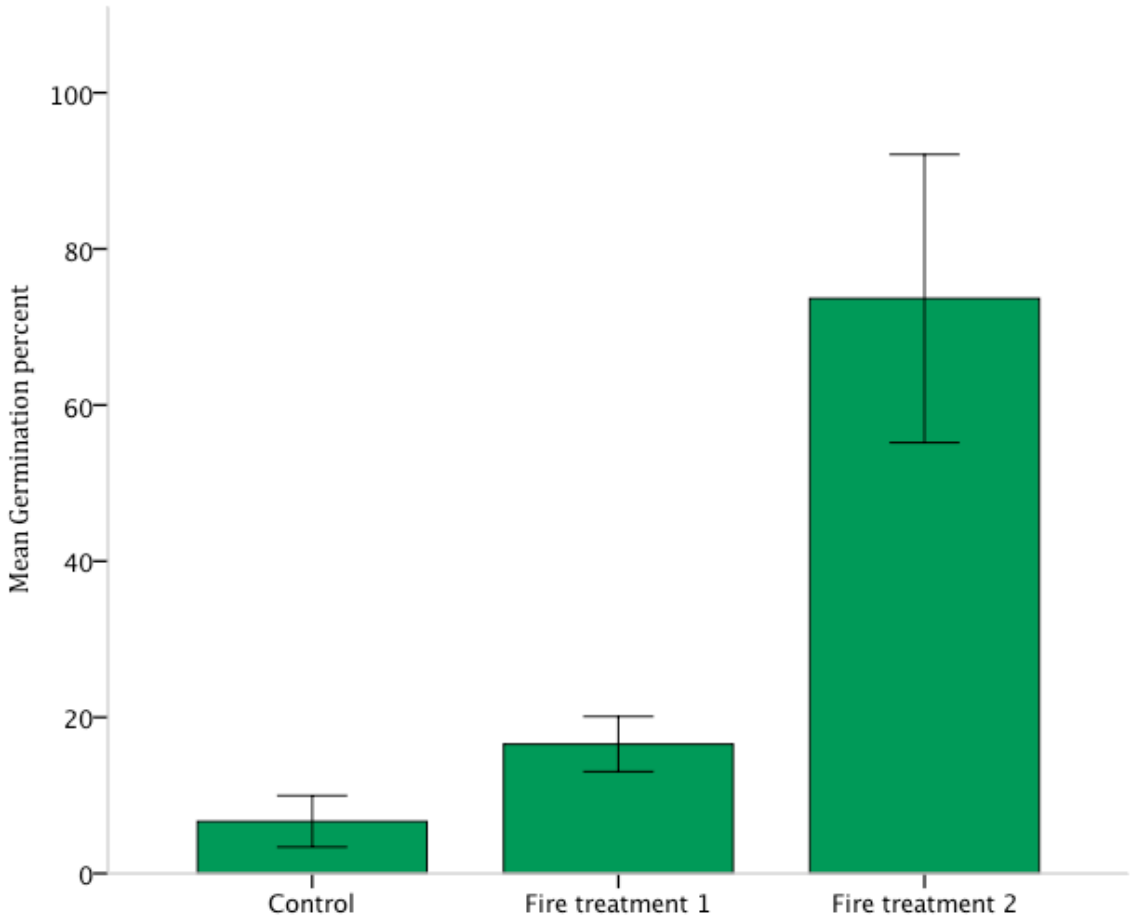

Figure 3.7. The mean germination of L. ambigua seed (%) in petri dishes with wet sand when exposed to; Fire treatment 2 – max 146°C in smoker for 100 minutes, Fire treatment 1 – max 60°C in smoker for 30 minutes, Control – left in the petri dishes for the same amount of time. The bars represent ± 95% confidence interval

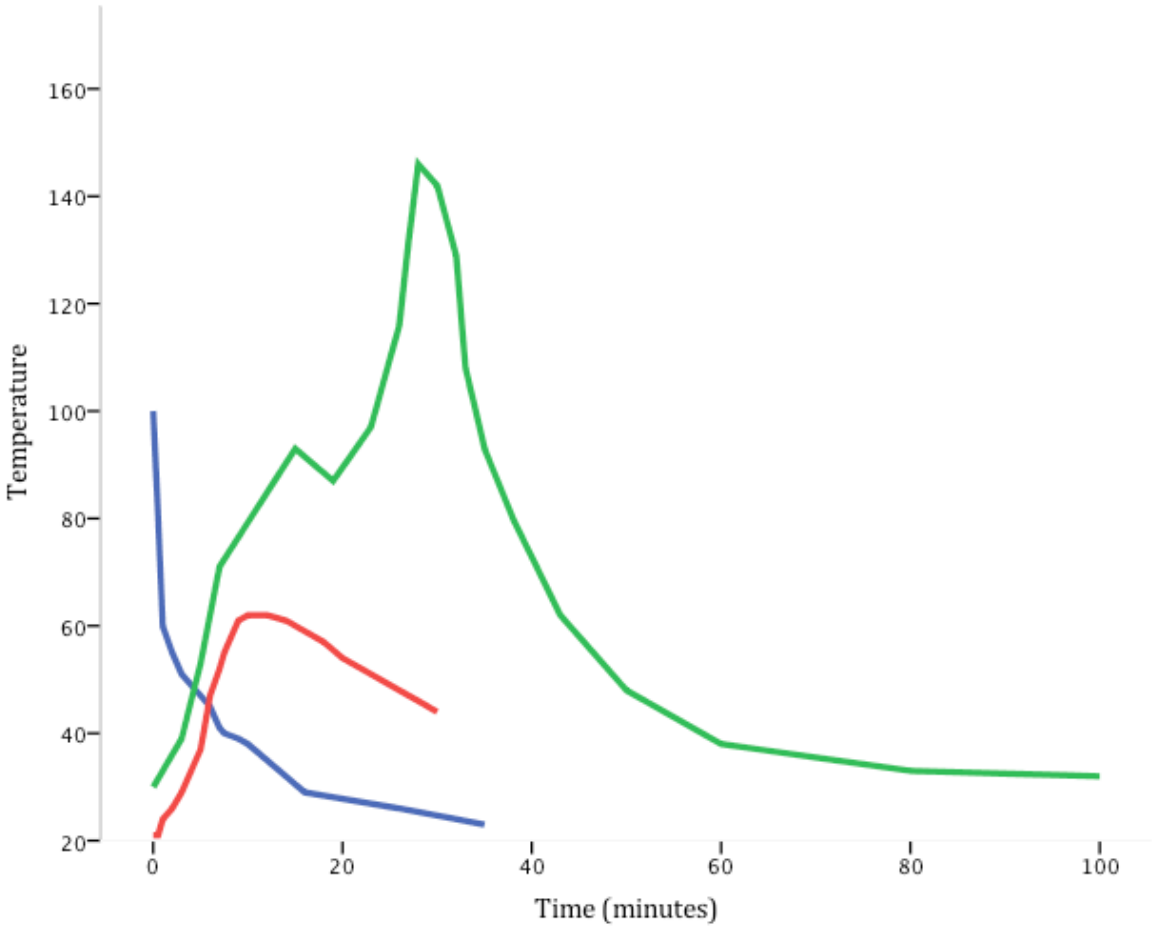

Figure 3.8. The temperature (°C) recorded inside a petri dish for each of the fire simulation experiments and the boiling water and sand experiments. Blue represents boiling water in sand, Red represents fire treatment 1 and Green represents fire treatment 2.

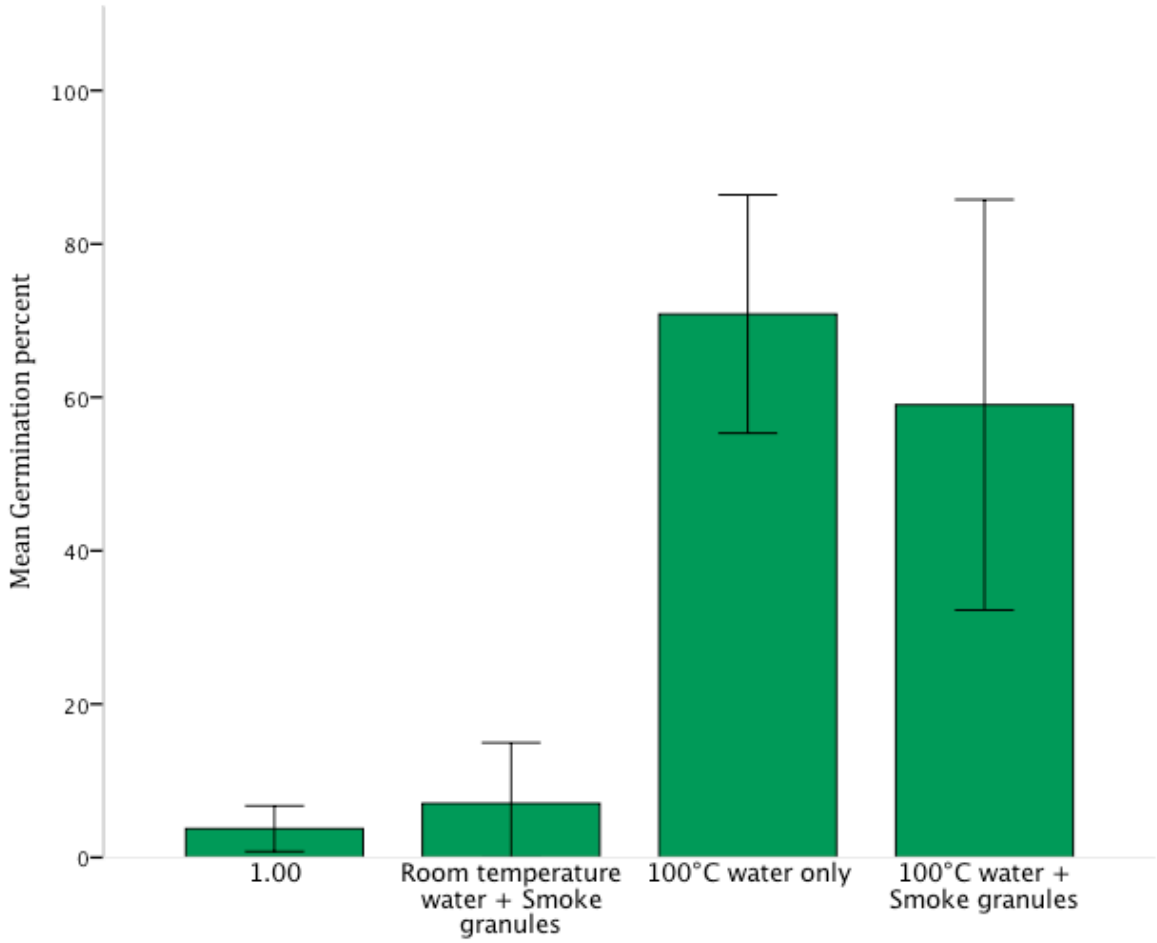

Figure 3.9. The germination of L. ambigua (%) in petri dishes in sand when exposed to smoke granules and/or water at 100 °C. The bars represent ± 95% confidence interval.

3.3.4 The effect of freeze-thaw cycles on seed germination

The temperature of the freezer reached as low as -25°C and fluctuated as high as -9°C. The soils and seed samples though ranged from -1°C to -8°C during the freezing stage and reached between 13°C to 19°C during the thawing stage. No difference in germination or seed death was seen as a result of exposing seed to freezing temperatures (Figure 3.10).

