Propagation and *ex situ* conservation of *Protea roupelliae* subspecies *hamiltonii*

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Declaration

I, **Refilwe F. Kai** (Student number: **741786**), am a student registered for the degree of **MSc (Dissertation)** in the academic year **2019**.

I hereby declare the following:

I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong. I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise. I have followed the required conventions in referencing the thoughts and ideas of others. I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

20 June 2019

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Abstract

With nearly 10% of the world's flora in South Africa and approximately 30 000 species of higher plants, *Protea roupelliae* subsp. *hamiltonii* is one of the 15.5% of species in South Africa threatened with extinction. This is attributed to its reduced seed production and amplified by its slow growth. The aim was to study the germination and *ex situ* conservation of this subspecies to contribute to improving its long-term conservation. The objectives concerning germination were: (a) to understand the germination and viability of the canopy-stored seeds (serotiny) in relation to their age on the plant, (b) to establish the relationship between the size of the plant canopy and the number of infructescences produced per plant, (c) to investigate if there is a relationship between seed predation (granivory) and seed age, and (d) to observe the number of viable (mass range of 0.017 g to 0.029 g) seeds produced per seed age class (1-3 'years' of age).

Ex-situ conservation aspects studied were (e) the effect of seed pre-storage water content variation on post storage behaviour and germination, and (f) optimising *in vitro* propagation methods for this subspecies using six different types of media with, varying strengths of nutrients, and different types of growth regulators.

Protea roupelliae subsp. *hamiltonii* cones were collected and recorded according to their ages. The plant canopy and volume of the plants were measured and recorded. The number of cones produced were very poorly correlated with plant canopy area (r^2=0.0963). The seeds were then removed from the cones, counted and weighed before being categorised by cone age and seed mass. The mean number of seeds per cone was 104.62, 107.67 and 100.00 for the three seed ages respectively with viable seeds (mass range of 0.017 g to 0.029 g) of 3199 for year three seeds which was not significantly different to year one seeds at a seed count of 3062. The two-year-old seed count of 2525 was significantly less than both one and three-year-old seeds. The 1-year old seeds of *Protea roupelliae* subsp. *hamiltonii* had a higher germination percentage of 86%, compared with the 2- and 3-year-old seeds, which both had a 56% germination. The granivory of the seeds affects the germination of seeds due to the damage caused to the seed embryo. Granivory was more prevalent in three-year-old seeds at 8.56%, followed by one-year old seeds with 4.81%, and the least being 2.58% for two-year-old seeds. Thirty-eight plants were sampled with a yield of 221 cones; 69 were 1-year old, 64 were 2-year old and 88 were 3-year old cones. The total number of seeds produced per age group was 7219 (1-year old cones), 6891 (2-year old cones) and 8800 (3-year old cones).

The seeds were shed at water contents varying from 0.0471-0.0938 $g.g^{-1}$ d.m.b. The water content extremes resulted in a variation of results yielding uncertain protocols regarding seed storage at low temperatures (ambient-70^{o}C). In an attempt to find optimum water contents for low temperature (^{o}C) storage, seeds were equilibrated to 50% relative humidity (RH) prior to storage and the coefficient of variation of seed water content decreased from 19.58% to 13.20% for all the seeds combined. Seeds were stored for 6 and 12 months at different temperatures; -70^{o}C, -20^{o}C, 4^{o}C, 25^{o}C and a control of no storage treatment. Seeds stored for 6 months showed higher variation in water content across all storage conditions/treatments than those stored for 12 months. Seeds stored at -70^{o}C for 12 months showed less change in their water content resulting in less variation and a 60% germination, which was lower than that of the control seeds.

In vitro propagation methods using leaf and shoot axillary meristems were tested using two different media containing half ($^{1}/_{2}$) strength and quarter ($^{1}/_{4}$) strength Murashige and Skoog (MS) medium. Total contamination was observed on tissues disinfected using Sodium Hypochlorite and Tween-20® only, on both media. Leaf explants disinfected with the antioxidant solution and 0.1% Tween-20® method showed no contamination, but subsequently turned black and showed no development, while the shoot explants on the same decontamination regime became contaminated (100%) after 2 weeks. The use of the zygotic embryo for *in vitro* propagation via indirect somatic embryogenesis and organogenesis was assessed. Four media comprising of full and half ($^{1}/_{2}$) strength Murashige and Skoog (MS) medium powder and exogenously added 0.5 mg. l^{-1} Picloram or 0.5 mg. l^{-1} 2.4D were used. All the media were augmented with 3 g. l^{-1} Gelzan® and 30 g. l^{-1} sucrose to a pH of 5.6-5.8. No development was observed on explants cultured on the half and full-strength medium without plant growth regulators, but explants cultured on half strength medium with 0.5 mg. l^{-1} 2.4D and 0.5 mg. l^{-1} Picloram showed callus growths with no further development.

Micropropagation of the *Protea roupelliae* subsp. *hamiltonii* plant using leaf and shoot meristems is a process that should be standardized to achieve the conservation aims regarding plant population diminishment in nature. Continued efforts in the conservation of *Protea roupelliae* subsp. *hamiltonii* are encouraged to increase seedling establishment, field re-introduction and eventually population growth. Efforts should be amassed to further test the effect of seed water content variation pre-storage, on the successful short and potentially long-term storage of the seeds. *In vitro* propagation of the *Protea roupelliae* subsp. *hamiltonii* plant

should be optimised, with more consideration on the decontamination method for this systemically contaminated plant species.

Keywords: *ex situ* conservation, low temperature, *Protea roupelliae* subsp. *hamiltonii*, seed storage.

“Trust in the Lord with all your might, and lean not on your own understanding”

-Proverbs 3:5

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Glossary

2,4D	-	2,4 – Dichlorophenoxyacetic acid
$Ca(NO_3)_2$	-	Calcium Nitrate
CV	-	Coefficient of variation
d.m.b	-	Dry mass basis
ddH_2O	-	Double distilled (Millipore) water
DWAF	-	Department of Water Affairs and Forestry
FS	-	Full strength
HS	-	Half strength
IBPGR	-	International Board of Plant Genetic Resources (now called Biodiversity International)
IUCN	-	International Union for the Conservation of Nature and Natural Resources
MGT	-	Mean Germination Time (The average time it took the seed to germinate
MS	-	Murashige and Skoog
NGO	-	Non-Government Organisations
QS	-	Quarter strength
RH	-	Relative humidity
SANBI	-	South African National Biodiversity Institute
TPA	-	Transvaal Provincial Administration

1 General Introduction & Rationale

1.1 Conservation

Conservation is defined as the management of an environment, the resources and values it contains to prevent its exhaustion (Heywood and Iriondo, 2003) or premature culmination. It focuses on environmental quality, the sustainable use of natural resources and their allocation and protection (Allaby, 1992; Goldsmith, 1983). Conservation safe guards an entity and keeps it in its natural state for as long as possible, therefore embracing the philosophies of protection, sustainable utilisation and maintenance, restoration and enhancement of the natural environment (Heywood and Oriondo, 2003). It is optimistic (Heywood and Iriondo, 2003) and works towards the improvement of desolate species and their habitats. Primarily due to the impact of human activity, conservation has become a global imperative (Benson, 2002), and affords a future for those species threatened with extinction.

Biological conservation particularly aims to maintain the natural ecosystem in a balanced state by protecting the fauna and flora (and other biological systems) whilst taking into account environmental factors such as water scarcity and increased/decreased environmental temperatures (Ratcliffe, 1977; Ingram *et al.*, 2012). An ecosystem is deemed a cluster of biological organizations, which interact with the physical environment (Odum, 1966), and is considered balanced when the interaction between the biological entities and the physical environment are not disturbed or hindered. The scientific commitment to conserve the Earth's biodiversity heritage is a formidable obligation (Gaston and Jennings, 1996) because the requirements to conserve threatened species outweigh available conservation resources (Myers *et al.*, 2000).

The conservation sciences have been described as 'crisis research' (Bottrill *et al.*, 2008); where hasty decisions are made with incomplete or misunderstood knowledge of the system/ species under consideration. These views have been attributed to the lack of knowledge regarding the biology of many of the threatened species (Soule and Orians, 2001; Maclaurin and Sterelny, 2008), thus sometimes resulting in 'blind' conservation. Conservation studies are, however, not new nor are they impromptu, they have contributed to the processes of well-founded sustainable ecosystem maintenance. Conservation studies pre-date the concept of sustainable biosphere management (Heywood and Iriondo, 2003), with the past 26 years showing a major evolution in our understanding of environmental inter-relatedness and the considerable role

that sustainable use of biological diversity plays towards the goal of conservation (Mace *et al*., 2018).

Increased efforts are evident and are supported by global groups such as, but not limited to, the International Union for the Conservation of Nature and Natural Resources (IUCN) and national groups e.g. the South African National Biodiversity Institute (SANBI). These entities especially the IUCN work with governments, Non-Government Organisations (NGO) and academics towards developing sustainable conservation goals.

1.1.1 Biological diversity

Biological diversity (biodiversity) has been described as the number and variety of living organisms present in a particular habitat at a specific time (Reid and Miller, 1989; Wilson, 1992; Heywood and Watson, 1995; Hubbell, 2001; Rands *et al.,* 2010) and summarised by Barbault (2011) as 'the living tissue of the Earth'. The variation in diversity is contributed to by the different biological resources that make up the Earth's heritage (Gaston and Jennings, 1996; Patidar *et al*., 2013). Humanity's main concern is the decline and impoverishment of the resources at various levels of organization (Noss, 1990), *viz*. the various trophic networks and species relatedness (Barbault, 2011). Globally, the resources and therefore biodiversity are declining irreversibly and rapidly (Roe *et al.,* 2019).

The decline of resources was reported (2003 and 2006) to be at a rate 10-fold faster than in the 1800's and a 100-fold compared with the average natural rate (Heywood and Iriondo, 2003; Sweedman and Merrit, 2006). The natural rate is that which has not been affected intensively by human interactions with the environment. Currently, biodiversity decline has been reported to be at a rate 10-fold higher than in 2006 (Roe *et al.*, 2019). Natural ecosystems are disappearing due to reckless human development in many places, and the consequence is the concomitant loss of biodiversity (Reed *et al*., 2011). Therefore, increased conservation efforts using various strategies are greatly required. According to the IUCN, 8321 plant species were added to the red list of endangered species during the period of 1996-2004 (Sarasan *et al*., 2006), increasing the number of critically endangered plant species by 60% (Engelmann, 2011). Amongst numerous other factors such as ocean acidification, alien species introduction (Cruz-Cruz *et al.,* 2013) and other anthropogenic environmental impacts (Rands *et al.*, 2010), this can particularly be attributed to land-use changes such as conversion of tropical forests for timber production and urbanisation (Gibson *et al*., 2011) to sustain the continued growth of the human population (Chape *et al*., 2008; Rands *et al.,* 2010).

Conservation biologists through activities such as enclosing certain habitats, education and awareness, *ex-situ* programs, removing invasive species, and legislation, have made progress in reducing the loss of biodiversity (Salafsky *et al*., 2008). Other instances, using the abovementioned activities, have yielded improved situations or are at least at 'a work in progress' level of success. A local example would be the conservation of *Protea roupelliae* Meisn. subsp. *hamiltonii* Beard ex Rourke that started with enclosing the population by erecting a fence to mitigate its diminishment by herbivores and the removal of pine trees which hindered the growth of the endangered species. Through numerous projects carried out by researchers from the University of the Witwatersrand, awareness of this critically endangered species has been raised and *ex situ* programmes initiated.

1.1.2 *In situ* and *ex situ*

Plant conservation requires a variety of tools (Pence, 2010) and has been approached in several different ways. The combination of methods in which plant conservation can be carried out has an impact on the outcome of the conservation goals. It can be carried out *in situ* or *ex situ* or in combination but using one method exclusively is usually not enough for most plants/communities (Pence, 2010). The main objective of *in situ* conservation is to maintain viable population(s) of the plants in their natural environment with minimum human intervention (Heywood, 2014). Thus, it is necessary to mitigate the threats to which the plants are subjected in their niche and take into consideration that the population is adapted to the specific environment of its habitat (Heywood, 2014).

In situ conservation methods concentrate on the protection of the endangered species by management of wild species and their habitats. In particular, it concentrates on the recovery of viable populations within their natural surroundings (Hawkes *et al*., 1997) and thus encourages natural reproduction and the process of evolution to continue (Qualset *et al*., 2000) thereby maintaining or even increasing genetic diversity (Blackmore and Oldfield, 2017). This approach to conservation is efficient for some species (Sarasan *et al*., 2006), but not necessarily for slow growing and low seed or propagule producing plants (Pence, 2010; Reed *et al*., 2011), such as *Protea roupelliae* subspecies *hamiltonii*. Another draw-back associated with *in situ* conservation is the land needed for sustainable preservation and conservation which can be addressed using *ex situ* conservation.

Conservation of whole plants or parts thereof, out of their natural habitat is termed *ex situ* conservation (Engelmann and Engels, 2002). This is achieved through the preservation of

material in botanical gardens, fields, *in vitro* storage, seed banks and gene banks (Li and Pritchard, 2009). Clonal material from *in vitro* banks can be used to restore rehabilitated land while seeds from seed banks aid in re-establishing populations and genetic diversity. The conservation of plants and their seeds away from their natural habitat in a controlled environment, can protect genetic integrity for many years. Therefore, efforts to manage and protect species should not be conducted in isolation, but rather that these two approaches (*in situ* and *ex situ*) should be used holistically (Hawkes *et al*., 1997) to enhance and supplement one another (Pence, 2010; Reed *et al*., 2011).

1.2 *Protea roupelliae* subsp. *hamiltonii*

1.2.1 Background

Protea roupelliae subsp. *hamiltonii* is one of more than 300 species in 14 genera of *Proteaceae* in South Africa (Lamont *et al*., 1985, Rebelo, 1995). The *Proteaceae* are found across South Africa with their highest concentration located in the Western Cape (Cape Floristic Region) (Hannah *et al.*, 2005) with four genera found outside the winter rainfall areas (Collins and Rebello, 1987; Rebelo, 1995), in the northern Limpopo and Mpumalanga provinces (Valente *et al*., 2010). *Protea roupelliae* subsp. *hamiltonii* is a slow growing prostrate shrub with an average height of 34 cm (Schmidt *et al*., 2002). It has a serotinous woody growth form and like many in the family *Proteaceae*, (Bond, 1984) retains its seeds on the parent plant for years (0-30 years) (Lamont *et al.*, 1991; Midgley and Bond, 2011).

Protea species generally grow in nutrient impoverished soils (Lamont *et al*., 1985; Milewski and Cowling, 1985; Esler *et al.*, 1989; Witkowski, 1990; Cowling and Witkowski, 1994; Cowling *et al.*, 1994), particularly those low in phosphorus (Witkowski and Mitchell, 1987). In this regard Milberg and Lamont (1997) hypothesised that plants in nutrient poor soils produce bigger seeds than their counterparts in nutrient rich soils as an adaptation to enhance their establishment. It is therefore logical to assume that increased mass is directly proportional to increased nutrients that assist during seedling establishment and development e.g. *Protea repens* (Witkowski, 1989; Witkowski, 1991). However, Tarlton (2013) demonstrated that seeds of *P. roupelliae* subsp. *hamiltonii* with mass greater than 0.029 g had lower germination values than seeds in the mass range of 0.017 g - 0.029 g.

Observations of *P. roupelliae* subsp. *hamiltonii* seeds, have noted that some are woodier than others. The woodier seeds tend to be heavier, but not necessarily of superior quality (see above). Seeds become heavier as they mature, but this increase in mass is not always related to embryo

and storage tissue development as some seeds become woody. In such seeds, the seed coat becomes sclerified with the thickening creating heavier seeds, which are not necessarily vigorous seeds, i.e. large seeds with large storage reserves. The large sclerified seeds are in some instances parthenocarpic, *viz*. are empty non-viable seeds (Willson and Burley, 1983 *loc sit* Fuentes and Schupp, 1998) or are dormant (in some species).

Protea roupelliae subsp. *hamiltonii* has several different inflorescence colours, ranging from light pink, yellow pink, yellow with pink to deep pink (Tarlton, 2013). The parent plant produces flowers within inflorescences, which then mature into infructescences often referred to as cones, which contain and retain the seeds within the plant canopy (canopy seed storage, or serotiny). The seeds are released after certain environmental cues such as fire (Lamont *et al*., 1991), but also in some species when the cones reach a certain age (Bond, 1985).

