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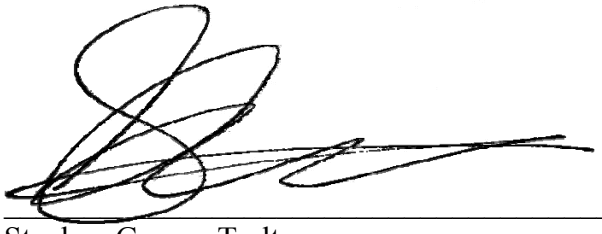
Conservation and propagation of the critically endangered *Protea roupelliae* ssp. *hamiltonii.*

Stephen Tarlton
2011

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



22nd day of July 2011

Stephen George Tarlton

Abstract

The critically endangered *Protea roupelliae* ssp. *hamiltonii* persists as a single population (of 124 individuals in 2005) within the 26ha Dr Hamilton Reserve in Mpumalanga province, South Africa. Between 2000 and 2005, no recruitment had been observed. In order to aid the recovery of this species, aspects of its reproduction and conservation were studied, namely; (a) the relationship between the demographics and the reproductive capacity of the population, (b) the relationship between achene mass and germination, (c) methods of propagating the species *ex-situ*, (d) the *ex-situ* seed storage of the species and (e) methods of establishing seedlings *in-situ* (the reserve) in order to augment the small population. The reason for the decline of the species has been attributed to the reported high levels of herbivory before an antelope proof fence was erected. The survival and reproductive capacity of the species is sensitive to herbivory as browsing decreases canopy size and both achene production and germination were found to be significantly related to canopy size. The number of cones produced per plant (R^2= 0.532; P<0.005), number of achenes filled with embryos (filled achenes) (R^2=0.178; P=0.014), the mass of the achenes produced within a cone (R^2=0.127; P=0.041) and the germination percentage of filled achenes (R^2=0.200; P=0.009) increased with the plant canopy area. While the number of days for 50% of the achenes to germinate (T_{50}) (R^2=0.231; P=0.005), peak day of germination (R^2=0.208; P=0.008) and mean days until germination (R^2=0.270; P=0.002) decreased with plant canopy area. However, the percentage of filled achenes (seed set) did not increase with plant canopy area (R^2=0.044; P=0.241). Each plant had a degree of autonomy in the mass of the achenes produced which was significantly different between plants (P<0.05). The mass of individual achenes from various plants was not related to the germination (rate or viability) of that achene. However, the germination percentage of filled achenes (R^2=0.133; P=0.037), the peak value of germination (Czabator 1962) (P.V.) (R^2=0.403; P<0.001), the T_{50} (R^2=0.209; P=0.0074) and the mean number of days for achenes to germinate (R^2=0.178; P=0.014) were all significantly related to the mean mass of the achenes produced within cones harvested from different plants. The achenes not filled with an embryo had a higher variation in mass and were significantly lighter than filled achenes (P<0.05). Mean seed set (n=33 cones) was 36.08±2.31% (±S.E) filled achenes per cone. By sorting achenes using a simple achene mass based selection method, a sample containing 93.67±1.46% filled achenes was selected per plant. Overall, the filled achenes had high germination percentages ranging from 57.67% to 97.00%, however, germination was sensitive to various pre-treatments. Soaking achenes in water for 48 hours before germination decreased the P.V. from 3.69 to 1.22 and germination percentage from 94.06% to 82.11%

compared with un-soaked achenes. However, when embryos were excised from the seed coat after 48 hours soaking, both the P.V. and the germination percentage increased further to 6.03 and 97.00% respectively. Excised embryos did not germinate normally *in-vitro* when plated on various media but dedifferentiated into callus on all media tested (including growth regulator free media). The most effective regeneration via adventitious somatic embryogenesis (but not statistically significant) occurred on a growth regulator free medium (containing 2.21g.l^{-1} Murashige & Skoog (1962) salts with vitamins, 30.00g.l^{-1} sucrose and 3.00g.l^{-1} Gelrite® with a pH of 4.20) producing a mean of 4.66±1.09 (±S.E.) embryos per explant. Secondary somatic embryogenesis also occurred on a growth regulator free medium (containing 2.21g.l^{-1} Murashige & Skoog (1962) salts with vitamins, 30.00g.l^{-1} sucrose and 3.00g.l^{-1} Gelrite® with a pH of 5.20). The somatic embryos developed into plantlets (generally un-rooted) on media containing a high gibberellic acid to cytokinin ratio (3:1 by mass) and a growth regulator free medium (containing 2.21g.l^{-1} Murashige & Skoog (1962) salts with vitamins, 30.00g.l^{-1} sucrose and 3.00g.l^{-1} Gelrite® with a pH of 5.20). Limited success (5.77%) was achieved in rooting shoots (from somatic embryos) and transferring them to a greenhouse environment. Other vegetative propagation techniques were attempted, including the rooting of stem cuttings and direct shoot organogenesis but these were unsuccessful. As the achenes of *Protea* species are not long lived *in-situ*, the *ex-situ* conservation of achenes (and therefore the species) was tested. Achenes had low water contents (9.27±0.10%) and were therefore categorised as orthodox seeds (Roberts 1973). Germination percentages were high in all tested storage regimes (Ambient, 25°C, 4°C, -70°C and -196°C), including those at ultra-low storage temperatures and after 18 months ranged between 86.81% and 92.23%. Evidence of loss of germination vigour was found in achenes from the Ambient and 25°C storage regimes. After 12 months of storage, the quality of stored achenes under all storage regimes was thoroughly tested. Those achenes germinated successfully and produced seedlings that grew well under greenhouse conditions. Those seedlings were of high enough quality to be transplanted back into the Dr Hamilton Reserve, where growth and survival continued successfully. This showed convincingly that seed storage is an effective tool in the future *ex-situ* conservation of *Protea roupelliae* ssp. *hamiltonii.* Population augmentation was effective when planting achenes directly *in-situ* or transplanting *ex-situ* propagated seedlings *in-situ* (transplants), but varied between the two methods. Planted achenes had a low seedling emergence (less than an estimated 10% of planted, viable achenes emerged) and survival after one year was 76.41% and 69.81% after two years. However, 96.44% of the transplants survived after the first year and 95.37% after the second year. Additionally the leaf production rate of transplanted seedlings was superior to seedlings that originated from planted achenes. Through this project 1707 *Protea roupelliae* ssp. *hamiltonii* transplants have been planted into the Dr

Hamilton Reserve. Although these individuals have not yet reached a reproductive size, the population numbers *in-situ* have increased considerably. Intensive intervention has worked in aiding the recovery of the *Protea roupelliae* ssp. *hamiltonii* population *in-situ,* however new threats to the population have been identified and still need to be mitigated.

Key words: critically endangered species, propagation, reproductive output, population augmentation, seed storage.

We are as gods
and have to get good at it

Stewart Brand, *Whole Earth Discipline, 2009*

In memory of my Father,
Roger Tarlton 1945-2003



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Abbreviations and nomenclature

°C	Degrees Celsius
%	Percent
®	Registered
ABA	Abscisic acid
BAP	Benzylaminopurine.
C.V.	Coefficient of variation.
Cone	Inflorescence/infructescence of *Protea* species
µmol m-2.s-1	Micro Einsteins per square meter per second
Filled achene	An achene containing an embryo
$g.l^{-1}$	Grams per litre
GA^3	Gibberellic acid.
IBA	Indole-3-butyric acid
$mg.l^{-1}$	Milligrams per liter
$ml.l^{-1}$	Milliliters per liter
MS	Murashige & Skoog (1962) salts with vitamins
pH	Hydrogen ion concentration
P.V.	Peak value of germination (Czabator 1962).
ROS	Reactive oxygen species
S.D.	Standard deviation.
S.E.	Standard error
T_{50}	Time taken for 50% of a seed lot to germinate.

Chapter 1

1. Introduction

The aim of conservation is to prevent the extinction of species, their genes and the processes they perform. The risk of extinction is greater in small populations than in large populations (Pimm *et al.* 1988). The impact of population size is so high that small populations, even in a perfectly constant environment, face the risk of extinction from demographic anomalies alone (Pimm *et al.* 1988). In such situations reproduction is paramount, the only consequence for a species with persistent reproductive failure, for any reason, is extinction (Bond 1994). Therefore issues surrounding the reproduction of critically endangered species consisting of single small populations, such as *Protea roupelliae* ssp. *hamiltonii* have a crucial role in reducing risk of their extinction.

1.1. *Protea roupelliae* ssp. *hamiltonii*

Southern Africa holds about 360 species of Proteaceae in 14 genera (Rebelo 1995). Only four of the 14 genera are found outside the winter rainfall area of the Cape Floral Kingdom (Rebelo 1995). The species of the genus *Protea* occur in two main regions in Africa namely the winter rainfall Cape Fynbos region (about 70 species) and the tropical Central African Brachystegia belt (about 54 species) (Beard 1993). Many *Protea* species in southern Africa (29 out of 86 *Protea* species) are threatened and have an IUCN rating of "vulnerable", "endangered" or "critically endangered" (SANBI website: http://posa.sanbi.org 2010). The *Protea* species that occur in the summer rainfall region of South Africa are essentially in a transitional region, containing fewer species (of which 12 species are endemic) and are therefore of special interest (Beard 1993).

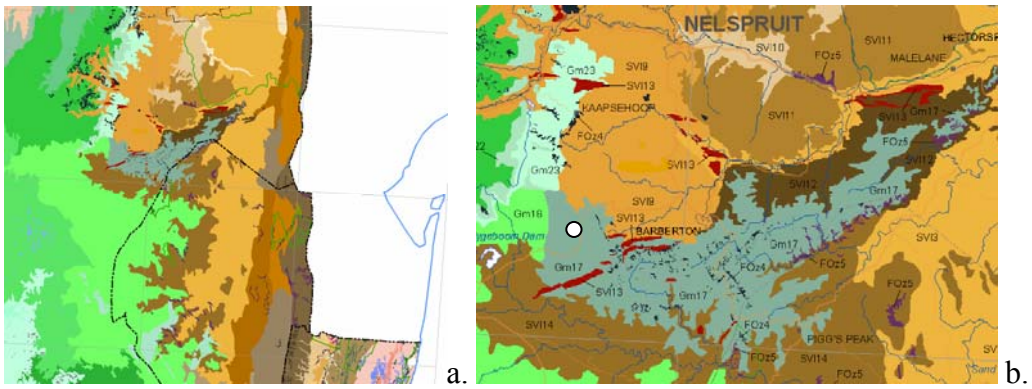
P. roupelliae ssp. *hamiltonii* was discovered in 1957 by Dr P. D. Hamilton (Rourke 1980). In contrast to the ubiquity of the typical subspecies (*P. roupelliae* ssp. *roupelliae*) which is one of the most widespread in the genus, *P. roupelliae* ssp. *hamiltonii* is critically endangered. The Protea Atlas provided a detailed IUCN rating of "critically endangered": A2ac, B1a(ii)b(i,ii,iii,iv,v)+2a(ii)b(i,ii,iii,v), C2a(i,ii) (Rebelo 2006), indicating that:

- Through direct observation, the population numbers as well as area of occupancy/extent of occurrence has decreased by 80% within three generations (A2ac).
- It is a single declining population that occurs in an area less than 100km^2 and occupies an area less than 10km^2 (B1ab+B2ab).

- There are less than 250 individuals and less than 50 of those are mature (reproductive). All individuals of the species exist in a single population (C2ai,ii).

It is thought to be a naturally rare species, originally consisting of several scattered colonies (Rourke 1980). The only remaining population is conserved within a small 26ha reserve in Mpumalanga, South Africa. *P. roupelliae* ssp. *hamiltonii* is one of the 12 species endemic to the Barberton Montane Grassland (GM17) vegetation unit (Figure 1.1), which forms part of the Barberton Centre of Plant Endemism (Mucina & Rutherford 2006; Steenkamp *et al.* 2005).

The extent of the Barberton Montane Grassland (GM17) vegetation unit stretches from high lying grassland around Kaapmuiden and Malelane in the northeast to Piggs Peak in Swaziland to the south (Figure 1.1) (Mucina & Rutherford 2006). In the west, this vegetation unit occurs along the high mountains above Barberton towards and including Nelshoogte and Dr Hamilton Nature Reserve (Mucina & Rutherford 2006). The precipitation primarily occurs between November and March and varies from 950mm in the west to 1470 mm in the east (Mucina & Rutherford 2006). Frosts are infrequent and occur for about ten days of the year (Mucina and Rutherford, 2006). Hot dry winds blow from August to October (Mucina & Rutherford 2006). The conservation status of this vegetation unit is classified as vulnerable as about 40 percent has been transformed by forestry plantations, however 26 percent of the Barberton Montane Grassland (GM17) is conserved within nature reserves (Mucina & Rutherford 2006).



a. b.

Figure 1.1. Maps showing the extent of the Barberton Montane Grassland (GM17) depicted as grey, (a) its position within South Africa and in relation to other vegetation units, (b) the position of the Dr Hamilton Reserve within Barberton Montane Grassland vegetation unit depicted as a white circle (from Mucina and Rutherford, 2006).

1.2. Propagation of Proteaceae

Proteaceae are considered to be inherently difficult to propagate by seed and by vegetative means (Malan 1992). Historically it has been repeatedly published that South African Proteaceae achene

germination was “unsatisfactory” (Van Staden 1966; Vogts 1960; Thorns 1943). The successful propagation via the rooting of cuttings and the use of tissue culture techniques in *Protea* species has also been met with extensive horticultural challenges (Malan 1995; Malan 1992). While described techniques are now available for the propagation of horticulturally important species but the propagation techniques available for Red Data Proteaceae species are limited (Laubscher *et al.* 2009). Consequently there is a need for the development of propagatory approaches for endangered Proteaceae so that they may be adequately conserved (Laubscher *et al.* 2009). Furthermore, *ex-situ* seed storage can only be effective if a reliable method of seed germination or plant regeneration/production from the stored tissues (such as seeds) is possible.

1.2.1. Fruits of *Protea* species

The diaspores of *Protea* species are single seeded indehiscent fruits known as achenes (Wilson *et al.* 2006; Rebelo 1995). The achenes are supported in broad, flattened infructescences surrounded by bracts (known as cones), where they are held within the canopy of the plant (canopy seed storage; Lamont *et al.* 1991) until dispersal, which is often cued by fire (Coates-Palgrave 1991). *Protea* species (and most of the Proteaceae) may produce many achenes per cone however, this high achene production may be misleading and by no means translates into a high seed set, which is generally low in Proteaceae (Collins & Rebelo 1987). Many of the achenes produced are not filled with an embryo and this can make breeding programs for Proteaceae difficult (Collins & Rebelo 1987; Rebelo & Rourke 1986). In addition, with no apparent change in appearance some cones may be sterile, producing no filled achenes at all (Hargreaves *et al.* 2004; Collins & Rebelo 1987).

1.2.2. Dormancy mechanisms in Proteaceae

The dormancy mechanisms in Proteaceae have not been fully established. The mechanisms in *Protea* achenes are thought to ensure that the germination of the seed crop is spread over an extended period, so that the entire seed crop does not germinate at any one time (Deall & Brown 1981). Germination inhibitors cannot fully explain the dormancy within the Proteaceae and the lack of germination in *P. compacta* has been attributed to the lack of endogenous germination promoting compounds such as cytokinins and gibberellins within the achenes (Brown & Van Staden 1975). It was concluded that the synthesis of these promoting compounds are however, oxygen dependant (Brown & Van Staden 1975). An increase in atmospheric oxygen concentration during incubation is met with a considerable increase in the germination percentage of *P. compacta, P. magnifica, Leucadendron daphnoides* and *Leucadendron tinctum* (Brown & Dix

1985; Deall & Brown 1981; Brown & Van Staden 1973a; Brown & Van Staden 1973b). The restriction of oxygen to the embryo may be due to a chemical barrier, a physical barrier or a combination of the two.

Chemical dormancy

The chemical dormancy mechanism within *Protea* species may be based on the reported presence of a coumarin like compound (similar Rf value and same UV absorbance peak to a coumarin standard) found in the seed coat and embryos of the Proteaceae: *P. compacta, P. barbigera, Leucospermum cordifolium* and *Leucadendron daphnoides* (Van Staden & Brown 1972). This coumarin like compound was the only identifiable germination inhibitor but it was not the only compound present that significantly inhibited the germination of lettuce bioassays (Van Staden & Brown 1972). In addition, the presence of the plant growth regulator abscisic acid (ABA) was excluded from both the seed coat and embryo tissue of the tested Proteaceae species (Van Staden & Brown 1972).

Phenolics and coumarin like compounds

The inhibitory action of coumarins has been found to be greater with the presence of other phenolic compounds (Li *et al.* 1993), which may be the additional unidentified inhibitory compounds found within the seed coats and embryos of Proteaceae (Van Staden & Brown 1972). The hypothesis that dormancy is imposed by phenolic compounds in *Protea* species is supported by the study of flower colour polymorphism within a species. White morphs which produced smaller quantities of pigment and related compounds throughout the plant (such as phenolics) had a significantly higher germination percentage than their pink pigmented counterparts (Carlson & Holsinger 2010). The germination rate as opposed to final germination percentage of *Protea* achenes is affected by various germination treatments (Le Maitre 1990). Similarly phenolic germination inhibitors are reported to affect the rate of germination but not necessarily the final germination percentage (Kuiters 1989). One of the interesting factors concerning the dormancy of *Protea* species is the lack of germination and the lack of decomposition of achenes whilst held for long periods in cones exposed to seasonally wet and warm environments (Rebelo & Rourke 1986). This may be due to phenolic compounds in the seed coat of achenes preventing precocious germination in the same manner that phenolics in caryopses are related to depressing pre-harvest sprouting of grains (Gatford *et al.* 2002; Weidner *et al.* 1999). In sunflowers the anti-fungal effect of applied coumarin compounds depressed the microbial activity that causes the rotting of flower heads and stalks (Prats *et al.* 2006).

Coumarin and its derivatives are phenylpropanoids and therefore synthesized from phenylalanine and the hydroxylation of cinnamic acids (Debeaujon *et al.* 2007). There are many coumarins found in plants and their physiological role is not well understood. At high concentrations they are thought to be inhibitory but at low concentrations they exert a stimulatory role on plant growth (George *et al.* 2008). Coumarins act via the inhibition of lipases that convert lipids into sugars (especially reducing sugars) and also interfere with the metabolism of one of the most important plant growth regulators within the seed, indole acetic acid (IAA) (Miller *et al.* 1975; Sirois & Miller 1972). Many coumarins and their derivatives have an antioxidant capacity, scavenging superoxide, hypochlorous acid and hydroxyl radicals (Fylaktakidou *et al.* 2004; Payá *et al.* 1992). The rate of coumarin (scopoletin) oxidation with H_2O_2 was thought to be so efficient that it was used as an estimation of H_2O_2 production in tissues (Andreae 1955). This antioxidant effect of coumarins may decrease the oxidation of unsaturated fatty acids, protecting those fatty acids present in *Protea* achenes from oxidation (Yu *et al.* 1999; Vickery 1971).

Physical dormancy

In *Leucospemum cordifolium* desiccation of fruits followed by wetting breaks the oxygen impermeable exo- and endotesta, allowing oxygen to reach the embryo, thereby lifting physical dormancy (Brits *et al.* 1993). The germination in *P. compacta, P. magnifica, Leucadendron daphnoides* and *Leucadendron tinctum* increased with the removal of portions of the seed coat and lower germination temperatures, but decreased with the length of time achenes were soaked (Brown & Dix 1985; Deall & Brown 1981; Brown & Van Staden 1973a; Brown & Van Staden 1973b). Therefore the seed coat of these species was also thought to possibly provide a mechanical restriction (Brown & Van Staden 1973b). Additionally the decrease in germination of *Protea* species may be imposed in response to soaking due to a thin film of water that forms around the achene/embryo, physically decreasing the rate of gas diffusion (Brown & Van Staden 1973a; Brown & Van Staden 1973b). Germination was therefore considered to be inhibited by the decreased diffusion of gasses across the seed coat to the embryo. Gasses also dissolve better in water at colder temperatures, corresponding to an increase in germination of *P. compacta* and *Leucadendron daphnoides* at low temperatures (Brown & Dix 1985; Brown & Van Staden 1973a; Brown & Van Staden 1973b).

Chemo-physical dormancy

Van Staden & Brown (1972), found the coumarin like compound not only in the embryos of the tested Proteaceae species but also the seed coats. Some phenolic compounds are efficient reducing agents and when present in seed coats are, in some cases, capable of diminishing the oxygen

concentration within the seed coat (and therefore the embryo) to anoxic conditions (Debeaujon *et al.* 2007; Kähkönen *et al.* 1999). This has been reported in sugar beet, where phenolics in the seed coat form a chemo-physical barrier to oxygen, especially if seed are soaked (Coumans *et al.* 1976), similar to that found in *Protea* species (Deall & Brown 1981). This seed coat imposed dormancy by phenolic compounds increases sensitivity of the embryo to anoxic conditions, as seen in barley (*Hordeum vulgare*), where the dormancy is broken by dry storage (Lenoir *et al.* 1986). Therefore, when present in the embryo, the oxidation of phenolic compounds may be necessary for dormancy alleviation (Oracz *et al.* 2007).

As oxygen availability seemed to restrict germination in Proteaceae, oxygenating treatments were tested in a range of South African Proteaceae (Brown & Van Staden 1973a; Brown & Van Staden 1973b). Apart from providing oxygen for respiration, oxygen and H_2O_2 are reactive oxygen species (ROS) compounds capable of oxidising organic molecules (Benson & Bremner 2004), exposure to H_2O_2 may therefore be detrimental. However, it may also oxidise germination inhibiting endogenous phenolic compounds. Soaking Proteaceae fruits in 1% H_2O_2 did not significantly increase the germination of *Protea* species (except *P. caffra* where germination increased significantly) but did significantly increase the germination percentage of 13 out of 15 Proteaceae species with nut like achenes (Brits 1986). Interestingly, out of the 29 Proteaceae species tested with 1% H_2O_2, no significant decrease in germination percentage was reported (Brits 1986). A hydrogen peroxide concentration of 1% may have been too low for a significantly marked change in germination as it was found that a 10% H_2O_2 produced a significant increase in germination of fruits of *Leucadendron tinctium* (Brown & Dix 1985). Therefore the increased germination of Proteaceae fruit with increased oxygen or the addition of H_2O_2 may aid the oxidation of coumarin like compounds, decreasing their inhibitory action (Mayer & Poljakoff-Mayber 1978).

1.2.3. Germination

Germination is a process in which a quiescent structure reinitiates growth (Nonogaki *et al.* 2007). The germination of orthodox seeds is a triphasic event in relation to the fresh weight of the seed, which includes imbibition, the lag phase and protrusion of the radical or plumule (Hartmann *et al.* 2002). Therefore the word ‘germination’ may depict a variety of different stages of growth. Seed physiologists consider germination to be the period from imbibition to the point where embryo growth is initiated and protrudes through the seed coverings (Nonogaki *et al.* 2007). For a seed quality analyst germination is complete once the root, hypocotyl and cotyledons have developed

sufficiently to be examined (Nonogaki *et al.* 2007; ISTA 2003). Additionally, growers understand germination to only have occurred once the seedling penetrates the soil surface (Nonogaki *et al.* 2007). In this study the term germination *sensu stricto* was used as the period from the start of imbibition to the point at which embryonically derived tissues emerge from any enclosing tissue (Perino & Come 1991). Generally the protrusion of the radical or plumule is the first external sign that the seed is viable and that germination had been initiated (Fenner & Thompson 2005).

The vigour of a seed or germination vigour is the condition of the seed that determines the maximum potential for seedling establishment. A seed with high vigour allows rapid germination over the full range of environmental conditions naturally experienced by the species (Pollock *et al.* 1972). As a seed ages the degree of vigour can decrease from this genetic and physiological maximum until the seed is dead or non-viable (Pollock *et al.* 1972; Berjak & Villers 1972). Seedlings from high vigour seeds have been shown to contain more ATP, express a higher crop yield in agriculture as well as an ability to out compete seedlings with a lower level of vigour (TeKorny & Egli 1991; Ching 1973; Pollock *et al.* 1972). Vigour may be determined by calculating the peak value of germination (P.V.) (Czabator 1962). The peak value represents highest individual daily quotient when the cumulative germination percentage of a seed lot is divided by the number of days it takes to reach this percentage (Ranal & DeSantana 2006; Czabator 1962). Peak value has been used to calculate the germination vigour of *P. neriifolia* achenes (Le Maitre 1990).

1.2.4. Vegetative propagation

Plants that are produced vegetatively (including through *in-vitro* techniques and stem cuttings) are clones of a select genotype. This is undesirable in an ecological setting, where there is emphasis on the conservation of high genetic diversity of plants (Loyola-Vargas & Vazquez-Flota 2006; Nichols 2005; Lammi *et al.* 1999), but can be highly desirable in the agricultural, horticultural and forestry industries. Populations that have a low genetic base are at risk of extinction through decreased fitness, making them more susceptible to the cascading, synergistic effects of many drivers such as habitat destruction, pest (disease and invertebrate) infestations and climatic disturbances (Markert *et al.* 2010; Chen *et al.* 2009; Brook *et al.* 2008). Although undesirable, many conservation initiatives have originated from a small genetic base, in some cases a single founder plant individual, and have still achieved the objective of creating self sustaining populations (Maunder 1992).

In vitro propagation is a means to mass produce disease-free plantlets in a relatively short period, provided an optimum protocol is followed for a particular species, variant or clone (George 1993). To a degree, the *in-vitro* production of propagules is independent of environmental events such as seasons, pollination, drought and other disasters and hence plants can be multiplied throughout the year (Liao *et al.* 2006). Increasingly micropropagated explants are being used in international reintroduction restoration efforts (Liao *et al.* 2006; Benson *et al.* 2000; Maunder 1992). As plants produced *in vitro* have passed through decontamination procedures, international resistance surrounding the complex phytosanitary regulations is reduced, facilitating the transport of such plants (Maunder 1992). Unfortunately, each species requires a specific protocol to effectively propagate the species. Empirically determining this protocol is often difficult, time consuming and expensive (George, 1993). This investment however, is often offset by the reward that this biotechnology affords the species.

Micropropagation is achieved in five broad but distinct steps (Stages 0-4), with each stage often having obstacles that have to be overcome (George, 1993). Each stage builds on the accomplishment of the previous stage and for the successful propagation of a plant species this has to be achieved consecutively in each of the following stages:

Stage 0: Selection and preparation of the parent material: The parent material has to be of the variety (genotype) typical of the species and free from disease. A chemical pre-treatment is often necessary to decontaminate the parent plant from micro-organisms in preparation for stage I.

Stage 1: Establishing an aseptic culture: Selected explants are transferred *in-vitro*. At this stage the material should undergo some growth and be free of obvious contaminants.

Stage 2: Multiplication of propagules: New outgrowths, capable of giving rise to intact plants, are produced and separated from the culture. Some of these propagules may be used in further cycles of propagation, increasing explant numbers.

Stage 3: Preparation for growth in a natural environment: The explants need to acquire the necessary structures for self-sustaining growth in soil.

Stage 4: Transfer to the natural environment: This stage is known as hardening, as plants are gradually "hardened" off or adapted to the conditions of the external *ex-vitro* environment.

The rooting of *Protea* stem cuttings is the propagation method of choice in commercial situations where specific and selected genotypes are required in large numbers (Malan 1992). Propagation using rooted stem cuttings has also been recommended for the conservation of rare and endangered Proteaceae on the Agulhas plain in the Western Cape (Laubscher *et al.* 2009).

However, difficulties in producing such plants arise as cuttings from some *Protea* species are difficult to root. The protocol for rooting Proteaceae cuttings depends heavily on the species or cultivar propagated (Coetzee & Littlejohn 2007; Malan 1995).

1.3. Conservation of *Protea roupelliae* ssp. *hamiltonii*

1.3.1. Conservation and dynamic environments

The continued decline of plant biodiversity will have a greater impact on human society than any other type of biodiversity loss (Schatz 2009). Many of the *in-situ* conservation management prescriptions to date have assumed a stable environment, however it is becoming increasingly clear that natural environments are anything but stable (Kramer & Havens 2009). The environmental conditions that species have to adapt to, in order to survive, are changing faster now than ever before. Environments change not only physically through habitat degradation and fragmentation but also biologically through the globalisation of pests and disease as well as by the introductions of alien species (Kramer & Havens 2009). The reactions of species to either adapt or go extinct in the face of events such as climate change are unknown. Climate change however, has the potential to threaten not only species that are currently rare but relatively common and widespread species, which are currently unaffected by habitat loss (Pimm 2008).

In recent years, due to limited budgets, there has been much debate on whether the emphasis of conservation research should be placed on single species or on ecosystems (Lindenmayer *et al.* 2007). As these two concepts are inherently linked with individual species creating and maintaining ecosystem processes, the protection of species is imperative in the conservation of ecosystem processes (Nott *et al.* 1997). Both ecosystem and single-species orientated research have advantages and disadvantages with a combination of the two being ideal (Lindenmayer *et al.* 2007). A single species approach yields an understanding of relationships between a species and its environment, and is useful in providing information for policy and management of threatened, keystone and invasive species (Lindenmayer *et al.* 2007). Along with small population size, other species specific attributes such as slow life histories and small geographic range contribute considerably to the decline of a species (Purvis *et al.* 2000).

1.3.2. *Ex-situ* and *in-situ* conservation

Ideally species should be conserved within their natural habitat along with the associated ecological processes however, habitat loss and the breakdown of such processes are becoming commonplace world-wide and South Africa is no exception (Berjak *et al.* 2010; Kramer &

Havens 2009). *Ex-situ* conservation of a species is a safeguard against the destruction of the original *in-situ* population. Unfortunately more and more *ex-situ* conservation efforts are being depended on to restore populations that have gone extinct in their natural habitats (Menninger *et al.* 2006). *Ex-situ* conservation of plant species may be achieved through various methods, for example botanical gardens or the storage of propagules. *Ex-situ* conservation of most species that produce orthodox seed is achieved through germplasm conservation in the form of seed storage (Engelmann 2000), which is estimated to be vastly cheaper than *in-situ* conservation (Li & Pritchard 2009). Due to the poor seed longevity of some species at conventional temperatures (>-20ºC), cryogenic seed storage is expected to become increasingly important in the future of *ex-situ* conservation (Li & Pritchard 2009; Walters *et al.* 2004).

There are however limitations to both botanical gardens and seed banks. The populations of the species within gardens are often small and therefore have a limited genetic diversity. Botanical gardens are also costly to run and ineffective in providing optimal conditions for each species. Detailed records of the conserved species are also needed. For example *P. roupelliae* ssp. *hamiltonii* was first cultivated from seed in 1963 at the Kirstenbosch Botanical Garden and plants were raised in the late 1970's however these plants were assumed to have died (Weiersbye *et al.* 2000; Rourke 1980). As a consequence of the time involved and resources necessary, many of the *ex-situ* conservation approaches/methods have not and cannot be fully developed and tested in their entirety. For example many years are needed to fully test the efficacy of seed storage from the harvest and storage of germplasm to the successful reintroduction of each species. Due to reports of rapid deterioration and loss of viability of *Protea* achenes, the seed storage of *P. roupelliae* ssp. *hamiltonii* may be unpredictable (Holmes & Newton 2004; Le Maitre 1990; Van Staden 1978a; Van Staden 1978b) especially at ultra-low temperatures.

The *in-situ* reintroduction of *ex-situ* conserved/produced propagules is not always straight forward but many conservation initiatives have recommend some form of reintroduction (Hoekstra *et al.* 2002) and the conservation initiatives for *P. roupelliae* ssp. *hamiltonii* are no exception in also prescribing population augmentation (Weiersbye *et al.* 2000; Green 1995). In many cases the reintroduction and transplantation of propagules fails, prompting warnings that, if untested, this method of conservation should not be relied upon (Kay 2008; Fahselt 2007)

1.3.3. Conservation and identifiable threats to *P. roupelliae* ssp. *hamiltonii*

Historically there were three populations of *P. roupelliae* ssp. *hamiltonii*, now however, the 26ha of Dr Hamilton Nature Reserve conserves the only known extant population (Weiersbye *et al.* 2000). A second population of *P. roupelliae* ssp. *hamiltonii* was accommodated in a small reserve within the Nelshoogte State Forest. However, through mismanagement by the Department of Forestry, the population was declared extinct in 1984 (Weiersbye *et al.* 2000). The reserve was de-proclaimed and afforested, consequently destroying the only other known *P. roupelliae* ssp. *hamiltonii* habitat (Weiersbye *et al.* 2000). The extant *P. roupelliae* ssp. *hamiltonii* population is conserved, along with a population of the vulnerable *Leucospermum gerrardii* and other endangered species, within the Dr Hamilton Nature Reserve (Coetzer 2008; Dayaram 2007). Most of the *P. roupelliae* ssp. *hamiltonii* individuals persist in a highly clumped distribution on a small spur between two valleys.

