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Function, Ecology and Evolution of Seed Size in *Hakea* (Proteaceae)

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

Date ……14/12/2015…………….

Abstract

Southwestern Australia is one of the global biodiversity hotspots for its richness in endemic plants including grevilleas and hakeas. The genus *Hakea* (Proteaceae) has an evolutionary history of *c.*18 million years, and displays exceptional high functional diversity with adaptation to nutrient impoverished soil, granivory, droughts and recurrent fires. Seeds play a critical role in the life cycle of a plant. Research into function, ecology and evolution of seed size will provide critical insights into evolution, adaptation and diversification of flora in Southwestern Australia subject to nutrient impoverished and water deficient soil. In this thesis, I first assembled a time-based phylogeny for *Hakea* (Proteaceae) with 82 species, reconstructed ancestral state for six functional traits and determined their evolutionary trajectories in response to the advent or increasing presence of fire, seasonality, aridity, nectar-feeding birds and (in)vertebrate herbivores/granivores in Chapter 2. In Chapter 3, the interaction network between seed size and fecundity, postfire regeneration strategy, fruit size, plant height and serotiny (canopy seed storage) among 82 species of *Hakea* was investigated using Structural Equation Modelling (SEM) and correlated trait evolution analysis. From this analysis suggested that the ancestral *Hakea* arose *c*. 18 million years ago and was broad-leaved, non-spinescent and insect-pollinated, with medium-sized, serotinous fruits and resprouted after fire. The results also showed the causal correlations between seed size and fruit size (strong) and fecundity (weak) in *Hakea*, and between fecundity and postfire regeneration strategy (strong), but not between seed size and regeneration strategy. Overall, evolutionary histories have had most control over seed size variation among *Hakea* species. In two glasshouse experiments, I explored the relationship between capacity of tolerating cotyledon damage and seed size by removing part of cotyledons in *Hakea* species with different seed size in Chapter 4, assessed whether the critical nutrients in cotyledon can be compensated by external nutrients, and whether seed mass is consistently associated with a seedling's

ability to cope with cotyledon damage in Chapter 5. I found that small-seeded *Hakea* species may gain competitive advantages over larger those with seeds due to earlier germination, faster seedling emergence, rapid true leaf emergence and quicker transfer nutrient to early seedling's growth. Removal of the cotyledons but addition of a balanced nutrient solution failed to restore complete growth of any experimental *Hakea* species, but the root: shoot ratio was maintained, as was the extension of roots for the four species with smallest seeds. The cotyledons provide the essential nutrients, N, P and K, to support early growth of *Hakea* seedlings but other nutritional roles of the cotyledons are also implicated. *Hakea* species with small seeds can only tolerate damage to the cotyledons better than large seeds when they have ready access to soil nutrients.

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ANOVA	one-way analysis of variance
BEAST	Software - Bayesian Evolutionary Analysis Sampling Trees
BF	Bayes factor
BiSSE model	Binary State Speciation and Extinction
Ca	calcium
Cu	copper
DI water	deionised water
DNA	deoxyribonucleic acid
Fe	iron
GTR	general time reversible
HDP	highest density probability
K	Potassium
Ma	Millen years ago
MANOVA	Multivariate analysis of variance
MC tree	MC tree
MCMC	Monte Carlo Markov Chain
Mg	Magnesium
Mn	manganese
MUSCLE	MUltiple Sequence Comparison by Log Expectation
N	Nitrogen
Na	sodium
NCBI	National Center for Biotechnology Information
P	Phosphorus
PR	proliferation rate
S	sulphur
SEM	structural equation modelling
SEM	structural equation modelling
SND	stigma–nectary distance
SPR	speciation promotional rate
SPSS	Statistical Product and Service Solutions
SR	stabilization rate
TR	Transition rate
Zn	zinc
λ	Pagel’s lambda

Chapter 1: Ecology of seed size, and ecology and adaptation of *Hakea*: A general introduction

1.1. Background

1.1.1. Natural variation of seed size in plants

Seed size is a keystone plant trait that reflects a selective advantage in the evolutionary history of plant species to provide a fitness advantage in early seedling development and is evolutionarily associated with other plant attributes (Westoby *et al.* 1992). The study of the function, ecology and evolution of seed size has engaged the attention of many evolutionary biologists (Hodgson & Mackey 1986; Mazer 1989; Westoby *et al*. 1992). It is suggested, therefore, that the importance of seed size in determining seedling establishment success will depend on the relationship between seed size and other life history characteristics during early seedling survival. Many authors have proposed models that predict the evolution of an optimal seed size that maximizes the fitness of the plant (Janzen1977; Schaal 1980; Waller 1982; Pitelka *et al*. 1983; Stanton 1984 a, b; Antonovics & Schmitt 1986; Wolf *et al*. 1986; WuIff 1986b; Thompson 1987).

Optimal seed size models are based on three main assumptions. The first is the existence of a trade-off between seed mass and other plant traits, e.g., fruit size, growth form (larger seeds in taller species) and fecundity (numbers of seeds per plant) (Olsson 1960; Werner & Platt 1976; Bradford & Smith 1977; Stanton 1984b; Giles & Lefkovitch 1985; Giles & Gtsson 1988; El-ahmir *et al.* 2015), longevity and plant height (Leishmn *et al.* 1995; Moles *et al.* 2004; 2005). In addition, seed mass is correlated with several other plant traits, including seed nutrient content (seed reserves) (Esler *et al.* 1989; Mustart & Cowling 1992), dispersal biology (Hughes *et al.* 1994), cotyledon size and specific leaf area. The second assumption is the existence of a positive association between seed mass and fitness. The dependence of seedling fitness on seed size has been extensively supported by empirical evidence in several studies. For instance, larger seeds benefit seedlings against herbivory (Bonfil 1998), drought (Leishman & Westoby 1994; Seiwa *et al*. 2002), competition (Rees 1995; Jakobsson & Eriksson 2000), shading (Hewitt 1998; Bond *et al*. 1999) and nutrient limitation (Jurado & Westoby 1992; Seiwa 2000). Thus, variation in seed size among plant species in different environmental conditions has been reported. The third assumption is of phylogenetic constraints or niche conservatism (Lord *et al*. 1995, Hodgson & Mackey 1986). Seed size can be considered like any other trait to be shaped both by the natural history of the species and by the evolutionary history of the lineage and selection may favour greater allocation of maternal resources to each offspring (Harper 1977; Willson 1983; Westoby *et al*. 1990). The following paragraphs will discuss all of these concepts.

Seed mass is inversely related to the number of seeds (i.e., there is a strong negative relationship between seed mass and the number of seeds produced from by plant) (Shipley & Dion 1992; Greene & Johnson 1994; Rees 1995; Eriksson & Jakobsson 1998; Turnbull *et al*. 1999; Jakobsson & Eriksson 2000, 2003; Aarssen & Jordan 2001; Henery & Westoby 2001; Levine & Rees 2002; El-ahmir *et al*. 2015). This relationship has been illustrated for a wide range of species in many different ecosystems (Arssen & Jordan 2001; Henery & Westoby 2001). For instance, species with smaller seed mass can produce more seeds from a given reproductive effort; such plants tend to have short life cycles and commonly have greater persistence in the soil seed bank (Thompson & Grime 1979; Thompson 1984, 1987; Leishman *et al*. 2000). Although small-seeded species produce more seeds than do larger-seeded species, large seeds on the other hand can provide the energy and nutrients necessary for successful seedling establishment under many harsh establishment conditions (Harper *et al*. 1970; Willson, 1983; Westoby *et al*. 1992, 1996; Leishman *et al*. 2000). Disagreeing with the reasoning that there should be a negative relationship between seed size and fecundity (greater seed production, smaller seeds, and vice versa), Maddox & Antonovics (1983) highlighted that the simple correlation between seed size and number of seeds per plant was positive because both seed size and seed number increase with plant size (i.e., small plants may be constrained to produce smaller seeds or few seeds, but large plants can produce more and larger seeds). Consequently, plant adult height represents the trade-off between height benefits (such as higher seed production) and the costs of producing seed size (Moles *et al.* 2005). Nevertheless, in some families (e.g., Caryophyllaceae, Fabaceae and Poaceae), plant height is strongly correlated with seed size (Thompson & Rabinowitz 1989; Leishman *et al* 1995). The strength and the importance of this relationship in different environments and in different families may vary. Seed size might also be associated with physiological characteristics, such as leaf photosynthetic rate (Black *et al.* 1976), resource availability (water, soil nutrients, light) (Nobel 1980; Westoby *et al.* 1990; Hammond & Brown 1995) and plant longevity (Leishman *et al.* 1995; Moles *et al.* 2005). Although McIntosh (2002) interpreted different correlations in the sizes of several reproductive characters where noted that the seed mass is not correlated with plant size, others indicated that positive or negative correlations between these variables have been associated with improved resource availability (Lamont *et al.* 1985; Venable 1992; Kuo *et al.* 1982). For example, smaller seeds are produced when the plant is grown on nutrient-enriched soils because large seeds are more difficult to fill, requiring a greater seed-filling rate or longer duration of filling (Duarte & Adams 1972), but when mineral nutrient are added, the fitness-maximizing seed size will change (usually increase) with the availability of resources (Baker 1972; Salisbury 1974; Foster 1986; Mazer 1989; Parrish & Bazzaz 1985; Wulff 1986; Wolfe 1995). Criticising this assumption, Smith & Fretwell (1974) and Haig & Westoby (1988) argued that improving the supply of resources must change the number of seeds produced instead of the seed size. Substantiating this criticism, two studies concluded that fecundity (seed quantity) was not related to seed size in some plant species if resource levels vary (Richards, Groom & Lamont 1997; Lamont *et al.* 1994). Plant reproductive response to resource availability likely depends on the mechanism of dispersal. Small seeds tend to be adapted for dispersal by wind, a mode in which small size and a greater number of seeds provide the ability to spread vast distances, which increases the probability of survival under hazardous conditions. On the other hand, large seeds are adaptations for dispersal by animals, which overcome various difficulties during seedling establishment. In general, seed size is thought to evolve as a compromise between these two counterposed selection pressures, more numerous seeds vs. seeds with a better chance of giving rise to an established seedling.

Variation in seed size among species might also be associated with habitat type. For example, some species in open habitats have smaller seeds, while others in arid or closed habitats have larger seeds (Mazer 1987; Mazer 1990). Studies have associated seed size with the survival rate of seedlings. Venable (1992) asserts that larger seed sizes are advantageous because they increase the survival of seedlings and grow with greater success from deep burial, thereby increasing reproductive success. Species with large and small seeds must be equally capable of replacing themselves by producing surviving offspring during the course of their life.

1.1.2. The relationship between seed size and cotyledon size

Seed size has been associated with cotyledon size (White & Gonzalez 1990; Bonfil, 1998). This relationship may be associated with differences among species in their timing of achieving maximum seed mass, as well as with differences in the environmental conditions that each species generally experiences during seed filling. For instance, Sung & Chen (1990) have demonstrated that cotyledon size and seed size both increase with greater assimilate supply during seed-filling, i.e., when assimilate availability from actual photosynthesis during seed filling plus reserve remobilization exceeds the demand from the growing plants. In general, larger seeds will have larger cotyledons containing more nutritional reserves to improve the early development of the growing plant and increase its chances of survival (Jurado & Westoby 1992; Westoby *et al.* 1992). Moreover, seed size has been shown to be related to embryo size and the amount of the cotyledon used for food reserves (Mayer & Mayber 1982).

The general correlation between seed size and seedling performance holds because of the correlation between seed mass and the functional morphology of cotyledons (Miquel 1987; Hladik & Miquel 1991; Kitajima 1996). The functional morphology of cotyledons is classified into three groups by the degree of cotyledon exposure and thickness: photosynthetic, photosynthetic-storage and storage, but the two major functions of cotyledons are photosynthesis (Marshall & Kozlowski 1976 a, b; Ampofo, Moore & Lovell 1976 a, b) and storage (Kitajima 1996). Some cotyledons serve strictly as storage organs of seed reserves, while others may develop into leaf-like photosynthetic organs (Garwood 1996). Large seed masses are often associated with cotyledons used for storage, while photosynthetic-type cotyledons occur most frequently among smaller-seeded plants (Ng 1978; Hladik & Miquel 1990; Garwood 1996; Kitajima 1996). Mainly, the functional morphologies of cotyledons can be classified based on cotyledon position (epigeal for those raised above ground, hypogeal for those that stay at or below ground level) or the degree of cotyledon enclosure by the seed coat after germination (phanerocotylar for those that become free of their seed coat, and cryptocotylar for those remaining at least partially enveloped by the seed coat) (Garwood 1983; Hladik & Miquel 1991).

Cotyledons play a critical role in seedling establishment, supporting their initial growth and development with their nutritional reserves (Milberg, & Lamont 1997). The essential nutrients in cotyledons are nitrogen, sulfur, phosphorus, potassium, calcium, magnesium, zinc, sodium, copper, manganese and iron. However, the levels of these nutrients are different between plants, especially N, P and the trace elements, depending on environmental conditions, including the availabilities of resources such as soil nutrients (Fenner & Lee 1989) and light, or on the duration of seed maturation, as some fruits and seeds usually mature within a few weeks, whereas others usually require many months. For instance, shade-tolerant plants usually have high nitrogen concentrations in their cotyledons (Kitajima 2002). High seed phosphorus concentrations are also found among Proteaceae species on impoverished-nutrient soils in Australia (Milberg & Lamont 1997), possibly because the phosphorus may facilitate initial root penetration or minimize the energy expended on soil nutrient uptake for an extended period (Leck *et al*. 2008).

The importance of photosynthates and reserves from cotyledons for seedling growth was demonstrated by the effects of removing cotyledons in many studies (Zhang & Maun 1991; Mulligan & Patrick 1985; Armstrong & Westoby 1993; Sonesson 1994; Andersson & Frost 1996; Lamont & Groom 2002; Milberg & Lamont 1997; Hanley & Fegan 2007). Damage to either or both cotyledons may negatively influence early seedling growth, limiting the ability of the seedling to develop a root system capable of accessing reliable moisture sources, or prevent the aboveground biomass from reaching a critical mass that ensures self-sustainability (Mulligan & Patrick 1985, Armstrong & Westoby 1993). Consequently, seedlings from bigger seeds with bigger cotyledons develop faster than those from smaller seeds (Bewley & Black 2012). The advantage of large cotyledons lies in their ability to provide energy and nutrients necessary for successful seedling establishment because of the larger store of carbohydrates in the seed's endosperm or cotyledons (Lamont & Groom 2002; Green & Juniper 2004). This initial energy and nutrient store within the cotyledon allows seedling growth through soil, litter and vegetation layers into more intensely lit environments above (Baker 1972; Ng 1978; Foster 1986). Thus, cotyledon physiology has significant consequences on the ability of a seedling to grow and survive (Hanley & May 2006 Hanley & Fegan 2007) because a new seedling derives energy from the cotyledon before photosynthesis occurs.

1.2. An overview of Australian genus *Hakea*

Hakea is a shrub genus containing over 150 species, spread throughout Australia but best represented in Mediterranean-climate southwestern Australia and is renowned for its great variation in leaf and fruit morphology, pollinators, climate and fire tolerances and susceptibility to herbivores and granivores (Groom & Lamont 1996a, 1997, 2015; Lamont & Groom 2002, 2015, in press; Hanley *et al*. 2009, Rafferty *et al*. 2010, El-ahmir *et al*. 2015).

1.2.1. Mediterranean-climate ecosystems and Southwest Australia as a biodiversity hotspot

Mediterranean-climate ecosystems are characterized by wet and cool winters; dry and hot summers; and vegetation such as woody shrubs, evergreen and sclerophyllus shrubs (Christensen 1985; Syphard *et al*. 2009). These ecosystems have the highest biodiversity in the world (Cowling, Holmes & Rebelo 1992; Arroyo *et al.* 1995) and harbour very high rates of endemism and many rare species (Cody 1986; Hopper 1992; Greuter 1994). Five regions of the world have this climate: the Mediterranean basin, Southwestern Australia, the South African Cape Region, the California-Baja region and central Chile (Cowling *et al.* 1996; Syphard *et al*. 2009). These ecosystems occupy less than 5% of unglaciated land and contain more than 20% of global flora (Cowling *et al.* 1996; Syphard *et al.* 2009). These Mediterranean-climate ecosystems are a worldwide conservation concern because of anthropogenic pressure and rapid climate change (Mooney *et al*. 2009).

Underwood *et al*. (2009) posited that changes in human population density have had a significant impact on biodiversity in the following Mediterranean-climate regions: South Africa, California-Baja, the Mediterranean basin and Australia. Their research showed that the richness of species in Mediterranean-climate ecosystems is threatened by increasing global population density and urbanization. To reduce this threat, the authors propose that governments should analyze human-environmental relationships and the synergic impact of these interactions (such as invasiveness). In contrast, another study posited that the most significant threats to Mediterranean-climate ecosystems are changes in hydrology and water balance, increasing temperatures, extreme temperatures, habitat loss and pests (Hulme 2005). A vulnerability assessment by Laurance *et al.* (2011) asserts that the Southwest Australian Mediterranean ecosystem is one of the most vulnerable terrestrial ecosystems. That assessment contradicts previous research that suggests that over-exploitation of the ecosystem, invasion, fragmentation and salinization are the greatest environmental threats to the Southwest Australian ecosystem.

There have been numerous studies on the biodiversity hotspot in Southwest Australia. Hopper & Gioia (2004) focused on the Floristic Region in Southwest Australia. This region has an island-like appearance because the ocean borders it on two sides and it is isolated by arid lands. This region makes it known as one of the world's five Mediterranean-climate regions (Hooker 1860; Hopper 1979, Hopper and Gioia 2004; Cowling *et al*. 1996, 2005; Dallman 1998) and one of the top 25 biodiversity hotspots in the world (Myers *et al*. 2000). It has a flat and weathered plateau composed of granite and small mountainous areas (Anand & Paine 2002). Nutrient-deficit soils in old landscapes dominate the region's maritime climate (Hopper *et al*. 1996 a, b).. These plants include sclerophyllous shrubs, herbs and trees as well as eucalypt forests, mallee, kwongan shrub lands and woodlands (Beard 1990). Species of threatened plants that require conservation also dominate the region. However, the authors posit that conservation of the Floristic Region is a daunting task for scientists and managers. Reasons for this conservation challenge include the abundance of plantations and the inability to manage hotspots (Hopper & Gioia 2004).

Prober *et al*. (2012) concur on the existence of a biodiversity hotspot in Southwest Australia. They refer to the Western Woodlands of Southwestern Australia as the largest Mediterranean-climate woodlands on the Earth. Wastson *et al* (2008) also reported that the region has a mosaic of shrub land and mallee that dominates almost 160,000 square kilometres of the land. Some articles have noted that the livestock grazing and agricultural clearing activities that characterize other woodlands in Mediterranean-climate areas have not affected the Southwestern Australian region (Yates *et al.* 2000; Judd *et al.* 2008; Watson *et al.* 2008). Consequently, this region provides an ideal model for evaluating the functioning of intact ecosystems and their adaptation to climate change for conservation purposes (Prober *et al*. 2012). Humans have converted most of these regions for land use and less than 3% of the woodlands have formal protections (Underwood *et al*. 2009). The article confirms that the most vulnerable of these woodlands is in southwestern Australia due to climate change, disturbance (such as fire) and human activity such as increased grazing, logging and mining (Prober *et al*. 2012).

Events such as repeated intense fires have killed woodland eucalypts, leading to permanent alternation of the woodland composition and local extinction (Prober *et al.* 2012). Syphard *et al*. (2009) observe that human ignitions or intentional fires have increased the frequency of fires in Mediterranean-climate areas with high population density. For instance, humans cause 95% of fires in California’s Mediterranean ecosystem (Keeley 2005). The increased frequency of these fires is a threat to the conservation of the region because some scrubland species cannot regenerate or reproduce after repeated burning (Espelta *et al.* 2008; Forysth & Wilgen 2008). The ecological threat of the fires demonstrates the need to alter fire patterns and determine the degree to which frequent fires affect vulnerable plant species in Mediterranean-climate ecosystems (Syphard *et al.* 2009).

1.2.2. Southwestern Australia as the diversity centre for *Hakea*

Hakea is a genus in the family Proteaceae. Australia has the world's greatest diversity of Proteaceae (Rao 1971). It is the only continent in which all five of the subfamilies (Grevilleoideae, Proteoideae, Sphalmioideae, Carnaronioideae and Persoonioideae) recognised by Johnson & Briggs (1974) are found. Many scientists have discussed the past and present distribution, including (Beadle 1966; Specht 1972), who stated that the family is adapted to drought conditions. These adaptations may also be due to poor nutrient supply. Grundon (1972) considers that heathland Proteaceae have adapted to low P and N requirements because the soils have very low available N, P, K and Ca; Mo, S, Cu, Zn and B may also be extremely low.

This family of woody evergreen plants includes tall trees, small trees and shrubs, some of which are prostrate. There are 79 genera and approximately 1700 species of Proteaceae world-wide, of which 46 genera and approximately 1100 species occur in Australia, and 37 genera and almost all of the species in them are endemic to Australia, making Proteaceae one of the most prominent flowering plant families in the continent of Australia (Douglas 1995). One of the major genera in this family is *Hakea* (subfamily Grevilleoideae). *Hakea* is a genus of 150 species of shrubs and small trees native to Australia. it is found throughout the country, with the highest species diversity in the Western Australia. Southwestern Australia is the richest area for *Hakea* species because *Hakea* can grow in a wide variety of environmental conditions, including coastal, tropical, desert and mountainous habitats (Young 2006). However, the largest diversity of this genus has been recorded in the southwestern region of Western Australia because this genus has evolved to survive in low-nutrient soils, e.g. low-phosphorus soils.

Southwestern Australia is the richest area for *Hakea* species due to a number of reasons. First, *Hakea* species are known for their ecophysiological adaptation (Rundel, Dickie & Richardson 2014). Their adaptation helps these species to persist in environments that are nutrient-deficient. Some ecophysiological characteristics of the species that enables them to proliferate in such environments include wind dispersal of the seeds and serotinous follicles (which are retained on mature seeds on the plant for an extended period) (Midgley *et al.* 1991; Lamont *et al.* 1991; Groom & Lamont 1996). These features work similarly to the serotinous cones of pine species found in temperate climates. The cones act as buffers against nutrient deficiency and fire. The serotinous follicles in the *Hakea* species work the same way as in the pines (Rundel, Dickie & Richardson 2014). They protect the plant population from fires and enable the species to occup and persist in new locations with high efficiency (Bradstock *et al.* 1994).

Although Rundel, Dickie & Richardson (2014) acknowledge the richness of the *Hakea* species in Southwest Australia, they posit that acacias are more efficient in their adaptation because they accumulate seed stores and do not require wind to disperse their seeds. Acacias need animals for dispersal and harbour nitrogen-fixing symbionts to survive in nutrient-poor soils. Nevertheless, the serotinous follicles help *Hakea* species to invade and adapt to woodlands, similar to the nitrogen-fixation characteristics in acacias. Rundel *et al.* (2014) assert that many tree species that invade oligotrophic soils are able to fix nitrogen. This characteristic is associated with the alders' ability to become settled in soils that are inhospitable for other native plant species. Alders' nitrogen fixation helps them to grow in different soils that range from silty clay loams to ashes over lava flows. These plant species increase nitrogen uptake by up to 90-fold. Thus, symbiotic nitrogen fixation facilitates the invasion other plant species in nutrition-deficient soils. The serotinous follicles that characterise *Hakea* plant species contribute to the successful invasion of trees in Mediterranean-climate ecosystems and represent an investment of expendable energy in nutrient-poor tissues that protect P-rich seeds on P-poor soils (Lamont *et al.* 1991).

Second, the richness of *Hakea* species in the Mediterranean-climate ecosystems of Southwestern Australia may be due to insect pollination (Phillips, Hopper & Dixon 2010). Insect pollination could be important because it may reduce florivory (Cronk & Ojeda 2008). A morphological data review by Mast *et al.* (2012) showed that bird pollination is primitive in *Hakea*, and multiple shifts to insect pollination have occurred in the lineages of extant *Hakea* species. Pollinator diversity thus contributes to the diversity of *Hakea* species in Southwestern Australia as well as the invasion of these species in to other Mediterranean-climate ecosystems, such as South Africa and New Zealand. Hanley, Lamont & Armbruster (2009); McCall & Irwin (2006); Poot & Lambers (2008) agree that *Hakea* support of mammal, bird and insect pollinators makes it ecologically important. Pollination as well as the Hakea leaf form and nutrient strategies have helped these plant species to invade and adapt to nutrient-deficient soils in Mediterranean-climate ecosystems.

Third, the diversity of *Hakea* species in Southwestern Australia may also be due to their high rate of photosynthesis. For example, according to Lambers, Brundrett, Raven & Hopper (2010), *Hakea prostrata* has a relatively high rate of photosynthesis and high leaf phosphorus content. This species is able to allocate this P to leaf cells that are photosynthetically active rather than to vascular bundles and epidermal cells (Shane *et al.* 2004c). This P allocation pattern helps the plant to adapt to phosphorus-deficient soils.

Lambers *et al.* (2011) confirm that the phosphorus-impoverished soils in Southwestern Australia affect the way in which plants acquire and utilize phosphorus in this infertile landscape. Proteaceae plant species have two traits that help them to acquire soil phosphorus: mycorrhizal association and root clusters (proteoid roots). Over 65% of *Hakea* species have been reported to produce proteoid roots (Venkata Rao 1967; Lamont 1972; Lamont 2003). The root system develops root clusters that provide a unique plasticity in the development of branches and roots (Purnell 1960). One of these possible changes is to initiate the formation of root clusters at highly branching roots (Reich, Oleksyn & Wright 2009). These root clusters have specialized physiology and structure that maximizes the acquisition of phosphorus from soils with low P or with insoluble complexes of phosphorus such as iron phosphate and rock phosphate (Lamont 1972 a, b; Lambers *et al.* 2006). These root clusters are also found in other families (such as Cyperaceae’s dauciform roots (Davies *et al*. 1973; Lamont 1974; Shane *et al.* 2006b) and Casuarinaceae (Reddell *et al*. 1997), Fabaceae (Lamont 1972b) and Restionaceae (Lambers *et al*. 2006)). The root clusters help *Hakea* to adapt to the Southwestern Australian ecosystem by mining the soil directly instead of via mycorrhizae (Skene 1998; Lamont 1973, 1981, 1982, 1983). In Proteaceae, this mining produces compound or simple root clusters where ephemeral rootlets arise from the mother root (Shane & Lambers 2005). The total surface area of such roots is dependent on root length and width, as well as the number, length and width of root hairs (Lamont 2003). For example, *Hakea prostrata* has these root clusters. Its rootlets produce many root hairs that help entrap organic matter and soil. The cluster roots form carboxylates that replace phosphorus bound to iron- or aluminium-rich soil particles, leading to phosphorus release (Lambers *et al.* 2011). Unlike mycorrhizae and root nodules, these cluster roots are more advantageous in environments where P is unavailable because compound cluster roots (such as proteoid roots in Proteaceae family) have a high carbon cost. This high carbon cost is associated with reduced leaf growth in phosphorus-rich environments (Denton *et al.* 2007).

Regardless of the low P concentrations, *Hakea* species are able to invade phosphorus-impoverished soils in Southwestern Australia due to their high photosynthesis rates (Wright *et al*. 2004; Denton *et al*. 2007). This high rate is due to a high photosynthetic phosphorus-use-efficiency (PPUE) (Lambers *et al.* 2012). This characteristic enables *Hakea* species to replace the phospholipids with non-phospholipids in the leaf development phase (Conn & Gilliham 2010; Cowan 2006). This replacement does not compromise the photosynthesis process for the plants. An analysis of the leaf P and soil concentrations and photosynthesis rates shows that mature leaves have lower phospholipid levels than younger or expanding leaves. Younger leaves had greater leaf P compared with mature leaves but had lower photosynthetic rates compared with the mature leaves in *Hakea prostrata*. In addition, younger leaves had 46.5% galactolipids and 7.5% sulfolipids whereas mature leaves had 77.7% of galactolipids and 12.8% of sulfolipids. The mature leaves, however had lower concentrations of phospholipids (9.6%), compared with 46% in younger leaves. On the other hand, the mature leaves have higher concentrations of sulfolipids and galactolipids (Groom & Lamont 2010; Reich, Oleksyn & Wright 2009). These differences were caused by the increase in photosynthetic rates from the young leaves to the mature leaves and therefore that changes in activity associated with age of leaf must be due to changes in the chloroplasts. The findings confirm that *Hakea* invests quite sparingly in phospholipids, which explains the high photosynthetic rate per leaf for plants in phosphorus-impoverished soils (Lambers *et al.* 2012).

Hakea species are successful in Southwestern Australia because of their distinctive photosynthesis rates, lipid concentrations and phosphorus-use efficiency. The plant species have low mature leaf phosphorus and high photosynthetic phosphorus-use efficiency (PPUE). The phospholipid levels decrease with leaf maturity because the plant replaces the phospholipids with nonphospholipids during leaf development (Lambers *et al.* 2012; Tjellström *et al.* 2010). This conversion increases the rate of photosynthesis, leading to lower phosphorus deficiency in the mature leaves. This explains the adaptation of *Hakea* plant species to phosphorus-deficient soils in Southwestern Australia. Additionally, there are many possible explanations for why Southwestern Australia is the area of highest species richness in genus *Hakea*, including their ability to produce large numbers of seeds (Neser 1977; Kluge & Richardson 1983), the high degree of protection afforded to the seeds by the woody follicles (Fugler 1983), high seed longevity in the canopy (Neser 1968), high germinability and rapid germination (Richardson & Van Wilgen 1984), efficient dispersal (Hall 1979) and high nutrient content of seeds (Mitchell & Allsopp 1984).

1.2.3. Morphology and function attributes in *Hakea* (stems, leaves, florescence, flowers, fruit, Seed)

Hakea is an evergreen small tree or shrub with alternate dentate leaves. The leaves may also be deeply divided or leathery, with numerous paired flowers on the head (Barker *et al*. 1999b). The leaves lack stipules and petioles. The blade appears leathery, with both the lower and upper surfaces appearing similar. *Hakea* stems have forked hairs. The flowers in the head may appear loose and have axillary umbels or short racemes (Black 1995). Flowers on the short stalks appear in pairs sharing a common bract. The flowers of the *Hakea* species are perfect. The ovary is superior, is made up of one cell that contains two ovules and appears hairless and smooth. The style of the flower has a simple terminal pollen pretense, and the stigma is quite small (Young 2006).

The perianths of the local species can be white, cream or pink (Young 2006). The Perianth appears as curved claws which may resemble an egg; the limb is globular (Black 1995). *Hakea* flowers also have four tepals (Young 2006). The tepals appear concave. In addition, the flowers have four stamens that attached to the tepals. The stamens are inserted into the sepals during fertilizationwhile the nectary appears as a broad gland located adjacent to the ovary (Black 1995).

Hakea species produce woody follicles that vary greatly in size (2040-450 mg) (Groom & Lamont 1997). Their seeds are highly nutritious (Groom & Lamont 1998; Groom & Lamont 2010). Specialist granivores have evolved among beetles [e.g., *Erytenna consputa* (Curculionidae) (Kluge 1983), *Aphanosperma occidentalis* (Cerambycidae) (Britton 1969)] and moths [e.g., *Carposina autologa* (Carposindae) (Gordon 1993)] whose larvae bore into the fruits to reach the seeds. By far the most destructive granivore of hakeas in southwestern Australia is Carnaby's black cockatoo, *Calyptorhynchus latirostris* (Stock *et al.* 2013, Groom & Lamont 2015). Two mechanisms of dealing with granivores have been observed: small, camouflaged fruits that are difficult to detect visually (Groom *et al*. 1994b) and large, exposed fruits that resist attack mechanically (Groom & Lamont 1997, 2015).

Hakea seeds are wing-like and broad. The body of the seeds has warts that appear on the outer surface. In addition, there is no septum located between the seeds. This means that the follicle does not have a septum. Once the fruit is picked, the seed will be released from ripe fruits after several days to several weeks under warm dry conditions. *Hakea* are normally propagated from seed, but some species can be successfully struck from cuttings (Young 1997). No special treatment is needed for germination, and seedlings usually emerge within three to six weeks after being sown directly into the ground or into pots or dishes for planting out later (Groom & Lamont 1998). It is also possible to germinate *Hakea* seeds on moist filter paper.

1.3. Evolutionary history and phylogeny of *Hakea*

Phylogenetic information is useful in the analysis of the co-occurrence of different species. This is because it provides a way for explaining the patterns of diversity and distribution of species (Emerson & Gillespie 2008; Vamosi, Heard, Vamosi & Webb 2009). The Mediterranean-climate ecosystem of Southwestern Australia is rich in species. However, species from this ecosystem are not represented well in phylogenetic studies. The ecosystem is rich in *Hakea* species, but there is limited phylogenetic research into fire-regeneration (Cardillo 2012). The genus *Hakea* has its world-wide centre of biodiversity in southwestern Australia (Barker *et al*. 1999), having evolved approximately 10 million years ago on soils that were increasingly becoming nutrient-impoverished (Mast *et al.* 2012). The genus lends itself to study of the relative importance of seeds in determining seedling traits that relate to seedling fitness. Southwestern Australian *Hakea* species have a wide range of seed sizes (from 5-500 mg; Groom & Lamont 1996). Preliminary observations indicate that seed size within southwestern Australian species is phylogenetically conserved; thus, the role of phylogeny as a factor influencing seed and hence cotyledon size needs to be taken into account. The phylogeny of the genus *Hakea* is currently incomplete, with the most recent attempt (Mast *et al.* 2012) restricted to 55 of the 150 extant taxa. This section aims to discuss phylogenetic information concerning the influence of fire strategy, seed size, serotinous seeds, pollination and cotyledon function and type on the patterns of diversity and distribution of *Hakea* plant species.

1.3.1. Fire strategy

Fire is an evolutionary force that has altered vegetation communities around the world (Bowman *et al.* 2009; Bond, Woodward & Midgley 2005). The Southwestern Australian ecosystem experiences frequent fires with a recurrence interval of ten to fifteen years (Cowling *et al*. 1990; Enright *et al.* 1998a,b; Groeneveld *et al*. 2002). The majority of previous studies have acknowledged the fire-adaptive traits of plant species in Mediterranean climate regions (He *et al.* 2011, Keeley *et al.* 2011). Mediterranean flora have evolved fire-resistance characteristics that are specific to their particular fire regime. DellaSala *et al.* (2004); & Syphard *et al.* (2009) describe these characteristics as resprouting, smoke- or heat-cued germination, seed banking and fire-stimulated flowering. For instance, Cardillo (2012) and Clarke, Knox & Butler (2010) acknowledge that flora in these regions cope with recurrent fires in two main ways: resprouters and non-resprouters (reseeders). The resprouters can regenerate from epicormic buds or lignotubers, while reseeders are killed in the fire and populations regenerate solely from seedlings. Resprouters are resistant to fire, making them more persistent and stable (Bell *et al.* 1993; Lamont & Wiens 2003; Clarke *et al*. 2013). However, reseeders are not resistant to fires and are easily killed, are susceptible to local extinction and are more variable compared with the resprouter population (Cardillo 2012). This coping mechanism has led to the invasion of fire-adapted flora including acacia, pines and *Hakea* species in to Mediterranean-climate ecosystems. This invasion complicates fire management practices because invasive shrubs are a threat ecosystem conservation measures (Wilgen *et al.* 2010)

Fire is a principal factor interacting with the vital attributes of plant species (Satterthwaite *et al.* 2002; Menges & Quintana-Ascencio 2004; Keeley *et al.* 2011). Fire has long been known to result in a seasonal increase of nutrient enrichment to the soil (Anderson & Menges 1997). Historical and current fire regimes are thought to have influenced the distribution and extent of several *Hakea* species. Fire frequency and intensity may be related to biomass production and fecundity (Safford & Harrison 2004). High seed production may also increase the potential for long-distance dispersal (Nathan & Muller-Landau 2000). Carpenter & Recher (1979) once attempted to link fire-response strategies with reproductive features, such as fecundity. They suggested that non-resprouting plants devote more energy toward the production of seed as a reproductive strategy than do resprouters because resprouters can also survive by self-replacement. Lamont &Wiens (2003) also reasoned that non-sprouting species should produce more and smaller seeds than resprouters. In addition, several investigations have demonstrated that resprouting species have fewer flowers, fruits and seeds per unit plant size, but they may have larger seeds than non-resprouters (Groom & Lamont 1996, 2011; Lamont & Barrett 1988; Low & Lamont 1990; Lamont *et al.* 1998; Lamont & He 2012). However, on the contrary, Bell (2001) reported that resprouters tend to have more flowers and offer greater rewards for pollinators to produce more seeds.