Dr. P.D. Hamilton first discovered this subspecies in 1957 and even then, it was rare in nature and scarcely seen in gardens (Rourke, 1982). *P. roupelliae* subsp. *hamiltonii*, unlike its taller tree form relative, *Protea roupelliae* subsp. *roupelliae*, is currently only found at the Dr. Hamilton Nature Reserve; a pine infested grassland in Mpumalanga, South Africa. This subspecies was already listed as critically endangered by the IUCN in 1997 and continues to be critically endangered according to SANBI (SANBI, 2017).

1.2.2 Seeds (achenes) of *Protea roupelliae* subsp. *hamiltonii*

The achenes of *P. roupelliae* subsp. *hamiltonii* are held in closed infructescences for several years (Bond, 1985). These closed infructescences act as a ‘storage vessel’ for the seeds until they are opened by fire (Midgley and Bond, 2011) and the seeds dispersed by wind. The storage of the seeds in the infructescence allows for their maturation before they are shed (Rimbawanto *et al.,* 1988), and forms a ‘canopy-stored’ rather than ‘soil stored’ seed bank. The seeds are covered in trichomes and are shed with varying mass ranging from 0.010 g to 0.034 g (Tarlton, 2013). There is evidence that seeds with a mass lower than 0.018 g are not viable (Tarlton, 2013). The *P. roupelliae* subsp. *hamiltonii* seeds are stored in the cone at an approximate water content range of 0.047 g - 0.094 g on a dry mass (DM) basis, indicating that the seeds are orthodox in behaviour (Roberts, 1981; Tarlton, 2013). Orthodox seeds (Roberts, 1973) attain their tolerance to desiccation during development in a process called maturation drying (Bewley and Black, 1994). This results in these seeds being shed at low water contents, which are in equilibrium with the prevailing relative humidity (RH) (Berjak and Pammenter, 2002),

and allows the seed to tolerate dry environments and low temperatures for protracted periods (Berjak and Pammenter, 2002).

Some species produce seeds that never develop into fully mature viable seeds and this is characterised as embryo abortion. Seed abortion is a very common phenomenon (Bawa and Webb, 1984) in the plant kingdom. It can be attributed to lack of nutrients in the soil or lack of water, thus curbing the developmental process (Bawa and Webb, 1984). Dormant seeds on the other hand, generally (but not always), have a fully developed embryo but will not germinate even under favourable environmental conditions (Hartmann *et al*., 1997). Such seeds are usually inhibited from germination in one or two ways, such as the presence of impermeable seed coats or germination inhibitors in the seed coat (Hartmann *et al*., 1997).

1.2.3 *Ex situ* conservation of *Protea roupelliae* subsp. *hamiltonii*

The *ex situ* conservation and propagation of *P. roupelliae* subsp. *hamiltonii* has been explored using various methods including seed storage (including cryopreservation), seed germination, the setting of cuttings and plant tissue culture. The latter included somatic embryogenesis, as well as direct and indirect organogenesis (Tarlton, 2013). These were considered in the framework of restoration and rehabilitation of the Dr. Hamilton Nature reserve.

The main intention of conservation is to protect the genetic diversity of a species, therefore seed banking and conventional propagation are perceived as efficient methods to protect *Protea roupelliae* subsp. *hamiltonii*. However, this species is critically endangered and seed production may not be sufficient to maintain a long-term viable population. Consequently, other investigations such as seed germination and *in vitro* propagation of the species were initiated.

Seed preservation is a vital component for the conservation of genetic diversity and biodiversity. In this context, it is pivotal to establish the viability of the seed material to be stored and to maintain that viability throughout storage. Tarlton (2013) showed that seeds of *P. roupelliae* subsp. *hamiltonii* stored at ambient temperatures (variable laboratory ambient) and at a constant 25^{o}C lost vigour over 18 months of storage. Before storage, the cumulative germination was 95% for both treatments but dropped to 85% and 90% respectively. In contrast, in the -196^{o}C storage regime, there was a decrease in vigour after only 6 months. The achenes in the 4^{o}C and -70^{o}C storage regimes were less effected (95% to 88% and 87% respectively) in their vigour in comparison with the 25^{o}C and the ambient temperature storage regimes.

Tarlton (2013) also showed that the germination peak value (P.V.) of the 4°C storage treatment increased as the storage time increased, and at the 25°C storage treatment the cumulative germination percentage P.V. was higher than all the other storage treatments. It is important to note that he also observed that the coefficient of variation of the water content before storage was higher than after storage. It was thus hypothesised that this was responsible for the discrepancies in storage response.

Micropropagation using *in vitro* culture techniques has been shown to be more advantageous than conventional vegetative propagation (amongst others Kozai, 1991). It allows material to be mass-produced under sterile conditions, which are monitored and adjusted to suit and accommodate the growing plantlets. Even with the known fact that micropropagation techniques produce cloned plants, *in vitro* techniques are perceived to be more reliable and predictable (Illg, 1991). Modern technologies and methodologies have also allowed for the storage of *in vitro* tissues and cells in both the medium- and long-term (Kasso and Balakrishnan, 2013), and as such add to the array of approaches used in *ex situ* conservation. The *in vitro* propagation of the *P. roupelliae* subsp. *hamiltonii* has previously been explored (Tarlton, 2013). That study showed that micropropagation is possible using achenes as initiating explants. Other explants such as apical meristems and shoot meristems were not tested, nor were other developmental processes such as embryogenesis explored in depth. Secondary embryogenesis was the focus of the present study since Tarlton (2013) showed that zygotic embryos can undergo both primary and secondary somatic embryogenesis. The latter had a higher conversion rate to *in vitro* plantlets. Secondary embryogenesis allows unorganised tissue (callus) to de-differentiate further and elicit a more vigorous response from the re-differentiated embryos.

1.3 Rationale for the present study

Plants form part of the Earths' natural wealth and conserving them is significant to the economy, ecology, as well as medical and scientific research (Patidar *et al.,* 2013). It is important to remember that the total loss of plants would result in missed opportunities to gain new knowledge, and diminished plant biodiversity may impact the natural ecosystem. Consequently, studying and concentrating resources and efforts on conserving plant species, which are on the brink of extinction such as cycads and some *Proteaceae* species, will increase our present knowledge and to an extent emphasise the significance of conservation (Cousins and Witkowski, 2017).

This study forms part of a broader programme considering the diverse ways of conserving the various plant populations in the Dr. Hamilton Nature Reserve. *Protea roupelliae* subsp. *hamiltonii* species is slow growing and in 2000 Weiersbye *et al.* (2000) reported that the population was not reproductive. Based on monitoring data from DWAF (Department of Water Affairs and Forestry), this species should have been extinct by 2005 (Figure 1.1) (*loc sit* Weiersbye *et al.*, 2000).

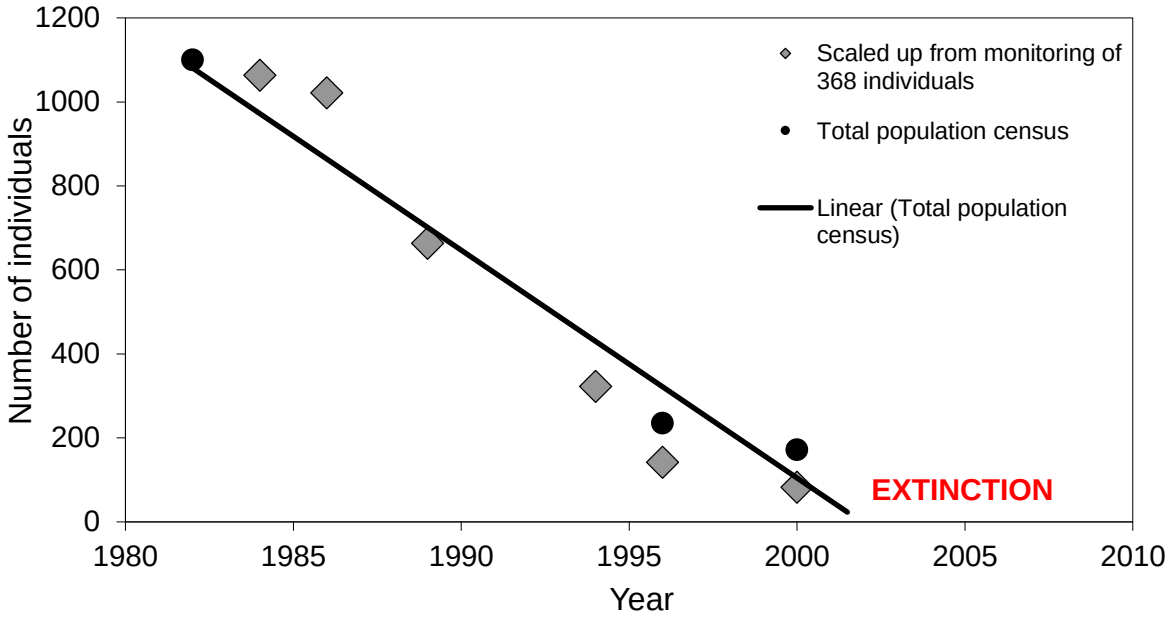

Figure 1.1: Population decline of *Protea roupelliae* subsp. *hamiltonii* in the Dr Hamilton Nature Reserve over a period of 20 years. Monitoring data is from R. Green (DWAF), census data for 1982 is from the TPA (Transvaal Provincial Administration), and census data for 1997 and 2000 is from the Restoration & Conservation Biology Research Group at Wits and R. Green (DWAF). (Weiersbye *et al.,* 2000).

However, at that time, this single extant population had 124 plants remaining and these showed some evidence of seed set but no recruitment (Czypionka, 2006). This cemented the necessity for the application of the basic protocols of conservation, *viz*. enclosing the population to mitigate herbivory, and to control the impact of alien species. Bond (1984) stated that with many serotinous plant species, the probability of seed loss to predators and seed decay is directly proportional to the time spent on the plant canopy. This has been confirmed for a range of species such as *Banksia baxteri*, *Banksia speciosa* and *Banksia coccinea* (*Proteaceae*) (Witkowski *et al.*, 1991), but there was no data in this regard for *P. roupelliae* subsp. *hamiltonii.* This can be attributed to the previous gradual reduction in seed production from 1980-2000, with very few plants producing seeds in 1994 (E.T.F. Witkowski *pers. comm*). It is thus

important to have data relating to this specific subspecies in order to expand on the knowledge regarding seed serotiny.

1.4 Aims and objectives

In an effort to better understand the biology of *Protea roupelliae* subsp. *hamiltonii* in the context of its successful conservation, research was conducted with the following three aims:

- To improve understanding of the reproductive ecology of the seeds/achenes of *Protea roupelliae* subsp. *hamiltonii*
- To investigate preservation methods for the *ex situ* conservation of *Protea roupelliae* subsp. *hamiltonii* germplasm
- Establish both direct and indirect micropropagation techniques for *Protea roupelliae* subsp. *hamiltonii*

The specific objectives were to:

a. Investigate the relationship between seed mass and seed viability
b. Assess the vigour and viability of the seeds as they age on the plant (canopy seed storage or serotiny)
c. Assess four *ex situ* seed storage regimes
d. Assess the viability and vigour of the seeds, with known initial water content, in response to seed storage regimes
e. Investigate whether seed embryo or vegetative tissues are more effective explants *in vitro* when using the following regeneration techniques:
    - Indirect somatic embryogenesis
    - Indirect morphogenesis via secondary embryogenesis
    - Direct shoot organogenesis

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2 Seed germination ecology and seedling emergence of *Protea roupelliae* subsp. *hamiltonii*

2.1 Abstract

Seed ecology and plant physiology are important aspects in the conservation of endangered species such as the serotinous *Protea roupelliae* subsp. *hamiltonii*. These aspects can be affected by factors such as predation, herbivory, annual rainfall and others. Presently there is only one population of this subspecies that is being monitored and it is under conservation protection, while research is being conducted to understand the factors which affect its recovery over time.

With the aim of understanding the factors that govern the decline and success of this species, the size of the plant canopy area and volume and the number of cones in relation to canopy size were investigated. The relationship between seed mass and seed viability of *Protea roupelliae* subsp. *hamiltonii* was assessed using seed weight relations to seed viability and vigour, accompanied by how seedling emergence of the species is affected. The seed predation within the population was also recorded and its prevalence assessed across three seed ages, i.e. seeds within serotinous cones 1, 2 and 3 years of age.

The recovery of the *Protea roupelliae* subsp. *hamiltonii* population since the last report in 2010 has shown an increase in the adult plant population from 36 to 45 individuals. However, predation occurrence increased from negligible amounts to 4.81%, 2.58% and 8.56% for the 1, 2 and 3-year-old seed ages respectively.

The mean (±S.E.) canopy area and volume of the adult plants decreased from 0.422 ± 0.030 m^2 and 0.0878 ± 0.0077 m^3 in 2010, to only 0.085 ± 0.05m^2 and 0.0020 ± 0.0017m^3 in 2013, respectively. However, in 2013, the plants had 5.82 ± 8.37 (mean ± S.E.) cones per plant, which was higher than the 3.34 ± 0.41 cones recorded in 2010. The total number of cones per plant showed a weak relationship of R^2 = 0.2546 to plant canopy area, with the one-year cones showing the best relationship with of R^2 = 0.3727 in comparison with 2-year and 3-year cones (R^2 = 0.1434 and R^2 = 0.0932 respectively). Herbivory on the leaves was recorded at less than 30% for the entire sampled population in 2013.

The population is slowly recovering despite the increasing levels of seed predation, which are likely related to the greater numbers of cones, making them more apparent to seed predating insects. However, a concern is the decreasing adult plant sizes.

Keywords: Germination rate, plant size, seed age, seed mass, seed predation, seed viability

2.2 Introduction

2.2.1 Seed

Apart from their primary role in plant reproduction and evolution, seeds play important roles as sources of medicine and as part of the diet for humans and animals (Bewley, 1997). It is for these reasons that seeds are being exploited and their developmental understanding being vigorously researched to help preserve them for future use.

A seed is a structure which originates from a fertilised ovule (Vicente-Carbajosa and Carbonero, 2004). It comprises an immature plant and other maternally derived tissues enclosed in a protective outer covering (Harada, 1997; Koornneef *et al*., 2002; Vicente-Carbajosa and Carbonero, 2004). A seed is the mature plants' means of reproduction and dispersal, and thus its approach of increasing its survival. It is the precursor and a chance at independence for the next generation (Baskin and Baskin, 2004; Waterworth *et al*., 2015), although some plants can also reproduce and disperse from vegetative growth. Seeds are valuable; they allow for the successful transfer of genetic material from the parent (mature) plant to the subsequent generation (Waterworth *et al*., 2015). They carry the plant's embryo, the very mechanisms that will give rise to a seedling, and protect it between the process of seed maturation and seedling establishment (Koornneef *et al*., 2002).

Maturation of seeds is a critical process in seed development due to the many unique processes that occur (Harada, 1997). During seed maturation, storage products are accumulated in the various embryo and storage tissues, desiccation tolerance is acquired, and concomitantly premature germination is suppressed (Harada, 1997; Wobus and Weber, 1999), and any further embryo development is arrested. The desiccation phase of orthodox seeds (maturation drying), which is also characterised by the seed embryo entering a quiescent state, marks the end of maturation development (Gutierrez *et al*., 2007).

The developmental phases associated with seed maturation can differ and are expressed in the storage behaviour of the resultant seed, *viz*. orthodox or recalcitrant (Roberts, 1973). Orthodox seeds undergo the described maturation which ends with a desiccation and cold tolerant propagule, which is shed from the parent plant at low water contents. As a consequence, orthodox seeds can over winter and indeed survive for protracted periods. Humans have taken advantage of these characteristics and maintain seed in cold and dry storage. Recalcitrant seeds on the other hand, undergo little or no desiccation at the end of seed maturation and do not acquire desiccation tolerance. Such seed is shed from the parent plant at high water contents

and can be sensitive to low temperatures (Berjak and Pammenter, 2002). Recalcitrant seeds are therefore not storable under standard storage (cold and dry) regimes. The classification of seed according to their storage behaviour is therefore critical in developing an optimum method of *ex situ* conservation per species (Hong and Ellis, 1996).

Successful plant production via seed (healthy) begins with germination followed by seedling establishment and survival, which are vulnerable stages in a plants' life cycle (Wilson and Witkowski, 1998). These steps are important in a plants' life, and can have substantial impacts on plant population dynamics and consequently on species richness.

2.2.2 Seed quality

The quality of a seed is related to particular characteristics that control its performance when sown or post storage. It reflects the seeds overall value and should measure up to the expectations of the end user (Hampton, 2002) in certification, production, sales and importing (Hampton, 2015). Characteristics such as water content and equilibrium relative humidity (RH) of the storage environment contribute to seed quality, while factors such as weight and vigour affect the successful germination of the seed.