Rare and endangered Proteaceae may not fill their climatic envelope partially due to dispersal limitation (Witkowski & Lamont 2006). Additionally there have been estimations that it would take up to approximately 500 years for a population of a serotinous *Protea* species to advance 1km through natural seed dispersal and establishment (Manders 1986). It is therefore doubtful that a *Protea* species will be able to track impending rapid climate change. Through natural progression, *P. roupelliae* ssp *hamiltonii* would effectively not be able to advance from the confines of the Dr Hamilton Reserve due to the roughness of the substrate, even if corridors through the surrounding alien pine forests were created (Bond 1988). Fortunately the pollinating vectors (Sugarbirds and Sunbirds) would be able to genetically connect populations (formed by possible reintroductions) over large distances (Hockey *et al.* 2005; Hargreaves *et al.* 2004).

Apart from climate change, there are other threats to the *P. roupelliae* ssp. *hamiltonii* population in the Dr Hamilton Reserve. The Barberton region of South Africa is not only a centre of plant endemism (Van Wyk & Smith 2001), but is also rich in gold deposits. Gold was discovered in De Kaap Valley sparking a gold rush in 1884 and has supported lucrative gold mines in the area ever since (Otto *et al.* 2007; Norman & Whitfield 2006). The Dr Hamilton Reserve is on the edge of De Kaap Valley about 21km from the newly reopened historic Agnes gold mine which is now being mined “in a new bulk fashion” (Creamer 2010). It is noteworthy that one of the Areas of Special Interest for the Nelshoogte plantation, outlined in the Komatiland Environmental Report April 2006 – March 2007, is categorised under “mining.”

The Dr Hamilton reserve is small and is surrounded by plantations of *Pinus* species which are highly invasive (Nyoka 2003; Weiersbye *et al.* 2000). The effect that forestry has had on *P. roupelliae* ssp. *hamiltonii* cannot be ignored. Although forestry companies are currently supporting the conservation of endangered species in general within their plantations (Komatiland Environmental Report April 2006 – March 2007) the practice of forestry itself has led to the decline of the species directly, by planting over *P. roupelliae* ssp. *hamiltonii* populations and possibly indirectly by changing plant-herbivore interactions (Weiersbye *et al.* 2000). There are concerns that the hydrological cycle, the nutrient cycling and the intra-specific interactions are dysfunctional due to the surrounding afforestation and associated practices (Weiersbye *et al.* 2000). Pompom weed (*Campuloclinium macrocephalum*) has recently been identified within the Dr Hamilton reserve and is considered to be highly invasive in grasslands (E.T.F Witkowski & R. Green *pers. com.* 2008; Goodall *et al.* 2010).

1.4. Rationale for the study

In 2005 there were only 124 *Protea roupelliae* ssp. *hamiltonii* plants left in the single extant population (Czypionka 2006). This small population number increases the risk of extinction from relatively small disturbances. Additionally the genetics of the population can be adversely affected, as inbreeding depression occurs. Increasing population numbers to 500 individuals or establishing additional populations would help safeguard the species survival and decrease inbreeding (Traill *et al.* 2010). Although the plants were setting seed in 2005, no recruitment was observed (Czypionka 2006). A species with a small, single population that lacks recruitment remains at a high risk of total extinction (Pimm *et al.* 1988). *Protea* species are prone to low seed set, slow growth, high susceptibility to pathogens and seed predators, placing *P. roupelliae* ssp. *hamiltonii* at further risk of extinction (Rebelo 1995; Malan 1992). Factors that influence the reproductive capacity of the population, survival and seedling recruitment in this species need to be identified in order to focus conservation interventions and management efforts. The specific technologies needed to propagate and conserve this critically endangered species *ex-situ* need to be developed in detail and in doing so, it may be possible to mitigate the threat of total extinction faced by *P. roupelliae* ssp. *hamiltonii*.

1.5. Aims and objectives

The broad aim of this study was to investigate aspects of the life history, reproduction, seed storage and methods of the propagation of *Protea roupelliae* ssp. *hamiltonii* in order to enhance the *ex-situ* and *in-situ* conservation of the species.

The objectives were as follows:

a. To develop a better understanding of the natural regeneration of the *Protea roupelliae* ssp. *hamiltonii* population and some of the influencing factors *viz.* population size, stage and size structure, achene production and herbivory.
b. To relate the variation in size (mass) of the achenes produced by *Protea roupelliae* ssp. *hamiltonii* to achene germination and parent plant size.
c. To test various methods for the propagation of *Protea roupelliae* ssp. *hamiltonii* using seed and vegetative tissues as starting material.
d. To develop protocols for: (a) the *ex-situ* conservation of germplasm of *Protea roupelliae* ssp. *hamiltonii* and (b) the establishment of *P. roupelliae* ssp. *hamiltonii* seedlings to augment the population.

1.6. Dissertation structure

This first chapter is a general introduction which has been provided to give context and a rationale for the study. The succeeding chapters (Chapters 2 to 5) deal with aspects that may be necessary for conserving *P. roupelliae* ssp. *hamiltonii* and have been written in order to facilitate the writing of stand alone papers once the dissertation has been examined. Consequently there is a degree of repetition in the chapters but it has been kept to a minimum by referring to results and methods between chapters.

Chapter 2 provides demographic information on the natural *P. roupelliae* ssp. *hamiltonii* population, focusing on the reproductive capacity of individual plants and identifies possible threats to the species. Chapter 3 investigates the influence that achene mass has on the viability and germination of achenes, as well as investigating protocols for achene selection. Chapter 4 investigates propagatory protocols including: seed germination, rooting of stem cuttings and mass propagation using *in-vitro* techniques. Chapter 5 investigates the establishment of propagules *in-situ* and the storage behaviour of *P. roupelliae* ssp. *hamiltonii* achenes. Chapter 6, the final chapter, summarises the direct impact this study has had on the recovery of the species and poses recommendations on both the study of the species and future conservation management objectives.

Chapter 2

2. The natural regeneration of *Protea roupelliae* ssp. *hamiltonii.*

2.1. Abstract

The *Protea roupelliae* ssp. *hamiltonii* population has been recovering since the erection of an antelope proof fence in October 2003. In 2000 there was effectively no flowering and most of the population was in a functionally "juvenile" state. Flowering and the production of achenes filled with embryos was reported by 2005, however the population still lacked recruitment and most of the individuals (94.35%) still had small canopy areas (0-0.19m^2). By November 2008, the plants had significantly larger canopy areas and volumes than in 2005, there was evidence of natural recruitment and 71.23% of the population were reproductive. By 2009 a mean of 3.34±0.41 (±S.E.) cones were produced/plant. However, since observing small mammal herbivory in 2009, the mean cone production per plant decreased significantly in 2010 to 0.34±0.10 (±S.E.) cones/plant and only 13.01% of the population produced inflorescences. The activity of small mammalian herbivores had clearly negatively impacted on the reproductive output of the population in 2010. The reproductive capacity of a *Protea roupelliae* ssp. *hamiltonii* plants, in terms of both the numbers and the quality (rate of germination) of the achenes produced, was related to the canopy area of the plant. The total number of cones produced in a season (R^2=0.532; P<0.005), the number of filled achenes produced/plant (R^2=0.178; P=0.014) and germination percentage of filled achenes (R^2=0.200; P=0.009) increased with increasing plant canopy area. Whilst mean days until germination (R^2=0.270; P=0.002), the time taken for 50% of the achenes to germinate (R^2=0.231; P=0.005) and the peak day of germination (R^2=0.208; P=0.008) decreased with an increase in canopy area. Therefore conserving and maintaining large individuals is central to ensuring achene production and conserving the species. Recruitment has occurred since 2008, however the proportion of naturally recruiting newly established individuals remains small and herbivory has again regressed previously reproductive individuals to a non-reproductive state. It is feared that as older plants continue to die (nine plants died since October 2008; 6.16% of the 2008 population) and herbivory continues, the regenerative capacity of the population may decrease further. Therefore external intervention may need to be intensified for the long term survival of the population/species.

Key words: canopy area, herbivory, life history stage regression, population size, reproductive capacity

2.2. Introduction

2.2.1. History of *P. roupelliae* ssp. *hamiltonii* conservation

Protea roupelliae ssp. *hamiltonii* was originally thought to be a naturally rare species, consisting of several scattered colonies (Rourke 1980). Due to the rarity of the species and subsequent loss of all but a single known population, the single remaining population has been monitored for many decades and the available data are from 1982. In 1985, due to high levels of herbivory, a fence was erected around the central core of the population in order to exclude herbivores

however the fence (including the fence poles) was stolen two years later (R. Green *pers. comm.* 2010).

In the year 2000, the rate that individual plants were being lost was so rapid that it was predicted that the species may be extinct by the year 2005 (Weiersbye *et al.* 2000). The cause of the rapid decline in population numbers was thought to be the result of large herbivore activity, altered by the surrounding unpalatable plantation forests (Weiersbye *et al.* 2000). The impact of the increased herbivory was magnified by the life history strategy typical of *Protea* species namely: a slow growth rate, poor seed set and high susceptibility of the seeds produced being destroyed by seed predators or low reproduction in ageing plants (Holmes & Newton 2004; Weiersbye *et al.* 2000; Malan 1992; Collins & Rebelo 1987). This prompted the erection of a second fence surrounding the entire *P. roupelliae* ssp. *hamiltonii* population in October 2003. Although sections of the second fence have been stolen throughout the years, repairs and replacements have been promptly performed.

2.2.2. Present and future recovery of *P. roupelliae* ssp. *hamiltonii* population

Since the erection of this antelope proof fence in 2003 it was reported that the original population has recommenced flowering, setting seed and recovering (Czypionka 2006). However the rate, extent and ability of this population to recover was unknown. This subspecies has been largely unstudied and its regenerative ability and current state would dictate whether the population requires external intervention to increase population numbers. Analysing and expanding the available population data will assist in developing effective conservation practices for the species.

2.2.3. Information needed for recovery efforts

Schemske *et al.* (1994) argues that the information needed to construct an approach to the conservation of rare plants is not necessarily the product of genetic or autecological studies. The information from these studies is useful in endangered species conservation and future recovery programs (Evans *et al.* 2010; Dostálek *et al.* 2009; Lande 1988). However, this information is only appropriate in initial investigations if the relative impacts on vital rates of the population can be demonstrated (Schemske, *et al.* 1994; Lande 1988). As an initial assessment of the population, with the aim of developing an approach to recovering an endangered plant species, fundamental biological questions relating to the vital rates of the species should be answered. The following three questions are adapted from Schemske *et al.* (1994):

a. What is the status of the species with regard to individual and population numbers?

b. Which life history stages have the greatest effect on population growth and the persistence of the species?
c. What are the causes of variation in life history stages that have a major demographic impact?

Answering these questions will establish whether further conservation efforts are necessary and if so, place the emphasis of conservation efforts on various stages of the plants' life history, ensuring that individuals successfully pass from seed to reproductive adult (Figure 2.1) (Schemske *et al.* 1994).

Life history stages of long lived plants are related to the size of an individual plant and not necessarily the age of the plant (Garcia 2003). Furthermore an individual may regress to a more juvenile stage due to herbivory, fire or other disturbances (Figure 2.1) (Bond & Midgley 2001; Pfab & Witkowski 1999). Additionally it has been suggested that the study of the life history of serotinous Proteaceae may help predict future risks by understanding their response to a changing world (Cabral & Schurr 2010; Witkowski & Lamont 2006).

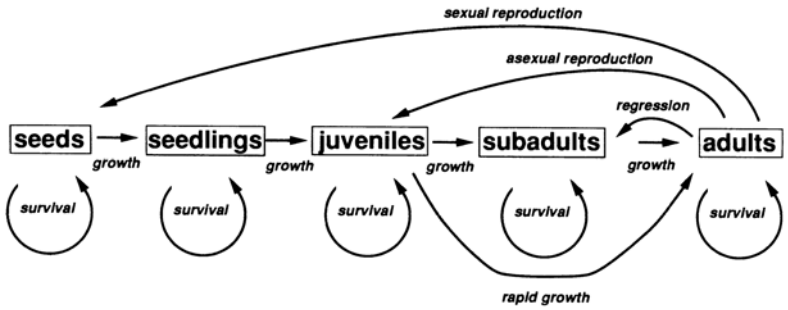


Figure 2.1. The life cycle of a hypothetical plant population where arrows indicate the possibility of individuals growing, surviving or regressing through the five life history stages over time and the possible reproductive strategies employed by the species (from Schemske *et al.*, 1994).

2.3. Aims and objectives

The aim was to develop a better understanding of the natural regeneration of the *Protea roupelliae* ssp. *hamiltonii* population and some of the influencing factors *viz.* population size, stage and size structure, achene production and herbivory.

The objectives were:

1. To understand the demographics of the *Protea roupelliae* ssp. *hamiltonii* population over a limited time in terms of:
    a. Population size.
    b. Mean canopy dimensions.

c. Cone production.
d. The variation (based on flower colour) within the *Protea roupelliae* ssp. *hamiltonii* population.

2. To study the relationship between plant canopy area and the reproductive capacity of a sample of reproductive *Protea roupelliae* ssp. *hamiltonii* individuals.
3. To identify life history stage classes of *Protea roupelliae* ssp. *hamiltonii* and utilise these to assess changes in the population over time.
4. To observe possible disturbances that may cause the reproductive capacity of the population to increase/regress.

2.4. Materials and methods

2.4.1. Plant demographics over time

Population counts and measurements that were used in this study were made by various people from 1982 – 2005 (Table 2.1).

In October 2008, the spatial positions (co-ordinates) of all adult plants were captured using a Trimble Recon Differential Global Positioning System (GPS). The system provided accuracy to 0.5m, which was necessary to locate the position of each individual adult plant that was alive in October 2008 on successive site visits, including small plants and the remains of dead plants.

Table 2.1. The year and names of researchers that monitored the *Protea roupelliae* ssp. *hamiltonii* population.

Year	Researcher	Fruiting season assessed
1982†	S. Venter	-
1985†	R. Green and K. Zunckel	-
1986†	R. Green and P. Raal	-
1987†	R. Green	-
1989†	F. Smith	-
1994†	R. Green	-
1996†	R. Green and N. Eccles	-
1997†	N. Eccles	-
2000†	I. Weiersbye, K. Balkwill and B. Patrick	1999/2000
2005 (October) ‡	E.T.F. Witkowski and A. L. Czypionka	2003/4
2008 (March)	This study	2006/7 and 2007/8
2008 (October)	This study	-
2009 (March)	This study	2008/9
2009 (November)	This study	2009/10*
2010 (November)	This study	2010/1*

† Weiersbye *et al.* (2000)
‡Czypionka, *unpublished data*
* Cone production estimated from inflorescences and inflorescence buds produced/plant.

Population count

The adult plants were visually assessed for survival (small plants that appeared dead were located by GPS and appeared as a dark-brown/black cluster of leaves due to phenolic oxidative browning (Chapter 4)), if plants appeared dead a small portion was removed from the basal stem and if there was no green tissue the plant was considered dead. New seedlings associated with adult plants (within 2m from an adult) were identified (excluding those planted in population augmentation experiments (Chapter 5) which were conducted simultaneously). Population data from 1982 were included and counts were performed in this study on October 2008, November 2009 and November 2010 (Table 2.1).

Canopy dimensions

The longest canopy diameter, the perpendicular canopy diameter and the height of the tallest part of the plant were measured. Canopy dimension data used were collected from 2000, October 2005, October 2008, November 2009 and November 2010 (Table 2.1). The following measurements were used to calculate the canopy area and canopy volume as follows:

$$\text{Area} = \pi\ (d_1/2)\ (d_2/2) = 0.7854\ d_1\ d_2$$

$$\text{Volume} = 4/3\ \pi\ (h_t/2)\ (d_1/2)\ (d_2/2) = 0.5236\ h_t\ d_1\ d_2$$

Where d_1= longest plant diameter, d_2= the perpendicular diameter and h_t = and the tallest part of each plant. The data were compared over the years using an ANOVA and a Tukey post hoc test.

Cone production

The flowering phenology of *Protea roupelliae* ssp. *hamiltonii* reported in a field guide (Rebelo 1995) is unsupported by direct observations from this study, as it has been observed that flowering occurs from mid October to December with peak flowering in November (see Chapter 6, Section 6.1.6a) (R. Green *pers. comm.* 2011; S. Tarlton *pers. obs.* 2008). Based on harvesting experiments performed on *P. neriifolia* by Van Staden (1978a), cones can only be considered mature by seven months from peak flowering. Identifying the years the cones were produced was possible by observing the colour of the cone and the position of the cone on the branching system (Le Maitre 1990; Esler *et al.* 1989). The canopy stored cones weather on the plant and become faded, appearing grey (Figure 2.2a) (Le Maitre 1990).

In March 2008 the cones produced from the 2006/7 fruiting season (>1 year old) were easily differentiated from those produced from the 2007/8 (<1 year old) fruiting season by colour, condition and position on a branch. Brown cones were therefore produced from the 2007/8 fruiting season and the grey, weathered cones were produced from the 2006/7 fruiting season

(Figure 2.2a). Additionally in March 2009, scars from where small mammals had removed or destroyed cones were also counted (Figure 2.2b and c). Cone production/plant was compared over the years with a Kruskal-Wallis test.

Plants producing inflorescences, inflorescence buds and cones were considered to be reproductive although the cones may have been sterile and may not have produced any viable achenes (S. Tarlton *pers. obs.* 2008; Hargreaves *et al.* 2004; Collins & Rebelo 1987). The number of reproductive plants per season from the 1999/2000 to 2010/1 fruiting season as per Table 2.1 was counted.



a.



b.



c.

Figure 2.2. (a) Older cones were identified by their grey colour (indicated by white arrows). Scars left on the plant where cones were either (b) partially destroyed or (c) completely removed by small mammals.

2.4.2. Flower (bract) colour variation

In November 2009 the bract colour of the flowering plants were categorised into five classes based on the position and extent of pink pigment appearing on the bracts of the inflorescences (Figure 2.3), and forming a cline from yellow to pink/almost red, as follows:

a. Bracts completely devoid of pink pigment and with flowers appearing yellow (Y).

b. Pink pigment lightly present on the margin of the thickened "spatulate" portion of the bracts (YYP).

c. Pink pigment on the margin of the thickened "spatulate" portion of the bracts but not in the centre of the bracts (YP).

d. Pink pigment present across the full, thickened "spatulate" portion of the bracts but not descending to the base of the bracts (P).

e. Pink pigment present over the total portion of the bracts with flowers appearing deep pink to almost red (DP).



Figure 2.3. Bract colour categories into which flowering plants were identified (a) yellow (Y), (b) yellow with pink (YYP), (c) light pink (YP), (d) pink (P) or (e) deep pink (DP). Inflorescences (f and g) were identified as colour classes yellow (Y) and deep pink (DP) respectively (bars approximately 1 cm).

2.4.3. Canopy area and reproductive capacity

On the 18th October 2008, a sample of 33 flowering plants present in the reserve were visually selected based on canopy size (specifically canopy area) to include the full range of flowering plants (three initial canopy area categories of small, medium and large were used to assist sampling). The cones containing achenes and the cones from which achenes had been dispersed on each plant were counted. Eleven cones produced from the 2007/8 fruiting season (approximately one year old) were harvested. One cone was harvested from each of the 11

different plants per size category, and hence a total 33 cones were harvested. Canopy area of each plant was measured as above. Each harvested cone was maintained in a brown paper bag for one month under ambient conditions, allowing the achenes to reach water contents in equilibrium with the ambient relative humidity and be more easily released from the cone.

On the 18th of November 2008 the achenes from each cone were removed from the receptacles and counted. On 1st December 2008 all the seeds from each cone were placed in germination conditions (below) with a maximum of 15 achenes per Petri dish for a period of 30 days. After 30 days achenes were dissected to ascertain if the achene was filled with an embryo or not.

Germination conditions

The standard germination environment occurred on a filter paper substrate in accordance with the international rules for seed testing (ISTA 2003). Achenes were placed in a Petri dish containing two 90mm disks of filter paper and covered by a third filter paper disk. The filter paper was wet with 6ml sterile ultra-pure water (milli-Q filtration and autoclaved at 120°C at 1.2 k.P.a.) and incubated at 25±2ºC and a 13 hour photoperiod with 200µmol m-2.s-1 photosynthetic photon flux density. The achenes were checked every second day for germination. Germination was considered complete when the radical emerged 2mm from the pericarp.

Germination

Germination was expressed by calculating the following parameters:

- The Peak Value of germination (P.V.) is the maximum quotient derived by dividing the cumulative germination percentage by the number of days taken to reach this percentage (Czabator 1962).
- The day of peak germination: the day that had the highest frequency of germinants.
- T_{50} was calculated as the time taken for 50 percent of the achenes that were filled with an embryo to germinate.
- The mean number of days until germination; the mean number of days germinable achenes from a cone took to germinate (the sum of the number of days each germinant took to germinate divided by the total number of germinants).

Analysis

Regression analyses were used to test relationships between canopy area and the reproductive capacity of the plants. The relationships tested included the canopy area of plants and:

a. The total number of cones on the plant (b and c below)

b. Number of cones produced per plant containing achenes.
c. The number of cones that were still attached to a plant from which achenes had dispersed.
d. The number of achenes per cone.
e. The number of germinable achenes per cone (achenes that germinated).
f. The number of achenes filled with an embryo.
g. The percentage of filled achenes that germinated.
h. The P.V.
i. The peak day of germination.
j. The T_{50}.
k. The mean number of days until germination.

2.4.4. Life history stage classes

The stage classes depicted by Schemske *et al.* (1994) (Figure 2.1) were adapted for *P. roupelliae* ssp. *hamiltonii*.

"Seedlings" were considered to be less than one year old. A description (in terms of size) of such seedlings includes a mean seedling height of 51mm, ranging from 31mm to 71mm. This and further quantitative descriptions of plants about one year old were obtained from achenes that were planted and emerged under herbivore enclosures in the Dr Hamilton Reserve during the population augmentation experiment (see Figure 5.13 and 15). Therefore individuals that are up to about 70mm in height may be considered in the seedling stage class.

"Juveniles" were considered to be non-reproductive individuals over one year of age. The population data from the fruiting seasons of 2007/8 to 2010/1 were examined to ascertain the minimum canopy area of plants producing cones. Individuals in this juvenile stage may have either (a) progressed from the seedling stage and would be typical young juveniles which had not yet reached reproduction size or (b) regressed from previously reproductive adult/sub-adult stages (below) due to herbivory.

'Sub-adults' and 'adults' were differentiated from each other with regard to the regression relationships between the canopy area and reproductive capacity of the 33 reproductive individuals sampled (above). A cut-off canopy area size was chosen through visual assessment and sequential statistical analyses (Mann-Whittney U tests). Where individuals smaller than this cut-off area (sub-adults) were significantly inferior to larger individuals (adults) with regard to reproductive parameters. The reproductive parameters chosen had highly significant relationships

between canopy area and both the number and quality of achenes produced by a plant, specifically; (a) the total number of cones produced, (b) the number of filled achenes/cone, (c) percentage of filled achenes that germinated/cone, (d) mean number of days until germination. Differences were tested with a Mann-Whitney U test.

2.5. Results

2.5.1. Population demographics over time

Population size

The species population number decreased from a measured high of 1040 in 1982 to 195 individuals 14 years later (Figure 2.4). The number of individual *P. roupelliae* ssp. *hamiltonii* plants within the population reached lowest population number of 124 individuals in 2005 and the population has been increasing since (Figure 2.4). In October 2008, the population totalled 146 and by 2010 this number had risen to 178 individuals (Figure 2.4). Details on the stage classes over time and mortality can be found below in Sections 2.5.4 and 2.5.5 respectively.

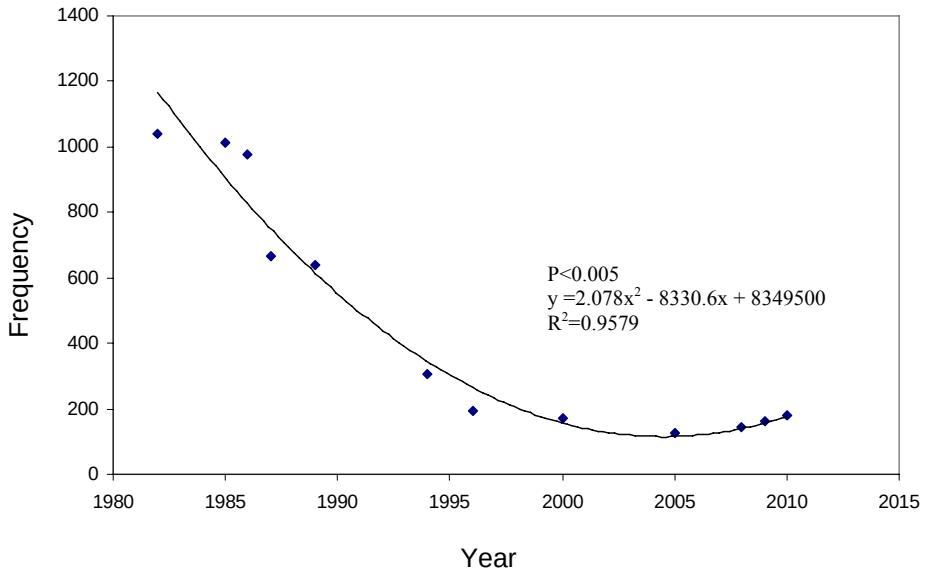


Figure 2.4. Total *Protea roupelliae* ssp. *hamiltonii* plants within the Dr Hamilton Reserve excluding those from augmentation experiments (Chapter 5), from 1982 to 2010.

Plant size and cone production

The mean (±S.E.) canopy area and volume of *P. roupelliae* ssp. *hamiltonii* population has risen over the period between 2000 and 2010 from 0.052±0.007m^2 to 0.422±0.030m^2 and from 0.0021±0.0002 m^3 to 0.0878±0.0077m^3 respectively (Figure 2.5).

The mean number of cones produced per plant peaked between the 2007/8 and 2009/10 fruiting season, with 487 cones in total produced in the 2009/10 season (the mean cone production per plant for 2007/8 season was not significantly different from that of 2008/9 or 2009/10 season (Tukey; P>0.05)) (Figure 2.6). The number of cones produced in the 2010/1 fruiting season (estimated from inflorescences and inflorescence buds and hence may be an over estimate) dropped significantly from the 2009/10 season (P<0.05) (Figure 2.6).

The number of plants producing cones had increased in the 2008/9 fruiting season to where 71.23% of the individuals within the population were reproductive (Figure 2.7). However this percentage dropped in the 2009/10 fruiting season and by the 2010/1 season only 13.01% of the population was reproductive (Figure 2.7).

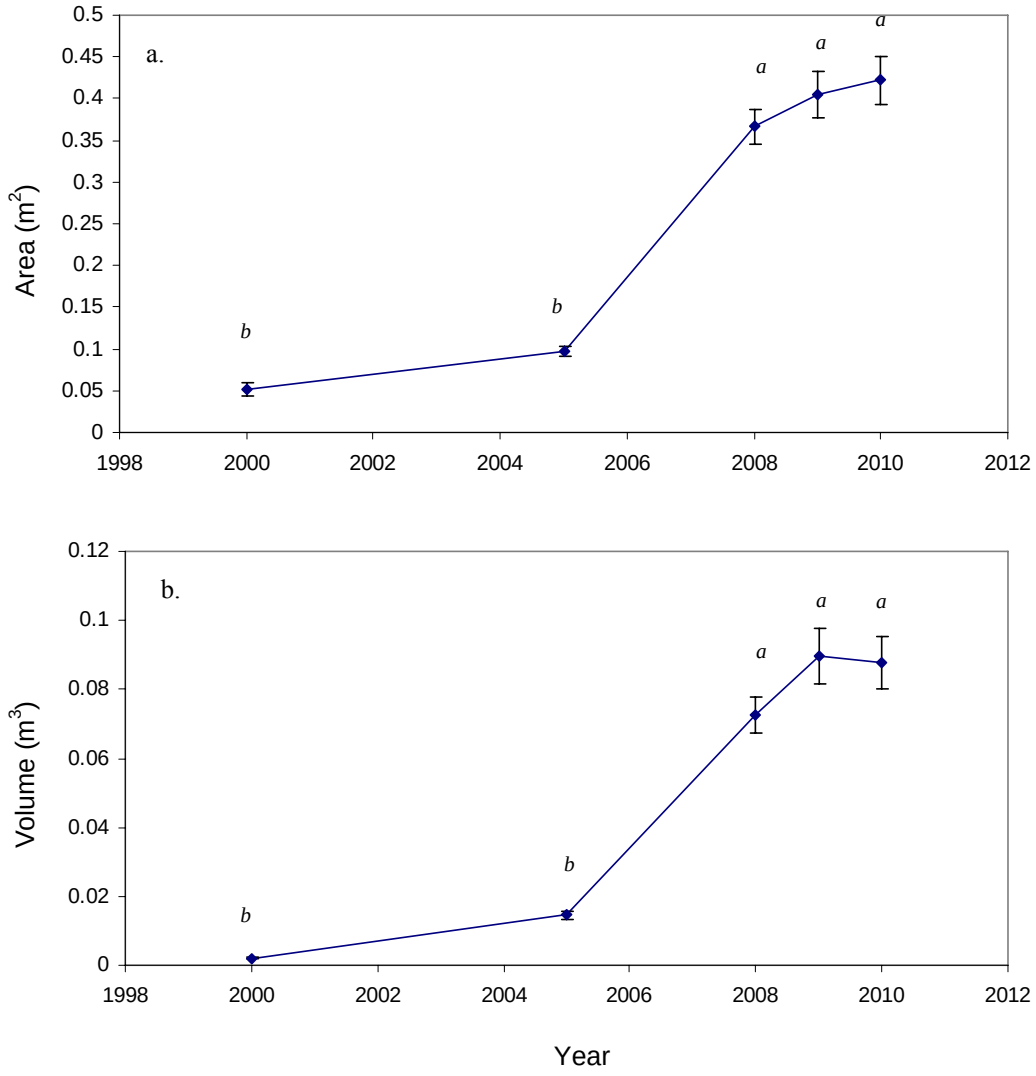


Figure 2.5. Mean (±S.E.) plant canopy (a) area (R^2=0.867; P=0.022) and (b) volume (R^2=0.874; P=0.020) from 2000 to 2010. Different letters indicate significant differences between years (P<0.05).

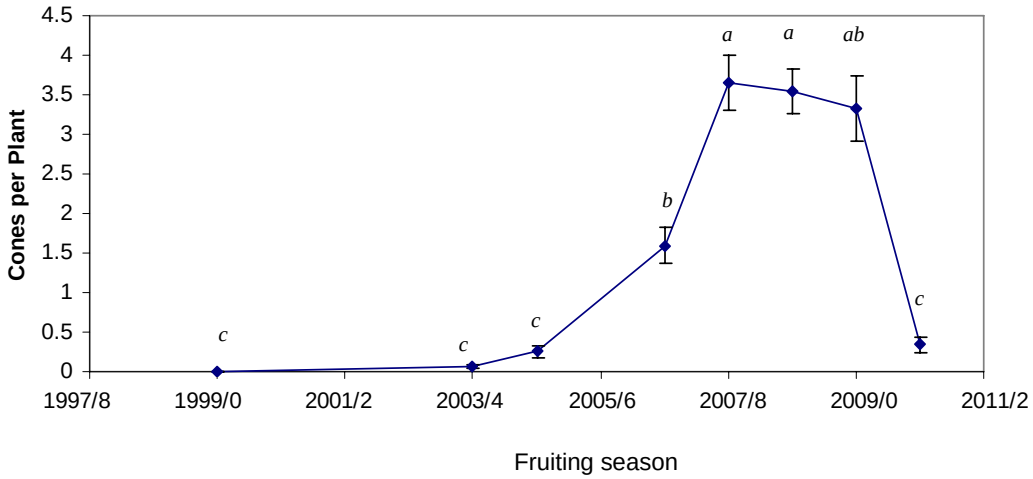


Figure 2.6. Mean (±S.E.) cone production per plant. Different letters indicate significant differences between years ($P<0.05$).