1.3.2. Variation of seed size in *Hakea*

Hakea seeds are wing-like, broad and have a wart-like outer surface. The seeds are born in serotinous follicles and are light enough to be dispersed by the wind (Barker *et al.* 1999b). Variation in the size of *Hakea* seeds is related to taxonomy. Taxonomic factors affect variation in seed size as well as persistence in soil (Venable 1992). The establishment of small rounded seeds is particularly dependent upon soil persistence because small seeds can avoid predation by incorporation into the soil. Conversely, larger seeds do not have this ability to escape predation (such as by invertebrates). Their large size makes the seeds susceptible and easily detectable within the soil. Therefore, small seeds are likely to be incorporated into soil and less likely to be consumed by predators compared with large seeds (Leishman *et al.* 2000).

Another aspect that may related to seed size is moisture stress. Leishman *et al*. (2000) observed a positive correlation between the seed size of herbaceous species and moisture stress. However, this correlation is not fund in shrubs or trees. Dry habitats may favour larger seeds because larger seeds desiccate more slowly. Conversely, Mazer (1989) did not find any evidence for association between moisture and seed size in plant species. In addition, Leishman *et al*. (2000) posit that seed size could be influenced by environmental factors such as habitat, competition and nutrient deprivation. Some habitats have species with larger seed sizes than do others. Seeds in temperate zones have approximately 4% variation in size between species. For instance, the Northern Territories in Australia have more seeds that weigh between 1 mg and 100 mg, whereas Southwestern Australia has higher frequency of seeds in the 0.1 mg to 10 mg range. Competition also influences seed size in adult plants. The results of manipulative experiments show that large-seeded plants are more successful in closed canopies compared with small-seeded species in different environments. This could be due to the dependence of small-seeded plants on disturbance and lower competition. Westoby, Falster, Moles, Vesk & Wright (2002) add that large-seeded species have higher survival rates in nutrient-deficient environments compared with small-seeded species. Proteaceae species have high seedling survival in nutrient-deficient environments because of their relatively large seed sizes (Schurr *et al*. 2012). These large sizes could explain why *Hakea* seedlings survive nutrient-deficient soil in Mediterranean-climate ecosystems.

1.3.3. Serotinous fruits

Two ecophysiological characteristics of *Hakea* that enable it to reproduce in the Mediterranean climate are wind dispersal and serotinous follicles. The serotinous follicles protects seeds from fires and enable the species to effectively occupy and persist in new locations (Rundel, Dickie & Richardson 2014). This characteristic represents serotiny, which refers to the ability to retain mature seeds in a closed fruit in a canopy (Cramer & Midgley 2009; Lamont *et al.* 1991). This retention is important because it prevents the early release of seeds that would germinate in non-favourable periods. Serotiny is a common characteristic among southern hemisphere flora in fire-prone ecosystems. Serotinous plants are able to remain alive and to allocate resources to maintain the closed fruits until a fire occurs. Tonnabel *et al*. (2012) posit that serotiny becomes crucial when competition hinders adult plants from establishing their seeds between fire occurrences. Strong serotiny helps plants to retain their seeds between fire occurrences and is the best strategy for soil seed dormancy. Low serotiny occurs when the inter-fire loss of fruit canopy occurs less than three years after production. Cramer & Midgley (2009) agree that the degree of serotiny influences plants' population growth rate and is tuned to maximize this growth rate during inter-fire periods.

The most common type of serotiny in some Australian Proteaceae (such as *Hakea*) is necriscence. Necriscence refers to the release of the seeds triggered by plant or stem death (Lamont 1991). Tonnabel *et al*. (2012) contrast this adaptation with pyriscence, which is a characteristic of cones that do not release their seeds prior to a fire event. Pyriscent cones typically have chemically altered resins with higher melting points that preventing the cone from opening without exposure to heat. Perennial serotinous plant species in Mediterranean-climate ecosystems, such as *Hakea*, have the ability to regenerate their protected buds because they can resprout once a fire has occurred..These adaptations enable the resprouters to experience numerous recruitment events, leading to higher survival rates during fire cycles (Pausas 2006).

Another study posits that the adaptation of *Hakea* plants is not primarily about the level of serotiny. Keeley *et al.* (2011) assert that plant populations do not adapt to fire but to the fire regime. This means that the population growth rate of the plant species has become sensitive to fire intervals and any variations that may affect the plant's maturation. These plants decrease their growth rate when a fire event occurs prior to maturation or when they have not accumulated sufficient seeds in their canopy for successful regeneration after the fire. Menges *et al.* (2006) supports this assertion, claiming that plants adapt to the average fire interval and any changes in the interval would increase the immaturity risk of the perennial plants. Both studies confirm that conservationists should focus on strategies that target fire intervals rather than the actual prevention of fire. Management strategies should therefore rely on a fire strategy that considers the fire interval, maximum variance, minimum variance and ability for the plant species to handle the changes that occur during fire cycles. This consideration would help conservationists to preserve the heterogeneity of Mediterranean-climate ecosystems (Tonnabel *et al*. 2012).

1.3.4. Pollination

Pollination is an important aspect of plant diversity. Althoff, Xiao, Sumoski & Segraves (2013) describe how the associations between plants and other species, such as seed predators, herbivores and nectar robbers, have contributed to plant evolution. These pollinators select for traits that may improve their pollination services. By causing floral tissue loss, florivores disrupt the mutualism between plants and pollinators. McCall & Irwin (2006) do not emphasize the resulting negative effects, but they establish that florivory is common in plant-pollinator interactions because it has the ability to influence the evolutionary and ecological dynamics of these interactions. The direct and indirect effects of florivores may include variations in nectar production, changing the attractiveness of flowers and reducing the time that pollinators spend foraging.

The reduction of florivory is very important for *Hakea* plant species in Southwestern Australia (Hanley *et al.* 2009). Cronk & Ojeda (2008) establish that insect pollination helps *Hakea* species to reduce florivory. Insect pollination also contributes to the diversity of *Hakea* species in Southwestern Australia and the invasion of species in other Mediterranean-climate ecosystems (Mast *et al*. 2012). The combination of pollination, leaf form and nutrient strategies is important because it helps *Hakea* species to adapt to nutrient-deficient soils in Mediterranean-climate ecosystems, such as Southwestern Australia (Hanley *et al.* 2009; Poot & Lambers 2008).

Pollinators define many trade-offs among traits and adaptations to different types of environments, as recognised by Harper *et al*. (1970). As such, they provide comprehensive links to key traits (seed size) and an important link to successful plant reproduction. In particular, it has been suggested that a reduction in pollinator service can directly influence reproductive output, decreasing the seed number and size (Agren 1996). The large flowers in *Hakea* species are usually visited by birds (e.g., honeyeaters) because the nectaries reward is suitable for a bird (e.g., *Hakea lorea*) (Young 2006). Indeed, birds require more energy than insects (Cronk & Ojeda 2008). In contrast, small axillary flowers held within a tight barrier of protective spines in many *Hakea* species are generally visited by insects (Barker *et al*. 1999; Hanley *et al*. 2009;).

1.3.5. Type, size and functions of cotyledons in *Hakea*

In the nutrient-impoverished soils of Southwestern Australia, species from the family Proteaceae (e.g., species in *Banksia, Hakea, Grevillea* etc.) produce significantly larger seeds than the other taxa (Pate *et al.* 1986) that are particularly enriched in N, P and Mg content (Kuo *et al.* 1982; Pate *et al.* 1986; Groom & Lamont 2010). Once germinated, these cotyledon-stored nutrients become the main source of nutrients (especially P) for early seedling growth (Stock *et al.* 1991; Milberg & Lamont 1997; Lamont & Groom 2002). The majority of Proteaceae seeds can rely on their cotyledons as the sole source of mineral nutrients for at least 200 days when grown in mineral deficient soils (Stock *et al*. 1991). Cotyledon size and function is an important factor that plays a role in the performance of plant species in nutrient-deficient soils. Milberg & Lamont (1997) investigated the significance of seed size in species with different cotyledonous in nutrient-deficient soils in Southwestern Australia. Their experiments involved removing the cotyledons from the seedlingswhich resulted in reduced the penetration of roots, increasing mortality and changed the growth rate; they reported a 30-50% decrease in seedling mass (Milberg & Lamont 1997; Lamont & Groom 2002).Their experiment was conducted on small-seeded Hakea *lasianthoides* species and large-seeded *Hakea psilorrhyncha*. They found that the growth rate in the *Hakea* species was unaffected because the cotyledon content did not change over time. Cotyledon content such as mineral nutrients (nitrogen, magnesium, potassium and phosphorus) shifted to the growing plant when the cotyledons were shifted from fertile to nutrient-deficient soils. The translocation of nutrients from the cotyledon to growing areas of the plant played a substantial role in the growth of the large-seeded *Hakea* species (Milberg & Lamont 1997). The findings confirm that larger-seeded species obtain nutrient support from cotyledons rather than the soil. This nutrient support enables *Hakea* spp. to survive nutrient-deficient soils in Mediterranean-climate ecosystems.

Lamont &groom (2002) concur on the nutritional support provided by cotyledons of perennial plant species. They assert that any damage to the cotyledon directly affects the survival of seedlings and plant fitness (seed size and number) in *Hakea* species. In particular, cotyledon damage lowers the seed number, owing to the decline in flower numbers. This low seed number reduces the fitness of the plant. Cotyledon damage also delays the initiation of flowering and the flower numbers are lower than expected (Hanley & May 2006; Hanley & Fegan 2007). Thus, cotyledon damage to *Hakea* plant species would directly affect the flowering phenology and plant size as well as the fitness of the species. In the southwestern Australian flora, the ability of seedlings to survive their first summer is critical to ensure the success of species that rely exclusively on seeds for the species' continual survival. However, information on how seedlings grow to achieve this is limited. Damage to either or both cotyledons may negatively influence early seedling growth, limiting the ability of the seedling to develop a root system capable of accessing reliable moisture sources or preventing above-ground biomass from reaching a critical amount that ensures self-sustainability (Mulligan & Patrick 1985; Armstrong & Westoby 1993). Thus, cotyledons have significant consequences on the ability of a seedling to grow and survive (Hanley & May 2006; Hanley & Fegan 2007).

The size of a seed has implications for how large the cotyledons will be post-germination, with cotyledon size positively correlated with seed size. Cotyledons are the primary energy and nutrient storage organs for seedling growth. This research has investigated the functional aspects of cotyledons as they relate to early seedling growth and biomass allocation to roots and shoots (a function of species fitness) in the Australian genus *Hakea* (Proteaceae).

1.3.6. The evolution and adaptation of *Hakea*

Nutrient-poor, fire-prone, Mediterranean-type regions with a prolonged hot, dry season and exposed to intensive pressure from pollinators, herbivores and granivores are characterised by high species richness and endemism (Cowling *et al*. 1996; Groom & Lamont 2015). *Hakea* is a shrub genus of over 150 species, spread throughout Australia but best represented in mediterranean southwestern Australia, and renowned for its great variation in leaf and fruit morphology, pollinators, climate and fire tolerances and susceptibility to herbivores and granivores (Groom & Lamont 1996a, 1997, 2015; Lamont *et al*. 2002, 2015, in press; Hanley *et al*. 2009; Rafferty *et al*. 2010; El-ahmir *et al*. 2015).

Hakea is a highly sclerophyllous genus (Lamont *et al*. 2015) with needle-leaved and broad-leaved species, both share equally high leaf densities attributed to their occupation of nutrient-impoverished soils, while their exceptional thickness is attributed to prolonged exposure to drought and/or heat to which they are better adapted (Groom *et al*. 1994a). Needle leaves are twice as thick as broad leaves implying that they have had better able to cope with drought and heat (Lamont *et al.* 2015). Previous molecular analysis indicates that *Hakea* originated in the early Miocene directly from non-fireprone, rainforest ancestors (Sauquet *et al.* 2009; Lamont & He 2012). This was post the Miocene 'hothouse' maximum and Australia had moved to the drier mid-latitudes. There was a general drying and cooling trend, punctuated by warm, wet periods and cold, dry periods ('glacials') (Kominz *et al*. 1998; Li *et al*. 2004; Macphail 2007). *Hakea* most probably originated in the sclerophyll shrublands of southwestern Australia where it continued to diversify strongly until the present (Lamont *et al.* in press). Gradually it speciated and migrated from the mid-Miocene onto recently-exposed, rocky substrates, sclerophyll forests and woodlands, and across the drier centre of Australia to the moister margins.

Many hakeas have spiny leaves with a sharp apex and/or acute, marginal teeth (Barker *et al*. 1999a; Hanley *et al.* 2009). Spiny *Hakea* leaves are more effective at deterring herbivory by kangaroos than broad leaves (Hanley *et al*. 2007), and moderately effective at deterring black cockatoos from reaching the woody fruits of hakeas that contain highly nutritious seeds (Groom & Lamont 2015). Macropods appeared from 17 Ma (Prideaux & Warburton 2010), soon after the evolution of *Hakea*. The median stem of black cockatoos (Cacatuidae, Calyptorhynchinae) is positioned at 21.5‒15 Ma and they speciated through the Neogene (White *et al*. 2011). Needle leaves lend themselves to termination by a sharp apex, so once they appeared selection pressure from vertebrate herbivores/granivores would have promoted the evolution and stabilization of sharp-tipped leaves among vulnerable lineages.

Pollinator-driven speciation has been invoked to explain plant richness in some biodiversity hotspots, since pollinator shifts usually provide effective barriers to gene flow, thereby contributing to the origin of new plant lineages (Van der Niet *et al.* 2014). Using a morphologically based phylogeny (Barker *et al.* 1999b). Hanley *et al.* (2009) concluded that insect pollination was ancestral in *Hakea* followed by repeated evolution of bird pollination. From their molecular phylogeny including 51 *Hakea* species and mainly from eastern Australia, Mast *et al.* (2012) concluded the reverse. Either interpretation is possible since it is now known that honeyeaters (Meliphagidae) originated 23.5 Ma, though they only radiated strongly from 15 to 5 Ma (Joseph *et al*. 2014). I hoped to resolve this disagreement by adding more West Australian species to our analysis. I expected bird pollination to stabilize quickly as Toon *et al.* (2014) showed for bird-pollinated legumes that this was an irreversible process.

Hakeas produce woody follicles that vary greatly in size (20–40,450 mg) (Groom & Lamont 1997). Their seeds are highly nutritious (Lamont & Groom 1998; Groom & Lamont 2010) and specialist granivores have evolved among beetles [eg *Erytenna consputa*, Curculionidae (Kluge 1983), *Aphanosperma occidentalis,* Cerambycidae (Britton 1969)] and moths [eg *Carposina autologa*, Carposindae (Gordon 1993)] whose larvae bore into the fruits to reach the seeds. By far the most destructive granivore of hakeas in southwestern Australia is Carnaby's black cockatoo (*Calyptorhynchus latirostris*), (Stock *et al.* 2013; Groom & Lamont 2015). Two ways of dealing with granivores have been observed: small, camouflaged fruits that are difficult to detect visually (Groom *et al*. 1994b) and large, exposed fruits that resist attack mechanically (Groom & Lamont 1997, 2015).

Recurrent fire has been proposed as one of the key factors to which many plants have had to adapt and can explain high species richness in fire-prone ecosystems (Cowling *et al*. 1996; Ojeda 1998; Simon *et al*. 2009; He *et al.* 2011; Lamont & He 2012). Whole-plant responses to fire can be placed into two regeneration syndromes: plants that survive fire and recover via dormant buds or meristems protected beneath the bark of trunks or underground organs, such as lignotubers (Clarke *et al*. 2013). In contrast, nonsprouters are killed by fire and population regeneration relies solely on seedlings. Both trait-types are well represented among hakeas (Groom & Lamont 1996b). Wells (1969) proposed that fire-response traits will have consequences on rates of species evolution. Nonsprouters live for a shorter time and have a higher fecundity than resprouters (Lamont & Wiens 2003; Pausas & Verdu 2005). Nonsprouters are therefore hypothesized to have higher speciation rates because they would have fast rates of molecular evolution as a result of the inverse correlation between generation time and replication-induced mutations (Laird *et al*. 1969; Ohta 1993). Indeed, Litsios *et al*. (2014) concluded that fire-killed Restionaceae speciated four times faster than resprouters in South Africa but not Australia. However, Verdu *et al.* (2007) reported that rates of speciation were not associated with fire response for five genera in four plant families present in fireprone environments. Lamont & Wiens (2003) argued that somatic mutations will be much more common among resprouters as these are dependent on plant longevity and may enable them to keep pace with nonsprouter speciation rates. Resprouters have an adaptive advantage over nonsprouters when a) fire is either frequent, rare or highly stochastic, b) conditions do not favor growth or seed production e.g. infertile soils or intense competition (fertile soils, high rainfall), and/or c) conditions do not favor seedling recruitment or adult survival (stony substrates, low rainfall, extreme summer drought) (Ojeda 1998; Lamont *et al.* 2011, 2013). Thus, promotion of resprouters may not follow a monotonic relationship with environmental gradients. As the climate became more seasonal through the Miocene, the accumulation of dry matter would have been promoted and fires would have become less stochastic and more likely to occur within the lifespan of the shorter-lived nonsprouters, essential for their promotion (Enright *et al.* 1998a; Groeneveld *et al*. 2008) but not necessarily at the expense of resprouters (Enright *et al*. 1998b; Groeneveld *et al*. 2002). Increasing occurrence of arid periods (glacials) would have created mosaics of deep sands and exposed laterites and granites (Glassford & Semeniuk 1995) favoring nonsprouters and resprouters respectively (Lamont & Markey 1995). Thus, existing knowledge does not enable prediction of the ancestral regeneration strategy of *Hakea*. Whatever trait was ancestral it can be speculated that some of its descendants must soon have transitioned to the other trait, and transition and stabilization rates in both would have been similar throughout the Neogene-Quaternary.

Serotiny (on-plant seed storage) is adaptive under conditions that restrict annual seed production (poor soils, low rainfall) in fire-prone environments (for cueing seed release) with a reliable wet season (for effective seedling recruitment) (Lamont *et al*. 1991; Cowling *et al*. 2005). While plant death from drought or disease is sufficient to induce general seed release among hakeas, postfire conditions are still required for optimal seedling recruitment (Causley *et al*. in press). Thus, I postulate that strong serotiny is the ancestral condition in *Hakea* and that stabilization will be the main evolutionary process for this trait in its subsequent diversification in response to intensifying fire and seasonality. Loss of serotiny will be a later development corresponding to the gradual appearance of fire-free habitats such as rock outcrops exposed during the glacials and migration/speciation into arid lands (lacking vegetation continuity to carry fire), and evolution of small-fruited, drought/herbivore-tolerant species with sufficient annual seed production to enable recruitment in any year (Hanley & Lamont 2001; El-ahmir *et al*. 2015). Hakeas that migrate to summer-rainfall grasslands (savannas) that developed in the late Miocene can also be expected to become nonserotinous (He *et al*. 2011; Lamont *et al*. 2013).

Studies that capture patterns of speciation associated with changes in environmental conditions provide compelling support for the key role of functional trait shifts in the process of evolution by natural selection (e.g. Stanton *et al.* 2000; Jetz *et al*. 2012; Jonsson *et al*. 2012). Natural selection can induce the evolution of novel traits whose fitness exceeds that of the incumbent trait (directional selection) or perpetuation of the current trait whose fitness exceeds that of a former or alternative trait (stabilizing selection) (Lemey *et al*. 2009, Lamont *et al*. 2013). Phylogenetic methods have been developed to investigate a wide range of questions regarding species evolution, including the inference of ancestral traits (He *et al*. 2011, 2012; Crisp *et al*. 2011) and to address the relationship between traits and rates of speciation (Litsios *et al*. 2014). While currently the origin and evolutionary trajectories of novel traits are emphasised the role of stabilization has been neglected and interpretations have often been *posthoc* rather than as hypothesised responses to stated agents of natural selection (He *et al*. 2012; Litsios *et al.* 2014). This is partly because of the ignorance of the advent or strength of the postulated selective forces. Significant questions remain: To what extent do directional and stabilizing processes contribute to trait proliferation? Do their contributions vary between attributes, traits and/or over geological time? Is the proliferation of a trait at the expense of its alternative traits? Can patterns of directional and stabilizing selection over time be interpreted in terms of the advent or changes in the intensity of particular agents of selection (Lamont *et al*. 2013)?

1.4. Objectives and organization of this thesis

This research focused on:

1. Reconstruction of the most likely ancestral state of key functional traits in relation to seed size and with adaptation to droughts, fire and granivory in *Hakea*, and exploration the directional and stabilizing evolution of those functional trait contributing to the diversification in *Hakea*.

2. Exploring the interacting network of seed size, postfire regeneration strategy, fecundity, fruit size, serotiny and plant height in the phylogeny of 82 *Hakea* species.

3. Experimentally examining the role of cotyledons in maintaining early seedling growth to investigate the role of cotyledons in early seedling growth and establishment (= *fitness*).

4. Experimentally examining the relative importance of cotyledons as a vital source of nutrients during early seedling growth.

The following hypotheses were tested:

H1: ancestral *Hakea* leaves were broad, reflecting their mesic heritage, that they were retained (or re-evolved) in temperate environments, but that needle leaves arose in the mid-Miocene and proliferated strongly through the late-Miocene to present.
H2: The environment and availability of pollinators are important in the evolution of pollination syndromes.
H3: fruit size has taken two directions in *Hakea*: fruits have become either smaller (and protected or cryptic within spiny foliage, Hanley *et al.* 2009) or larger and woodier and that these transitions are strongly unidirectional.

H4: Strong serotiny is the ancestral condition in *Hakea* and that stabilization will be the main evolutionary process for this trait in its subsequent diversification in response to intensifying fire and seasonality.

H5: There is a causal relationship between seed size, fecundity and postfire regeneration strategy in *Hakea*, and the driving force behind variation in seed size within the genus is adaptation to poor soils, recurrent fire and severe summer droughts.

H6: Small seeds confer survival advantages in nutrient-impoverished soils.

H7: Seed size contributes to the initial growth and establishment of seedlings and cotyledons; hence, by association, seed size can affect seedling development in early stages.

H8: Cotyledon damage during early stages of seedling development has the greatest effects on plant growth (Kitajima 2003). Alternatively, the large cotyledons may be more damage-tolerant than small cotyledons or *vice versa*. Additionally, the amount of cotyledon damage may cause different effects depending on cotyledon size and whether reserves remaining in the cotyledons at the time of damage still contribute to survival and further seedling growth.

H9: Cotyledon reserve has been identified as main resource to improve the early development of the growing plant (Milberg & Lamont 1997; Milberg *et al.* 1998; Stock *et al.* 1991; Lamont & Groom 2002). Evidence remains equivocal regarding the link between seed size and critical cotyledon elements.

To achieve these objectives of study, the work began with a basic examination of the role of cotyledons (or the effects of their absence) on seedling fitness (i.e., growth attributes) in the Australian-native genus *Hakea* (family Proteaceae), a genus known for its diverse range of seed-sizes (variation up to 500 mg between species), the majority of which inhabit the nutrient-impoverished soils and landscapes of southwestern Australia. The proposed research used southwestern Australian species from phylogenetically (i.e., evolutionary) different groups. In Chapters 2–5, the importance of cotyledons for seedling fitness within this genus was assessed on various levels.

Chapter 2 focuses on the ancestral traits for six attributes (with 15 trait states) and determined their evolutionary trajectories in response to the advent or increasing presence of fire, seasonality, aridity, nectar-feeding birds and invertebrate / herbivores /granivores. In addition the traits of the putative ancestor and the relative contribution of transition and stabilization processes to the frequency of alternative traits over geological time to account for trait representation among the extant species was identified in this chapter to evaluate the impact of changing environmental conditions on trait evolution and stabilization and their contribution to diversification in *Hakea* to provide insights on the factors and processes explaining high species richness in this prominent Australian genus.

Chapter 3 focuses on the interdependence of seed size, fecundity and postfire regeneration strategy in *Hakea*. The evolutionary relationships of these traits was assessed, assuming that seed sizes of extant taxa have remained relatively stable since their time of origin (within the past 10 million years). Relationships between seed and seedling fitness traits were overlaid onto a phylogeny (a depiction of evolutionary relationships between species) of southwestern Australian *Hakea* species, to investigate the evolutionary context for why there is such a large variation in seed size within the genus. Chapter 4 investigates the effect of cotyledon removal on the growth of Southwestern Australian *Hakea* species. To find out the relationship between cotyledon damage response and cotyledon size (and hence by association, seed size), six *Hakea* species from taxonomically diverse groups were grown. Chapter 5 addresses the critical nutrients that can compensate for cotyledon loss in *Hakea* species. Experiments involving either the removal of cotyledons or the addition of soil nutrients were conducted to examine the relative importance of cotyledons as a vital source of nutrients on early seedling growth. In addition, the role of cotyledons in early seedling growth was examined in this chapter, in terms of biomass allocations and growth rates as a measure of the importance of cotyledon size (and hence seed size) on seedling establishment and the ability to access underground resources (e.g., summer soil moisture) and hence survival.

1.5. References

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Chapter 2: Evolution of functional traits and effects on diversification in *Hakea*

2. 1. Abstract

Trait proliferation through phylogenies is the outcome of the evolution (innovation and transition) of novel traits and their subsequent fixation (stabilization) in response to novel agents of selection. To examine the relative contribution of transition and stabilization processes to species diversification, I assembled a time-based phylogeny for the Australian genus *Hakea* (Proteaceae), reconstructed its ancestral traits for six attributes (with 15 trait options) and determined their evolutionary trajectories in response to the advent or increasing presence of fire, seasonality, aridity, nectar-feeding birds and (in)vertebrate herbivores/granivores. The ancestral *Hakea* arose *c*. 18 million years ago (Ma) and was broad-leaved, non-spinescent and insect-pollinated, with medium-sized, serotinous fruits and resprouted after fire. Of the subsequent 190 diversification events that led to the 82 extant species analysed, 50–93% involved the retention or re-evolution (reversal) of the ancestral traits. Needle leaves appeared by 14 Ma and accounted for 35% of diversification events throughout the Neogene/Quaternary consistent with increasing seasonality and aridity. Spinescence arose 12 Ma among the needle-leaved lineages consistent with the advent of vertebrate herbivores, and contributed to 42% of diversification events. Bird-pollination appeared by 14 Ma in response to the advent of the Meliphagidae in the early-Miocene, accounting for 29% of subsequent diversification events. Small and large woody fruits evolved from 12 Ma as alternative defenses against granivory, and contributed to 47% of events equally spread between transition and stabilization processes. Succumbing to fire evolved by 14 Ma and accounted for 50% of events as fire became less stochastic at similar transition and stabilization rates as resprouters. Loss of serotiny began in the late Miocene and stabilized in the Pliocene but only contributed 8% of events as rare non-fireprone habitats became available. Trait innovation and stabilization in these functional traits promoted the overall species diversification rate by 15 times such that only three species now retain the ancestral phenotype and even hese involved trait reversals. My approach holds great promise for

understanding the processes responsible for speciation of organisms generally when the ancestral condition can be identified and the likely selective agents are understood.

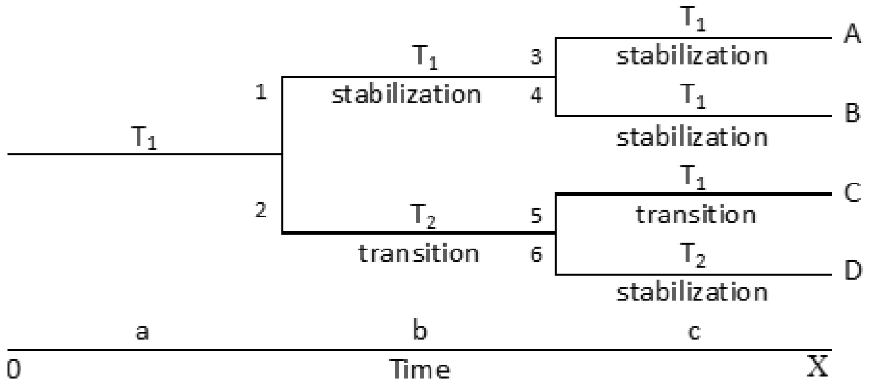
2. 2. Introduction

Studies that capture patterns of speciation associated with changes in environmental conditions provide compelling support for the key role of functional trait shifts in the process of evolution by natural selection (e.g. Stanton *et al*. 2000; Jetz *et al.* 2012; Jonsson *et al*. 2012). Natural selection can induce the evolution of novel traits whose fitness exceeds that of the incumbent trait (directional selection) or perpetuation of the current trait whose fitness exceeds that of a former or alternative trait (stabilizing selection) (Lemey *et al*. 2009, Lamont *et al*. 2013). Phylogenetic methods have been developed to investigate a wide range of questions regarding species evolution, including the inference of ancestral traits (He *et al*. 2011, 2012; Crisp *et al.* 2011) and to address the relationship between traits and rates of speciation (Litsios *et al.* 2014). While currently the origin and evolutionary trajectories of novel traits are emphasised the role of stabilization has been neglected and interpretations have often been *posthoc* rather than as hypothesised responses to stated agents of natural selection (He *et al*. 2012; Litsios *et al.* 2014). This is partly because of the ignorance of the advent or strength of the postulated selective forces. Significant questions remain: To what extent do directional and stabilizing process contribute to trait proliferation? Do their contributions vary between attributes, traits and/or over geological time? Is the proliferation of a trait at the expense of its alternative traits? Can patterns of directional and stabilizing selection over time be interpreted in terms of the advent or changes in the intensity of particular agents of selection (Lamont *et al*. 2013)?

2. 2.1. Consideration of theory and concepts

Each alternative state of a species attribute is termed a trait. Increase in occurrence of a given trait through a phylogeny is defined as trait proliferation (He *et al*. 2011; Lamont *et al*. 2013). The fraction of total diversification events that result in the presence of that trait is the trait proliferation rate (this may also be given on an absolute basis per unit time, as for species diversification). Trait proliferation results from two evolutionary processes: diversification or transition - a new trait arises during the event, and stabilization - the trait is conserved during the event. Transition rate (TR) is the fraction of events in which the trait arises relative to the maximum number in which that trait could occur, while stabilization rate (SR) is the fraction of events in which the trait is retained relative to the maximum number in which that trait could occur. Thus, the (net) trait proliferation rate (PR) = TR + SR. Generally, for a pair of opposing traits, 1 and 2, evolving in a clade with a total of $n/2$ nodes and thus n diversification events for the period of interest, $PR_1 = TR_{2/1} + SR_{1/1}$, $TR_{2/1} = \Sigma(2 \rightarrow 1 \text{ events})/n$, and $SR_{1/1} = \Sigma(1 \rightarrow 1 \text{ events})/n$. Trait reversals are successive transitions that return the phenotype to the previous trait state in the lineage, e.g, broad leaf ➔ needle leaf ➔broad leaf, as an inverse function of the stability through time of the selective pressure for that trait. The concepts of rates of diversification, proliferation, stabilization and transition, and reversals are illustrated with a concrete example in Fig. 2.1.

To what extent the introduction of novel traits promots species diversification depends on whether the associated agent of selection is an adjunct to the extant agents (extinction is not induced) or replaces the extant agents (extinction is induced). Usually the new agent of directional selection operates in a different spatial or temporal dimension than the existing agents and becomes a supplementary force that initially retards speciation and then promotes it once an adapted genotype has evolved followed by rampant speciation into the 'vacant niche' now available as the new trait proliferates (Lamont *et al*. 2013). The premise here is that the more habitats (niches) a given area can be divided into, the greater the opportunities for novel genotypes to arise. Accepting that new, alternative traits supplement rather than replace ancestral traits as options, the contribution of novel traits to speciation can be calculated as the inverse of the percentage contribution of the ancestral trait to all subsequent diversification events (Y): speciation promotional rate (SPR) = 100/Y. Thus, the smaller Y, the greater the contribution of novel traits to the subsequent diversification events.



Species diversification rate: (Spp @ c – Spp @ a)/[Spp @ a x (a + b + c)] = (4 – 1)/(1 x X)

Six diversification events over time X:

Trait (state)	Genetic process involved	Within trait	Between traits	Trait proliferation rate
1	1→1, stabilization rate	3/4	3/6	4/6
1	n→1, transition rate	1/4	1/6	
2	2→2, stabilization rate	1/2	1/6	2/6
2	n→2, transition rate	1/2	1/6	

where n = one or more alternative states to the evolved one.
Speciation promotory rate (increase in species diversification rate due to the presence of novel trait 2) is given by 1/(4/6) = 1.50.

Figure 2.1: Hypothetical phylogeny showing the evolution of four species from six diversification events with proliferation of two alternative traits, 1 (ancestral) and 2 (novel), of a given character over time, due to both stabilization (trait retained during diversification event) and transition (new trait attained during diversification event) processes. Diversification rate is relative to the starting number of species/lineages and the time interval, while proliferation, stabilization and transition rates are relative to the maximum number of events in which that trait could occur. Note that the two transitions that yield Sp C is an example of a reversal (to T_1).

In this Chapter, using a time-based phylogeny for *Hakea* I assembled (El-ahmir *et al*. 2015, Chapter 3), I reconstructed the ancestral traits for six attributes (with 15 trait states) and determined their evolutionary trajectories in response to the advent or increasing presence of fire, seasonality, aridity, nectar-feeding birds and vertebrate/herbivores / granivores. I paid particular attention to identifying traits of the putative ancestor and the relative contribution of transition and stabilization processes to the frequency of alternative traits over geological time to account for trait representation among the extant species. My objective was to evaluate the impact of changing environmental conditions on trait evolution and stabilization, and their contribution to diversification in *Hakea* to provide insights on the factors and processes explaining high species richness in this prominent Australian genus.

2. 3. Materials and Methods

2. 3.1. Phylogenetic reconstruction

I built a time-based *Hakea* phylogeny (El-Ahmir *et al*. 2015, Chapter 3). Briefly, I included 82 *Hakea* species, each with eight gene sequences extracted from NCBI (Mast *et al*. 2012), combined with new sequences that I generated. Outgroup included *Grevillea juncifolia*, *Finschia chloroxantha* and *Buckinghamia celsissima* and their DNA sequences were obtained from NCBI. I set the calibration point for the origin of the subfamily Grevilleoideae (to which *Hakea* belongs) at 70.6 Ma based on the fossil *Lewalanipollis rectomarginis* used by Sauquet *et al* (2009). I used BEAST v2.1.0 to estimate phylogeny and divergence times under a strict clock model (Drummond *et al.* 2006), and further details on the methods are provided in El-Ahmir *et al*. (2015).

2. 3.2. Trait data

I collated leaf shape and spinescence from Barker *et al.* (1999a); Hanley *et al.* (2009), personal field observations and images from database of State Herbarium of Western Australia (http://www.flora.sa.gov.au). Needle leaves were rounded in cross-section with a length: width ratio of >20:1. Heteroblastic species, with seedling leaves initially broad becoming needle by the end of the first growing season (Lamont 1976) or seasonally broad to needle (Groom *et al*. 1994a), were also recognized. Blunt leaves had a mucro or marginal teeth with length: width ratio <1:1 while sharp leaves were >2:1.

For pollinator types, Hanley *et al*. (2009) showed that stigma–nectary distance (SND) in *Hakea* is a reliable predictor of pollinator class (also adopted by Mast *et al.* 2012). All known or putative insect-pollinated species have a SND <13 mm and all known or putative bird-pollinated species have a SND >13 mm. This is supported by the shortest bill length of the principal bird pollinators in Australia (family Meliphagidae) being 12 mm (Paton & Ford 1977) while no known insect pollinator in Western Australia can touch the nectary and pollen presenter simultaneously if the SND >12 mm. I therefore assigned species with SND <13 mm to the insect-pollination class and >13 mm to the bird-pollination class. I took SND from Hanley *et al*. (2009) and Mast *et al.* (2012). Approximate SND for the remaining species were obtained from pistil lengths in Barker *et al.* (1999a).