2.2.3 Seed germination

Germination in orthodox seed is the resumption of embryo growth from the quiescent structure (Nonogaki *et al.*, 2007; Hopkins and Huner, 2009). The process is the precursor to seedling establishment and is characterised by the uptake of water (imbibition), triggering a series of growth programmes that lead to the protrusion of the radicle. The latter is evidence of seed viability, which is then followed by seedling establishment (Bewley and Black 1994), regulated by the seed vigour (the rate at which the germination process proceeds). Germination is an important process during the life cycle of a plant and for its success, viable and vigorous seeds are required (Müller *et al*., 2016). It is therefore important that seed quality is assessed before conservation strategies can be implemented.

2.2.4 Seed of *Protea roupelliae* subsp. *hamiltonii*

The seeds (achenes) of *P. roupelliae* subsp. *hamiltonii* exhibit orthodox storage behaviour. Orthodox seeds are stored optimally at low temperatures and at low water contents [2 - 6% wet mass basis (w.m.b.)] under dry conditions (Vertucci and Roos, 1990). In nature, the achenes of *P. roupelliae* subsp. *hamiltonii* are stored in the parent plants' canopy for a minimum of a year and a maximum of ca. 3 years. Canopy seed storage or serotiny describes an adaptive trait which allows achenes to be released after certain cues (fire/smoke etc.) into a more receptive

and germination conducive environment. However, serotiny exposes seeds to granivory while still on the parent plant (which is known as pre-dispersal seed predation). Granivory is the attack/parasitism on achenes by small insects. Understanding how both granivory and serotiny affect the seed of *P. roupelliae* subsp. *hamiltonii* takes seed biology research a step closer to better understanding this plant species.

This study aimed to understand the reproductive ecology of the seeds/achenes of *Protea roupelliae* subsp. *hamiltonii* starting with the removal of the infructescence from the parent plant and by assessing seed germination and seedling survival *ex situ*.

The objectives were:

1. To revise the status of the *Protea roupeliae* subsp. *hamiltonii* population in 2013 in terms of plant size, cones production and herbivory, and more specifically to:
    a. Determine the number of cones produced per plant
    b. Assess whether the number of cones produced per plant was related to plant canopy area
    c. Measure the degree of herbivory on the plant
2 To synthesise the vigour and viability data of the seeds as they age on the plant (serotiny) in terms of:
    a. Germination per seed age (1 - 3 years)
    b. Seedling establishment per seed age (1 - 3 years)
    c. Pre-dispersal seed predation per seed age (1 - 3 years)

2.3 Materials and methods

2.3.1 Study site

The Dr Hamilton Protea Reserve is a small 26 ha area situated in the Nelshoogte region of Barberton, Mpumalanga in South Africa. The reserve is within the Barberton Greenstone Belt, which falls within the bounds of the Barberton Centre of plant endemism (Tarlton, 2013; Williamson, 2016). The centre of endemism has about 2210 plant species covering an area of ~ 4000 km^2 (Williamson, 2016). Twenty-nine percent of this area has been transformed by species of *Pinus* and *Eucalyptus* commercial forestry plantations (Lotter *et al.*, 2002 *loc sit* Williamson, 2016). Pine plantations grow on the borders surrounding the whole of the Dr Hamilton Reserve and can become a threat to the *Protea* species. Plants within part of the reserve were enclosed using an antelope proof fence, but at the time of sampling (2013) the

fence had gaps caused either by the antelopes or human interference. Fencing the plants was first attempted in 1985 due to high levels of herbivory on the foliage, but it was stolen in 1987 (R. Green *pers. comm.* 2010 *loc sit* Tarlton, 2013). The present fence, much larger area fenced, was erected in 2003. The population started recovering with evidence of flowering and seed setting, after cessation of mammal herbivory on the foliage of the plants (Czypionka, 2006; Tarlton, 2013).

2.3.2 Cone variation/categorisation

Cones were collected according to age categories on the 28th July 2013. The physical appearance of the cones provided the category and nodal scars on the branches separated the growth of the current growing season (year 1) with that of the previous season (year 2), and then another nodal scar separated year 2 and year 3's growth. The younger cones (year 1) were bright orange and the seeds were still strongly attached to the cone (Figure 2.1a), while the three-year-old cones were grey and the seeds were loose (Figure 2.1c). The second-year cones were still orange but not as bright as the first-year cones and the seeds were weakly attached to the cones (Figure 2.1b). Some of the 3-year-old cones had dropped from the parent plant (passive dispersal) and were collected from the ground under the shrubs. The observed amount of herbivory/damage to the plant cones was assessed externally on each cone and the percentage damage data collected and synthesised.

Figure 2.1: *Protea roupelliae* subsp. *hamiltonii* seeds in infructescences (cones) of different ages, *viz*. (a) year 1 cone, (b) year 2 cone, (c) year 3 cone, collected from the Dr. Hamilton Nature Reserve in Barberton, South Africa on 28th July 2013.

2.3.3 Plant canopy area, volume and inflorescence (Cone) sampling

At the time of sampling the reserve had ~ 45 cone producing plants of which 38 were randomly selected and sampled. The height, maximum diameter and the diameter at 90° to the maximum diameter were measured using callipers and a 1.5 m long ruler. These measurements were recorded on a data sheet and used to calculate the plant canopy area and volume. The cones were sampled per age category and placed in brown paper bags as a collective per age category for transporting to the University of the Witwatersrand, Johannesburg.

2.3.4 Seed categorisation and granivory

Upon arrival at the University of the Witwatersrand, the cones were maintained under ambient laboratory conditions for 7 days to allow them to dry out further. This also assisted in loosening the seeds from within the cones, especially for those from the one-year-old cones since they were more tightly packed into the cones than the 2 or 3-year-olds which had already dried in the field. Each cone was opened, and the number of seeds counted. The 22910 seeds collected were inspected for granivory and affected seeds separated from the intact ones, categorised as predated and counted.

2.3.5 Seed germination

To determine the initial germinability of the seeds, 50 seeds per age category were placed between filter paper in plastic 50 mm diameter Petri dishes; 10 seeds per Petri dish. They were set to germinate under growth room conditions (25^0C and 14 h photoperiod at ~ 320 -µmol m^{-2} s^{-1} photosynthetic photon flux density) and watered every second day with 8 ml of distilled water. Germination, which was defined as radicle protrusion from the seed, was recorded daily from the first sign of germination until maximum germination was reached (a period of 45 days). The germinated seeds were then planted into 125 ml polystyrene cups (one seed per cup) containing acidic, nutrient-poor soil with a mean pH (KCl) of 3.91 ± 0.19 (±S.E.), also collected from the Dr Hamilton Reserve on 28th July 2013, from the 0 – 3 cm depth.

2.3.5.1 Seedling establishment

The seedlings were grown in the green house at the University of the Witwatersrand, Johannesburg under a 15 minute, three times a day, watering regime. Established seedlings for this study were recorded after 50 days and analysed. After recording seedlings remained in the green house for the *in vitro* studies (see Chapter 4). This regime was in place for 2 months, but was later modified due to water logging. In the adapted regime the seeds were manually watered every second day. This treatment resulted in sufficient watering with no water logging observed. The seedlings started thriving and were maintained thereafter for the *in vitro* studies (see Chapter 4).

2.3.6 Data analysis

Data were analysed and presented using regression to illustrate the relationship between the plant canopy volume and the number of cones produced per m^3 of a plant. An analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used to compare the percentage seed germination data between the three seed age classes. Germination value (GV), which is

an index combining rate and completeness of seed germination (Czabator, 1962) was calculated using the following equation:

$$GV = MDG \times PV$$

Where MDG = Mean daily germination

PV = Peak value

Mean germination time (MGT) (days), described as the length of the interval from commencement of imbibition to radicle protrusion, was also calculated using the following equation (Mavi *et al.*, 2010):

$$MGT = \sum (nT) / \sum$$

Where n = number of seeds newly germinated at time T

T = number of hours from the beginning of the germination trial

$\sum$ n = number of total germinated seeds at the end of the germination trial. The statistical tests were performed using Rstudio® Version 1.1.463 (RStudio, 2018) at a 0.05 significance level.

2.4 Results

2.4.1 Plant canopy area, canopy volume and number of cones per plant

The 2010 data were obtained from (Witkowski, 2010). The canopy area and volume of *Protea roupelliae* subsp. *hamiltonii* plants decreased from 0.422 m^2 and 0.0878 m^3 to only 0.085 m^2 and 0.0020 m^3 after 3 years (from 2010 to 2013; Table 2.1), however, the average cone production per plant was higher in 2013 than in 2010 (Table 2.1).

Table 2.1: Canopy area and volume per plant, and cones per plant of *Protea roupelliae* subsp. *hamiltonii* plants in 2010 and 2013 (mean ±S.D).

	2010	**2013**
Canopy area/plant (m^2)	0.4220 ±0.0300	0.0850± 0.0500
Canopy volume/plant (m^3)	0.0878±0.0077	0.0020±0.0017
Cones produced/plant	3.34±0.4100	5.82±8.3700

The number of cones produced by the plants were more positively related to the canopy area for the year-one cones (Figure 2.2a), than year-two (Figure 2.2b) and year-three cones (Figure 2.2c). The relationship for the total cone production was also weaker than for the year-one cones (Figure 2.2d). The relationship between the canopy area and the canopy volume of the plant canopies was strong, with $R^2 = 0.9134$ (Figure 2.3).

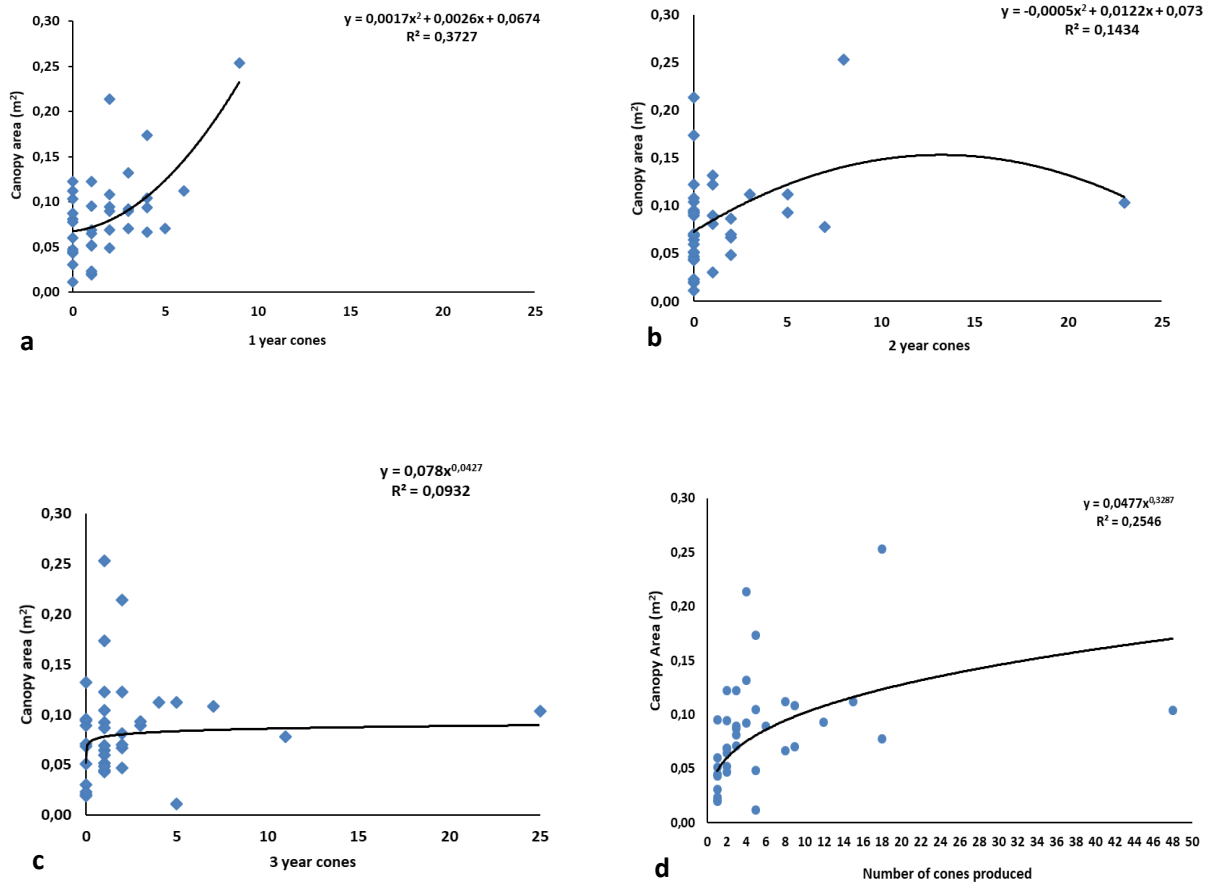

Figure 2.2: Relationships between the number of cones produced per year and the canopy area of the *Protea roupelliae* subsp. *hamiltonii* plants individually (a-c) and combined (d) in 2013.

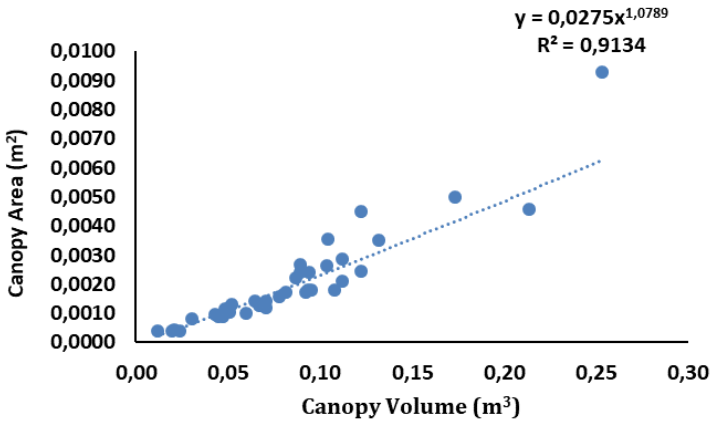

Figure 2.3: The relationship between canopy volume and the canopy area of *Protea roupelliae* subsp. *hamiltonii* fitted with a power trendline which was the best fit with an $R^2 = 0.9134$ (n = 38), which signifies the strong relationship between the area and the volume of the plant canopies.

2.4.2 Seed categorisation and granivory

The *Protea roupelliae* subsp. *hamiltonii* seeds displayed a statistical association between the three seed mass categories across the three seed ages (Table 2.2). The plants produced an overall higher percentage of seeds in the mass category < 0.017g and the year 1 seeds had the highest number of seeds in the ≥ 0.017g mass category.

Table 2.2: Numbers (with %s in brackets) of *Protea roupelliae* subsp. *hamiltonii* intact seeds categorised by mass (excluding predated seeds) in each of the age categories, 1-3 years ($x^2 = 273.75$; df = 4; $p < 0.05$).

Seed size	Year 1	Year 2	Year 3	Total
<0.017g	3789 (55.1%)	4150 (61.8%)	4841 (60.2%)	12780 (59.1%)
≥0.017g	3062 (44.6%)	2525 (37.6%)	3199 (39.8%)	8786 (40.6%)
>0.029g	21 (0.3%)	38 (0.6%)	7 (0.09%)	66 (0.3%)
Total	6872 (31.8%)	6713 (31.0%)	8047 (37.2%)	21632 (100%)

The overall predation for the *Protea roupelliae* subsp. *hamiltonii* seeds was 5.58% and seeds from the oldest cones, the year-three cones, showed the highest predation percentage (Table 2.3). The seeds acquired from the year-one cones showed a predation percentage higher than the seeds collected from the year-two cones (Table 2.3).

Table 2.3: Numbers of predated and non-predated seeds, and percentage seed predation, of *Protea roupelliae subsp. hamiltonii* according to seed ages from the 1-3-year-old cones ($x^2 = 273.75$; df = 2; $p < 0.05$).

Seed categorisation	Year 1	Year 2	Year 3	Total
Predated	347	178	753	1278
Non-predated	6872	6713	8047	21632
Total seeds	7219	6891	8800	22910
Predation (%)	4.81%	2.58%	8.56%	5.58%

The number of predated seeds was significantly higher in year-two seeds with a mass of ≥ 0.017g. Seeds from year-three cones with a mass < 0.017g had the highest predation percentage (Table 2.4). The overall predation was higher in seeds with a mass < 0.017g (Table 2.4).

The proportion of seeds collected in 2006 (from Czypionka, 2006) according to the 3 different mass categories of < 0.017 g, ≥ 0.017 g and > 0.029 g were not statistically associated to those collected in 2013 (Table 2.5). Seed germination percentages were higher in 2013 than in 2006, with no statistical association between the two years (Table 2.5).