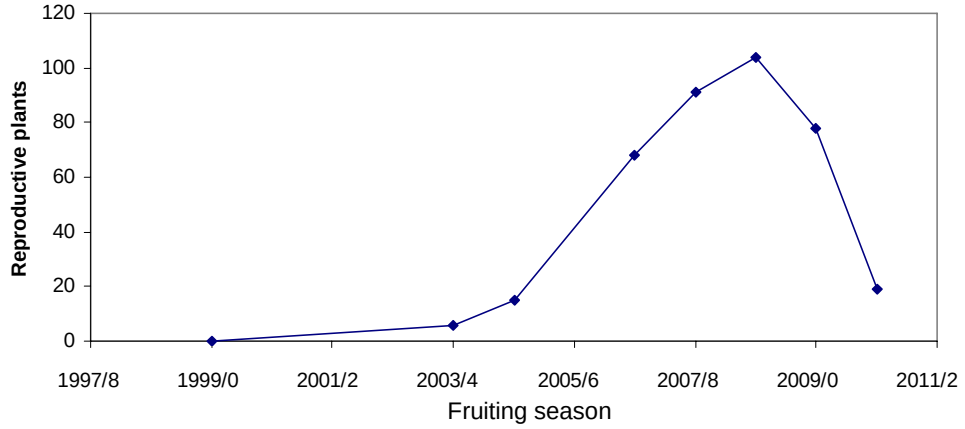


Figure 2.7. The number of reproductive plants within the Dr Hamilton Reserve.

2.5.2. Flower (bract) colour

In November 2009, 29.38% of the plants produced open inflorescences (bract colour could not be categorised accurately from closed inflorescence buds). The majority of the flowering plants (42.55%) were classified as the intermediate category of light pink (YP) (Figure 2.8) and a similar percentage of the flowering plants were classified as yellow (Y) (6.38%) and deep pink (DP) (4.26%) (Figure 2.8).

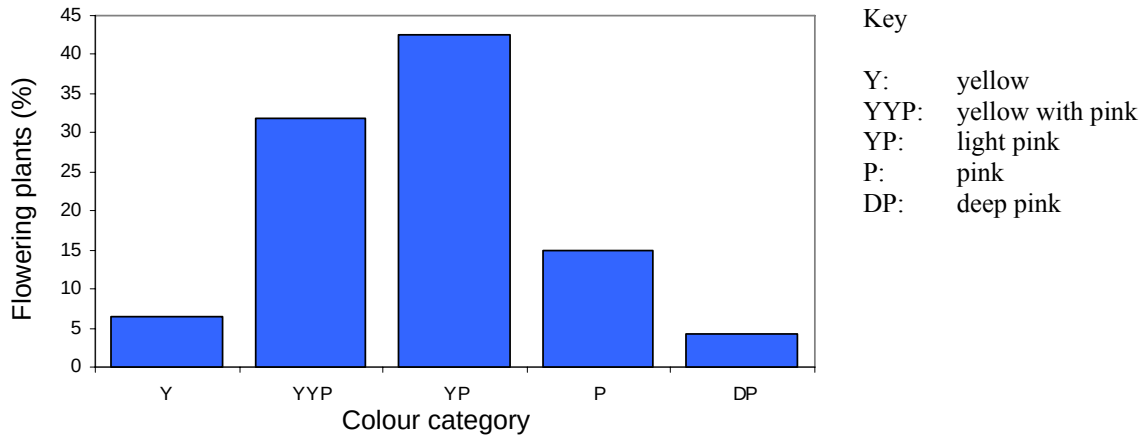


Figure 2.8. Percentage of flowering plants in each of the flower (bract) colour categories (November 2009; based on n=47 plants).

2.5.3. Canopy area and reproductive capacity

Each of the 33 cones harvested, set viable seed and were therefore fertile. The mean number of achenes that geminated per cone was 36.61±3.21 (mean±S.E.). The canopy areas of the plants (n=33) were related to their reproductive capacity in terms of the production and germinability of achenes that they produced (Figures 2.9-11). Cone production (Figure 2.9), achene production (Figure 2.10a), and the production of filled achenes (Figure 2.10b) increased with plant size (canopy area). This corresponded to there being more germinable achenes in cones that were harvested from plants with larger canopy areas than those harvested from plants with smaller canopy areas (Figure 2.10e).

The germination percentage of the filled achenes increased with canopy area (Figure 2.11a). The P.V. was not significantly related (P=0.065) but tended to increase with the canopy area of the plant (Figure 2.11b). The other germination parameters including T_{50}, the peak day of germination and the mean number of days for an achene to germinate decreased with canopy area, confirming that achenes from plants with larger canopy areas germinated more rapidly than those from smaller plants, with smaller canopy areas (Figure 2.11c-e). The fact that the majority of these achenes germinated readily under growth room conditions indicated no inherent dormancy. As such, the achenes can therefore be classified as quiescent.

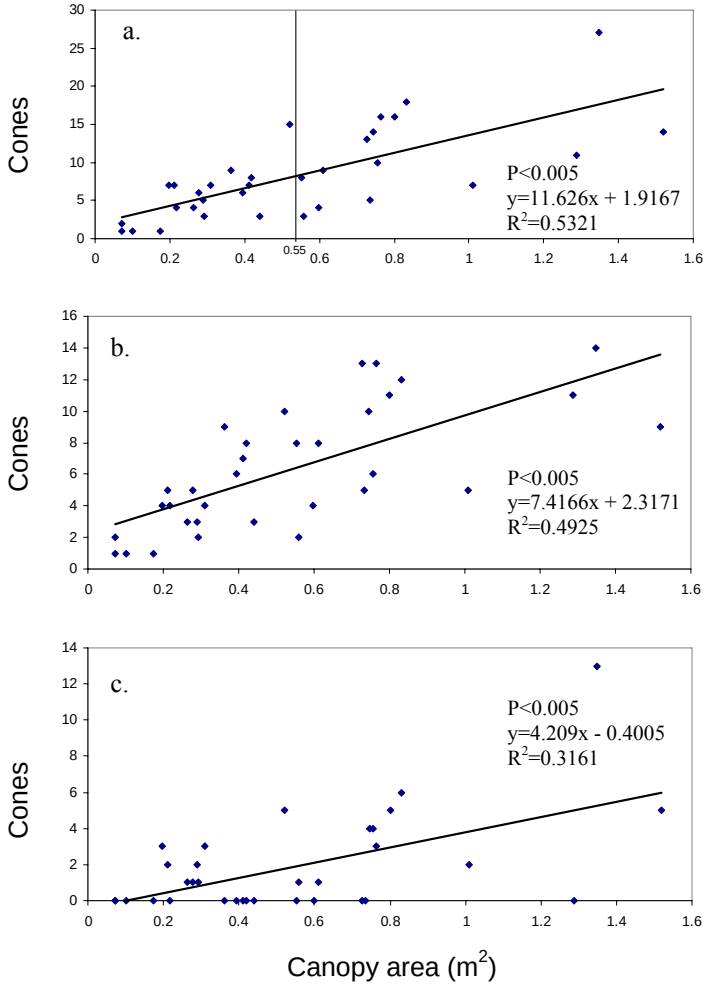


Figure 2.9. Linear regression relationships between the canopy area of 33 plants in October 2008 and (a) the total number of cones per plant (note that the vertical line represents the cut-off from sub-adult to adult stages; see text for more details), (b) the number of cones containing achenes per plant and (c) cones from which achenes had dispersed per plant.

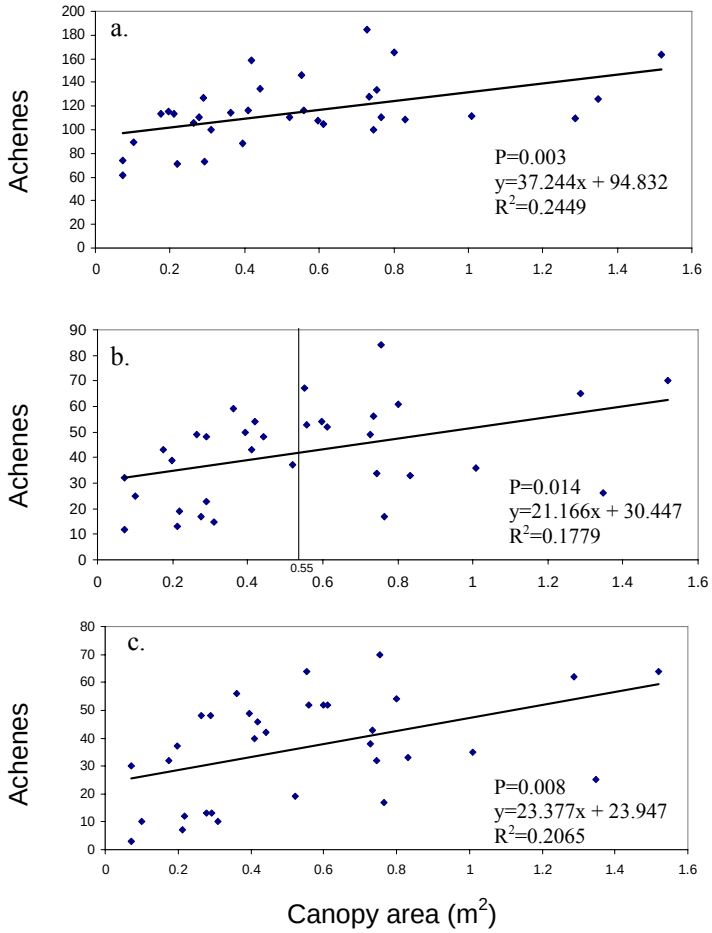


Figure 2.10. Linear regression relationships between the canopy area of 33 plants in October 2008 and (a) the total number of achenes within the sampled cone, (b) the number of achenes filled with an embryo within the sampled cone (note that the vertical line represents the cut-off from sub-adult to adult stages; see text for more details) and (c) the number of achenes within the sampled cone that germinated..

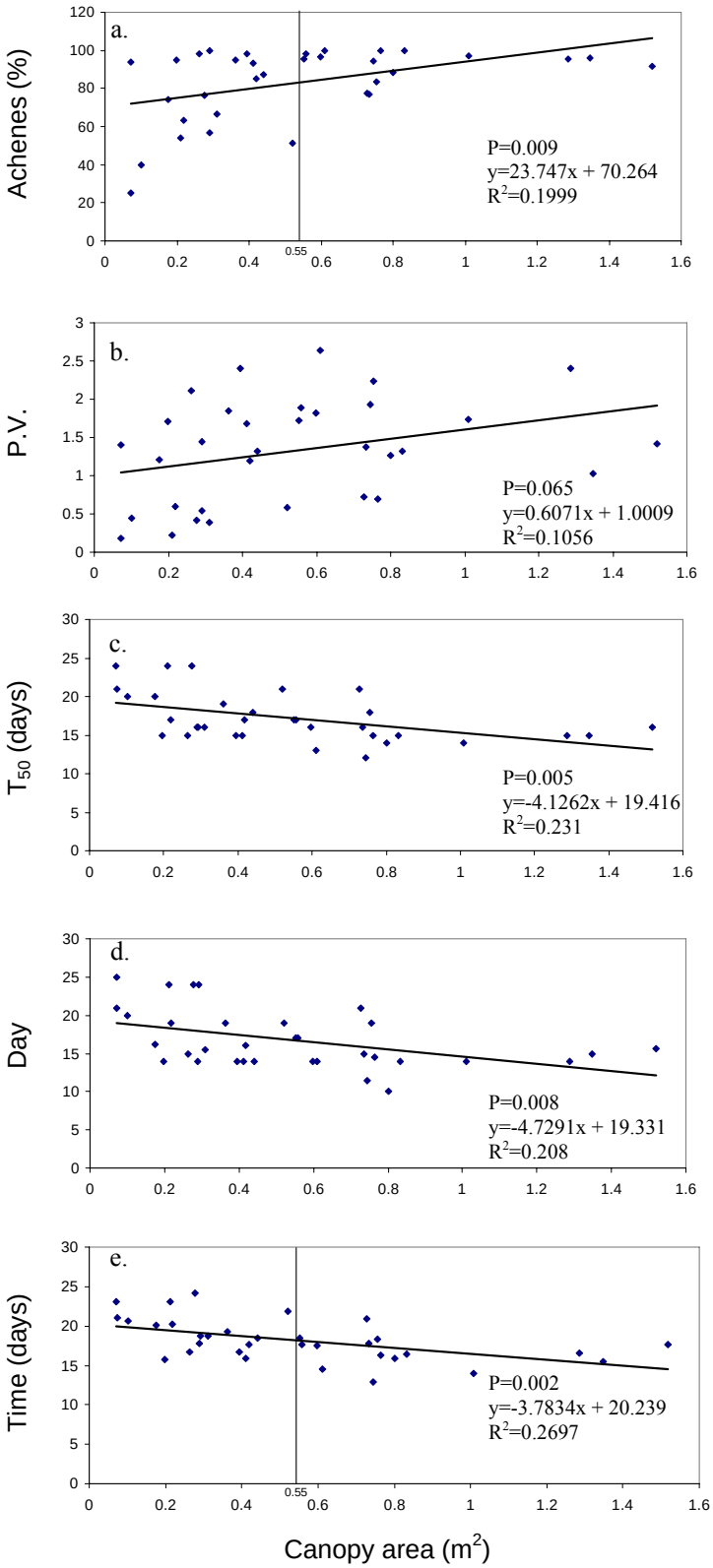


Figure 2.11. Linear regression relationships between the canopy area of 33 plants in October 2008 and (a) the percentage of achenes filled with an embryo that germinated, (b) the P.V., (c) T_{50}, the peak day of germination and (e) the mean number of days until germination. Note that the vertical line represents the cut-off from sub-adult to adult stages (see text for more details).

2.5.4. Life history stage classification

Defining life history stages

Seedlings

As above.

Juveniles

The smallest plant to produce a cone between the 2007/8 and 2010/1 fruiting season was 0.0470m^2. Therefore plants older than one year but smaller than 0.0470m^2 were considered functionally juvenile within the population, regardless of whether they were young individuals or previously reproductive and had regressed to this small canopy area.

Sub-adults and adults

Results from the regression analyses (Figures 2.8a, 2.9b, 2.10a and 2.10b) were used to identify the boundary between adults and sub-adults. Plants with a canopy area <0.550m^2 produced significantly fewer cones and fewer filled achenes than plants with a canopy area >0.550m^2 (Table 2.2). Filled achenes produced by plants with a canopy area <0.550m^2 had significantly lower germination percentages and germinated slower than those from plants with a canopy area >0.550m^2 (Table 2.2). Therefore, individuals that had a canopy area ≥0.047m^2 and <0.550m^2 were classified as sub-adults and all plants with a canopy area ≥0.550m^2 were classified as adults.

Table 2.2. Reproductive parameters (mean±S.E.) used to differentiate Sub-adults from Adults

Parameter	Sub-adults n = 18 (≥0.047 and <0.55m^2)	Adults n = 15 (≥0.55m^2)	Z value; P value
Total number of cones per plant	5.33±0.82	11.67±1.62	-3.1636; 0.0015
Filled achene per cone	34.78±3.66	50.47±4.70	-2.5128; 0.0119
Percentage of filled achenes that germinated	75.15±5.33	92.70±2.00	-2.5850; 0.0097
Mean days until germination	19.44±0.60	16.69±0.51	3.0370; 0.0024

Population size and stage class structures over time (2000 - 2010)

In the year 2000, most (90%) of the population was in the smallest of the measured size classes (0–0.09m^2; Figure 2.12a) and most of the population (76%) was classified as being in the juvenile stage class (Figure 2.12f). Individual plants had increased in canopy size by 2005, but 94% of the population was still occupying the first two size classes (0-0.19m^2; Figure 2.12b). Although these plants still had a relatively small canopy area, the majority of plants (80%) had become reproductive sub-adults (Figure 2.12g). From 2005 to 2008 many plants had grown into the subsequent size classes (Figure 2.12b and c) and more individuals progressed to become adults and sub-adults (Figure 2.12g and h). In October 2008, although the seedling and juvenile stage classes made up only 16% of the population (the lowest combined percentage from 2000 to 2010),

recruitment was observed and 2008 had the most seedlings between the 2000 and 2010 period (Figure 2.12h). In November 2009 and 2010, the percentage of individuals in the seedling and juvenile stage classes (non-reproductive) increased to 29% of the population in 2010. As the population expanded within the non-reproductive stage classes the percentage of individuals in the sub-adult and adult stage classes consequently dropped. Interestingly there was only a net gain of two individuals in the reproductive stage classes over the 2008 to 2010 period.

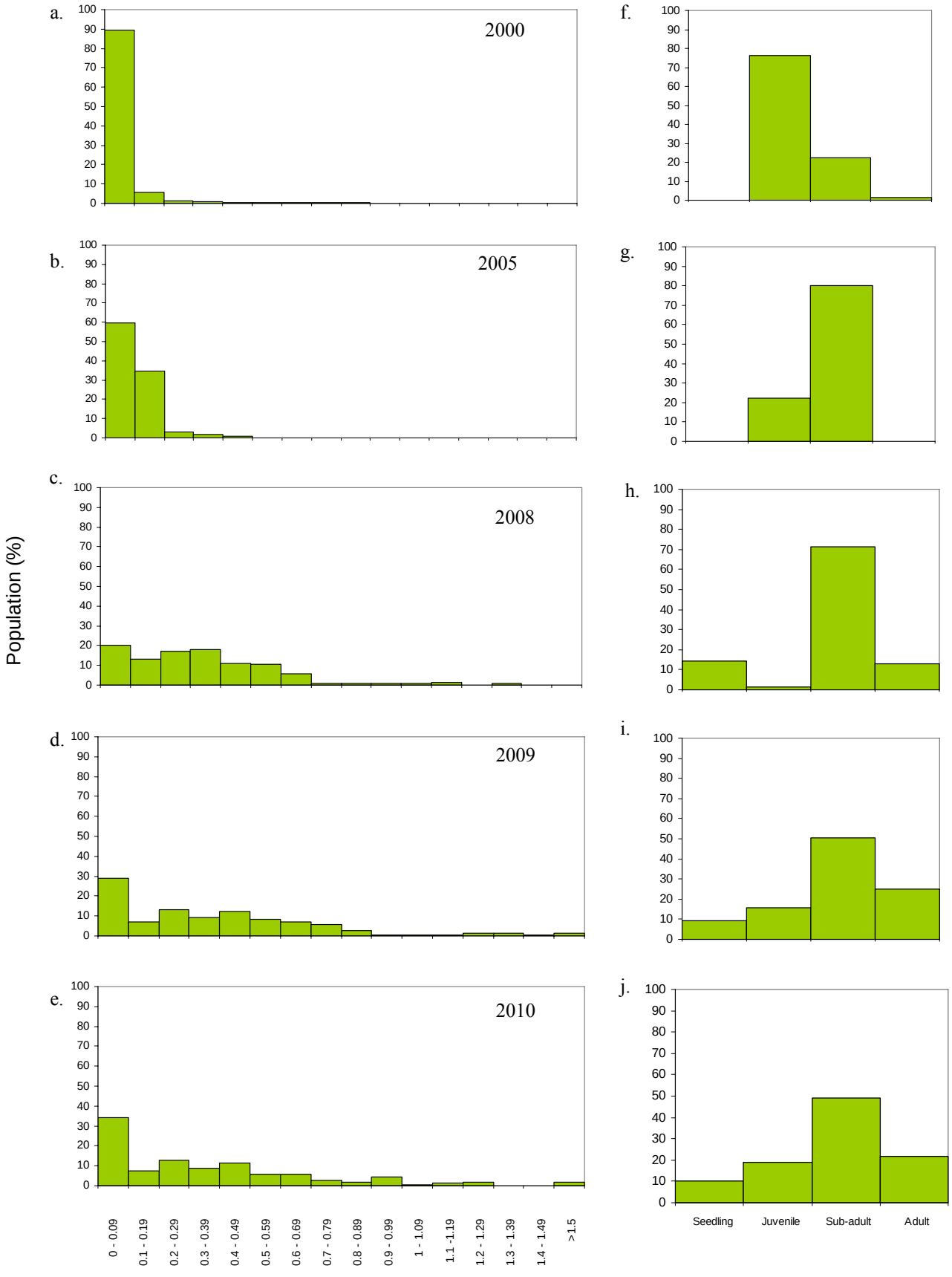


Figure 2.12. Percentage of the population within canopy area size classes (a – e) and life history stage classes (f –j) for the years sampled (a and f) 2000, (b and g) October 2005, (c and h) October 2008, (d and i) November 2009 and (e and j) November 2010.

2.5.5. Threats to the population

From October 2008, when the spatial co-ordinates of the population were captured, to November 2010 nine plants died. In March 2009 one plant was confirmed dead, and in November 2010 another eight plants had died. Therefore, of the 146 plants counted in October 2008, 6.16% had died by November 2010. All nine plants were in the sub-adult stage class.

In 2000 a mean of 22.04±1.80% of each plant had been browsed by large herbivores (Weiersbye *et al.* 2000). In March and November 2009, damage from small herbivores was observed which had not occurred during sampling in 2008 (Figure 2.13a to c). It was evident that 30.65% of the total number of cones produced in the 2008/9 fruiting season had been removed by small mammals by March 2009. During monitoring in November 2009 it was observed that inflorescences and inflorescence buds were also being consumed, in many cases damage was restricted to the base of the inflorescence (receptacle) rather than developing embryos (Figure 2.13d).

The plant stems were also consumed by small mammals that removed tissue from the outer portion of the stem and apical buds, generally avoiding the inner stem tissue and old leaves (Figure 2.13e to g). This was particularly notable in November 2009 after the dry season when 11 (6.88% of the total population) plants were found to have portions of tissue removed from stems and one plant was consumed (almost entirely) and had already died. Of the eight plants that died in 2010, four had tissue removed from their stems in November 2009 (Figure 2.13g and h).

Invertebrate herbivores were also observed feeding on the plants however, their impact was limited (Figure 2.14a to c). Damage from invertebrates seemed to occur only on the leaves of the plants.



Figure 2.13. The flowers (a) in November 2008, the cones present before herbivory in January 2009 (b) and the loss of cones due to herbivorous small mammals in March 2009 (indicated by arrows) (c). Herbivores appear to favour the receptacle of the flower (November 2009) (d). Small mammal herbivory occurred on the periphery of stems (observed in November 2009) (e and f). An individual plant resprouting from the base (g and h) (indicated by arrows) after sustaining chronic herbivory observed in November 2009 however, this individual had ultimately died by November 2010.


a.

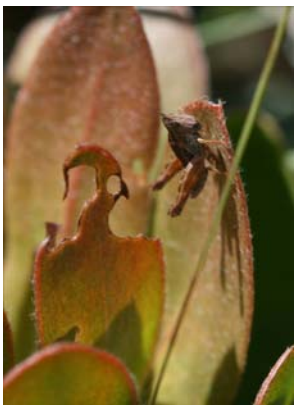
b.

c.

Figure 2.14. Lepidopteron (a) and Orthopteron (b and c) herbivores observed feeding on the leaves of *P. roupelliae* ssp. *hamiltonii.*

2.6. Discussion

2.6.1. Population demographics

Over the recorded period, the *P. roupelliae* ssp. *hamiltonii* population decreased rapidly reaching its lowest recorded numbers in 2005 (Figure 2.4). In October 2003 an antelope proof fence was erected around the reserve and from 2005 the number of individuals within the population started to recover (Figure 2.4).

For the period post 2000, the mean canopy area and volume of the plants increased, as did the mean number of cones produced per plant and the number of plants producing cones (Figure 2.5a, b, 6 and 7). However, from the 2008/9 fruiting season the number of plants producing cones decreased considerably (Figure 2.7) and in the 2010/1 fruiting season the number of cones produced per plant dropped significantly compared to the year before (Figure 2.6). This rapid drop in cone production was not reflected in a significant drop in canopy area (Figure 2.5-7). The additional stress imposed by herbivores can severely limit seed production in woody plants (Rockwood 1973), which is seen in *P. roupelliae* ssp. *hamiltonii* by the drop in cone production since herbivory was observed in March 2009 (Figure 2.6 and 7). This indicates that population recovery may not follow an extrapolation of the fitted curve in Figure 2.4 under current management and intervention may need to be intensified in order to assist regeneration.

2.6.2. Canopy area and reproductive output

The percentage of fertile cones produced by *P. roupelliae* ssp. *hamiltonii* may be considered high as each of the 33 cones harvested from different plants, set viable achenes. This was considerably higher than many other *Protea* species (Collins & Rebelo 1987) and indeed higher than the typical

sub species, *P. roupelliae* ssp. *roupelliae*, which only set seed in 54.5% of the 11 cones assessed (Hargreaves *et al.* 2004).

The number of cones, the number of achenes and the quality of the achenes (in terms of germination rate) produced by *P. roupelliae* ssp. *hamiltonii* plants increases with the canopy area of the plant (Figure 2.9-11). The majority of superior, germinable achenes are best produced only once a plant is of larger canopy area (Figure 2.11). Therefore it may be argued that resources obtained by a plant may be best utilised by increasing plant size as opposed to the production of few cones with inferior achenes. Many species of Proteaceae are andromonoecous. Andromonoecy within the individual flowers of inflorescences (cones) has been suggested as a possible explanation for the low seed set in other Proteaceae (Collins & Rebelo 1987). However, no evidence of distinctly male individual flowers within these Proteaceae inflorescences exists (Ladd & Connell 1994; Walker & Whelan 1991). Plant size dependant sex allocation is also reasonably common in plants (De Jong & Klinkhamer 2005). Unfortunately, data on pollen production of *P. roupelliae* ssp. *hamiltonii* flowers on small plants (or indeed any plants) were not collected but based on their poor production of germinable achenes, the role of a flowering plant with a small canopy area may be chiefly as a pollen donor (De Jong & Klinkhamer 2005).

The fruits of Proteaceae are of high nutrient content and therefore costly to produce in the nutrient poor soils generally favoured by Proteaceae (Groom & Lamont 2010; Esler *et al.* 1989). From this perspective, it may be necessary for the high cost of producing numerous, high quality achenes to be incurred by large well established plants (Figures 2.9-11).

2.6.3. Size and stage classes over time

Protea species are known to have comparitively slow life histories, with seedlings taking a number of years to reach flowering stage (Coetzee & Littlejohn 2007). With none of the seedlings recruited in 2008 reaching flowering stage class by 2010, *P. roupelliae* ssp. *hamiltonii* was no exception. Based on reports that there were no signs of recruitment in 2000 (Weiersbye *et al.* 2000) and the plants were previously large and reproductive (R. Green *pers. comm.* 2008), most of the plants in the population in 2000 were therefore in a secondary juvenile stage class (Figure 2.12f). This was prior to the exclusion of large herbivores in 2003 and the plants had regressed to this stage class due to extensive herbivory and maintained as such by continuous herbivory (Weiersbye *et al.* 2000; Pfab & Witkowski 1999; Schemske *et al.*, 1994). This juvenile state of the population corresponds to the lack of cone and achene production during this period (Figure 2.6

and 7). The constant damage caused by herbivores may be likened to an intense version of commercial harvesting of Proteaceae flowers. The harvesting of Proteaceae inflorescences decreases the following season's production of cones in many Cape (Agulhas) species including *P. obtusifolia, Leucadendron coniferum, L. meridianum* (Mustart & Cowling 1992). The commercial harvesting of only 29% of the blooms of *Banksia hookeriana* (Proteaceae) was sufficient to decrease the canopy area and canopy volume but more importantly decreased flowering by 35.1% (Witkowski *et al.* 1994).

The inverse-j shaped curve of the canopy area classes generally signifies a "healthy" population with more, smaller individuals replacing the aging population of larger adults (Rao *et al.* 1990). Throughout the monitored period this inverse j-shaped curve was maintained in the size class distribution of the *P. roupelliae* ssp. *hamiltonii* population (Figure 2.12a-e). However, recruitment was not observed in 2000 or 2005, consequently along with a trend of decreasing population numbers, this population could not have been considered sustainable (Figure 2.4) (Czypionka 2006; Weiersbye *et al.* 2000). By 2005 most of the plants were in the sub-adult stage class, producing flowers and filled achenes but there were no adults, therefore few cones were produced and the achenes produced were of low germination vigour (Figure 2.11 and 12g) (Czypionka 2006). Although most of the population was in a sub-adult stage in 2005 (therefore capable of reproduction) there was no recruitment until 2008 (Figure 2.12h). Recruitment was reinitiated however, when there were large individuals in the adult stage and could imply that seedling recruitment depends on the greater number and the superior quality of achenes produced by these adult individuals. Recruitment has been occurring since 2008 and the shape of the stage class distribution had not displayed the inverse-j shape associated with a healthy population by 2010 (Figure 2.12j) (Rao *et al.* 1990). Although there has been a considerable increase in the percentage of the population in seedling and juvenile stage classes, it will take a considerable period before there is sufficient natural recruitment for an inverse-j shaped stage class distribution to manifest (Figure 2.12).

2.6.4. Threats to the population

The longevity of *P. roupelliae* ssp. *hamiltonii* plants is unknown. Proteaceae are reported to have a tendency to die suddenly at any age or season for no apparent reason (Thorns 1943), but with 50% of the deaths occurring in 2010 having evidence of herbivore damage it may be expected that herbivory hastens the process. The exclusion of large herbivores has allowed individuals to grow and become reproductive (Figures 5 and 6). However from 2009 when herbivory was observed

the number of reproductive individuals has decreased considerably (Figure 2.7). The significant decrease in cone production (Figure 2.6) and reproductive individuals (Figure 2.7), that coincided with the observed increase in herbivory (possibly by rodents), indicates that herbivory affected the reproductive output and therefore the regenerative ability of the population. Proteaceae species do have defences against herbivory. Their leaves contain phenolic compounds and once mature the leaves become increasingly sclerophyllous (Coetzee *et al.* 1997). Additionally, similar to *Grevillea pyramidalis* (Proteaceae), long chain phenolic lipids which have been found to inflict severe dermatitis on contact, have been extracted from the heartwood of *P. roupelliae* ssp. *roupelliae* (Bullock & Drewes 1989; Occolowitz & Wright 1962). The presence of these phenolic lipids may explain the herbivore avoidance of the heartwood tissue in *P. roupelliae* ssp. *hamiltonii* (Figure 2.13e and f).

The activity of small mammals is effective enough to mediate the suppression of woody vegetation and maintain grassland and savanna landscapes (Weltzin *et al.* 1997). Rodent behaviour has been found to significantly reduce the recruitment of Proteaceae (Bond 1984). Small mammal species richness and density follows the successional age of Proteaceae dominated fynbos stands following fire (Willan & Bigalke 1982). Therefore in the regeneration of the *P. roupelliae* ssp. *hamiltonii* population, it is imperative that the threat from small mammals is taken seriously. Small mammalian herbivores do not only pose a threat in terms of the damage sustained by the adult plants resulting in depressed cone production (Figure 2.6 and7), but they also decrease the number and probability of propagules surviving and establishing *in-situ* (Bond 1984).

The *P. roupelliae* ssp. *hamiltonii* population is small. The genetic consequence of the population size alone infers a threat to the persistence of the population (Schemske *et al.* 1994; Lande 1988). From the range of inflorescence colour morphs present in the flowering population in November 2009, it may be possible that this long lived species may still hold a degree of genetic variation with regard to bract colour (Figure 2.8). However, the true extent of the genetic bottleneck may only be elucidated through genetic studies. It is therefore essential that the genetic makeup of each individual is conserved so that it can contribute to recruitment and genetic diversity of the population. Additionally it is essential that the population numbers exceed 500 individuals as soon as possible in order to limit the effects of inbreeding depression (Traill *et al.* 2010).

2.7. Conclusion

Although the *Protea roupelliae* ssp. *hamiltonii* population was recovering, this recovery was slow and may slow further due to threats to the population that still exist. These threats are in the form of (a) the presence of small mammal herbivores and (b) aging of older plants which may decrease reproductive output without sufficient recruitment to maintain the population.