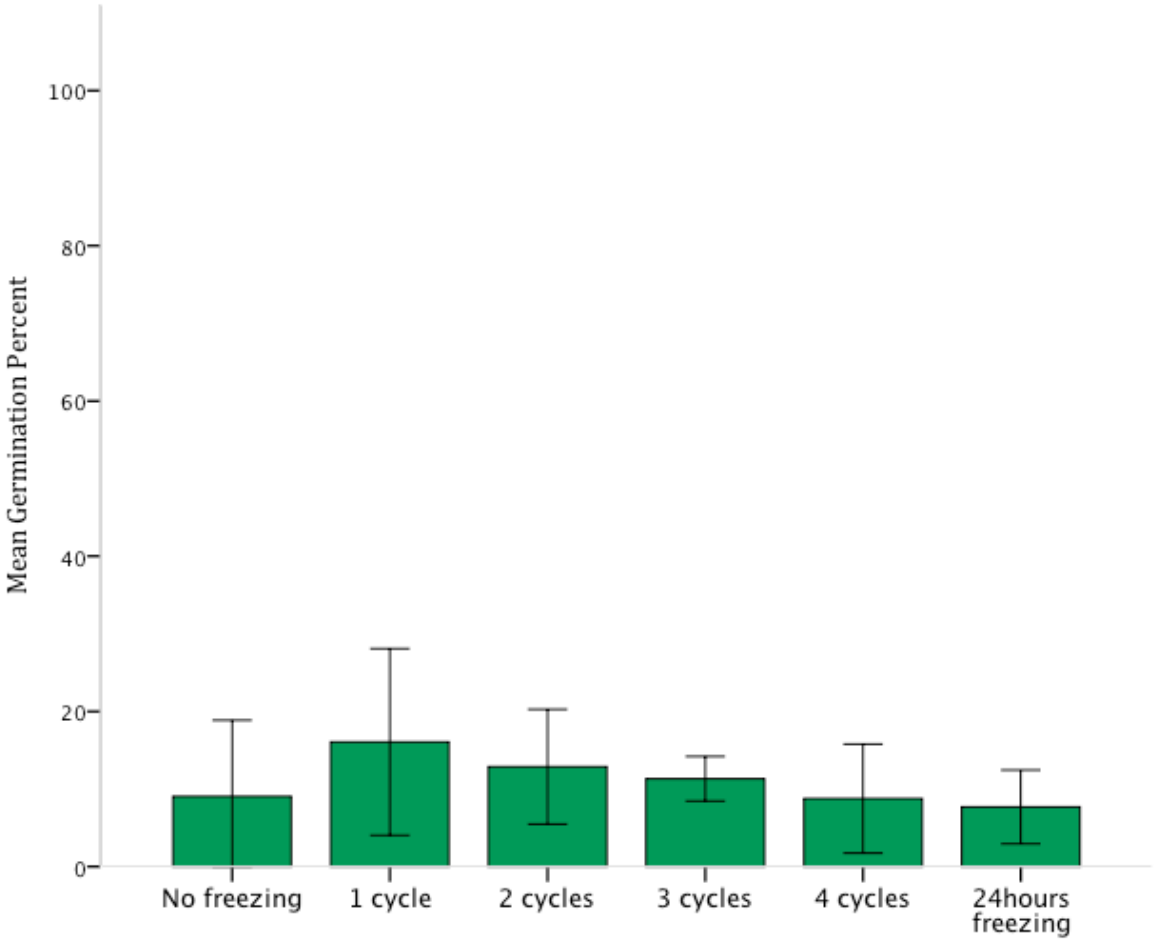

Figure 3.10: Seed germination of L. ambigua (%) under different exposure to freeze thaw cycles. The bars represent ± 95% confidence interval.

3.3.5 Effect of scarification regimes on seed germination and death

Both the speed of the rotating plates and the number of passes through the scarifier significantly affected germination of *L. ambigua* seed (p Value 0.000 for both). The most severe physical treatment (Four passes at speed 4) produced the highest germination of nearly 70% (Figure 3.11). This treatment also caused the highest seed death of over 17% (Figure 3.12). The number of passes also had a significant effect on seed death (p Value =0.033). At the highest speed, 3 and 4 passes caused a large increase in the seed death.

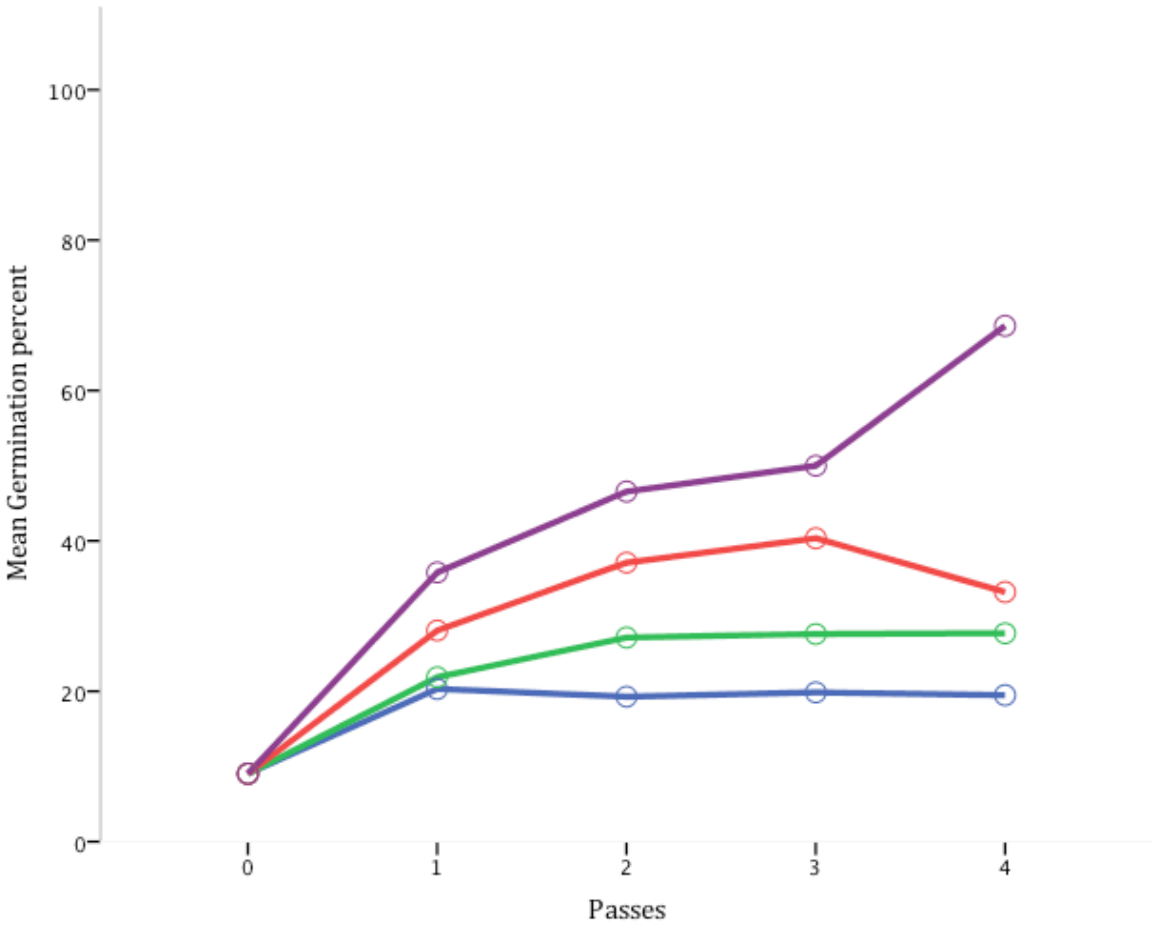

Figure 3.11. Mean germination of L. ambigua seed (%) in response to the interaction between the number of passes and the speed of the scarifying machine. S [(blue)] (green) S [(red)] S [(purple)].

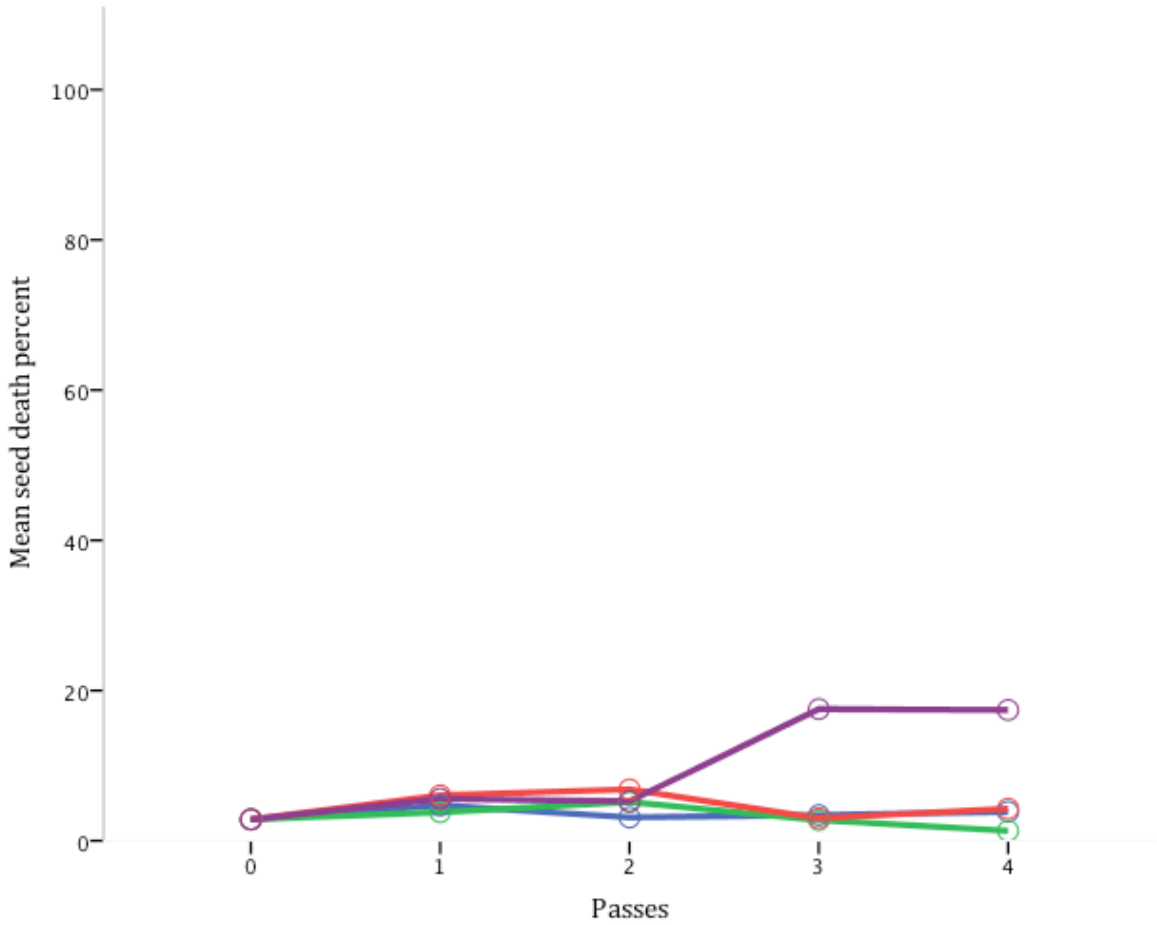

Figure 3.12. Average seed death of L. ambigua seed (%) in response to the interaction between number of passes and the speed of the scarifying machine. S [(blue)] (green) S [(red)] S [(purple)].

3.4 Discussion

The seed of *L. ambigua* has a very high level of hard seed dormancy with an average of 88% hard over the course of these experiments. Before broad acre sowings can proceed, a commercially viable method of breaking hard seed dormancy must be identified. The effectiveness of any particular method of rupturing dormancy is dependent on the composition of the seed coat, and there can be considerable variation between species as to the optimal treatment (Kimura and Islam, 2012). *L. ambigua* seed could be induced to germinate by the range of treatments applied in this series of

experiments, as summarised in Table 4.3. Applications of moist heat, simulated fire regimes and scarification techniques all significantly improved the germination level.

Table 3.3: Germination means for treatments applied to reduce hard seed in L. ambigua showing level of germination (%), non-viable seed and germination velocity.