Table 2.4: Predated seeds (%) of *Protea roupelliae* subsp. *hamiltonii* categorised by their mass across the different seed ages. ($x^2 = 18.827$; df = 2; $p < 0.05$).

	Year 1	Year 2	Year 3	Total
<0.017g	279 (80.4%)	113 (63.5%)	536 (71.2%)	928 (72.6%)
≥0.017g	68 (19.6%)	65 (36.5%)	217 (28.8%)	350 (27.4%)
Total	347 (27.2%)	178 (13.9%)	753 (58.9%)	1278 (100%)

Table 2.5: Comparisons of seed mass class compositions between 2006 (from Czypionka 2006) and 2013 ($x^2 = 0.229$; df = 2; $p > 0.05$) in the recovering *Protea roupelliae* subsp. *hamiltonii* plants, and their percentage germination ($x^2 = 0.138$; df = 2; $p > 0.05$), showing no overall statistical differences over the 7 years (2006-2013).

	2006		2013	
Seed size	**Number of seeds collected**	**Seeds germinated (%) (n)**	**Number of seeds collected**	**Seeds germinated (%) (n = 50)**
<0.017g	1515 (58.7%)	4% (303)	12780 (59.1%)	6%
≥0.017g	1045 (40.5%)	42% (209)	8786 (40.6%)	67%
>0.029g	20 (0.8%)	0% (4)	66 (0.3%)	0%
Total	2580 (100%)	46% (516)	21632 (100%)	73%

2.4.3 Seed germination and seedling establishment

The seed germination percentage of *Protea roupelliae* subsp. *hamiltonii* were associated with seed age; the year 1 seeds had a higher germination percentage than the year 2 and 3 seeds (Table 2.6). The seedling establishment, which was recorded 50 days after germination and analysed using the successful germinants only, also displayed an association between seed age, with year 3 seeds having established at a higher percentage than the year 2 and year 1 seeds. Although the one-year seeds had a higher germination percentage, only 14% of the seeds managed to successfully establish into seedlings (Table 2.6). Unfortunately, after 12 months all the seedlings had died.

Table 2.6: Seed germination (%) and seedling establishment (%) of *Protea roupelliae* subsp. *hamiltonii* seeds (n=50) according to their age categories ($x^2 = 12.66$, df = 2, $p < 0.05$; $x^2 = 33.172$, df = 2, $p < 0.05$, respectively).

	Year 1 (%)	**Year 2 (%)**	**Year 3 (%)**	**Total (Overall %)**
Seeds germinated	43 (86%)	28 (56%)	29 (58%)	100 (67%)
Seeds not germinated	7 (14%)	22 (44%)	21 (42%)	50 (33%)
Established seedlings	6 (14%)	17 (61%)	23 (79%)	46 (46%)
Seedlings not established	37 (86%)	11 (39%)	6 (21%)	54 (54%)

Fifty *Protea roupelliae* subsp. *hamiltonii* seeds were set to germinate according to their age categories. The first sign of germination was observed on day 20. Year 1 seeds had a significantly higher germination percentage than the year 2 and year 3 seeds after 45 days (post-

hoc Bonferroni test). The final germination percentages for the two older seed categories were not significantly different ($F_{2,147} = 31.95$, $P < 0.05$) (Figure 2.4).

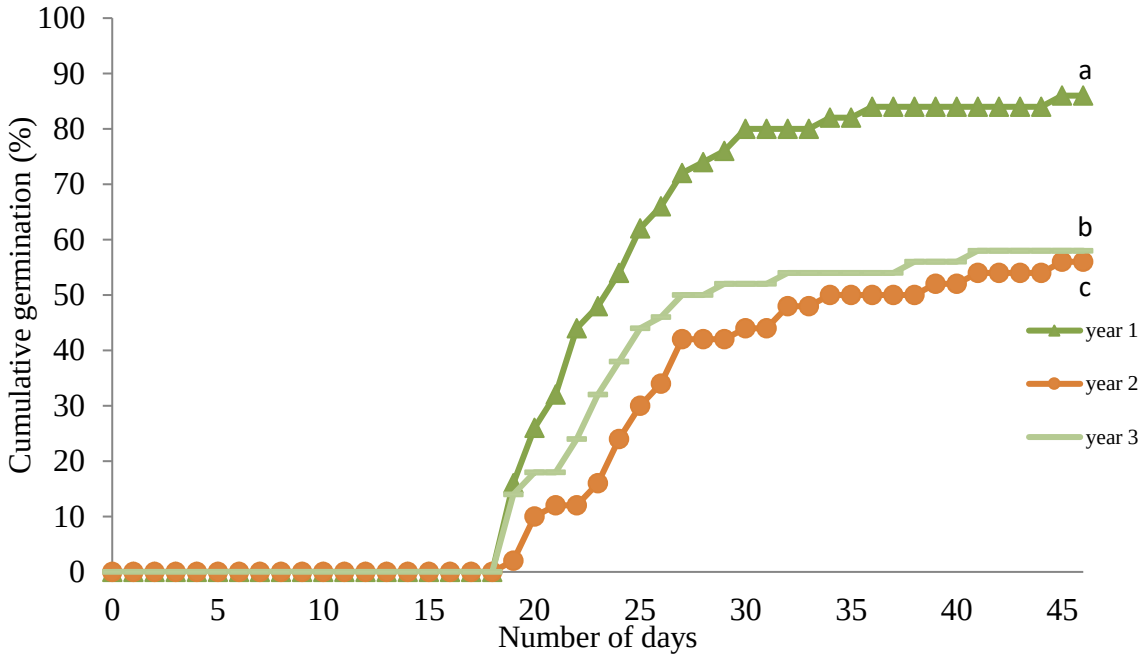

Figure 2.4: Cumulative germination percentages for 1, 2 and 3-year-old seeds of *Protea roupelliae* subsp. *hamiltonii* (n=50/year) during a 45-day germination trial ($F_{2,147} = 31.95$, $P < 0.05$).

The germination value was higher for the year 1 seeds, followed by the year 3 seeds (Table 2.7). Year 2 seeds stopped germinating earlier than year 1 and year 3, reaching final germination of 56% at 35 days. Year 1 seeds germinated until day 45 reaching a final germination of 86%, while year 3 seeds reached a final germination of 58% after 42 days (Table 2.7).

Table 2.7: Germination value and the measures used to compute this value for the 3 different seed age categories of *Protea roupelliae* subsp. *hamiltonii*.

	Seed age (years)		
	1	**2**	**3**
First day of germination	Day 20	Day 20	Day 20
% of initial germination (S.E.)	16% (±1.02)	2% (±0.45)	14% (±0.56)
Peak value	2.581	1.500	1.786
Mean daily germination	1.830	1.191	1.234
Germination value	4.722	1.787	2.204
Last day to germinate	45	35	42
Final germination %	86	56	58

The mean germination time for *Protea roupelliae* subsp. *hamiltonii* seeds was higher for year 2 seeds and the same for year 1 and year 3 seeds, however, the standard error was higher in year 1 seeds (Table 2.8).

Table 2.8: The mean germination time (±S.E.) of *Protea roupelliae* subsp. *hamiltonii* seeds calculated using the formula from Mavi *et al.* (2010).

	Seed age (years)		
	1	2	3
Mean (±S.E.) germination time (days)	26 ± 9.91	29 ± 7.29	26 ± 7.63

2.5 Discussion

2.5.1 Plant canopy area, canopy volume and number of cones per plant

The comparison of the results over time for the recovering *P. roupelliae* subsp. *hamiltonii* population indicated an improvement in the population in terms of seed production, showed by an increase in the average number of cones produced per plant (Table 2.1). However, the plants become smaller over time, evinced by the decrease in average canopy area and volume (Table 2.1). Nonetheless, the increase in seed production confirms a positive outcome regarding its conservation since there was no seed production reported in 2000 (Weiersbye *et al.*, 2000). The decrease in the plant size comes with an increased cone production, suggesting that the plants expend most of its energy and nutrients to produce cones and seeds rather than grow larger in size. Thus, contributing directly to the increase of the plant population. There are a number of studies (Witkowski, 1990; Witkowski and Lamont, 1996) that have shown that a high proportion of limited nutrients, such as nitrogen and phosphorus, are allocated to seed production at the expense of plant size, in the species of the Proteaceae in South Africa and Australia.

The canopy area of the plants in this extant population exhibited a positive relationship to the number of cones produced per year. The one-year-cones had a higher R^2 value than the two and three-year-cones (Figure 2.2a-c). The three-year cones showed the poorest relationship; the cones have been on the plant for ca. 3 years and this could have affected the relationship. Overall, the number of cones produced per plant canopy area has a positive R^2 value (R^2 = 0.2546). Nonetheless in Figure 2.2d, it is evident that cone production is highly variable, with for example, one medium-sized plant producing the highest number of cones by far.

The plant canopy volume was strongly related to plant canopy area as one would expect (Figure 2.3) with an R^2 = 0.91. Plant cone production influences the recovery of plants since without cones there are no seeds to germinate and therefore the population will begin to dwindle over time when there is no recruitment of new seedlings. Serotinous cone production evolved in the South African *Protea* genus 28 million years ago (mya) (Lamont and He, 2012; de Gouvenain *et al.*, 2019). This characteristic exposes plant seeds to higher levels of granivory due to being exposed for longer periods of time on the plant canopy. This could result in many seeds being damaged before they are released from the parent plant particularly by host-specific seed predators, which cause moderate to high predation rates (Jeffs *et al.*, 2018). This vulnerability to granivory could influence the plant population dynamics (Jeffs *et al.*, 2018).

2.5.2 Seed categorisation and granivory

Of the 22910 seeds of *P. roupelliae* subsp. *hamiltonii* collected and classified into age and size classes (Table 2.3), all were potentially exposed to granivory, with 5.58% being attacked by granivorous insects (Table 2.3). The seeds from the three-year cones had the highest granivory percentage (Table 2.3). Due to the statistical association ($x^2 = 273.75$; df = 4; $p < 0.05$) between the three seed years, it can be hypothesised that the amount of damage caused to seeds is affected by the time spent on the canopy. However, the two-year seeds overall had predation less than that of the one and three-year seeds, and this could be due to the maturation process being undergone by the seeds making them unpalatable to the insects, or climate fluctuations (War *et al.*, 2016).

When granivory takes place, the seed is consumed and killed *in situ* on the parent plant (Jeffs *et al.*, 2018), therefore directly influencing the proportion of viable seeds maintained and dispersed (Fenner and Thompson, 2005; Crawley, 2013a *loc sit* Jeffs *et al.*, 2018). Granivory has been reported to influence the amount of canopy-stored seeds in many *Banksia* (*Proteaceae*) species (e.g. Cowling *et al.*, 1987; Witkowski *et al.*, 1991; Witkowski *et al.*, 1994), and the similar negative effect can also be seen with *Protea roupelliae* subsp. *hamiltonii* (Table 2.3). The decrease of canopy-stored seeds due to granivory in turn has a negative impact on the plant populations; damaged stored seeds do not convert to seedlings therefore the plant population size will eventually dwindle.

The predated seeds weighed less than 0.029 g and this could be because they were predated on and therefore either lost dry matter through consumption, or development was prevented by predation and hence they did not grow to full size. The two-year cones had the highest predation in the highly germinable seed mass category of ≥ 0.017 g. This could have resulted in the year 2 seeds having the least highly germinable mass category of seeds. Overall, it is evident that granivory is negatively impacting the quality of the seeds of *P. roupelliae* subsp. *hamiltonii* plants, and it may increase further over time.

The 21632 un-predated seeds were categorised into mass classes in line with observations made by Tarlton (2013) and Cyzpionka (2006). The plants produced more seeds with a mass < 0.017 g (59.1%) and the least amount of seeds with a mass > 0.029 g (0.3%). The seeds within the highly germinable mass category were highest in the one-year cones (44.6%) followed by the three-year-cones at 39.8%. Minor changes in temperature and rainfall affects the population(s) of some plant species and this could be the cause for *P. roupelliae* subsp. *hamiltonii* plants

having produced more seeds capable of recruitment in new cones than in the older cones (Cochrane *et al.*, 2015).

The comparison of the numbers of seeds over time shows that the proportions in terms of their mass classes for this study (2013), were not significantly associated to those collected and classified in 2006 (Table 2.5). This allows the deduction that the plant produces these seeds categorically; whilst non-viable seeds do not contribute to the population increase of the species they may play a role in deterring insects/predators (Fuentes and Schupp, 1998). This is supported by the observation that seeds in mass category < 0.017 g had the highest granivory percentage of 72.6% in comparison with seeds of a mass ≥ 0.017 g at 27.4% (Table 2.4).

The seed quality (evidenced through seed germination) was also not significantly different during those two years (Table 2.6). The plant population is getting larger, but the proportion of highly germinable seeds to those which are not germinable or have a low germination rate, is not changing as the plant species is recovering.

2.5.3 Seed germination and seedling establishment

Seed germination is a fundamental aspect to every plants' establishment, survival and community development (Nonogaki *et al.*, 2018). The germinability of the seed depends on its quality, which translates to seed viability and vigour. Seed viability can be defined as the percentage of seeds that establish normal seedlings under ideal laboratory conditions in a standard germination test (van de Venter, 2001; Brits *et al*., 2015). The seed quality of serotinous seeds could be different across the cone ages due to the differing amount of time the seeds spent in the cone on the plant, but their proportion could be similar. This could be true for *P. roupelliae* subsp. *hamiltonii* as evidenced by the significant association in the proportion of germinated to non-germinated seeds across the age categories (Table 2.6).

The initial seedling establishment is key for determining and assessing plant population dynamics (Jeffs *et al.*, 2018), such as recovery. Seedling establishment is at times confused with increased rates of germination, but research on five Australian and South African members of the *Proteaceae* (*Protea lorifolia*, *Protea cynaroides*, *Leucadendron laureolum, Hakae sericea* and *Banksia laricina*) show that rapid germination of those Proteaceae seeds did not appear to be a significant indication of seedling establishment/fitness (Stock *et al.*, 1990). This seems to be true for *P. roupelliae* subsp. *hamiltonii* seeds; the year-one seeds with the highest germination percentage (86%) had the lowest seedling establishment (14%) (Table 2.6,

Figure 2.4), while the year-two and three seeds with insignificantly different germination rates of 58% and 56%, showed seedling establishment of 60.71% and 79.31% for the two seed ages (Table 2.6, Figure 2.4). Cernac *et al*. (2006) stated that successful seedling establishment relies on the presence and effective use of seed storage reserves, and reserves are accumulated during seed development and maturation (Gutierrez *et al.,* 2007); therefore, it can be assumed that a more mature seed has more resources for successful seedling establishment. This being the reason year-two and three seeds had a higher seedling establishment than year-one seeds and furthermore year-three to year-two seeds.

The germination value of the seeds expresses the speed and totality of germination as well as their interaction (Czabator, 1962; Ranal and Santana, 2006) (Table 2.7). The one-year seeds had a higher germination totality and rate compared with two and three-year seeds (Table 2.7), which followed the trend of the seeds final germination (Figure 2.4). In serotinous species such as *M. solisioides*, *M. hernandezii* and *M. napina*, Rodríguez-Ortega *et al.* (2006) found that the seed germination declined significantly with seed age in the first two species but increased slightly in *M. napina*. However, *P. roupelliae* subsp. *hamiltonii* seeds do not exhibit a clear increase or decrease pattern regarding germination according to seed age evinced in that the three-year seeds had a GV higher than the two-year seeds, but lower than the one-year seeds (Table 2.7).

Initially, all the seeds germinated from day 20 but the one-year seeds accumulated more germinants per day. Therefore, one-year seeds were more vigorous than the two and three-year seeds probably due to their advantage of not yet having started their maturation drying; the three-year seeds had a higher germination value than two-year seeds but lower than one-year seeds. The three-year seeds have reached its maximum/peak in maturation and therefore is more stable than the two-year seeds thus a higher germination rate. This is also evident when looking at the average time it took the seeds to germinate which was the least for one and three-year seeds. The two-year seeds took the longest time to germinate (radicle protrusion) (highest MGT). This could be attributed to the fact that when orthodox seeds approach their final developmental stages on the parent plant, they undergo maturation drying (Erickson and Merritt, 2016). Therefore, the two-year seeds were still in the process of maturing, consequently taking them longer to re-align metabolic processes that assist the germination progression.

The findings of this research provide background and the foundation for further research since it established the proportion of seeds produced by the plant according to the seed mass categories. It further elaborates on the susceptibility of the seeds with a mass < 0.017 g to granivory, although many of those were probably predated before they could mature to full seed size. It is also important to note that the one-year seeds germinate considerably faster with a significantly higher germination percentage than the two and three-year seed, but the establishment of the year-one seeds is low. Thus, the serotinous nature of the species is retaining robust seed in the canopy which can go on to successfully establish at the expense of the rate of the germination process.