Fruit size, as dry fruit weight, was obtained from Groom & Lamont (1997). If not available there, the three mean fruit dimensions were obtained from Barker *et al.* (1999), converted to volume and multiplied by mean fruit density in Groom & Lamont (1997). They were divided into four size classes: <1, 1–5, 5–10 and >10 g, such that the 1–5 g class accounted for about half of species. Postfire response/regeneration strategy was collated from Groom & Lamont (1996b); Barker *et al.* (1999a); Young (2006). Each species was assigned as either a nonsprouter or resprouter (with two species recognized to have both fire response forms in different populations). Level of serotiny was obtained from Groom & Lamont (1997) and images on the web (especially http://www.flora.sa.gov.au).

2. 3.3. Trait reconstruction through the phylogeny

I used MultiState in BayesTraits (Pagel & Meade 2006) to determine the most likely ancestral traits for the *Hakea* phylogeny. First, I tested which of the possible models (simple or complex, associated with uniform rates of 0~30) should be used via the log Bayes factor (log BF) recommended by Pagel & Meade (2006). I excluded morphological data for the outgroup in order to avoid potential biases in trait assignment because they do not adequately represent the associated clades (Mooers & Schluter 1999). I applied the best-fit model parameters to our MC tree in a Bayesian framework using MCMC sampling to search for optimal parameter estimates. The MCMC parameter searches consisted of 1,000,000 iterations with 25,000 discarded as burn-in. I used maximum likelihood parameter estimates as starting values in the MCMC analyses. I also used the continuous random walk (Model A) associated with the MCMC method to determine whether pairwise traits evolved in a correlated manner, and BayesFactor was used to determine the significance of correlation between any two traits (Pagel & Meade 2006). Trait reconstruction of fruit size was carried in Mesquite using a parsimonious procedure (Madison & Midford 2007). *Hakea* fruits were categorised into small (<1.0g), medium (1.0-5.0g) and large (>5.0g).

2. 3.4. Speciation and trait proliferation rates

Net species diversification rate was calculated as $(N_{i+t} – N_i)/(N_i.t)$, where N is the number of lineages at the start, i, and end, $i + t$, of the time interval, t (He *et al*. 2011) for the three geological periods/epochs in which *Hakea* has been recorded as well as overall. As plotting the relationship between cumulative lineages versus time showed the relationship to be linear I did not use the log form of this formula nor did I insert a dummy variable for conjectured extinction rates. The geological boundaries were set according to the International Commission on Stratigraphy (2009) (available from www. stratigraphy.org), while the start time in the Miocene was set at the time that *Hakea* first appeared. Following trait assignment to each node of the phylogeny, trait stabilization and transition rates (see Introduction) were determined for the three periods/epochs and overall by counting their number in each time interval. Where the ancestor was ambiguous this event was omitted from the counts as the process was unclear. They were then converted to the fraction that each process contributed to total proliferation within the trait and between all traits of that character. The number of reversals was also noted: i.e. a trait reverting to its immediate preceding trait. Individual speciation promotional rates (SPR) for the three geological periods were determined from the percentage of events retaining the ancestral trait, Y, where SPR = 100/Y (see Introduction).

Generally, $\mathrm{SPR}_n = \prod_{i=1}^{n} 100/Yi$ where Ys for the n attributes assessed are multiplied to give their total promotional effect on species diversification. SPR_n was converted to its fractional contribution to species diversification for the n attributes assessed: $(\mathrm{SPR}_n – 1)/\mathrm{SPR}_n$.

2. 4. Results

2. 4.1. *Hakea* time-based phylogeny

The Bayes MCMC analysis indicated that the *Hakea* stem arose 18.0 Ma [with the 95% highest density probability (HDP) at 15.8–20.2 Ma] and split into two clades (defined as clades A and B in Mast *et al*. 2012) 14.1 Ma with 95% HPD at 12.5–15.8 Ma. This approximate 10-12% error term about the mean continued through the rest of the phylogeny. The phylogeny was strongly supported by the branch posterior probability where 48 out of 81 branches were ≥ 0.70. The overall topology of *Hakea* phylogeny was consistent with that in Mast *et al*. (2012). Net species diversification rate in the Miocene greatly exceeded that in the Pliocene (9.6×) and Quaternary (13.5×) and the overall rate was dictated by the Miocene rate as it was the longest period (Table 2.1).

2. 4.2. Evolutionary trajectories for two leaf attributes

Trait reconstruction showed that the most recent ancestor (MRA) had broad leaves (P = 0.61) that were blunt-tipped with smooth margins (P = 0.88) (Fig.2. 2). The phylogeny split into needle (A) (P = 0.78) and broad (B) (P = 1.00) clades by 14.1 Ma. Heteroblasty arose 6.9 Ma. Both clades remained blunt-tipped (P = 0.69, 1.00) but sharp tips emerged in one branch of the A clade 12.7 Ma. While the transition rate for needle/heteroblastic leaves exceeded that of broad leaves in the Miocene, proliferation of broad leaves accounted for 60% of the diversification events (Table 2.1A). Broad leaf proliferation continued (mainly through stabilization) at the expense of needle but not of heteroblastic leaves through the Pliocene and Quaternary. Overall, 65% of total proliferations were of broad leaves (mainly stabilization), with 33 reversals to broad leaves, 30% to needle and 5% to heteroblastic (mainly recent transitions), with the overall transition rate of non-broad leaves 2.6 times broad leaves. The evolution of non-broad leaves increased the overall speciation rate by 54%, greatest in the Miocene. Spiny leaves proliferated at a similar rate as non-spiny leaves in the Miocene but the rate for spiny leaves declined slightly through the Pliocene and Quaternary due to reducing stabilization but increased slightly among non-spiny leaves due to increasing stabilization (Table 2.1B). Reversals were negligible. The transition rate for spiny leaves was twice that for non-spiny leaves, with their advent and proliferation increasing the diversification rate by 73%.

Table 2.1: Paired trait evolution in *Hakea* apportioned among stabilization (trait retained during diversification event) and transition (trait attained during diversification event) processes in each Epoch based on the molecular chronogram reported here. All node-to-node steps in the phylogeny were treated as diversification events. Where the ancestor was ambiguous this event was omitted from the counts as the process was unclear. Reversals refer to transitions back to the previous trait**.**

Epoch/period:		**Miocene (18—5.3 Ma)**		**Pliocene (5.3—2.6 Ma)**		**Quaternary (2.6—0 Ma)**		**Overall (18—0 Ma)**		
Species diversification rate:		3.23		0.23		0.07		4.48		
Trait that evolved	**Genetic process**	**Within trait (%)**	**Between traits (%)**	**Within trait (%)**	**Between traits (%)**	**Within trait (%)**	**Between traits (%)**	**Within trait (%)**	**Between traits (%)**	**Number of reversals**
A. Leaf shape										
Broad leaves	Stabilization	93.9	56.8	96.9	64.6	94.1	72.7	94.9	61.6	
(ancestral)	Transition	6.1	3.7	3.1	2.1	5.9	4.6	5.1	3.3	33
Needle leaves	Stabilization	87.1	33.3	83.3	20.8	50.0	4.6	84.4	25.2	
	Transition	12.9	4.9	16.7	4.2	50.0	4.6	15.6	4.6	10

Heteroblasty	Stabilization	0.0	0.0	50.0	4.2	0.0	0.0	25.0	1.3	
	Transition	100.0	1.2	50.0	4.2	100.0	13.6	75.0	4.0	0
			100.0		100.0		100.0		100.0	
Fisher Test (*P*, 2-tailed)		0.0367		0.0120		0.0013		<0.0001		
Speciation promotion rate			1.65		1.50		1.29		1.54	
B. Spinescence										
Non-spiny leaves	Stabilization	95.0	50.7	96.7	60.4	100.0	61.9	96.4	55.6	
(ancestral)	Transition	5.0	2.7	3.3	2.1	0.0	0.0	3.6	2.1	3
Spiny leaves	Stabilization	91.4	42.7	94.4	35.4	75.0	28.6	90.2	38.2	
	Transition	8.6	4.0	5.6	2.1	25.0	9.5	9.8	4.2	0
			100.0		100.0		100.0		100.0	

Fisher Test (*P*, 2-tailed)		0.6594		1.0000		0.1333		0.1686		
Speciation promotion rate			1.87		1.60		1.62		1.73	
C. Pollinators										
Insect pollination	Stabilization	93.3	68.3	87.9	63.0	100.0	52.9	92.3	65.3	
(ancestral)	Transition	6.7	4.9	12.1	8.7	0.0	0.0	7.7	5.4	47
Bird pollination	Stabilization	68.2	18.3	100.0	28.3	85.7	47.1	83.1	23.9	
	Transition	31.8	8.5	0.0	0.0	14.3	0.0	16.9	5.4	31
			100.0		100.0		100.0		100.0	
Fisher Test	(*P*, 2-tailed)	0.0068		0.3130		1.000		0.1383		
Speciation promotion rate			1.37		1.39		1.89		1.41	
D. Fruit size										

Small fruit	Stabilization	25.0	2.7	47.8	22.0	66.7	16.7	45.9	11.6	
	Transition	75.0	8.2	52.2	24.0	33.3	8.3	54.1	13.6	0
Medium fruit	Stabilization	91.3	57.5	90.0	36.0	100.0	50.0	92.3	49.0	
(ancestral)	Transition	8.7	5.5	10.0	4.0	0.0	0.0	7.7	4.1	8
Medium-large	Stabilization	71.4	13.7	33.3	2.0	0.0	0.0	55.0	7.5	
fruit	Transition	28.6	5.5	66.7	4.0	100.0	12.5	45.0	6.1	0
Large fruit	Stabilization	20.0	1.4	75.0	6.0	66.7	8.3	50.0	4.1	
	Transition	80.0	5.5	25.0	2.0	33.3	4.2	50.0	4.1	0
			100.0		100.0		100.0		100.0	
Fisher Test (*P*, 2-tailed)		<0.0001		0.0063		0.0016		<0.0001 (χ^2)		

Speciation promotion rate			1.59		2.50		2.00		1.88	
E. Fire response										
Resprouter	Stabilization	86.1	44.3	100.0	51.4	100.0	41.7	92.2	45.7	
(ancestral)	Transition	13.9	7.1	0.0	0.0	0.0	0.0	7.8	3.9	33
Nonsprouter	Stabilization	79.4	38.6	88.2	42.9	100.0	58.3	86.2	43.4	
	Transition	20.6	10.0	11.8	5.7	0.0	0.0	13.8	7.0	9
			100.0		100.0		100.0		100.0	
Fisher Test (*P*, 2-tailed)		0.5354		0.2286		1.0000		0.3969		
Speciation promotion rate			1.95		1.95		2.40		2.02	
F. Serotiny										
Strong serotiny	Stabilization	100.0	92.2	100.0	91.0	100.0	100.0	100.0	92.6	
(ancestral)	Transition	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0

Weak serotiny	Stabilization	0.0	0.0	100.0	9.0	–	0.0	63.8	4.2	
	Transition	100.0	7.8	0.0	0.0	–	0.0	36.2	3.2	0
			100.0		100.0		100.0		100.0	
Fisher Test (*P*, 2-tailed)		<0.0001		1.0000		–	–	<0.0001		
Speciation promotion rate			1.08		1.10		1.00		1.08	

Table 2.2: Trait evolution in *protea* apportioned among stabilization (trait retention) and transition (trait turnover) processes in each Epoch, collated from Lamont *et al.* Speciation rates based on natural log formula.

Epoch/period:		**Miocene (23—5.3 Ma)**		**Pliocene (5.3—2.6 Ma)**		**Quaternary (2.6—0 Ma)**		**Overall (23—0 Ma)**		
Speciation rate:		2.216		0.241		0.109		–		
Trait that evolved	**Genetic process**	**Within trait (%)**	**Between traits (%)**	**Within trait (%)**	**Between traits (%)**	**Within trait (%)**	**Between traits (%)**	**Within trait (%)**	**Between traits (%)**	**Number of reversals**
A. Fire response										
Resprouter	Stabilization	67.7	50.6	83.3	57.4	100.0	47.6	90.5	51.2	
	Transition	32.3	9.1	16.7	4.3	0.0	0.0	9.5	5.4	7
Nonsprouters (ancestral)	Stabilization	84.8	27.3	100.0	31.9	90.9	47.6	79.5	34.5	
	Transition	16.2	13.0	0.0	6.4	9.1	4.8	21.5	8.9	10
			100.0		100.0		100.0		100.0	

Speciation promotion rate			2.48		2.61		1.91		2.30	
B. Serotiny										
Serotiny	Stabilization	98.4	86.1	100.0	79.1	90.9	70.0	98.4	79.0	
(ancestral)	Transition	1.6	1.4	0.0	0.0	9.1	2.5	1.6	1.3	2
Nonserotiny	Stabilization	77.8	9.7	88.9	18.6	100.0	27.5	90.3	17.8	
	Transition	22.2	2.8	11.1	2.3	0.0	0.0	9.7	1.9	0
			100.0		100.0		100.0		100.0	
Speciation promotion rate			1.14		1.26		1.38		1.25	

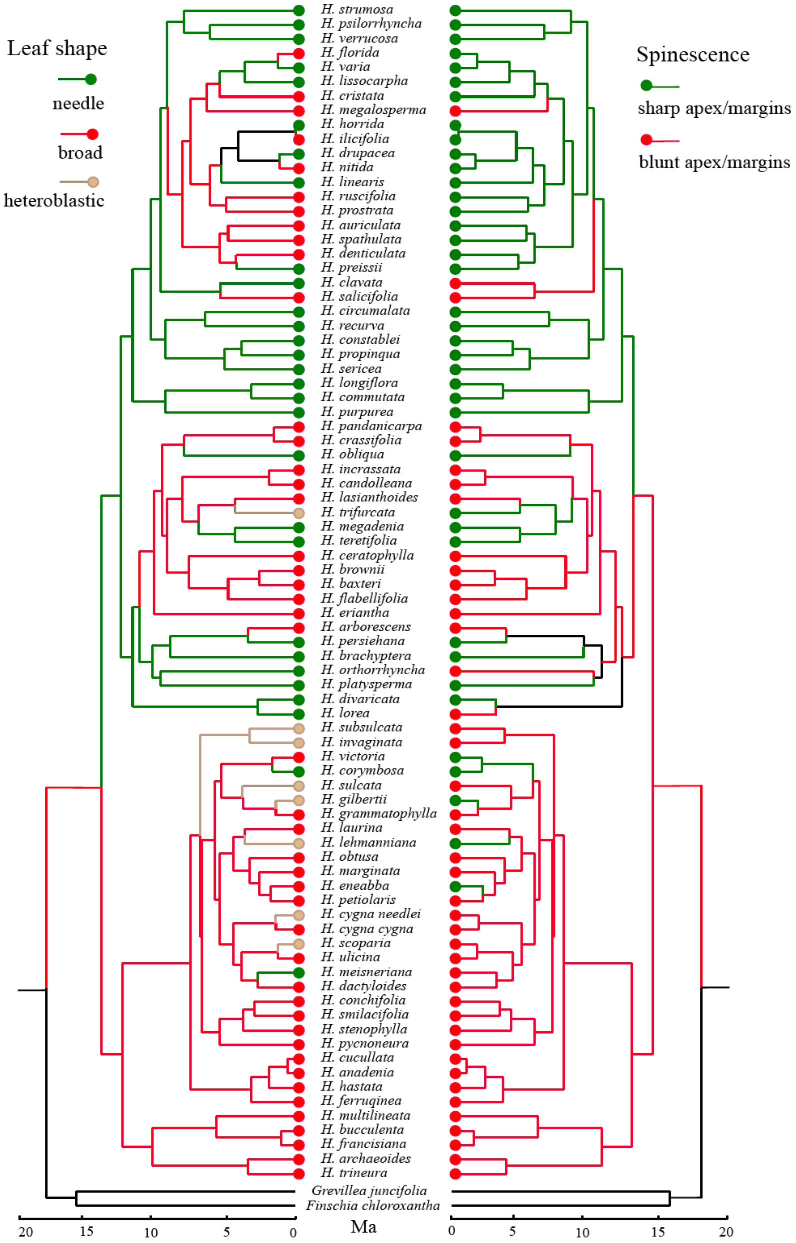


Figure 2.2: Reconstruction of leaf morphology traits through time in the genus *Hakea*. Left: leaf shape, broad, needle or heteroblastic (broad followed by needle). Right: leaf

spinescence, blunt/nil or sharp apices or teeth**.**

2. 4.3. Evolutionary trajectories for two reproductive attributes

The MRA of *Hakea* showed a high posterior probability (*P* = 0.75) of being insect-pollinated. The basal split of the genus was accompanied by a shift to bird pollination 14.1 Ma in clade B (*P* = 0.82) but retention of insect pollination in clade A (*P* = 0.98) (Fig. 2.3). A reversal occurred in clade B 12.6 Ma while pollination transitioned to birds 12.1 Ma in clade A that remained predominantly insect-pollinated. Overall, 78 reversals occurred (Table 2.1C). The switch to bird pollination was restricted to the Miocene with transitions accounting for 32% of bird proliferation events, and increasing stabilization through the Pliocene/Quaternary. Bird to insect transitions occurred in the Pliocene but not in the Quaternary. Overall transition rates for insect and bird pollination were similar, with bird pollination accounting for 30% of events and promoting speciation by 41%. The MRA had a high probability (by parsimony) of producing medium-sized fruits (1.0–5.0 g). Smaller (< 1.0 g) and larger (> 5.0 g) fruits first arose 12.1 Ma in clade A and smaller fruits appeared 6.5 Ma in clade B (Table 2-1D). In the Miocene, 19% of events involved transitions to other than medium-sized fruits but proliferation of medium-sized fruits predominated. Proliferation of small fruits (46% of events) dominated in the Pliocene, through both transitions and stabilization, and proliferation of non-medium-sized fruits contributed 150% to the stimulation of diversification events. In the Quaternary and overall, proliferation of medium and non-medium fruits contributed equally to all diversification events. Only medium fruits were sometimes the outcomes of reversals; all other transitions were unidirectional with medium ➔ small accounting for 30 events, medium□medium-large / large for 11 events, and medium➔medium-large➔large for 8 events. Overall, 24% of all events involved transitions to non-medium fruits and their proliferation accounted for an 88% increase in the speciation rate.

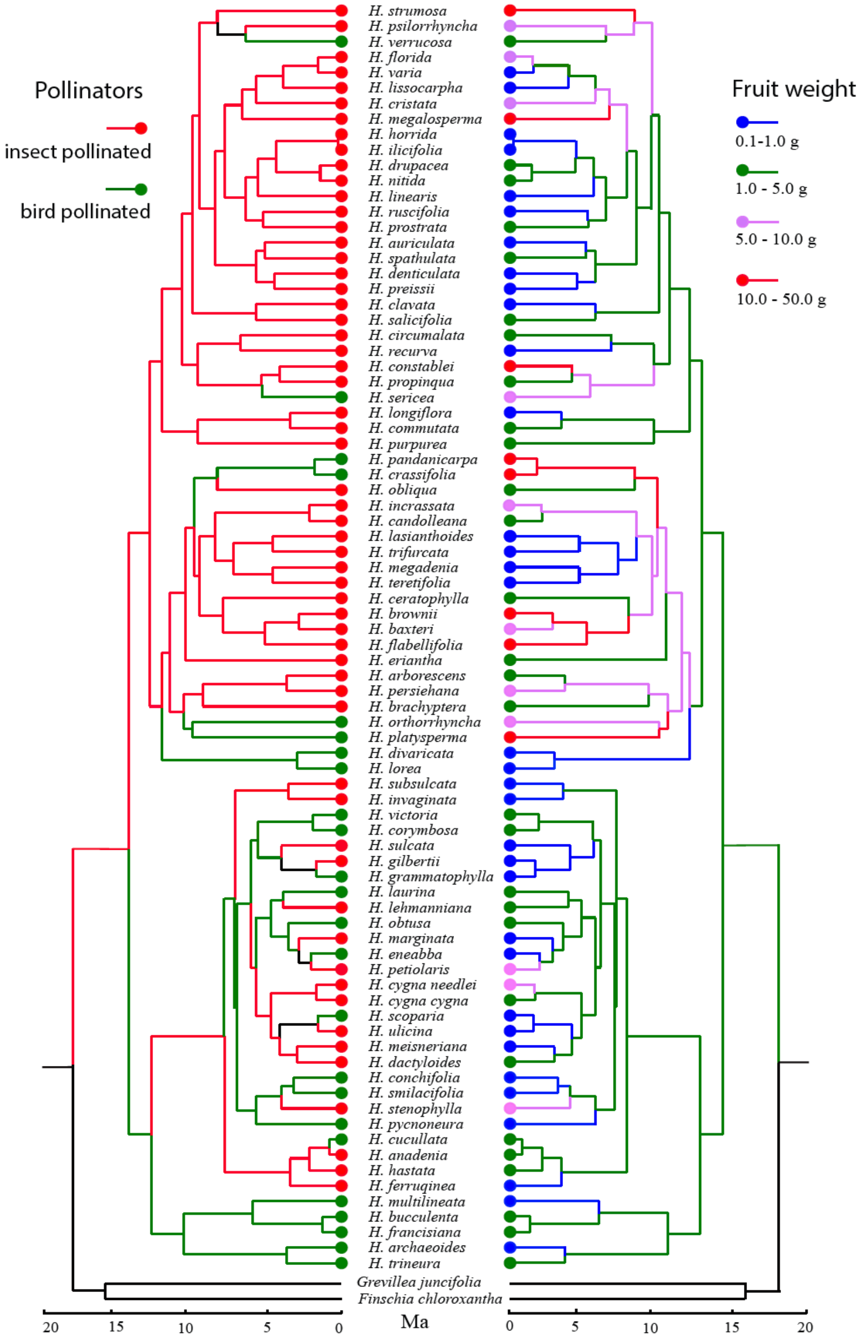


Figure 2.3: Reconstruction of reproductive biology traits through time in the genus *Hakea*. Left: insect or bird pollinated. Right: four classes of fruit size by weight.

2. 4.4. Evolutionary trajectories for two fire-adapted attributes

Postfire regeneration of the MRA was via resprouting though the posterior probability was not strong (*P* = 0.62). The ancestor of clade A was a resprouter (*P* = 0.73), while clade B was a nonsprouter (*P* = 0.86) (Fig. 2. 4). By 12.7 Ma, nonsprouters also evolved in clade A. By the end of the Miocene, diversification events were spread almost uniformly between resprouters and nonsprouters (Table 2.1E). Transitioning to nonsprouters remained strong in the Pliocene but ceased among resprouters. Transitioning ceased in the Quaternary with nonsprouting promoting 140% more speciation through stabilization in that period. Overall, proliferation among resprouters and nonsprouters was similar with the advent of nonsprouters doubling the speciation rate due to similar high rates of stabilization, though transitions to nonsprouting approached twice that for resprouting. Reversals were common among resprouters but only 20% of reversals involved nonsprouters. Serotiny was the MRA with *P* = 1.00 and both major clades remained serotinous (*P* = 1.00). There was an isolated occurrence of weak/nil serotiny 12.1 Ma and five more subsequent origins in clade A but non-serotiny never arose in clade B. Stabilization among moderately/strongly serotinous lineages dominated trait proliferation throughout hakea's history with limited transition to weak/non-serotiny in the Miocene followed by stabilization in the Pliocene and absence of proliferation in the Quaternary. Overall, stabilization of serotiny was the main process with proliferation of non-serotiny accounting for 7% of events and it increased speciation by 8%. All transitions were unidirectional.

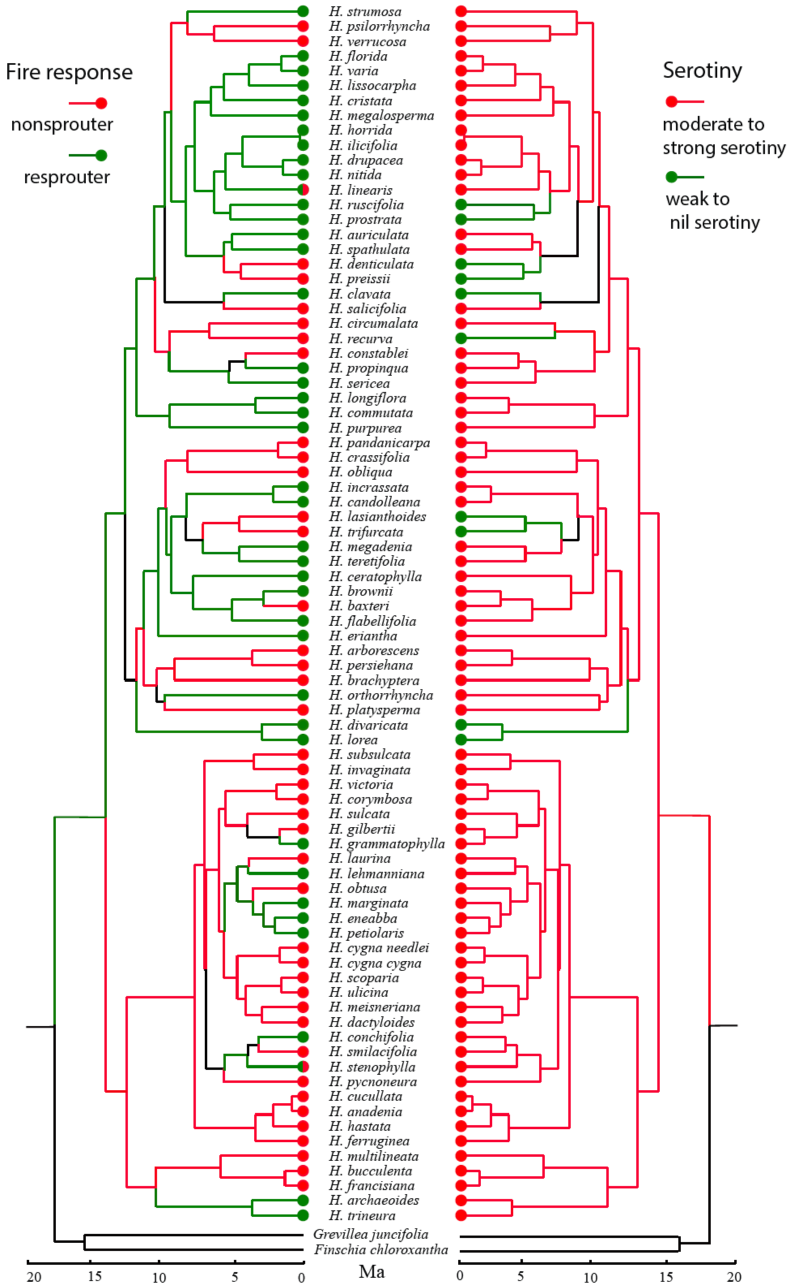


Figure 2.4: Reconstruction of fire-adapted traits through time in the genus *Hakea*. Left: nonsprouter (seedlings only) or resprouter. Right: strongly or weakly/nil serotinous.

2. 4.5. Promotion of species diversification

The overall speciation promotional rate (SPR_6) induced by the advent of novel traits was given by 1.54 × 1.73 × 1.41 × 1.85 × 2.02 × 1.08 = 15.16. Thus, 93.4% of diversification events (ignoring reversals) can be attributed to the presence of at least one non-ancestral trait. Three species possessed the six ancestral traits (*H. candolleana*, *H. ceratophylla*, *H. eriantha*), all in the same subclade of 14 species, but they included two reversals (nonsprouter➔resprouter, medium-large➔medium fruits). Thus, 96.3% of extant species lack at least one ancestral trait. One species (*H. divaricata*) had five of six traits in the advanced condition.

2. 4.6. Correlated evolution between traits

Correlation analysis using the Bayes Factor (BF) showed no relationship between any pairs of attributes ($BF < 1.0$) except leaf shape and spinescence, with needle leaves more likely to be spiny ($BF = 5.3$).

2. 5. Discussion

Trait reconstruction of the ancestral *Hakea* phenotype shows it to have been broad-leaved, non-spinescent and insect-pollinated, with medium-sized, serotinous fruits and resprouting after fire. Resprouting and serotiny confirm that the associated vegetation was fireprone and experienced a reliable postfire wet season by 18 Ma (Lamont & Enright 2000; Lamont *et al*. 2013). It is clear that *Hakea* changed radically at the level of fire-related adaptations, including woodiness of their fruits, when migrating from the non-fireprone environment of its parents (nonsprouting and nonserotinous), whereas leaf form (broad, non-spinescent) and reproductive biology [insect-pollinated, medium-sized (1–5 g) fruits] were initially conserved. Nevertheless, within 4 My, two (sub)clades had evolved with quite different syndromes of traits: one (A) that retained resprouting but possessed needle leaves many of which developed sharp apices, was bird-pollinated and where the largest woody fruits (>10 g) were produced, and the other (B) that became nonsprouting but all other attributes were dominated by their ancestral traits. The final outcome was almost equal representation of broad and needle leaves and spiny and blunt leaves, significant (30%) presence of bird-pollination, almost equal representation of small (<1g) and large (>5g) fruits, equal representation of resprouting and nonsprouting, and limited presence (10%) of weak/non-serotiny. Only three of 82 extant species retain all six ancestral traits and even two of these traits were the outcome of reversals. At the genus level, of 15 possible pairs of correlated evolution between attributes, only needle and sharp-pointed leaves were associated through time (attributable to their morphological links). What processes accounted for the independent evolution of the advanced states of these attributes? And to what extent did they account for the strong speciation rate in this clade (>150 species in 14 Ma)?

The species diversification rate of *Hakea* was highest by far in the Miocene than in the more recent epochs. The Miocene was a period of great climatic upheavals and the speciation rates among banksias in Australia (He *et al*. 2011) and proteas in South Africa (Lamont *et al.* 2013) (both genera also in Proteaceae) were also an order of magnitude higher then. The same pattern applies to proliferation of traits, with all alternative traits of the six examined highest in the Miocene (obtained by multiplying the percentage contribution to species diversification of each trait by the diversification rate on a time basis). This was also true for growth form (clonal/non-clonal), serotiny (present/absent), dead florets (retained / shed) and dead leaves (retained / shed) among banksias (He *et al.* 2011), and fire response (killed / resprout) and serotiny among proteas (Lamont *et al.* 2013). Epicormic resprouting was a Pliocene / Quaternary phenomenon arising from lignotuberous ancestors in the Miocene as for *Protea*, but it arose five times in the Miocene among banksias where it was probably ancestral to lignotubers (Lamont *et al.* 2011). The tree form with large leaves among proteas arose in the late Miocene but peaked in the Pliocene and followed the invasion of the savanna grasslands 12.7 Ma (Lamont *et* *al*. 2013). This eastward migration was made possible by the simultaneous evolution of resprouting and nonserotiny from nonsprouting and serotiny in the Cape shrublands with both peaking in the Pliocene. Parallels exist with the resprouting, nonserotinous *H. lorea*/*divaricata* that are best represented in the semi-arid, summer-rainfall region of central-north Australia, whose stem can be traced to 12 Ma most likely arising from resprouting, serotinous parents in the shrublands of SW Australia (Lamont *et al*. in press). In contrast to South Africa, this never led to prolific speciation in the vast region now invaded.

2. 5.1. Transition versus stabilization processes

While trait initiation (transition) is a vital step in speciation its incorporation into the clade (stabilization) is just as important. That proliferation of a trait through the phylogeny is rarely a function of the transition rate is strongly supported here. Taking leaf shape as an example (Table 2.1A), the transition from broad to needle leaves overall occurred at >2.6 times the rate as the reverse transition, yet stabilization of broad leaves occurred at 2.3 times the rate as needle leaves. The net result was the proliferation of broad leaves at 1.85 times the rate of needle leaves because the ratio of stabilization to transition events among broad leaves was five times the rate for needle leaves. In theory, only one initiation step is required for incorporation of a new trait into the clade provided it stabilizes quickly and is not subject to reversals. Thus, the ratio of stabilisation to transition events is a function of the strength of directional selection. The invasion of the savanna grasslands by *Protea* is a rare example of unidirectional selection associated with a single transition followed by almost universal stabilization (Lamont *et al.* 2013). In practice, the same trait arises numerous times through the phylogeny (Figs. 2.2 – 2.4) while reversals depend on the trait (Tables 2.1, 2.2). For *Hakea* leaf shape, 77% of the 43-recorded reversals were for the recovery of broad from needle leaves rendering transitions to needle less effective and reflecting unstable selective forces. The relative contribution of transition and stabilization events to proliferation depends on both the trait and the time period under consideration. Overall, collating the data in Tables 2.1 and 2.2 show that 30% of (net) trait proliferations in a given epoch involved stabilization (replication) of the (existing) trait only, transitions contributed 1–20% of proliferation events 37% of the time, 21–40% of events 16% of the time, 41–80% of events 11% of the time, and 100% of events 7% of the time (Fig. 2.5). Collectively, transitions account for 0–100% of proliferation events in any period, averaging 20%, for all traits examined here.

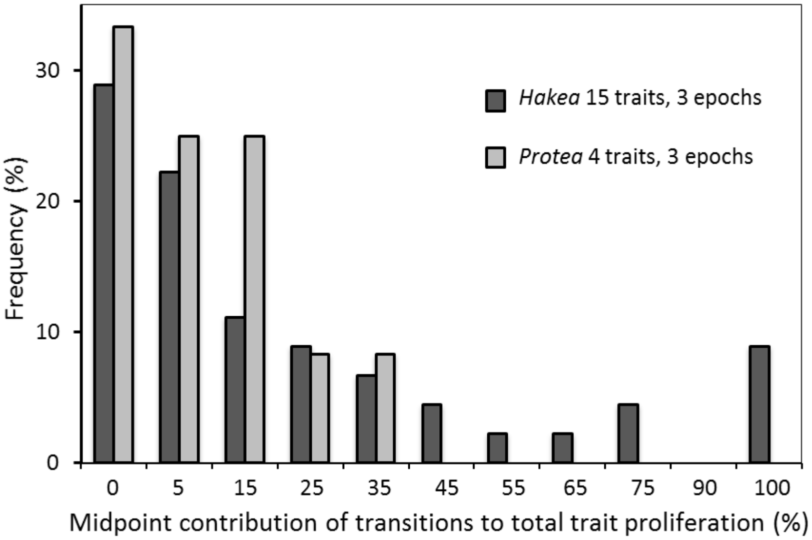


Figure 2.5: Contributions of transitions to total trait proliferation among six attributes and 15 traits over three epochs for *Hakea* and *Protea* (collated from Tables 2.1 and 2. 2).

2. 5.2. Evolutionary trajectories for leaves

By the time *Hakea* separated from its non-fireprone ancestors 18 Ma (Lamont & He 2012), Australia (and much of the world) was experiencing declining levels of rainfall, temperatures and metabolically active atmospheric gases and increasing seasonality. In addition, the opening up of the vegetation would have exposed them to high light intensity and diurnal temperatures (Jordan *et al*. 2005) compared with closed forests. If needle leaves increase fitness to such constraints, and currently they account for 45% of species so this genus has a strong propensity to produce them, they should have evolved early in its history and proliferated through stabilization. Indeed, within 4 Ma, a needle-leaved clade (A) had arisen with strong stabilization leading to 56% of its extant species being needle-leaved (Fig. 2.2). Evolution of needle leaves was greatly delayed in clade B and was mainly expressed through the appearance of heteroblastic species over the last 5 Ma (all from broad-leaved ancestors). The latter appeared so recently that there have been no opportunities for reversals unlike needle leaves where reversals to broad have been frequent (Table 2.1). These reversals confirm the lability of leaf form among isobilateral leaves as demonstrated ontogenetically by *H. trifurcata* whose juvenile leaves are needle, a few becoming broad at the start of the growing season in adult plants and needle again as the dry summer approaches (Groom *et al*. 1994a). The persistence, indeed dominance, of broad leaves and reversals to them require some explanation. Clearly, leaf form is not the only way of dealing with drought, such as deep root systems (Groom & Lamont 2015), while broad leaves among hakeas are still highly sclerophyllous (Lamont *et al*. 2015) with thick cuticles, sunken stomata and a tannin-filled hypodermis (Lamont *et al*. 1987; Jordan *et al*. 2005, 2008; Groom & Lamont 2015). They are often narrow or strap-shaped rather than truly broad, such as *H. grammatophylla* in the 'deadheart' of Australia, and all are vertically oriented. In addition, broader-leaved species have retreated to the moister parts of the landscape or subregions (Groom & Lamont 1996a). In fact, the frequent reversals in both directions are consistent with climatic oscillations that became characteristic of the Pliocene/Quaternary and their evolutionary tracking.