Treatment	*Germination %*	*Seed death%*	*Hard%*	*Germination velocity %*
Untreated	*9.04 95%CI; (0-18.8)*	*2.84 95%CI; (0-6.3)*	*88.11 95%CI; (76.8-99.4)*	*0.83*
Freeze-thaw 1 cycle from: -9℃-14℃	*16.07 95%CI; (4-28.1)*	*0.57 95%CI; (0-2.3)*	*83.36 95%CI; (71.6-95.12)*	*11.04*
Heat 100°C dry heat for 3 hours	*25.88 95%CI; (12.5-39.3)*	*21.70 95%CI; (0-2.3)*	*52.42 95%CI; (44.4-60.5)*	*5.69*
Wet heat +smoke 100℃ water added to sand and smoke granules	*59.02 95%CI; (32.3-85.8)*	*0*	*40.98 95%CI; (14.2-67.7)*	*0.29*
Wet heat 100℃ added to sand	*70.85 95%CI; (55.3-86.4)*	*0*	*29.15 95%CI; (13.6-44.7)*	*0.29*
Fire simulation 140℃ max in moist environment and aerosol smoke incubation 100 minutes	*73.64 95%CI; (34.7-56.2)*	*6.46 95%CI; (0-16.5)*	*19.81 95%CI; (7.8-32)*	*0.278*
Scarification 2900 rpm, with 4 passes	*68.61 95%CI; (49.1-88.2)*	*17.42 95%CI; (0-42.3)*	*13.97 95%CI; (2.5-25.5)*	*19.31*

3.4.1 Difference between applications of wet heat and dry heat

The most remarkable result in this study was the very high germination response following exposure of seed to intense heat applied through water. This was significantly greater than that recorded for the application of dry heat. The moist heat differed to the dry heat in 4 ways; sand was used to buffer temperature, water was the conduit of the heat, the periods of exposure to the intense heat were shorter and in some treatments the active chemical compounds of smoke were applied.

Short intervals of intense heat applied through moist sand may more closely resemble the thermological pattern of a fire. In previous studies *Acacia saligna* and *Acacia longifolia*, which are endemic to fire prone environments, exhibited optimum germination levels at 100°c and 140°c respectively (Kimura and Islam, 2012). The optimum treatment of *A. saligna* had the seeds exposed to dry heat for 30 minutes while the *A. longifolia* treatment involved just 1 minute of exposure. Ellis (1973) reported good germination levels for two legume species with typically poor seed survival after heat exposure (*Medicago sativa* and *Trifolium pretense)* by exposing them to 104°c for only four minutes. Therefore potentially limited exposure to intense heat is beneficial, irrespective of the association of the species with fire disturbance.

Applying heat through water and sand to create a moist environment may be responsible for the increased germination in comparison to the dry heat methods. Patane and Gresta (2006) recorded an improved germination response of agricultural hard seeded legumes species *Astragalus hamosus* and *Medicago orbicularis* when they placed the seeds in hot water baths at temperatures of 70°C and 80°C. However, typically, studies aimed at identifying the best method to break dormancy for

agricultural implementation focus on applications of dry heat because they are generally less energy intensive.

In contrast, ecological studies investigating post fire regeneration of native species, often examine wet heat as a method to improve germination. Herrantz *et al* (1998) tested a number of dry heat and wet heat regimes on a range of legumes native to a fire prone ecosystem in Spain. Although there was a lot of interspecific variation recorded for these experiments, for the majority of species the hot water treatment was more effective at increasing germination. That study applied the hot water directly to a Petri dish containing the seeds, with no sand included to buffer.

Enright and Kintrup (2001) did experiment with both wet and dry heat treatments applied to seed collected from a fire prone ecosystem in south eastern Australia, that were buffered by soil. This study however was focused on determining seedling emergence, and species richness, not germination. Both wet and dry heat methods improved the density of seedlings and the richness compared to a control, however no difference was detected between these methods.

In this study the germination of *L. ambigua* seed when exposed to smoke granules, with no application of heat was effectively the same as for the control. Enright and Kintrup (2001) also included a treatment with an aqueous solution of smoke with no heat applied. This treatment (in contrast to our data) produced the highest seedling emergence and species richness in the study. However, through analyses of the species richness in each treatment it is evident that the proportion of legumes is substantially higher in the treatments where heat is applied. Therefore, although fire is considered the major disturbance regime in the native range of *L. ambigua*, the fynbos, and a wide

range of legumes have reported to increase germination in response to fire, we need to consider whether this is solely a product of the heat or whether the smoke is influential (Rutherford *et al.*, 2011).

3.4.2 Germination in response to fire and smoke

The maximum percentage germination was recorded when the seed of *L. ambigua* was exposed to a combination of fire and aerosol smoke at temperatures above 140°C in a moist environment. This was developed to simulate a fire. A wide range of plant species from the fynbos have been reported to increase their germination in response to fire (Enright and Kintrup, 2001). Seeds from these species have evolved to respond to either the physical or chemical cues provided by the heat and smoke the fire produces (Staden *et al.*, 2000). Further, in a number of species the interaction of particular levels of smoke and heat produced the best germination response suggesting that the effect could be additive or synergistic for the two factors (Crosti *et al.*, 2006). This is supported by my results, where greatest germination response is recorded when *L. ambigua* seed was exposed to a combination of heat and smoke. However the germination levels were effectively the same for exposure to boiling water, with and without the application of smoke extract. Van staden *et al* (1989) hypothesized that aerosol smoke may be more effective at stimulating germination than smoke extract. However Pennacchio *et al* (2007) argued that both aersol and granulated extracts are equally effective.

Exposure to cold smoke applied through granulated smoke extract did not improve germination of *L. ambigua* seed. The importance of smoke as an ingredient in breaking seed dormancy of plant species from fire prone environments was demonstrated by

Dixon *et al* (1995). This study improved the germination of 45 species native to fire prone environments by exposing them to cold smoke without the presence of heat by allowing smoke to cool before piping it into smoke tents. That study however did not include any members of the legume family. The responsible chemical compound within smoke *butenolide 3-methyl-2H- furo(2,3-c)pyran-2-one* has since been isolated and described by Flematti *et al* (2004). Since then the active smoke chemical has been demonstrated to enhance the germination of many genera from fire prone environments (Pennacchio *et al.*, 2007).

Roche *et al* (1998) extended the studies of Dixon *et al* (1995) and found that while germination of the legume *Hibbertia amplexicautis* improved with the application of cold smoke, it was less than when heat was also applied. Van staden (1989) similarly reported that while hard seeded legumes increased germination in response to smoke, it is unknown whether the physical fracturing and dessiccation of the hard seed coat was also required. Therefore it is unclear whether smoke is required in order to achieve the optimum germination response for *L. ambigua* (or other legumes), but it is clear from our results that heat and moisture are required.

3.4.3 Germination in response to changes in temperature

Valuable information on the response of seeds of *L. ambigua* to heat was gained from this study. High temperatures were required to increase germination and seeds appear to have a high temperature tolerance. These characteristics are quite novel in comparison to other perennial temperate legumes commonly encountered in agriculture.

Heat can cause the palisade layer to soften and separate from the mesophyll layer which lies underneath, and consequently cracks in the seed coat appear (Baskin and Baskin, 2001). High levels of temperature and length of exposure generally result in damage to the embryo and increased seed death (Patane and Gresta, 2006). The response of seed to temperature can be very specific to the species as the structure and physiognomy of the seed is very influential (Taylor and Ewing, 1988). The seed of *L. ambigua* displayed a comparatively high tolerance to heat and high temperatures were required to break dormancy.

The germination of *L. ambigua* decreased at 120°C as the time of exposure increased, as a clear consequence of the high level of seed mortality in these treatments. Therefore it seems that the majority of *L. ambigua* seed cannot tolerate temperatures above 120°C for longer than an hour. However this threshold is high in comparison to many other legume species. *M. Sativa* seed, for instance, experiences increased seed death in response to exposure to temperatures over 80°c for 30 minutes (Kimura and Islam, 2012).

Both the temperature and the time of exposure are thought to be factors in determining the effectiveness of a heat treatment to soften seed (Baskin and Baskin, 2001). However if the temperature is not high enough the seed coat will not rupture regardless of how long the seeds are exposed. For example Baskin and Baskin (2001) reported that *Trifolium pratense* and *Melitotus officinalis* seeds subjected to 50°C for 21 hours showed no increase in germination. Similarily *L. ambigua* seed showed no increase in germination at 60°C as the time exposed was increased. High temperatures were required to elicit a germination response in the dry heat experiment with the peak germination recorded at 100°C. In the simulated fire experiment, over three times

the germination response was recorded when the temperature was above 140°C, compared to 60°C. This suggests that 60°c, which is commonly reported as the maximum soil surface temperature in summer (Quinlivan, 1968), was not high enough to damage the seed coat of *L. ambigua.* Potentially this could also restrict seed softening *in situ* in response to environmental cues.

However for Mediterranean legumes, in natural settings it is reported that the majority of hard seed dormancy is broken by exposure to cycles of diurnal fluctuating temperatures (Quinlivan, 1968). This is discussed in greater detail in section 4. Similarly some studies into the artificial rupturing of seed coats report that the changes in temperature have more influence on breaking seed dormancy than exposure to extreme temperatures *per se* (Tiryaki and Topu, 2014).

The freeze-thaw method exposed the seeds to cycles of large fluctuations in temperature, aiming to shrink and swell the seed until the seed coat became brittle and eventually cracked (Baskin and Baskin, 2001). Kimura (2012) reported that exposing *M. sativa* seed to -80°C was more effective at increasing its germination than exposing it to heat treatments of 80°C. For *L. ambigua* seed no increase in germination or seed death was recorded when exposed to cycles of freezing and thawing. It is possible that the seeds were not exposed to a large enough temperature differential. The sand buffered the temperature changes and in some instances the temperature only moved between-1 °C and 13°C. Tiryaki *et al* (2014) proposed a method were optimal germination for *Lupinus albus* and *T. pratense* was achieved when seeds were exposed to a freezing temperature of -80°C followed by heat at 90°C for 5 seconds, to increase the temperature disparity. Although it is impossible that *L. ambigua* seed (or any other seed) would be faced with a similar temperature regime in nature, investigating the

mechanical effect of temperature changes on seed coat integrity may be useful to increase our understanding of the seed physiognomy and provides insight into how the seed may respond to environmental cues in the environment

3.4.4 Practical recommendations

Fire and smoke are commonly used to propagate native seed in nurseries, and to re-vegetate natural ecosystems in the heathlands of both South Africa and Australia (Dixon *et al.*, 1995). Additionally, smoke treatments can enhance the germination of some non-fire prone, species and is often applied to common vegetables (Enright and Kintrup, 2001). However exposure to fire has not previously been applied as a tool to break dormancy in broad scale agriculture and further investigation into the practical implications of large-scale adoption of this method would be required.

Scarification is the most common method to rupture seed coats of legumes in modern agriculture because of its wide availability and application to large quantities of seed (Patane and Gresta, 2006). Mechanical scarification, with four passes through the machine rotating at 2900 rpm, produced germination high enough to ensure successful stand establishment. A much more rapid germination velocity was also noted for scarification treatments, indicating that the seed coat possibly had multiple ruptures in response to this technique (Mott and McKeon, 1979). This is important to ensure uniformity of stand establishment (Ewing *et al.*, 2006).

Although the process of scarification is promising, other machinery and types of abrasive surfaces need to be investigated to find a viable method of rupturing the seed coat reliably, with reduced handling times and reduced losses. Often commercial seed

processing machines will employ a diamond chip covered disc to scarify and this could improve efficiency, as the quantity of seed required increases.

However currently as it stands the optimal scarification method identified in this study is time demanding and labor intensive because of the number of passes required. In the best-case scenario, this will significantly add to the cost of seed. In the worst case, it may make the production of germinable seed at a commercial level unviable.

Historically expensive seed severely limits the adoption of legume cultivars in dryland agriculture (Rolston, 2003). This expense can be offset in the future, if seed can be harvested from the stand and the seed used for future plantings or sale. However many regions are not suitable for high seed production (RIRDC, 2014). Alternatively the risk of investing in expensive seed can be reduced if there is a potential for farmers to only incur the cost once, and then have the stand regenerate from hard seed, similar to how an annual ley farming system operates. To assess the potential of this option we need to determine how the seed of *L. ambigua* responds to environmental induced softening when left in the field, and this is explored in section 4.

Section 4 Field softening of the seed of *Lebeckia ambigua*

4.1 Introduction

The seeds of *Lebeckia ambigua,* like many legumes, develop an impermeable "hard" seed coat that imposes dormancy by preventing the imbibition of water and therefore the immediate germination of the seeds when wet (Gama-Arachchige *et al.*, 2013). Dormant seeds form a reserve bank of viable seed until their dormancy is released by the softening of the seed coat (Ehrman and Cocks, 1996).