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3 Low temperature storage of *Protea roupelliae* subsp. *hamiltonii* seeds

3.1 Abstract

Conserving seeds at low temperatures has proven to be progressive for many seeded plants, particularly those at risk of extinction such as *Protea roupelliae* subsp. *hamiltonii*. Seed batch quality post storage is affected by pre-storage water contents as well as storage humidity and temperatures during storage. The water content of *Protea roupelliae* subsp. *hamiltonii* seeds were equilibrated over a saturated solution of Ca $(NO_3)_2$ in a sealed desiccator for 40 days before storage at four different temperatures, *viz.* -70°C, -20°C, 4°C and 25°C for 6 and 12 months simultaneously.

The overall coefficient of variation (CV) of the water contents decreased from 20.8, 19.2 and 18.8 to 8.0, 15.5 and 14.6 per seed age (1-3 years) respectively when equilibrated over a saturated solution of Ca $(NO_3)_2$ before storage. After 6 months of storage there was no significant ($p > 0.05$) difference between storage treatments in water content, while the variation around the means increased in comparison to post equilibration. The germination percentage (CV) post 6-months storage increased from 66% (27.8%) to 73% (18.1%) and 69% (28.0%) under -70°C and 25°C, respectively. Under -20°C the overall mean germination percentage (CV) decreased to 60% (20.4%) and remained similar under 4°C at 66.5% (36.2%).

After 12 months of storage there was a significant ($p < 0.05$) difference between the water content of the treatments, -70°C (a), -20°C (b), 4°C (b) and 25°C (c), while the variation around the means increased in comparison to post equilibration. Subsequently, 12-months storage resulted in a decrease in germination percentage (CV) from 66% (27.8%) to 60.9% (14.7%), 60.4% (17.8%) and 57.9% (21.0%) under -70°C, 4°C and 25°C, respectively. Under -20°C the overall mean germination percentage (CV) increased slightly to 67.1% (17.6%).

The germination percentage of the seeds post equilibration and storage decreased for many of the seeds, especially after 12 months of storage, however, the variation around those means decreased. The water contents of *Protea roupelliae* subsp. *hamiltonii* seeds have a confounding effect on their behaviour at low temperatures, causing increased ambiguity on the quality of a seed batch post storage.

Keywords: *Ex situ* conservation, low temperature storage, seed storage, water content variation.

3.2 Importance of seeds and why they should be stored

Seed production is an important process, not only for plant population dynamics (Pfab & Witkowski, 1999; Venter & Witkowski 2013; Payne *et al.*, 2016), but also as a major food source for various animals, including humans (Bewley, 1997; Evans *et al*., 2011). The seeds are not only important as they are the major plant tissue harvested by humankind and animals; they are also dispersal mechanisms and are utilised as reproductive propagation propagules (Shewry *et al.*, 1995). They constitute the main system for plant propagation and are a convenient and reliable propagule for storage of plant species germplasm; particularly orthodox seeded species (Mycock *et al.*, 1995; Rajjou and Debeaujon, 2008). The amount of seed being consumed (by herbivores and processed for human consumption) and that being produced naturally are not equal. The former outweighs the latter, leaving an ecosystem with less seeds to be propagated into a new generation (Cullen *et al*., 2001).

Some plants do not produce enough seeds if any at all, and this can be attributed to global climate change (Davis, 1989; Graham *et al*., 1990 *loc sit* Primack and Miao, 1992) as well as other causes such as landscape fragmentation leaving small unviable populations (Lamont *et al.*, 1993). The amount of viable seeds produced at any one time, has an impact on the survival of the plant species, its viability and richness making it important to safeguard seed for the future.

Seed storage is an *ex situ* approach to conservation, where the germplasm of the plant is preserved long term outside its original habitat (Bonner, 1990; Hong *et al*., 1996). It is not only for the benefit of human consumption; seed storage is also at the heart of conservation efforts (Lozano *et al*., 2012), since it affords researchers and conservation biologists instantaneous access to possible new sources of nutrition, medicine and genetic material (Schoen and Brown, 2001). Seeds can be a gateway to facilitate scientific study, which will provide an insight into conserving the remaining natural populations of the species, especially in cases of critically endangered species. Significantly, from a conservation viewpoint, the conserved seeds are exempt from habitat destruction, predators and diseases and thus can be used to rehabilitate suitable habitats (Schoen and Brown, 2001).

There are numerous seed banks around the world such as the Royal Botanic Gardens, in Kew, Great Britain and the U.S. National Seed Storage Laboratory in Fort Collins (Vertucci and Roos, 1990). Seed banks store seeds under cold and dry conditions (Schoen and Brown, 2001) and are currently the most useful approach to preserving germplasm of plants, including that

of wild species (Treuren *et al.,* 2018). The quality of a seed batch during storage can remain at the initial level or it can decline to a level that makes the seed unviable after storage (Simic *et al.,* 2007). The goal of these banks is to minimise and hopefully maintain the highest viability and vigour of a wide range of seed species for an indefinite amount of time (Hong *et al.,* 1996; Vertucci and Roos, 1990; Agrawal, 1988; Harrington, 1972) to preserve them for future use (Schoen and Brown, 2001). This is done for varied reasons such as biodiversity conservation, agro-biodiversity and economic purposes (Rajjou and Debeaujon, 2008).

3.2.1 Seed storage conditions

Successful seed storage protocols afford researchers the opportunity to preserve copious amounts of propagules (Lazar *et al.,* 2012). The response of seed to the storage environment (storage behaviour) depends on a few factors such as the quality, seed water content at harvest, as well as the storage temperature and humidity of the storage environment (Withers, 1991). The former probably being the principal factor (Mills, 1996) since viability during storage cannot be gained only maintained (Delouche, 1968). The seed must therefore be under conditions which protect it from heat, moisture and oxygen (de Candolle, 1832 *loc sit* Justice and Bass, 1978; Naik and Chetty, 2017). The moisture content (preferably low) of a seed during storage is more important than maintaining low temperatures because high moisture accelerates seed respiration during storage, resulting in loss of nutrient reserves and making them more susceptible to storage microorganisms (Delouche, 1968; Mondal *et al.,* 1981; Fairey *et al.,* 1999). It is advisable to store seeds at constant temperatures of below 5^{o}C or sub-zero to maintain longevity (Fairey *et al.,* 1999) and avoid deterioration. Deterioration is a natural process resulting in the breakdown of organic matter caused either by physical, chemical or biological processes such as the use of the seed endosperm and ATP (energy) forming biomolecules by other life forms (Mills, 1996), and it can be considerably diminished through careful seed storage management. The optimal storage environment also has no or minimal light stimulation. In many species, light stimulation can trigger seed germination therefore dark or low light levels are recommended to halt pre-germination processes (Fairey *et al.,* 1999).

Agrawal (1988) mentioned the two principal factors that influence seed viability during storage as temperature and relative humidity. Thereafter, Roberts and Ellis (1989) showed that water content and oxygen are two other important factors. Studies (Simic *et al*., 2004 *loc sit* Simic *et al.,* 2007) have shown that factors such as environmental conditions during seed harvesting, granivory, diseases, oil content of seeds, seed moisture content, storage longevity, air temperature and relative air humidity in storage and biochemical injury of seed tissue also play

a key role After harvest, the viability of an orthodox seed should remain constant until the next growing season or longer, depending on the storage conditions (Berjak *et al.*, 1989). Its longevity in storage increases as the temperature and water content decreases (Fairey *et al.*, 1999; Hay *et al*., 2003). Therefore, these two conditions should be monitored and kept in balance to achieve minimal decline in seed vigour and viability.

3.2.2 Low temperatures

Under ambient temperature, seeds age at a faster rate than they would at lower temperatures. It is important to know that seed ageing is accompanied initially by the loss of vigour and ultimately the loss in viability (Roberts and Ellis, 1984), especially under ambient conditions (27^{o}C). Thus, the storage temperature (combined with seed water content) plays a major role in the maintenance of the vigour and viability of the seed after storage (Agrawal, 1988).

3.2.3 Relative humidity

The humidity of the environment before and during storage of a seed plays a significant role in its post storage behaviour. This is due to the hygroscopic nature of seeds; they absorb (absorption) or lose (desorption) moisture from, or to, the environment until an equilibrium is reached between the seed and the environment (Delouche, 1968). In seed banks, orthodox seeds are generally dried to equilibrium at 15% relative humidity at 15^{o}C, and then they are stored in airtight containers at -21^{o}C. Reaching equilibrium can be a time laborious process; it does not occur instantaneously (Delouche, 1968). *Protea roupelliae* subsp. *hamiltonii* sheds seeds with a high-water content range (Czypionka, 2006) (0.0398 – 0.1032 g.g^{-1} d.m.b) accompanied by high coefficient of variations around the seed water content mean (19.58). This can be remedied by controlling the RH of the air used to dry the seed (Smith, 1992) thereby controlling the water content of the seed before storage.

Relative humidity within an environment can be maintained using a saturated salt solution such as [Ca $(NO_3)_2$]. Specific saturated salt solutions are chosen to control the RH of the air to a given value which is dependent on the temperature at which the closed system is at. This salt solution at 27^{o}C keeps a closed environment at 50% RH. When orthodox seeds are placed in an environment with high relative humidity, the seeds undergo absorption and their water contents become comparatively similar. The rule of thumb by Delouche (1968) states that the best storage condition is when the storage temperature (^{o}F) and percentage RH add up to 100.

3.2.4 Seed storage intervals

Generally, seed lots undergo monitoring tests to estimate longevity of seeds in medium and long- term storage. These tests are required for the effective management of seed banks, but frequent monitoring tests can cause a decline in seed lots of critically endangered and extinct species and have a risk of losing seed genotype of wild species (Ellis *et al.,* 2018). Due to the conservation status of *P. roupelliae* subsp. *hamiltonii* 6- and 12-month storage regimes will be investigated and compared with one another for future long-term storage reference of this species.

This study aimed to show that decreasing the water content variation of seeds before storage can have a positive effect on the seed quality after storage. The equilibrated seeds were subjected to four seed storage regimes (-70^{o}C, -20^{o}C, 4^{o}C and 25^{o}C) in their different seed age classes and the quality of the seed lots were assessed after 6 and 12 months. The seed water content, germination percentages and their associated variations after storage were used as a key enlightenment to germination index of the seeds in their different age classes and overall.

3.3 Materials and methods

3.3.1 Seed storage treatments

Equilibrium relative humidity (eRH)

A saturated solution of [Ca $(NO_3)_2$] was prepared, poured into a glass desiccator (7.5l) and left sealed for 3 hours in a growth room at 27^{o}C. This allowed for the environment within the desiccator (7.5l) to reach an equilibrium relative humidity of 50%. The relative humidity was measured using an iButton®. Seeds were treated according to Table 3.1 before storage; this was done to achieve the hypothesised narrow range of seed water contents.

Storage

The trichomes on *P. roupelliae* subsp. *hamiltonii* seeds within a mass range of 0.017 g and 0.029 g were removed and the seeds were placed on a steel plate over a saturated solution of calcium nitrate [Ca $(NO_3)_2$] and sealed in a glass desiccator. Seeds were separated according to their age categories (see Chapter 2, Section 2.3.2) per desiccator. The seeds were removed from the desiccator after 40 days and stored under 4 different temperature regimes of -70^{o}C (freezer), -20^{o}C (freezer), 4^{o}C (fridge) and 25^{o}C (incubator). After 6- and 12-months storage, 10 of each of the 50 seeds per age category and temperature treatment were used to determine the water content of the seeds by individually placing them into weighing boats of known weights, weighed and placed into glass petri dishes half filled with activated silica gel. The

seeds were then placed in an 80°C oven for 48 hours. After 48 hours the seed were removed from the oven and allowed to cool with the glass petri dish lid on, at room temperature for 15 minutes. The seeds were then weighed in the weighing boats, their dry mass subtracted from the wet mass and divided by the dry mass to find the seed water content ($g.g.^{-1}$ d.m.b.). The remaining (40 seeds/treatment combination) seeds were left to equilibrate with the atmosphere (relative humidity) at room temperature. The seeds were then set to germinate under growth room conditions (see earlier - Chapter 2, Section 2.3.5). The seeds were watered every two days and their germination assessed every day after the first sign of germination.

Table 3.1: Preparation of *Protea roupelliae* subsp. *hamiltonii* seeds for storage, with time lines.

Time	**Procedure**
Day 0	Place 1200 seeds (400/age category) in a desiccator filled with a saturated solution of Ca $(NO_3)_2$ (replicate 1); 400 seeds per desiccator.
Day 40	Remove repeat 1 seeds from the desiccator; 50 seeds per sample per age category and place them in their storage treatments of -70°C, -20°C, 4°C and 25°C. Repeat day 0 with another set of seeds (replicate 2).
Day 80	Repeat day 40 with repeat 2 seeds. Repeat day 0 with another set of seeds (replicate 3).
Day 120	Repeat day 40 with replicate 3 seeds.
Month 6	Remove first 50 seeds per age category of replicate 1 seeds from storage.
Month 6 + 40 days	Remove first 50 seeds per age category of replicate 2 seeds from storage.
Month 6 + 80 days	Remove first 50 seeds per age category of replicate 3 seeds from storage.
Month 12	Remove second set of 50 seeds per age category of replicate 1 seeds from storage.
Month 12 + 40 days	Remove second set of 50 seeds per age category of replicate 2 seeds from storage.
Month 12 + 80 days	Remove second set of 50 seeds per age category of replicate 3 seeds from storage.

Data analysis

The data collected (see Table 3.1) were analysed by calculating descriptive statistics such as means, standard deviations and coefficient of variation. Analysis of variance (ANOVA) tests (one-way and two-way ANOVAs) were performed on the water content data (before and after

storage) and further analysed with a Tukey HSD post-hoc test. T-tests were used to analyse water content means before and after equilibration over Ca $(NO_3)_2$. The above data analysis was conducted using R studio® Version 1.1.463 (RStudio, 2018).

3.4 Results

Equilibrating the seeds over Ca $(NO_3)_2$ (RH = 50%) for 40days increased the water content of the seeds but decreased the variation (standard deviation represented by the bars) in the seed samples (Figure 3.1). There was no significant difference (represented as different letters above the bars) between the water content means across the 3 seed ages before the seeds were equilibrated ($F_{2,27}$ = 0.423 P > 0.05), but there was a large standard deviation per mean. After equilibration there was also no significant difference between the means ($F_{2,27}$ = 0.213, P > 0.05,) (Figure 3.1).

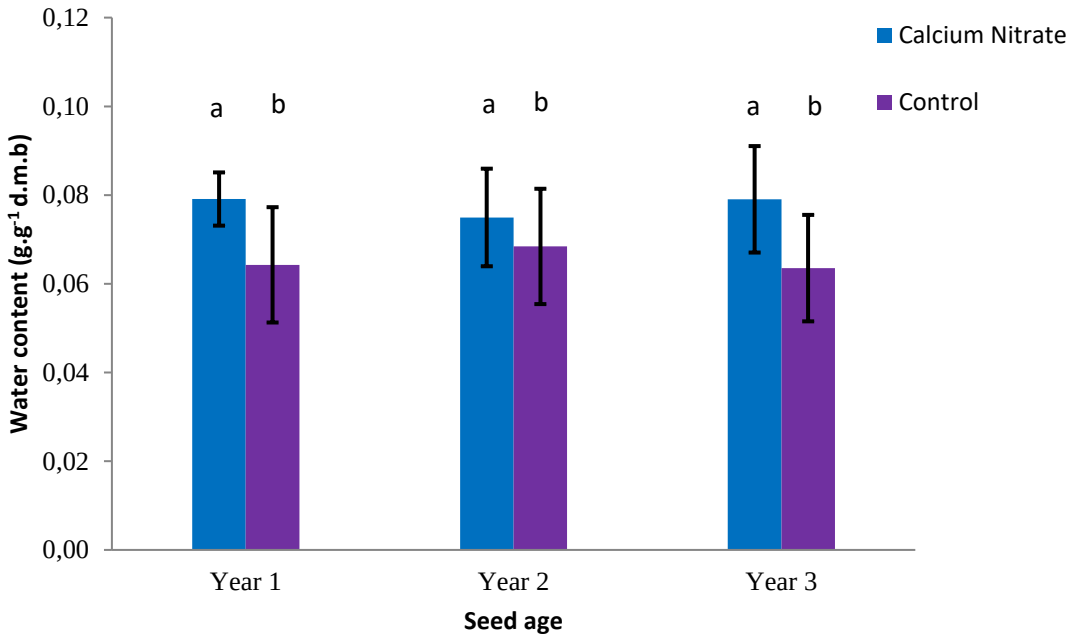

Figure 3.1: Initial water content means (±standard deviation) of *Protea roupelliae* subsp. *hamiltonii* seeds before storage (Control) and after equilibrium over Ca $(NO_3)_2$ salt solution. Different letters above the bars indicate significant differences between seed ages and treatments (Tukey HSD, P < 0.05).