The original decline of the species was thought to have resulted from the altered herbivore behaviour brought about by the afforestation of surrounding grasslands into plantation forests (Ferguson *et al.* 2003; Weiersbye *et al.* 2000). Unfortunately the species has again been impacted by herbivores, although this was not reflected in the size/stage class structure of the population, cone production per plant has significantly decreased. Large adults may be essential for the production of sufficient high quality achenes necessary for seedling establishment. It is therefore imperative that large individuals (adults) remain in the population and that herbivory does not suppress the superior cone and achene production of these individuals. It may be necessary to place exclosures around individuals or alter the fire regime in order to decrease the destructive activity of small mammals, restoring the reproductive capacity of the population (Willan & Bigalke 1982). The seedling and juvenile stage classes, although increasing, remained a relatively small percentage of the population. These small individuals are probably more susceptible to mortality especially from herbivory (Bond 1984); ideally their percentage should be much higher in order to replace the ageing adult population and increase population numbers (Ranta *et al.* 2006). In order to achieve this ideal population structure, population augmentation may be recommended to assist and fast track recruitment and growth of seedlings (Kay 2008).

Chapter 3

3. Achene mass: a predictor of seed set and germination in the critically endangered *Protea roupelliae* ssp. *hamiltonii.*

3.1. Abstract

Protea species produce cones containing achenes that are either filled or unfilled with an embryo and these may appear identical. By individually weighing achenes from five *Protea roupelliae* ssp. *hamiltonii* plants, it was established that filled, germinable achenes (25.3±0.2 mg) were significantly heavier than unfilled achenes (17.3±0.3mg) (mean±S.E.). Mean achene mass was significantly different between plants ($P<0.05$), with less within than between plant variation. This lower within plant achene mass variation enables the identification of a demarcation in achene mass for each plant, above which most of the achenes (93.67±1.46%) were filled and may be selected for various applications. Most of the within plant variation in achene mass was associated with the difference between filled and unfilled achenes and there tended to be more mass variation across unfilled achenes than for filled achenes. No relationships between the mass of individual achenes and the germination parameters calculated (including T_{50}, Mean days until germination and P.V.) were detected. By analysing the mass of achenes produced in a single cone from different plants (n=33), it was elucidated that there was a positive, but weak, linear relationship between plant canopy size (area) and mean achene mass ($R^2=0.127$; $P=0.041$). There was a positive linear relationship between the mean mass per achene produced in each cone and the percentage of filled achenes per cone ($R^2= 0.454$; $P<0.001$), the percentage of filled achenes that germinated per cone ($R^2=0.133$; $P=0.036$) and the peak value of germination ($R^2=0.403$; $P<0.001$). Whilst there was a negative linear relationship between mean mass per achene produced in each cone, the time it took for 50% of the seeds to germinate ($R^2=0.209$; $P=0.007$) and the mean days until germination ($R^2=0.148$; $P=0.014$). Therefore, measuring the mean mass per achene within a cone provides an indication of the number, the germination percentage and the quality (in terms of germination rate) of the filled achenes within that cone. The percentage of filled achenes/cone (seed set) was not related to the canopy area of the plant that the cone was harvested from ($P=0.241$). The seed set of *Protea roupelliae* ssp. *hamiltonii* was over 35% and of those at least 83% germinated.

Key words: achene mass, achene mass variation, achene selection, filled achenes, seed set.

3.2. Introduction

3.2.1. Achenes filled with an embryo or just woody tissue

Three types of achenes are produced by *Protea* species, these include an achene containing an embryo, a thickened plump woody achene not containing an embryo and a thin hollow achene not containing an embryo (Figure 3.1) (Collins & Rebelo 1987; Rebelo & Rourke 1986).

Many of the Proteaceae have a seed set so low that breeding programs based on seed production are difficult (Rebelo & Rourke 1986). *Protea* achenes take several months from fertilisation to maturity (about seven months for *P. neriifolia*) and are generally considered mature only after a

full year, when the next season's flowers open (Van Staden 1978a; Eliovson 1965). It is during this period of seed development that a wide range of species abort ovules (Wiens *et al.* 1987). The reasons for a plant to abort seed development include pollination limitation, genetic defects and a lack of sufficient resources, including light, mineral nutrients and water (Fenner & Thompson 2005; Stephenson 1992; Witkowski 1990; Bawa & Webb 1984). It is further thought that this unpredictable variation of seed set and low numbers of filled achenes act as a defence mechanism against seed predators; higher proportions of fruit filled with embryos are correlated to a greater degree of herbivory (Fuentes & Schupp 1998; Wright 1994).

Insufficient pollen transfer is not considered to be a sole limiting factor of seed set in Proteaceae (Vaughton 1991; Collins & Rebelo 1987; Whelan & Goldingay 1986; Paton & Turner 1985;). The seed set of hermaphroditic Proteaceae remains low irrespective of the major pollen vectors or the breeding system of the species (Collins & Rebelo 1987). This is true in *P. roupelliae ssp. roupelliae* which produced a mean seed set of about 24% in pollen supplemented flowers, slightly although not significantly, lower than naturally pollinated flowers (Hargreaves *et al.* 2004).

The mating system of *Protea* species was thought to be obligate out-crossing and many species are protandrous (Collins & Rebelo 1987; Horn 1962). However, *P. roupelliae* ssp. *roupelliae* has been found to be self-compatible (Hargreaves *et al.* 2004). Functional andromonoecy or androgyny has been proposed as a possible cause for the low flower to seed set ratio in Proteaceae (Collins & Rebelo 1987; Rebelo & Rourke 1986). However, it has been argued that the female role in selective ovule abortion is more likely and could be at evolutionary odds with androgyny (Walker & Whelan 1991). Androgyny would decrease the number of female flowers in an inflorescence, decreasing the opportunity for female choice and increase the chance that high quality pollen may be received by flowers with no female function (Walker & Whelan 1991). The role of female choice is further reinforced by indications that seed set control is imposed in the stages preceding pollen tube formation in *P. repens* and *P. eximia* (Van der Walt & Littlejohn 1996).

When there is a limited availability of resources a parent plant may abort more seeds. This is thought to be a quality control mechanism, ensuring that resources are not expended on inferior progeny (Fenner & Thompson 2005; Stephenson 1992). The seed set of *Banksia laricina* (Proteaceae) significantly increased with an addition of fertilizer, therefore it is thought that seed set in this species is resource limited (Stock *et al.* 1989). The lack of adequate resources was also shown in *P. compacta* having a lower seed production on highly infertile soils compared with the

higher seed set of closely related *P. obtusifolia* on less infertile soil (Esler *et al.* 1989). Additionally, *Leucospermum parile* showed an increase in inflorescence production with nitrogen supplementation (after the second year) (Witkowski 1990).

South African species belonging to the *Protea* genus have a mean (±S.D.) of 18.1±24.3 achenes/cone equating to a seed set of 8.3±6.5% (mean±S.D.) according to Rebelo & Rourke (1986) and 16.1±20.7 (mean±S.D.) achenes/cone (a seed set of 9.2±7.2%) according to Collins & Rebelo (1987). This low seed set is reflected in all hermaphroditic Proteaceae throughout Southern Africa and Australia with many inflorescences failing to set seed at all (Collins & Rebelo 1987).

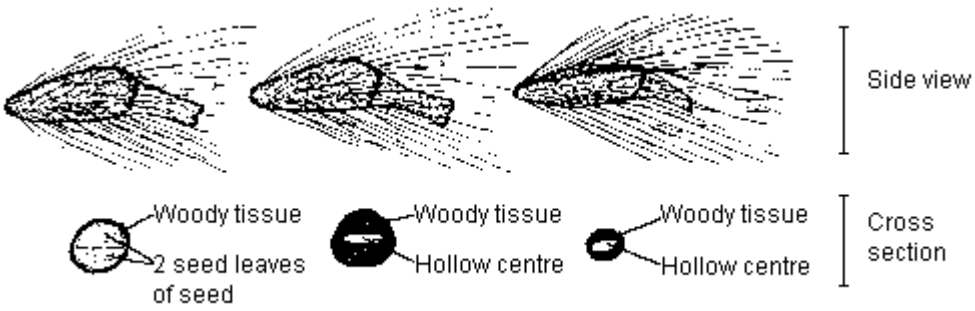


Figure 3.1. Seeds that are plump may contain an embryo or be hollow with a large mass of woody tissue and as such are unviable (*Protea* Atlas: http://protea.worldonline.co.za/seedsize.htm).

3.2.2. Achene mass

The mechanisms a plant employs to control the number and size of cells in a seed and subsequently a seed's size is not fully understood and is a rapidly expanding area of research in agriculture (North *et al.* 2010). The mass and size of a seed that a plant produces can have a major effect on the survival and growth of the ensuing seedling (Turnbull *et al.* 1999). There is evidence that seed mass can be associated with many of the plants attributes, including the size of the plants genome, growth form and height of the plant, the mode that the seed disperses, the environment in which it grows and the geographical range of the species (Leishman *et al.* 2000). In general, Proteaceae seed mass (per seed) decreases with the addition of nutrients (Groom & Lamont 2010; Witkowski 1990). Additionally phenotypic link has also been observed where *Protea* plants producing white flower morphs produce heavier achenes and have better germination than pink flower morphs (Carlson & Holsinger 2010).

The size of the seed can also dictate how a species relates to others in the community by affecting its competitive ability (Leishman 2001). Seedlings developed from large seeded species are larger,

they have access to more resources (stored in the seed), have a lower relative growth rate and are therefore better able to withstand adverse conditions for longer periods than those from smaller seeds. (Westoby *et al.* 1996). Larger seedlings are able to acquire more resources such as nutrients (Jurado & Westoby 1992), sunlight (Walters & Reich 2000; Grime & Jeffrey 1965) and water (Ramírez-Valiente *et al.* 2009; Leishman & Westoby 1994). However there are trade-offs for having large seeds. These trade offs include the greater probability of being consumed by seed predators, the distance of unassisted seed dispersal as well as the number of seeds produced/plant due to the greater energy expenditure incurred by the parent plant (Gómez 2004; Rees & Westoby 1997; Greene & Johnson 1993; Smith & Fretwell 1974) .

Within a species, variations in seed mass can be considerable, with coefficients of variation (C.V.) exceeding 20% (Michaels *et al.* 1988), such as in *P. repens* with a C.V. of 28% (Witkowski 1991). In many species, high variation in seed mass is displayed within, rather than among plants or populations (Michaels *et al.* 1988). This was also true in *Banksia marginata,* which showed pronounced variation within plants (42%) and infructescences (36%) and less variation among plants (6%) and among infructescences (7%) (Vaughton & Ramsey 1998).

Seed mass can be used as a measure of the amount of resources available to an embryo as in *P. repens,* where nitrogen and phosphorus contents of the achene were linearly related to seed size (Witkowski 1991). There are higher levels of nitrogen, phosphorus and magnesium in the seeds of Proteaceae species than in the seeds of other species of a similar in size (Groom & Lamont 2010; Pate *et al.* 1986). Small seeded Proteaceae are more enriched with mineral nutrients than species producing larger seed (Pate *et al.* 1986). Starch was universally absent in the seeds of 70 Proteaceae species examined by Pate *et al.* (1986) but oils were abundant in most species.

The extent of the reproductive investment by Proteaceae can be observed in partitioning of nutrients in *Banksia hookeriana* (Proteaceae), the seeds of which contributed 0.5% of the total above ground dry mass of the plant but represented 24% of the nitrogen and 46 % of the phosphorus contents (Witkowski & Lamont 1996). The accumulation of nitrogen and phosphorus in the developing cones of *P. compacta* and *P. obustifolia* occurred after flowering during the maturation of the achenes (Esler *et al.* 1989). It is thought that the larger seeds of *P. compacta,* which contained more nitrogen and phosphorus than those of the closely related *P. obtusifolia,* are necessary for seedling establishment in soils containing fewer nutrients (Esler *et al.* 1989). Although larger seeds have been shown to contain more nutrients (Witkowski 1991), no relationship between seed mass and days to germination in four other *Protea* species was found

(Carlson & Holsinger 2010). Nor has a relationship between seed mass and the relative growth rates of the seedlings in five members of the Proteaceae been observed (Stock *et al.* 1990).

3.2.3. Achene selection

The domestication of crops is considered the result of unintentional/non-deliberate selection over thousands of years (Tang *et al.* 2010). Through the archaeological record there is evidence of a dramatic increase in the grain size of cereals (Purugganan & Fuller 2009), indicating that seed size may be hereditary and can be selected. In conservation practices the selection of germplasm from a population has to ensure that the genetic diversity of the population collected has no artificial bias (Lefevre 2004). As *Protea* species typically have a seed set below 10%, there is a high chance that a randomly chosen achene will not be filled with an embryo. Conservation operations (with this study contributing to one) involving propagation from seed, including population augmentation, managed relocation, population recovery from *ex-situ* seed storage and micropropagation, invest resources into achenes whether they are filled with an embryo or not. These conservation methods also rely on the fact that achenes harvested from a specific genetic source provide a sufficient quantity of germinable seed (germplasm) needed for the genetic conservation of a species (Oldfield 2009). For this reason, an objective, non-destructive means of ascertaining whether an achene is filled with an embryo is imperative in conservation initiatives where one achene has to be chosen over another.

In most studies where achenes filled with an embryo are separated from unfilled achenes, the separation is done on 'plumpness' or in an even more subjective visual manner (Ellis & McCauley 2009; Kay *et al.* 2006). The visual appearance of filled and unfilled achenes can be identical (Figure 3.1). Determining if an achene is 'plump' in *Protea* species is further complicated by the fact that the achenes are covered with long trichomes and achenes that lack seed are often reinforced with extra woody tissue giving the illusion of an embryo containing achene (Figure 3.1) (Rebelo 2006; Eliovson 1965; Vogts 1958; Horn 1962). This extra woody tissue is thought to be an adaptation to protect against herbivores from easily selecting only filled achenes (Wright 1994).

The Protea Atlas (Rebelo 2006) suggests that achenes that are 'plump' and hard (reinforced with woody tissue) will not germinate but rather achenes that are 'plump' as well as pliable should be selected for propagation (Figure 3.1). This tactile method of selecting seeds on the basis of 'plumpness' in estimating seed presence has been used by Hargreaves *et al.* (2004) in *P.*

roupelliae ssp. *roupelliae* and Esler *et al.* (1989) in *P. compacta*. Horn (1962) used the method for selecting seeds from 19 *Protea* species, obtaining germination percentages from 21.3% to 75.9% and described it as ineffective. Recognizing a "plump" achene is a skill acquired through practice and is only gained when many achenes are available. The pressure required to test whether the achenes are both plump and pliable can damage the seed resulting in broken cotyledons and poor germination (S. Tarlton *pers. obs.* 2008).

Determining the presence of an embryo within an achene based on mass is underpinned by the assumption that an achene containing an embryo will be heavier than an achene not containing an embryo. Selecting achenes in an objective manner ensures that individual achenes are not discarded due to the effectiveness of predator defence mechanisms or achene polymorphism. Weighing each achene is an exercise that requires neither previous knowledge of achenes nor, strong sensitive fingers. However, the process is time consuming.

In both a tactile and a mass based selection process it is inevitable that a larger achene size will be selected. This will impose an artificial selection pressure and the resulting populations could well exhibit decreasing phenotypic plasticity and a decreasing ability to establish self-sustaining populations (Kramer & Havens 2009; Broadhurst *et al.* 2008).

3.3. Aims and objectives

The aim was to relate the variation in size (mass) of the achenes produced by *Protea roupelliae* ssp. *hamiltonii* to achene germination and parent plant size.

The objectives were:

1. To describe the mass distribution of achenes in relation to:
    a. Whether an achene was filled with an embryo or not.
    b. Variation within and among plants.
    c. The canopy area of the parent plant.
    d. The germinability of achenes.
2. To compare the accuracy of selecting filled achenes tactilely and on the basis of achene mass.

3.4. Materials and methods

3.4.1. Individually weighed achenes

Five randomly selected plants were measured (the longest canopy diameter and the perpendicular canopy diameter) and the cones produced in the 2006/7 fruiting season were harvested in March 2008. The cones were placed in brown paper bags and left in ambient laboratory conditions to dry for one month. The achenes were carefully removed from the receptacle and the achenes were weighed to within 0.1mg. Each achene was placed inside a 1.5ml microcentrifuge tube (Figure 3.2.a). The microcentrifuge tubes had both the lid and bottom perforated, contained a small piece of sterile cotton wool which had been autoclaved at 104 kPa at 120 ºC for 20 minutes. The achenes were irrigated (using a syringe) with 0.5 ml sterile ultra-pure water (milli-Q filtration and autoclaved at 104 kPa at 120ºC for 20 minutes).

All the achenes (in their individual microcentrifuge tubes) from each cone were placed into Ziploc® bags and maintained at growth room conditions (25±2ºC and a 14/10 hour photoperiod with a 200µmol m-2.s-1 photosynthetic photon flux density) and observed every second day for germination. The process of germination was considered complete once the radical had protruded 2mm from the pericarp. Achenes that had not germinated within 30 days were dissected in order to ascertain whether the achenes contained an embryo or not (Figure 3.2b and c).



a.

b.

c.

Figure 3.2. (a) An achene and wet cotton wool in a perforated 1.5ml microcentrifuge tube. A dissected achene filled with an embryo (b) and an achene not containing an embryo (c).

Table 3.1. Number of cones and achenes sampled per plant

Plant Number	Plant canopy area (m^2)	Number of cones weighed	Number of achenes
A13	0.264	4	495
162	0.475	3	360
29	0.291	1	142
A8	0.331	2	286
A27	0.683	5	399
Total		15	1682

Data analysis

Mass distribution

Achene mass size class distributions (SCD) were plotted using mass classes of 2.0mg intervals for (a) all achenes, (b) filled achenes and (c) achenes that germinated within 30 days (germinable). These mass class distributions were compared using Kolmogorov–Smirnov tests. The relationship between each mass class and the percentage of achenes filled (germinable and un-germinable) and achenes germinated was also explored using regression analyses.

The mean masses of achenes that were (a) filled and germinable, (b) filled with an embryo but did not germinate (un-germinable) and (c) unfilled with an embryo, were compared using an ANOVA followed by Tukey HSD (α=0.05). The coefficient of variation of mass for all achenes as well as for a, b and c (above) were determined.

Germination of achenes in relation to individual achene mass

The cumulative germination for all five plants combined was plotted. The time taken for 50% (T_{50}) of the filled achenes in each mass class to germinate and the mean number of days for all the filled achenes of a mass class to germinate was calculated. The Peak Value of germination (P.V.) was determined by identifying the maximum quotient derived by dividing the daily cumulative germination by the number of days taken to reach that percentage for each mass class (Czabator 1962). The correlation between mass classes and the T_{50}, mean number of days until germination and the P.V. was calculated.

3.4.2. Mass distribution of achenes from individual plants

The mean achene mass of the five plants was compared using an ANOVA and Tukey HSD (α=0.05) and the between plant coefficient of variation.

The canopy dimensions were used to calculate the canopy area as follows:

$$\text{Area} = \pi\ (d_1/2)\ (d_2/2) = 0.7854.\ d_1\ .d_2$$

Where d_1= longest plant diameter and d_2= the diameter perpendicular longest diameter of each plant.

The regression between the mean mass of the achenes produced (dependant variable) by each plant with that plants' (a) canopy area, (b) the number of cones it produced, (c) the percentage of

the achenes that were filled with an embryo, (d) the mean number of days the achenes from that plant took to germinate and (e) P.V. was determined.

Plants that produced heavy, intermediate and light achenes

The mean mass of the achenes produced by each of the five plants was calculated and compared with an ANOVA and a Tukey HSD (α=0.05) and three significantly different categories (heavy, intermediate or light) of achene producing plants were found. The categories heavy and light both consisted of single replicates. In order to maintain consistency in the number of replicates between categories, the plant with the highest number of achenes was used as the replicate for the intermediate category.

Mass size class distributions including the percentage of filled and germinable, filled and un-germinable as well as unfilled achenes per mass class were plotted for each of the three categories/plants. The cumulative germination and the final germination percentage after 30 days were also compared between plants.

3.4.3. Achene mass from plants with a range of canopy areas

On the 18th October 2008, 33 flowering plants (in three categories of small, medium and large were visually selected based on canopy size (see Chapter 2, Section 2.4.3). Note, these plants and achenes are distinct from those in Sections 3.4.1 and 3.4.2.

On the 18th of November 2008 the seeds from each cone were removed from the receptacles. The achenes from each cone were counted and weighed collectively to an accuracy of 0.1mg allowing for the mean achene mass to be calculated for each cone. On 1st December 2008 all the seeds from each cone were placed in the germination conditions as described in Chapter 2, Section 2.4.3.

Data analysis

Regression analyses were used to test the following relationships, between:

Canopy area and:

a. Mass of all achenes contained in each cone.
b. Mean achene mass of each cone.
c. Percentage of filled achenes per cone.

Mean achene mass and:

a. Percentage of achenes filled with an embryo.
b. The percentage of achenes that germinated.
c. The percentage of filled achenes within each cone that germinated.

Mean achene mass and:

a. P.V.
b. T_{50}.
c. The mean number of days for the filled achenes to germinate.
d. The peak day of germination.

3.4.4. Selection of filled achenes

Selection according to mass

The selection of filled achenes according to the mass of the achene assumed that a heavy achene contained an embryo and a light achene did not. Utilizing the plotted mass class distributions (described above), cut-off mass classes were identified. Starting with the heaviest mass classes and progressively selecting lighter mass classes until no fewer than 90% the selected sample of achenes filled with an embryo, this mass class would be the cut-off mass class. A cut-off achene mass class was found for:

a. Achenes from all plants combined.
b. The three plants of significantly different mean achene masses combined.
c. Each of the three plants of significantly different achene masses (individually).

Other values calculated included the percentage of filled achenes that were below the cut-off mass (and therefore not selected) and the percentage of total achenes (filled and unfilled) that were selected.

Tactile assessment of achenes

The tactile assessment was based on a "plump" feeling achene being filled with an embryo whereas an achene that did not feel "plump" was not filled. Achenes were considered "plump" if the seed-containing region was not corrugated, did not collapse under gentle pressure nor felt hard. These plump feeling achenes were manually selected for the seed storage treatments (Chapter 5). 10337 achenes were tested for "plumpness" and those that were found to be plump were placed in storage treatments, and germinated (under standard germination conditions as described in Chapter 2 Section 2.4.3). Those that did not germinate were dissected to verify the

presence of an embryo. The percentages of the total achenes selected and the percentage of those that were filled with an embryo were used to compare the efficacy of a tactile assessment versus the selection of filled achenes according to achene mass.

An ANOVA was used to compare (a) the percentage of achenes selected on a plant by plant basis according to the achene mass method (above) with (b) the percentage of achenes selected by the tactile method, (c) the seed set of the 33 plants with a range of canopy areas and (d) the seed set of the five plants with individually weighed achenes.

3.5. Results

3.5.1. Individually weighed achenes

The shape of the overall achene mass distribution (Figure 3.3a.) resembled a bimodal distribution, the "lighter" peak was considerably reduced when the un-filled achenes were removed from the distribution (Figure 3.3b). Achenes filled with an embryo were significantly heavier (25.33±0.20mg) than those that were un-filled (17.29±0.28mg) (mean±S.E.) (Figure 3.4a). The percentage of filled achenes was 43.6%.

The final germination percentage of all the achenes combined (filled and unfilled) from the five plants was 41.9 % after 30 days (Figure 3.6). Only 43.6% of the achenes were filled with an embryo, but their viability was high, as 96.1% of the achenes filled with an embryo were germinable.

There were no significant relationships between the achene mass class distributions from these five plants and the germination parameters measured (P.V., the mean days until germination and the T_{50}) (Figure 3.7). Figure 3.7 should be interpreted in conjunction with Figure 3.3 and 3.5 as the two lightest mass classes contained very few germinable achenes, in fact mass classes of 10-11.9mg and 12-13.9mg contained only 1 and 4 achenes respectively.

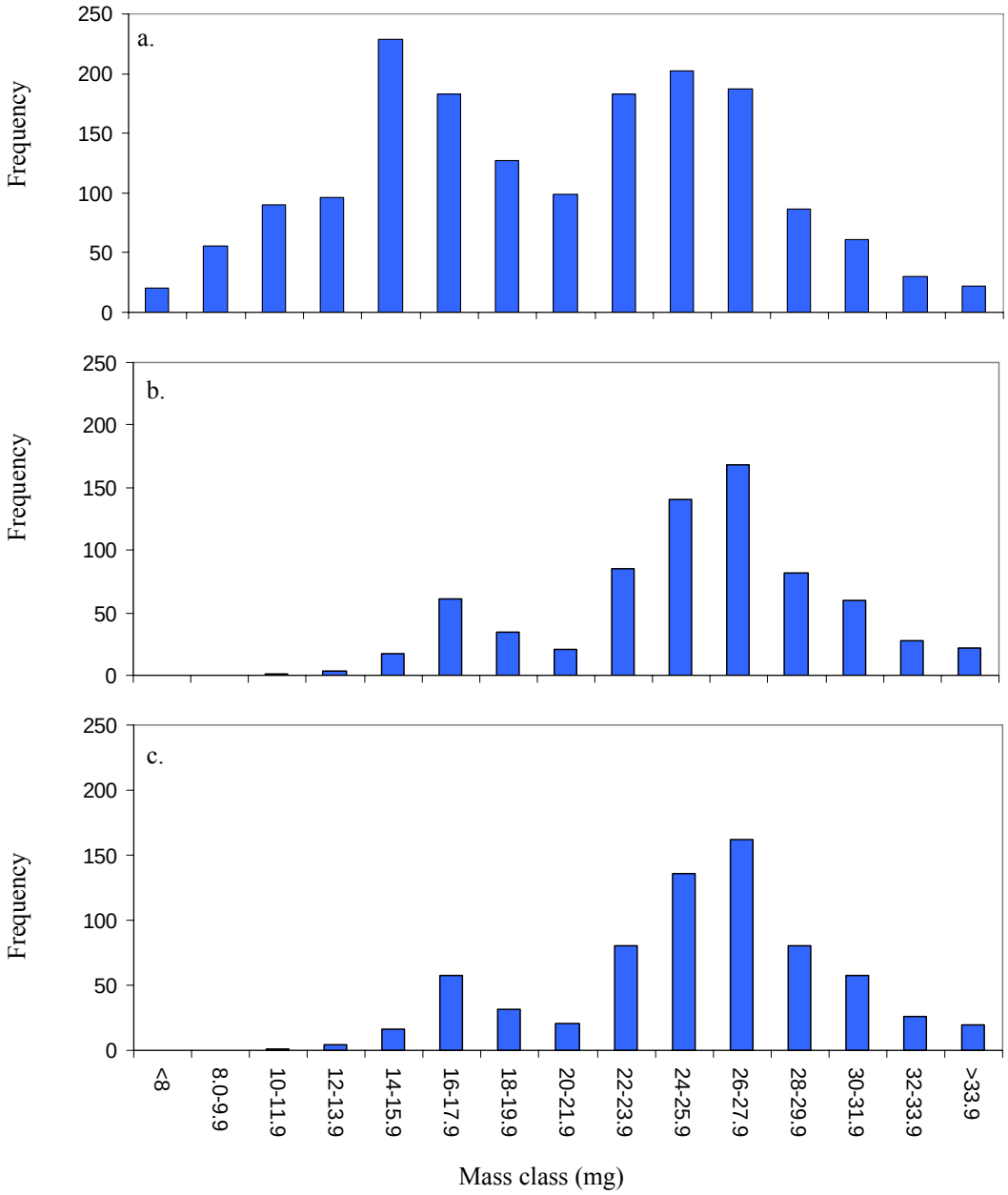


Figure 3.3. The frequency distribution of the 1682 individual achenes from 5 plants, (a) combined, (b) only the achenes filled with an embryo and (c) only those that germinated. The Kolmogorov–Smirnov test showed a significant difference in the mass distributions of a and b ($D=0.347$; $P<0.001$) but no significant difference between b and c ($D=-0.095$; $P>0.1$).

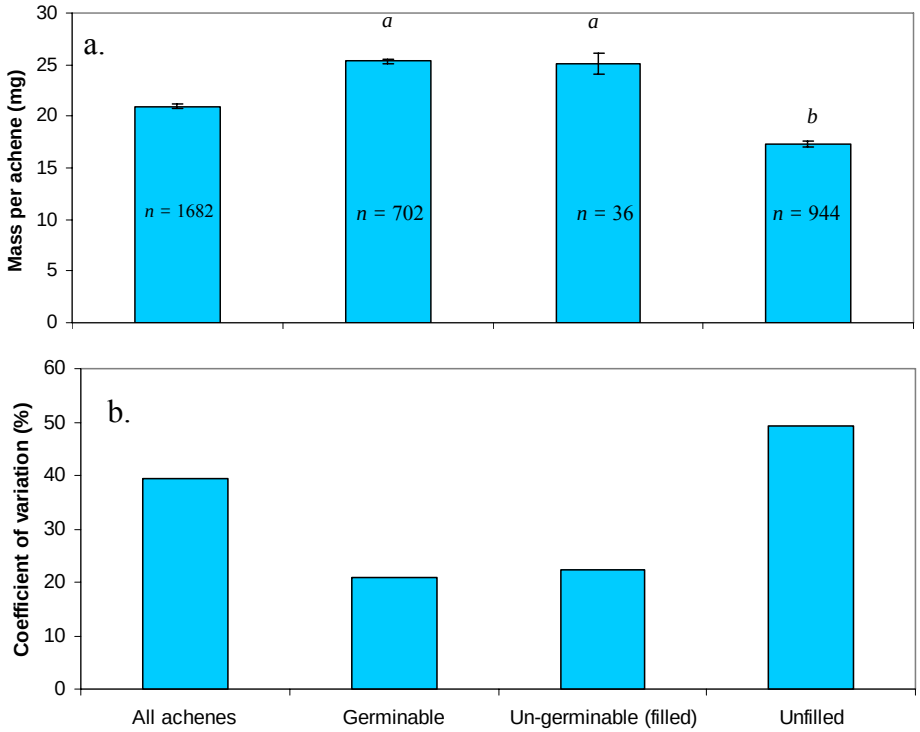


Figure 3.4. (a) Mean (± SE) achene mass and (b) coefficient of variation of all *Protea roupelliae* ssp. *hamiltonii* achenes in relation to whether they were filled with an embryo and germinable, filled and un-germinable or were un-filled. Differing letters indicate a significant differences (Tukey HSD; $P<0.05$).

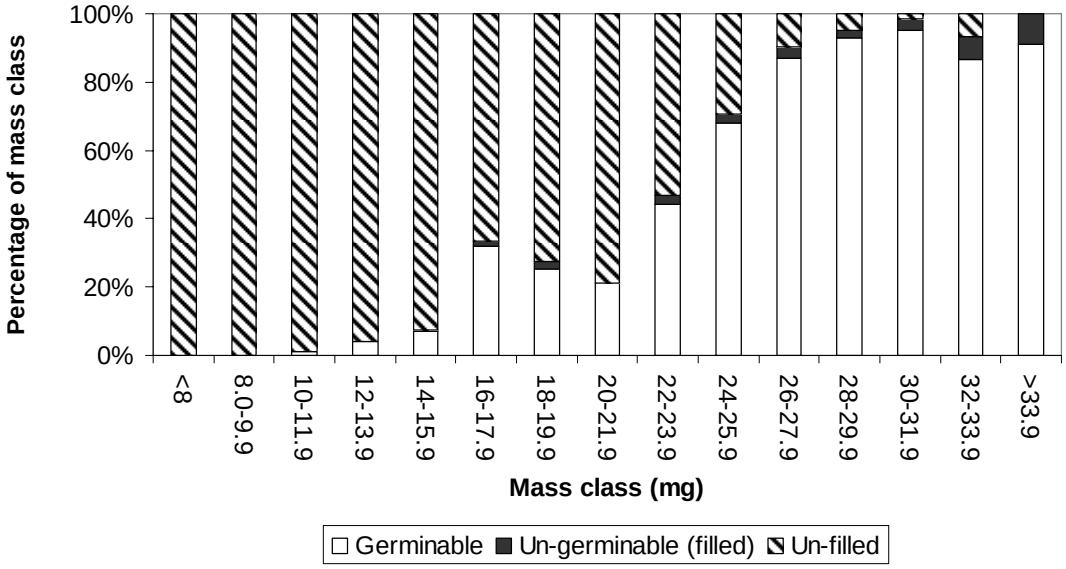


Figure 3.5. Percentages of achenes that were either filled and germinable, filled and un-germinable or un-filled, as a percentage of each weight class. The percentage of achenes that were filled (germinated and un-germinated) increases linearly with achene mass class ($R^2=0.8662$, $P<0.0001$) as did the percentage of germinated seeds ($R^2=0.8565$, $P<0.0001$).