In field settings, softening occurs when the hard seed coat is ruptured in response to a series of well-ordered environmental triggers, however an understanding of the mechanisms involved and how they interact is still not complete (Taylor, 2005). The process appears to be acutely temperature dependent but light and moisture have been identified as physiological processes that can also impact the breakdown of hard seed (Baskin and Baskin, 2001, Hanbury *et al.*, 1999). Taylor (1981) proposed that two distinct temperature dependent processes are required. This two-stage model involved a preconditioning stage of high temperature, followed by cycles of fluctuating temperatures. The optimal temperature regime required to break dormancy has been shown to be specific to each species (Taylor, 2004).

Further there can also be considerable variation within species (Taylor and Ewing, 1988). Genetic factors and environmental conditions during seed development can, be responsible for differences in the breakdown patterns between different seed populations even when the conditions experienced during softening are the same (Taylor, 2005). In particular the location of the maternal plant and the year of production can have considerable impact on hard seed breakdown (Taylor and Ewing,

1988). Taylor (1996) reported the maternal environment to be potentially more important in determining the rate of seed softening than the conditions of seed softening for cultivars from the *Medicago* genus. It has been shown that the latitude, elevation, day length, presence of herbicide, length of growing season, soil moisture and soil nutrients of the parent plant can directly influence the germination dynamics of a number of plant species (Baskin and Baskin, 2001). The percentage of hard seed of *Trifolium subterraneum* has been observed to reduce as the parent plant is subjected to more stressful conditions (Taylor and Ewing, 1988).

In this context, *L. ambigua* seed has been collected from plant populations geographically separated by 250km, therefore it is possible that these populations have diverged from each other to display different ranges of phenotypic variation. Seed collected from different provenances may have been subjected to an isolated evolutionary niche, leading to differences in hard seed behavior based on their ancestral growing conditions (Bischoff *et al.*, 2006). Loi *et al* (1999) investigated the hard seed breakdown of four provenances of *Biserrula pelecinus* seed and found that only one accession acted significantly different. In contrast Revell *et al* (1999) demonstrated that *Ornithopus compressus* seed originally collected from two different plants within the same geographical area displayed vastly different hard seed dynamics. As well as phenotypic variation, geographic provenance variation can also be genomic if the genetic isolation is for a long enough period of time for the genomes to differentiate (Linhart and Grant, 1996).

Implementation of legume cultivars with a high persistence of hard seed is considered a critical component to the success of the ley farming systems of southern Australia (Puckridge and French, 1983). A number of techniques have been developed to

evaluate the hard seed characteristics of annual legume cultivars over the last 70 years to identify and select cultivars with an ideal hard seed break down response. Aitken (1939) was the first to demonstrate a softening of *T. subterraneum* seed in response to fluctuating temperatures by inserting the seed into fly-wire mesh pockets and placing them in the field. Quinlivan (1968) used temperature-regulated cabinets to effectively soften seeds of *T. subterraneum,* demonstrating that the temperature fluctuations in isolation of other physiological variation could drive seed softening. No previous studies have investigated the hard seed break down of a perennial legume species in response to field conditions. However, by applying the methods developed to investigate the hard seed dynamics of annual legumes to the seed of *L. ambigua* we aim to determine the specific breakdown response in this species and the variation for this trait within the collected germplasm.

4.2Method

4.2.1 Collection of germplasm and establishment in plots in WA

Seeds of *L. ambigua* were collected by Murdoch university researchers from four sites (Figure 4.1) along the western cape of South Africa in November 2007, where it was found naturally growing. The annual rainfall and GPS coordinates were recorded for each location and soil pH was determined using a CSIRO universal field kit (Table 4.1) The seed was then grown under quarantine conditions within the Murdoch University CRS glasshouse.

Fifteen plants were transplanted from the glasshouse into Murdoch University research plots (32°04'04.3"S 115°50'24.8"E) as separate, spaced plants in the winter of 2008.

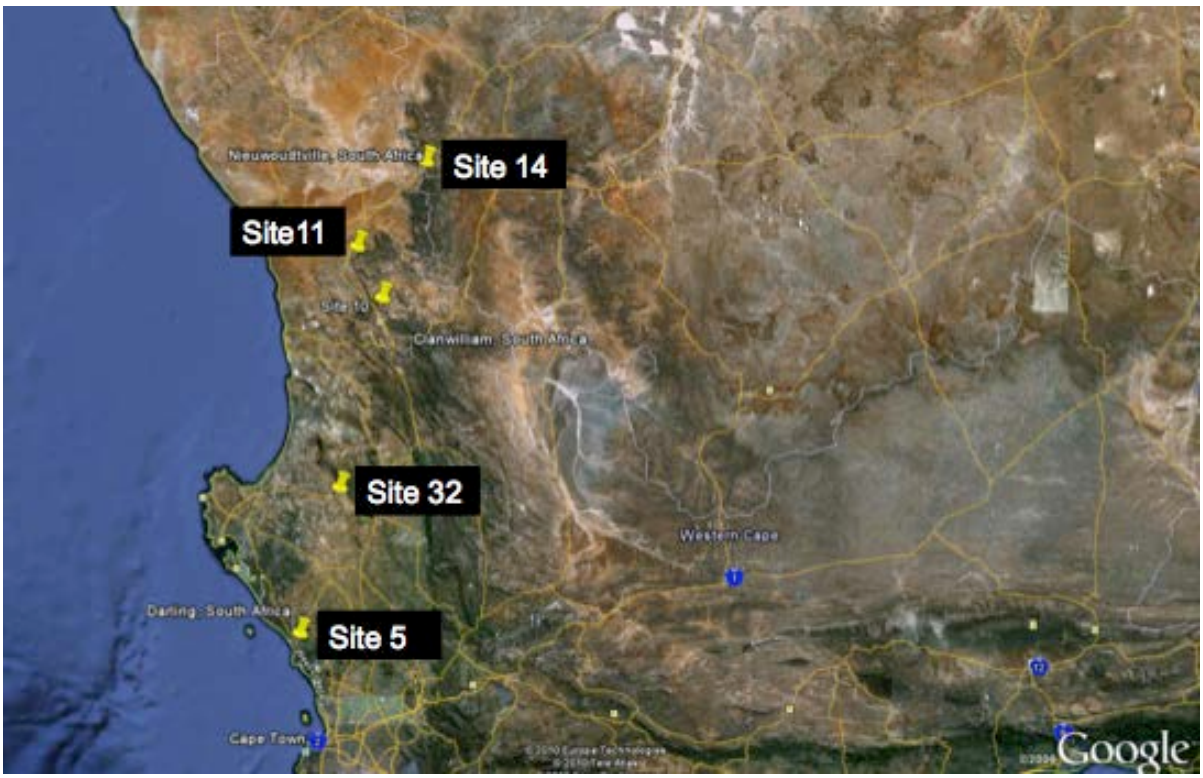

Figure 4.1. The location of the collection sites in South Africa. The red lines indicate the latitude and how that relates to locations in Western Australia.

The spaced plants were rainfed and left unfertilized which allowed assessment of their persistence in Australian conditions. Seed was then harvested by hand from the mature plants in December, 2008. The seed was dehulled and scarified. The seed from nine plants was selected and hand sown into quadrants in July 2012. Each subsequent quadrant has the seed lineage recorded. Consequently the original provenance of every adult plant in the quadrant is traceable. One application of 3:1 super potash was applied by hand during establishment of the quadrants and the plants that persisted were solely rainfed see Figure 4.11 for rainfall pattern. No herbicide or pesticide was used in the quadrants. Weeds were routinely removed from the plot to reduce the competition for water and reduce the fuel load in summer.

4.2.2 Harvesting

Eighteen mature individual adult plants within the established quadrants were targeted for seed collection in 2014. The selected plants were distributed throughout the different quadrants so that the hard seed breakdown of the different provenances could be compared. Each selected plant, its original collection site and lineage is listed in Table 4.1.

Table 4.1. The accession lines of L. ambigua in the Murdoch plots and environmental information on original collection sites.

South African collection site	Coordinates	pH ($BaSO_4$-CSIRO field kit	Annual rainfall	Parent plant	Re- plant block	Individual number
5	33° 29'21";18° 19'36"	6.5	400-450mm	4.3	9	8,11,14
11	31° 47'59";18°37'16"	5.5	200mm	2.2	1	1,2,3
				2.4	3	5,6,7
14	31° 26'47";19° 8'41"	5.5	350-400mm	1.9	4	9,10
32	32°50'36";18°37'99"	6	400mm	2.1	7	15,16
				2.5	2	4
				2.9	6	17
				3.2	10	12,13,18

To collect the seed from the plants, a black plastic sheet was placed underneath each individual on 18/12/2013 while the pods were still maturing (Figure 4.2). On the 10/1 /2014 any material that had fallen from the plant onto the sheet was swept into bags and consequently captured. This method ensured that a high percentage of the total dropped pods and shattered seed was captured, and that there was a high probability that the seed collected had reached maturity. Of the 18 plants targeted for collection only 12 were subsequently used in the study. Issues with either the capture or the

cleaning of the seed, resulting in insufficient seed to conduct a robust experiment were encountered for plants 5,6,7,14,17and 18.

A soft seeded form of *Ornithopus sativus* cv Emena that was also growing in the experimental plot was hand harvested from several plants selected at random on the 17/12/2013. This provided a comparison species for the field softening studies.

Harvested seed of *L. ambigua* that had not naturally shattered was separated from the pods by rubbing it between two corrugated rubber surfaces. The seed was then separated from other incidentally collected material, such as plant debris, through the use of a wave aspirator. The cleaned samples of naked seed were then weighed and sub sampled using an Encott 2 way sample divider.

Figure 4.2. The method of collecting the seed from L. ambigua in the Murdoch plots in January 2014. Any pods dropped from the plant were captured by the sheet and then collected.

4.2.3 Field softening trials

The sub samples were counted to get an estimate of the total seed captured for each plant and this number was used to estimate how many seeds to allocate to each repetition. The seed harvested from the *O. sativus* plants was not separated from pods, but was broken into individual pod segments. For each individual plant the seeds were then randomly sorted into 5 treatments with 3 replicates using a seed counter.

The counted seeds were placed into pockets of flywire micromesh. There were 12 flywire strips (not including treatment 1 which was never placed in the plot) each containing a repetition of each individual plant along its length in a random order. Each strip was labeled with a treatment number (2-5) and a replicate number (1-3) and buried to 1cm in the Murdoch plot on 23/1/2014, in a random order (Figure 4.3). Chicken wire was used to cover the strips of seeds to prevent interference from animals and the perimeter was sprayed with Regent on the 29/1/2014 to prevent ant predation.

Figure 4.3. The flywire strips lightly buried in the field. (Left) and covered with chicken wire (Right).

Strips of seeds were exposed to the field conditions and then sampled at treatment intervals. The amount of time that the seeds were exposed to the field conditions is

recorded in Table 4.2. When each treatment was sampled the seeds were placed on a moist filter paper in a tray with excess water, in a 15°C room, to provide conditions for germination. The seeds were checked for germination each week until germination ceased. Seeds were considered to have germinated if they had imbibed and a soft radicle at least 2mm in length had protruded. Any seed that had not imbibed or germinated was considered to be viable but dormant.

Table 4.2. Sampling times for each treatment

Treatment	**T1**	**T2**	**T3**	**T4**	**T5**
Season Sampled	Initial	Autumn	Winter	Spring	Autumn
Date Sampled	10/1/2014	19/3/2014	11/7/2014	17/10/2014	27/2/2015
Weeks exposed	0	8	24	38	57

Weather data was collected from Jandakot airport weather station (32.11°s, 115.87°E). This station is within 4km of the experimental site. A thermocron temperature and moisture logger was buried next to the seeds at the same depth. Unfortunately the device was faulty and did not record any data. For this reason soil temperature information is supplied by the previous work of Nutt (2012).