The mean water content of the seeds before equilibration to 50% RH over Calcium Nitrate was significantly lower, but had a higher variability (as shown by the higher standard deviation) than after equilibration (Figure 3.2).

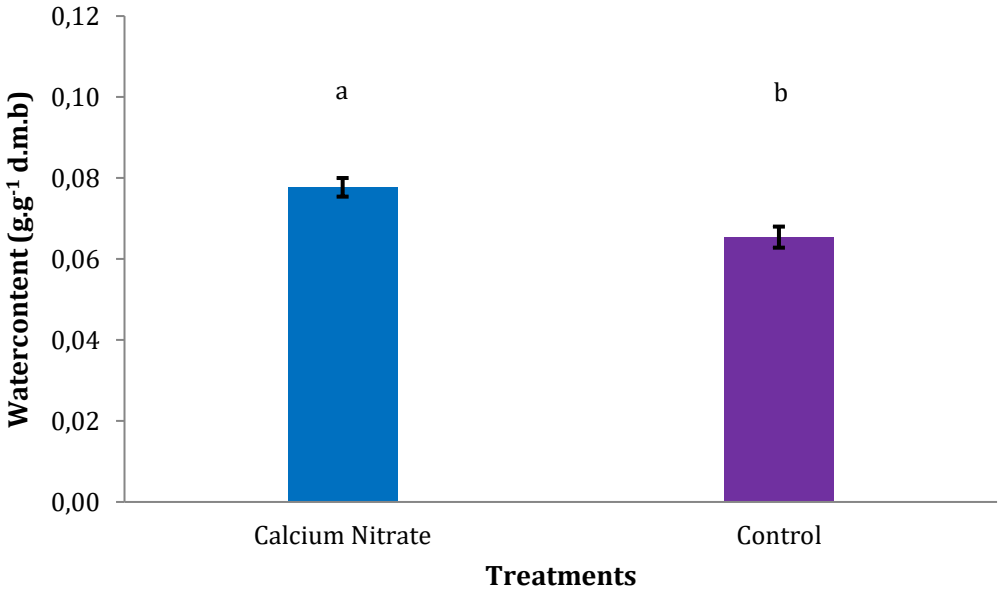

Figure 3.2: Overall (mean ± standard deviation) water content of *Protea roupelliae* subsp. *hamiltonii* seeds before and after 40-day equilibration over a saturated solution of Ca $(NO_3)_2$. (t = 31.421; P < 0.05, df = 59). *The bars represent the standard deviation around the overall means of the seed water contents and the different letters above the bars indicate significant differences between the water content means of the different treatments.*

The coefficients of variation (CV) around the seed water contents were lower after 50% RH equilibrium for all the seeds; one-year seeds had a calculated lower percentage than two and three-year old seeds. The variation in the percentage was similar for the two and three-year-old seed, but higher in the latter seed category (Table 3.2).

Table 3.2: Coefficient of variation (CV) of *Protea roupelliae* subsp. *hamiltonii* seed water content means between seed ages, before and after 40 days equilibration over a saturated solution of Ca $(NO_3)_2$.

	Coefficient of variation (%)	
Seed Age (years)	**Before Equilibration**	**After Equilibration**
1	20.80%	7.96%
2	19.18%	15.51%
3	18.77%	16.15%

After 6 months of storage at -70°C, -20°C, 4°C and 25°C, the mean water content of the seeds was significantly different between some of the storage treatment (Figure 3.3). However, the water content of seeds stored at -20°C and 4°C were not different. Although the different seed years in each storage category were not significantly different to one another all the year 1, year 2 and year 3 seeds across the storage treatments were significantly different to one another, except those at -20°C and 4°C.

After 12 months storage the different seed years in each storage category were not significantly different to one another in terms of their water content (Figure 3.4), but all the year 1, year 2 and year 3 seeds across the storage treatments were significantly different except those at -20°C and 4°C (Figure 3.4).

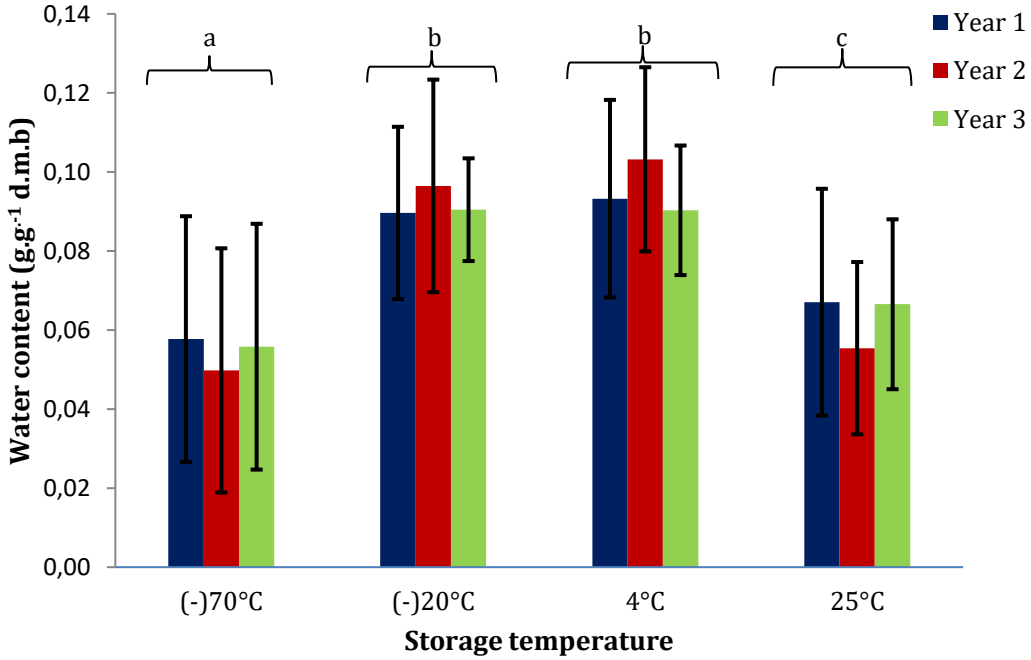

Figure 3.3: Water content of *Protea roupelliae* subsp. *hamiltonii* seeds after 6-months storage under different storage temperatures, *viz.* -70°C, -20°C, 4°C and 25°C. A two-way ANOVA showed a significant difference in seed water contents between the different storage regimes ($F_{3,196} = 82.514$; $P < 0.05$), except between -20°C and 4°C. However, there were no significant difference in the seed water contents between the seed ages within the same storage regimes ($F_{2,147} = 1.964$, $P > 0.05$) Nonetheless, there was a significant interaction in seed water contents between the temperature regimes and the seed ages ($F_{6,588} = 3.461$; $P < 0.05$).

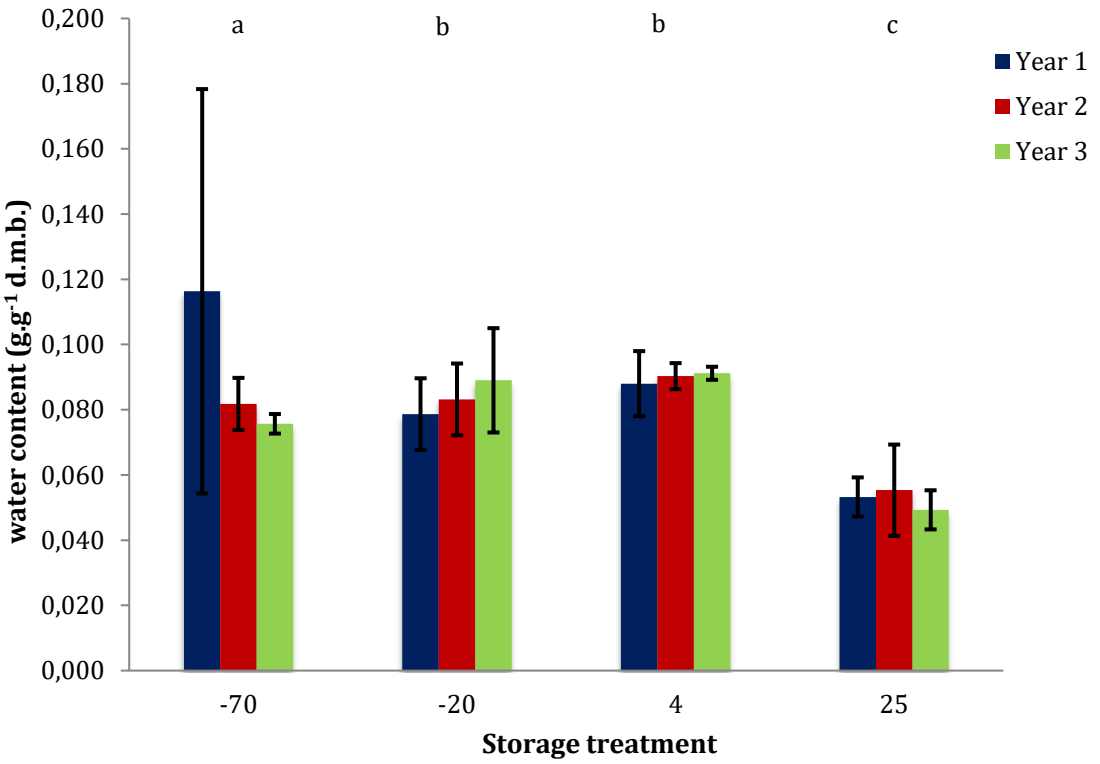

Figure 3.4: Water content (mean ± standard deviation) of *Protea roupelliae* subsp. *hamiltonii* seeds after 12-months of storage under different storage temperatures, *viz.* -70°C, -20°C, 4°C and 25°C. A two-way ANOVA showed a significant difference in seed water contents between the different storage regimes ($F_{3,196} = 82.502$; $P < 0.05$) besides between -20°C and 4°C. There was no significant difference in the seed water contents between the seed ages within the same storage regime ($F_{2,147} = 1.964$, $P > 0.05$) and there was a significant interaction in seed water contents between the treatments and the seed ages ($F_{6,588} = 3.461$; $P < 0.05$).

The water content of the seeds (Table 3.3) before equilibration (a) had higher variation values which decreased after equilibration (b); year 1 seeds decreased the most (from 20.8% - 7.96%). The water content CV after 6 months of storage increased under all 4 storage regimes, with the highest increase at 25°C (Table 3.3) from 13.21% to 27.25%. The water content CV after storage was overall lowest after 12 months at -70°C (Table 3.3).

After storage the seeds were germinated. Seeds stored for 6 months had an overall higher germination percentage than the control seeds and those stored for 12 months under the different storage regimes. The year 1 seeds showed the highest germination percentage after storage at -70°C for 6 months (Table 3.4). After 12 months of storage, seeds stored at -70°C, -20°C and 4°C retain viability close to the control, while at 25°C the viability dropped (Table 3.4).

The variation associated with the germination means of the seeds after 6 months of storage was highest for year 2 seeds stored at -70°C and lowest for year 1 seeds under the same temperature (Table 3.5). The least variation after 12 months of storage was observed at -70°C for year 1 seeds and the highest variation was associated with the year 3 germination after storage at 25°C (Table 3.5).

Table 3.3: Coefficient of variation (CV, %) of *Protea roupelliae* subsp. *hamiltonii* seed water content before (Control) and after storage treatments (-70°C, -20°C, 4°C and 25°C). *a-water content before equilibration; b-water content after equilibration.

	***Control (%)**		**-70°C (%)**		**-20°C (%)**		**4°C (%)**		**25°C (%)**	
Seed Age (year)	**a**	**b**	**6 months**	**12 months**	**6 months**	**12 months**	**6 months**	**12 months**	**6 months**	**12 months**
1	20.8	7.96	17.60	10.16	17.87	15.30	15.23	17.90	22.03	22.83
2	19.18	15.51	21.47	18.10	24.47	19.10	19.37	19.10	31.80	17.20
3	18.77	16.15	17.20	17.20	11.23	23.20	16.77	16.37	28.63	23.10
Mean CV	19.58	13.21	18.75	15.15	17.86	19.20	17.12	17.79	27.25	21.04

Table 3.4: Germination percentage of *Protea roupelliae* subsp. *hamiltonii* seeds per age category, before (Control), and after 6 months storage and after 12 months storage under four temperature treatments, *viz.* -70°C, -20°C, 4°C and 25°C.

	Control (%)	**-70°C (%)**		**-20°C (%)**		**4°C (%)**		**25°C (%)**	
Seed Age (year)		**6 months**	**12 months**	**6 months**	**12 months**	**6 months**	**12 months**	**6 months**	**12 months**
1	86.0	74.2	46.3	67.5	66.3	55.0	66.3	68.1	48.8
2	56.0	74.9	70.0	70.8	55.0	69.2	48.8	73.3	55.0
3	56.0	70.0	66.3	65.8	80.0	75.3	66.3	65.8	70.0
Overall	66.0	73.0	60.9	68.1	67.1	66.5	60.4	69.1	57.9

Table 3.5: Total germination coefficient of variation (CV, %) of *Protea roupelliae* subsp. *hamiltonii* seeds before (control) and after storage treatments (-70°C, -20°C, 4°C and 25°C)

	Control (%)	***-70°C (%)**		***-20°C (%)**		***4°C (%)**		***25°C (%)**	
Seed Age		**6 months**	**12 months**	**6 months**	**12 months**	**6 months**	**12 months**	**6 months**	**12 months**
1	13.3	7.7	11.7	13.6	12.4	28.3	17.9	15.3	22.8
2	37.0	32.5	18.2	23.6	20.1	18.2	19.1	29.5	17.2
3	33.2	14.2	14.2	24.0	20.4	62.1	16.4	39.3	23.1
Mean CV	27.8	18.1	14.7	20.4	17.6	36.2	17.8	28.0	21.0

3.5 Discussion

Seeds of *P. roupelliae* subspecies *hamiltonii* are shed with a variation in water content per seed lot (Figure 3.1). Such variation is responsible for the observed ambiguity in the germination trials before and after storage or during monitoring tests in long term storage (Tarlton, 2013). Kearns and Toole (1939) attribute viability differences among seeds of the same age to heterogeneity of individual seeds within a seed lot. This interpretation implies that significant differences in quality levels exist among individual seeds within a lot. Other differences, such as the variation in water content, are not necessarily due to any innate deficiencies; this variation is attributed to the hygroscopic nature of the seed which is influenced by the relative humidity and temperature of the air (Silva and Rodovalho, 2016).

The water content variation in this serotinous species was reduced, especially in year 1 seeds followed by the year 2 and year 3 seeds with less than 20% of the variability lost (19.13 % for year 2 seeds and 13.95% for year 3 seeds). The decrease in water content variability resulted in an increase in the overall water content of the seeds (from 6.5% to 7.7%) (Figure 3.2), which occurred due to the hygroscopic nature of the seeds. The conditions from the International Plant Genetic Resources Institute (IPGRI) (1994) for orthodox seed storage require a seed water content of 3% - 7% (van Hintumm and Menting, 2003). Therefore, the water contents of *P. roupelliae* subsp. *hamiltonii* after equilibration fell within the regulatory range (Figure 3.2).

The water content variation of *P. roupelliae* subsp. *hamiltonii* seeds prior to storage was identified by Tarlton (2013) as an aspect affecting post storage water content variation and possibly viability more than the storage environment itself. Therefore, the pre-requisite to maintain a low seed water content variability pre and during storage was heeded as an attempt to retain the vigour and viability of the seeds in storage. After 6 months of storage, *P. roupelliae* subsp. *hamiltonii* seeds exhibited increased water content standard deviations (Figure 3.3) corroborated by the increased CV values (Table 3.3) and the germination variability (CV) (Table 3.5) was high at 4^{o}C and 25^{o}C. It is important to note that storage conditions also influence seed viability, and that this depends entirely upon individual species (Bourgeois *et al.,* 2019). However, 25°C and probably 4^{o}C are considered high for seed storage since seed viability decreases as storage temperature increases (Chala and Bekana, 2017); every 5°C increase in storage reduces the seeds initial viability by 50% (Harrington and Douglas, 1970 *loc sit* Chala and Bekana, 2017). Six months storage is a very short period and therefore it is possible that the seeds were still equilibrating to the storage environment because reaching RH

equilibrium is a slow process which is affected by the temperature of the environment (Delouche, 1968) hence the water content variability. It is however possible that the storage vessels were not air-tight, and the seed had gained water from the environment. The increased germination variability at 25°C and 4°C can be attributed to the ageing of the seeds in storage which is associated with increased storage temperatures.