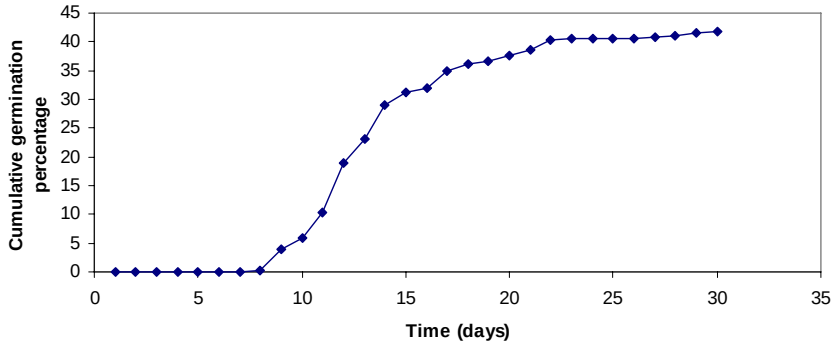


Figure 3.6. Cumulative germination percentage of all achenes (including unfilled achenes) from the five plants combined.

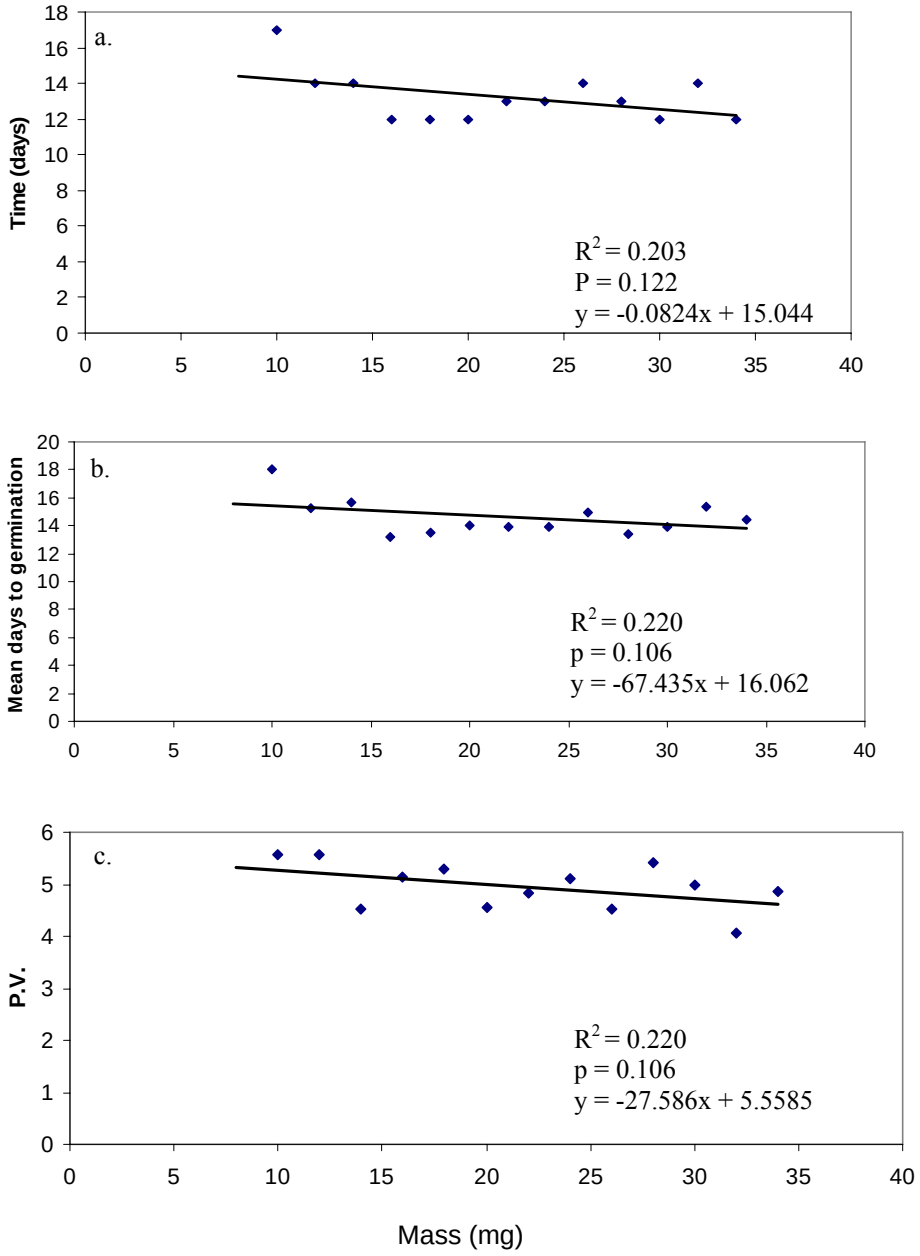


Figure 3.7. Regressions between the achene mass class and (a) the time taken for fifty percent of the filled achenes to germinate (T_{50}), (b) the mean number of days for achenes of each mass class to germinate, as well as (c) the peak value of germination (P.V.). No significant relationships were found ($P<0.05$).

3.5.2. Achene mass distribution of achenes from individual plants

Different *P. roupelliae* ssp. *hamiltonii* plants produced achenes of significantly different mass (Figure 3.8a.). The mean coefficient of variation for all five plants was 25.39% for all the achenes, 9.15% for germinable filled achenes, 8.80% for un-germinable filled achenes and 14.49% for the unfilled achenes. The mean mass of the achenes was not significantly related to the canopy areas of the five plants measured (Table 3.2).

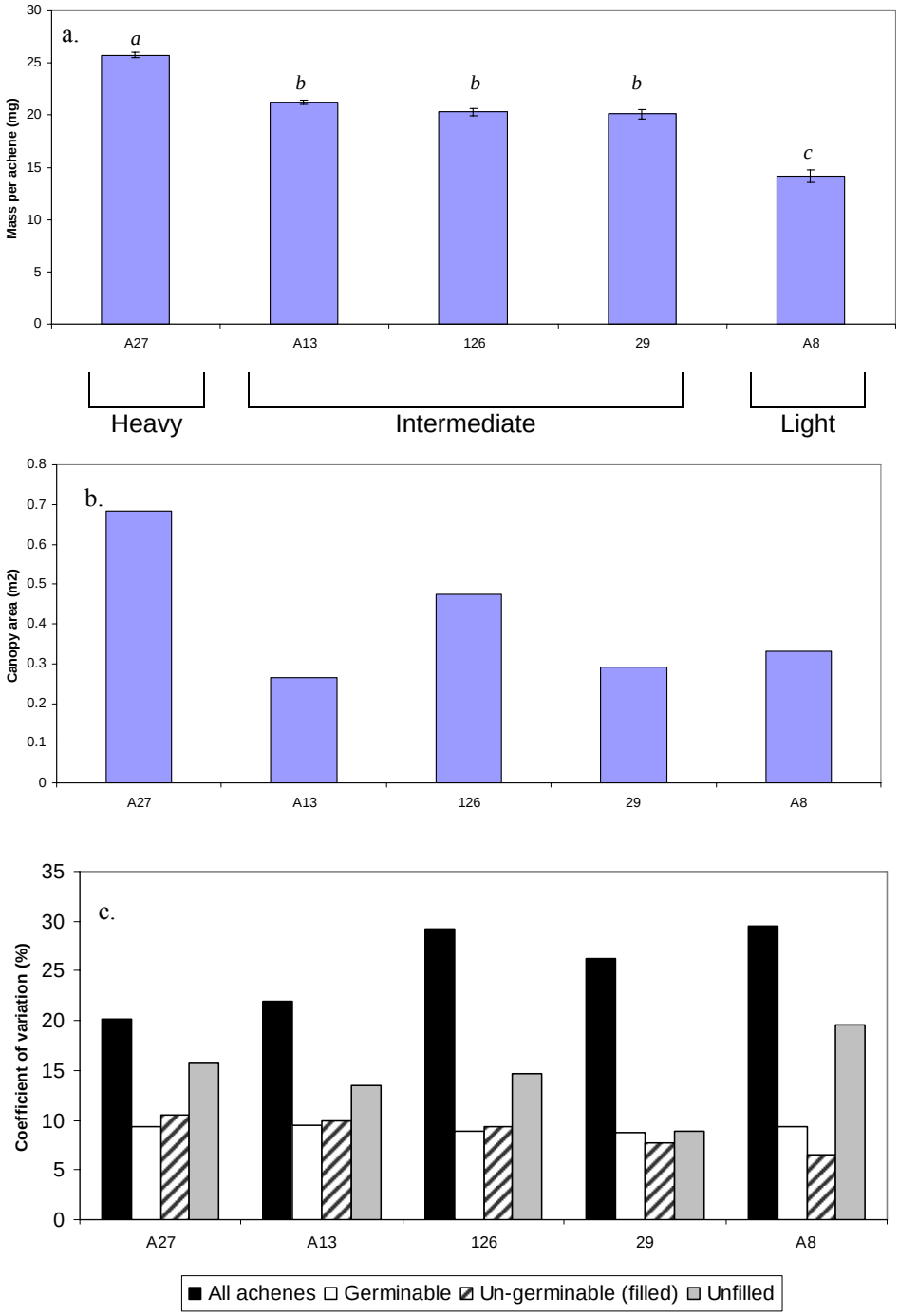


Figure 3.8. The five plants in terms of their (a) individual mean (±SE) achene mass, (b) the canopy areas and (c) the coefficient of variation of the mass of (i) within all the achenes, (ii) achenes that were germinable (filled), (iii) un-germinable (filled) and (iv) the un-filled with an embryo in all five plants. Differing subscript letters indicate plants with significantly different mean achene mass ($P<0.05$).

Table 3.2. Linear regression analyses of the relationships between mean plant achene mass and plant physical attributes as well as germination parameters of the five plants from which individual achenes were weighed. No significant regressions were found ($P<0.05$).

Attribute	R^2 value	P value	Regression equation
Canopy area	0.4202	0.2368	y = 27.099x - 0.141
Number of cones produced	0.4204	0.1637	y = 945.52x - 12.784
percentage of the achenes filled	0.0001	0.9835	y = -40.404x + 45.105
Mean days until germination	0.3599	0.2849	y = 154.56x + 10.737
P.V.	0.2921	0.3470	y = -68.044x + 6.284

Mass distribution and germination of heavy, intermediate and light achene producing plants

The quasi-bimodal distribution of the achene mass classes was present for each plant individually (Figure 3.9a). When only the filled achenes were plotted (Figure 3.9b), it became apparent that the shape of the distribution of each plant may have been the product of two unimodal distributions, namely (a) the filled achenes and (b) the un-filled achenes.

Filled achenes may occur over a wide range of masses (from 10 to over 34mg) but each plant showed a degree of autonomy with regard to the mass of the achenes they produced (Figure 3.10). When the achenes from multiple plants were combined (Figure 3.3a and 3.5), a range of "light" mass classes (16.0 –25.9mg) were found to contain a large number of both filled and un-filled achenes. Most of the filled achenes found in these lighter mass classes were from one individual plant (A8) that produced lighter achenes (Figure 3.9b and 3.10c) and therefore were not individual, out-lying achenes. When each plant was considered independently, the range of mass classes in which filled achenes occurred was more limited (Figure 3.9), than when data from all the measured plants were combined (Figure 3.3a).

Although the mass of the achenes these three plants produced was significantly different, the cumulative germination and the final germination percentages of the three plants were similar (Figure 3.11).

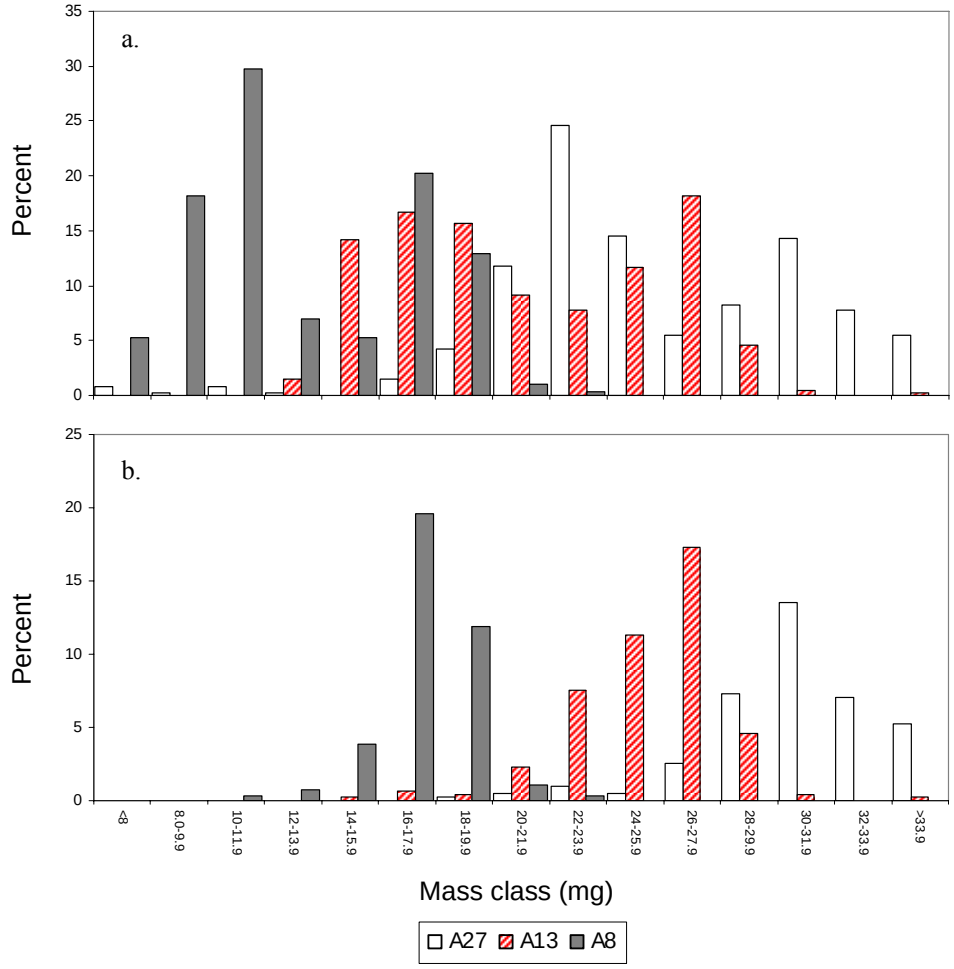


Figure 3.9. Mass distribution of plants with heavy (Plant A27), intermediate (Plant A13) and light (Plant A8) achenes, for (a) all the achenes and (b) filled achenes as a percentage of the total number of achenes from each plant in each mass class.

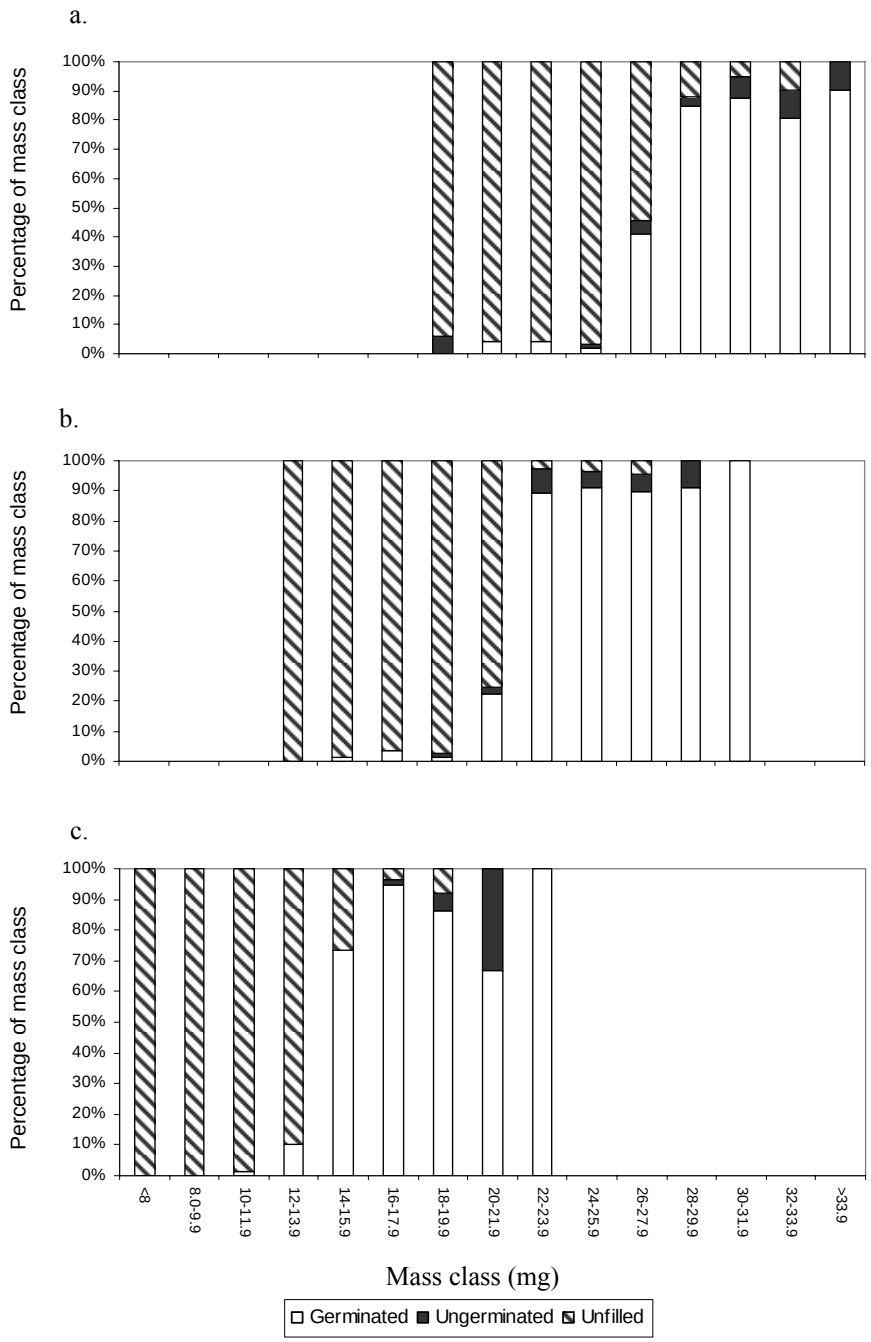


Figure 3.10. The percentage of germinable, un-germinable (filled) and un-filled achenes in each achene mass class according to plants with an overall achene mass categorised as (a) heavy, (b) intermediate and (c) light.

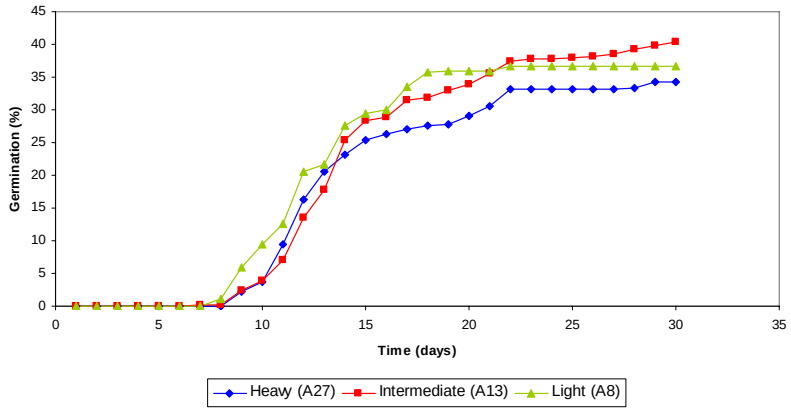


Figure 3.11. Cumulative germination percentages (of all achenes including unfilled achenes) from plants that produced heavy (plant A27), intermediate (plant A13) and light (plant A8) achenes. The final germination percentage for the three categories was 34.3%, 40.4% and 36.7% respectively.

3.5.3. Achene mass from plants with a range of canopy areas

The mean (±S.E.) percentage of filled achenes per cone was 36.08±2.31% and the germination percentage was 31.2±2.61%. Of the filled achenes produced in each cone, a mean (±S.E.) of 83.1±3.42% germinated. A significant relationship was found between the mean achene mass of each cone and the P.V., T_{50} and the mean number of days until the germination of the achenes (Figure 3.14).

The total mass of the achenes within a cone was significantly related to the canopy area of the plants from which they were harvested (Figure 3.12a). The mean mass per achene of each cone was significantly related to the canopy area of the parent plant (Figure 3.12b). The percentage of filled achenes per cone (seed set) was not significantly related to the canopy area of the parent plant (Figure 3.12c).

The mean achene mass of each cone was significantly related to the percentage of filled achenes per cone and the percentage of achenes that germinated per cone (Figure 3.13a and b). Plants that produced cones with a higher mean achene mass had a higher percentage of filled achenes germinating (Figure 3.13c)

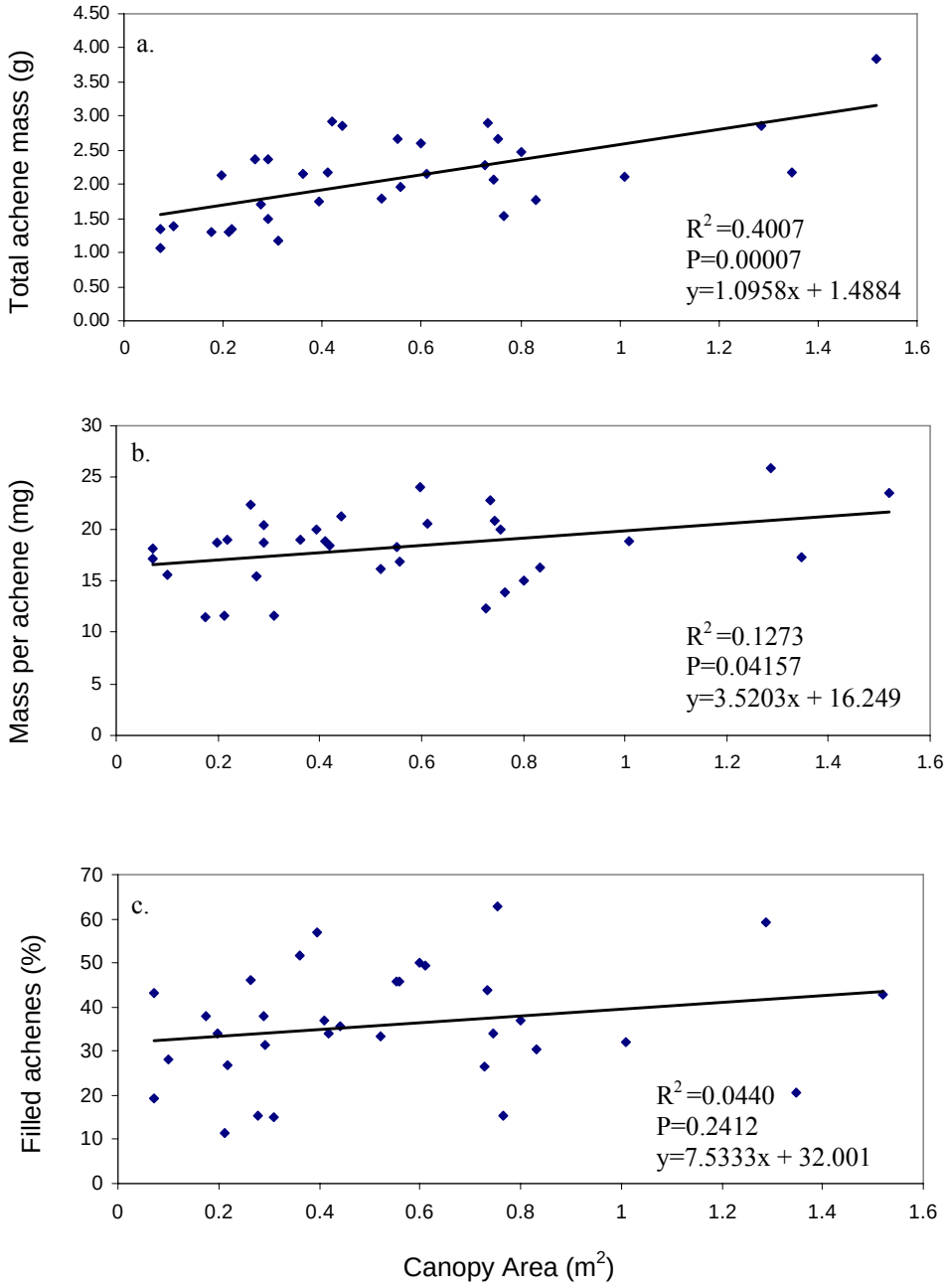


Figure 3.12. Linear regression relationships between the canopy area of 33 plants and (a) the total mass of achenes within a cone, (b) the mean mass of the achenes produced per cone, (c) the percentage of filled achenes in a cone (seed set). Significant regressions correspond to $P<0.05$.

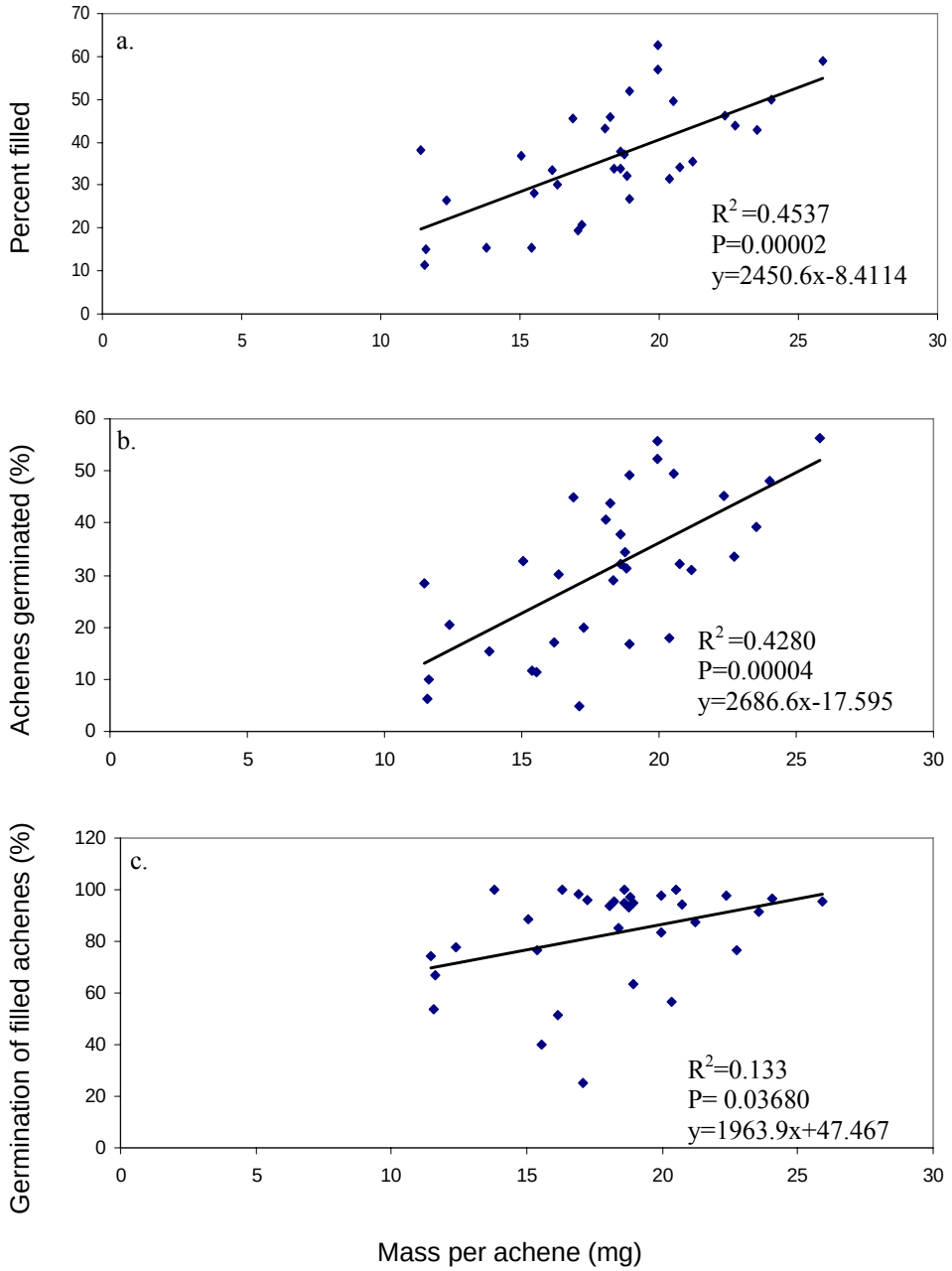


Figure 3.13. Linear regression relationships between the mean mass of the achenes produced per cone from different parent plants and (a) the percentage of filled achenes produced per cone, (b) the percentage of achenes that germinated, (c) the percentage of achenes filled with an embryo that germinated. Significant regressions correspond to $P<0.05$.

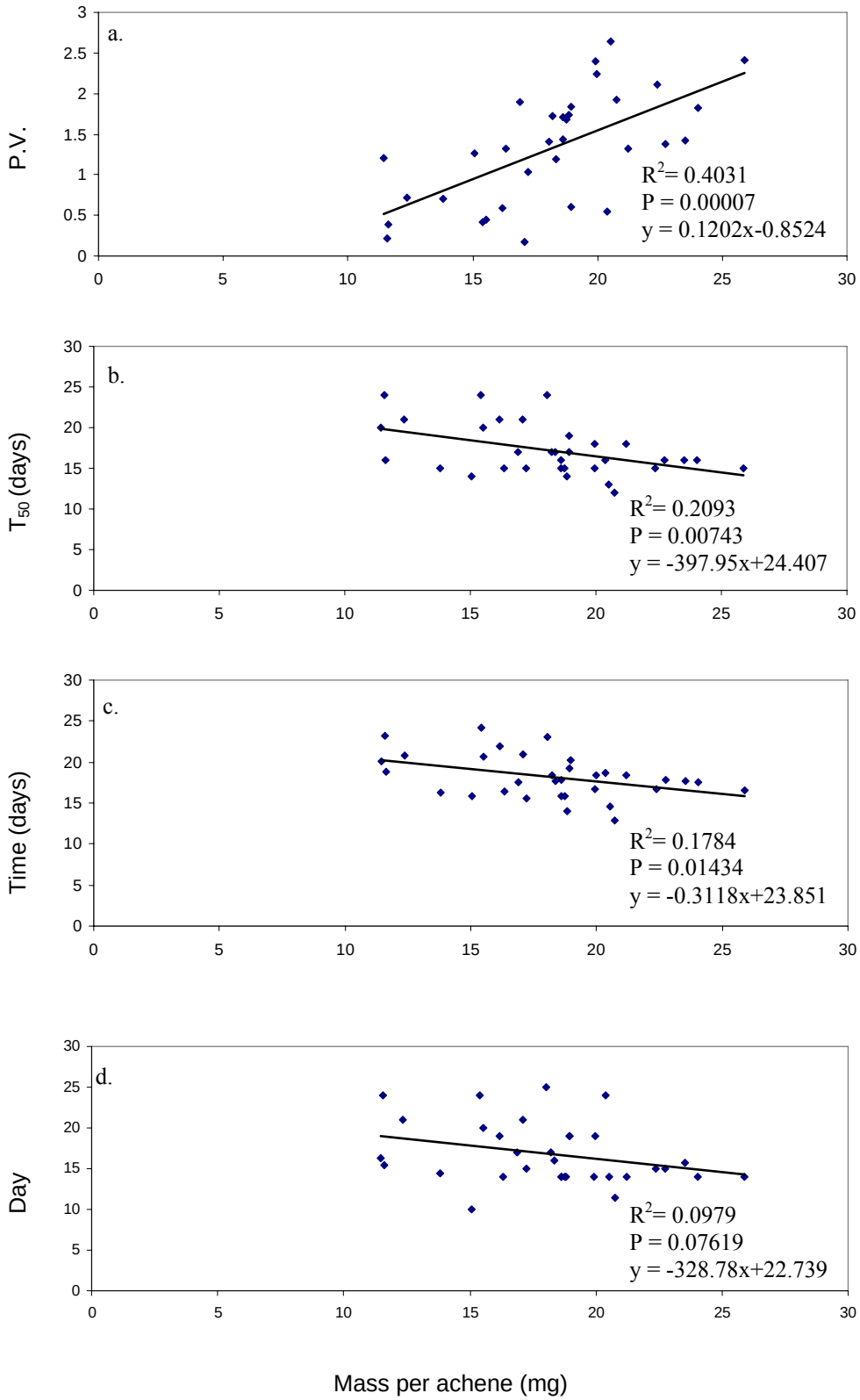


Figure 3.14. Linear regression relationships between the mean achene mass produced per cone and the following germination parameters; (a) the P.V., (b) the time taken for 50% of the filled achenes to germinate, (c) the mean number of days for achenes to germinate (d) the day that had the highest percentage of achenes germinating. Significant correlations correspond to $P<0.05$.