4.2.4 Statistical treatment and data analysis

The mean percentage of germinated seed for each adult *L.ambigua* plant was calculated across the 3 replicates for all of the five times of sampling. This data was analysed as a "repeated measures analysis of variance" experiment with SPSS version 22 (IBM, 2013)

where the time exposed to the field conditions is applied as the repeated factor. This provided a pattern of hard seed breakdown for each individual *L. ambigua* plant.

The null hypothesis states that if the provenance had no affect on hard seed breakdown, the means of all 4 provenances will be equal across the different times of sampling. This analysis of variance assumes that the dependent variable has a normal distribution, all recorded data from different individuals should be independent and that factors within should demonstrate sphericity (Ramsey and Schafer, 2012). The sphericity of the analysis was violated as per Mauchley's test so the degrees of freedom were recalculated using Greenhouse Geiser epsilon (Ramsey and Schafer, 2012).

4.3 Results

4.3.1 Patterns of hard seed breakdown

All of the *L. ambigua* plants displayed a high percentage of initial hard seed, and an incremental pattern of breakdown over time, as the seed was exposed to field conditions. A small reduction in hard seed for some of the provenances was recorded in the first summer-autumn period of exposure (Figure 4.4). However the major reduction in percentage hard seed for all the provenances was detected over the following spring-summer period.

O. sativus seed produced in the same location under the same conditions recorded between 50 and 65% germination when sampled in the first 24 weeks. After that period no germination was detected for *O. sativus* and the remaining seed was presumed to be non-viable.

Variation in the hard seed breakdown pattern was observed from the provenances from which the original seed was collected (Table 4.3). A repeat measures analysis of variance (Table 4.4) produced a significant p Value for the time of sampling, the provenance and the interaction between the two.

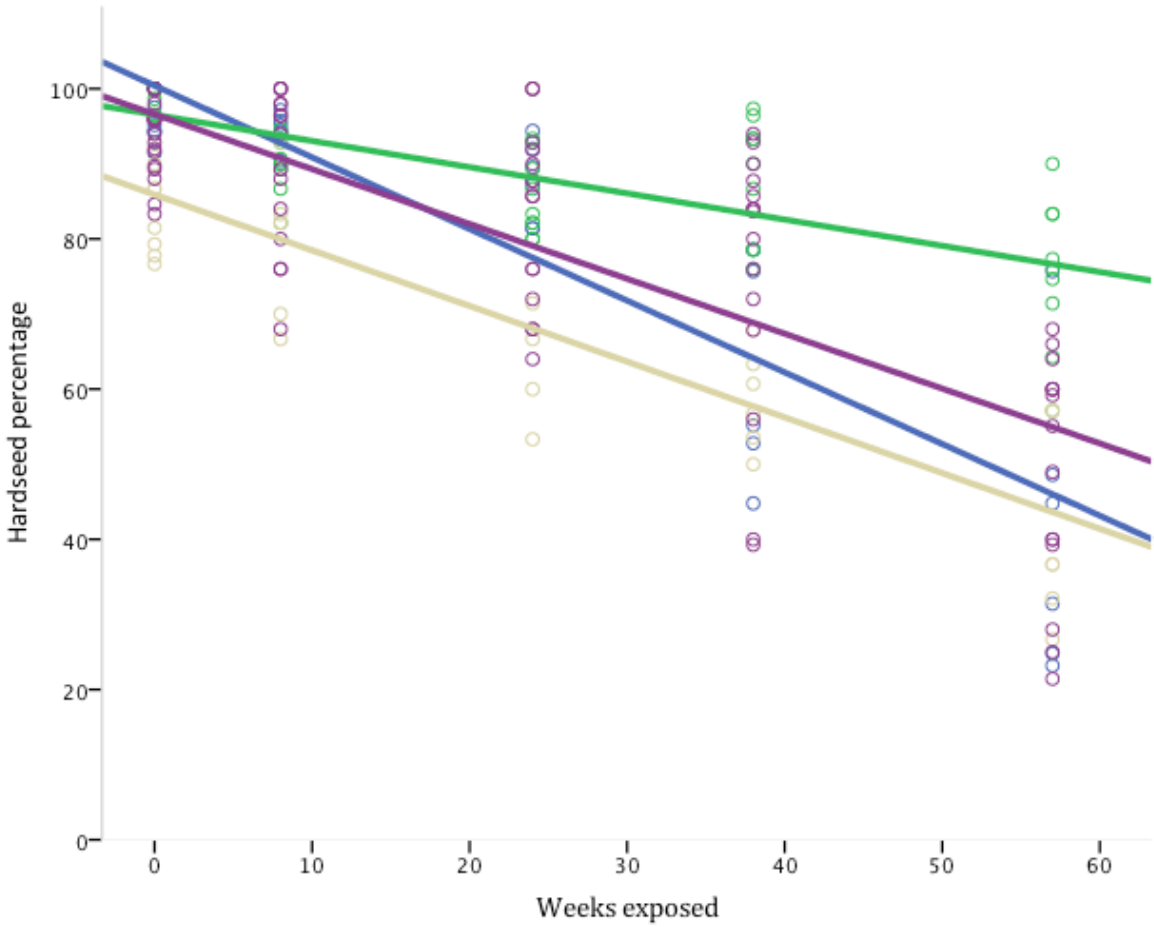

Figure 4.4. Hard seed breakdown pattern for different provenances of L. ambigua. Gold is sample site 14; Blue is sample site 5; Purple is sample site 32; Green is sample site 11.

A significant p value for the interaction between provenance and sampling time provides strong evidence that the patterns of breakdown are not equal for all of the provenances. Through this analysis we can be confident that the mean hard seed percentage of one or more of the provenances was not statistically equal. Post hoc tests to statistically compare each provenance are not possible with this analysis model (M

Scheiner and Gurevitch, 2001). Provenance 14 showed the lowest level of initial hard seed in the population and the lowest level throughout the experiment.

Table 4.3. The R^2 values of each provenance determined by the data on Figure 4.4

Provenance	***R^2***
5	*0.747*
32	*0.537*
14	*0.687*
11	*0.582*

Table 4.4. Analysis of variance on repeated measures model. Greenhouse Geisner applied to the degrees of freedom accounts for a lack of sphericity.

Source	***Type III Sum of squares***	***Sphericity assumed df***	***Mean square***	***F***	***Sig***	***Greenhouse Geisner df***	***Corrected P***
Time of sampling	*10994.879*	*4*	*2748.72*	*32.19*	*0.00*	*2.868*	*0.00*
Provenance	*8328.723*	*3*	*2776.241*	*9.633*	*0.00*	*-*	*0.00*
*Time of sampling *provenance*	*2846.441*	*12*	*237.203*	*2.778*	*0.002*	*8.603*	*0.007*
Error (Time of sampling)	*11613.081*	*136*	*85.39*			*97.498*	

4.3.2 Variation within provenances

The percentage of hard seed recorded for the fourth time of sampling after 56 weeks of burial was very different between the two individual plants originating from Provenance 5 (Figure 4.5). However no significant difference was seen between the hard seed breakdown for the rest of the times of sampling.

Considerable variation in the persistence of hard seed was detected within provenance 32. At the original collection site of 32, two plants that provided seed had distinctly different appearances and were subsequently labeled 32.1a and 32.1b. These were 50m apart in their original habitat. Plant 4, 12 and 13 are derived from the progeny of 32.1b while plants 15 and 16 parentage can be traced to 32.1a. The individuals sampled from 32.1a recorded consistently higher levels of hard seed than those of 32.1b across all of the times of sampling (Figure 4.6).

Whilst provenance 14 displayed the lowest initial level of hard seed, the two plants sampled from this provenance showed differences in breakdown over the first autumn period. However the level of hard seed was similar by the end of the following summer (Figure 4.7).

Provenance 11 showed a significantly higher persistence of hard seed over the time exposed compared to the other provenances. This provenance came from the most arid of the collection sites (Table 4.1). The three plants sampled for this provenance showed similar levels of residual hard seed throughout the time exposed to the field environment (Figure 4.8).

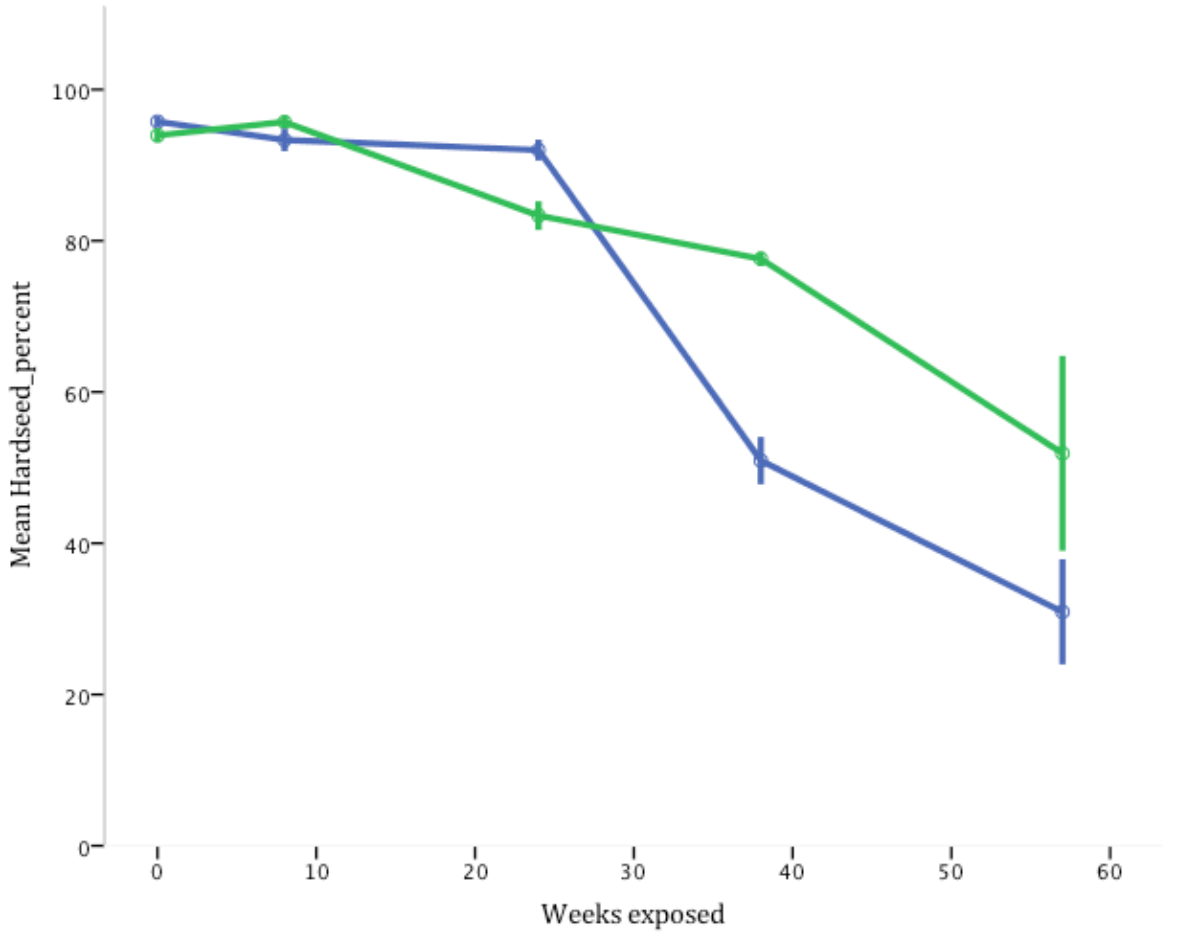

Figure 4.5. Cumulative hard seed breakdown percentage calculated over time exposed to field conditions for the seed of indiviually spaced plants; Plant number 8 (■) Plant 11(■) which are the progeny of seed collected from site 5 in South africa. ± 1 standard error.