Tarlton (2013) found that seeds stored at -70°C for 12 months displayed rapid early growth rates *ex situ.* This study established that seeds stored at this temperature had the lowest germination variation with more than 50% final germination. All seeds maintained their viability after 12 months storage with the exception of those stored at 25°C showing evidence of decreased vigour (Table 3.4). The maintained viability came with increased germination variations. Therefore, it is reasonable to accept that storage at -70°C is possibly the most beneficial regime for long term storage of *P. roupelliae* subsp. *hamiltonii* seeds.

The year 1 seeds after -70°C storage had increased water content levels (Figure 3.4), this could be due to experimental and technical negligence (as above). This result however, did not impact the year 2 and year 3 seeds and the non-significant difference between the means of the different ages (Figure 3.4).

The variability in seed water contents after storage can be seen to influence the germination results obtained (Table 3.4 and Table 3.5). However, equilibrating them to a set RH indicated that the variation in the water content of the *P. roupelliae* subsp. *hamiltonii* seeds harvested from the wild can be reduced. Therefore, it is evident that together with the correct storage environment, maintenance of low seed water content in storage can result is a successful protocol for the long-term storage of *P. roupelliae* subsp. *hamiltonii* seeds. Edging conservation, a step closer towards successfully preserving the germplasm of the *P. roupelliae* subsp. *hamiltonii* species.

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4 Efforts towards micropropagating *Protea roupelliae* subsp. *hamiltonii* exploiting somatic embryogenesis.

4.1 Abstract

Tissue culture research on *Protea* species has been of limited value, but if effective would provide an alternative means of increasing populations of declining or critically endangered species such as *Protea roupelliae* subsp. *hamiltonii*. Seeds of *P. roupelliae* subsp. *hamiltonii* were germinated under growth room conditions at 25ºC and 14 h photoperiod at ~ 320-µmol m^{-2} s^{-1} photosynthetic photon flux density and subsequently moved to a greenhouse to establish the seedlings. The established seedlings were treated fortnightly with a systemic fungicide (1 $ml.l^{-1}$previcure®) and a foliar fungicide (2 g. l^{-1} Dithane®) interchangeably to reduce systemic and surface contamination on the seedlings.

Shoots with 4 young leaves were excised and decontaminated using 2% sodium hypochlorite and 0.1% Tween-20® (decontamination method 1). Leaf axillary meristem and shoot axillary meristem were excised, plated on to two different MS (Murashige and Skoog) media (pH 4.2), *viz.* half strength and quarter strength media, and incubated under growth room conditions. Explants had 100% contamination after 2 weeks and were discarded.

Shoots with mentioned characteristics were excised and decontaminated using an antioxidant solution consisting of 100 $mg.l^{-1}$ ascorbic acid, 1500 $mg.l^{-1}$ citric acid and $1ml.l^{-1}$ Tween-20® and 70% ethanol (decontamination method 2). The leaf axillary meristem and shoot axillary meristem were excised and further decontaminated with new antioxidant solution, 1% sodium hypochlorite, 70% ethanol and 1% cicatrin® solution before being plated on media (see above). Shoot tip explants had 100% contamination by week 2 and were discarded, while leaf tip explants exhibited blackening which reached 100% at week 3 with no growth observed.

Trichomes of seeds of *P. roupelliae* subsp. *hamiltonii* were removed and the seed coats removed under sterile conditions. The uncoated seed (zygotic embryo) was decontaminated using sodium hypochlorite, 70% ethanol and an antibiotic antimycotic Sigma® solution before being plated onto four different MS media (pH5.6-5.8), *viz.* full strength, half strength, half strength with 0.5 mg. l^{-1} 2.4D and half strength with 0.5 mg. l^{-1} Picloram (auxin) and incubated under growth room conditions. 40% of explants on half strength medium with 0.5 mg. l^{-1} 2.4D had a mass of clear cells after 6-weeks, which showed no further growth. The explants plated

on medium with Picloram showed a mass of clear cells as well as an opaque apical structure growing from the mass of cells.

Systemic contamination of *P. roupelliae* subsp. *hamiltonii* is a critical factor affecting its micropropagation. It needs to be meticulously approached in order to make advancements with the *in vitro* conservation of this species. Exogenously adding auxins (0.5 mg.l^{-1} 2.4D and 0.5 mg.l^{-1} Picloram) to medium for the *in vitro* propagation of *P. roupelliae* subsp. *hamiltonii* embryonic axes produced positive results of callus growth, which need to be further investigated due to the lack of further development after the callus.

Keywords: Auxins, Contamination, Media, Phenolic exudates, Tissue culture.

4.2 Plant propagation

Plant propagation can be achieved through vegetative means (*in vivo*) or through *in vitro* micropropagation techniques. The former is the use of vegetative propagules of a plant to produce cultivars and clones. This is achieved naturally (*in vivo*) from bulbs, rhizomes, tubers and stolons or artificially with methods such as grafting, cuttings, budding and tissue culture (Mudge and Brennan, 1999).

Vegetative propagation is used extensively in agriculture, forestry and horticulture to retain genetically desirable plants and to exploit genetic gains, in addition to its role in plant conservation programmes (Negash, 2003; Ali *et al.,* 2008; Anis *et al.,* 2012). Vegetative propagation is also considered a beneficial regeneration method over sexual propagation, since limited harvesting of vegetative tissues does not result in the death of the plant (Anis *et al.,* 2012). This is advantageous in conserving threatened species which are dwindling due to issues such as environments with limited resources, *viz*. nutrients, light and growing space (Anis *et al.,* 2012). The development of the mother plant is still guaranteed after part of the vegetative tissue has been removed, irrespective of the success or failure of the vegetative technique implemented.

Alternatively, plants can be propagated *in vitro* using tissue culture techniques. The propagation of plants using tissue culture emerged as a promising technique in the early to mid-20th century, and it is now recognised as a well-established practise (George, 1993; Idowu *et al.,* 2009; Anis *et al*., 2012). This technology underwent a number of evolutionary stages, *viz*. scientific curiosity, research tool, novel applications and ultimately mass application (Idowu *et al.,* 2009), and has now developed into a science of immense possibilities in many fields such

as medicine, agriculture and biological research aiding in human health, large-scale plant production (micropropagation) as well as environmental and plant sciences (Anis and Ahmad, 2016). Plant tissue culture aims to manipulate plant tissues, organs or cells *in vitro* and then grow these components back into complete plants. Table 4.1 provides a summary of some of the advantages and disadvantages of conventional vegetative propagation and plant propagation *in vitro*.

Table 4.1: A comparison between plant tissue culture and conventional vegetative propagation.

	Types of propagation	
	Vegetative (Conventional)	Tissue culture (Micropropagation)
Time intense	✓	✓
Space consuming	✓	
The resultant plant success (establishment) is inconsistent	✓	✓
Can produce multiple plantlets/seedlings from 1 parent explant (mass propagation) (Saxena and Dhawan, 1999)	-	✓
Produces clones of the parent plant (Reilly and Washer, 1977)	✓	✓
The clones produced are more vigorous (Davidson, 2019)	-	✓
Can increases genetic variability in species population (Negash, 2003; Ali *et al.*, 2008; Anis *et al.*, 2012), which avoids species genetic depression	-	✓
Preserves a superior genotype per parent plant (Reilly and Washer, 1977)	✓	✓
Environmental conditions can be controlled and kept aseptic (Ahloowalia *et al*., 2004; Kumar and Reddy, 2011; Dagla, 2012)	-	✓
Has more than one pathway (Phillips, 2004)	✓	✓

Evolutionary changes can be introduced, and species can adapt to their environments	✓	✓
Conserves declining species populations (Idowu *et al*., 2009)	✓	✓
Commercially beneficial for species in the export industry (Ahloowalia *et al*., 2004; Anis *et al*., 2012)	✓	✓

4.2.1 Media composition

Tissue culture technology is dependent on the type of medium the explant is grown on. In order to survive, the portions of plant (explant) removed from the mother plant require a continuous source of nutrients (macro-, micro-nutrients and vitamins), carbohydrates, water, appropriate pH and plant growth regulators (amongst others) and these are supplied via a medium that may be solid, semi-solid or aqueous. As each species has a different set of requirements for optimal growth, the derivation of a plant tissue culture medium requires empirical investigation which is time consuming and costly. However, as this technology has evolved, a number of generic media have been formulated and in this regard the medium described by Murashige and Skoog (1962) is widely used particularly for herbaceous plants.

Investigations by Tarlton (2013) demonstrated the initiation of morphogenic callus from intact zygotic embryos of *P. roupelliae* subsp. *hamiltonii* using growth regulator free medium, which contained half strength (HS) Murashige and Skoog (1962) salts with vitamins (MS) (2. 21 $g.l^{-1}$), sucrose (30.00 $g. l^{-1}$) and 3.00 $g. l^{-1}$ Gelzan®. The media used in the present investigations were adapted from Tarlton (2013). *Protea* species thrive in nutrient poor soils, therefore the low nutrient levels in the medium support the natural growth of this plant species. As a consequence, quarter strength (QS) MS medium was also tested.

4.2.1.1 Plant growth regulators

Plant growth regulators play a role on the developmental morphology of the tissues. The different concentrations of the auxins and cytokinins exogenously added to the axenic tissue culture media direct the development of the tissue into a whole plant body (Skoog and Miller, 1957; Su *et al.*, 2011). An increased concentration of auxins results in root proliferation, whereas a higher concentration of cytokinins favours shoot production (Idowu *et al.*, 2009). A

balance between both the PGR's supplied to the explant can induce an unorganised mass of cells (callus).

4.2.2 Explants/organs

The type of explant that can be used as a tissue source depends on their age, size, how they are obtained as well as the overall quality of the parent plant (Murashige, 1974; George, 1993). The manner in which the explant is plated onto the medium is also important for successful induction (George, 1993). There are several commonly used types of explants (shoot tip, embryo, root tip, young leaf tip and inflorescence node) and their derivation plays a part on the effect that the plant growth regulators have on the development and growth of the plant (Murashige, 1974; Su *et al*., 2011). Exogenously applied hormones can support callogenesis, organogenesis or embryogenesis (George, 1993) when applied to a responsive explant in certain concentrations. These factors work together in completing tissue culture propagation whether it be through organogenesis or embryogenesis.

4.2.3 Organogenesis

In vitro organogenesis is the regeneration of organs either directly from the explant or indirectly from callus (George, 1993). Indirect organogenesis starts with inducing a mass of unorganised cells called callus before re-differentiation into plant organs. This is achieved by exogenously incorporating plant growth hormones into the culture medium. Direct organogenesis is the development of new shoots, roots and even flower initials from a large plant piece (explant) that is cultivated *in vitro* (George *et al*., 2008). This process is manipulated to not produce callus tissue by correctly balancing the exogenous plant growth regulators (PGR's). In this regard the organ/tissue from which the explants are derived has an influence on their morphogenic potential (George *et al*., 2008).

4.2.4 Embryogenesis

Somatic embryogenesis is the *in vitro* formation of embryos before the formation of a new plant (Razdan, 2003), it too can be achieved directly or indirectly via a callus stage. As opposed to zygotic embryogenesis which follows the formation of a zygote (fusion of gametes), somatic embryos are formed when somatic (sporophytic) plant cells undergo embryogenic processes and differentiate into embryos (von Arnold *et al.,* 2001; Razdan, 2003; George *et al*., 2008) to redevelop bipolar plant structures (Mendez-Hernandez *et al.,* 2019). Somatic embryo production was first recognised in 1958 as an important pathway to generate plants from carrot cell culture systems (Zimmerman, 1993). Since then, various stages of somatic embryo

development have been documented, and are described for dicotyledonous plants as follows (George, 1993);

- Pro-embryos: small aggregates of meristematic cells from which somatic embryos will arise
- Globular stage: larger and globular aggregates of cells not yet having a definite embryo-like shape
- Heart shape: a characteristic three lobed stage where cotyledonary initials are separated from the root meristem
- Torpedo stage: an elongated form of the heart-shaped embryo where distinct shoot and root meristems are discernible
- Plantlet: discernible small "plantlet" with primary root and shoot

Consequently, somatic embryogenesis has been used extensively to better understand seed developmental biology.

In addition to the medium and suitable explants for the success of tissue culture, there are 5 stages involved in the i*n vitro* propagation of plants (Debergh and Maene, 1981 *loc sit* George, 1993), *viz.*

- stage 0 : mother plant selection and preparation
- stage I : establishing an aseptic culture
- stage II: production of suitable propagules
- stage III: preparation for growth in the natural environment
- stage IV: transfer to the natural environment

These stages are complementary and the omission of one could lead to unsuccessful *in vitro* propagation.

4.3 Tissue culture of *Protea roupelliae* subsp. *hamiltonii*

In vitro propagation of South African *Proteaceae* was first investigated in the early 1980's. The information available from that work is limited to the initiation of callus from the hypocotyls and cotyledonary tissue of *Leucospermum cordijolium* seedlings (Van Staden *et al*., 1981). *Protea* species have proven to be generally difficult (although not impossible) to propagate *in vitro*, either by seed or via vegetative propagules (Malan, 1990; Brown *et al*., 2004), however, it has been successfully applied for many species such as *Stirlingia latifolia*

(R.Br.) Steudel (Blueboy), *Leucospermum* species (George, 1993; Anis *et al*., 2012) and the rare and endangered *Grevillea scapigera* (*Proteaceae*), which produced rooted plants from shoot tip explants (Bunn and Dixon, 1992).

Limited success has been reported for other *Protea* species such as *P. cynaroides* and *P. roupelliae* subsp. *hamiltonii* due to the inability to induce rooting in the former and phenolic oxidation in the latter (Wu *et al*., 2007; Tarlton, 2013), amongst other things. Phenolic oxidation and systemic contamination of many *Protea* species results in much of the *in vitro* material being discarded.

The response of different plants from similar genera or closely related species to already existing protocols can be varied, and this has been emphasised with advancing research in this field as can be seen with *Protea* species. This type of response to the *in vitro* environment is not unique to the *Proteaceae* and there are various other species which have not responded to existing protocols (Idowu *et al.,* 2009). Consequently, careful selection of explant material has been prioritised for the successful establishment of Proteaceous plants in tissue culture. Concomitant advancement of our knowledge and the techniques associated with plant tissue culture has led to a pool of *in vitro* options which need to be optimised for every species according to the chosen explant and predicted outcome (Idowu *et al.,* 2009).

This chapter aimed to establish the use of both direct and indirect micropropagation techniques for *Protea roupelliae* subsp. *hamiltonii* with the following objectives:

To investigate whether seed embryo or vegetative tissue are more effective as explants *in vitro* when using the following regeneration techniques:

i) Indirect somatic embryogenesis

ii) Indirect morphogenesis via secondary embryogenesis

iii) Direct shoot organogenesis

4.4 Materials and methods

Media composition

Media was prepared aseptically according to the below tables and kept at 4°C until used.

Table 4.2: Media for micropropagation using leaf axillary meristem and the shoot axillary meristem of *Protea roupelliae* subsp. *hamiltonii* (*pH 4.2) HS = half strength Murashige and Skoog (1962) medium; QS = quarter strength Murashige and Skoog (1962) medium.

Components	Medium 1	Medium 2
MS (Murashige and Skoog (1962)	HS	QS
Sucrose ($g.l^{-1}$)	30	30
Gelrite ($g.l^{-1}$)	3	3
Picloram ($mg.\ l^{-1}$)	-	-
2.4D ($mg.\ l^{-1}$)	-	-

Table 4.3: The composition of media used to initiate organogenesis from isolated zygotic axes of *Protea roupelliae* subsp. *hamiltonii* (*all media were at pH 5.6-5.8) FS = full strength Murashige and Skoog (1962) medium; HS = half strength Murashige and Skoog (1962) medium.

Components	Medium 1	Medium 2	Medium 3	Medium 4
MS	FS	HS	HS	HS
Sucrose ($g.l^{-1}$)	30	30	30	30
Gelrite ($g.l^{-1}$)	3	3	3	3
Picloram ($mg.\ l^{-1}$)	-	-	-	0.5
2.4D ($mg.\ l^{-1}$)	-	-	0.5	-

The *in vitro* culturing for *P. roupelliae* subsp. *hamiltonii* was as follows:

Stage 0: Mother plant selection and preparation

Seeds (150) were germinated according to section 2.3.5. The 46 established seedlings were sprayed with a systemic fungicide (1 $ml.l^{-1}$previcure®) and a foliar fungicide (2 $g.l^{-1}$ Dithane®) every fortnight for three months to reduce the systemic (Phytophthora) and foliar contamination of the plant material. The fungicides were sprayed on plant foliage until run off.