While broad leaves may be spinescent, such as *H. cristata*, needle leaves that are already rigid and with a sclerified apex can readily be transformed into strongly piercing structures through elongation and thinning of the mucro. This morphogenetic link explains the evolutionary correlation through time of needle and spinescent leaves. Thus, following a small delay, one branch of the A clade became spinescent at 12.7 My (Fig. 2.3). The broad-leaved B clade remained essentially non-spinescent. If spinescence is effective against herbivores (macropods) and florivores/granivores (emus, cockatoos) (Hanley *et al*. 2007, Groom & Lamont 2015) the delay in its appearance cannot be attributed to their absence as all were present by this time but they speciated gradually and their selective effects would have intensified over time. It is of interest that transitioning to spiny leaves was most marked in the Quaternary, a time when modern cockatoos evolved in SW Australia, though their ancestors were present from the early Miocene (Joseph *et al*. 2014). Overall, 42% of proliferation events were of spiny leaves, with stabilizations at ten times the rate of transitions. Reversals were negligible indicating strong directional selection. Why more events did not yield spiny leaves is partly attributable to morphological constraints, the fact that all *Hakea* leaves are highly unpalatable and not grass-like (Rafferty *et al.* 2010) anyway, and ability of vertebrates to learn to overcome physical deterrents (Hanley *et al.* 2007; Lamont, unpublished).

2. 5.3. Evolutionary trajectories for flowers and fruits

Bird-pollinated flowers evolved from insect-pollinated flowers with the split of the genus 14.1 Ma (Fig. 2.3). This resolves the disagreements over which was the ancestral condition (Barker *et al*. 1999b; Hanley *et al.* 2009; Mast *et al*. 2012) caused by misidentifying the basal lineages or not including sufficient (representative) insect-pollinated lineages from SW Australia where the clade most probably arose (Lamont *et al.* in press). Honeyeaters

(Meliphagidae) were already present in Australia at the time *Hakea* originated, but those birds only diversified strongly in the mid to late-Miocene, especially among such major pollinators as *Phylidonyris, Anthochaera, Lichmera* and *Lichenostomus* (Joseph *et al.* 2014). In fact, apart from *H. cucullata* in the Quaternary, the only time flowers increased their size to accommodate bird pollinators was in the Miocene, at 4.75 times the rate of the reverse (Table 2.1). For the Pliocene/Quaternary it was stabilization processes only. The greater levels of stabilization among insect-pollinated lineages (2.73 times that among birds) throughout their history explains their current greater abundance and suggests that they have been a greater selective force, possibility associated with their greater reliability rather than morphological diversity that would have favored greater transition rates.

Of note are the transitions from bird to insect pollination in the Miocene / Pliocene and the large number of reversals (78), 60% of which were insect➔bird➔insect. This is significant on two counts: trait reversibility and fluctuating selection. Bird-pollination is regarded as an evolutionary 'dead-end' because specialization of floral structures for birds may be irreversible (Futuyma & Moreno 1988; Toon *et al*. 2014) so shifts from bird to insect pollination are rare (van der Niet & Johnson 2012). This hypothesis derives from hummingbird pollination systems that require specialised floral structures (Tripp & Manos 2008). However, specialised floral structures are not essential for honeyeaters because they are generalist pollinators and not obligate nectarivores. This lack of specialization in the honeyeater pollination system implies minimal floral structure specialization – simple elongation of the pistil is sufficient (Hanley *et al*. 2009). As a result, reversal shifts from bird to insect are possible in situations when bird pollinators become scarce. The evolutionary tracking of the great climatic fluctuations, with their profound effects on the abundance of both birds and insects that occurred from the mid-Miocene can explain the remarkable number of reversals in both directions. Such great flexibility in pollinator shifts may provide insights in explaining the mechanisms that promoted explosive speciation in *Hakea* but even more so in its younger sister, *Grevillea* with 150 of 362 species bird-pollinated (Ford *et al.* 1979).

Morphological variation in fruit structure among hakeas is exceptional and transition rates away from the ancestral medium-sized ancestor equalled their stabilization rates through each of the three epochs (Fig. 2.3, Table 2.1). Two groups of granivores, insects and parrots, were already present when *Hakea* emerged though it is unknown when the specialist beetle and moth granivores evolved. Nevertheless, the transition to small (camouflaged) fruits, which appear better protected against insects than cockatoos (B Lamont, unpublished), was strongest in the Miocene and continued throughout the period, by far the highest (13.6% of all events) among all traits we assessed. Large woody fruits, particularly effective against cockatoos (Groom & Lamont 2015), followed a similar path. There were no reversals among small and large fruits indicating strong unidirectional selection for these extremes, and included medium➔ medium-large➔ large occurring eight times.

The pattern for medium fruits was quite different: low transition rates ceasing altogether in the Quaternary, stabilizations at 12.2 times the rate of transitions and with reversals back to medium. If small and large fruits so enhance fitness against granivores, why did over 50% of proliferation events involve medium fruits? The possible answer is that 1–5 g is already in the medium-strong serotiny category that must already have considerable resistance to predispersal granivores as long-stored seeds are otherwise vulnerable. Since larger fruits are less efficient at translocating nutrients from the fruit to the seeds, there may also be a nutrient-supply issue (Groom & Lamont 2010). Large fruits produce large seeds and so plants may be constrained to produce fewer of them (El-ahmir *et al*. 2015) that may reduce the chances of self-replacement after disturbance. Small fruits are usually weakly serotinous and these run the risk of their seeds failing to recruit in the hostile prefire environment (Causley *et al.* submitted) whereas large seeds will produce large seedlings with a greater chance of success (Lamont & Groom 2013). Finally, large fruits require a strong supporting stem, often associated with cauliflory, that may not be an option for many species.

2. 5. 4. Evolutionary trajectories for fire-related traits

The twin ancestral traits of resprouting (adaptive in the presence of severe, periodic disturbances where recruitment opportunities are limited) and serotiny (ensuring seeds are released when those limited recruitment opportunities are optimal) demonstrate that *Hakea* arose in a fireprone environment (Fig. 2.4). We can also surmise that fires were of overall moderate frequency (> 5 - 45 y intervals) but highly stochastic. If fires were at the high frequencies associated with savanna grasslands (<5 y intervals) the plants would have stayed nonserotinous (Lamont *et al.* 2013). If fire intervals exceeded their longevity then, upon death in the absence of fire, serotinous seeds would have been released onto a hostile seedbed and rarely yielded recruits (Causley *et al*. 2016). However, within 4 Ma, a nonsprouting clade (B) had arisen. The outcome was strong transitioning to both fire-response types in the Miocene and all epochs were dominated by stabilizations (Table 2.2E) against a background of almost universal proliferation by serotinous descendents (Table 2.2F). Of note are both the continuing transitions to nonsprouting in the Pliocene and its steady increase in stabilization rate throughout *Hakea*'s history (from 38.6% to 58.3% of all fire-response proliferations). It is likely that the trend of increasing aridity and seasonality and declining atmospheric oxygen and carbon dioxide (He *et al*. 2012) led to less frequent, but more reliable, fires and promotion of nonsprouting (Lamont *et al.* 2011, 2013). Today, resprouters are better represented in the more fireprone, strongly seasonal northern sandplains of SW Australia and nonsprouters in the less fireprone parts, especially on deeper soils where recruitment and adult survival are more likely (Lamont & Markey 1995; Groom & Lamont 1996a).

Transitions to non/weak serotiny were rare but five of the six independent origins were in the late Miocene and proliferation was restricted to the Pliocene (Fig. 2.4, Table 2.1).

Explanations vary but include migrations to frequently-burnt savanna grasslands (*H. divaricata* lineage) or rarely-burnt aridlands (*H. recurva*), exposure of novel firefree rock outcrops to which some species adapted (*H. clavata*), and presence in forests with reliable winter rains ensuring recruitment interfire (*H. trifurcata* lineage) (Hanley & Lamont 2001). This pattern has limited parallels with *Protea* in South Africa where one transition to nonserotiny in grasslands was followed by increasingly extensive stabilization there (Table 2.2) that failed to occur in Australian grasslands. However, in both super-regions stabilization of the ancestral condition, serotiny, was by far the dominant process and reversals were negligible. By contrast, the well known lability in fire responses (He *et al*. 2011; Litsios *et al*. 2014) was expressed in both *Protea* and *Hakea*, although 80% of reversals were to resprouting in *Hakea* in the Miocene, perhaps reflecting periods of increased or more stochastic fire frequencies as the clade moved to other parts of Australia. The historic levels of fire frequency coupled with the severity of seasonal droughts serve well to interpret the relative abundances and distributions of resprouters and nonsprouters among hakeas, ericas and proteas (Ojeda 1998; Lamont *et al*. 2013), and this no doubt lies behind the conclusion of Litsios *et al.* (2014) that a more heterogeneous / mesic climate explains why Restionaceae nonsprouters are proportionately so well represented in South Africa compared with Australia, that can be more parsimoniously interpreted as their evolution in a less fire-prone landscape (Lamont *et al*. 1985, 2011; Bond *et al.* 2003).

2. 5.5. Promotion of speciation by non-ancestral traits

There are two ways of considering the role of traits in speciation. One is to compare how they have *contributed to* all diversification events (Maddison & Midford 2007) and the other is to estimate to what extent non-ancestral traits have *promoted* these diversification events. The latter is hypothesis-driven and based on the premise that without trait innovation and subsequent stabilization these diversification events would not have occurred. Thus, it relies on being able to identify both the ancestral state and its pathway through the phylogeny in order to ascertain trait reversals. This means that any extinction events cannot be incorporated into the analysis, unlike claims of the BiSSE model, but we argued earlier (see Introducion) that most new traits are adjunct to, rather than displace, ancestral traits anyway. Nevertheless, entire but unknown lineages characterized by certain historically maladapted traits may be missing (or only represented by long branches in the chronogram) if there have been radical environmental shifts that no amount of adjustment, in the absence of fossil evidence, can correct for. However, there is little evidence of extinctions as a significant evolutionary process in the SW Australian flora over the last 10 My (Hopper 2009).

For the six *Hakea* attributes examined, proliferation of the non-ancestral trait promoted speciation by 1.08 (weakly serotinous) to 2.02 (fire-killed) times. Overall, this increased speciation by 15.2 times, equivalent to 93.4% of diversification events. Two of the ancestral traits possessed by the three species of identical phenotype to the putative original phenotype were the result of reversals so that even these were the outcome of trait diversification when the ancestral condition would have been temporarily lost. Reversals were particularly prevalent among pollination types (41% of all events) and fire-response types (22%) but absent altogether from the serotiny types (Table 2.1). They represent the net effect of a) the constancy of directional selection, b) the lability of opposing traits, and c) time available for further transitions to occur. Certainly, the high level of lability among fire-response types is consistent with previous studies on *Protea* (Table 2.2), *Banksia* (He *et al*. 2011) and Restionaceae (Litsios *et al.* 2014) and prevented the identification of the ancestral state in the last two. Clearly, without adaptive responses to seasonal drought, aridity, changing fire regimes, novel habitats, nectar-feeding birds, marsupial herbivores and insect/cockatoo granivores, already operating in its Miocene beginnings, *Hakea* would have remained a relict species somewhere in the wetter parts of SW Australia. By contrast, its remarkable genetic/morphological malleability in the face of these strong selective agents has resulted in an exceptionally diverse clade (Hanley *et al*. 2009, Groom

& Lamont 2015) and distributed throughout Australia. We might now wonder to what extent the cumulative contributions by transitions and stabilizations to trait proliferation among critical plant attributes, in response to an array of environmental constraints introduced in the Miocene, has led to the explosive radiation of such speciose genera as *Grevillea, Acacia, Melaleuca* and *Eucalyptus,* that currently dominate Australia's sclerophyll flora, and the floras of many other parts of the world subject to similar selective forces.

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Chapter 3: Seed size, fecundity and postfire regeneration strategy are interdependent in *Hakea* [1]

3. 1. Abstract

Seed size is a key functional trait that affects plant fitness at the seedling stage and may vary greatly with species fruit size, growth form and fecundity. Using structural equation modelling (SEM) and correlated trait evolution analysis, we investigated the interaction network between seed size and fecundity, postfire regeneration strategy, fruit size, plant height and serotiny (on-plant seed storage) among 82 species of the woody shrub genus, *Hakea*, with a wide spectrum of seed sizes (2–500 mg). Seed size is negatively correlated with fecundity, while fire-killed species (nonsprouters) produce more seeds than resprouters though they are of similar size. Seed size is unrelated to plant height and level of serotiny while it scales allometrically with fruit size. A strong phylogenetic signal in seed size revealed phylogenetic constraints on seed size variation in *Hakea*. Our analyses suggest a causal relationship between seed size, fecundity and postfire regeneration strategy in *Hakea.* These results demonstrate that fruit size, fecundity and evolutionary history have had most control over seed size variation among *Hakea* species.

3. 2. Introduction

Seed size is a key trait in the life history of plants that affects fitness at the seedling stage and is often correlated with other attributes important in their evolution and ecology

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[1] Abbreviation in this chapter: SWA: southwestern Australia; SEM: structural equation modeling; NCBI: National Center for Biotechnology Information; MCMC: Monte Carlo Markov Chain; BF: Bayes factors.

(Westoby *et al.* 1992). Seed size among angiosperms varies from 1 × 10^{-5} g to 3 × 10^{4} g (Harper *et al.* 1970). Many factors have been shown to influence seed size, such as resource availability (Westoby *et al.* 1990; Hammond & Brown 1995), growing conditions (Vaughton & Ramsey 1998; Pluess *et al.* 2005), and plant growth form, longevity and height (Leishman *et al.* 1995; Moles *et al.* 2005). For example, by analysing seed mass data for 13,000 species, Moles *et al.* (2005) concluded that there is a close association between seed size and plant height that is likely the result of the scaling of seed size to plant height (Kang & Primack 1999). In addition, seed size variation may also be subject to phylogenetic constraints on seed development, such that closely related species may have similar seed sizes (Kang & Primack 1999).

Recurrent fire is a prominent phenomenon in ecosystems with Mediterranean-type climates, such as those in SWA. Recent research points to a significant role for fire in shaping the evolution of plant functional traits in these fire-prone ecosystems (He *et al.* 2011; Keeley *et al.* 2011). However, studies of how fire might have influenced seed size variation are scarce. Plants in fire-prone ecosystems can be divided into different functional groups in terms of their overall response to fire. In the simplest scheme, the fire response of plant species entails nonsprouters (killed by fire, and populations regenerate solely from seedlings) and resprouters (resprout after fire from roots, rhizomes, lignotubers or major stems of the pre-fire plants) (Bell *et al.* 1993; Lamont & Wiens 2003; Clarke *et al.* 2013). This divergence of life form and postfire regeneration strategy in fire-prone environments can be expected to have significant implications for seed size variation through direct or indirect interactions.

Carpenter & Recher (1979) first proposed that fire-response strategies are linked with reproductive features, such as fecundity. Nonsprouters should invest more resources in seed production than do resprouters because resprouters have the ability to survive via self-replacement. By comparing species pairs, Lamont & Wiens (2003) showed that nonsprouting species indeed have greater seed set on a per ovule basis than resprouters, but it is by no means universal (Lamont 1985; Enright *et al.* 2007). An improvement in resource availability usually leads to greater seed production mainly because the plants are larger (Geritz *et al*. 1999; Rautio *et al.* 2005), but the reverse may also be true (Groom & Lamont 2011). In addition, there is much support for a trade-off between fecundity and seed size (Greene & Johnson 1994; Turnbull *et al*. 1999; Jakobsson & Eriksson 2000), though this relationship must be set in the context of other life-history traits. Working in fire-prone sclerophyll shrublands, Esther *et al.* (2011) showed that the two most important interactions affecting population viability were seed size–seed production and seed size–regeneration strategy. Resprouters always did well, but the success of nonsprouters depended on their having many or large seeds.

Nonsprouters might opt for many small seeds as these have a greater probability of reaching favourable habitats further from the parents than larger seeds (Losos & Leigh 2004). Since small seeds produce small seedlings they are likely to be more drought-prone (Enright & Lamont 1992; Richards & Lamont 1996). Where both fire-response types produce few seeds they are expected to be larger as seedling survival is dependent on quickly developing a strong root system, possible only from larger seeds, to avoid the effects of drought (Gómez 2004; Lamont & Groom 2013). Heavy seeds may also gain a competitive advantage over small seeds due to their earlier germination (Dubois & Cheptou 2012). Resprouters typically produce few seeds, and they invest less in reproductive organs relative to the storage functions that help them re-establish quickly after fire, irrespective of seed size, and thus there should be a discernable relationship between fire response and seed size.

Serotiny (prolonged storage of seeds on the plant) is characteristic of fire-prone, sclerophyll vegetation in the Southern hemisphere (Lamont *et al.* 1991). Mature seeds are retained in the crown and seed release is usually cued by heat from fire. Empirical observations suggest serotinous species usually produce large fruits (Lamont *et al.* 1991; Stock *et al.* 1991). Serotinous seeds take longer to mature (1–3 years) and therefore can receive more resources during seed filling (Lamont *et al.* 1991; Stock *et al.* 1991; Groom & Lamont 2010). Secondly, serotinous species usually have large, woody fruits on stout stems to protect their seeds against predators and temperature extremes. Given a fixed number of seeds per fruit (e.g., two seeds in each follicle, typically in Grevilloidae), larger fruits can support and nurture larger seeds.

The endemic Australian genus *Hakea* (Proteaceae) is known for its wide range of seed sizes (2–500 mg) among its 150 extant species (Groom & Lamont 1996a), 100 of which inhabit the nutrient-impoverished soils of SWA, characterised by hot, dry summers and frequent fire (Groom & Lamont 2015). Species are either killed by fire or resprout from lignotubers or sometimes epicormic buds or lateral roots (Groom & Lamont 1996b). Growth form varies from creeping sub-shrubs to trees rarely >5 m tall. All possess woody fruits that vary in size by >3 orders of magnitude and in degree of serotiny from zero to ~10 years (Groom & Lamont 1997) and on-plant seed storage varies from close to zero (some resprouters) to thousands (large nonsprouters) of seeds (Groom & Lamont 1996a, b). Much study have looked into the relationship of fruit size and seed size (Groom & Lamont 1997), fruit size and postfire regeneration strategy (Groom & Lamont 1996b), serotiny and fruit size (Groom & Lamont 2010), and generated significant insights into the ecology of seed size variation in *Hakea.* However, as the majority of those studies investigated a simple relationship between seed size and another functional trait, it is not clear how these functional traits interact in a network of ecological setting and in an evolutionary context.

In this Chapter, I used SEM analysis and correlated trait evolution analysis to explore the interacting network of seed size, postfire regeneration strategy, fecundity, fruit size, serotiny and plant height in a phylogenetic context including 82 species. The objective was to identify the driving force behind variation in seed size within a genus adapted to poor soils, recurrent fire and severe summer drought.

3. 3. Material and Methods

3. 3.1. Trait data and structural equation modelling analysis

I focused on seed size and five functional and life history traits that are expected to influence seed size in *Hakea*. Trait data were collated from the literature (Groom & Lamont 1996a; Barker *et al*. 1999; Young 2006; Kew 2008; Hanley & Sykes 2009; Mast *et al*. 2012). A total of 82 species covering morphological variation in the genus and distribution range, and with relatively even numbers of resprouters and nonsprouters, was investigated (Table S1).

I first used SEM analysis to generate and explore models that infer the causal relationships between seed size and putative interacting traits. SEM extends the basic correlation approach to path analysis by directly testing the goodness of fit of the model to the data, calculates correlation coefficients, and separates total effects into direct and indirect effects (Hox & Bechger 1998). Models can be modified by deleting pathways that are not correlated, therefore optimising the fit of the model. The modelling process in SEM analysis is based on a priori and theoretical knowledge and begins with a consideration of expected relationships based on the mechanisms predicted to operate in the system. We began by building a conceptual SEM model of the expected multivariate relationships based on prevailing theory of the interactions between seed size and functional or life history traits, and then refined the model by deleting the uncorrelated pathways. Seed size and another five functional or life history traits for each of the 82 species were included in the SEM model (Fig 3.1): 1) plant height, 2) postfire regeneration strategy, 3) fecundity (on-plant seed store), 4) serotiny, and 5) fruit size. Fecundity was estimated as the number of fruits stored on plants at least 15 years since the last fire. Each fruit supported two seeds though very occasionally one of these may abort. Seed and fruit size (dry mass) were continuous data while height, regeneration strategy, fecundity and serotiny were categorical. The working hypotheses were based on the following predictions:

H_1: Nonsprouters produce more seeds or larger seeds than resprouters (Esther *et al.* 2011) and have greater investment in seeds (Lamont & Wiens 2003; Losos & Leigh 2004; Dubois & Cheptou 2012; Lamont & Groom 2013);

H_2: There is a negative correlation between fecundity and seed size (Greene & Johnson 1994; Turnbull *et al*. 1999; Jakobsson & Eriksson 2000);

H_3: Resprouters have lower fecundity (Carpenter & Recher 1979), and therefore a larger trade-off in resource limited systems;

H_4: Strongly serotinous species produce larger seeds than non-weakly serotinous species (Lamont *et al.* 1991; Stock *et al*. 1991; Groom & Lamont 2010);

H_5: Taller plants produce larger seeds (Kang & Primack 1999; Moles *et al*. 2005);

H_6: Larger fruits possess larger seeds (since all fruits contain two seeds) as suggested by allometric logic (Primack 1987).

SEM was performed in SPSS AMOS 18.0.0 (Analysis of Moment Structures, SPSS Inc., Chicago, USA). The conceptual model was examined using a likelihood approach, and non-significant pathways were later deleted, and the model with the remaining pathways was retested. The significance of correlations was taken as one-tailed, $P \leq 0.05$ because our predictions were directional.

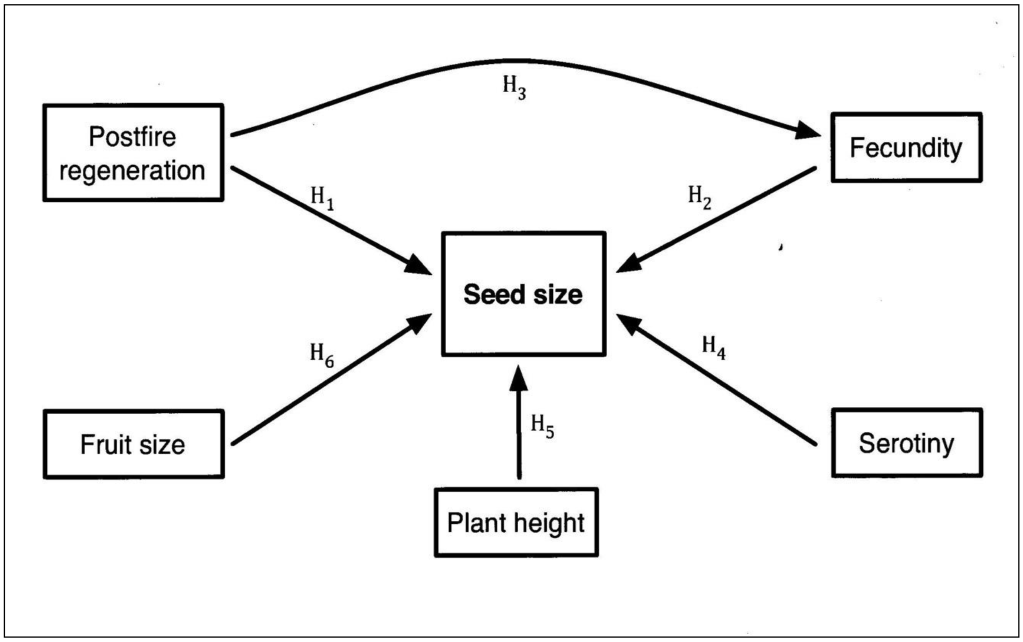

Figure 3.1: Conceptual model showing working hypotheses on the interaction between seed size and other functional and life history traits. See text for hypotheses H_1–H_6.

3. 3.2. Phylogenetic analysis of correlated evolution between seed size and other traits

Trait correlations were further tested in a phylogenetic context with divergence time as branch length. We built a *Hakea* phylogeny of the 82 species using gene sequences extracted from NCBI (51 species), combined with new sequences generated in this study (31 species). For the newly generated sequence for each species we amplified 8 DNA regions: the nuclear ribosomal internal transcribed spacers (ITS) and plastid *matK*, *rbcL*, *trnL* intron, and *trnL-trnF* intergenic spacer, *atpB*, *atpB-rbcL* intergenic spacer, and *rpl16* intron, were produced following standard protocols (GenBank accession numbers shown in Table S2) (Sauquet *et al.* 2009). *Grevillea juncifolia*, *Finchia chloroxantha*, *Buckinghamia celsissima*, *Banksia serrata* and *Persoonia lanceolata* (all Proteaceae) DNA sequences were chosen as outgroup for the *Hakea* phylogenetic analysis (Table S2).

The sequences were aligned and edited using the computer software MUSCLE (Edgar 2004).

BEAST v2.1.0 (Drummond & Rambaut 2007) was used to estimate phylogeny relationships and divergence time under a strict clock model (Drummond *et al.* 2006) that provided phylogenetic topology consistent with previous studies (Mast *et al.* 2012; Sauquet *et al*. 2009). The dataset was partitioned by genes, with each partition unlinked and set to a general time reversible (GTR) model with γ -distributed rate heterogeneity. We set the calibration point for crown Proteaceae at 70.6 Ma as suggested by Sauquet *et al.* (2009) based on the fossil *Lewalanipollis rectomarginis* described by Khan (1976). Yule prior was used for rates of cladogenesis and ran analyses of 10 million generations, sampling every 1000 generations. The program Tracer (Rambaut *et al*. 2014) was used to visualize the posterior distribution of trees and estimate the appropriate burn-in. Consequently, a 2.5 million generation burn-in was determined. Tree Annotator v1.6.1 (Drummond & Rambaut 2007) was used to generate a maximum credibility tree (MC tree) based on this analysis.

The degree of phylogenetic signal in the six traits in *Hakea* was tested using Pagel’s lambda (λ) based on 1000 *Hakea* phylogenies generated from BEAST in above analysis. A value of 0 indicates no significant phylogenetic signal in the trait, while a value of 1 indicates complete phylogenetic patterning. Pagel’s λ estimation and significance tests were conducted in the R package ‘Geiger’ Geiger’ (Harmon *et al*. 2008).

BayesTraits continuous random walk (Model A) was used to determine the relationships between pairwise *Hakea* traits, as illustrated in the conceptual model (Pagel and Meade 2006). BayesTraits uses a Monte Carlo Markov Chain (MCMC) procedure to calculate the harmonic means of different pairs of *Hakea* traits based on the MC tree. Bayes factors (BF) were used to determine IF significant phylogenetic correlations between two traits (BF < 2: weak; 2 < BF < 5: moderate; BF 5~10: strong). The study excluded the outgroup taxa in these analyses to avoid introducing bias in estimates of trait relationships that might occur when a single taxon is used to represent a much larger group (Mooers & Schluter 1999).

3. 4. Results

Seed size showed wide variation among the 82 *Hakea* species, and both resprouters and nonsprouters had a wide range of seed weights. For example, among resprouters, *H. oleifolia* seeds weigh 5 mg while *H. flabellifolia* seeds weigh >156 mg. The nonsprouting *H. sulcata* has a seed weight of 3 mg but *H. platysperma* weighs > 509 mg. However, resprouting species had lower fecundity than nonsprouters when adjusted for plant size. Nonsprouting species produced on average more than 100 fruits per plant, while resprouters averaged half this number.

Seeds of resprouting species were slightly lighter than those of nonsprouters (34.5 ± 34.1 mg vs 40.0 ± 76.6 mg, mean ± standard deviation, respectively), but fire response had no direct effect on seed size variation in *Hakea* ($P = 0.36$; Table 3.1). Larger seeds were not associated with taller plants ($P = 0.255$), and serotinous species did not necessarily have larger seeds than weakly- or non-serotinous species ($P = 0.240$; Table 3.1). Deleting these non-significant pathways, the final SEM analysis revealed a direct causal correlation between the postfire regeneration strategy and fecundity (resprouters store fewer seeds) with a direct effect of 0.55 ($P < 0.001$), and a significant trade-off between fecundity and seed size (direct effect = - 0.12; $P = 0.047$), such that species with more seeds had smaller seeds. Strong positive correlations were observed between fruit size and seed size with a direct effect of 0.78 ($P < 0.001$), i.e., heavier fruits have larger seeds (Fig 3.2).

Table 3.1: Standard direct effect and associated probability of the hypothesised interaction pathways in the conceptual model. (*)Star indicates hypothesis supported.

Dependent variable		**H_1**	**H_2^***	**H_3^***	**H_4**	**H_5**	**H_6^***
Seed size		Postfire response		Fecundity	Serotiny	Plant height	Fruit size
Fecundity			Postfire response				
	Standardised direct effect	0.030	0.550	-0.150	-0.058	0.044	0.779
	P (one-tailed)	0.360	<0.001	0.047	0.240	0.255	<0.001

The topology of our *Hakea* phylogeny, which included 82 species, was consistent with one reported earlier by Mast et al. (2012) which included 55 species were included (Fig. S1). We detected a strong phylogenetic signals for seed size with a λ of 0.82, implying closely-related *Hakea* species tend to be more similar in seed size than expected by chance (Fig. 3.3). Similar results were recovered for postfire regeneration strategy and (especially) serotiny, and, to a lesser extent, fruit size. Plant height and fecundity were less constrained by phylogeny with λ much less than one. From the trait data and time-calibrated phylogeny, associated evolution between pairwise traits was noted in *Hakea* using Bayesian MCMC analysis (Fig. 3.4). The analysis revealed significant evolutionary correlations between postfire regeneration strategy and fecundity (BF = 8.6), and between seed size and fruit size (BF = 4.6). Seed size and serotiny are also appear to be correlated (BF = 2.7). Seed size showed a weak association with fecundity (BF = 1.5) and with postfire regeneration strategy (BF = 1.1). Plant height was unlikely to have been related to seed size during the evolution of the genus (BF = 0.8).

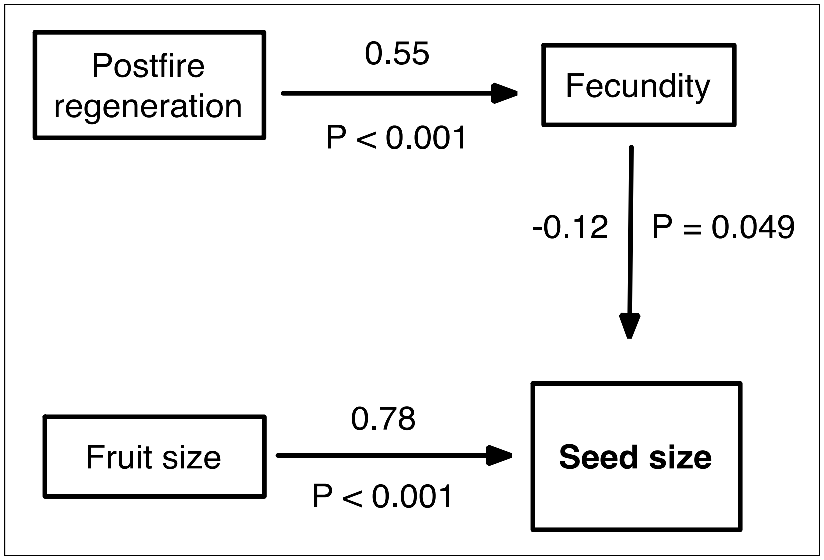

Figure 3.2: Simplified structural equation modelling analysis showing the significant interacting pathways between seed size, fecundity, postfire regeneration strategy and fruit size. Numbers above the lines are the standardised direct effects.

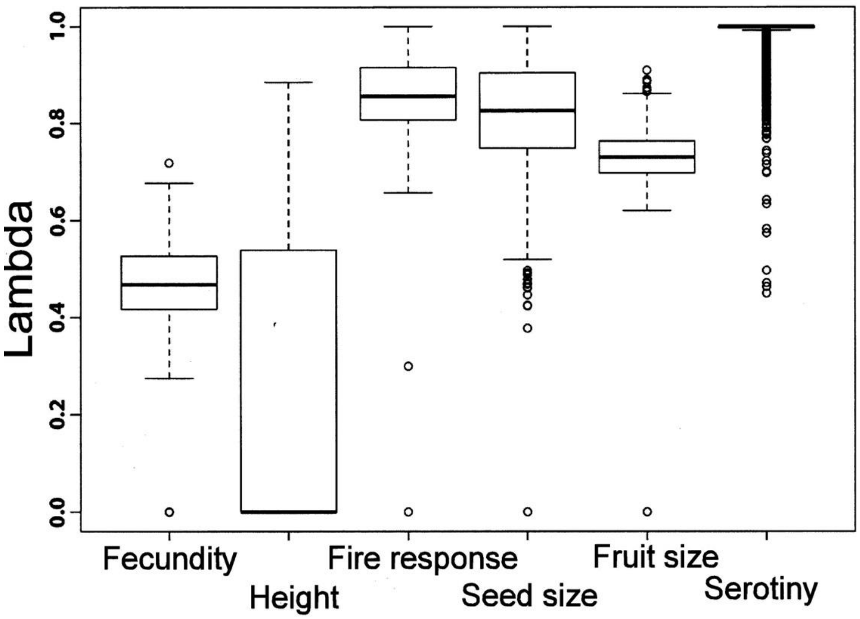

Figure 3.3: Distribution of lambda values among 1000 simulations indicating the degree of phylogenetic constraints on six traits in *Hakea*. Means are shown by thickened horizontal lines, standard deviations are bounded by boxes and ranges are connected by broken lines, and circles are outliers.

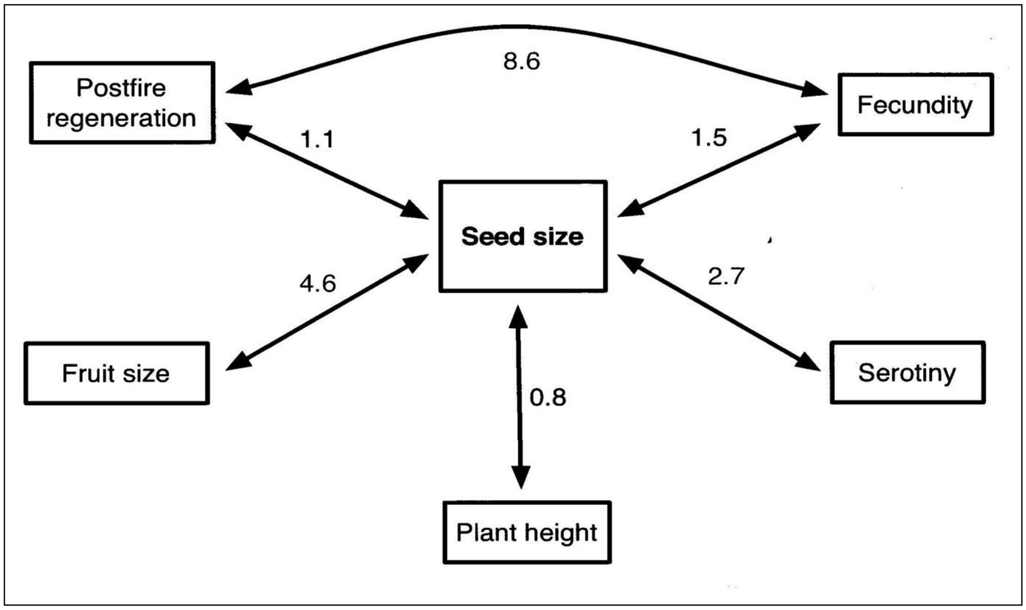

Figure 3. 4: Evolutionary associations between pairwise traits in *Hakea*. Numbers beside the arrows are Bayes factors (BF), with BF > 2 indicating strong association.