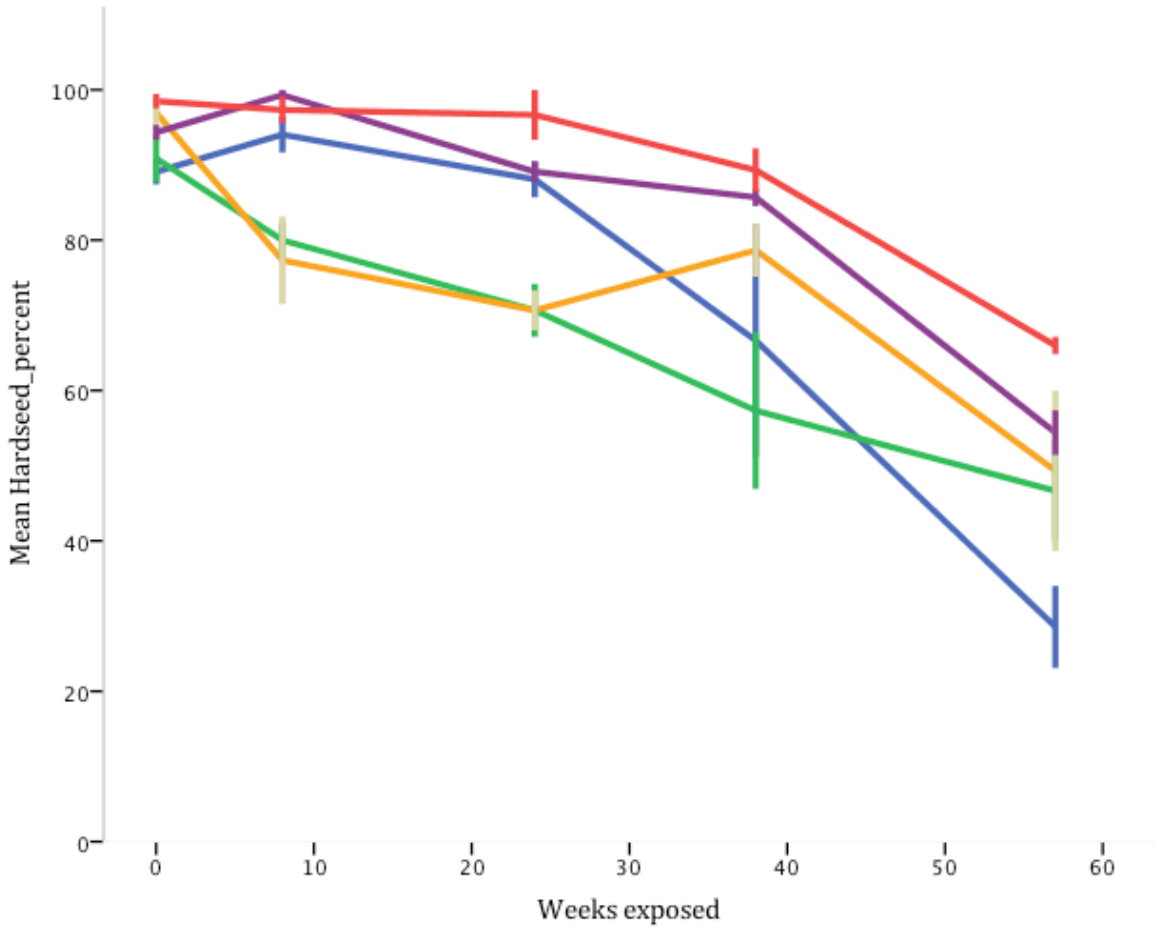

Figure 4.6. Cummualtive hard seed breakdown percentage calculated for time exposed for seed of individual spaced plants; Plant 4 (■), Plant 12 (■), Plant 13 (■), which are the progeny from seed originally collected from site 32.1b in South Africa and individually spaced plants Plant 15 (■), Plant 16(■)which are the progeny from seed originally collected from site Provenance 32.1a in South Africa ± 1 standard error.

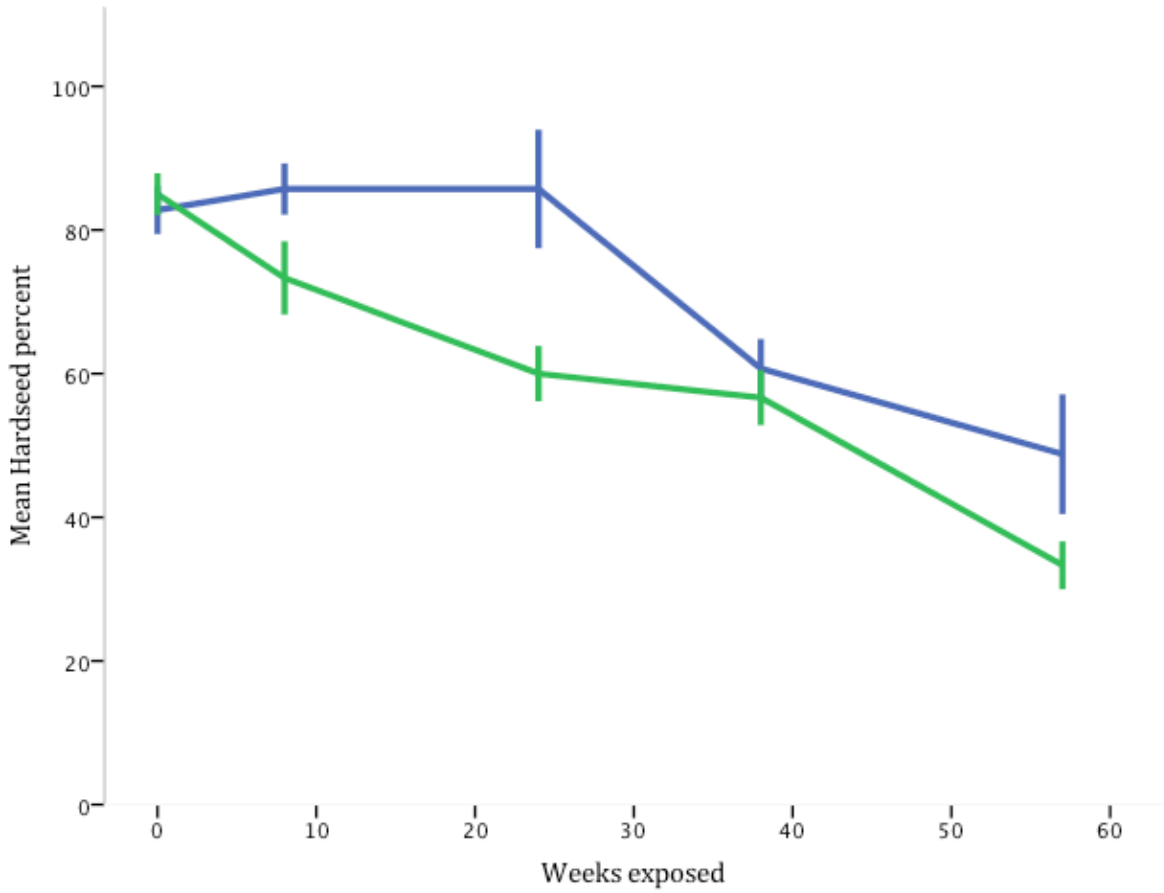

Figure 4.7. Cummulative hard seed breakdown percentage calculated over time exposed to field conditions for the seed of indiviually spaced plants; Plant 9 (■), Plant 10 (■)which are the progeny of seed collected from site 14 in South Africa. ± 1 standard error.

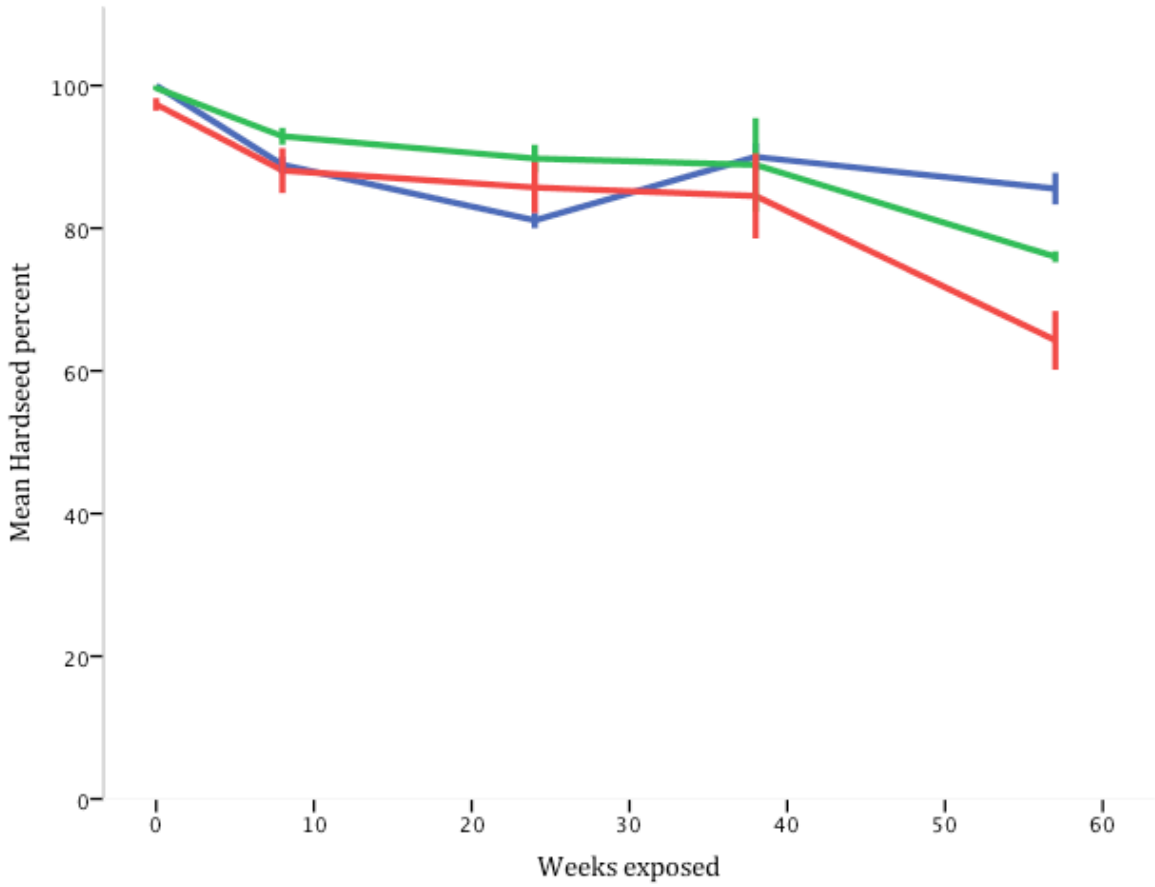

Figure 4.8. Cummulative hard seed breakdown percentage calculated over time exposed to field conditions for the seed of indiviually spaced plants; Plant 1 (■) Plant 2 (■) Plant 3 (■) which are progeny of seed originally collected from site 11 in South Africa. ± 1 standard error.

3.3.2 Field conditions that impact seed softening

The environmental conditions that could be responsible for driving the breakdown of hard seed were recorded. The greatest diurnal fluctuations in soil temperature were observed in the January-March period (Figure 4.9). During this period the soil temperature ranged from a minimum of 20°C to a maximum of 53°C 1cm below the soil surface where the seeds were placed. The winter–spring period displayed a less pronounced soil temperature fluctuation. Burial at only 1cm buffered the temperature extremes considerably, compared to the soil surface, particularly during the warmer months.