3.5.4. Selection of achenes

Selection according to mass

It was possible to select a sample of achenes that containing over 90% filled achenes based on the mass of the achenes alone regardless of whether the data of all the plants were combined or plants were analysed individually (Table 3.3). Furthermore it was possible to select a higher percentage of the filled achenes and a higher percentage of the total achenes produced by plants when selecting on a plant-by-plant basis (Table 3.3).

Table 3.3. Minimum cut-off mass classes, above which a sample of achenes contained over 90% filled achenes. Cut-off mass classes were identified for the five plants combined, three plants per seed mass category combined and each plant per seed mass category separately.

Plants achenes are selected from	Cut-off mass(mg)	Percentage of filled achenes above cut-off mass (selected)	Percentage of filled achenes not selected	Percentage of total achenes selected
Combined plants				
All plants	24.0	90.93	50.34	23.15
Heavy, intermediate and light	26.0	90.25	47.26	23.80
Heavy (Plant A27)	28.0	92.31	12.58	35.84
Intermediate (Plant A13)	22.0	96.59	7.91	42.90
Light (Plant A8)	14.0	92.11	2.80	39.96

Selection methods

Assuming that the percentage of filled achenes (seed set) was constant in all achene samples, the percentage of filled to unfilled achenes selected according to mass on a plant-by-plant basis (39.5±2.0%) was not significantly different from the percentage of achenes selected through tactile selection (44.2±1.1%, mean±S.E.) (Table 3.4). Nor was the percentage of filled achenes selected according to mass on a plant-by-plant basis significantly different from the seed set of both the 33 plants with a range of canopy areas (36.1±2.31%, mean ±S.E.) and all five of the plants that had their achenes individually weighed and germinated (44.29±3.72%, mean±S.E.) (ANOVA and Tukey post hoc test; $P<0.05$).

Table 3.4. A comparison of two methods for selecting filled achenes; by mass and tactile assessment.

	Percentage (±S.E.) of achene selection filled with an embryo	Percentage (±S.E.) of total achenes selected
Selection according to mass		
All plants combined	90.93	23.15
Individual plants (mean)	93.67±1.46	39.50 ±2.02
Tactile selection	92.26±0.78	44.20±1.08

3.6. Discussion

3.6.1. Seed set of *Protea roupelliae* ssp. *hamiltonii*

A seed set (mean±S.E.) of 44.29±3.72% (five plants weighed and germinated) and 36.1±2.31% (33 plants of varying canopy area) was found and could be considered low for most plant species, but was high for *Protea* species which generally have a mean seed set of 9.2±7.2% (±S.D.) (n=41 species) (Collins & Rebelo 1987). This seed set was still high when compared with the summer rainfall tree form subspecies (*Protea roupelliae* ssp. *roupelliae*) with a seed set of about 30% for open pollinated cones (Hargreaves *et al.* 2004).

3.6.2. Achene mass distribution

Most plant species, including *Banksia* (Vaughton & Ramsey 1998), have a greater coefficient of variation within a plant than among plants of a population (Michaels *et al.* 1988). This was not the case with *P. roupelliae* ssp. *hamiltonii* as the total variation of achene mass within each plant was less than that among plants (Figure 3.4b and 3.8c) although only five plants were studied. As a consequence there was a clearer, more defined difference in each plant between filled and unfilled achenes with regard to mass (Figure 3.5 and 3.10).

The variation in the mass of unfilled achenes was more than twice that of the filled achenes for combined plants (Figure 3.4) and in individual plants unfilled achenes had a mean C.V. 5.3% higher than the mean C.V. of filled germinated achenes (Figure 3.8c). This higher mass variation in unfilled achenes may be explained by the fact that some unfilled achenes are reinforced with extra woody tissue (Figure 3.1) and may enhance their role in avoiding seed predator avoidance (Wright, 1994).

There were no significant relationships between the mass of individual achenes and their germinability (T_{50}, mean days until germination and P.V.) when individually weighed achenes were not associated with their parent plants (Figure 3.7). Therefore there was no short term advantage for achenes of *Protea roupelliae* ssp. *hamiltonii* to be of higher mass with regard to germination. This is consistent with other investigations considering the influence of seed size in relation to germination in other Proteaceae species for example; *Protea aurea, P. lacticolor, P. punctata, P. subvestita* (Carlson & Holsinger 2010), *P. lorifolia, P. cynaroides, Leucadendron laureolum, Hakea sericea, Banksia laricina* (Stock *et al.* 1990) and *Banksia cunninghamii* (Vaughton & Ramsey 2001).

With a small sample size of five plants there were no significant relationships between the mean mass of the achenes and the canopy area, cone production as well as the germination parameters calculated for each plant (Table 3.2). However, with a larger sample size (n=33), the mean mass of achenes produced by different plants was significantly related to the P.V., the T_{50} and the mean day till germination (Figure 3.14a-c). Additionally, the mean mass of the achenes per cone was related to the percentage of filled achenes in a cone that germinated within 30 days (Figure 3.13c). If these relationships hold for horticulturally valuable *Protea* species, the simple process of weighing and counting the achenes of a harvested cone may allow the industry to select cones that have a higher percentage of filled achenes (Figure 3.13a) with better viability and higher vigour. Although the canopy area of a plant may be significantly related to the total mass and the mean achene mass of the achenes produced by a plant, there was no relationship between canopy area and seed set (Figure 3.12c). This variation in the germination between plants indicates that the maternal effect that *P. roupelliae* ssp. *hamiltonii* plants hold over their achenes is high (Gutterman 2000), even between plants from the same population (see Chapter 2). This ensures that, as with other *Protea* species, the germination of the seed crop from *P. roupelliae* ssp. *hamiltonii* is spread over an extended period (Deall & Brown 1981).

Cones of *Protea roupelliae* ssp. *hamiltonii* plants vary in colour from light green/white though pink to almost red (Chapter 2). Carlson and Holsinger (2010) found that cones with white bracts produced achenes that were 10% heavier and 3.5 times more likely to germinate, whilst achenes produced from plants with pigmented (pink) bracts were less susceptible to herbivory. This indicates there may be a genetic or phenotypic link between achene mass and germination (Carlson & Holsinger 2010). Additionally their study demonstrates that although achenes from plants with pigmented bracts have a low germination percentage and are lighter (in mass), discarding them in a selection process would predispose the resulting population to higher levels of herbivory (Carlson & Holsinger 2010). Therefore in order to maintain the phenotypic variation and protection from herbivory this variation may afford the population, it is essential that if achenes are selected according to mass, each plant is considered individually.

Filled achenes may be found in mass classes ranging from 10 mg to over 34mg and this variation in mass may have implications for seed dispersal. The distance that a *Protea* achene is dispersed is related to the tuft size of the trichomes on the seed and the wing loading (seed mass) (Bond 1988). Heavier *Protea* achenes are dispersed significantly shorter distances during primary dispersal (Bond 1988), whereas the length of the trichomes (wind interception) has been shown to

have a greater effect during the secondary dispersal phase (Schurr *et al.* 2005; Bond 1988). During secondary dispersal achenes act as "tumble seeds" and through this mechanism, it has been reported that the achenes of *P. repens* can travel up to 500m depending on the substrate and wind conditions (Bond 1988). As a dwarf shrub it is likely that the stature of *P. roupelliae* ssp. *hamiltonii* would have a greater effect on primary dispersal than the mass of the achenes it produces.

3.6.3. Selection of achenes; tactile versus mass based methods

Horn (1962) used the tactile method for separating "plump" achenes and commented on its efficacy, saying "we are inclined to ascribe the main cause for the unsatisfactory germination in Proteaceae to ineffective means of separating fertile from infertile seed." However, the method of tactile selection has not been improved upon since his use. Tactile selection is quick, simple, requires no equipment and the method was found to be effective in this present study by selecting a sample of achenes with an accuracy of 92.26%. With this high accuracy there may be no reason for the development of another method of selection, however the mechanisms used in a tactile selection may not be favourable for its use in all situations. As achenes are either 'plump' or not, no subtle differences in the feel of the filled achenes can be accepted. The consequence of selecting only "plump" feeling achenes in restoration or *ex-situ* conservation of a species may be to decrease the phenotypic variation of the F1 population's achene production.

The selection of achenes according to mass was shown to be a feasible alternative and possibly a more effective method, albeit only true for individual plants where the coefficient of variation was relatively low (Figure 3.4b and 3.8c). The necessity to ascertain the mass distribution of both filled and unfilled achenes so that the cut-off masses could be identified did make this method more time consuming and cumbersome. The "dip" in the quasi-bimodal distribution allowed for separation between the mass classes of filled and unfilled achenes. For individual plants most of the cut-off mass classes (Table 3.3 and Figure 3.10) were at or, one mass class heavier than this "dip." In individual plants the cut-off mass classes were simple to identify and only excluded an average of 7.2% of the available filled achenes (Table 3.3). The "cut off" mass classes were chosen in order to select a sample containing 90% filled achenes, this could be considered unnecessarily stringent for undomesticated, critically endangered species where filled achenes may be of great value (Kay 2008). Selection by mass is based on a continuous range of masses and not the binary "plump" versus "un-plump" found in tactile selection, as a result the cut-off mass can be lowered in order to include a buffer of unfilled achenes. By including a buffer of

unfilled achenes all the filled achenes may be selected whilst excluding most of the unfilled achenes.

Combining the achene mass data of all five plants (where there a was higher coefficient of variation in achene mass) the cut-off mass class was in the centre of the second peak (Figure 3.3) and only selected 49.7% of the available filled achenes (Table 3.3.). Examination of Figure 3.9a and b. shows that the cut-off mass of 24.0mg (Table 3.3.) would completely exclude the achenes (filled and unfilled) produced by the plant that produced light achenes (Figure 3.10). Therefore the selection of achenes by mass for various plants combined cannot be advocated in the conservation of an endangered species such as *P. roupelliae* ssp. *hamiltonii*, as it is wasteful of filled achenes and would decrease the genetic diversity of the achenes selected (Way 2003).

3.7. Conclusion

3.7.1. Achene mass and germination

The mass class distribution of all the achenes combined and individual plants showed a quasi-bimodal distribution, which changed into a quasi-unimodal distribution when the unfilled achenes were removed from the sample (Figure 3.3 and 3.9). This indicated that unfilled achenes were not randomly spread throughout the achene mass distribution of the population or individual plants. In fact the mean mass of achenes filled with an embryo was significantly higher than those without an embryo (unfilled), regardless of whether or not the achene was germinable (Figure 3.4). This increased mass of filled achenes explained the highly significant relationship between the mean achene mass and both the percentage of filled achenes per cone as well as the germination percentage of achenes per cone from the 33 plants (Figure 3.13a and b).

The mass of germinable achenes ranged from about 10mg to over 34mg (Figure 3.3b) however, the mass of individual achenes did not have a significant influence on germination of the achenes (Figure 3.7). The achene mass variation was lower in single plants than when data was combined from various plants, and therefore it may be assumed that sampling a single cone from a plant may give a good indication on the mass of all the achenes produced from that plant. The canopy area of plants (n=33) was significantly related to the total and mean achene mass and the mean achene mass in turn was related to the germinability of the achenes (Figure 3.13c and 3.14). This indicated that the parent plant has an effect on the mass and germination of the achenes but the effect that the actual mass of each achene has on germination is limited and reiterates the impact

the parent plant has on the germinablity of the achenes. The performance of the achenes may additionally be linked to the phenotype of the plant (Carlson & Holsinger 2010).

3.7.2. Achene selection

The method employed for the selection of filled achenes would therefore depend on the end use of the germplasm. Guidelines for *ex-situ* conservation through seed storage are stringent, aiming to incorporate the greatest possible genetic diversity of the population (Way 2003). Both methods of selection tested here rely on an assessment of the phenotype and do have some advantages but can exclude the extremes in both the "feel" and mass (lower end) of filled achenes. However, not knowing if sufficient germplasm (diversity and volume) has been collected may be detrimental to the conservation of the species (Way 2003). In a species such as *P. roupelliae* ssp *hamiltonii* where the surviving population is small and it is unknown if the species is self compatible, over harvesting may adversely affect the seed bank *in-situ*. Therefore a rapid method of assuring that sufficient filled achenes had been harvested from a sufficient number of plants would need to be employed in the field. For an "in-the-field" estimate of numbers, the accurate, rapid, technologically undemanding tactile selection achenes would be preferential. However, for making decisions concerning the genetic integrity of *ex-situ* seed banks and establishing populations *de-novo*, the more objective and possibly more inclusive mass based method of sorting achenes would be advisable.

Chapter 4

4. The propagation of *Protea roupelliae* ssp. *hamiltonii* via the rooting of stem cuttings, achene germination and micropropagation.

4.1. Abstract

The propagation of Proteaceae plants has received considerable attention due to the economic importance of members of this family in the horticulture industry. The cues and techniques for the maximum germination of achenes, the successful rooting of cuttings and tissue culture plantlet generation are considered to differ greatly between *Protea* species. The *ex-situ* plant production of the critically endangered *Protea roupelliae* ssp. *hamiltonii* was investigated in order to enhance the conservation of the species. Three methods were investigated *viz.* (a) semi-hard wood stem cuttings (b) germination of zygotic embryos (c) micropropagation through (i) direct organogenesis and (ii) indirect morphogenesis.

a. The rooting of semi-hard wood stem cuttings failed due to excessive phenolic oxidative browning of the cuttings.
b. The germination percentage of "plump" (presumably filled) *P. roupelliae* ssp. *hamiltonii* achenes that received no pre-treatment reached 92.88% within 27 days at a temperature of 25±2ºC with a peak value (P.V.) of 3.69. Soaking achenes in water for 48 hours without removing the pericarp decreased both the vigour (P.V. of 1.22) and germination percentage (82.11%). Removing the pericarp surrounding the embryo after 48 hours of soaking markedly increased both the germination percentage (97.00%) and vigour (P.V. of 6.03).
c. i. Micropropagation via the rooting of shoots was unsuccessful due to endogenous micro-organism contamination associated with the field material and phenolic oxidative browning.
c.ii. Zygotic embryos plated on various nutrient media failed to germinate normally. However morphogenic callus was initiated and adventitious roots, shoots and somatic embryos formed on all media tested, albeit very slowly (within ten weeks). The most effective medium (although not significantly different from other media) for adventitious somatic embryogenesis from intact zygotic embryos was composed of a growth regulator free medium containing Murashige & Skoog (1962) salts with vitamins (2.21g.l^{-1}), sucrose (30.00g.l^{-1}) and Gelrite® (3.00g.l^{-1}) with a pH corrected to 4.20. Zygotic embryos plated on this medium had a mean somatic embryo production of 4.66±1.09 (±S.E.) embryos per growing explant. However, significantly more somatic embryos were produced on plated zygotic embryonic axes (6.58±1.31 (±S.E.) embryos/explant) than intact zygotic embryos and no growth (somatic embryos or callus) occurred on excised cotyledons. Few adventitious somatic embryos developed into discernable shoots on a medium with a high gibberellic acid to cytokinin ratio (3:1 by mass). When somatic embryo explants were subcultured onto growth regulator free medium (above) corrected to a pH of 5.20, the primary somatic embryos underwent secondary embryogenesis. Secondary embryos were of higher quality (morphologically) than primary embryos and developed into plantlets with shoots and roots, but more often without roots on the same medium. Limited success (5.77%) was achieved in the rooting and hardening of shoots from converted embryos.

The propagation of *Protea roupelliae* ssp. *hamiltonii* plants via the germination of achenes without soaking is effective whilst conserving desired genetic diversity. The number of plants propagated through achene germination is however, limited to the number of available achenes. Propagule multiplication through indirect morphogenesis was achieved, which allowed for the

mass propagation of the species. However, more research is needed to improve micropropagatory protocols for this species.

Key words: Achene germination, excised embryos, micropropagation, phenolic oxidative browning, secondary somatic embryogenesis

4.2. Introduction

4.2.1. Propagation from seed

Protea plants produced from seed/achenes are slow to reach flowering age, therefore the use of seed by the horticultural industry is limited to the development of new cultivars and the production of rootstocks (Malan 1992). However, in an ecological setting where the conservation of genetic diversity is imperative, the use of sexually derived seed is the preferred method of preservation and propagation.

Germination

Agriculturally satisfactory techniques for breaking dormancy in *Protea* species are considered to be established (Malan 1995). The achenes of serotinous Proteaceae are thought to have a low temperature requirement for germination (Mitchell *et al.* 1985). In commercial Proteaceae species a germination percentage of 80% should be achievable by incubating seeds at 5 to 12ºC for 20 to 40 days in moist conditions (Malan 1995). Although most research has been performed on winter rainfall species or species of high horticultural value, it is still recommended that seed is sown during autumn or winter in summer rainfall areas of South Africa (Matthews 1993; Eliovson 1965; Vogts 1958). Observations of very young *Protea roupelliae* ssp. *hamiltonii* seedlings were made on November 21 in the Dr Hamilton Reserve (Figure 4.1) (S. Tarlton *pers. obs.* 2009). November is the beginning of spring, which is associated with an increase in both moisture and temperature in the Barberton Montane Grassland vegetation unit in which the Dr Hamilton Reserve is located (Mucina & Rutherford 2006).



Figure 4.1. *Protea roupelliae* ssp. *hamiltonii* seedling found on the 21st of November 2009 having recently germinated in the Dr Hamilton Reserve with the emergence of only the epigeal cotyledons (bar equates to approximately 5mm).

Scarification and soaking

The soaking of *Protea* seeds was suggested as a means of leaching out possible germination inhibitors (Vogts 1960) and improving germination (Matthews 1993). However, soaking seeds of *Protea compacta* and *P. barbigera* decreased the germination percentage in relation to the number of days soaked, even when soaked in running water (Brown & Van Staden, 1973a; Brown & Van Staden, 1973b). Removing some of the pericarp of various parts of the achene increased the germination rate of *P. compacta* seeds significantly. The full removal of the seed coat from the embryo resulted in rapid germination but the excised embryos started rotting after 10 days (Brown & Van Staden, 1973a; Brown & Van Staden, 1973b). Brown and Van Saden (1973 a & b) concluded that the seed coat imposed mechanical restrictions and could be partially responsible for the dormancy of *P. compacta.* However, the apparent lack of oxygen reaching the embryo imposed by the seed coat and water availability during incubation had a much greater influence on germination.

4.2.2. Vegetative propagation

Cuttings

Cuttings from *Protea* species are generally a terminal semi-hard wood cutting of about 15-20cm, treated with 4000mg/dm^3 Indol-buteric acid for 5 seconds (Coetzee & Littlejohn 2007; Malan 1995). The cuttings are then set in a well aerated medium with 23ºC bottom heat and hourly mist of 1-5 minutes for rooting (Coetzee & Littlejohn 2007; Malan 1995). The subsequent rooting of cuttings occurs within about 16 weeks (Coetzee & Littlejohn 2007). Microbial contamination and phenolic browning have to be avoided to ensure the success of cuttings in *Protea* species (Coetzee & Littlejohn 2007; Reid *et al.* 1989). Not all *Protea* species are easy to root, for instance very slow rooting of cuttings occurs in species such as *P. longifolia, P. holosericiea* and *P. magnifica* (Malan 1995).

Tissue culture

Plant tissue culture allows for mass propagation of plants independent of biotic and abiotic events such as seasons, pollination, drought and other disturbances or disasters, therefore plants can be multiplied throughout the year (Liao *et al.* 2006). Botanical gardens routinely employ micropropagatory techniques when working with rare and endangered species that are difficult to propagate through conventional methods (Fay 1992).

The concept of employing micropropagation techniques for the conservation of *Protea* species is not novel (for example Van Staden *et al.* 1981). However, the Proteaceae and in particular *Protea* species have proven difficult to regenerate *in-vitro* as difficulties (see below) seem to arise in every one of the five stages of tissue culture outlined by George (1993). It is therefore an expensive exercise to develop tissue culture protocols for members of this family (Malan 1995). Indeed Malan (1992) states that *Protea* species are "probably the most difficult and slowest plants of all the Proteaceae to propagate."

The potential for mass propagation of Proteaceae through tissue culture for the horticultural industry is now considered to have been over-estimated in the past due to: (a) the cost of technique development, (b) the cost of synthetic growth regulators needed to be applied to the mother plant to induce axillary bud sprouting, (c) the longer period *in-vitro* plants need before flower production compared with plants grown from cuttings and (d) the relatively small annual requirement for Proteaceae plants (Malan 1995). The establishment of *Protea* species in culture often suffers from high levels of endogenous contamination (even when parent plants are maintained in greenhouses and fungicides are applied prior to harvesting) and phenolic oxidative browning/blackening of culture resulting in death of the explant (Coetzee & Littlejohn 2007; Wu & du Toit 2004; Watad *et al.* 1992). The subsequent multiplication of buds is often hampered due to the difficulty of achieving bud break (Watad *et al.* 1992; Ben-Jaacov and Jacobs 1985). In addition, vitrification (glassy, succulent appearance) has been observed in all stages of culture in *Protea obustifolia* (Watad *et al.* 1992). Rooting of shoots has also been difficult in plants from the *Protea* genus, to such an extent that some researchers have resorted to micrografting *in-vitro* derived *P. cynaroides* shoots onto seed derived rootstocks (Wu *et al.* 2007b).

In spite of these difficulties, shoot proliferation has been successfully performed with *P. repens*, *P. obtusifolia* and *P. cynaroides* (Coetzee & Littlejohn 2007), while somatic embryogenesis has been reported in many *Protea* species (Wu *et al.* 2007a; Rugge 1995; Watad *et al.* 1992). The conversion of somatic embryos into plantlets has been achieved (Wu *et al.* 2007a), however rooted shoots have yet to be planted out into soil successfully and more research is needed on this aspect of the mircropropagatory process (Coetzee & Littlejohn 2007).

Parent material and propagation methods for conservation purposes

New, actively growing shoot tissue is generally the preferred tissue for *in-vitro* propagation and this was also the case for *P. obtusifolia* (Watad *et al.* 1992). However, endogenous contamination can make it extremely difficult to bring shoot tissue into culture, especially if vigorous growth

cannot be initiated (George 1993; Bhojwani & Razdan 1983). Zygotic embryo tissue can be free of endogenous pathogens (Bhojwani & Razdan 1983) and has been successfully used by Wu *et al.* (2007a & b) in the propagation of *P. cynaroides*.

There is limited information on the propagation of rare and endangered Proteaceae (Laubscher *et al.* 2009). A balance needs to be found between the benefit of the propagation technique in terms of numbers of propagules that can be produced and the genetic diversity that is introduced, destroyed or maintained by that technique. Furthermore, the availability of both plant tissue and the technology to utilise that tissue has to be taken into account. The extant *P. roupelliae* ssp. *hamiltonii* population is polymorphic with regard to flower colour, ranging from yellow to deep pink (see Chapter 2) and with each morph having differing evolutionary advantages (Carlson & Holsinger 2010). To conserve these morphs and propagate the species clonally, emphasis needs to be placed on the genotype and phenotype of the parent material.

The addition of the synthetic auxin 2,4-D and the length of time cultures are kept in the callus phase increases the chance of somaclonal variability (Collin & Edwards 1998). In some species morphogenesis and the regeneration phase seems to select against high levels of variation. In this way there may be high levels of variation in a callus culture but embryos and shoots may be variation free although the reverse is also possible in other species resulting in embryos and shoots with high variability (Collin & Edwards 1998). The level of variation that can be introduced by the callus and subsequent embryogenesis is therefore unknown, however, genetic techniques such as nuclear simple sequences repeat (SSR) markers may be used to ensure that the genome has not been altered by the *in-vitro* situation.

Tissue culture has the potential to mass propagate plants clonally and indirect methods may be employed to introduce genetic variation that will be incorporated into the progeny. Due to the genetic and epigenetic changes that arise from somaclonal variation it has been advised against introducing plants with altered genetics to an already threatened population unless under extreme circumstances (Benson *et al.* 2000; Kaeppler *et al.* 2000; Fay 1994; Jacobsen & Dohmen 1990). Through natural selection a population would have adapted genetically to a given habitat. Therefore, introducing individuals with an altered genome that may not be as well adapted to the habitat would dilute the beneficial genes which may be seen as genetic pollution. However, in a small population, such as that of *P. roupelliae* ssp. *hamiltonii,* where the population might have reached a genetic bottleneck, it has been hypothesised that the genetic diversity can be restored via somaclonal variation (Jacobsen & Dohmen 1990).

In the last few years the *P. roupelliae* ssp. *hamiltonii,* plants have produced achenes containing viable zygotic embryos (see Chapter 2), allowing zygotic tissue to be utilised in this present study. However, the availability of naturally produced zygotic embryos has only occurred recently (2005) and their production *in-situ* is inconsistent (Chapter 2). Therefore although plant zygotic embryos are a preferable propagule in terms of maintaining the genetic diversity of the population, in the future, after a major disturbance/disaster the population may have to be restored from a few founding individuals or a limited stock of *ex-situ* stored germplasm. Additionally in most reintroduction efforts, large numbers of propagules are necessary due to the associated high mortality rates of propagules in these situations (Maunder 1992). In events that necessitate reintroduction, the numbers of propagules produced via *in-vitro* methods may outweigh the threat of possible genetic instability or loss of genetic diversity.

4.3. Aims and objectives

The aim of this chapter was to test various methods for the propagation of *Protea roupelliae* ssp. *Hamiltonii* using seed and vegetative tissues as starting material.

The objectives were:

1. To establish the germination parameters for *Protea roupelliae* ssp. *hamiltonii* achenes/embryos both *in-vitro* and under standard conditions.
    a. To determine the effect of pre-soaking achenes on germination
    b. To determine the effect on the germination of embryos excised from the pericarp after pre-soaking achenes.
2. To establish parameters for the rooting of stem cuttings of *Protea roupelliae* ssp. *hamiltonii*.
3. To investigate methods of generating/regenerating *Protea roupelliae* ssp. *hamiltonii* plantlets *in-vitro* via:
    a. Direct shoot organogenesis.
    b. Indirect morphogenesis.
4. To establish rooting and ‘hardening’ requirements for *in-vitro* derived shoots to withstand greenhouse conditions.

4.4. Materials and Methods

4.4.1. General materials methods

The following general materials and methods (a – d) apply to various experiments:

a. Greenhouse conditions

The greenhouse was located at the University of the Witwatersrand, Johannesburg. An automated Microjet watering system watered the plants three times a day for 10 minutes to soil saturation.

b. Growth room conditions

The growth room at the University of the Witwatersrand was maintained at standard growth room conditions: 25±2ºC with a 14/10 hour photoperiod and 200µmol m-2.s-1 photosynthetic photon flux density.

c. Standard germination conditions

The germination conditions were as described in Chapter 2 Section 2.4.3. Five achenes/embryos were germinated per Petri dish.

- Embryos with pericarps (i.e. achenes) that did not germinate after the allotted germination period were dissected to ascertain if the achene was filled with an embryo or not.
- Germination of excised embryos was considered complete once geotropism was identified in the radical (Figure 4.2).



Figure 4.2. Isolated *P. roupelliae* ssp. *hamiltonii* embryos with emerging radicals displaying geotropism.

d. Media composition

The following media were used and are henceforth referred to by the number assigned (Table 4.1). All media comprised the basal medium of Murashige & Skoog (1962) salts with vitamins (MS) plus other additives dissolved in ultra-pure water (milli-Q filtration), which were autoclaved at 104 kPa at 120ºC for 20 minutes. The pH of the media was adjusted prior to autoclaving.

Table 4.1. Composition and pH of media used in the micropropagation and germination of *Protea roupelliae* ssp. *hamiltonii*.

Medium number	Constituent	Concentration	Rationale for using medium
1	MS (1962) – half strength Sucrose Gelrite®	2.21g.l^{-1} 30g.l^{-1} 3g.l^{-1} pH 5.50	Adapted from: • Wu *et al.* (2007b) for the germination of *P. cynaroides* zygotic embryos. • Wu *et al.* (2007a) for the germination of *P. cynaroides* zygotic embryos and the induction of direct somatic embryogenesis from *P. cynaroides* cotyledons.
2	MS (1962) – full strength Sucrose Gelrite®	4.42g.l^{-1} 30g.l^{-1} 3g.l^{-1} pH 5.50	Based on Medium 1 and used to test the effect of increased nutrients.
3	MS (1962) – quarter strength Sucrose Gelrite®	1.105g.l^{-1} 30g.l^{-1} 3g.l^{-1} pH 5.50	Based on Medium 1 and used to test the effect of decreased nutrient concentrations.
4	MS (1962) – half strength Sucrose Gelrite®	2.21g.l^{-1} 30g.l^{-1} 3g.l^{-1} pH 4.20	Based on Medium 1 and used to test the effect of a low pH.
5	MS (1962) – quarter strength Sucrose Gelrite®	1.105g.l^{-1} 30g.l^{-1} 3g.l^{-1} pH 4.20	Based on Medium 1 and used to test the effect of both a low nutrient content and a low pH.
6	MS (1962) – half strength Gibberellic acid (GA_3) Sucrose Gelrite®	2.21g.l^{-1} 1 mg.l^{-1} 30g.l^{-1} 3g.l^{-1} pH 5.50	Adapted from: • Wu *et al.* (2007a) for the germination of *P. cynaroides* primary, somatic embryos.
7	MS (1962) – half strength Benzyl aminopurine (BAP) Giberellic acid (GA_3) Activated charcoal meso-inositol Ethylenediaminetetraacetate (EDTA) Sucrose Gelrite®	2.21g.l^{-1} 2 mg.l^{-1} 6 mg.l^{-1} 3 g.l^{-1} 100mg.l^{-1} 50mg.l^{-1} 30g.l^{-1} 3g.l^{-1} pH 5.00	Adapted from: • Wu *et al.* (2007b) and Wu & du Toit (2004) for the establishment of *P. cynaroides* shoot culture. GA_3 to cytokinin ratio adapted from: • Authors above for *P. cynaroides* culture. • Rugge (1995) for the shoot culture of *P. repens.* • Watad *et al.* (1992) for bud sprouting and elongation in *P. obtusifolia.*

4.4.2. Stem cuttings

Ten, 20cm terminal shoots were selected (Malan 1992) from *P. roupelliae* ssp. *hamiltonii* plants growing at the forestry station (in the forester's garden) at Nelshoogte. The shoots were sprayed with an antioxidant solution of ascorbic 100mg.l^{-1} acid and 1500mg.l^{-1} citric acid (Wu & du Toit 2004) until it dripped off and was allowed to dry. They were then sprayed with 70% ethanol until it dripped off and were harvested. The basal 10cm of leaves were striped. The cut end of the stem was dipped in 70% ethanol for 30 seconds (Crick 1999) and the last 5mm from the base of the stem was removed. The basal 10cm of stem was then dipped into a 4 g.l^{-1} Indole-3-butyric acid (IBA) solution for five minutes (Malan 1995). The cuttings were then pushed into moist soil

collected from the forester's garden and immediately transported to Johannesburg (4 hours away), and maintained under greenhouse conditions (above). Cuttings were left for 16 weeks before they were checked for rooting.

4.4.3. Zygotic embryo/achene germination trials

As *P. roupelliae* ssp. *hamiltonii* is a critically endangered species, sample numbers were kept low in order to decrease the impact of harvesting on the population *in-situ.* The following germination parameters were collected:

a. Cumulative germination percentage.
b. The peak value (P.V.) of germination (the maximum quotient derived by dividing the daily cumulative germination by the number of days taken to reach that percentage) (Czabator 1962).
c. The time it took for 50 percent of the seeds to germinate (T_{50}).
d. The peak day of germination.
e. The mean number of days taken for germination to occur.