3.5. Discussion

Structural equation modelling and Bayesian MCMC analysis showed that seed size was most significantly associated, in a co-evolutionary sense, with fruit size where larger fruits support larger seeds (H_6). This relationship is clearly causal as a) the number of seeds per fruit is fixed (two) so that only seed size can vary, b) the pericarp acts directly as a source of nutrients for seed filling (Groom & Lamont 2010), c) larger fruits have a better vascular supply for seed filling (Lamont & Barrett 1988), and d) larger (woody) fruits are an adaptive response to the greater vulnerability of larger seeds to granivores (Stock *et al.* 1991).

However, the relationship between seed and fruit size breaks down when serotiny (prolonged on-plant seed storage) is considered. Although seed size increases with stronger serotiny through evolutionary time (second only to fruit size), the follicle:seed weight ratio of strongly serotinous species is six times that of weakly serotinous species without any difference in seed weight (Groom & Lamont 1997). This is not so when a wider range of genera is collated (Groom & Lamont 2010) therefore it must be a special feature of *Hakea*. It appears that protecting the seeds from granivorous cockatoos, during their prolonged storage on the plant, has taken precedence over any potential benefits of larger seed size. Nevertheless, Groom & Lamont (2010) show that in SWA the phosphorus concentration of strongly serotinous species is 40% higher than in weakly serotinous *Hakea* species. This confirms that the seed size-nutrient content relationship is not crucial to the ability of hakeas to recruit inter-fire as occurs with weakly serotinous species, in contrast to the anti-herbivore role of their phenolic content (Hanley & Lamont 2001).

Despite a recorded seed weight range of 2 to 500 mg, nonsprouters (38 mg) and resprouters (36 mg) had similar mean seed mass i.e., there was no relationship between regeneration strategies and seed size, therefore hypothesis H_1 (nonsprouters produce larger seeds than resprouters) was not supported by the analysis. Just over half the species in both fire-response types had seeds weighing >20 mg, a size considered to contain sufficient nutrient resources to ensure adequate root extension for survival of the initial summer drought in the poor soils of SWA (Lamont & Groom 2013). The remaining species must rely on drought-tolerance traits (Richards & Lamont 1996). Seed size conservatism within a species contrasts with huge differences in seed number associated with variations in plant age and size, and nutrient and water availability (Stock *et al.* 1989; Lamont *et al*. 1991; Lamont *et al*. 1994; Susko & Lovett-Doust 2000). Thus it seems that a given seed size is embedded in the adaptive biology of each species by strong selection pressures (e.g., resource availability) and shows little phenotypic plasticity.

SEM analysis supported our expectation of a relationship between postfire regeneration strategies and fecundity ($P < 0.001$, hypothesis H_2). Further, the two traits have coevolved, as revealed by the Bayesian MCMC analysis. Given that both more and larger seeds may be adaptive among nonsprouters, as they regenerate solely from seeds after fire and their seedlings establish in nutrient-impoverished environments (Richards & Lamont 1996; Esther *et al*. 2011), this fire-response type opts for more rather than larger seeds. Extensive demographic studies on hakeas and related woody species in SWA have shown that postfire recruitment patterns conform to biased lotteries, with the demographic component paramount followed by biotic components, such as seedling size (Lamont *et al*. 1999). Given a fixed seed size, the best option to ensure population viability of fire-killed species is through a large seed store. This is achieved via faster growth rates, earlier time to maturity, more flowers/plant, more seeds/ovule, higher seed viability and finally more seedlings/parent compared with resprouters (Lamont *et al*. 1999). In contrast, low fecundity among resprouters may be best related to the accumulation of deleterious somatic mutations, a random, time-dependent process unrelated to seed size and to which nonsprouters are immune (Lamont & Wiens 2003, but see Dickinson & Grant-Downton 2009).

Using structural equation modelling and Bayesian MCMC analysis, we show that seed size is traded off with species fecundity (H_3). For example, *H. flabellifolia* seeds weigh 156 mg and it produces only one or two fruits per plant. In contrast, *H. pycnoneura* and *H. scoparia* have seed weights of only 5.9 mg but >100 fruits per plant. Apart from a trade-off with fecundity, further phylogenetic analysis revealed that seed size in *Hakea* might also be constrained by speciation patterns in the genus, i.e., closely related species tend to have similar seed sizes. For example, in *Hakea*, the Ulicina group has relatively small seeds while the Ceratophylla group has large seeds (Barker *et al*. 1999). Interestingly, fire response and serotiny, both considered adaptations to fire-prone environments, are shown here to have phylogenetic signals. It is likely that seed size in *Hakea* might have tracked selection pressure from fire as well. These processes are one explanation for the apparent trade-off between the size of seed stores and seed size, and phylogenetic constraints on seed size. They provide insights as to why the relationship is not strong for either of them because of the over-riding interactions with resource limitations and other selective pressures in fire-prone environments.

Global variation in seed size is associated with divergence in plant growth form (Moles *et al*. 2005), with taller plants sproducing larger seeds, which is assumed to reflect a trade-off between likelihood of survival to maturity (low in tall plants) and offspring size. However, plant height has no direct effect on seed size in *Hakea* (H_5). Drawing parallels with other congeneric pairs, resprouters are the slowest, and least likely, to mature but they are rarely the tallest (Lamont & Wiens 2003; Merwin *et al*. 2012) and seed size is no different from nonsprouters. It is true that larger seeds have a lower wing/mass ratio than smaller seeds among hakeas (Lamont & Enright 2000) and thus might benefit from a greater release height. On the other hand, long-distance dispersal is facilitated by wind vortices that lift and carry seeds from the ground in postfire habitats of SWA making seed size less relevant to their dispersal potential (He *et al*. 2004, 2009). It is also worth noting that plant height variation in *Hakea* is small (0.5–5 m) and may not be sufficient to promote divergence in seed size.

3. 6. Conclusions

The synthesis of powerful SEM analyses and robust phylogenies, by which multiple trait data sets are compared, revealed causal relationships between seed size and fruit size (strong) and fecundity (weak) in *Hakea*, and between fecundity and postfire regeneration strategy (strong), but not between seed size and regeneration strategy, plant stature or serotiny. Large seeds are supported/ protected by large fruits and have a weak trade-off with fecundity that is much lower among resprouters even though these do not have larger seeds. All relationships are constrained to some extent by their evolutionary history, with seed size correlated with fruit size and serotiny through evolutionary time.

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Chapter 4: Small-seeded *Hakea* species tolerate partial loss of cotyledons better than large-seeded congeners

4. 1. Abstract

Rapid growth after emergence is crucial for seedling establishment and early growth in the nutrient-poor and water-limited soils of Southwest Australia, a region characterised by a Mediterranean climate. The aim of this study was to determine the effect of cotyledon loss on the early growth of seedlings of species from the Australian genus *Hakea* that had epigeal germination. The growth of six *Hakea* species of different seed sizes with partially or completely removed cotyledons at four days after emergence was monitored over 90 days in a glasshouse. All seedlings perished by the fifth week when both cotyledons were completely removed, irrespective of seed size. Partial removal of the cotyledons caused a significant delay in seedling growth, measured as stem height, root length, and dry biomass of large seeded species. The growth of small-seeded species was less impacted by cotyledon damage, particularly when the cotyledon loss was not more than 50%. In conclusion, small-seeded species might tolerate partial cotyledon loss through the early emergence of true leaves for photosynthesis to support seedling growth

4. 2. Introduction

Cotyledons play an important role in seedling development, particularly during early growth, as these structures provide a major proportion of the nutrients and minerals needed for seedling growth until emergence of the first true leaves. In some species, cotyledons also have photosynthetic capabilities and serve as a significant source of carbohydrates (Kitajima 1996; Milberg & Lamont 1997; Kitajima 2003). Before germination, the main function of cotyledons is to absorb and store resources from the endosperm (Murray 2013). Cotyledons transfer reserve materials (lipid, carbohydrate, and mineral nutrients) into developing shoots and roots during and after germination (Ashcroft & Murray 1979; Hocking 1980).

In accordance with the functional morphology, three types of cotyledons were distinguished (Ibarra-Manríquez *et al.* 2001; Kitajima 2003): photosynthetic cotyledons, which rise above the ground, semi-photosynthetic reserve cotyledons, and reserve storage cotyledons. Reserve cotyledons primarily remain below ground level. Cotyledons have been associated with two main functions during seedling development: the storage and subsequent provision of reserve materials, and the development of photosynthetic capacity. Therefore, the partial or total loss of cotyledons represents a major loss of stored reserves (Kennedy *et al.* 2004) as well as capacity to photosynthesise (Kidson & Westoby 2000; Kitajima 2003). Drought, pathogen infection, and herbivory can result in the partial or complete destruction of cotyledons. Cotyledon damage or loss has negative impacts on seedling development and survival (Kitajima 2003; Hanley & Fegan 2007), and if seedlings reach maturity it can reduce reproductive output (Zhang et al. 2011). Several studies have focused on the response of seedlings to cotyledon losses (Lamont & Groom 2002; Kitajima 2003; Hanley *et al.* 2004; Hanley & May 2006; Hanley & Fegan 2007; Lamont & Groom 2013), revealing that the damage or loss of one or both cotyledons during early development reduces the growth rate of the plant (Fenner & Thompson 2005). Seedlings without cotyledons as a source of nutrient supply might suffer higher mortality than those with intact cotyledons (Matson & Waring 1984). The timing and amount of cotyledon damage should influence seedling development, as seedling dependence on the cotyledon would gradually decline as nutrients are consumed and the seedlings become autotrophic (García-Cebrián *et al.* 2003). However, results from empirical observations are mixed. For example, 100% mortality was observed when both cotyledons were removed soon after emergence in *Hakea*. However, Bonfil (1998) reported 60% mortality of *Quercus laurina* when the cotyledons were removed after the true leaves were fully exposed and expanded. Li & Ma (2003) showed that complete cotyledon removal in another *Quercus* species reduced seedling survival to 50%, which was significantly lower than seedlings without cotyledon removal, but the removal of half of the cotyledons had no effect on seedling survival in the same study. In some plant species, the remnant cotyledon may be able to compensate for the loss of photosynthetic capacity (Zheng *et al.* 2011) while seed mass may also play a role in mitigating the loss of the cotyledons.

The seedlings of many Proteaceae rely on the cotyledons as the sole source of mineral nutrients for up to 200 days after emergence in mineral-deficient soils (Stock *et al.* 1990; Stock et al. 1991). Previous studies on early seedling growth in *Hakea* have reported a 30-50% decrease in seedling mass resulting from the removal of cotyledons (Lamont & Groom 2002). Groom & Lamont (1998) reported that a 70% reduction of total plant mass for the largest-seeded species occurred when the cotyledons were removed from *Hakea* species in sand culture lacking nutrients, with both studies also showing that the removal of both cotyledons had no effect on total plant mass for small-seeded species; this supports the hypothesis that seed mass might determine the tolerance of a species to cotyledon damage. It is clear that cotyledons have significant consequences on the growth and survival of a seedling prior to the emergence of the true leaves (Hanley & May 2006; Hanley & Fegan 2007). Previous studies have involved two treatments with the removal of one or two cotyledons, mimicking the cotyledon damage from herbivory or physical forces (such as wind and frost). However there are no studies concerning the extent of the damage or loss of cotyledons that a seedling could tolerate. It is also not clear how seed size (cotyledon size) determines the capacity of seedlings to tolerate cotyledon damage, although this knowledge is important in explaining ecological adaptation, particularly in nutrient-deficient environments.

Despite the importance of cotyledons in the regeneration of plant life (Grime 2006), some of the factors affecting seedling survival, including the interaction between seed size, seedlings and herbivores, are not completely clear (Hanley & May 2006). Using Australian *Hakea* species as a model, the objectives of this study were to: 1) reveal the effects of the partial or complete removal of cotyledons on the early growth of seedlings to determine to what extent *Hakea* species tolerate cotyledon damage; and 2) explore the role of seed size (cotyledon size) in mitigating the negative impact of the partial or complete removal of the cotyledons on the early growth of seedlings.

4. 3. Materials and Method

Six *Hakea* species were selected to represent the wide phylogenetic diversity, with each species representing a different species group within the genus (Table 4.1). *Hakea* species have epigeal (above ground) germination. These six species represent the wide range of seed size present in this genus, from 8 mg in *H. francisiana* to over 500 mg in *H. platysperma*. The seeds of all six species were obtained from a commercial seed supplier (Nindethana Seed Services, Albany, Western Australia). The species used in this study are serotinous, and seeds readily generate without requirement of pre-treatment. *Hakea* seeds were first geminated on filter paper-lined Petri dishes in an environmentally controlled germination cabinet with constant 15 °C ambient temperature. At least 100 seeds per species were germinated to ensure the availability of a sufficient number of seedlings per species for late growth. The germinants were subsequently transplanted into tall pots made from PVC irrigation pipe (50 or 100 cm tall, 5 cm in diameter) to encourage root growth. The pots were filled with deionised water-washed white sand containing no nutrients and soluble minerals. All germinants were placed at the same depth (i.e. 1 cm below the soil surface) so that no seedlings had access to greater soil moisture.

The glasshouse experiment commenced on the 2nd of July 2013 and finished on the 25th of the November 2013, a duration of 146 days. The seedlings were grown till the cotyledons expanded. Once the emerged cotyledons flattened after 4 to 7 days, the cotyledon manipulations were initiated, as the time of cotyledon removal is important in determining whether the seedling will survive. The removal of cotyledons later than seven days was less effective because most of the essential substances had been transported from the cotyledons to the remainder of the plant or the primary leaves had emerged and further growth was independent of the cotyledons, while the early removal of the cotyledons would cause death from the wound impact (Lamont & Groom, 2002).

The manipulation treatments involved either 1) 0% cotyledon removal (control), 2) 25% of each cotyledon removed, 3) 50% of each cotyledon removed, 4) 75% of each cotyledon removed, and 5) 100% of both cotyledons removed. The cotyledons were carefully excised using a sterilised razor blade. All seedlings were manually treated with deionised water every three days to ensure sufficient soil moisture, while no additional nutrients were introduced.

The emergence of the true leaves was monitored for all treatments. Three sets of harvests (initial, mid-experiment and final) were collected. Every 30 days after cotyledon manipulation, 5 seedlings in each treatment, including control, in each of the six species were randomly selected and harvested. During harvest, the plants were cleaned before oven-drying at 60 °C for 48 h, when the following parameters were recorded: dry mass of the roots, dry mass of the stems, and dry mass of the leaves. In addition, the root length (length from ground level to tip of the longest root) and stem height (length of the longest stem) were also measured, as the two variables may also be impacted by removal of cotyledon (Groom & Lamont 2015).

Table 4.1: The mean seed size (from Groom & Lamont 1996) and informal taxonomic group (from Barker *et al.* 1999) of the *Hakea* species used in the present study.

Species	Cotyledon area mm²	Seed size (mg)	Taxonomic group
H. francisiana	74.8 ± 8	8 ± 2	Strumose
H. petiolaris	88 ± 10	16 ± 2	Petiolaris
H. cucullata	102 ± 12	29 ± 3	Cucullata
H. prostrata	117 ± 13	60 ± 7	Prostrata
H. pandanicarpa	232 ± 23	100 ± 12	Ceratophylla
H. platysperma	597.7 ± 24	501 ± 60	Platysperma

4. 4. Data analysis

Linear mixed effects models were used to test for differences among control, and partial or total removal of the cotyledons (0%, 25%, 50%, 75%, and 100%). General linear model and MANOVA were used to examine the effects of seed size on tolerance of the partial removal of cotyledon with seed size as fixed factor, harvest time as covariates, growth of seedlings (root length, stem height, root dry biomass, stem dry biomass and leaf dry biomass) in treatments with 25%, 50% and 75% removal of cotyledon as dependent variables. Three dimensional column charts were used to visualise the growth comparisons in different treatments. Data analysis was implemented using SPSS 22.0 (SPSS Inc., Chicago). Significance was taken at $P < 0.05$.

4. 5. Result

Cotyledon removal significantly impacted the seedling growth in all six *Hakea* species. All seedlings of the six species with both cotyledons completely removed (100% removal) died within 30 days (before the first harvest) (Table 4.2), while all seedlings with partial cotyledon removal survived. The surviving seedlings showed that medium to severe cotyledon damage (50% and 75% removal) significantly delayed the emergence of the first true leaves ($P < 0.05$) (Table 4. 2). Longer delays were observed for seedlings with higher proportions of cotyledon removal and seedlings from species with large seeds (Table.4.2).

Seed size has a significant effect on the growth of seedlings after partial removal of cotyledons. For all five growth parameters (root length, root dry biomass, stem height, stem dry biomass and leaf dry biomass), MANOVA analysis revealed a significant effect from seed size after partial removal of cotyledon over 90 days of growth ($P < 0.05$, Table 4.3). This suggests different tolerances to partial removal of cotyledon within the six study species. the effect of seed size on growth was similar in seedlings with 25% removal of cotyledon ($P > 0.05$, Table 4.3), whereby most measures of growth after partial removal of cotyledons were not significantly different to those of seedlings with intact cotyledon in the six study species (Fig. 4.1- 4.5).

Table 4. 2: The time (days) of true leaf emergence for all treatments (the days were calculated from the emergence of the cotyledon).

Species	Control	25%	50%	75%	100%[*]
H. francisiana	8 ± 1	8 ± 1	11 ± 1	12 ± 1	-
H. petiolaris	4 ± 1	4 ± 1	6 ± 1	8 ± 1	-
H. cucullata	12 ± 1	16 ± 1	16 ± 1	20 ± 2	-
H. prostrata	12 ± 1	17 ± 2	17 ± 2	20 ± 2	-
H. pandanicarpa	22 ± 2	25 ± 2	27 ± 2	28 ± 3	-
H. platysperma	20 ± 2	22 ± 3	25 ± 3	28 ± 3	-

*Seedlings with both cotyledons completely removed died within 30 days before the first harvest.

Growth (except stem height) after 75% removal of cotyledon in all six species was similar regardless of the difference in seed size ($P > 0.05$, Table 4.3), where growth of seedlings was severely impacted by partial removal of cotyledon irrespective of seed size (Figs. 4.1- 4.5). Seed size significantly affected growth of seedlings in the treatment with 50% removal of cotyledon ($P < 0.05$, Table 4.3), suggesting different tolerances to 50% removal of cotyledon within the studied species.

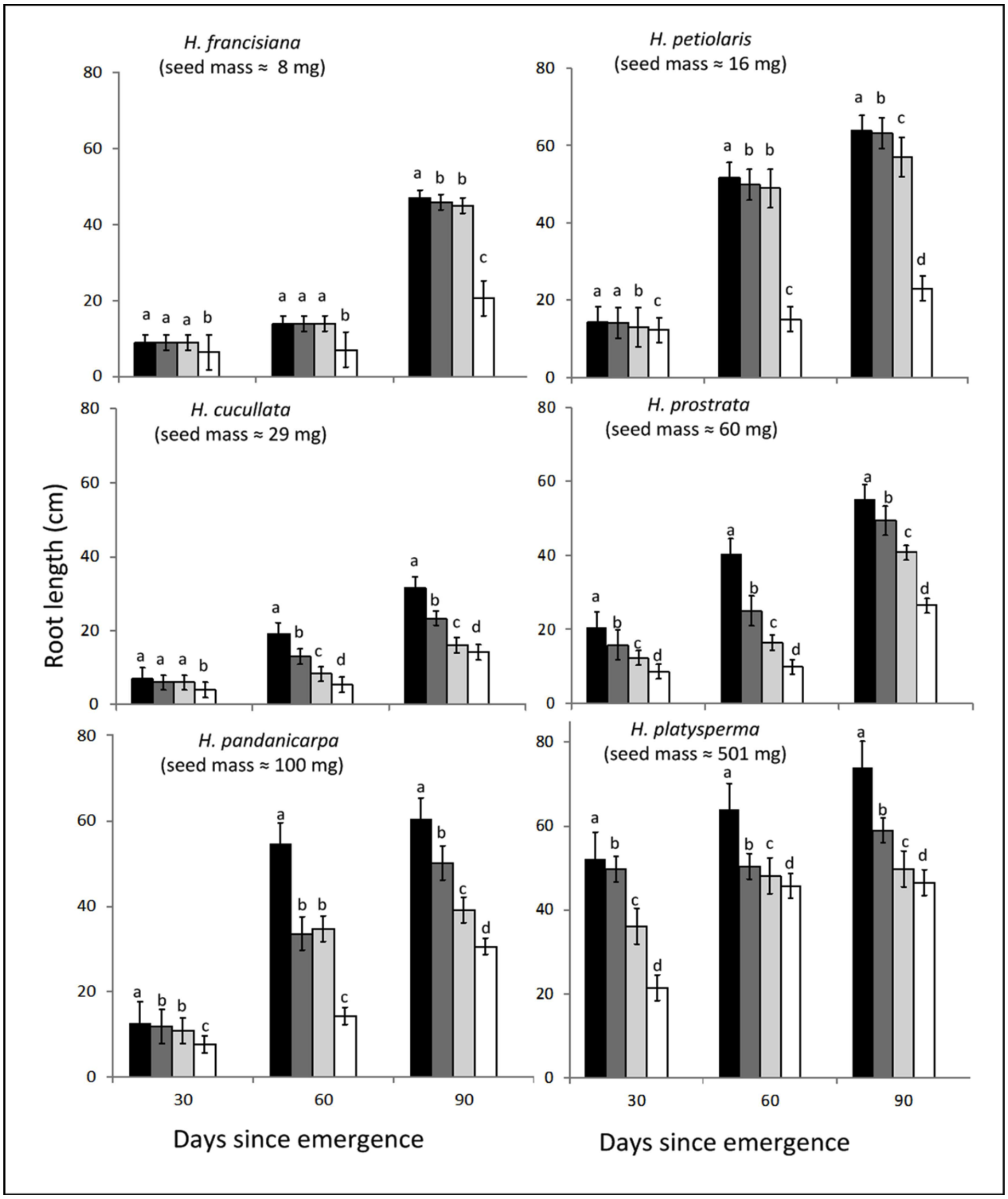

Figure 4.1: Shows the impact of 25%▩ , 50%▨ , and 75%□ , of cotyledon damage on root length compared with control■ . Means ± S.E. Different letter indicates significant differences (P > 0.05) between treatments for each harvest.

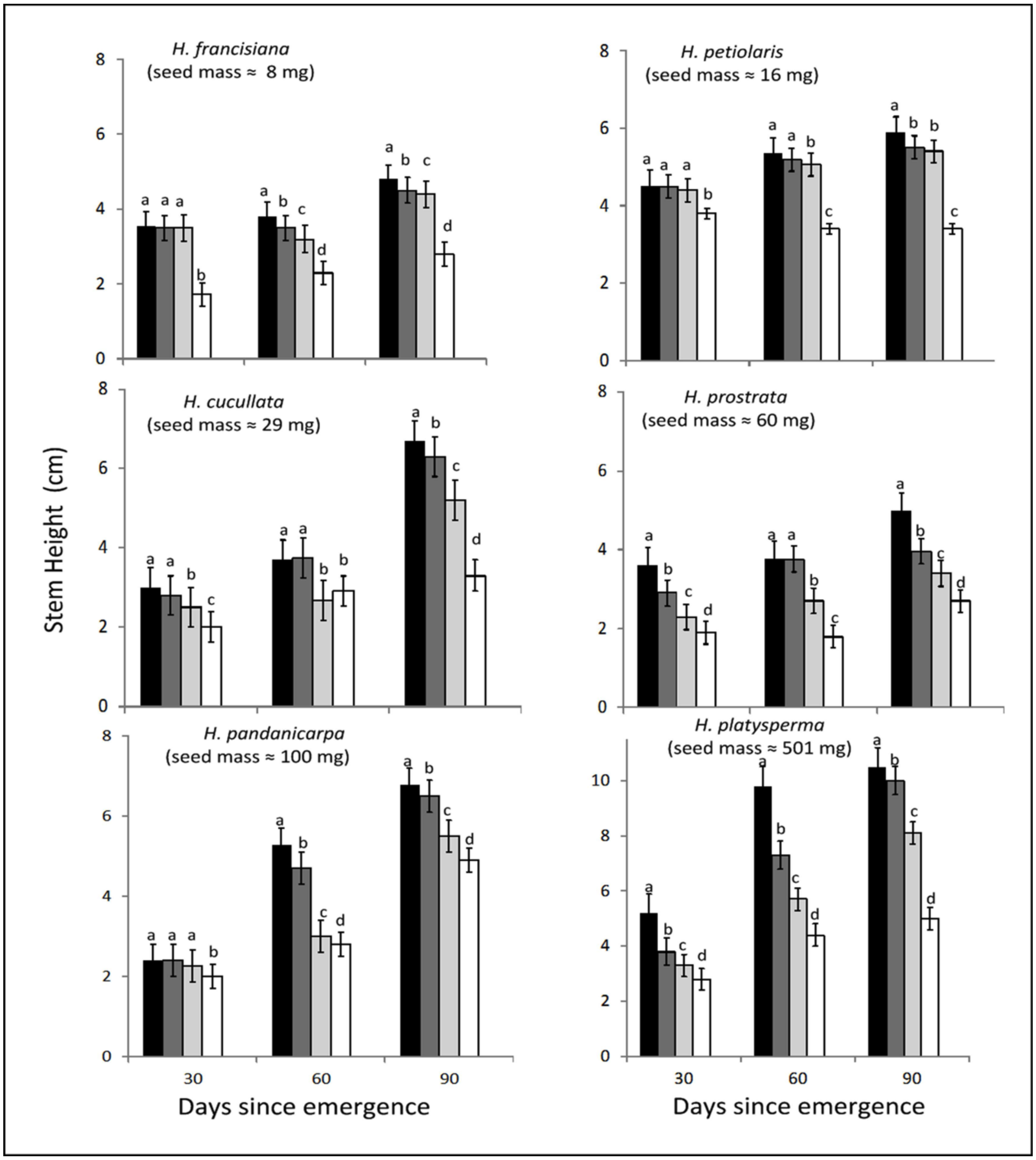

Figure 4.2: Shows the impact of 25% ▓ , 50% ░ , and 75% □ , of cotyledon damage on stem height compared with control ■ . Means ± S.E. Different letter indicates significant differences (P > 0.05) between treatments for each harvest.

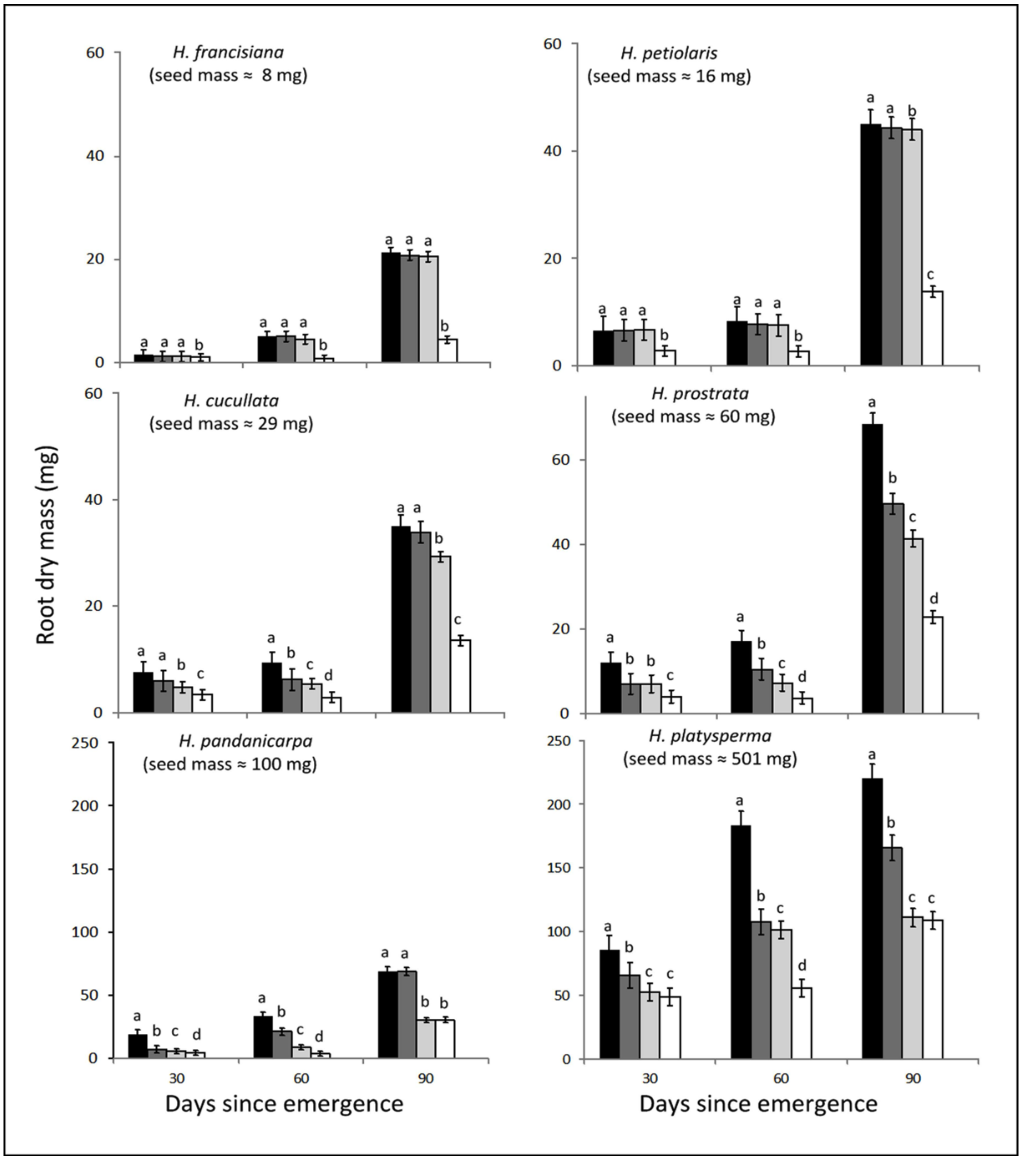

Figure 4.3: Shows the impact of 25% ▩ , 50% ▨ , and 75% □ , of cotyledon damage on root dry mass compared with control ■ . Means ± S.E. Different letter indicates significant differences ($P > 0.05$) between treatments for each harvest.

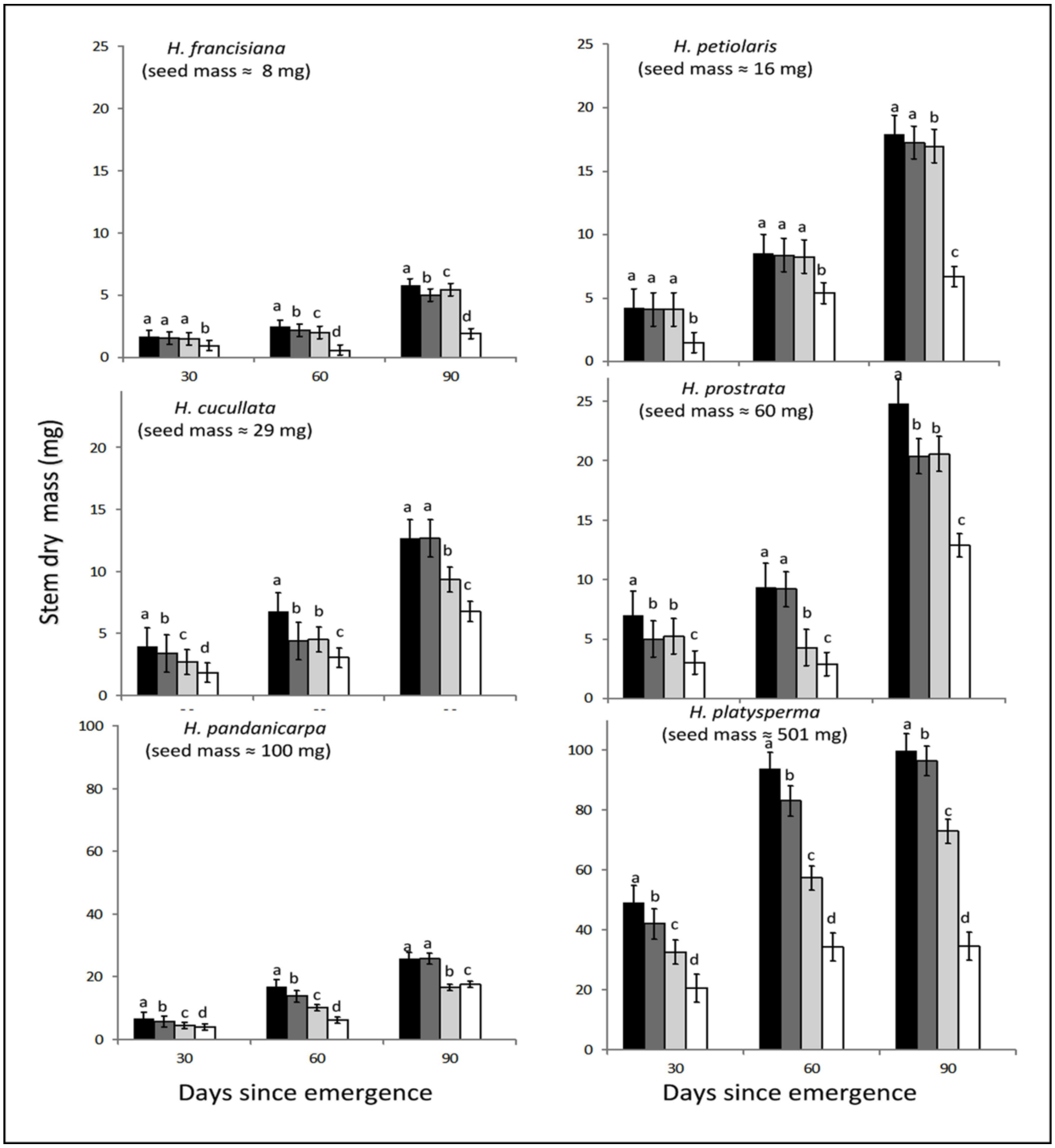

Figure 4.4: Shows the impact of 25% ▩ , 50% ░ , and 75% □ , of cotyledon damage on stem dry mass compared with control ■ . Means ± S.E. Different letter indicates significant differences ($P > 0.05$) between treatments for each harvest.