Stage I: Establishing an aseptic culture (and reduction of phenolics)

-Shoot and leaf tips

Method 1

Shoots of pre-treated *P. roupelliae* subsp. *hamiltonii* maintained in the greenhouse were excised and placed in a beaker containing 2% sodium hypochlorite and 0.1% Tween-20® for 10 minutes (from green house to the laboratory). The explants were rinsed three times with Milli-Q H_2O for 30 seconds each rinse and blotted dry on sterile filter paper. The leaf axillary meristem and the apical meristem were excised on sterile filter paper with precision to have a major vein on each explant. The explants were then plated (in a laminar flow cabinet) separately on medium 1 and medium 2 (Table 4.2) before being incubated under growth room conditions (Section 2.3.5).

Method 2

Shoots of *P. roupelliae* subsp. *hamiltonii* maintained in the greenhouse were excised and placed in a beaker containing an antioxidant solution comprising 100 mg. l^{-1} ascorbic acid, 1500 mg. l^{-1} citric acid and 1 ml. l^{-1} Tween-20® in sterile ultra-pure water (Wu and du Toit, 2004) for approximately 10 minutes (from greenhouse to the laboratory). Under sterile conditions, the antioxidant solution was decanted and replaced with fresh solution and swirled for 10 minutes. This was followed by rinsing the explants in 70% ethanol for 30 seconds and blotting dry on sterile filter paper. The leaf axillary meristem and the apical meristem were excised and then re-placed into fresh antioxidant solution for 10 minutes and blotted dry on sterile filter paper. Afterwards, the explants were soaked in 1% sodium hypochlorite for 10 minutes, rinsed three times with Milli-Q H_2O and soaked in 70% ethanol for 1 minute. Finally, they were transferred into 1% cicatrin® solution for 5 minutes and blotted dry on sterile filter paper. The material was plated on medium 1 and medium 2 (Table 4.2). The material was then incubated under growth room conditions (Section 2.3.5).

-Zygotic embryos

Seeds of *P. roupelliae* subsp. *hamiltonii* were dehusked and soaked in sterile Milli-Q H_2O for 20 minutes. The seed coat was removed under sterile conditions and the seed was placed in 1% sodium hypochlorite for 5 minutes, rinsed three times with Milli-Q H_2O and soaked in 70% ethanol for 1 minute. The explants were then transferred into antibiotic antimycotic Sigma® solution for 5 minutes, blotted dry on sterile filter paper, plated onto the medium in Table 4.3 and incubated under growth room conditions (Section 2.3.5).

4.5 Results

Stage 0: Mother plant selection and preparation

Established seedlings (n = 46) of *Protea roupelliae* subsp. *hamiltonii* were maintained in the green house (Figure 4.1a and b). After 3 months 15% became susceptible to a systemic fungal contamination (Figure 4.1c).


a

b

c

Figure 4.1: Newly established *Protea roupelliae* subsp. *hamiltonii* seedlings (a) a three-month-old seedling maintained under greenhouse conditions (b) and a seedling which succumbed to systemic fungal infection (c).

Stage I: Establishing an aseptic culture (and reduction of phenolics)

Leaf axillary meristem explants were plated on HS and QS medium after surface sterilisation and they turned black with no development observed (Figure 4.2). The explants displayed no phenolic exudation (Figure 4.2).

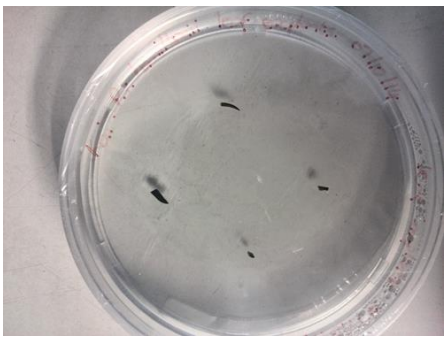
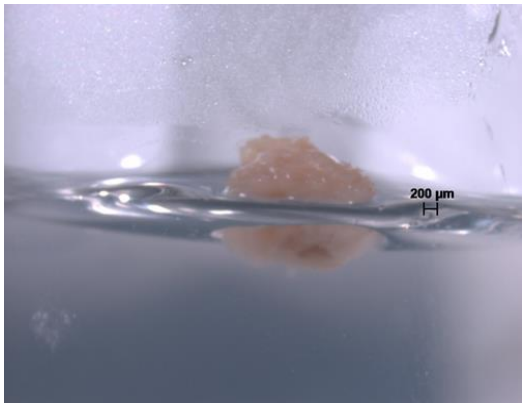


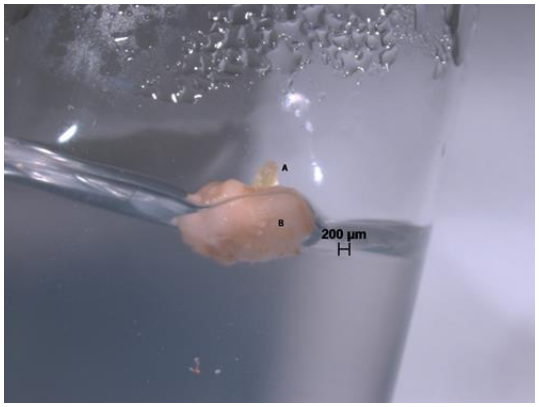
Figure 4.2: Explants of leaf axillary meristems from *Protea roupelliae* subsp. *hamiltonii,* pre-treated, decontaminated and cultured on half strength MS (Murashige and Skoog) medium

Seed zygotic embryos of *Protea roupelliae* subsp. *hamiltonii* were plated on four FS medium and HS medium containing no plant growth regulators, 0.5 mg.l^{-1} Picloram and 0.5 mg.l^{-1} 2.4D (section 4.3). Indirect somatic embryogenesis using seed embryogenic axes on growth medium with 2,4D (Figure 4.3a) developed a clear mass of cells, however, these cultures did develop

any further. Explants cultured in the presence of Picloram showed an apical structure in addition to the mass of clear cells with no further growth (Figure 4.3b).



a.



b.

Figure 4.3: Development of a zygotic embryo of *Protea roupelliae* subsp. *hamiltonii* plated on half strength MS (Murashige and Skoog) medium containing 0.5 $mg.l^{-1}$ Picloram (a) and 0.5 $mg.l^{-1}$ 2.4D (b).

The use of decontamination method 1 (Section 4.3) was not successful in providing sterile material for the micropropagation of tissue culture, and therefore the apical meristem explants were all contaminated after week 1 followed by the leaf axillary meristem after 2 weeks (Table 4.4).

Table 4.4: Contamination and callus formation of the leaf axillary meristem and the apical meristem explants of *Protea roupelliae* subsp. *hamiltonii* cultured on half and quarter strength MS (Murashige and Skoog) medium after decontamination method 1 (Section 4.3)

	Leaf axillary meristem		**Shoot tip**	
	Contamination (%)	Callus formation (%)	Contamination (%)	Callus formation (%)
Week 1	50	-	100	-
Week 2	100	-	discarded	-

The leaf explants had no contamination after decontamination method 2 (section 4.3), but all the samples turned black by the third week and no callus formation was observed. Shoot explants were totally covered by contamination at week 2 as a result there was no callus formation data for those explants (Table 4.5). The contamination in the zygotic embryo explants increased from 20% to 30% through the 6-week period with 40% forming callus (Table 4.5).

Table 4.5: Cumulative contamination of the *Protea roupelliae* subsp. *hamiltonii* leaf and shoot explants using decontamination method 2 (section 4.3) and the callus formation for the leaf, shoot and zygotic embryo explants over a 6-week period

	Leaf			**Shoot tip**			**Zygotic embryo**	
	Contamination (%)	Callus formation (%)	Percentage darkening (black)	Contamination	Callus formation	Percentage darkening (black)	Contamination	Callus formation
Week 1	0	0	0	50	0	-	20	0
Week 2	0	0	50	100	0	-	20	0
Week 3	0	0	100	-	0	-	20	0
Week 4	0	0	100	-	0	-	30	0
Week 5	0	0	100	-	0	-	30	30
Week 6	0	0	100	-	0	-	30	40

4.6 Discussion

The success of micropropagation relies on selecting suitable parent plants and maintaining them in good condition by excluding endogenous and external contaminants (Davidson, 2019) before the explants to be used *in vitro* are excised, amongst other things. The seedlings of *P. roupelliae* subsp. *hamiltonii* were successfully established in a greenhouse (Figure 4.1a, Figure 4.1b) while continuously applying fungicides to eliminate external and endogenous contamination (Figure 4.1c). *Protea roupelliae* subsp. *hamiltonii* seedlings are reported to be predisposed to endogenous microbial contamination as much as the rest of the *Protea* genus (Tarlton, 2013), this was evidenced by the continuous fungicide application which however still resulted in some of the seedlings dying from microbial contamination (Figure 4.1c).

Preceding explant inoculation is surface sterilisation (Mohapatra and Batra, 2017), which should eliminate external contaminants on the explants. Explants of *P. roupelliae* subsp. *hamiltonii* were excised and moved to the laboratory for surface sterilisation. Decontamination

method 1 resulted in 100% contamination of leaf axillary meristems and shoot apical meristems (Table 4.4). Shoot apical meristems are pluripotent stem cells from which all other plant cells are derived. They are tightly packed sets of cells and due to that they are more resistant to infection, allowing them to be more useful as explants in tissue culture techniques (George *et al.,* 2008). However, shoot apical meristems of *P. roupelliae* subsp. *hamiltonii* proved to be more susceptible to contamination than the leaf axillary meristems (Table 4.4).

Due to the high levels of phenolic exudation from the explants and endogenous microbial contamination on *P. roupelliae* subsp. *hamiltonii* seedlings (Tarlton, 2013), more rigorous decontamination procedures were applied together with methods to reduce the production of secondary metabolites, and this resulted in the leaf explants dying (Figure 4.2). Phenolic exudation of explants is visible as browning of tissues due to enzymatic responses to wounding (Chuanjun *et al.,* 2015). This can result in the death of an explant or failure to regenerate *in vitro* (Chuanjun *et al.,* 2015). The consequences of stubborn contamination can be seen in the establishment of other Proteaceae such as *Persoonia gunnii* and *Persoonia muelleri* (Offord *et al.,* 2015). The use of their apical and axillary shoots required rigorous decontamination methods, which led to the explants turning black and dying (Offord *et al.,* 2015), similar to *P. roupelliae* subsp. *hamiltonii* leaf explants.

Proteaceae plant tissue culture attempts using the shoot tip as an explant has been demonstrated to be laborious due to their hairy surface which attracts and traps environmental pathogens (George *et al.,* 2008) as was seen with *P. roupelliae* subsp. *hamiltonii* shoot tips, which still had contamination after more rigorous decontamination methods (Table 4.5). The amount of time it took the shoot tips to be completely covered by contamination was longer after decontamination method two, therefore this method should be further optimised in order to achieve less contamination from this plant organ, while considering the slow growing characteristic of the plant (Coetzee and Littlejohn, 2007).

However, the use of zygotic embryos showed limited success (Figure 4.3). The zygotic embryo showed no development in full strength medium, undoubtedly due to the plant's native habitat being in nutrient deficient soils. This explant had an undefined mass of cells (callus) in half strength (HS) MS medium in the presence of 2.4D as well as an undiscernible apical growth in the presence of the Picloram growth hormone in HS MS medium. The zygotic embryo responded to the exogenous addition of auxins and more so to Picloram than 2.4D. The

developed structures showed no further development even after being sub-cultured into fresh medium.

This set of experiments was restricted by the fact that the species under consideration is endangered and therefore the amount of starting material was limited. Nonetheless, positive research strides on the tissue culture of *P. roupelliae* subsp. *hamiltonii* using leaf and shoot apical meristems were made. The decontamination method used delayed contamination for the shoot meristems *in vitro* therefore it should be possible to completely eradicate contamination and hence the use of zygotic embryos in the micropropagation of *P. roupelliae* subsp. *hamiltonii* is reassuring given the current limited success observed during this study.

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5 Conclusion

Understanding the fundamental biology of one species in detail before implementing conservation policies will further enhance our broader understanding of declining fauna and flora. The main objective of many conservation strategies is to increase plant population size in order to aid its survival and persistence. Thus, investigating seed behaviour (both germination and storage) becomes a priority and this generally requires a large number of seeds. This objective was severely hindered when working with *P. roupelliae* subsp. *hamiltonii* as it is on the brink of extinction.

Previous data showed that no seed was recorded on the field plants between 2000 - 2005 and fortunately, recruitment (albeit slow) was evident with the increase in seed set from that time until 2013, when the field work for this study was undertaken. More three-year-old seeds were harvested from the *P. roupelliae* subsp. *hamiltonii* plants in comparison with the two-year-old seeds. The serotinous nature of the species allows for more robust individuals to survive on the parent plant (3 years) and successfully establish (Table 2.3). This is at the expense of the loss of seed vigour (year 1 seeds are more vigorous than year 3 seeds) (Figure 2.4) and this may well account for the slow recruitment of the species (D. Mycock *pers. comm.*). The one-year-old seeds were also higher in number than the two-year-old seeds; probably a reflection of the broader environmental conditions in the field when the seed was set (D. Mycock *pers. comm.*).

The association between seed mass and seed viability was shown by the much higher percentage germination (67%) of seeds with the optimal mass of 0.017 - 0.029 g, compared with only 6% germination for seeds with a mass < 0.017g and 0% for seeds which weighed > 0.029g. The viability of the seeds was more for the one-year seeds, with no significant difference between two- and three-year seeds. However, the vigour of the three-year seeds was higher indicating their completed maturation and therefore these are better able to sustain a seedling during establishment than the less mature seeds. This improves the recruitment opportunities for the plant, ultimately positively affecting the number of seeds produced. This greater reproductive potential would allow for the re-establishment of a viable and stable population in the wild and if successful also provide more resources for the science of seed conservation in general, but particularly for the *Proteaceae*.

Other avenues of research such as micropropagation were investigated with the attempt at contributing to potentially increasing the population of *P. roupelliae* subsp. *hamiltonii*. However, the *in vitro* propagation using leaf and apical shoot meristems was unsuccessful due to the high contamination levels. This investigation led to a limited breakthrough regarding the decontamination of shoot meristematic tissues of *P. roupelliae* subsp. *hamiltonii*. The use of zygotic embryos in the presence of exogenously added auxins was successful to a certain degree (callus formation). The success of *in vitro* propagation is a potential contributing factor to the recovery of this subspecies population, because the laboratory propagated plants could be reintroduced into the field and therefore aid in population increase. While the micropropagation of this subspecies is still being investigated, it is wise to also look at the storage of the seeds.

In line with the objectives of the storage experiment, the equilibration of the *P. roupelliae.* subsp. *hamiltonii* seed to the set RH of 50% greatly condensed the variation around the seed water content mean. Although this resulted in an elevated water content average above that of the seed from the wild, it was within the range recommended by genebank standard operating procedures for long-term storage. Despite this pre-treatment, the collected data however, showed a range of responses to the storage regimes. This is probably a reflection of the inherent germination variability (see Chapter 2) of a wild species and was also impacted by the limited numbers (replications) necessitated by the fact that the species is endangered.

Nevertheless, and most importantly, the combined results demonstrate that the seed of *P. roupelliae* subsp. *hamiltonii* can be stored successfully at sub-zero temperatures. This is a

significant and meaningful step toward the *ex situ* conservation of the species.The potential for plant population recovery is thus improving even more, and it is apparent that less variable post low temperature storage seed behaviour is achievable since the one-year seeds accomplished it after 12 months of incubation.

Further research on the water content variability and investigations into more types of media to be used for the micropropagation of *P. roupelliae* subsp. *hamiltonii* will drive research to understanding this subspecies further. The understanding of the water content variability will assist with the successful development of a protocol to store the seeds. The use of *in vitro* established plantlets, probably from zygotic embryo derived plants, could result in less systemic contamination and should also be investigated.

Conservation of rare species is not a crisis discipline and is therefore being approached in a stepwise manner, beginning with understanding the basics such as the water content variability during storage and *in vitro* propagation decontamination of explants to increase our knowledge, which will have an impact on the combined *(ex situ* and *in situ)* conservation goals of plant species such as *Protea roupelliae* subsp. *hamiltonii*, and the *Proteaceae* in general thereafter.