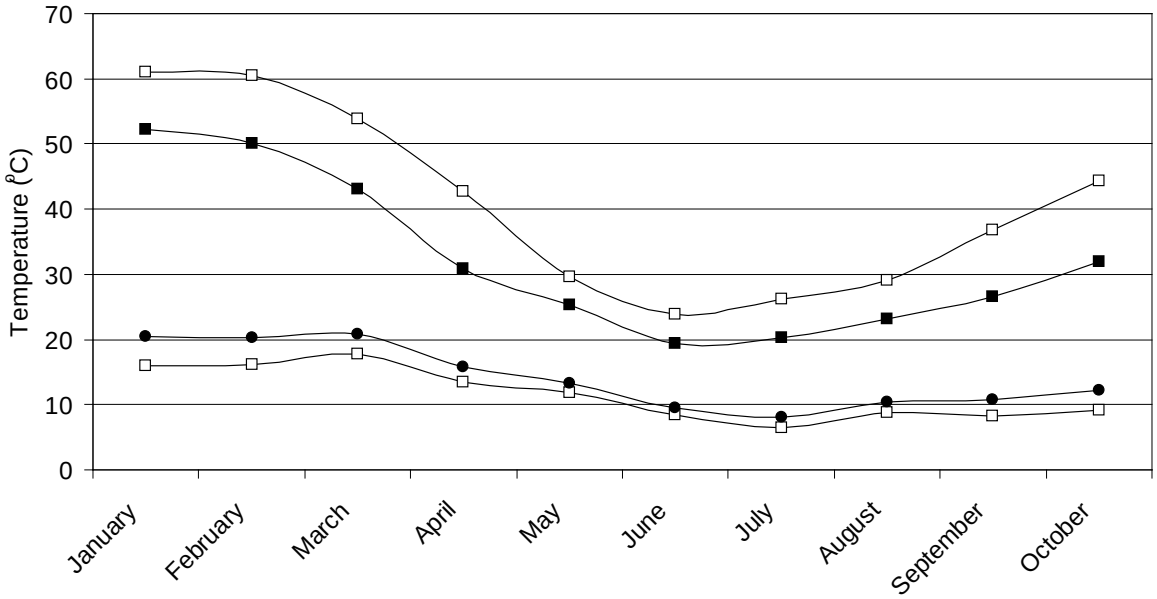

Figure 4.9. Average daily soil maximum and minimum soil temperatures, temperatures at the soil surface (□) and 1cm below the surface (■/□.Data collected from Medina (32.23°s,115.80°E) 2005 (Nutt, 2012)

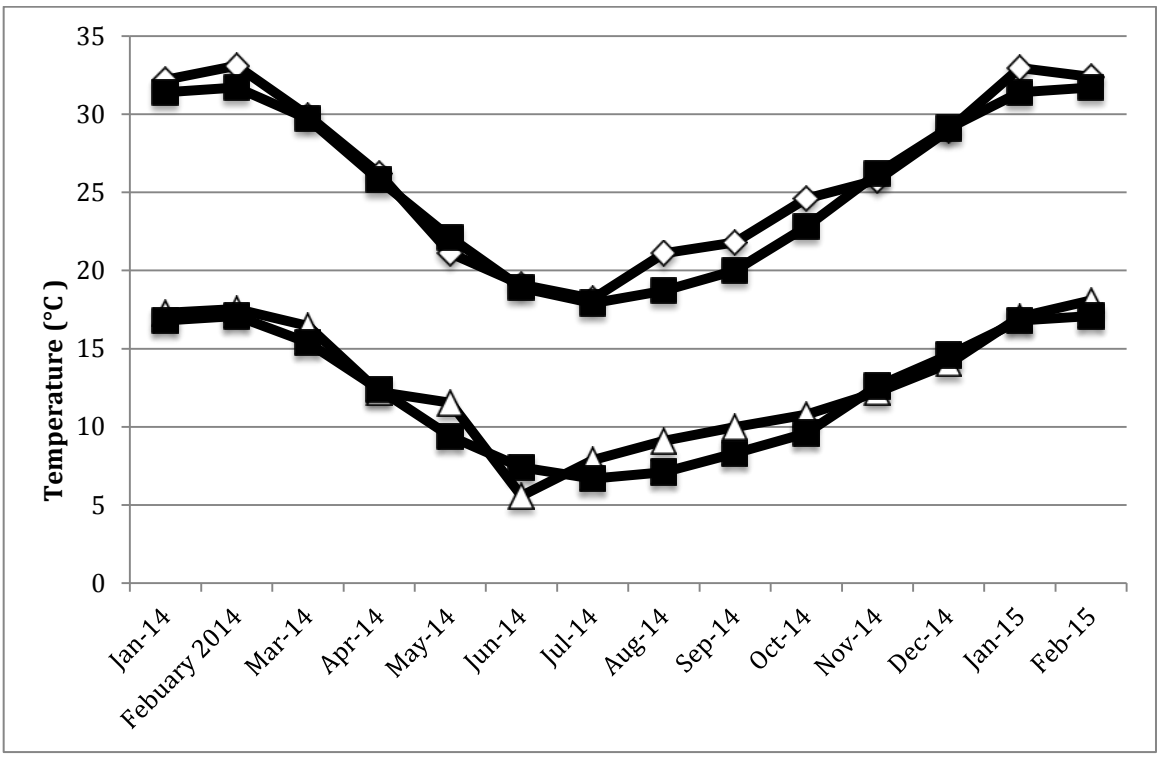

Figure 4.10. The monthly average maximum and minimum air temperature recorded for Jandakot Airport weather station in 2014/2015 (□) compared to the long term average (■).

The average monthly minimum and maximum air temperatures encountered in the trial period were very close to the long-term average recorded for the weather station (Figure 4.10). No rain was recorded for the summer months of 2014. Isolated rain

events occurred during the autumn period of 2014 and the summer period of 2015 (Figure 4.11). The year the seeds were produced (2013) recorded very little rain for June, but high rainfall for spring.

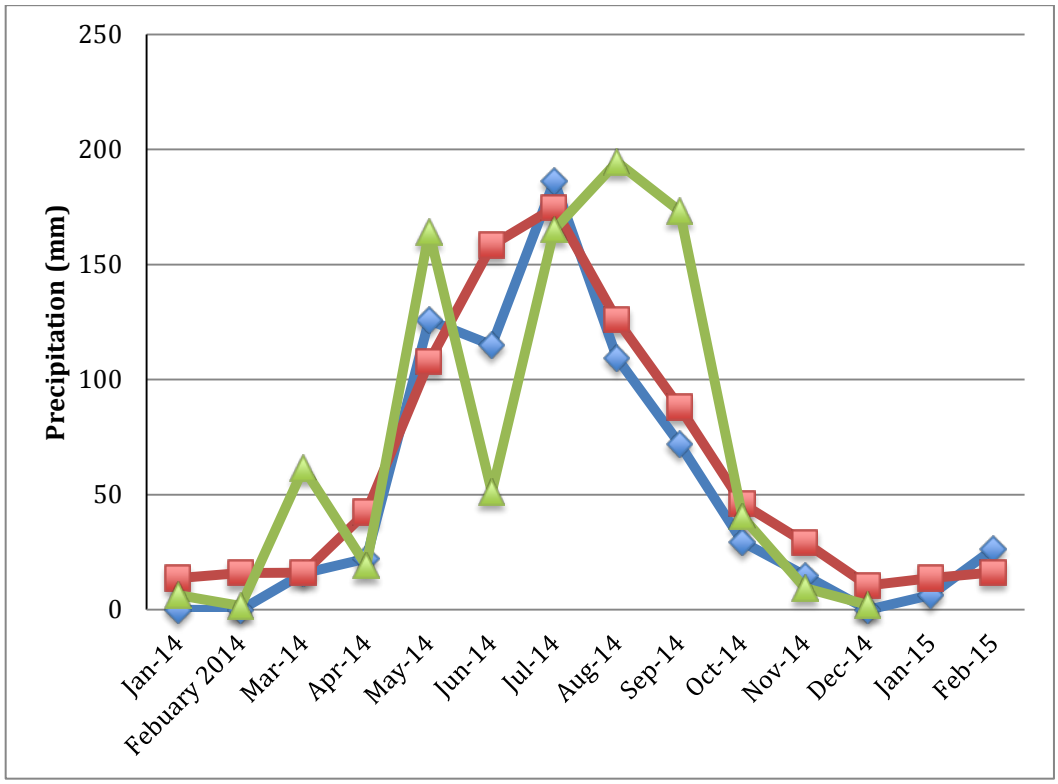

Figure 4.11. Total monthly rainfall 2014/2015 (■) and 2013 (■) for Jandakot airport weather station compared to the long-term average (■).

4.4 Discussion

In this study, the seed population of *L. ambigua* was determined to have an initial hard seed percentage of 93% (averaged across all provenances) that reduced to 53% after 57 weeks of exposure to field conditions. In comparison *Bituminaria bituminosa,* another perennial legume that is in the process of agricultural domestication, has a primary dormancy of 70%. This lasts for a period of 3 months before germination proceeds at near 100% (Castello *et al.*, 2013). The initial level of hard seed in *L. ambigua* is considerably higher than that encountered in perennial legumes *Medicago*

sativa (Kimura and Islam, 2012) and *Trifolium repens* (Harris *et al.*, 1987) and similar to *Lotus corniculatus* (Ollerton and Lack, 1996) which are all commonly used in agriculture.

No studies of the hard seed breakdown in response to field softening have been reported with these perennial legumes. This is likely due to the environmental influence on hard seed breakdown is not normally required for adequate agricultural management of perennial legumes. However the breakdown of the hard seed of *L. ambigua* can be compared to annual legume species, which are used in ley farming systems. Nutt (2012) reported that over the course of a year under field conditions, seed of Erica and Margurita cultivars of *O. sativus (French serradella)* dropped between 20-30% from their initial hard seed levels of 90%. Loi *et al* (1999) found that over the first year of field softening *Biserrula pelecinus cv* Casbah seed remained 90% hard, reducing only 8% from the initial level. In comparison, in the same study, *Trifolium subterraneum cv* Dalkeith recorded an initial hard seed percentage of 98% but this dropped to 18% over 12 months.

The hard seed level of *L. ambigua* dropped to 83% by winter after 24 weeks of field exposure during the first summer-spring softening period. Comparatively, Casbah and Dalkeith had hard seed percentages of 98% and 31% respectively at the beginning of winter in their first year of field exposure (Loi *et al.*, 1999). However in that study the seed was not placed into the field setting until March, and therefore had no exposure to the summer softening environment. Further, the seeds were buried to a depth of 2cm, not 1cm as in our study. *L. ambigua* therefore seems to uniquely behave more as a hard-seeded annual legume in its seed ecology, than in the expected manner of a domesticated perennial legume.

4.4.1 Genetic variation based on site of collection

The pattern of hard seed breakdown exhibited large variation between the seeds of different individual adult plants of *L. ambigua*. The statistical analysis showed that this variation was dependent on the provenance from which the original seed was collected.

As environmental conditions can vary extensively within a natural range, geographical separation of discrete plant populations can lead to phenotypic and genetic variation within a species (Morley-Bunker, 1980, Loha *et al.*, 2006). The Cape Floristic Region in particular has a highly varied landscape, giving rise to a large and rich biodiversity with high levels of localised endemism (Sprent *et al.*, 2010, Reinten and Coetzee, 2002). Fitness related traits such as seed phenology are thought to be the most strongly influenced by localized environmental factors, often displaying the largest genetic differentiation (Linhart and Grant, 1996). Bischoff *et al* (2006) concluded that germination, and the associated regulations which synchronise germination, are traits that will vary the most among geographically separated populations.

Differences in germination among provenances can be inflated by heterogeneous environmental conditions (Bischoff *et al.*, 2006). In this study the provenance of *L. ambigua* collected from the lowest annual rainfall displayed the highest persistence of hard seed over the course of field exposure. The importance of hardseedness in annual plants is potentially increased in sites were the rainfall is less reliable, as it provides insurance against sub-optimal seasons (Smith *et al.*, 1995). Comparatively, retaining more hard seeds can be disadvantageous to plant establishment if the environment has consistent and sustainable rainfall each year (Hanbury *et al.*, 1999). This result supports the view that differentiation between geographically separated

subpopulations is primarily the result of different selection regimes imposed by each environment (Linhart and Grant, 1996).

Local adaptation of fitness-related traits has been observed over a distance of only a few metres (Linhart and Grant, 1996). In this study two adult *L. ambgiua* plants within provenance 32 yielded quite different patterns of hard seed breakdown, despite growing less then 50m from each other. Large phenotypic and genetic variation within a small localized area may be explained by an additional fitness benefit conferred through the occurrence of many genotypes as opposed to one general purpose genotype (Joshi *et al.*, 2001). Differences in life history and breeding systems can separate plants into discrete populations even though they share the same geographic space (Linhart and Grant, 1996). For example, different flowering times will remove the possibility of cross pollination between individuals (Linhart and Grant, 1996).

Both cross-pollination and self-compatibility are potentially occurring among populations of *L. ambigua* (Section 2). No information was collected that would allow an estimation of the levels of cross-pollination in these experiments. It is possible that a level of cross pollination between 2009 and 2014 could have lead to new genetic compositions in the plants from different provenances.