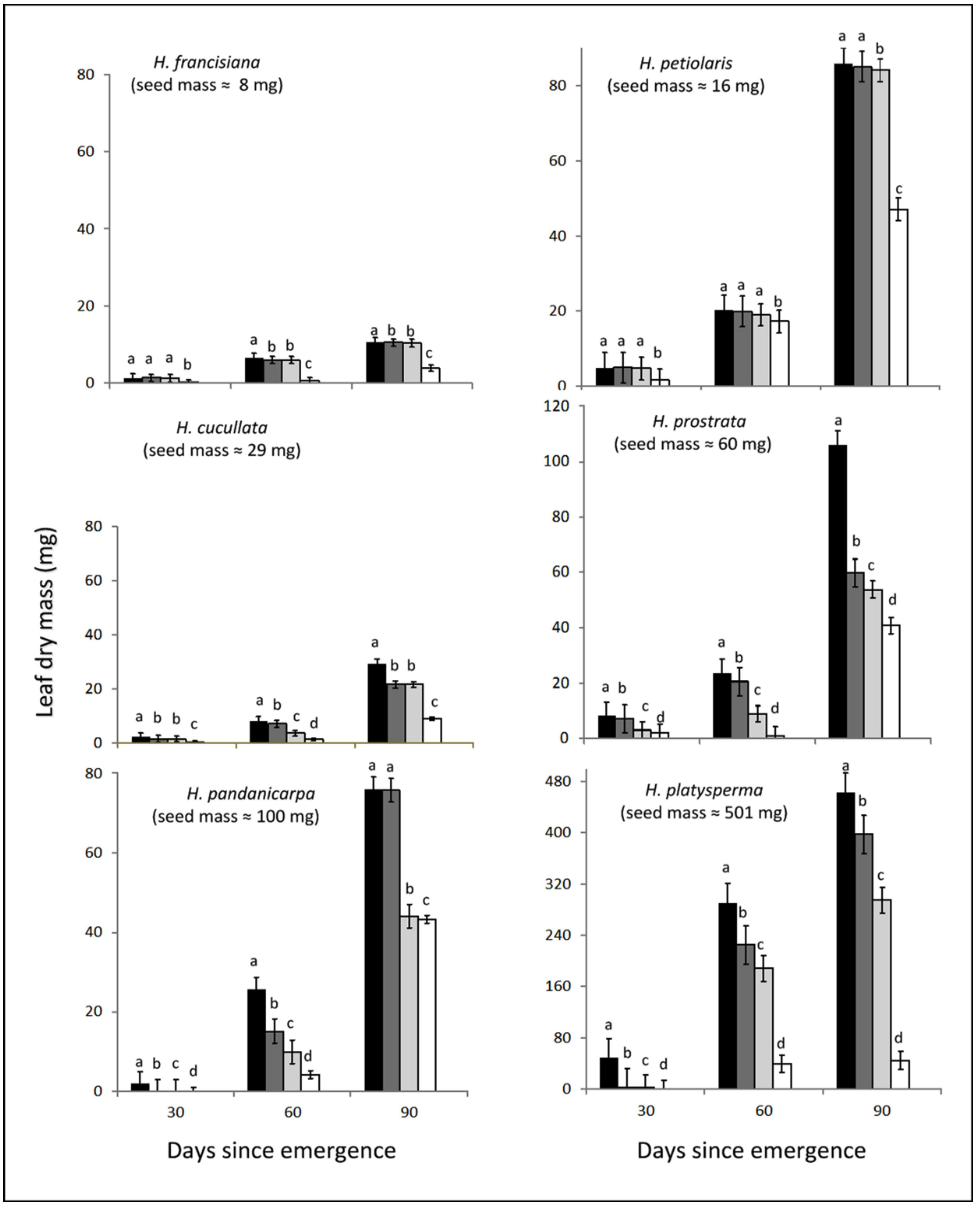

Figure 4.5: Shows the impact of 25% ▩ , 50% ░ , and 75% □ , of cotyledon damage on leaf dry mass compared with control ■ . Means ± S.E. Different letter indicates significant differences (P > 0.05) between treatments for each harvest.

Figure 4.6: Shows the impact of 25%, 50%, of cotyledons damage on lateral root, compared with control.

Table 4. 3: General linear model and MANOVA analysis on effect of seed size on growth of seedlings after partial cotyledon removal.

	Treatment (% cotyledon removal)	*F*	*P*
Root length	25%	1.970	0.092
	50%	11.718	< 0.001
	75%	1.781	0.126
	Over all*	3.782	< 0.001
Stem height	25%	1.008	0.418
	50%	3.394	0.008
	75%	3.584	0.006
	Over all*	3.330	< 0.001
Root dry mass	25%	2.070	0.077
	50%	10.547	< 0.001
	75%	0.736	0.599
	Over all*	4.460	< 0.001
	25%	0.336	0.890

Stem dry mass	50%	2.822	0.021
	75%	1.307	0.269
	Over all*	1.893	0.025
Leave dry mass	25%	0.336	0.890
	50%	2.820	0.020
	75%	1.307	0.270
	Over all*	1.890	0.025

* All three treatments (25%, 50%, and 75%)

4. 6. Discussion

The partial and complete experimental removal of the cotyledons negatively impacted the early growth of seedlings for the six *Hakea* species examined, and the extent of impact differed depending on the severity of damage and seed size (cotyledon size). Small-seeded species showed some tolerance to minor to medium cotyledon damage (25% removal). While even minor damage had a significant impact on the growth of large-seeded seedlings. Severe cotyledon damage (50% and 75% removal) resulted in a significant delay in seedling growth and development, irrespective of seed size. The cotyledons are vital for the survival of *Hakea* seedlings, as none of the seedlings from the six species survived the total loss of the cotyledons.

These results were consistent with the majority of previous studies, showing that damage to cotyledons had indirect effects on fitness through reducing rate of plant biomass. We demonstrated that damage to both cotyledons negatively influences early seedling growth, limiting the ability of the seedling to develop a root system to access reliable moisture and nutrient sources, or preventing the aboveground biomass from reaching a critical mass that ensures self-sustainability (Armstrong & Westoby 1993; Milberg & Lamont 1997; Fenner & Thompson 2005). For example, Hocking & Steer (1989) reported that cotyledon removal resulted in reduced growth and leaf numbers per plant during the early growth of oilseed and sunflower seedlings. Similarly, Frost & Rydin (1997) observed a large negative effect of cotyledon removal on the biomass and seedling survival of *Quercus robur.*

It is interesting to note that the impact of cotyledon damage, particularly severe damage, on root extension and root biomass accumulation was greater than on shoot growth in all studied species. This observation indicates a critical role for the cotyledon during root growth in water-limited environments. In water-limited environments, such as Southwest Australia, the rapid descent of a taproot reaching a reliable underground water table is critical for the survival of seedlings during the dry summer (Groom & Lamont 2015). Partial cotyledon removal significantly impacted root extension in at all experimental species (Fig.4.1). Minor to medium cotyledon removal had a relatively smaller impact on root extension in small-seeded species, such as *H. francisiana* and *H. petiolaris*. *Hakea* species are generally serotinous, and seeds are released after fire and germinate in the following winter (El-ahmir *et al.* 2015; Chapter 3). Severe damage significantly reduced the root length in all experimental species, indicating that these seedlings are unlikely to survive the first summer after germination.

The species of *Hakea* differed in susceptibility to partially damaged cotyledons, whereas the dry biomass (root, stem and leaf biomass) of larger cotyledons (*H. platysperma*) was almost ten times higher than that of smaller cotyledons (*H. francisiana*) for 25% and 50% cotyledon removal and two times higher than that of smaller cotyledons for 75% cotyledon removal. The seedlings from large-seeded species might have higher survivorship under cotyledon damage through rapid growth, as the remaining cotyledons were bigger with larger nutrient reservoirs in these species compared with small-seeded species. This result is supported by evidence showing that seedlings from large-seeded species have higher rates of survival than seedlings from small-seeded species in the field (Leishman *et al*. 2000; Westoby *et al*. 2002). However, in the present study, we demonstrated that small-seeded species are more resistant to cotyledon damage, therefore providing counter-evidence to the common hypothesis concerning a positive relationship between seed size and seedlings recovery from cotyledon damage (Armstrong & Westoby 1993; Agren, 1996; Harms & Dalling 1997; Green & Juniper 2004). Indeed, MANOVA analysis revealed a significant effect from seed size after partial removal of cotyledons, suggesting differing tolerances to partial removal of cotyledons in *Hakea.* The growth of two small-seeded species (*H. francisiana and H. petiolaris,* mean mass = 8 and 16 mg, respectively) was unaffected or only slightly impacted after 25 % and 50 % removal of the cotyledons. For large-seeded *H. platysperma* (seed mass = 501 mg), minor to medium damage caused a significant reduction in root length (Tables 4.3, Figs 4.1 – 4.5. Previous studies also observed that the seedlings of small-seeded species such as *Senecio jacobaea* (Asteraceae) and *Cerastium holosteoides* (Caryophyllaceae) were unaffected after cotyledon damage (Hanley *et al.* 2004).

There are two possible explanations for the greater tolerance of seedlings to partial cotyledon damage in small-seeded species. Small-seeded species might rely on the remaining cotyledon, with increased photosynthetic activity, for rapid shoot and root growth as compensation for cotyledon damage. Zheng *et al.* (2011) reported that when one or both cotyledons are damaged or lost during early development of the plant, the net photosynthetic rate of the remnant cotyledon increases in response to damage. In addition, leaf emergence and development was more rapid in seedlings from small-seeded species than from large-seeded species. As observed in the present study, the average time from cotyledon emergence to the expansion of the first true leaf was 8 days for small-seeded *H. francisiana*, and 20 days for large-seeded *H. platysperma* (Table 4.2). The growth of the seedling is not allometrically related to see size, small seeded species grow at greater rate with earlier emergence of true leave for photosynthesis , therefore small-seeded plants might have greater ability to acquire resources for growth from resources mobilised from the storage in remaining cotyledons. Green & Juniper (2004) observed that larger-seeded species did not disproportionately invest more biomass in resprouting after damage compared with smaller-seeded species. Thus, it might be an important physiological factor that the seedlings derived from small seeds have a more extensive and efficient metabolic translocation of nutrients from the remaining parts of cotyledons to the plant as occurs in seedlings of normal sizes. Thus, the remaining cotyledon of small-seeded plants shows a normal amount of function.

In conclusion, small-seeded species are more resilient to cotyledon damage than large-seeded species. Seedlings from small-seeded species have better tolerance to minor or medium cotyledon damage than large-seeded species, thereby gaining an advantage in environments with predation pressures or frequent disturbance resulting in damage to the cotyledons. The cotyledons contribute to the energy demand of *Hakea* seedlings at an early stage. The resources transferred from cotyledons to the remainder of the seedling during this period are important for survival. Partial damage to the cotyledons, as consequence of herbivory or physical forces, such as frost and wind in actual field settings, could significantly reduce the seedling viability in terms of biomass accumulation, root extension, and significant delay in true leaf emergence. Damage to the cotyledons will consequently reduce the survival of seedlings in the dry summer.

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Chapter 5: Seed size and seedling growth: can removal of the cotyledons be compensated for by nutrient addition to soil?

5. 1. Abstract

Loss of the cotyledons as a consequence of herbivory or other physical forces may have a major effect on seedling growth and survival. We investigated whether seed mass was associated with the ability of seedlings to survive cotyledon damage, and if removal of the cotyledons could be compensated for by the addition of critical nutrients to the soil, in the Australian genus, *Hakea* (Proteaceae). For six *Hakea* species with a range of seed masses, seedlings with the cotyledons removed after germination were grown in washed sand and watered weekly with the following treatments: 1) balanced nutrient solution; 2–4) full nutrients with nitrogen (N), phosphorus (P) or potassium (K) omitted; and 5) deionised (DI) water only. The survival levels, root lengths, and dry biomass of the seedlings were determined after 90 days in a greenhouse. When the seedlings were treated with a balanced nutrient solution following removal of the cotyledons, survival was 95–98% for all species, but 0% when supplied with nutrient solutions lacking N or P or with DI water only. Lack of added K resulted in 0% survival of the three species with smallest seeds but 70-90% survival of the largest-seeded. Removal of the cotyledons but addition of a balanced nutrient solution failed to restore complete growth of any species, but the root: shoot ratio was maintained, as was the extension of roots for the four species with smallest seeds. In conclusion, the cotyledons provide the essential nutrients, N, P and K, to support early growth of *Hakea* seedlings but other nutritional roles of the cotyledons are also

implicated. *Hakea* species with small seeds can only tolerate damage to the cotyledons better than large seeds when they have ready access to soil nutrients.

5. 2. Introduction

Cotyledons are essential for the early growth of seedlings until they become autotrophic (Milberg & Lamont 1997). Nutrients stored in cotyledons are subsequently translocated to the rapidly developing seedling axis during early growth. During the first season, the roots of seedlings must reach soil with sufficient moisture and nutrients to support growth, whereas the plumule must reach light to begin photosynthesis (Soriano *et al*., 2013). Additionally, reserved nutrients that include carbohydrates, organic nitrogen, phosphorus compounds, and inorganic ions are transported from the cotyledons into the developing seedling (Kitajima 2002; Lamont & Groom 2002; Kitajima & Myers 2008).

The extent of dependence of seedlings on cotyledon nutrient reserves may be related to seed size and nutrient translocation efficiency. Seedlings from small seeds are dependent on external resources soon after their germination with the rapid development of roots and the early emergence of leaves (Daws *et al*. 2007). Groom & Lamont (1998) reported that the addition of a balanced nutrient solution to small-seeded species with intact cotyledons increased their growth 2.5-fold compared with those that received distilled water only. By contrast, the seedlings from large seeds may use the seed resources more slowly and rely on the seed reserves longer (Green & Juniper 2004). Large seeds may have an advantage under adverse environmental conditions, including nutrient-impoverished soils, most likely because the seedlings are less reliant on soil nutrients for early growth (Milberg & Lamont 1997; Vaughton & Ramsey 2001; Poorter & Rose 2005). Seedlings from large-seeded species frequently survive and perform better than those from small-seeded species because the large-seeded species have extra nutrient reserve in the cotyledons to promote root growth (Lamont & Groom 2013; Groom & Lamont 2015).

However, the loss of the cotyledons as a consequence of herbivory or physical forces (e.g., strong wind, frost) could have a severe effect on those species that rely on their cotyledons for nutrients to support early growth. Several studies have focused on the role of seed size in affecting seedling establishment in response to the early loss of the cotyledons, stem or true leaves (Witkowski & Lamont 1991; Leishman & Westoby 1994; Milberg & Lamont 1997; Paz & Martinez-Ramos 2003). However, much less is known about the association between seed size and cotyledon damage and the ability of a seedling to respond to the damage under nutrient- or water-limited conditions.

Damage to the cotyledons during the early stage of seedling development may have significant effects on subsequent plant growth (Kitajima 2003) and reproduction (Hanley & May 2006; Yi & Liu 2014). Herbivore attack is the primary cause of cotyledon damage that affects the survival of seedlings (Hanley 1998; Moles & Westoby 2004; Fenner & Thompson 2005). .Variation in seed size is considered to be a primary factor that responds evolutionarily to herbivore attack. For some species, the production of a large number of small seeds could ensure reproductive success despite the high mortality that results from herbivore attack (Hoshizaki *et al*. 1997). It is equally plausible that small-seeded seedlings are less sensitive to cotyledon damage because these seedlings can use nutrients in the soil at a much earlier stage than the seedlings with large seeds. However, the larger seeded species have more total resources in their seeds that are more slowly deployed (Kidson & Westoby 2000). Therefore, they are slower to acquire nutrients from the soil and are more sensitive to damage to the cotyledons at an early stage.

In general, damage to seedlings from herbivore attack or physical forces can be compensated for by regrowth. The seedlings can recover from the damage in several ways; for example, a seedling can recover from the lost leaf area by using stored assimilates to increase the photosynthetic rates of the remaining leaves, or can decrease the rate of leaf senescence, or can produce new leaves (Quentin *et al*. 2010). However, the cotyledons cannot regrow when they are lost before the true leaves are produced, and damage to either or both cotyledons limits the ability of seedlings to develop root systems to access reliable moisture and nutrient sources and prevents the accumulation of aboveground biomass from reaching a critical mass to ensure seedling survival (Groom & Lamont 2015). While the cotyledons have significant consequences for the ability of a seedling to grow and survive (Hanley & May 2006; Hanley & Fegan 2007), it is less clear whether the nutrient reserves in the cotyledons can be compensated for by nutrient amendment to the soil after they are damaged .

Hakea (Proteaceae) is a plant genus endemic to Australia. This genus is known for the wide range of seed sizes (2–500 mg) among the 150 extant species, 100 of which occur in southwestern Australia, a region characterised by nutrient-impoverished soils and hot, dry summers (Groom & Lamont 1996). Hakeas possess phanerocotylous epigeal seedlings (i.e., the cotyledons emerge from the seed coat, protrude aboveground and are photosynthetically active) that is the most common type of seedling in the flora of southwestern Australia (Lamont & Milberg 1997). Milberg & Lamont (1997) noted that early removal of cotyledons caused death of *Hakea* species and suggested that the cotyledons had an important nutritional function in the early establishment of their seedlings. The seedlings of hakeas are unable to survive if both cotyledons are removed < 7 days after germination.The primary reserves of nutrients that are present in the cotyledons of *Hakea* species include nitrogen (N), phosphorus (P), potassium (K), sodium (Na), magnesium (Mg), copper (Cu), calcium (Ca), sulphur (S), iron (Fe), manganese (Mn) and zinc (Zn). N and P are the most important nutrients supplied by the cotyledons (Lamont 1995; Lamont & Groom 2002), whereas K, Ca and Mg are more likely to be obtained from the soil (Stock *et al*. 1990). P has an important functional role in energy transfer and in metabolic regulation, whereas most N is used for the synthesis of enzymes that are necessary during seedling development, including those of the photosynthetic cotyledons and the first leaves (Kitajima 1992). The other nutrients such as K, Mg, and S have supportive roles for the growth and survival of seedlings that inhabit nutrient-impoverished soils (Ramage & William 2002).

In the nutrient-impoverished soils of southwestern Australia, species in the family Proteaceae (such as banksias, hakeas, grevilleas) produce significantly larger seeds than the rest of the flora, and are particularly enriched in N and P (Groom & Lamont 2010). Further, seeds in the WA Proteaceae are larger and store more nutrients than those species in eastern Australia or the Cape of South Africa (Lamont & Groom 2013, Groom & Lamont 2015). After germination, these cotyledon-stored nutrients become the primary source of nutrients for early seedling growth (Stock *et al*. 1990; Lamont & Milberg 1997; Milberg & Lamont 1997; Lamont & Groom 2002). The majority of Proteaceae seedlings survive for up to 300 days without nutrient supplementation when grown in washed sand (Stock *et al.* 1990). Lamont & Groom (2002) proposed that the role of cotyledons in seedling development could be replaced by the addition of mineral (rather than organic) nutrients to the soil, and they found that the addition of N, P, or N + P after cotyledon removal had limited benefit for the seedlings but that the addition of P + N + K + Mg + S restored the growth of the seedlings to those with intact cotyledons. It is not clear whether the seedlings rely only on N and P in the cotyledon reserve or they acquire some N and P from soil at an early stage of growth, and how seed mass affects the capacity to access additional nutrients remains to be determined.

In southwestern Australia, seedling survival of the first summer is critical for the successful establishment of those species that rely exclusively on seeds for regeneration (as opposed to those species that resprout from the trunk or rootstock after disturbance). Although cotyledon nutrient reserves were identified as the primary resource to improve the early development of the growing plant (Milberg & Lamont 1997; Milberg *et al*. 1998; Stock *et al*. 1991; Lamont & Groom 2002), it remains equivocal whether seedlings can survive and establish solely on the nutrients in the soil, particularly N and P, and whether the role of seed size is important in the acquisition of nutrients after the cotyledons are damaged. In this study, using the genus *Hake*a as a model system, we investigated whether the loss of cotyledons could be compensated for by the addition of critical nutrients to the soil, and whether seed mass was consistently associated with the ability of a seedling to survive cotyledon damage. Therefore, the objective of the study was to explore the capacity of species varying in seed size to cope with the stress of cotyledon herbivory at the early stage of growth.

5. 3. Materials and methods

Six *Hakea* species were used in this experiment (Table 5.1). These species were chosen because they have diverse phylogenetic backgrounds and a wide spectrum of seed sizes that ranged from 8 mg (*H. francisiana*) to 500 mg (*H. platysperma*).

Seeds were sourced from a commercial seed supplier (Nindethana Seed Services, Albany, Western Australia). They were germinated in filter-paper lined petri dishes in environmentally controlled germination cabinets at 15°C with a 12–12 hour light-dark cycle (though light is not required for their germination). The germinants were transplanted into long pots constructed of PVC irrigation pipe (100 cm tall, 5 cm diameter) to encourage root growth. The pots were filled with white silica sand that had been washed with deionised water. All germinants were planted within the top 1 cm of the soil. They were grown until the cotyledons had expanded (4–7 days) and then 50 seedlings of each species had their cotyledons removed (both cotyledons were sliced off with a razor blade at the nodes) with an equal number left as intact controls. These seedlings were subjected to a nutrient omission trial.

Table 5. 1: *Hakea* species used in the experiment, their mean (± SD) seed mass (Groom & Lamont 1996) and cotyledon area after germination, and the informal taxonomic group to which the species belongs (Barker *et al.* 1999).

Species	Cotyledon area (mm^2)	Seed mass (mg)	Taxonomic group
H. francisiana	75 ± 8	8 ± 2	Strumosa
H. petiolaris	88 ± 10	16 ± 2	Petiolaris
H. cucullata	102 ± 12	29 ± 3	Cucullata
H. prostrata	117 ± 13	60 ± 7	Prostrata
H. pandanicarpa	232 ± 23	100 ± 12	Ceratophylla
H. platysperma	598 ± 24	501 ± 60	Platysperma

A balanced nutrient solution, as reported by Poot and Lambers (2008), was effective in sustaining the growth of a wide range of species in the family Proteaceae, including those of *Hakea*. The solution comprised as follows: 200 µM $Ca(NO_3)_2$, 100 µM K_2SO_4, 4 µM KH_2PO_4, 54 µM $MgSO_4$, 0.24 µM $MnSO_4$, 0.10 µM $ZnSO_4$, 0.018 µM $CuSO_4$, 2.4 µM H_3BO_3, 0.030 µM Na_2MoO_4, and 40 µM Fe-EDTA. Four nutrient solutions were prepared as follows:

1) Full (balanced) nutrient solution (F): with all the above nutrients included;

2) Nitrogen-omitted solution (N−): the full nutrient solution without $Ca(NO_3)_2$;

3) Phosphorus-omitted solution (P−): the full nutrient solution without KH_2PO_4;

4) Potassium-omitted solution (K−): the full nutrient solution without K_2SO_4.

Note that Ca was also omitted from the N− treatment while 1.96% of K used in the Full solution remained in the K− treatment (Table 5.2). All solutions were made as a 10× stock, which was then diluted with deionised water before being applied to the seedlings. For each of the six species, the seedlings were subjected to the treatments as specified in Table 5.2. All seedlings were watered with deionised water before the cotyledons were removed before further treatment. In each treatment, the individual seedlings were flushed once a week with 50 mL of a diluted nutrient solution or the identical amount of deionised water.

Table 5. 2: **Nutrient solutions used in the experiment.**

Treatment	Nutrient solution	Code	Concentration (µM)			
			NO_3	H_2PO_4	K	Ca
Control [1]	Deionised water	Con+	0	0	0	0
Control [2]	Deionised water	Con−	0	0	0	0
Complete[2]	Full	Full	400	4	204	200
N-omitted[2]	N−	N−	0	4	204	0
P-omitted[2]	P−	P−	400	0	204	200
K-omitted[2]	K−	K−	400	4	4	200

[1] Cotyledons intact; [2] Cotyledons removed.

The survival of the seedlings was monitored following treatment. Additionally, the emergence of true leaves was monitored for all surviving seedlings. The seedlings that turned dry and brown and that had no new growth for 10 days were recorded as “dead”. Every 30 days from emergence, 5 surviving seedlings were harvested per treatment for the following measurements: root length, shoot and root dry mass. At harvest, the entire plant was removed from its container, and the roots and shoots were washed with water until the sand particles were completely removed. Any residual cotyledons were removed from those seedlings in Con+ to facilitate the comparison of growth with those seedlings with cotyledons removed at the start of treatment. The cleaned plants were separated into roots and shoots. The length of the root system was measured. These organs were dried at 60°C for 48 hours before being weighed. The date of the harvests varied depending on the emergence time of the seedlings, so that growth time was consistent for all species and treatments. The small-sized seeds emerged early, whereas the large-sized seeds required more than a week before the emergence of their cotyledons.

Size and biomass of seedling parts harvested after 90 days of growth were analysed by ANOVA to test for differences between the control (Con+) and full nutrient (Full) treatments, and full nutrients with key elements omitted (K− only, because 100% mortality was recorded for the treatments Con−, N− and P −, see Results). MANOVA were used to examine the effects of seed size on tolerance of the removal of cotyledon while compensated with nutrients with seed size as fixed factor, harvest time as covariates, and growth of seedlings (total biomass, root length, ratio of root mass vs. shoot mass) in treatments Con+, Full and K− as dependent variables. Statistics was implemented using the software package of SPSS (SPSS Inc., Chicago, Illinois) with significance accepted at $P < 0.05$.

5. 4. Result

For the six species, all seedlings with intact cotyledons survived when treated with DI water for 30 days, whereas 100% mortality was recorded for seedlings with their cotyledons removed and receiving DI water only. Most death occurred within 4–10 days after removal of the cotyledons. By the first harvest, there was 95–98% survival of seedlings of all species with the cotyledons removed that received the full nutrient solution. For all species, seedlings with the cotyledons removed that received the N− or P− nutrient solutions did not survive. 70–90% of seedlings of the three largest-seeded species survived with the K− solution but there was 100% mortality for the three smallest-seeded species by the end of the experiment.

Compared with those in Con+, emergence of the first true leaf was delayed for the Con− treatment (Table5.3). For the species with smallest seeds, the first leaf emerged in 5−8 days after the cotyledons expanded in seedlings with intact cotyledons (with DI water), whereas for species with large seeds, the first leaf emerged 12−22 days after the cotyledons expanded. Emergence of the first leaf was delayed for 16−20 days in the other treatments with those from large seeds taking the longest (Table 5.3). Growth and development of surviving K− seedlings were delayed 3 times longer than those in Con+, which was twice as long as for those with the cotyledons removed but treated with the full nutrient solution.

Table 5. 3: Time (days) to emergence of the first true leaf (± SE) in all treatments.

Species	Con+	Full	K−	*P* value (t-test)	
				Con+ vs Full	Con+ vs K−
H. francisiana	8 ± 1	20 ± 4	–	0.001	–
H. petiolaris	5 ± 1	16 ± 4	–	0.001	–
H. cucullata	12 ± 1	20 ± 3	–	0.001	–
H. prostrata	14 ± 1	22 ± 4	43 ± 5	0.007	< 0.001
H. pandanicarpa	24 ± 2	32 ± 5	65 ± 4	0.017	< 0.001
H. platysperma	22 ± 2	34 ± 6	62 ± 6	0.004	< 0.001

For all species, seedlings that survived after the cotyledons were removed and treated with nutrients (both Full and K−) grew significantly slower and accumulated less biomass than those with intact cotyledons (Table 5.4, Fig. 5.1). The only exceptions were *H. prostrata* and *H. pandanicarpa* that were not significantly different between Full and K−.

Table 5. 4 Summary of ANOVA using a 2-tailed, mixed-effect model for growth of the six *Hakea* species seedlings (harvest time as covariate) for the control (Con+), full nutrient solution (Full), and potassium-omitted (K−) solution for dry biomass and root length after 90 days. Where significant, the left-hand treatment exceeded the right-hand treatment.

Trait and species	**Mean**				**Con+ vs Full**		**Full vs K−**		**Con+ vs K−**	
	Con	Full	% Reduction	K-	*F*	*P*	*F*	*P*	*F*	*P*
Total biomass (mg)										
H. francisiana	31.1 ±2	17.8	4.5	died	7.2	0.012	−	–	–	–
H. petiolaris	138 ±5	47.0	66	died	13.3	0.001	–	–	–	–
H. cucullata	70.8 ±2	22.7	68	died	10.3	0.003	8.8	0.006	28.3	< 0.001
H. prostrata	170 ±5	71.3	58	35.6	4.5	0.043	1.7	0.198	8.9	0.006
H. pandanicarpa	120 ±5	50.3	58	26.4	5.5	0.026	2.3	0.118	10.9	0.003
H. platysperma	577 ±7	189.4	67	74.7	7.9	0.009	4.4	0.023	14.0	< 0.001
Root length (cm)										
H. francisiana	12.6 ±1	11.0	13	died	0.4	0.514	–	–	–	–
H. petiolaris	30.9 ±2	27.1	12	died	0.6	0.463	–	–	–	–
H. cucullata	20.2 ±2	16.6	20	1.7	0.5	0.500	19.5	0.000	22.3	< 0.001
H. prostrata	34.2 ±2	18.0	47	17.5	4.9	0.035	0.3	0.588	4.8	0.037
H. pandanicarpa	29.3 ±2	14.8	49	8.9	7.2	0.012	1.7	0.071	15.2	< 0.001

H. platysperma	42.6 ±2	27.9	52	17.9	7.4	0.011	5.5	0.026	25.6	< 0.001
Root mass (mg)										
H. francisiana	17.8 ±2	13.6	24	died	1.6	0.210	–	–	–	–
H. petiolaris	40.0 ±2	23.4	52	died	1.1	0.290	–	–	–	–
H. cucullata	35.7 ±2	12.4	65	0.7	9.0	0.005	7.9	0.009	28.3	< 0.001
H. prostrata	67.5 ±5	25.9	62	15.9	5.0	0.033	1.5	0.226	7.9	0.009
H. pandanicarpa	72.3 ±5	27.5	62	14.7	6.9	0.013	26	0.118	13.7	< 0.001
H. platysperma	281 ±5	131.7	53	48.1	7.0	0.013	8.7	0.006	20.8	< 0.001
Root: shoot mass ratio										
H. francisiana	1.4 ±0.2	1.2	14	died	3.5	0.074	–	–	–	–
H. petiolaris	1.5 ±0.2	1.0	50	died	0.2	0.890	–	–	–	–
H. cucullata	2. ± 0.2	1.2	40	died	0.0	0.990	–	–	–	–
H. prostrata	1.7 ±0.2	0.6	65	0.8	1.0	0.330	0.0	0.995	0.7	0.410
H. pandanicarpa	1.7 ±0.2	1.2	29	1.3	2.1	0.160	0.5	0.500	0.0	0.880
H. platysperma	1.91±0.2	1.3	31	1.81	7.7	0.010	0.0	0.870	7.9	0.009

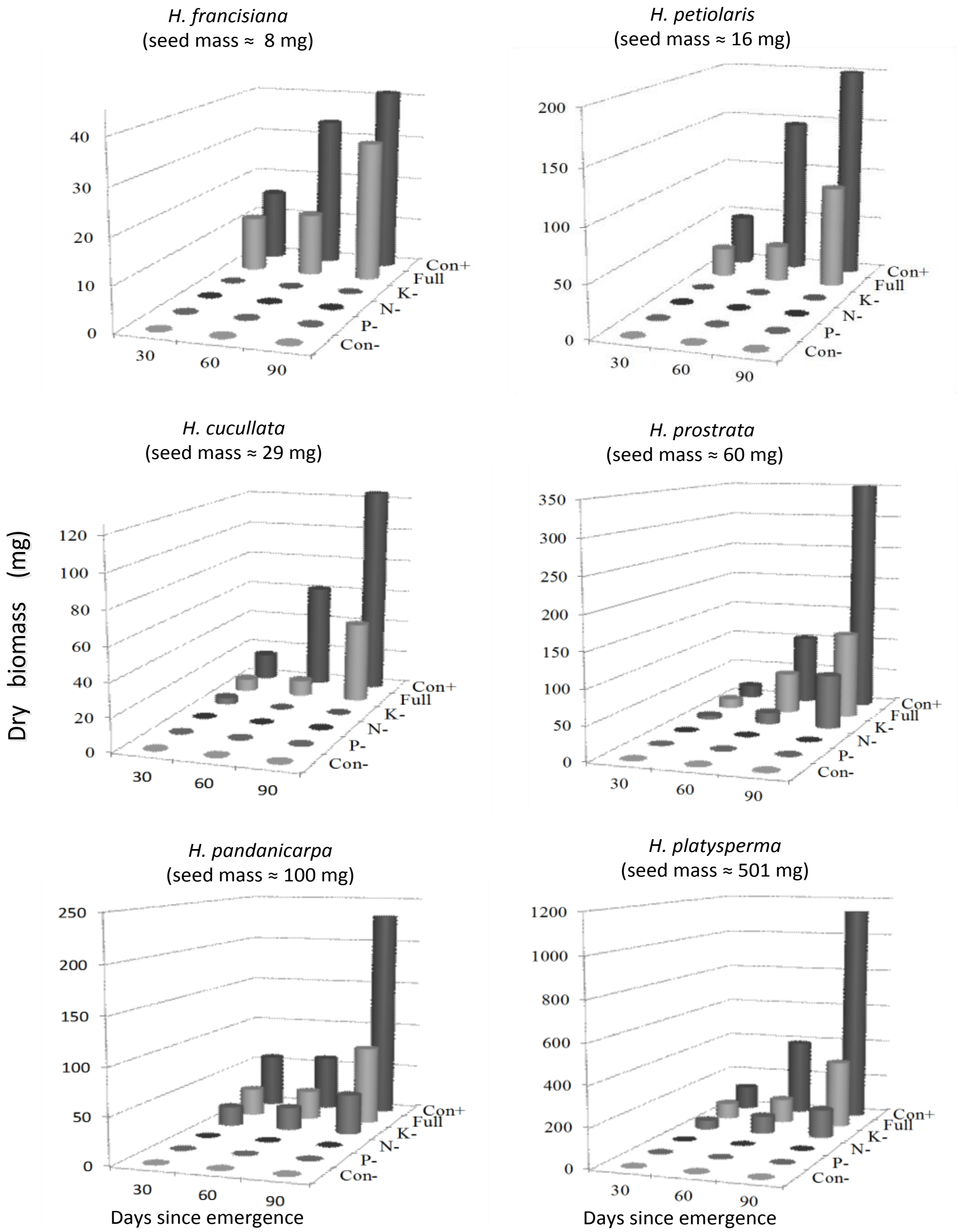


Figure 5.1: Accumulation of biomass over 90 days of growth in each treatment regime following cotyledon removal.

Root lengths of seedlings treated with the full nutrient regime were unaffected in the three species with smallest seeds, whereas root lengths were significantly reduced in the three species with largest seeds (Table 5.4, Fig. 5.2). Root lengths for the K− nutrient treatments were shorter than Full, with the exceptions of *H. prostrata* and *H. pandanicarpa* where there were no significant differences. Root mass was significantly reduced in seedlings treated with full nutrients after cotyledon removal compared with those with intact cotyledons (Table 5.4). With the exception of the large-seeded *H. platysperma*, all species treated with Full or K− (if they survived after cotyledon removal) maintained their root: shoot mass ratios (Table 5.4, Fig. 5.3). MANOVA tests indicated that seed size significantly interacted with nutrient supply after cotyledon removal ($P < 0.01$).