Standard germination trials

Table 4.2. Description of the treatments under the standard germination conditions (above) on a filter paper substrate.

Treatment	Sample size (plump achenes/embryos)	Pre-treatment (before being placed in the germination environment)	Duration in germination environment (days)
No pre-treatment	800	none	35
Soaked achenes	100	Achenes were soaked in 2% sodium hypochlorite solution containing Tween-20 1ml.l^{-1} for 5 minutes and soaked in 500ml sterile ultra-pure water for 48 hours under growth room conditions (above). Then rinsed in sterile ultra-pure water.	70
Excised embryos	100	Achenes were soaked as described above. Embryos were carefully removed from the seed coat and other coverings with a scalpel. Any embryos that were badly damaged by this process (i.e. missing cotyledon tissue) were discarded.	30

In-vitro germination trials

Decontamination of embryos

Achenes were soaked in 2% sodium hypochlorite solution containing 1ml.l^{-1} Tween-20 for 10 minutes and washed three times in sterile ultra-pure water. The achenes were then soaked in sterile ultra-pure water for 48 hours at 25±2ºC. The achenes were rinsed in 70% ethanol for 30 seconds and blotted dry on sterile paper. The seed coat was sliced open with a scalpel and the embryo was carefully excised. Excised embryos were placed in a 2% sodium hypochlorite

solution for five minutes and rinsed three times in sterile ultra-pure water. They were then placed in 70% ethanol for two minutes before being rinsed in sterile ultra-pure water.

Plating embryos

Embryos were plated on one of 7 media treatments (Table 4.3) with a maximum of four embryos per 400ml magenta jar and incubated under growth room conditions. Germination was checked every second day (as described above for excised embryos)

Table 4.3. Number of excised zygotic embryos plated on each medium

Medium	Number of embryos
1	66
2	79
3	68
4	70
5	65
6	66
7	110

4.4.4. Direct shoot organogenesis

The preparation of shoots (Stage 0)

Seeds that germinated in the seed storage experiments (see Chapter 5) were planted into two seedling trays, each containing 98, 100ml tubes filled with soil collected from the Dr Hamilton Reserve. The seedlings were maintained under greenhouse conditions (above) for 6 months (Figure 4.3).

Decontamination of parent material

The seedlings in both trays were sprayed twice a week with foliar fungicide (2 $g.l^{-1}$ Dithane) and a systemic fungicide (1$ml.l^{-1}$ Sporgon) for six weeks until the material was harvested for the *in-vitro* experiments. Fungicides were sprayed on leaves of plants until the solution dripped off.

BAP pre-treatment

Two weeks before harvesting, one tray of seedlings received a BAP pre-treatment as described by Rugge (1995). Seedlings were sprayed twice a week for two weeks with 200mgl^{-1} BAP until the solution dripped off. The remaining tray of seedlings received no exogenous growth regulators.

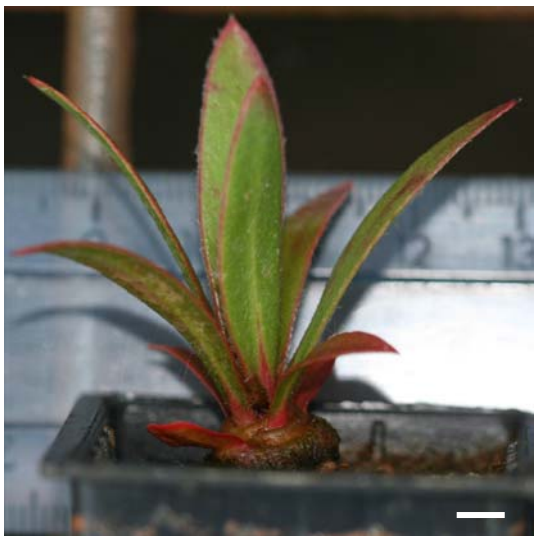

a.

b.

Figure 4.3. Seedlings maintained under greenhouse conditions that had been sprayed with systemic and foliar fungicide (a) and a six month old seedling in a 100ml growth tube (b) (bar equals 5mm).

Establishment of an aseptic culture (Stage 1)

Decontamination of explants

Shoots from the greenhouse established plants were excised at soil level and placed in an antioxidant solution described by Wu and du Toit (2004), consisting of sterile ultra-pure water, 100mg.l^{-1} ascorbic acid, 1500mg.l^{-1} citric acid and 1 ml.l^{-1} Tween-20. The antioxidant solution containing the shoots was stirred with a magnetic stirrer for 30 minutes, the solution was then poured off and replaced with fresh antioxidant solution and stirred for a further 30 minutes. Each shoot was then rinsed for one minute in 70% ethanol and blotted dry on sterile paper. The leaves were removed as close to the stem as possible and 5mm of tissue was removed from the cut surface of the stem. Shoots that had stems thicker than 10mm were bisected longitudinally, smaller shoots remained intact.

All shoots were placed into fresh antioxidant solution for 30 minutes, after which they were blotted dry on sterile paper and then soaked in a 1% sodium hypochlorite solution for ten minutes. The shoots were rinsed three times in ultra-pure water and soaked in a 70% ethanol solution for one minute, after which they were transferred to a 10 ml.l^{-1} Antibiotic Antimycotic Solution (Sigma-Aldrich) for five minutes.

The shoots were blotted dry on sterile paper and placed cut side down on Medium 7 in 400ml magenta jars (Figure 4.4) and cultured under standard growth room conditions (see above). Shoots were checked after 10 days for growth (bud break or elongation), phenolic oxidative browning

and contamination. The shoots pre-treated with BAP pre-treatment were compared with untreated shoots.



Figure 4.4. Shoots plated cut side down on Medium 7.

4.4.5. Indirect morphogenesis (Stage 2)

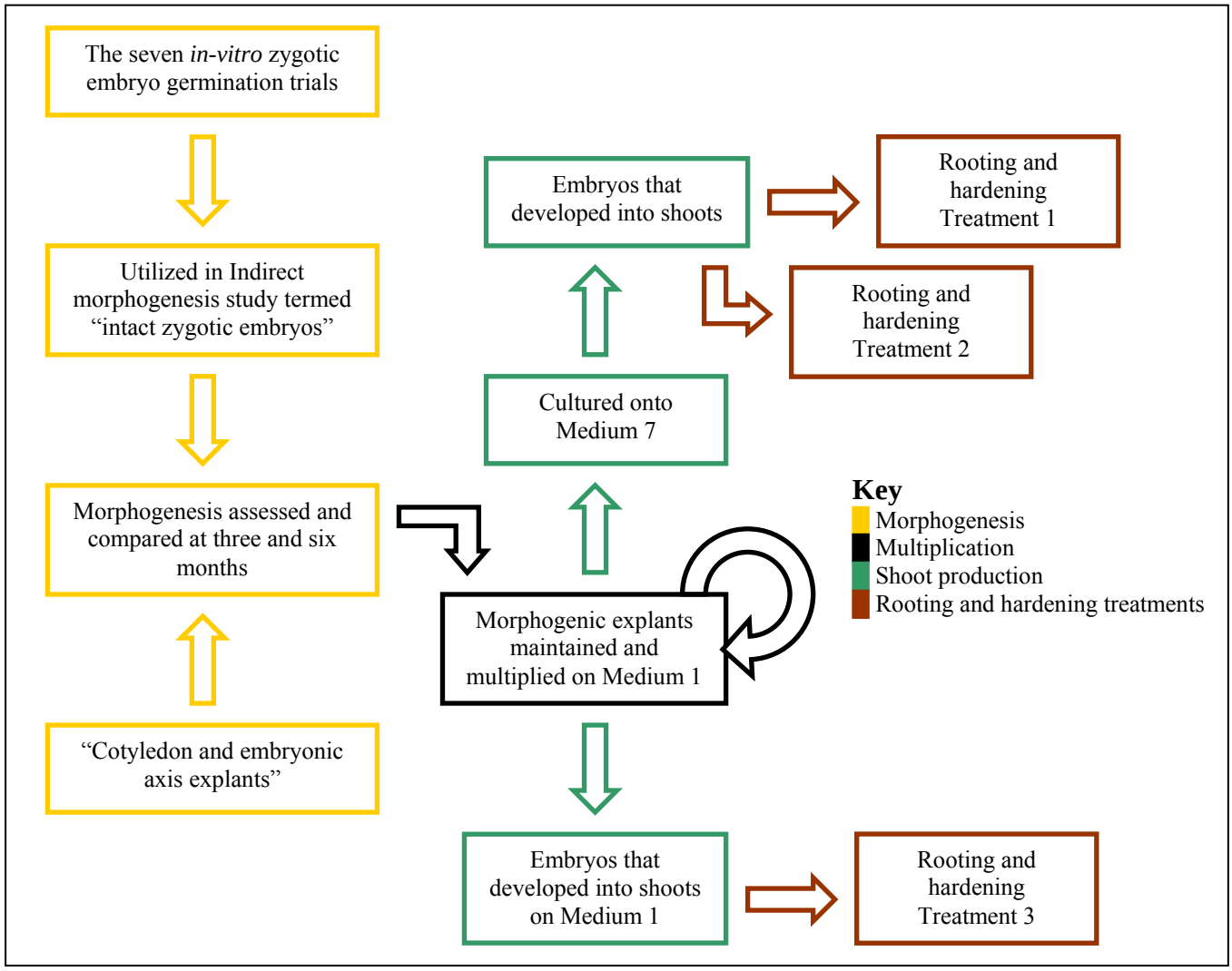


Figure 4.5. Depiction of the origin and protocol used to study morphogenesis from zygotic embryo explants.

Intact zygotic embryo explants

No germination occurred in the *in-vitro* germination trials (above), hence the plated embryos on their respective media were utilised for this morphogenic study. Stages 0 and 1 were therefore as described above.

Explants were maintained on the seven media on which they were plated in the germination experiment (Table 4.3). They were subcultured onto fresh medium at three and again at six months from plating, at which point the growth of the explants was evaluated and the following were assessed:

a) Growth of the embryo tissue
b) Presence and colour of callus
c) Presence of somatic embryo like structures.
d) Number of roots
e) Number of buds

The mean embryo, root and bud production was compared between media using a Kruskall-Wallis test and Mann-Whitney U tests were used as post hoc tests to compare between the seven media treatments.

Cotyledon and embryonic axis explants

Zygotic embryos were excised, decontaminated (see *in-vitro* germination trials) and were plated onto Medium 1 (in 400ml magenta jars). After two weeks in culture the cotyledons became less brittle and were aseptically removed from the rest of the embryo at the cotyledonary node. Both the embryos without cotyledons (n=40) and the removed cotyledons (n=80) were re-plated onto Medium 1 and maintained under growth room conditions.

Both types of explant (cotyledons and embryonic axes) were subcultured and assessed for growth at three and six months in culture. The mean somatic embryo, root and bud production per explant was compared with those produced by intact zygotic embryos (cotyledons attached) on Medium 1 using Mann-Whitney U tests.

4.4.6. Rooting and hardening of *in-vitro* derived shoots (Stages 3 and 4)

After six months in culture all zygotic embryo explants that showed signs of growth were plated on Medium 1 for two weeks before a subset of the somatic embryos were removed and plated onto Medium 7. The remaining tissue was subcultured every three months onto Medium 1.

The explants plated onto Medium 7 remained on Medium 7 for 3 months and were re-cultured on Medium 7 for a further two weeks, after which developing embryos were utilised in rooting and hardening Treatments 1 and 2 (below). Embryos that developed into shoots but not roots after successive subcultures on Medium 1 were used in Treatment 3 (below).

Treatment 1

75ml pots were filled with Dr Hamilton Reserve soil (moistened with 100ml of ultra pure water) and placed into 400ml magenta jars and then autoclaved at 104 kPa at 120ºC for 30 minutes. Shoots (n=52) that were initiated on Medium 7 were isolated and the medium was carefully washed from the shoot. Shoots were planted into soil in the pots with one to four plantlets in each pot. The jars containing the pots were placed in growth room conditions for six weeks, during which, twice each week the lids of the jars were removed under the laminar flow for five minutes. After six weeks, the survival of the shoots was evaluated.

After six weeks, the jar-lids were replaced by perforated (two holes with a diameter of 5mm) Parafilm® and relocated to ambient laboratory conditions. After a period of two week the survival was evaluated, and the plantlets were relocated to the greenhouse at the University of the Witwatersrand for one week. The surviving plantlets were planted into 340ml perforated polystyrene cups containing soil from the Dr Hamilton Reserve and maintained under greenhouse conditions. Survival was evaluated every two weeks for a total of 19 weeks.

Treatment 2

The same procedure was followed as in Treatment 1 until six weeks of age, when the jar-lids were replaced by perforated (two holes with a diameter of 5mm) Parafilm® and returned to the growth room. The plantlets were checked and watered regularly and after two weeks the plantlets were planted out into 340ml perforated polystyrene cups as for Treatment 1.

Treatment 3

Foil boats (50ml) were made by pressing aluminium foil over a rubber stopper, extra rigidity was provided by a small piece of autoclave tape. Two holes were pierced in the base of each boat. Each boat was filled with a mixture of two parts soil from the Dr Hamilton Reserve to one part vermiculite and placed in a 400ml magenta jars, wet with 50ml of ultra pure water and autoclaved at 104 kPa at 120ºC for 30 minutes. Shoots that were initiated on medium 1 (n=96) failed to produce roots were carefully placed in a $0.5g.l^{-1}$ IBA solution for 5 min (due to the surface tension of the solution on the leaf trichomes, the shoot floated stem down in the solution). The shoots

were rinsed in sterile ultra-pure water and planted in the soil containing boats (Figure 4.6). The jars were placed in standard growth room conditions for 13 weeks. The water within the jars was checked every two weeks and replenished when needed. Survival was assessed every two weeks and at the end of 13 weeks the soil-vermiculite mixture was washed off the shoots and examined for rooting.

All plantlets (with and without roots) were planted into 340ml perforated polystyrene cups containing soil from the Dr Hamilton Reserve and maintained under greenhouse conditions where survival was evaluated every two weeks for a further six weeks.



Figure 4.6. Shoots that developed on medium 1 without roots planted in foil boats filled with soil from the Dr Hamilton reserve and vermiculite as described in treatment 3.

4.5. Results and discussion

A synthetic overview of the outcomes from each of the methods carried out in this chapter can be seen in Figure 4.7.

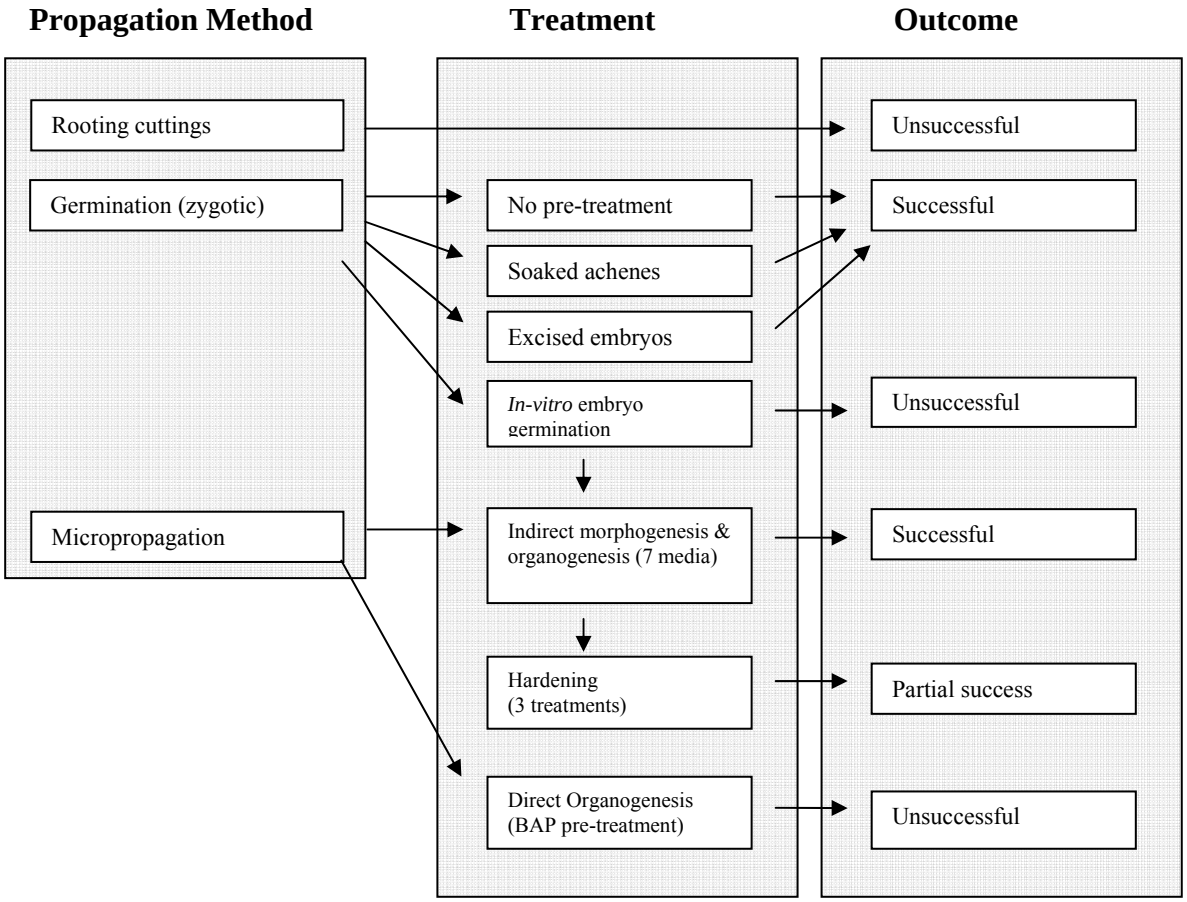


Figure 4.7. Synthetic diagram of the outcomes of this chapter, in terms of whether or not successful propagation was achieved.

4.5.1. Stem cuttings

All cuttings underwent phenolic leaf browning/blackening and were dead within two weeks. Blackening is a post-harvest problem in some commercially important *Protea* species of the cut flower industry (Ferreira 1985). Blackening is the result of the polymerization and oxidation of hydroxyphenols and tannins (Whitehead & De Swardt 1982) and may be brought about by carbohydrate stress and or water stress (Doorn 2000; Bieleski *et al.* 1992; Whitehead & De Swardt 1982).

Many protocols exist to prevent leaf blackening and extending vase life of flower stems for the cut flower industry (Doorn 2000), including the application of ethanol vapour (Crick 1999), antioxidant treatments (Jones & Clayton-Greene 1992), refrigeration (Doorn 2000; Ferreira 1985),

glucose pulse (Stephens *et al.* 2001), the maintenance of the stems in the light and controlled atmospheres (Jones & Clayton-Greene 1992).

Ethanol has been shown to reduce browning, but at high concentrations ethanol speeds up the rate of browning considerably (Crick 1999). The browning experienced in this study may have been caused by the over application of ethanol, as ethanol was sprayed directly onto the cutting and not applied as vapour as recommended by Crick (1999) resulting in considerably more ethanol coming directly in contact with the cutting. Although an antioxidant solution containing both ascorbic acid and citric acid (Wu & du Toit 2004) was used, the application may not have been sufficient to depress the oxidative reaction. Dipping stems in antioxidant solution of 1.5 mg l^{-1} diphenylamine has been shown to significantly decrease oxidative browning in *P. neriifolia* (Jones & Clayton-Greene 1992). As browning is a result of carbohydrate stress and 2.5% glucose in vase water has been shown to considerably extend the vase life of flowering stems, a pulse of glucose before the application of IBA may be beneficial in suppressing blackening in cuttings (Stephens *et al.* 2003; Stephens *et al.* 2001). In this present study IBA was applied as an aqueous solution and may have dispersed over the test period. It is possible that the application of IBA as a powder may have been more effective in combination with a glucose pre-treatment (Jones & Clayton-Greene 1992).

Due to the excessive phenolic oxidation and subsequent death of the explants, it was not possible to ascertain whether *Protea roupelliae* ssp. *hamiltonii* is an easy to root species. However, the experiment did show that *P. roupelliae* ssp. *hamiltonii,* like other *Protea* species, has high propensity for browning. This influenced the methods of micropropagation, experiments where wounding of the explants was kept to a minimum and antioxidant treatments were extensively utilized.

4.5.2. Zygotic embryo/achene germination trials

Standard germination trials

The excised embryos germinated most rapidly and with the highest germination percentage of all treatments (97% at 19 days), followed by achenes that had no pre-treatment (92.88% at 27 days) and soaked achenes (82% at 69 days) (Figure 4.8a). Additionally, excised embryos had a higher P.V., and lower T50, peak day of germination and the lower mean days until germination than the other treatments (Figure 4.8b-e).

These high germination percentages (for *Protea* species) were achieved at growth room conditions (25±2ºC) without the low temperature incubation requirement described by Malan (1995). The germination incubation temperature above 20°C did not negatively affect the germination of *P. roupelliae* ssp. *hamiltonii* to the same degree as reported by Brown and Van Staden (1971 & 1973b) in winter rainfall *Protea* species. This may be due to the fact that *P. roupelliae* ssp. *hamiltonii* persists in a summer rainfall region, where the moisture needed for germination is coupled with an increase in temperature (Mucina & Rutherford 2006).

The germination of achenes without a pre-treatment posed the least risk of damage from excising the embryos or exposing embryos to microbial contamination, as achenes/embryos that did not germinate in the other two treatments (excised embryos and soaked achenes) developed fungal contamination. Brown and Van Staden (1973a & b) noted that seeds removed from their seed coat germinated rapidly for the first ten to twelve days after which the embryos started rotting, resulting in a final germination percentage of only 30±8%. In the present investigation a very low percentage of the excised embryos (3%) did not germinate which could be due to mechanical damage (caused during the removal of the pericarp). The higher germination percentage of *P. roupelliae* ssp. *hamiltonii* excised embryos could be attributed to the decontamination treatment applied before soaking, the use of sterile ultra pure water, the use of filter paper instead of sand and a rigorous laboratory technique.

Oxygen availability

A decrease in the germination percentage of *P. compacta* achenes once soaked was reported by Brown and Van Staden (1973a & b), similar to that observed in *P. roupelliae* ssp. *hamiltonii* (Figure 4.8). The *P. compacta* seeds used by those authors had been soaked for increments of four days in both running and distilled water, the germination percentage dropped from 45±8% and 49±11% with no soaking to 0% in distilled water and 9±2% in running water after a soaking period of 12 days. Those authors attributed the reduction in germination to the decrease in the diffusion of oxygen to the embryo over a wet surface. Germination was increased by replacing the atmosphere in the germination flasks with oxygen (Brown & Van Staden 1973b). Germination also increased when seeds were incubated at 5°C (Brown & Van Staden 1973b). A decrease in germination in response to soaking or even an abundance of water has been found in many species. The embryos of such species are sensitive to anoxic conditions and the water alone (as in barley (*Hordeum vulgare*)), or in conjunction with the seed coverings, decreases the flow of oxygen to the germinating embryo (Essery *et al.* 1954). Such responses are known as water sensitivity (Essery *et al.* 1954). In many cases an increased concentration of atmospheric oxygen

increases germination in water sensitive species such as spinach (*Spinacia oleracea*) (Heydecker & Orphanos 1968), barley (*Hordeum vulgare*) (Ellerton & Perry 1983; Vlamis & Davis 1943), sugar beet (*Befa vulgaris*) (Perry & Harrison 1974) and mustard (*Sinapis alba*) (MacDonald & Gordon 1978). The partial or total removal of the seed coat also increases the germination in these species (MacDonald & Gordon 1978; Perry & Harrison 1974; Heydecker & Orphanos 1968). In *Datura ferox* and *D. stramonium* it was hypothesized that water becomes trapped in the space formed by the seed coat, thereby limiting the diffusion of gasses (Reisman-Berman *et al.* 1989). In species that are sensitive to low levels of available oxygen it is consistent that germination is improved at lower temperatures, <12°C for spinach (Řreggen 1984), as gas solubility increases with decreasing water temperature.

The rapid germination of the excised *P. roupelliae* ssp. *hamiltonii* embryos, as with the decreased germination percentage of the soaked achenes, may also be attributed to the change in diffusion rate of oxygen (Lenoir *et al.* 1986). Without any seed coverings atmospheric oxygen was able to freely reach the embryo tissue unimpeded. Additionally the increased rate of germination of the excised embryos indicated that the slow germination of the soaked achenes was not due to physical injury during the imbibition process.

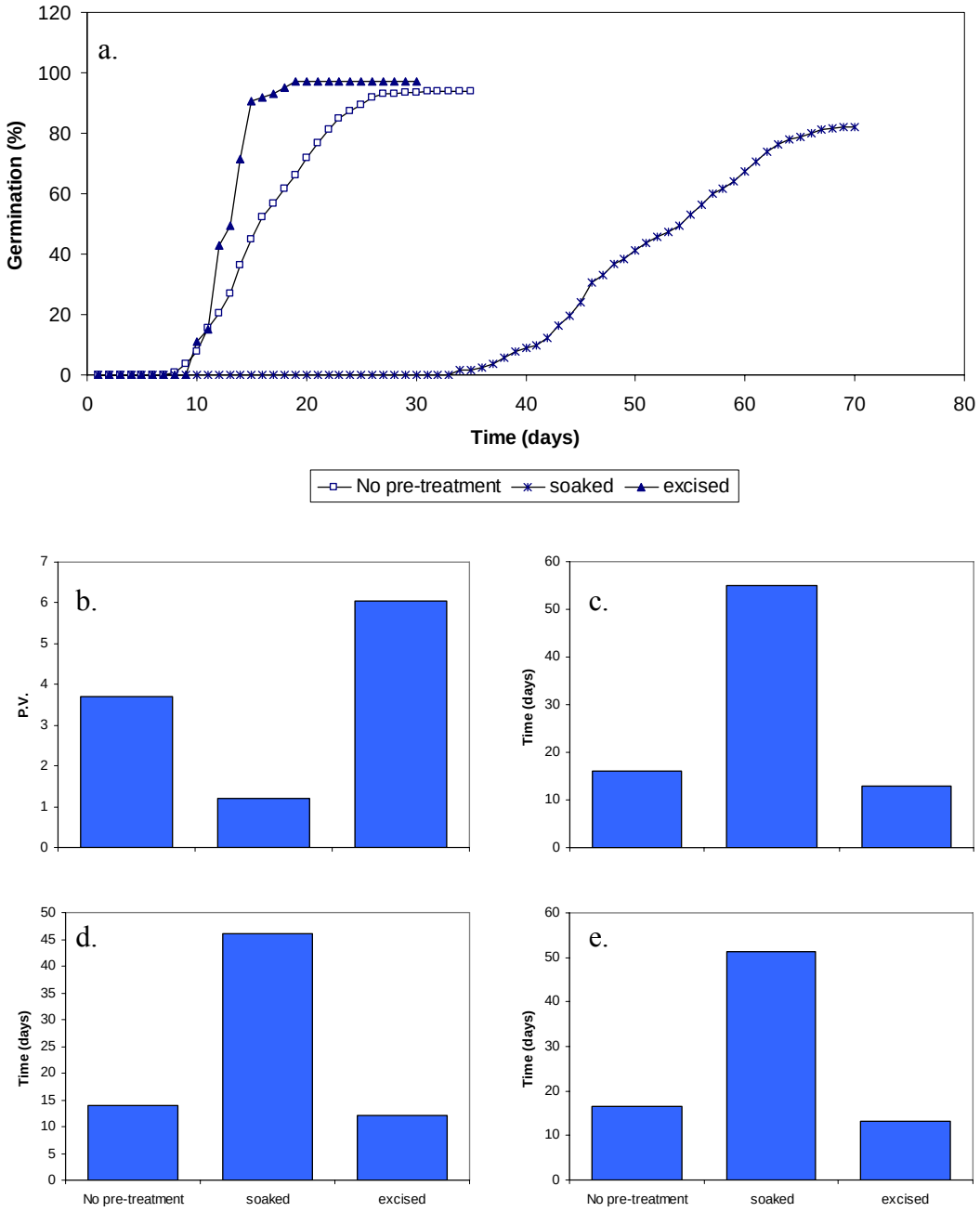


Figure 4.8. The germination parameters of achenes that received no pre-treatment or were soaked and excised embryos including (a) cumulative germination percentage, (b) the P.V., (c) the T_{50}, (d) the peak day of germination and (e) the mean number of days until germination.

In-vitro germination trials

All zygotic embryos failed to germinate normally *in-vitro.* This included embryos plated on the same medium (Medium 1, with no growth regulators) that Wu *et al.* (2007 a & b) reported successful germination of *P. cynaroides* embryos.

Soaking the achenes only delayed the germination process on moist filter paper and did not inhibit germination altogether. Furthermore the removal of the pericarp after soaking (as performed for this *in-vitro* germination experiment) resulted in rapid germination on moist filter paper with no abnormal physical appearance (Figure 4.2 and 4.8). It would therefore be expected that germination should occur rapidly *in-vitro* as with the excised embryos on moist filter paper (see above).

In general Proteaceae grow in nutrient-poor soils (Cowling & Witkowski 1994) with a pH range of 3.5 – 6.5 (Coetzee & Littlejohn 2007; Matthews 1993), and this is indeed true for the soils of the Dr Hamilton Reserve which had a mean pH (KCl) of 3.91 ± 0.19 (±S.E.) (Dayaram 2007). *Leucadendron* 'Safari Sunset' explants perform better in media with half strength MS nutrients than media containing full or three quarter strength MS, although lower MS concentrations were not tested (Perez-Frances *et al.* 1993). Additionally, it has since been advised to supply low concentrations of nutrients to explants of *Protea* cultivars (Poupet *et al.* 2004). Many media for the culture of Proteaceae species contain half strength MS including media used for species from the Proteaceae genera *Leucadendron, Protea, Leucospermum, Telopea, Synaphea* and *Banksia* (Willyams *et al.* 2008; Rugge 1995; Niccol *et al.* 1994; Perez-Frances *et al.* 1993; Offord & Campbell 1992; Kunisaki 1989). However, no incidence of normal germination was recorded on any of the presently tested MS media with concentration range (4.42 – 1.105 $g.l^{-1}$), including media with a low pH (pH 4.2).

Medium 7 with a high GA_3 to cytokinin ratio was also expected to promote germination (Wu *et al.* 2007a; Rugge 1995). The effect of GA_3 on *Protea* species can be species specific, GA_3 has been shown to increase the germination of *P. aristata, P. eximia* and *P. neriifolia* but suppress the germination of *P. repens* (Rodríguez-Pérez *et al.* 2007; Rodríguez-Pérez 1993). However, many of the media (Medium 1-5) on which excised embryos failed to germinate normally, contained no growth regulators. The lack of germination *in-vitro* may be due to the impaired diffusion of oxygen reaching the embryo, as the wet *in-vitro* environment with dissolved salts and sucrose further decreases the solubility of gases (George 1993).

4.5.3. Direct shoot organogenesis

Contamination of shoot explants

All shoots exhibited contamination within ten days of plating (Figure 4.9a) and no growth of the tissues had manifested in this short period. *Protea* species are known to be prone to endogenous

microbial contamination and *P. roupelliae* ssp. *hamiltonii* seedlings appear to be just as susceptible as the rest of the genus (Watad *et al.* 1992). The application of fungicides to seedlings grown in the greenhouse was frequent, however they were not effective, possibly due to the slow growth of the greenhouse seedlings at this age (see Chapter 5) or the use of incorrect fungicides during stage 0. In the future using younger vigorously growing seedlings (less than three months old) as parent material, that are treated with the appropriate fungicide(s) from germination may be effective in decreasing contamination (George 1993). More rigorous decontamination procedures may also be necessary, however these procedures would need to be executed with regard to the slow growth of the species, the propensity of *Protea* species to undergo phenolic browning, vitrification, the ease of callus formation and the difficulty associated with bud break (Coetzee & Littlejohn 2007; Wu & du Toit 2004; Malan 1992; Watad *et al.* 1992).


a. b.

Figure 4.9. Shoot culture showing contamination after ten days (a) and two shoots considered to have undergone oxidative browning (b) and for comparison, on the left a shoot showing no browning (bar approximately equates to 4mm).