The pattern of genetic variation is of fundamental importance in adapting a plant for agronomic use (Loha *et al.*, 2006). A detailed analysis of the within species variation allows us to approximate the limits of hardseedness for *L. ambigua* and relating this variation to the original collection sites identifies the ideal seed sources for the targeted agricultural areas and management strategy (Quinlivan, 1968).

4.4.2 Environmental influence on the expression of hard seed dormancy

The ancestral growing conditions of *L. ambigua* have ostensibly encouraged the production of high levels of hard seed. Hard seed dormancy is known to be an important aspect of a species fitness providing a range of ecological advantages and is a commonly encountered feature of annual legumes that have evolved in regions with Mediterranean-type climates (Enright *et al.*, 2014). The environmental regulation of hard seed breakdown can prevent germination during unfavorable conditions, instead encouraging the majority of seeds to synchronise their germination within a season that provides them with the greatest chance of survival and subsequent reproductive success (Baskin and Baskin, 2001).

The environmental factors that influence the proportion of hard seed and its break down can vary greatly between years and locations. In this study the growing conditions of the seed and the environment of softening were identical. However identifying how the seed interacts with the environmental variation is important to predict how the softening might proceed under different environments.

i) Maternal environment

There is strong evidence, from previous studies of annual legumes, that the persistence of hard seed can differ from year to year, based on the conditions of seed production (Taylor and Ewing, 1988). For annual *Medicago* species, the growing environment of the maternal plant has been shown to have a very large influence on the presence of initial hard seed (Taylor, 1996). In our study for the year of seed production (2013) the trial site received double the amount of rain in September compared to the long term

average. High rainfall in spring has been shown to be very influential on the initial levels of hard seed in populations of *T. subterraneum* (Taylor and Ewing, 1988). However this same correlation was not reported for *O. compressus* or *B. pelecinus* cultivars (Loi *et al.*, 1999).

Although this study only evaluated one year of seed production at one site to improve the understanding of the pattern of hard seed breakdown in *L. ambigua*, this trial should be repeated over a number of years (and perhaps with seed produced from different sites) to evaluate the level of influence of the maternal environment.

ii) Seed environment

It has been reported that, for most legume species, the majority of seed softening in the field is in response to exposure to two different temperature dependent processes. This two-stage model requires a preconditioning stage of high temperature followed by cycles of fluctuating temperatures. The initial high temperature subjects the seed to a level of thermal degradation which in isolation does not make the seed permeable, but conditions it to be more responsive to the fluctuating temperatures which follow (Hanbury *et al.*, 1999). Following this, the changes in temperature expand and contract the seed in a uniform manner that fractures the palisade cells at the strophiole, making the seed coat permeable (Taylor, 1981).

In southern Australia, the highest temperatures and range of fluctuations is over the summer- autumn period, where the soil surface temperatures can cycle between 70°c and 15°c diurnally if the soil is bare (Quinlivan, 1968). This regulates the majority of germination to proceed in the winter months (Hanbury *et al.*, 1999) when the majority

of the annual rainfall occurs (Caccetta *et al.*, 2010). However three of the four provenances of *L. ambigua* tested showed considerable break down of hard seed over the winter- spring period. It has been found that the best period for establishing *L. ambigua* is during the spring and not the winter. This is thought to be primarily because the seedlings are slow to establish and easily out-competed by winter dominant annuals. Further unlike annual species *L. ambigua* does not need to reach maturity and produce seed in the first spring to secure a genetic future, it will have an opportunity to set seed it future years. Winter–spring softening is unusual as at this point the fluctuation in soil temperatures are not great and the high temperatures that could be involved in preconditioning are likely to have occurred a long time previously. *L. ambigua* thus appears unusual in this regard.

The environmental conditions that could trigger a spring softening are not currently well understood. Potentially other environmental triggers are more important in breaking down hard seed in *L. ambigua*, and the process is less temperature dependent. This hypothesis could be tested by using temperature regulated cabinets to isolate the effect of temperature and identify the species requirement for a preconditioning temperature (Hanbury *et al.*, 1999).

Light and moisture can also influence the breakdown of hard seed dormancy (Baskin and Baskin, 2001). Taylor and Revell (1999) reported that light had an inhibitory effect on hard seed breakdown of *Ornithopus compressus*. In that study the rate of breakdown was increased in situations where the seeds were covered by soil and shielded from sunlight. However no mechanism was identified through which this inhibitory effect might operate. Equally, the increase in breakdown rate might have been the result of the insulating effects of burial. Burial can markedly alter the temperature and relative

humidity to which seeds are subjected as the depth is increased (Taylor and Ewing, 1988). Nutt (2012) also found that burial to 1cm increased rates of softening for *O. compressus*, and he postulated that this potentially could be explained by burial modifying the dynamics of seed moisture. The buried seeds would have retained a higher moisture content than those on the soil surface. Conversely, Loi (1999) found that across a range of annual legume species, the rate of softening reduced with deeper burial. Therefore the rate of softening in the response to burial seems to be species specific and associated with how reliant the seed is on fluctuating temperatures to break down the hard seed coat (Taylor and Ewing, 1988). Different depths of burial were not examined in this study, but in agricultural environments burial of seed is a common occurrence as result of livestock activity and soil disturbance through cropping (Wisheu *et al.*, 2000). Further investigation is required of the effects of burial of *L. ambigua* seeds on hard seed ecology.

4.4.3 Can *Lebeckia ambigua* regenerate from hard seed in a perennial ley

Perennial forage legumes are not widely used in ley farming systems (Cocks, 2001). Instead the perennial legume seed is artificially softened, if required, each time the perennial pasture phase is established (Ward, 2006). Conversely, the key indicators of success for annual pasture legume species in farming systems are the level of hardseededness at the end of the first summer and the number of years the seed remains hard (Loi *et al.*, 1999).

High levels of seed dormancy over summer are beneficial for all legumes because germination in response to isolated summer rain is highly likely to be detrimental to

survival in Mediterranean-type environments. This is because it is unlikely that a sufficient amount of rain will follow to sustain growth (Loi *et al.*, 1999). Isolated summer rains that trigger germination events are termed "false breaks". Historical rainfall records suggest that in southern Australia false breaks are frequent, occurring on average one in every three years (Chapman and Asseng, 2001). Our results indicate that only 17% of the *L. ambigua* seed population would potentially be lost to isolated rainfall events in the first summer.

In annuals a persistent seed bank of residual hard seed is also required to spontaneously regenerate the legume after the crop interval (Puckridge and French, 1983, Taylor, 2004). A reserve of 53% dormant *L. ambigua* seed after one year of softening is comparable to that of the hardseeded french serradella cultivars, and significantly higher than the successful sub clover cultivar Dalkeith. However whether enough seed of *L. ambigua* is released from dormancy at a favourable time to regenerate and form a useful pasture phase is unclear. This will depend on how much seed is produced, and the uniformity of release from dormancy. These factors are likely to be different between an annual and a perennial species.

As a small shrub, *L. ambigua* develops a sward with only a 2-3 individual plants per square metre. In comparison, the growing habit of annual legumes allows them to grow with significantly higher amount of plants per square metre (as many as 3000 for *B. pelecinus*), which can effectively carpet the field. Annual legumes also appear to produce much more seed per square metre than *L. ambigua* and that seed is more uniformly spread throughout the paddock. It is currently unclear how many seeds of *L. ambigua* will be required to be produced to sustain a sward. However, as a perennial

plant, *L. ambigua* could potentially accumulate seed over 4-5 years of growth, and require fewer plants to re-establish the pasture after perturbation.

The release from dormancy of *L. ambigua* seed is very gradual. Germination generally occurred in successive intervals without any large increases at any time point. In the natural environment this may be a prudent strategy, providing some insurance against any one timing strategy while also reducing within species competition (Smith *et al.*, 1995). However in agricultural systems it is considered that uniformity of germination is important, such that the pasture can regenerate with an adequate legume content (Nichols *et al.*, 2007). It remains to be seen whether this paradigm applies to hard seeded perennial legumes.

Section 5 Conclusion

The seed ecology of *Lebeckia ambigua* is a product of the ecological niche it occupies and the selection pressure it has faced throughout its evolution. *L. ambigua* is unusually highly fecund. Conventionally, high fecundity is a trait more associated with an annual life strategy and low fecundity is considered a major obstacle to the implementation of perennial plants in agriculture (DeHaan and Van Tassel, 2014). However, the fynbos is an environment characterized as having poor soil fertility, low rainfall, periods of drought and frequent fire disturbances. In this environment the probability that *L. ambigua* will successfully recruit is increased by the producing lots of seed, combined with exogenous physical dormancy to regulate germination (Wisheu *et al.*, 2000).

A high proportion of *L. ambigua* seed exhibits hard seed dormancy. Whilst ecologically sound this needs to be overcome to ensure rapid and reliable stand establishment in broadscale agriculture (Ewing *et al.*, 2006). The optimal scarification technique identified in this thesis yielded good germination results. However, this technique is time and labour intensive which could increase the cost of seed in a commercial setting. High priced seed is a major barrier to the adoption of dry land legume cultivars (Rolston, 2003). Therefore we need to further investigate methods to break seed dormancy, to reduce the cost of scarification.

It may however be possible to exploit the hard seed dynamics of the species so that the farmers will only have to establish the stand once. Potentially the subsequent perennial forage phase could be regenerated through a hard seed bank in the same manner that is used in annual ley farming systems. This is a novel management approach for perennial legumes, however as discussed the breeding system of *L. ambigua* has a number of traits that are analogous to that of an annual legume.

The pattern of hard seed breakdown displayed by *L. ambigua* is comparable to that recorded for *O. sativus* and *T. subterraneum,* annual legumes which are successfully implemented in annual ley systems. Variation in hard seed behavior is detected between provenances of *L. ambigua*. This potentially allows for efficient selection of a more appropriate and predictable breakdown. However further research incorporating more environmental situations, over longer periods and a better understanding of the events that drive spring softening are required in order to be confident that enough seed will germinate at the right time to successfully regenerate a perennial forage phase.

Interesting information was also gained into the response of *L. ambigua* seeds to heat. High temperatures are required to increase the germination of *L. ambigua* seed and fire simulation experiments produced the best germination response. These characteristics are quite novel in comparison to other perennial temperate legumes commonly encountered in agriculture. This possibly is as a consequence of evolution in a fire prone environment (Pierce and Cowling, 1991). Ecological studies into the post fire regeneration of native vegetation have found that the seeds of a number of legume species have evolved to trigger germination in response to the physical and chemical cues that the fire provides (Staden *et al.*, 2000). Fire can also indirectly improve germination by removing the above ground vegetation, so that soils are left bare and experience larger diurnal temperature fluctuations. Burning crop stubble is a commonly employed management technique employed in the Wheatbelt (Walsh and Newman, 2007). Therefore it is worth considering whether these routine burnings might be incorporated into a regeneration strategy.

A lack of productive perennial legumes adapted to the Wheatbelt is a major threat to the regions economic and environmental sustainability. Dear and Ewing (2008) conclude that when screening new forage plants for agricultural candidacy the establishment must be low risk and low cost. Traditional methods of establishing perennial forage legumes require a high economic outlay on seed each time the phase is established. A perennial ley would be superior to contemporary methods of perennial legume management as the new concept incorporates the low input, low risk aspects of annual ley farming which makes that enterprise successful in the challenging and highly variable environment of the Wheatbelt (Bathgate and Pannell, 2002).

Section 6. References

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Section 7 Appendix

A) Seed histology method

Seed were lightly scarified on the side not containing the lens region, using medium coarse sand paper. Seeds were soaked in a 20%(v/v) Hydrogen Chloride solution for 24hours then washed with distilled water and allowed to soak for 2 hours before fixing with glutaraldehyde 3%(v/v). They were then dehydrated in an ethanol xylene series,embedded in paraplast and fixed with a toloudine blue 0.05%(w/v). Paraplast units were then placed in a freezer for a week to set. Before sectioning the units were placed in Silken fabric softener for four hours to reduce the brittleness. Seeds were then sectioned and baked at 40°C on slides.