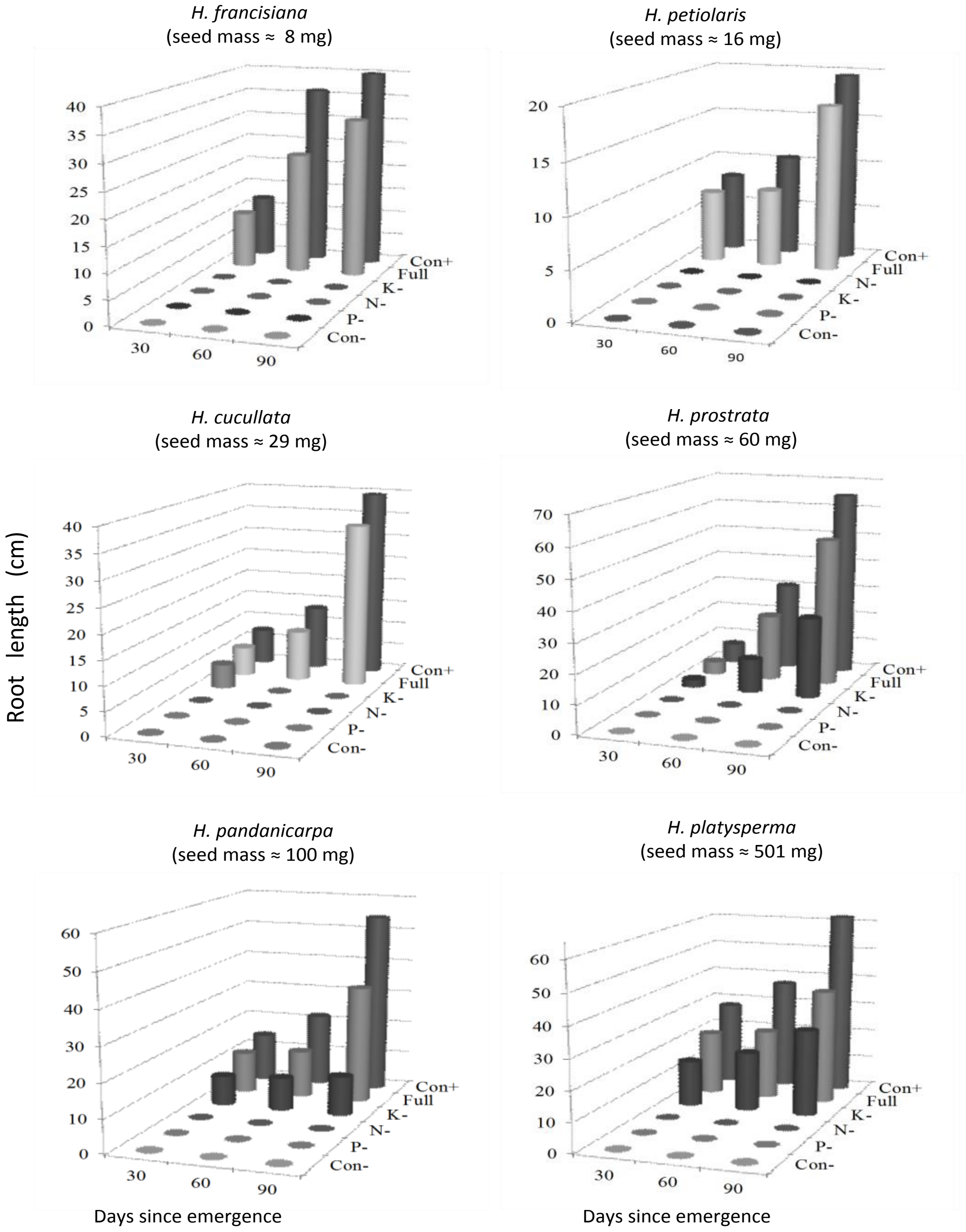
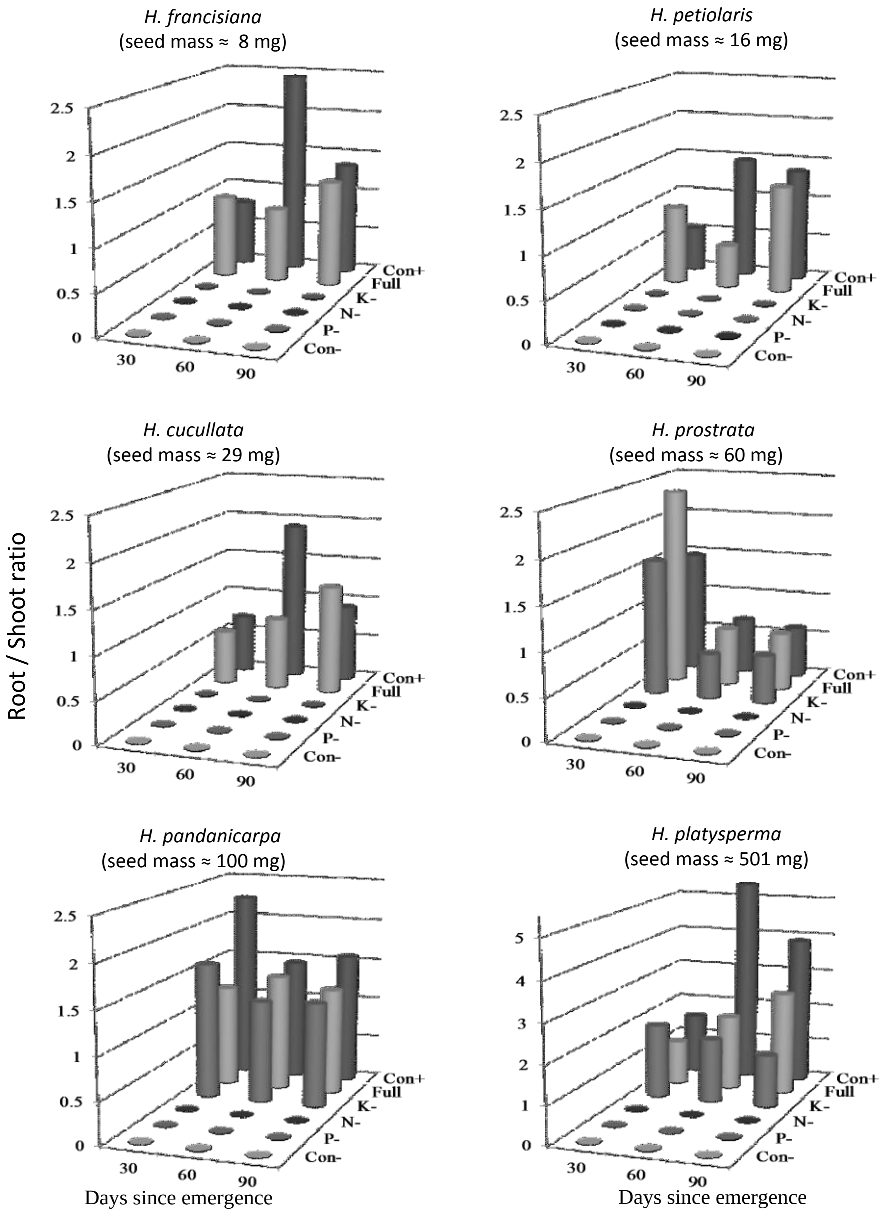


Figure 5.2: Accumulation of root length over 90 days of growth in each treatment regime following cotyledon removal.



H. francisiana
(seed mass ≈ 8 mg)

H. petiolaris
(seed mass ≈ 16 mg)

H. cucullata
(seed mass ≈ 29 mg)

H. prostrata
(seed mass ≈ 60 mg)

H. pandanicarpa
(seed mass ≈ 100 mg)

H. platysperma
(seed mass ≈ 501 mg)

Figure 5.3: Root: shoot mass ratio over 90 days of growth in each treatment regime following cotyledon removal.

5. 5. Discussion

In general, the survival of seedlings approached 100% when a balanced nutrient solution was supplied after removal of the cotyledons (and 0% when they were not), and cotyledons were more important for survival as a source of P and N than of K. However, growth of the seedlings (accumulation of biomass) could not be compensated completely by addition of a balanced nutrient solution. It is unresolved whether a) a suboptimal or toxic level or form of some nutrients was supplied preventing full recovery, b) cotyledons (located at the base of the new shoot) are a more efficient source of nutrients than an initially small root system that must first absorb nutrients then shunt them to the shoots, and/or c) the cotyledons also supply chemical resources other than inorganic nutrients such as hormones and carbohydrates. Regarding the first possibility, assuming a water-holding capacity of 10%, available P was only 1% of that recorded in typical sand at Eneabba (Lamont & Groom 2002) suggesting that levels of applied P might have been too low for full restitution. The second possibility seems unlikely as Lamont & Groom (2002), working on other *Hakea* species (with seed mass intermediate to those used here), showed that nutrient solutions could be devised that cancel out the cotyledon loss effect.

Regarding the third possibility, the addition of full nutrients after the total loss of the cotyledons might only partially compensate for the initial function of the cotyledons, which is to transfer reserved nutrient materials from the cotyledons to the developing shoots and roots during and after germination. Here, the cotyledons are also photosynthetic organs, and carbohydrate supplied from the cotyledons greatly increases the growth of seedlings and their ability to survive herbivory in some species (Zheng *et al*. 2011). Kitajima (2003) demonstrated that the loss of photosynthetic capacity in the cotyledons could have more effect on seedlings than the loss of the nutrients that are reserved in the cotyledons. Zheng *et al*. (2011) reported that the increased longevity of the cotyledons and the photosynthesizing area of the remnant cotyledon after partial cotyledon damage was a strategy to maximize the photosynthetic compensation for the damaged cotyledon. It appears that compensation with a full supply of nutrients after the total loss of the cotyledons could not replace the photosynthetic function of the cotyledons that would supply carbon compounds for translocation to the new shoot and roots.

The maintenance of root system length after cotyledon removal, at least among the four species with smallest seeds, despite an overall reduction in root mass, is intriguing (Table 5.5, Fig5.2). This suggests that under (additional) nutrient stress, root extension is maintained in an effort to reach water (indirect response) or nutrients (direct response) that will serve to rebalance growth. It is interesting to note that smaller-seeded species rely on soil sources of nutrients much earlier than large-seeded species in response to their low internal nutrient reserves (Milberg & Lamont 1997; Lamont & Groom 2002). In water-limited habitats, such as in southwestern Australia, the rapid elongation of the taproot increases the chances of maintaining contact with soil water during the first summer drought (Richards & Lamont 1996; Milberg & Lamont 1997), which is the key to successful seedling recruitment following wildfire. The small-seeded species rely less on nutrient reserves in the cotyledons and therefore may have a greater ability to acquire resources from the soil (Milberg & Lamont 1997). The extra (external) resources available to seedlings from small seeds promote early emergence of the true leaves and faster relative growth rates to utilize light energy as rapidly as possible (Jurado & Westoby 1992). In addition, small-seeded species may have better fine root development or more efficient physiological methods of up take due to their ability to drought-tolerant more than large-seeded ones (Milberg and Lamont 1997). While previous studies have shown that cotyledon removal from small-seeded species has little detrimental impact compared with large-seeded species (Milberg & Lamont 1997; Lamont & Groom 2002) this was not the case here as the roots did not have adequate access to essential nutrients compared with soil (as used in previous studies) so that the species with small seeds were just as adversely affected as were those with large seeds

Young plants of woody shrub species raised in pots under low nutrient conditions typically have a root: shoot ratio on a mass basis of ~ 0.43 reducing to ~ 0.30 in the presence of nutrient amendment (Reynolds & D'Antonio 1996) yet it averaged 1.78 at 90 days for the six *Hakea* species studied here. This can be attributed to two factors that promoted root growth (longer root, though possibly thiner) at the expense of shoot growth: the complete absence of soil nutrients and the deep (100 cm long) pots that promoted root elongation, simulating soil conditions in nature. Thus, the three small-seeded species average a root lenght of 21.2 cm and the three large-seeded species averaged 35.4 cm. This deep and rapid penetration of the soil is consistent with other results for large-seeded species (reviewed by Lamont & Groom, 2013) and supports the contention that nutrients translocated to the seedling serve first to promote photosynthesis by the shoot and then much of the fixed carbon is used to support elongation of the tap root that increases the chances of the young plant maintaining contact with moist soil during the first summer drought. That cotyledon nutrients serve primarily to support root growth was evidenced by the maintenance of the root: shoot ratio among five species and root length among four species when the cotyledons were removed, suggesting that this strategy is used to enhance soil uptake of nutrients when the cotyledon source fails. Only loss of cotyledons from the largest-seeded species was this strategy ineffective as the loss of nutrients was too great.

Our results show that cotyledons are an essential source of N and P for 90-day-old seedlings but not always of K. Thus, all decotyledoned plants died when supplied a balanced nutrient solution lacking P or N, whereas the four larger-seeded species survived in a solution lacking K, though none grew anywhere near as well as the intact plants (Table

5.4, Fig 5.1). However, it is worth noting that there was residual K (2% of full) in the K− treatment (from KH_2PO_4). Poot & Lambers (2008) suggested that a K concentration of 200 **μ**M is required for normal growth in *Hakea*, so that the residual K of 4 μM in K− solution would have negligible effect on growth, as evident by total death of the small-seeded species in the K− treatment. Either K is shunted earlier than P or N into the seedling (as it is in ionic form rather than bound organically that may be mobilized first) so that it is not as critical by the time cotyledons were removed or K is not as essential as P and N for early growth. Both interpretations are supported by two of the large-seeded species whose mass and root length were not different between the balanced and K omission treatments. This provides some support for the contention of Stock *et al.* (1990) that K is usually supplied (later) from postfire ash when seedling recruitment is most likely (Lamont *et al.* 1993) but the cotyledons were a major source of K in other studies (Milberg *et al.* 1998) while Groom & Lamont (2002) noted it depended on the K fertility of the soil: cotyledon-sourced K was not important in soils that were not K-limited.

Although the survival of decotyledoned seedlings was assisted by access to additional nutrients in the soil, the growth of seedlings was still significantly retarded by removal of the cotyledons (Table 5.4, Fig 5.1). The growth of seedlings was severely delayed when the cotyledons were removed that would place the seedling at a disadvantage when stressed by factors such as competition. Following the loss of cotyledons, even with the additional supply of all essential nutrients, we observed a significant delay in the growth of biomass accumulation and in the time to the emergence of the first true leaves for photosynthesis (Table 5.2). It remains unclear whether this was because the nutrient solution supplied did not meet the specific requirements of the species we used (possibly inadequate P) or because other essential functions of the cotyledons (e.g. providing organic compounds) were now prevented. Our study further shows that seed size is linked to seedling size through the nutritional role of the cotyledons whose size (ability to store nutrients or photosynthesise) is a function of seed size (Table 5.1). The small-seeded species were better able to tolerate the loss of cotyledons by maintaining root growth but only when they had access to soil nutrients (Table 5.4).By contrast, the species with large seeds were more severely affected by loss of the cotyledons because they depend on the nutrients in the cotyledons for survival and early growth and make little use of soil nutrients, especially N and P, at the early stages of growth (Lamont & Groom 2002). Thus, large-seeded species will be more sensitive to herbivory or other causes of cotyledon loss.

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Chapter 6: Thesis summary

Plant species inhabiting southwestern Australia have evolved a range of adaptive features to tolerate nutrient-poor soils, hot and dry summers and frequent fire. Of these, nutrient impoverished soil is associated with a divergence in seed size that influences many plant traits, such as cotyledon size and seedling growth, and plant functions, including biomass allocation. *Hakea* species have had to adapt effectively to survive the nutrition-impoverished and dry soils in Southwestern Australia. Seed mass affects the initial size of the seedlings and the amount of reserves that seedlings have for establishment.

In this study, seed size and early growth relationships in *Hakea* species were monitored to investigate the functional aspects of cotyledons as they relate to early seedling growth and biomass allocation to roots and shoots (a function of species fitness) in the Australian genus *Hakea* (Proteaceae). This role was assessed on various levels. First, the relative contribution of transition and stabilization processes to species diversification was tested by using a time-based phylogeny for the Australian genus *Hakea* (Proteaceae), reconstructed its ancestral state for six functional traits (with 15 trait options) and determined their evolutionary trajectories in response to the advent or increasing presence of fire, seasonality, aridity, nectar-feeding birds and (in)vertebrate herbivores / granivores. Second. Relationships between seed and seedling fitness traits were overlaid onto a phylogeny (a depiction of evolutionary relationships between species) of southwestern Australia *Hakea* species, to investigate an evolutionary context for why there is such a larger variation in seed size within the genus. Third, seedling growth was also examined in terms of biomass allocations and growth rates as a measure of the importance of cotyledon (and hence seed size) on seedling establishment, ability to access underground resources (e.g., summer soil moisture) and hence survival. Finally, the relative importance of cotyledons was examined as a vital source of nutrients on early seedling growth.

One of the major contributions of this study is the demonstration of evolutionary adaptation in *Hakea* by revealing the directional and stabilizing process contributing to the species proliferation, and the impact of changing environmental conditions on trait evolution and species diversification, and how diversity of seed size confers fitness in seedling establishment. The results provide important insights on the factors and processes explaining high species richness in this prominent Australian genus with adaptation to nutrient impoverished soil, drought, frequent fire and fierce granivors. Below I summarise the major results and conclusions of this study and identify further research that should be taken to gain a better understanding of the ecology, evolution and function of seed size variation.

The study described in Chapter Two provides a well-supported backbone phylogeny for *Hakea* based on the branch posterior probability where 48 out of 81 branches were ≥ 0.70. The overall topology of *Hakea* phylogeny was consistent with that in Mast *et al*. (2012). That proliferation of a trait through the phylogeny is rarely a function of the transition rate is strongly supported in this chapter. My data is consistent with the study of Mast *et al*. (2012). The reconstructions imply that the most recent ancestor (MRA) had broad leaves that were blunt-tipped with smooth margins. Broad leaf proliferation continued (mainly through stabilization) at the expense of needle but not of heteroblastic leaves through the Pliocene and Quaternary. This may be due to the ratio of stabilization to transition events among broad leaves being five times the rate for needle leaves. Also the reconstructions indicate potential reversals from bird- to insect syndromes when bird pollinators become scarce, but retention of bird pollination once the honeyeaters were present to exert selection pressure on flower morphology. Overall transition rates for insect and bird pollination, transitioning from resprouters to nonsproutrs were similar, with bird pollination accounting for 30% of events and promoting speciation by 41%. This is because the principal pollinators of *Hakea* are birds, (Barker *et al*., 1999b; Hanley *et al*., 2009), which appear to have been present in Australia from at least the Miocene (Hanley *et al*., 2009), making reasonable the inference of bird pollination in a 10-Ma-old MRCA of *Hakea*. In addition, Transitioning from resprouters to nonsproutrs is strong in the Pliocene, but it is ceased in the quaternary. The continuing transitions to nonsprouting in the Pliocene and its steady increase in stabilization rate throughout *Hakea*'s history (from 38.6% to 58.3% of all fire-response proliferations). It is likely that the trend of increasing aridity and seasonality and declining atmospheric oxygen and carbon dioxide (He *et al*. 2012) led to less frequent, but more reliable, fires and promotion of nonsprouting (Lamont *et al*. 2011, 2013). Stabilization transitions among moderately/strongly serotinous lineages dominated trait proliferation throughout hakea's history with limited transition to weak/non-serotiny in the Miocene followed by stabilization in the Pliocene and absence of proliferation in the Quaternary.

Chapter Three describes my investigation into the interaction network between seed size and fecundity, postfire regeneration strategy, fruit size, plant height and serotiny (canopy seed storage) in a phylogenetic context among 85 *Hakea* species endemic to SWA. First, seed size is negatively correlated with fecundity despite the resprouting species having a lower fecundity than non-resprouters. This pattern could be due to resprouters having the ability to survive via self-replacement. Resprouters typically produce few seeds, and they invest less in reproductive organs relative to the storage functions that help them re-establish quickly after fire. Moreover, Lamont & Wiens (2003) showed that non-resprouting species have greater seed output than resprouters. Although there is some evidence from seed mass differences, where nonsprouters species generally have larger seed mass than their congeners (Knox & Clarke 2005; Lasso *et al*. 2009;

Nzunda & Lawes 2011), my study could find no relationship between regeneration strategies and seed size. It is clearly that the seeds of resprouting species were slightly lighter than that of non-resprouters.

Second, seed size was unrelated to plant height and serotiny, while it showed an allometric relationship with fruit size. This is because larger fruits have a better vascular supply for seed filling and may also have a greater resource supply (Haig & Westoby 1988). Plant height was unlikely to have been related to seed size during the evolution of *Hakea* because the plant size variation in *Hakea* is small (0.5 - 5 m) and may not be sufficient to promote divergence in seed size. Although seed size increases with stronger serotiny through evolutionary time, the follicle: seed weight ratio of strongly serotinous species is six times that of weakly serotinous species without any difference in seed weight (Groom & Lamont 1997).

Thirdly, a strong phylogenetic signal in seed size was detected, implying phylogenetic constraints on seed size variation in this genus. For example, fire response and serotiny, both considered adaptations to fire-prone environments, are shown here to have phylogenetic signals. It is likely that seed size in *Hakea* might have tracked selection pressure from fire as well. Thus, causal correlations between seed size and fruit size (strong) and fecundity (weak) in *Hakea* were found, as well as between fecundity and postfire regeneration strategy (strong), but not between seed size and regeneration strategy, plant stature or serotiny.

The partial and complete experimental removal of the cotyledons negatively affected the early growth of the seedlings for the six *Hakea* species examined, as revealed in Chapter 4. Kennedy *et al*. (2004) suggest that cotyledon damage represents a major loss of stored reserves, especially for early seedling growth, and has great effects on plant growth. These effects are clearly manifested in my studies, where cotyledon damage at early seedling stage not only resulted in reduced growth during the establishment phase for all six species but also negatively affected true leaf emergence. There was significant correlation between cotyledon damage and true leaf emergence, where the greater the proportion of cotyledon damage, the more delayed was the emergence of true leaves, particularly for large-seeded species. This may be because the young true leaves are not self-sufficient and are dependent upon translocation from cotyledons to support new leaf growth for several days until photosynthetic capacity has sufficiently developed (Bisognin *et al.* 2005).

Additionally, root growth was more affected than shoot growth; the important physiological fact in this experiment is that the seedlings derived from small seeds have a more extensive and efficient metabolic translocation of nutrients from the remaining part of cotyledons to the plant as occurs in the normal seedling. However, growth of seedlings can be severely delayed if cotyledons are removed or damaged (Wallace & Eigenbrode 2002), which puts the seedling at disadvantage in hazardous conditions, such as competition. Additionally, this study confirms that seedlings from larger seeds are more severely affected by cotyledon removal than seedlings from smaller seeds because larger seeds have better resources, enhanced reserves in large seeds and their translocation from cotyledons to seedling can reduce reliance of seedling on external resources (Vaughton & Ramsey 2001). Thus, small-seeded *Hakea* species are better able to tolerate partial cotyledon damage than large-seeded species.

The major focus of Chapter 5 was to investigate whether seed mass was associated with the ability of seedlings to survive cotyledon damage, and if removal of the cotyledons could be compensated for by the addition of critical nutrients to the soil in *Hakea* (Proteaceae). This study found that addition of a balanced nutrient solution following cotyledon removal failed to completely restore the growth of any species. Although large seed size is frequently linked to enhanced seedling survival and regeneration success, particularly in highly nutrient-deficient habitats (Moles & Westoby 2004; Hanley *et al.* 2007), my results suggest that *Hakea* species with small seeds can only tolerate damage to the cotyledons better than large seeds when they have ready access to soil nutrients because the small-seeded species may rely less on the nutrient reserves in the cotyledons and therefore may have a greater ability to acquire resources from the soil (Milberg & Lamont 1997). Another explanation may be that the extra resources available to small-seeded seedlings are completely converted into an investment in early seedling growth and in the earlier emergence of the true leaves (Fenner 1983; Jurado & Westoby 1992; Wulff 1986).

It is important, however, to emphasize that seedlings from larger seeds are more severely impacted by cotyledon removal than seedlings from smaller seeds because larger seeds have more resources, and the enhanced reserves in large seeds and their translocation from cotyledons to seedlings can reduce seedling reliance on external resources (Green & Juniper 2004). This can be observed in chapter 3 as a quicker transfer of nutrients from small-seeded cotyledons to seedlings in early growth than from larger cotyledons. Additionally, the study results show that cotyledons are an essential source of N and P for early seedling growth, but not always of K. More than 90% of the stored P and 80% of the stored N was eventually translocated from the cotyledons to the seedlings to ensure the establishment of seedlings in their nutrient-impoverished habitats. The seedlings of the large-seeded species were more tolerant of a potassium deficiency in the soil after total cotyledon loss, with 70% to 90% survival in the K^- treatment . Thus, my data are more consistent with Lamont & Groom (2002) who found that N and P are the most important nutrients supplied by the cotyledons, whereas K, Ca and Mg are more likely to be obtained from the soil (Stock *et al.* 1991).

6.1. Overall conclusions

My study provides comprenhesive analysis on evolution, ecology and function of seed size in *Hakea*, with clear implication in their adaptation to nutrient-poor and moisture-deficient soil, recurrent fire and fierce granivores. Overall conclusions are as follows:

1. *Hakea* evolved with medium size woody fruits (i.e, medium seed size since there is allometric relationship between fruit size and seed size in *Hakea*). Smaller and larger woody fruits evolved from 12 Ma as alternative defenses against granivory
2. Overall, evolutionary histories have had most control over seed size variation among *Hakea* species.
3. Small-seeded species are able to produce more seeds for a given amount of energy than are large-seeded species, whereas large-seeded species have seedlings that are better able to tolerate many of the stresses encountered during seedling establishment.
4. Timing of germination and emergence play a critical role for small-seeded species. The tendency for the seeds of small-seeded species to germinate more quickly, for their seedlings to emerge more rapidly and for their true leaves to emerge earlier suggests that these species are well-placed to have their seedlings rapidly emerge and take advantage of favourable establishment conditions.
5. The cotyledons provide the essential nutrients, N, P and K, to support the early growth of *Hakea* seedlings but other nutritional roles of the cotyledons are also implicated. Large seeds will produce large seedlings with a greater chance of success Seedlings of small-seeded species depend more on external resources than do those of large-seeded species due to quick nutrient transfer from cotyledons, while larger seeds have better resources. Small-seeded *Hakea* species are better able to tolerate partial cotyledon damage than large-seeded ones by maintaining root growth, but only when they had access to soil nutrients.

6.2. Recommendations for future study

1. Further research on the field performance of *Hakea* species differing in seed mass and seedling morphology will be necessary to understand the consequences of the fitness differences observed here.
2. Further investigation, possibly with labelled elements, is needed to detail the efficiency of nutrients translocation from cotyledons to seedlings.
3. New research efforts should focus on experiments to show whether the concentration of K is critical in determining the differential response of the six species. A range of nutrients and nutrient availabilities may provide useful information on species adaptation to nutrient-impoverished soils.
4. In this study the N-omitted solution also omits Ca, as there was no other chemical in the mix that provided Ca. Future studies of nutrient omission should use Sodium Dihydrogen Phosphate NaH_2PO_4 instead of Calcium Nitrate Ca $(NO_3)_2$.
5. Further studies should investigate the effects of cotyledon excision on mature plants' growth and reproduction, and offsprings' fitness.

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Supplementary

S 1. The *Hakea* maximum clade credibility dated phylogeny from the BEAST analysis with branch lengths relative to time. Posterior probability values for each clade are given above the branches. Horizontal purple bars represent 95% highest posterior density (HPD) intervals of divergence dates (mya) for each node.

S 2: List of *Hakea* species investigated and trait data. Fecundity: (average fruits stored per plant; 1 = 1-2, 2 = 3-5,3 = 6-10,4 = 11-50, 5 = 51-100, 6 = >100); Plant height: 1: <1 m, 2: 1-3 m, 3: > 3m. Serotiny: serotinous: seeds retained on plant > 3 years, Non/weakly serotinous: seeds retained on plant < 3 years ; "-": data not available.

Species	Seed size (mg)	Fruit size (g)	Fecundity	Serotiny	Plant height	Post fire regeneration
Hakea ambigua	11.2	1.96	4	Serotinous	2	Nonsprouter
H. anadenia	11.1	0.92	4	Serotinous	1	Nonsprouter
H. arborescens	61.0	5.36	-	-	3	Nonsprouter
H. archaeoides	27.0	1.93	-	-	3	Resprouter
H. auriculata	39.6	0.96	3	Serotinous	1	Resprouter

H. baxteri	39.9	9.14	3	Serotinous	2	Nonsprouter
H. brachyptera	35.2	7.24	4	Serotinous	1	Nonsprouter
H. brownii	81.9	17.00	1	Serotinous	2	Resprouter
H. bucculenta	12.4	1.86	6	Serotinous	2	Nonsprouter
H. candolleana	31.4	3.11	1	Serotinous	1	Resprouter
H. ceratophylla	23.1	1.35	2	Serotinous	1	Resprouter
H. circumalata	21.6	1.13	4	Non/weakly Serotinous	1	Nonsprouter
H. clavata	12.1	0.32	4	Non/weakly Serotinous	1	Resprouter
H. commutata	7.2	2.55	4	Serotinous	2	Resprouter
H. conchifolia	30.1	0.41	1	Serotinous	1	Resprouter
H. constablei	75.3	9.87	-	-	3	Nonsprouter

H. corymbosa	6.8	1.57	4	Serotinous	1	Nonsprouter
H. pandanicarpa subsp crassifolia	100.3	32.69	3	-	2	Nonsprouter
H. cristata	85.2	8.86	3	Serotinous	2	Resprouter
H. cucullata	29.0	4.43	4	Serotinous	2	Nonsprouter
H. cygna cygna	27.0	1.76	3	Serotinous	1	Nonsprouter
H. cygna needlei	35.5	5.27	2	Serotinous	1	Nonsprouter
H. dactyloides	23.1	3.97	-	-	3	Nonsprouter
H. denticulata	40.0	0.57	3	Non/weakly Serotinous	1	Nonsprouter
H. divaricata	39.0	0.64	-	-	3	Nonsprouter
H. drupacea	17.1	3.36	4	Serotinous	2	Resprouter
H. eneabba	19.1	0.92	3	Serotinous	1	Resprouter

H. eriantha	14.0	0.64	-	Non/weakly Serotinous	2	Resprouter
H. ferruqinea	6.7	1.93	5	Serotinous	2	Nonsprouter
H. flabellifolia	156.2	13.31	1	Serotinous	1	Resprouter
H. florida	30.4	6.06	4	Serotinous	2	Resprouter
H. francisiana	9.7	1.92	6	Serotinous	3	Nonsprouter
H. gilbertii	10.3	0.28	4	Serotinous	1	Resprouter
H. grammatophylla	11.6	0.7	-	-	2	Nonsprouter
H. hastata	7.4	0.69	4	Serotinous	2	Nonsprouter
H. horrida	11.1	0.86	4	Serotinous	1	Resprouter
H. ilicifolia	6.4	0.63	1	Serotinous	2	Resprouter
H. incrassata	60.1	10.32	2	Serotinous	1	Resprouter

H. invaginata	5.7	0.83	6	Serotinous	2	Nonsprouter
H. lasiocarpha	11.2	0.38	1	Serotinous	1	Resprouter
H. lasianthoides	20.1	0.41	4	Non/weakly Serotinous	2	Nonsprouter
H. laurina	20.2	0.32	5	Serotinous	3	Nonsprouter
H. lehmanniana	20.7	0.31	2	Serotinous	1	Resprouter
H. linearis	11.1	0.41	4	Serotinous	2	Resprouter
H. lissocarpha	23.9	0.59	2	Serotinous	2	Resprouter
H. longiflora	16.7	0.12	1	Non/weakly Serotinous	1	Resprouter
H. lorea	98.0	3.54	-	-	2	Resprouter
H.; marginata	3.7	0.66	2	Serotinous	1	Resprouter
H. megadenia	5.2	0.69	-	-	2	Resprouter

H. megalosperma	109.1	13.43	1	Serotinous	1	Resprouter
H. meisneriana	5.3	0.42	4	Serotinous	2	Nonsprouter
H. multilineata	12.6	3.82	6	Serotinous	3	Nonsprouter
H. nitida	18.2	2.41	5	Serotinous	1	Resprouter
H. obliqua	26.9	7.11	4	Serotinous	2	Nonsprouter
H. obtusa	6.9	1.93	6	Serotinous	2	Nonsprouter
H. orthorrhyncha	43.8	5.01	6	-	2	Resprouter
H. pandanicarpa subsp pandanicarpa	100.0	32.69	3	Serotinous	2	Nonsprouter
H. pandanicarpa subsp crassifolia	100.3	32.69	3	-	2	Nonsprouter
H. persiehana	84.8	2.99	-	-	3	Nonsprouter
H. petiolaris	16.0	6.30	5	Serotinous	3	Resprouter

H. platysperma	508.8	40.45	3	Serotinous	2	Nonsprouter
H. preissii	12.5	0.42	5	Non/weakly Serotinous	2	Nonsprouter
H. propinqua	80.0	8.84	-	-	2	Resprouter
H. prostrata	60.0	1.41	2	Non/weakly Serotinous	2	Resprouter
H. psilorrhyncha	68.6	3.29	4	Serotinous	2	Nonsprouter
H. purpurea	18.0	3.24	-	-	1	Resprouter
H. pycnoneura	5.9	0.69	6	Serotinous	1	Nonsprouter
H. recurva	20.3	0.69	4	Serotinous	3	Nonsprouter
H. ruscifolia	30.0	0.87	2	Non/weakly Serotinous	2	Resprouter
H. salicifolia	20.2	3.67	-	-	3	Nonsprouter

H. scoparia	5.1	0.70	6	Serotinous	2	Nonsprouter
H. sericea	31.7	3.87	4	-	2	Resprouter
H. smilacifolia	9.9	0.06	4	Serotinous	1	Nonsprouter
H. spathulata	58.6	1.14	2	Serotinous	1	Resprouter
H. stenophylla	61.0	7.31	5	Serotinous	3	Nonsprouter
H. strumosa	62.7	14.45	3	Serotinous	1	Resprouter
H. subsulcata	5.4	0.66	4	Serotinous	2	Nonsprouter
H. sulcata	2.7	0.02	4	Serotinous	1	Nonsprouter
H. teretifolia	9.4	0.76	-	Non/weakly Serotinous	1	Resprouter
H. trifurcata	13.6	0.12	4	-	2	Nonsprouter
H. trineura	11.1	0.86	-	-	2	Resprouter

H. ulicina	6.4	0.83	-	-	2	Nonsprouter
H. varia	9.4	0.42	3	Serotinous	2	Resprouter
H. verrucosa	10.0	2.43	4	Serotinous	2	Nonsprouter
H. victoria	21.2	2.43	4	Serotinous	2	Nonsprouter

S 3: Eight types of *Hakea* chloroplast DNA used in *Hakea* genus phylogenetic reconstruction

Species	**atpB**	**atpB-rbcL**	**ITS**	**matK**	**rbcL**	**rpl6**	**trnL**	**trnL-trnF**
Hakea anadenia	KJ872858.1	KJ872963.1	KJ872928.1	-	-	KJ872915	-	KJ872990.1
H. arborescens	JQ257301.1	-	-	JQ257232.1	-	JQ257429.1	-	-
H. archaeoides	JQ257276.1	-	-	JQ257207.1	-	JQ257404.1	-	-
H. auriculata	JQ257280.1	KJ872975.1	KJ872942.1	JQ257211.1	KJ872899.1	JQ257408.1	KJ873015	KJ873000.1
H. baxteri	JQ257286.1	-	-	JQ257217.1	-	JQ257414.1	-	-
H. brachyptera	JQ257262.1	-	-	JQ257193.1	-	JQ257390.1	-	-
H. brownii	KJ872875.1	KJ872969.1	KJ872932.1	KJ872879.1	KJ872894.1	KJ872923	KJ873030	KJ873011.1
H. bucculenta	JQ257275.1	KJ872955.1	KJ872931.1	KJ872882.1	KJ872896.1	JQ257403.1	KJ873033	KJ872992.1
H. candolleana	KJ872869.1	KJ872971.1	KJ872938.1	-	KJ872895.1	KJ872922	-	KJ873012.1
H. ceratophylla	KJ872868.1	KJ872972.1	-	-	KJ872901.1	KJ872921	KJ873027	KJ873010.1
H. circumalata	KJ872873.1	KJ872978.1	KJ872939.1	KJ872881.1	KJ872897.1	KJ872919	KJ873014	KJ872982.1
H. clavata	JQ257263.1	-	-	JQ257194.1	-	JQ257391.1	-	-
H. commutata	JQ257290.1	-	-	JQ257221.1	-	JQ257418.1	-	-
H. conchifolia	JQ257291.1	-	-	JQ257222.1	-	JQ257419.1	-	-

H. constablei	JQ257279.1	-	-	JQ257210.1	-	JQ257407.1	-	-
H. corymbosa	JQ257272.1	-	-	JQ257203.1	-	JQ257400.1	-	-
H. crassifolia	KJ872872.1	KJ872977.1	KJ872934.1	KJ872878.1	KJ872900.1	-	KJ873023	KJ873001.1
H. cristata	JQ257255.1	-	-	JQ257186.1	-	JQ257383.1	-	-
H. cucullata	JQ257271.1	-	-	JQ257202.1	KJ872886.1	JQ257399.1	KJ873026	KJ872991.1
H. cygna	KJ872863.1	KJ872964.1	-	KJ872883.1	KJ872902.1	KJ872917	KJ873035	KJ872994.1
H. needlei	KJ872862.1	KJ872960.1	-	-	KJ872903.1	KJ872911	KJ873038	KJ872989.1
H. dactyloides	JQ257268.1	-	-	JQ257199.1	-	JQ257396.1	-	-
H. denticulata	-	-	KJ872941.1	-	-	-	-	KJ872999.1
H. divaricata	JQ257278.1	-	-	JQ257209.1	-	JQ257406.1	-	-
H. drupacea	JQ257264.1	-	-	JQ257195.1	-	JQ257392.1	-	-
H. eneabba	KJ872860.1	-	KJ872948.1	-	-	-	-	KJ872996.1
H. eriantha	JQ257256.1	-	-	JQ257187.1	-	JQ257384.1	-	-
H. ferruqinea	KJ872861.1	KJ872962.1	-	-	-	KJ872916	KJ873024	KJ872985.1
H. flabellifolia	KJ872876.1	KJ872973.1	KJ872937.1	KJ872880.1	-	KJ872924	KJ873031	KJ872984.1
H. florida	JQ257265.1	-	-	JQ257196.1	-	JQ257393.1	KJ873017	KJ873002.1

H. francisiana	KJ872856.1	KJ872956.1	-	-	KJ872889.1	KJ872925	KJ873034	KJ872997.1
H. gilbertii	KJ872866.1	KJ872958.1	KJ872953.1	KJ872885.1	-	-	-	-
H.grammatophylla	JQ257282.1	-	-	JQ257213.1	-	JQ257410.1	-	-
H. hastata	JQ257292.1	-	-	JQ257223.1	-	JQ257420.1	-	-
H. horrida	JQ257288.1	-	KJ872944.1	JQ257219.1	-	JQ257416.1	-	-
H. ilicifolia	KJ872871.1	KJ872976.1	KJ872946.1	KJ872877.1	KJ872887.1	KJ872927	KJ873016	KJ873005.1
H. incrassata	JQ257257.1	KJ872970.1	-	JQ257188.1	KJ872892.1	JQ257385.1	KJ873029	KJ873008.1
H. invaginata	JQ257296.1	-	-	JQ257227.1	-	JQ257424.1	-	-
H. lasianthoides	JQ257284.1	-	-	JQ257215.1	-	JQ257412.1	-	-
H. laurina	JQ257293.1	-	-	JQ257224.1	-	JQ257421.1	-	-
H. lehmanniana	JQ257298.1	-	-	JQ257229.1	-	JQ257426.1	-	-
H. linearis	JQ257297.1	-	-	JQ257228.1	-	JQ257425.1	-	-
H. lissocarpha	-	-	-	-	KJ872890.1	-	KJ873018	KJ873006.1
H. longiflora	-	KJ872968.1	-	-	-	-	KJ873022	KJ872980.1
H. lorea	JQ257250.1	-	-	JQ257181.1	-	JQ257378.1	-	-
H. marginata	KJ872864.1	KJ872959.1	KJ872949.1	-	KJ872906.1	KJ872913	-	-

H. megadenia	JQ257294.1	-	-	JQ257225.1	-	JQ257422.1	-	-
H. megalosperma	JQ257248.1	-	-	JQ257179.1	-	JQ257376.1	-	-
H. meisneriana	KJ872859.1	KJ872967.1	KJ872930.1	KJ872884.1	KJ872908.1	KJ872912	KJ873028	KJ873009.1
H. multilineata	JQ257270.1	-	-	JQ257201.1	-	JQ257398.1	KJ873032	KJ872995.1
H. nitida	JQ257289.1	-	-	JQ257220.1	-	JQ257417.1	-	-
H. obliqua	JQ257285.1	-	-	JQ257216.1	-	JQ257413.1	-	-
H. obtusa	KJ872867.1	KJ872961.1	KJ872936.1	-	KJ872904.1	-	KJ873025	KJ872988.1
H. orthorrhyncha	JQ257266.1	-	-	JQ257197.1	-	JQ257394.1	-	-
H. pandanicarpa	JQ257267.1	-	KJ872933.1	JQ257198.1	-	JQ257395.1	-	-
H. persiehana	JQ257300.1	-	-	JQ257231.1	-	JQ257428.1	-	-
H. petiolaris	JQ257269.1	-	-	JQ257200.1	-	JQ257397.1	-	-
H. platysperma	JQ257287.1	-	-	JQ257218.1	-	JQ257415.1	-	-
H. preissii	-	-	KJ872940.1	-	-	-	KJ873020	KJ872998.1
H. propinqua	JQ257252.1	-	-	JQ257183.1	-	JQ257380.1	-	-
H. prostrata	JQ257254.1	-	-	JQ257185.1	-	JQ257382.1	-	-
H. psilorrhyncha	KJ872874.1	-	KJ872935.1	-	KJ872905.1	KJ872918	KJ873013	KJ873007.1

H. purpurea	JQ257281.1	-	-	JQ257212.1	-	JQ257409.1	-	-
H. pycnoneura	JQ257277.1	-	-	JQ257208.1	-	JQ257405.1	-	-
H. recurva	JQ257274.1	-	-	JQ257205.1	-	JQ257402.1	-	-
H. ruscifolia	JQ257295.1	-	-	JQ257226.1	-	JQ257423.1	KJ873019	-
H. salicifolia	JQ257258.1	-	-	JQ257189.1	-	JQ257386.1	-	-
H. scoparia	KJ872865.1	KJ872966.1	KJ872952.1	-	KJ872898.1	KJ872914	KJ873039	KJ872987.1
H. sericea	JQ257251.1	-	-	JQ257182.1	-	JQ257379.1	-	-
H. smilacifolia	KJ872857.1	KJ872965.1	KJ872951.1	-	KJ872907.1	KJ872910	KJ873036	KJ872981.1
H. spathulata	-	KJ872974.1	KJ872943.1	-	KJ872891.1	KJ872926	-	KJ872983.1
H. stenophylla	JQ257283.1	-	-	JQ257214.1	-	JQ257411.1	-	-
H. strumosa	JQ257259.1	KJ872979.1	KJ872947.1	JQ257190.1	KJ872893.1	JQ257387.1	KJ873021	KJ873004.1
H. subsulcata	JQ257299.1	-	-	JQ257230.1	-	JQ257427.1	KJ873037	KJ872993.1
H. sulcata	-	KJ872957.1	KJ872950.1	-	KJ872909.1	-	-	KJ872986.1
H. teretifolia	JQ257261.1	-	-	JQ257192.1	-	JQ257389.1	-	-
H. trifurcata	JQ257260.1	-	-	JQ257191.1	-	JQ257388.1	-	-
H. trineura	JQ257249.1	-	-	JQ257180.1	-	JQ257377.1	-	-

H. ulicina	JQ257273.1	-	-	JQ257204.1	-	JQ257401.1	-	-
H. varia	KJ872870.1	KJ872954.1	KJ872945.1	-	KJ872888.1	KJ872920	-	KJ873003.1
H. verrucosa	JQ257253.1	-	-	JQ257184.1	-	JQ257381.1	-	-
H. victoria	-	-	KJ872929.1	-	-	-	-	-
Grevillea juncifolia	AY837794.1	AF060731.1	DQ499129.1	AY823169.1	HM849807.1	JQ765093.1	AY823210.1	AY823215.1
Finschia chloroxantha	JQ257245.1	AF060742.1	DQ499133.1	JQ257176.1	DQ875861.1	JQ257373.1	AF482145.1	AF482190.1
Buckinghamia celsissima	JQ257247.1	AF060747.1	FJ468589.1	JQ257178.1	DQ875862.1	JQ257375.1	-	FJ626569.1
Banksia serrata	JQ257302.1	-	-	JQ257233.1	-	JQ257430.1	EU676055.1	EU676060.1
Persoonia lanceolata	JQ257241.1	-	EU676069.1	JQ257172.1	U79178.1	-	-	-

S 4: Significant differences of seedling growth between 25% and control at age 60 days.

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P	F	P	F	P
H.francisiana	0.92	0.36	3.43	0.081	4.68	0.06	2.44	0.15	1.53	0.25
H. petiolaris	0.05	0.82	4.072	0.07	3.88	0.08	2.05	0.18	0.88	0.37
H. cucullata	3.92	0.08	3.746	0.08	0.42	0.53	5.90	0.04	6.4	0.03
H. prostrata	5.99	0.04	5.157	0.05	13.8	0.005	12.8	0.007	0.81	0.2
H. pandanicarpa	5.96	0.05	0.72	0.42	11.41	0.009	5.72	0.04	non	non
H. platysperma	5.28	0.04	14.34	0.003	11.23	0.01	5.37	0.05	6.65	0.02

S 5: Significant differences of seedling growth between 25% and control at age 60 days **.**

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P	F	P	F	P
H.francisiana	0.30	0.59	2.8	0.13	3.54	0.084	11.57	0.009	49.35	0.0001
H. petiolaris	5.99	0.04	0.56	0.35	4.08	0.077	1.25	0.29	0.27	0.61
H. cucullata	31	0.005	0.8	0.06	5.6	0.051	11.6	0.009	5.59	0.05
H. prostrata	36.54	0.003	0.25	0.62	5.05	0.05	0.003	0.95	5.75	0.041
H. pandanicarpa	6.41	0.03	5.5	0.05	5.13	0.05	7.58	0.024	4.06	0.07
H. platysperma	18.2	0.002	6.37	0.03	7.63	0.024	5.92	0.045	3.89	0.07

S 6: Significant differences of seedling growth between 25% and control at age 90 days.

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P	F	P	F	P
H.francisiana	7.38	0.02	5.50	0.05	0.91	0.17	8.0	0.01	9.03	0.01
H. petiolaris	7.98	0.02	12.25	0.008	1.22	0.3	1.68	0.22	0.07	0.78
H. cucullata	5.88	0.04	5.23	0.04	4.53	0.06	0.51	0.49	7.84	0.02
H. prostrata	5.54	0.04	5.26	0.05	5.92	0.04	7.47	0.02	6.92	0.03
H. pandanicarpa	6.65	0.02	0.19	0.67	0.18	0.67	0.40	0.54	1.19	0.30
H. platysperma	13.55	0.006	0.02	0.87	6.61	0.02	5.11	0.04	6.09	0.03

S 7: Significant differences of seedling growth between 50 % and control at age 30 days.

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P v	F	P	F	P
H.francisiana	0.98	0.3	3.38	0.13	4.69	0.06	9.68	0.01	2.71	0.13
H. petiolaris	7.03	0.04	2.88	0.12	0.25	0.07	3.27	0.10	2	0.12
H. cucullata	3.27	0.10	20.9	0.001	8.51	0.01	16.92	0.003	10.4	0.01
H. prostrata	7.87	0.02	10.62	0.01	21.47	0.001	6.99	0.03	13.5	< 0.001
H. pandanicarpa	6.88	0.03	0.2	0.66	15.59	0.004	6.22	0.05	non	non
H. platysperma	37.20	<0.01	20.0	0.002	5.90	0.041	5.78	0.04	18.7	< 0.001

S 8: Significant differences of seedling growth between 50 % and control at age 60 days.

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P	F	P	F	P
H.francisiana	11.5	0.09	5.9	0.04	1.8	0. 23	25.8	< 0.001	86.5	< 0.001
H. petiolaris	31.31	< 0.001	6.2	0.03	10.8	0.08	2.7	0.13	0.4	0.5
H. cucullata	135.0	< 0.001	6.5	0.04	6.52	0.03	6.5	0.03	41.0	<0.001
H. prostrata	80	< 0.001	9.8	0.01	17.3	0.003	12.0	0.008	20.3	0.001
H. pandanicarpa	23.6	0.001	81.	<0.001	79.4	<0.001	9.25	0.01	15.5	0.004
H. platysperma	22.8	0.001	31.	<0.001	8.87	0.017	16.3	0.003	23.4	0.001

S 9: Significant differences of seedling growth between 50 % and control at age 90 days.

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P	F	P	F	P
H.francisiana	7.94	0.02	9.78	0.014	24.02	0.001	21.58	0.001	16.9	0.003
H. petiolaris	24.67	0.001	19.5	0.002	11.15	0.01	8.24	0.02	8.7	0.018
H. cucullata	43.76	< 0.001	6.81	0.03	22.07	0.001	7.58	0.04	1.92	0.02
H. prostrata	13.14	0.006	5.01	0.05	6.97	0.02	6.82	0.03	9.31	0.01
H. pandanicarpa	8.20	0.021	5.82	0.04	6.05	0.04	6.06	0.03	7.21	0.04
H. platysperma	36.3	< 0.001	4.66	0.06	17.35	0.003	8.49	0.01	5.71	0.04

S 10: Significant differences of seedling growth between 75 % and control at age 30 days.

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P	F	P	F	P
H.francisiana	19.7	0.002	83.22	< 0.001	9.79	0.01	6.06	0.03	199	< 0.001
H. petiolaris	1.85	0.21	8.515	0.019	27.4	< 0.001	74	< 0.001	43	0.0001
H. cucullata	19.4	0.002	78.44	< 0.001	13.0	0.006	101	< 0.001	41.6	0.0001
H. prostrata	18.6	0.002	26.51	0.0008	114	< 0.001	33.7	0.0003	49.8	0.0001
H. pandanicarpa	14.5	0.005	0.408	0.54	18.2	0.002	6.35	0.035	non	non
H. platysperma	351	< 0.001	32	0.0004	158	< 0.001	16.1	0.003	24.1	0.001

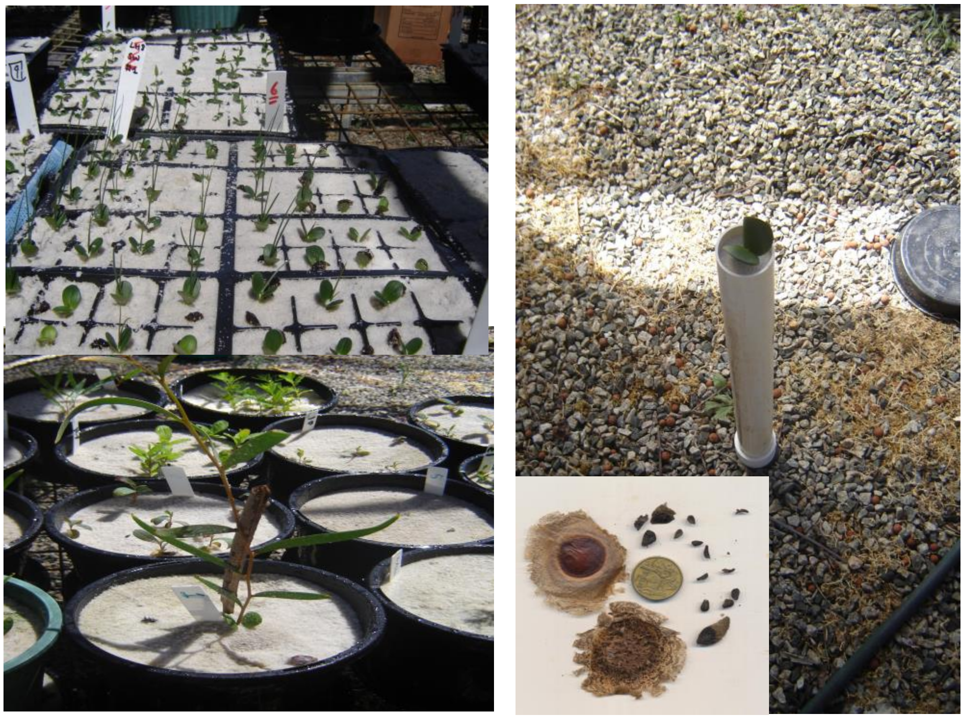
S 11: Significant differences of seedling growth between 75 % and control at age 60 days.

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P	F	P	F	P
H.francisiana	21.30	0.001	10	0.01	137	< 0.001	36.4	<0.001	307	< 0.001
H. petiolaris	66.11	< 0.001	6.9	0.029	149	< 0.001	7.74	0.02	108	< 0.001
H. cucullata	238	< 0.001	5.8	0.021	166	0.003	28.8	<0.001	213	< 0.001
H. prostrata	133.6	< 0.001	17.	0.003	42.4	0.0001	19.6	0.002	930	< 0.001
H. pandanicarpa	220.5	< 0.001	59.	<0.001	183	< 0.001	32.4	<0.001	35	< 0.001
H. platysperma	27.5	< 0.001	48.	<0.001	25.2	0.001	161.	<0.001	246	< 0.001

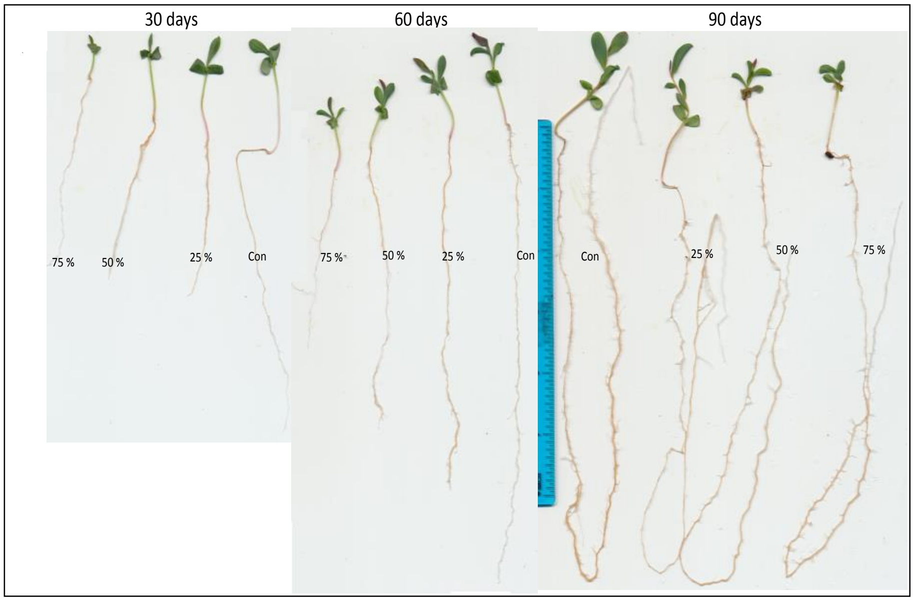
S 12: Significant differences of seedling growth between 75 % and control at age 90 days.

Species	Root length		Stem height		Root dry mass		Stem dry mass		Leaf dry mass	
	F	P	F	P	F	P value	F	P	F	P
H.francisiana	31.0	< 0.001	17.39	0.003	29.1	< 0.001	45.20	<0.00	18.6	0.002
H. petiolaris	68.3	< 0.001	52.08	< 0.001	74.1	0.00002	22.64	0.001	28.6	< 0.001
H. cucullata	0.00	28.43	35.03	< 0.001	7.29	0.027	11.56	0.009	36.6	< 0.001
H. prostrata	26.3	< 0.001	8.601	0.018	24.3	0.001	11.52	0.009	11.46	0.009
H. pandanicarpa	38.2	0.0002	12.03	0.008	5.99	0.042	4.31	0.07	4.4	0.06
H. platysperma	74.9	< 0.001	21.63	0.001	39.9	<0.001	22.85	0.001	76.7	< 0.001

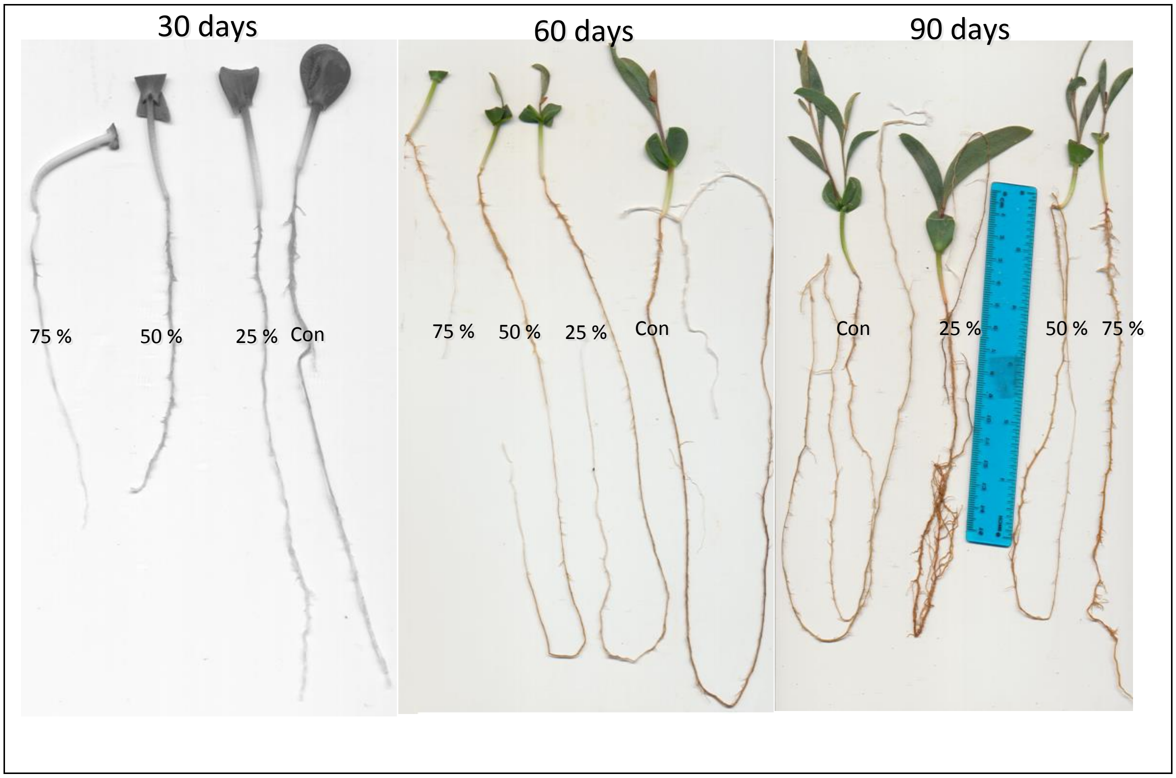
Appendicces.



Appendix A: Pots containing wash white sand (small 5 cm tall) pots, medium pots, and PVC irrigation pipe (50 or100 cm tall, 5 cm in diameter).

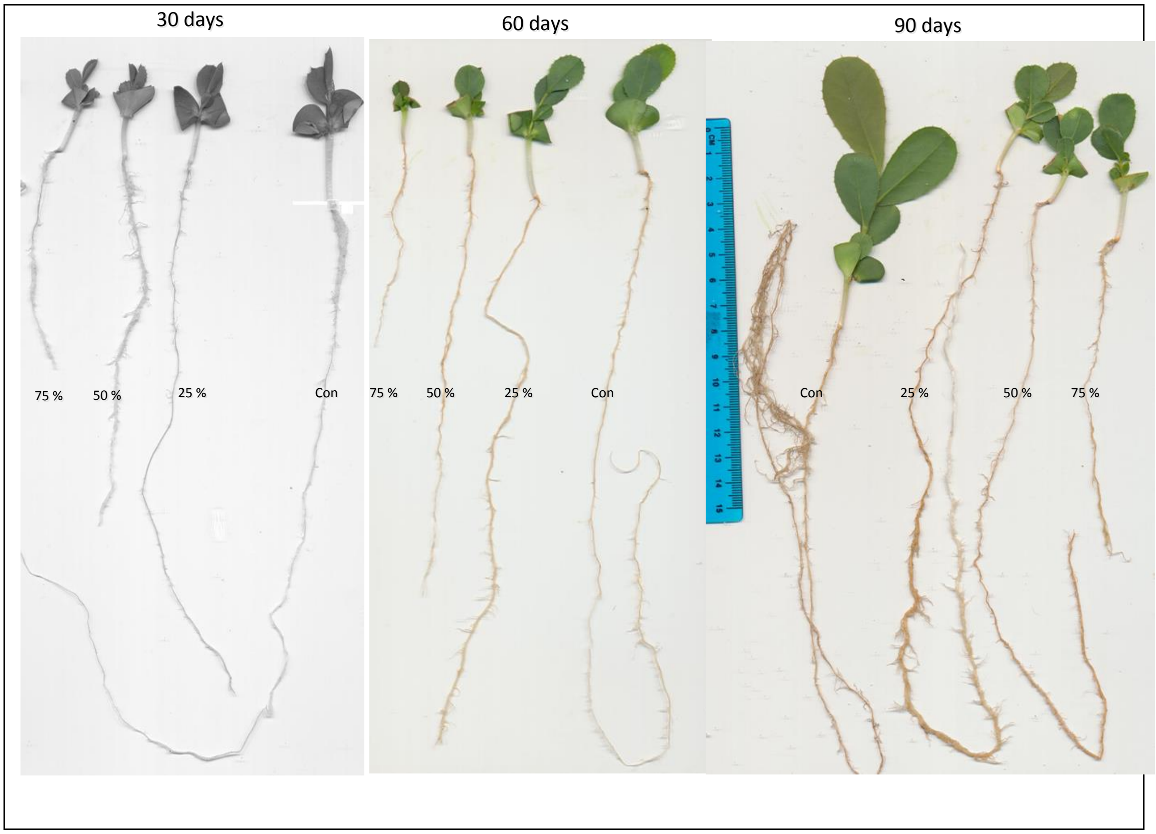


Appendix B: Three harvests. Shows the impact of 25 %, 50 %, and 75 % of cotyledons damage compared with control in *Hakea francisiana*.

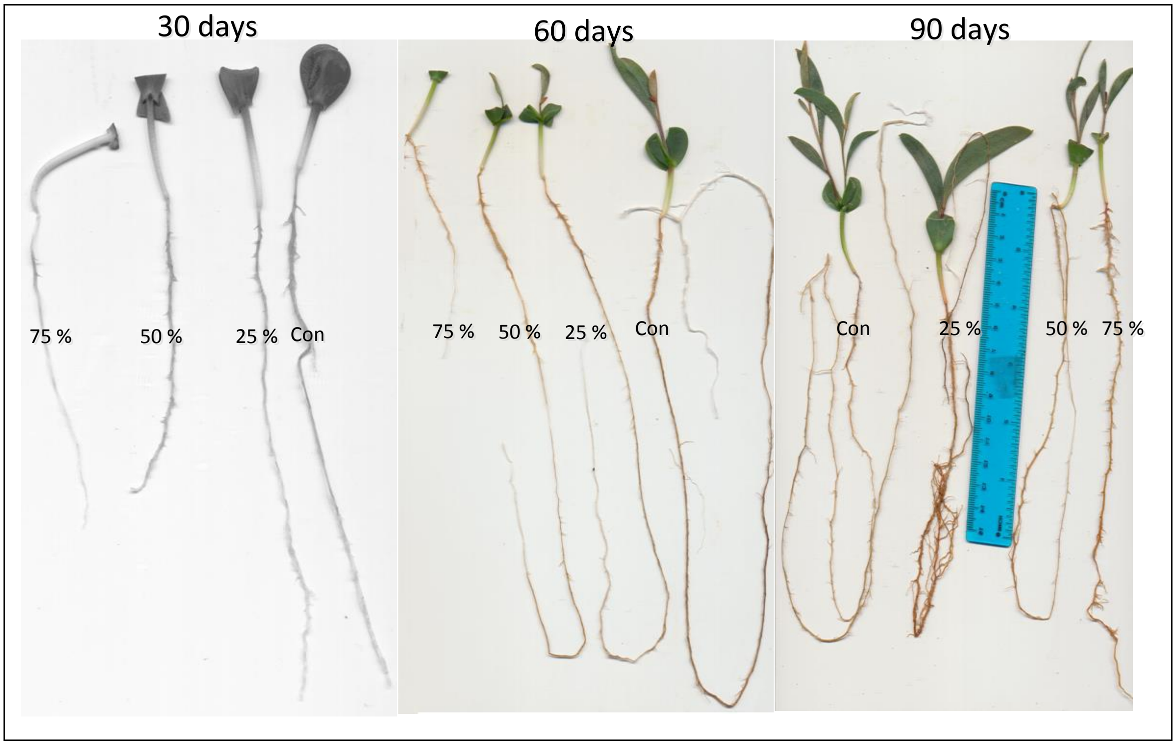


Appendix C:Three harvests. Shows the impact of 25 %, 50 %, and 75 % of cotyledon damage compared with control in *Hakea petiolaris.*

Appendix D: Three harvests. Shows the impact of 25 %, 50 %, and 75 % of cotyledons damage compared with control in *Hakea cuculata*.



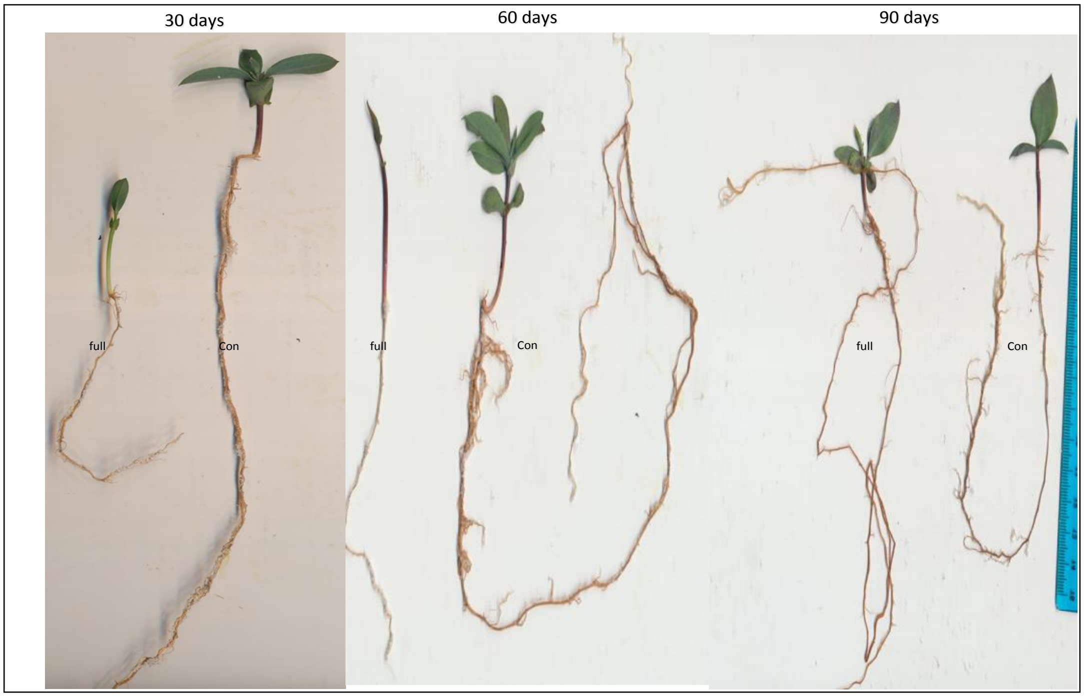
Appendix E: Three harvests. Shows the impact of 25 %, 50 %, and 75 % of cotyledons damage compared with control in *Hakea prostata*



Appendix F: Three harvests. Shows the impact of 25 %, 50 %, and 75 % of cotyledons damage compared with control in *Hakea pandanicarpa*.

Appendix G: Three harvests. Shows the impact of 25 %, 50 %, and 75 % of cotyledons damage compared with control on *Hakea platysperma*.

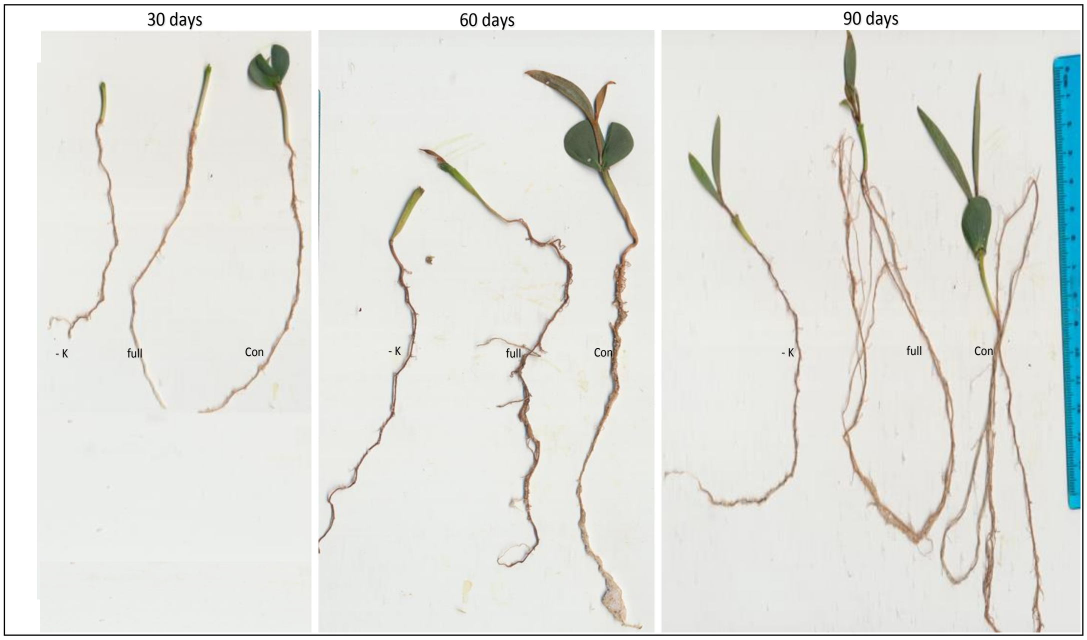
Appendix H: Three harvests. Shows the seedling growth development in treatment regime of addation full nutrient following total cotyledon removal compared with control in *Hakea francisiana*.



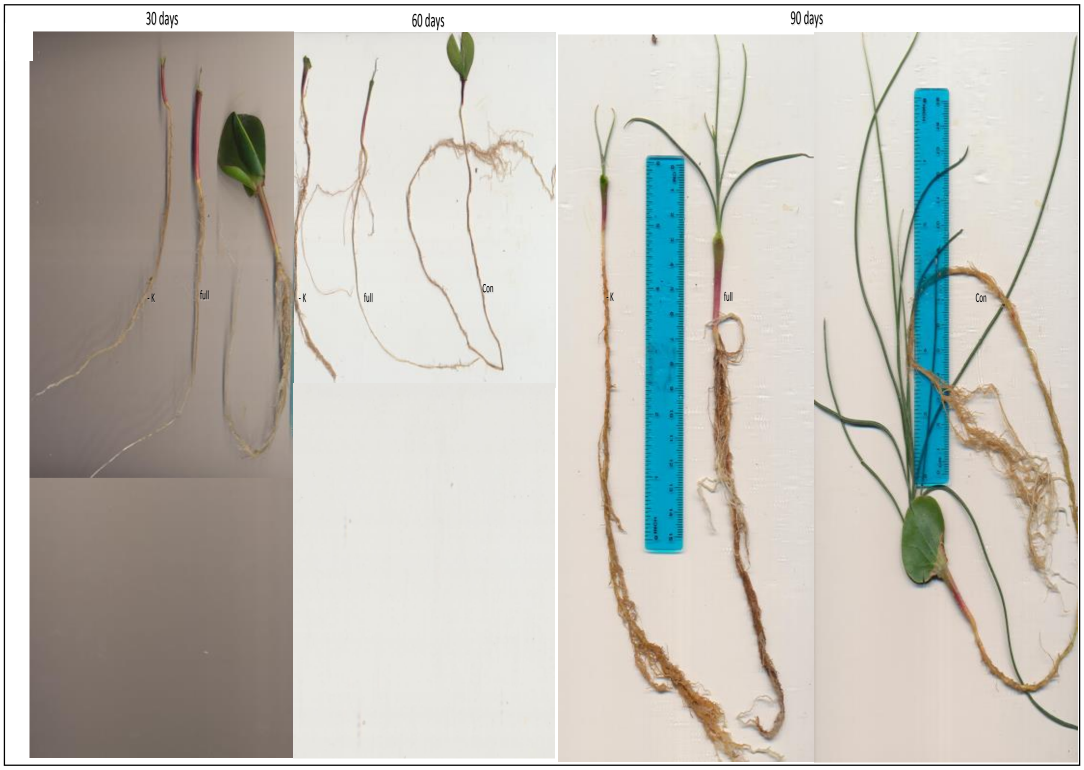
Appendix I: Three harvests. Shows the seedling growth development in treatment regime of addation full nutrient following total cotyledon removal compared with control in *Hakea petiolaris.*

Appendix J: Three harvests. Shows the seedling growth development in treatment regime of addation full nutrient following total cotyledon removal compared with control in *Hakea cucullata.*

Appendix K: Three harvests. Shows the seedling growth development in treatment regime of addation full nutrient and omission of potassum following total cotyledon removal compared with control in *Hakea prostrata*.



Appendix L: Three harvests. Shows the seedling growth development in treatment regime of addation full nutrient and omission of potassum following total cotyledon removal compared with control on *Hakea pandanicarpa*.



Appendix M: Three harvests. Shows the seedling growth development in treatment regime of addation full nutrient and omission of potassum following total cotyledon removal compared with control in *Hakea platysperma*.