BAP pre-treatment and phenolic oxidative browning of shoot explants

Phenolic browning was evident in shoots (Figure 4.9b), although the full extent of browning may not have been evident in the short assessment period. Of the 143 explants that did not undergo the BAP pre-treatment, only a single explant exhibited oxidative browning (0.699 %). On the other hand material that had the BAP pre-treatment exhibited a higher incidence of browning, with 11 of the 153 (7.19 %) explants considered to have browned. This is contrary to results found by Rugge (1995) in *P. repens* where the incidence of browning was reduced from 100% to 21% by pre-treating the parent plants with cytokinin. The low incidence of browning in both treatments was therefore attributed to the extensive use of a balanced antioxidant solution during Stage I as described by Wu and du Toit (2004), which was not used by Rugge (1995).

4.5.4. Indirect morphogenesis

Initiation of callus culture from intact zygotic embryo explants

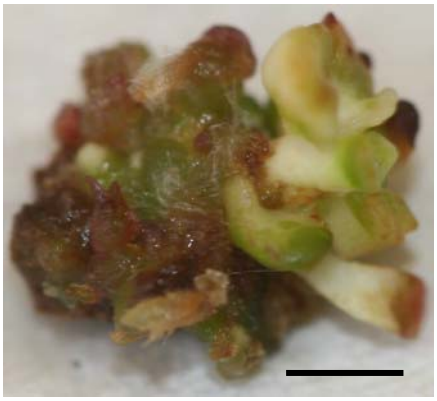
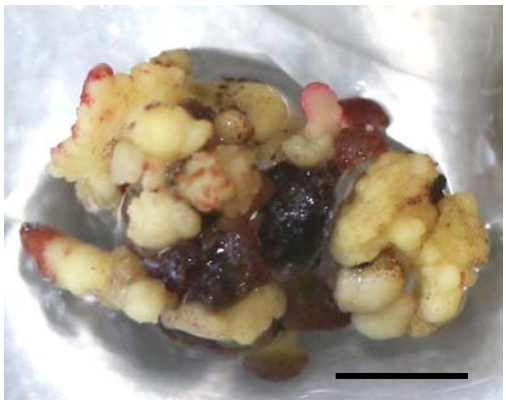
In terms of microbial contamination, the protocol followed for bringing tissue *in-vitro* (Stage 1) provided acceptably low levels of contamination with a mean percentage of 2.3% contamination after three months in culture.

Callus growth was initiated on many of the plated zygotic embryos (50.84±4.4%) (mean±S.E) (Table 4.4) however, growth was extremely slow. The slow rate and low percentage of growth may have been due to suboptimal culture conditions or the rigorous decontamination process resulted in low survival levels of the embryo tissue. The sensitivity of the embryos to low oxygen environments (seen in the soaked achenes above) may also have had a role in the survival and slow growth of the embryos in the wet *in-vitro* environment, although it is accepted that very slow growth is typical of *Protea* tissue *in-vitro* (Malan 1992). However, a benefit of the slow growth rate is that it may decrease the level of somaclonal variation in the callus cultures (Collin & Edwards 1998).

Table 4.4. Percentage of intact embryos that initiated callus growth after three months in culture.

Medium	Explants that initiated growth (%)
Medium 1	54.7
Medium 2	38.0
Medium 3	33.8
Medium 4	55.7
Medium 5	68.3
Medium 6	50.0
Medium 7	55.5

All zygotic embryos that showed signs of growth initiated callus on all media. Most clumps of callus were compact and hard (Figure 4.10a and b), however soft, crystalline callus also formed (Figure 4.10c). Wu *et al.* (2007a) working with *P. cynaroides* observed soft callus forming on media containing 2,4-Dichlorophenoxyacetic acid (2,4-D) and BAP however, no embryos were formed from that callus and ultimately the callus died. The soft crystalline callus that developed from *P. roupelliae* ssp. *hamiltonii* zygotic explants also had a tendency to die when subcultured but did eventually (>60 days) produce dense white structures within the callus (Figure 4.12c).


a.

b.

c.

Figure 4.10. Hard (a & b) and soft, crystalline (c) callus formed on medium 1 (bar approximately equates to 5mm).

Adventitious regeneration from intact zygotic embryo explants

Description of the regeneration process

After a period of 10 weeks in culture, the callus started to differentiate. Rhizogenesis was identified first, the roots had a furry appearance (presumably due to the presence of root hairs) and seldom grew longer than 2cm (Figure 4.11a). Caulogenesis was recognised by the small, fleshy leaflets that ranged in colour from green to dark pink (Figure 4.11b). However, the buds and roots that formed from the callus generally either died (particularly in the case of roots) or de-differentiated, reforming callus.

In a similar time frame, dense white structures also formed (Figure 4.11c). In order for these structures to be positively categorised as somatic embryos, they need to exhibit polarised root and shoot poles (Stafford & Warren 1991), a quality only identifiable once a root and a shoot has formed sufficiently during development. It has previously been observed that pro-embryonic tissue (tissue consisting of proliferating globular embryos) plated on media with inappropriate growth regulators may produce shoots without roots and therefore seem as though they have arisen from caulogenesis, while in actual fact the shoots originated from the embryogenic pathway (George *et al.* 2008). In such instances the only evidence that embryogenesis may have taken

place was the appearance of cotyledon-like first and second leaves (George *et al.* 2008; Phillips & Collins 1980).

The white structures (Figure 4.11c) that formed in this study were not removed from the callus. As tissue from *Protea* species are prone to phenolic browning, injuring the tissue was consciously avoided, therefore subculturing involved removing tissue from old media and plating the tissue straight onto fresh media without cutting or breaking the tissue where possible. When calli supporting these white structures were plated onto Medium 7 with a high GA_3 concentration, the structures became enlarged, turned green and fleshy. At this stage most of these developing structures were utilised in the rooting and hardening Treatments 1 and 2, where they often produced apparently normal shoots and the structures took on the appearance of cotyledonary leaves (Figure 4.20c). Roots did not develop from these structures and consequently they could be assumed to be shoot primordia. However, they were distinctly different from the buds that developed from the callus (Figure 4.11b) and in some respects these structures responded like somatic embryos; not undergoing organogenesis until a period of senescence (over six months in culture) or exposure to GA_3. Furthermore tissue forming from these white structures that developed from the callus had a similar colour and density as the secondary somatic embryos that developed later in the study which were capable of producing roots, albeit more rarely than shoots. The adventitious white structures that formed *in-vitro* in this study (Figure 4.11c) were therefore identified as somatic embryos, despite their lack of root production in conjunction with shoot production. It was assumed that the medium compositions on which these developmental processed were observed were not optimal for complete adventitious somatic embryogenesis (George *et al.*, 2008).

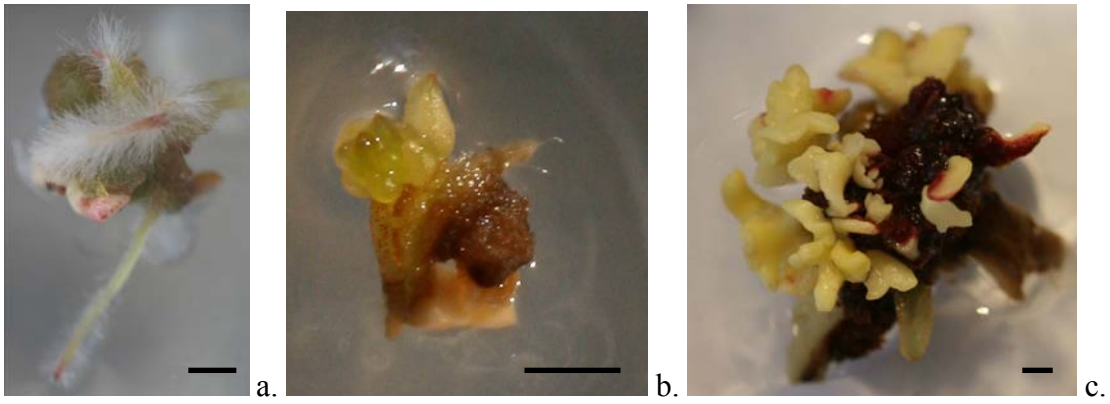

a. b. c.

Figure 4.11. Regeneration via (a) rhizogenesis, (b) caulogenesis and (c) embryogenesis (bar approximately equates to 5mm).

Rhizogenesis

Protea species *in-vitro* and *in-vivo* are known to produce cluster roots, also known as proteoid roots (Dinkelaker *et al.* 1995; Van Staden *et al.* 1981). These clusters of extensively branched swollen, short, lateral roots are reported to improve the mobilization of phosphorus, iron, zinc and manganese by their increased surface area and root induced chemical changes to the rhizosphere by the release of root exudates (Neumann & Martinoia 2002; Watt & Evens 1999). The development of proteoid roots *in-vivo* can be in response to a deficiency of phosphorus and iron (Watt & Evens 1999; Keerthisinghe *et al.* 1998). Conversely, supplementing the plant with nutrients (as would generally be the case *in-vitro*) suppresses root formation, which can only be re-stimulated by the application of exogenous auxin (Gilbert *et al.* 2000; Watt & Evens 1999; Keerthisinghe *et al.* 1998). This would explain the high level of rooting in the low nutrient Medium 3 (quarter MS) after three months (Table 4.5). However, after six months in culture, explants plated on Medium 3 had diminished rhizogenesis when compared with other media. Therefore rhizogenesis in *P. roupelliae* ssp. *hamiltonii* explants can only partially be explained by the lack of nutrient supply.

A slightly acidic pH has been associated with increased adventitious root formation in most species (George *et al.* 2008). This association was observed from cultures grown on media 4 (half MS) and 5 (quarter MS), both at pH 4.20 where the incidence of root formation (0.32±0.18 and 0.46±0.16 respectively) was higher than cultures on media 1 and 3 at pH 5.50 (a mean incidence of root formation of 0.29±0.12 and 0.12±0.06 respectively) (Table 4.5). The pH of a medium may also affect the uptake of nutrients by the explant (George *et al.* 2008) which could account for the increased incidence of rooting from cultures on Medium 5, which had both low pH and low concentrations of nutrients (Table 4.5).

In some species GA_3 is a promoter of rhizogenesis, especially when synthetic auxins are omitted from the medium (George 1993). In this respect the presence of a low concentration of GA_3 may have been the cause of increased root formation from explants plated on Medium 6 (Table 4.5).

Adventitious somatic embryogenesis

Somatic embryos are frequently initiated from *in-vitro* cultured zygotic embryos (Merkle *et al.* 1990). Additionally, recurrent cycles of somatic embryogenesis can be spontaneous and may be maintained in the absence of growth regulators (Merkle *et al.* 1990). In *P. roupelliae* ssp. *hamiltonii* somatic embryogenesis occurred in callus that had undergone some level of browning,

an occurrence also observed in *P. repens* (Rugge 1995) and numerous other species including *Haworthia* spp. (Mycock *et al.* 1997).

Somatic embryos developed from explants planted on all tested media including those containing GA_3. In most incidences GA_3 is known to suppress somatic embryo development (Bhojwani & Razdan 1983), which is analogous to the decreased embryo production seen in Medium 7 with a high concentration of GA_3 (Table 4.5). Explants planted on media 3, 4, 5 and 6 provided the highest production of embryos after 6 months (Table 4.5). All media with the exception of Medium 6, which had low levels of GA_3, were growth regulator free.

Explants planted on Medium 4, which contained half strength MS salts and a low pH, produced the highest mean number of embryos per plated zygotic embryo, after both three and six months in culture. The low pH of Medium 4 may have had a beneficial effect on somatic embryogenesis as pH is known to have a regulatory effect on morphogenesis (George *et al.* 2008). In carrot (*Daucus carota*) somatic embryogenesis, maintaining the multiplication of preglobular stage proembryo cultures on a growth regulator free medium at pH 4, was found to be as effective as the 2,4-D containing medium on which multiplication was generally achieved (Smith & Krikorian 1990a).

Table 4.5. Mean (±S.E) numbers of embryos, roots, leaves and buds that developed on each zygotic embryo that initiated growth after three months and six months in culture. Different superscript letters indicate significant differences between media ($P<0.05$)

	Medium number						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Three months in culture							
Embryos	0.40 ± 0.27	0.63 ± 0.27	0.35 ± 0.17	1.05 ± 0.35	0.93 ± 0.32	0.83 ± 0.29	0.30 ± 0.16
Roots	0.17 ± 0.08^{ab}	0.53 ± 0.19^{ab}	0.83 ± 0.24^{a}	0.28 ± 0.09^{ab}	0.35 ± 0.11^{ab}	0.14 ± 0.08^{b}	0.02 ± 0.02^{c}
Buds	0.11 ± 0.06^{ab}	0.23 ± 0.08^{ab}	0.17 ± 0.08^{ab}	0.31 ± 0.08^{a}	0.28 ± 0.07^{a}	0.10 ± 0.06^{ab}	0.12 ± 0.04^{b}
Six months in culture							
Embryos	2.10 ± 1.15^{bc}	1.13 ± 0.59^{bc}	3.04 ± 1.09^{ab}	4.66 ± 1.09^{a}	2.62 ± 0.69^{abc}	2.21 ± 0.58^{abc}	1.34 ± 0.34^{c}
Roots	0.29 ± 0.12	0.13 ± 0.08	0.12 ± 0.06	0.32 ± 0.18	0.46 ± 0.16	0.52 ± 0.24	0
Buds	0	0.07 ± 0.04	0	0.10 ± 0.06	0.10 ± 0.06	0.12 ± 0.06	0.14 ± 0.06

Variations in adventitious somatic embryogenesis

In *P. roupelliae* ssp. *hamiltonii,* most of the somatic embryos formed from hard callus and did not have the typical somatic embryo morphological appearance (Figure 4.12a). However, within the six months that morphogenesis was evaluated, on rare occasions somatic embryos were observed, originating from what appeared to be re-differentiated tissues (Figure 4.12b). This indicated the possibility of the initiation of somatic embryogenesis from differentiated tissues in this species.

Soft, crystalline callus cultures (Figure 4.10c) developed dense white structures within the culture, which had the appearance of globular embryos (Figure 4.12c) and seemed to develop into mature somatic embryos after culturing onto fresh medium at pH 5.20 (Figure 4.12d and e). It is possible that this culture (Figure 4.12c) consisted of proliferating pro-embryonic masses which would have been suited to a conventional suspension somatic embryo culture (Merkle *et al.* 1990; Terzi & Loschiavo 1990), capable of further increasing multiplication rates (Collin & Edwards 1998).

Additionally this culture (Figure 4.12c) may provide insight to the control of embryogenesis in *P. roupelliae* ssp. *hamiltonii.* Embryo cultures have the potential to decrease the pH of the surrounding medium during proliferation, the subsequent low pH further promotes embryo proliferation but inhibits embryo development (George *et al.* 2008). These embryos did not develop into heart and torpedo stages as long as the pH of the medium remained low (George *et al.* 2008). However, when these embryos were transferred to a high pH medium, the development of the embryos could have been initiated (George *et al.* 2008; Smith & Krikorian 1990a). This pH dependant embryo development could have been occurring in the *P. roupelliae* ssp. *hamiltonii* cultures. Where the proliferating embryo culture (Figure 4.12c) decreased the pH of the medium, and when the globular embryos were subcultured on to fresh medium with a higher pH, embryo development took place (Figure 4.12d and e).

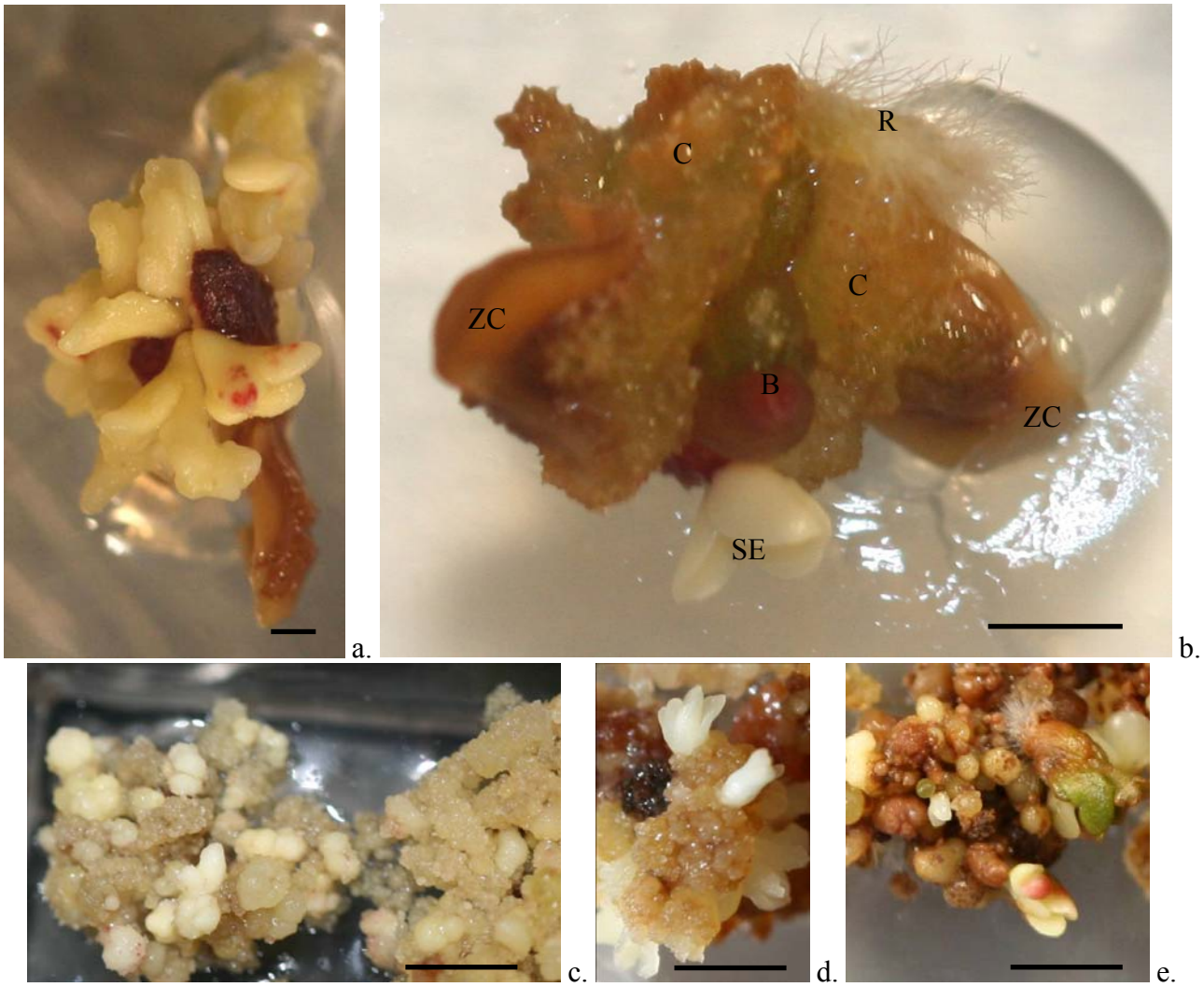


Figure 4.12. Somatic embryos that (a) regenerated from hard callus, (b) possibly arose from differentiated organs [where root (R), bud (B), somatic embryo (SE), zygotic cotyledons (ZC) and callus (C) formation is visible]. A soft callus culture containing white structures (c) gave rise to somatic embryos (d and e) (bar approximately equates to 5mm).

Indirect morphogenesis from zygotic cotyledon and embryonic axis explants

In many species it is possible to achieve multiple shoot and somatic embryo development originating at the cotyledonary node (Shyamkumar *et al.* 2004; Bhattacharjee *et al.* 2010) and cotyledonary explants from *Protea* species are also known to be morphogenic. Wu *et al.* (2007a) initiated somatic embryogenesis from both the zygotic embryos and excised cotyledons of *P. cynaroides* without the application of exogenous growth regulators. Conversely no embryos were initiated on *P. cynaroides* cotyledon explants plated on MS media supplemented with growth regulators (Wu *et al.* 2007a). The callus produced on cotyledonary explants of *P. neriifolia* was superior (although the manner of superiority was not given) over that produced on hypocotyl explants (Van Staden *et al.* 1981). However, the excised cotyledons of *P. roupelliae* ssp.

hamiltonii failed to undergo morphogenesis on a growth regulator free medium even after six months in culture.

In *P. roupelliae* ssp. *hamiltonii,* somatic embryo production was significantly higher after six months from zygotic embryos that had their cotyledons removed than those that were intact (Figure 4.13b). This increase could be attributed to a response to wounding of morphogenic tissue at the cotyledonary node or in response to a greater portion of morphogenic tissue being exposed to the medium (Merkle *et al.* 1990). Wu *et al.* (2007a) indicated that there may be a region of totipotent cells at the cotyledonary node of the somatic embryos of *P. cynaroides*. However, the same phenomenon was not mentioned regarding plated zygotic embryos from which embryogenesis was also initiated. Intact zygotic embryos of *P. roupelliae* ssp. *hamiltonii* showed an area of increased morphogenesis between the cotyledons (Figure 4.14). However, due to the close proximity of the cotyledonary node to the embryonic axis, it was possible that the increased embryogenesis could have been attributed to the increased ease of diffusion of nutrients from the media and atmospheric oxygen reaching the tissue of the embryonic axes.

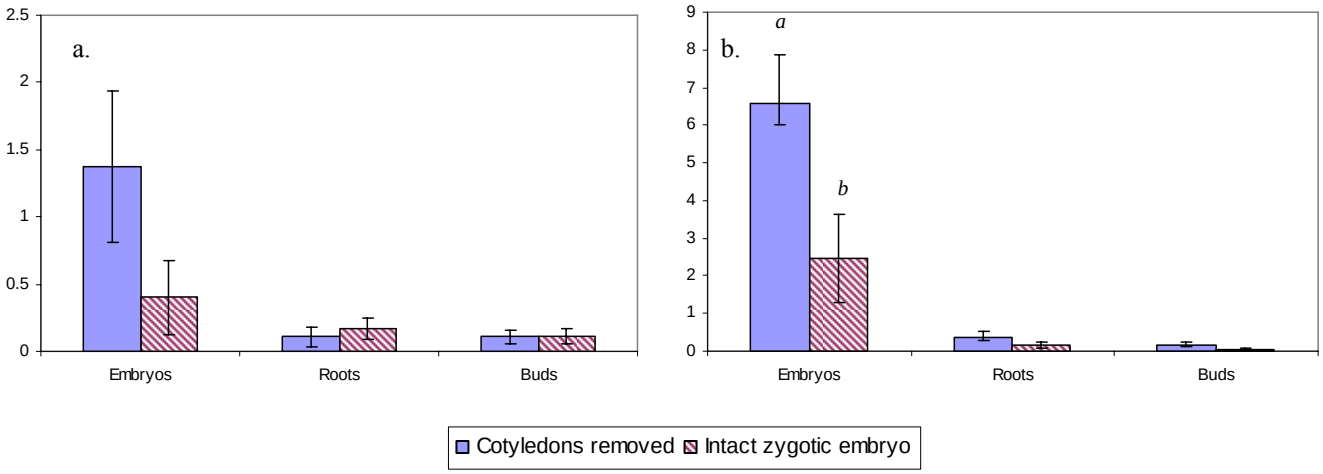


Figure 4.13. Comparison of the mean (±S.E.) numbers of somatic embryos, roots and buds that formed on zygotic embryos which were either intact or had cotyledons removed. Both were plated on the same media after three months (a) and six months (b). Differing subscript letters indicate significant differences ($P<0.05$).



Figure 4.14. Intact zygotic embryo explants with an area of differentiation between the cotyledons.

Primary somatic embryo development in response to a high GA_3 medium

GA_3 is reported to stimulate the normal development of *in-vitro* formed somatic embryos (Bhojwani & Razdan 1983) and this did occur when explants were plated on Medium 7 (high GA_3) (Figure 4.15), however many of the nascent embryos did not develop normally into plantlets. Apart from not forming roots, the white embryo tissue turned green and often became hyperhydric and leaves that were not hyperhydric, were elongated and pale (Figure 4.15). These misshapen leaves may have been developing cotyledonary leaves similar to those found during the regeneration of carrot embryos (Collin & Edwards 1998) but not all of the shoots with misshapen leaves developed normal leaves when planted out into the hardening Treatments 1 or 2 (Figure 4.20). Although these misshapen leaves may be part of the normal embryo developmental process, they did not form on embryos that developed on Medium 1. These abnormal leaves may be due to the inferior quality of adventitious somatic embryos that were formed on a suboptimal medium. Reducing the concentration of GA_3 may assist in decreasing the level of abnormal growth and elongation observed in Medium 7, yet GA_3 may still be necessary to induce shoot development (George 1993).



Figure 4.15. The converted embryos on Medium 7 displayed high levels of vitrification and extensive elongation.

Secondary embryogenesis

Rugge (1995) commented that primary somatic embryos formed directly and indirectly from the base of cultured *P. repens* shoots. That author went on to describe that the primary embryos failed to mature and gave rise to further cycles of embryogenesis and the secondary embryos occasionally matured into apparently normal plantlets (Rugge 1995).

Description of secondary embryogenesis

Regenerated somatic embryos and attached callus that arose from the zygotic embryo tissue in the above experiments were plated onto Medium 1. The embryos enlarged, turned dark and sometimes dedifferentiated to callus (Figure 4.16a). From these somatic embryos more nascent embryos formed (Figure 4.16a and b); only when embryos formed on easily identifiable somatic embryos (Figure 4.16c to f) was it clearly apparent that secondary somatic embryogenesis was occurring. However, secondary somatic embryogenesis may have been occurring much earlier in the tissue culture process (Figure 4.12b). Secondary embryos did form on all parts of the primary somatic embryos but embryogenesis was favoured at the shoot and root poles of the embryonic axis and rarely on the cotyledons (Figure 4.16). This development was presumably associated with the meristematic tissues of the poles.



Figure 4.16. Secondary somatic embryogenesis initiating on (a and b) aged and (c-f) developing somatic embryos (bar equates to approximately 1mm).

Maturation of secondary somatic embryos on growth regulator free medium

The secondary somatic embryos generally displayed the typical somatic embryo morphology and seemed to be of higher quality than primary somatic embryos. A higher proportion of the secondary embryos had two distinct cotyledons, identifiable root and shoot poles, additionally the margins of the embryos were smoother and better defined than those of the primary somatic embryos.

Many species need a maturation process to produce normal plantlets from somatic embryos, the treatment is often similar to the developmental processes of zygotic embryos (Bhojwani & Razdan 1983). A period of desiccation, synonymous with maturation drying during seed development, often allows the growth and maturation of *in-vitro* generated embryos and a higher proportion of embryo conversion (Merkle *et al.* 1990; Terzi & Loschiavo 1990). Desiccation can be performed osmotically by progressively increasing the sucrose concentration of the tissue culture medium or simply allowing embryos to dry in empty Petri dishes (Merkle *et al.* 1990). Multiple secondary embryos of *P. roupelliae* ssp. *hamiltonii* matured into shoots (and sometimes rooted plantlets) synchronously towards the end of three month subculture cycles on Medium 1 (Figure 4.17a). This period would have coincided with a decrease in the water potential of the medium as well as a change in the sucrose and MS salt concentration. Some point during the decrease in water potential within the *in-vitro* environment may have triggered the further maturation and subsequent development of the somatic embryos. The requirement for a period of desiccation was also observed when the embryos were excised, dried for an hour in the laminar flow cabinet and then left on moist filter paper where they greened rapidly (within three weeks) and initiated plantlet development (S. Tarlton *pers. obs.* 2010) (Figure 4.18).

Once growth was initiated shoots grew readily from the secondary embryos, however roots formed more rarely (Figure 4.17b to e). Root formation was poor but seemed to increase with successive cycles of embryogenesis. Rooting also seemed to be favoured from embryos that were not associated with callus. Poor root development is common in *Protea* shoot explants (Wu *et al.* 2007b) and has also been observed in seedlings derived from zygotic embryos. Furthermore seedlings originating from germinated zygotic achenes were sometimes observed lying un-rooted on the soil surface while leaf development seemed unaffected (S. Tarlton *pers. obs.* 2009).



Figure 4.17. Embryos that developed (a) leaves synchronously after a long period without subculturing, (b to e) both shoot and root poles, (f and g) shoots only and (h) shoots only that were still attached to callus.

Possible advantages of embryogenic cultures

The use of proliferating embryonic cultures is advantageous in that they have the potential to rapidly multiply embryonic propagules. These propagules are embryos which have the morphology and physiology similar to zygotic embryos, with the capability to form a complete plant (Merkle *et al.* 1990). A further advantage of somatic embryos is that in many instances the embryos can be delivered directly to the greenhouse or the field without the need for intermediate

transplanting steps (Merkle *et al.* 1990), which seems possible in *P. roupelliae* ssp. *hamiltonii* as somatic embryos develop readily on moist filter paper (Figure 4.18). Somatic embryogenesis also forms the basis of artificial seed production which has been perfected in alfalfa (*Medicago sativa* L.) (McKersie and Brown 2008). A further benefit of working with embryo tissue of *P. roupelliae* ssp. *hamiltonii* is that it seemed more resistant to phenolic browning when handled than callus or shoot explants.



Figure 4.18. Embryos produced through secondary embryogenesis that developed organs after one hour of desiccation and 3 weeks on moist filter paper under growth room conditions.

4.5.5. Rooting and hardening of *in-vitro* derived shoots in soil

The hardening process is required so that plantlets gain adequate stomatal function and increased production of cuticular waxes for transpirational control while simultaneously achieving successful rooting before being planted *ex-vitro* (for example the greenhouse).

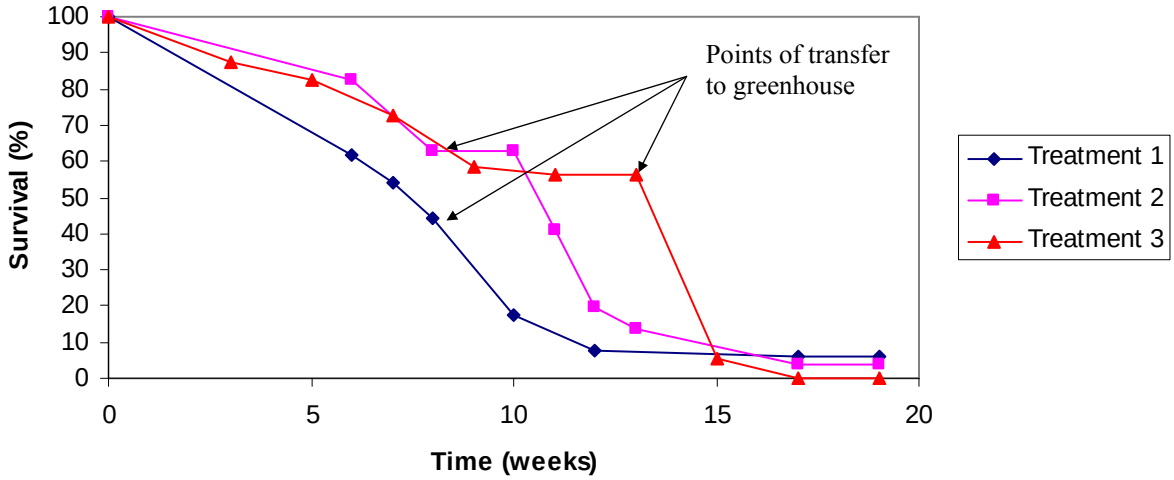


Figure 4.19. The percentage survival of *in-vitro* derived shoots hardened via three different treatments over 19 weeks (see text Section 4.4.6 for treatment details).

Rooting treatments 1 and 2

Presence of endogenous auxins in the explants was assumed as both the growth of callus and the formation of somatic embryos on growth regulator free media was possible (Terzi & Loschiavo 1990). Adding synthetic auxin to the seemingly already high levels of endogenous auxin could have reverted the shoot tissue back to callus and somatic embryo production, therefore IBA was not used in Treatments 1 and 2 to promote rooting. It was further considered that root development may have been promoted by the low nutrient conditions of the soil from the Dr Hamilton reserve (López-Bucio *et al.* 2003).

Hyperhydric shoots were not used in Treatments 1 and 2, but occasionally shoots became hyperhydric during the hardening treatment and died soon after. Whilst in the growth room, the development and growth of apparently normal leaves replaced the abnormal cotyledonary leaves that were produced on Medium 7 (Figure 4.20). Due to this leaf growth and development, it was assumed that some rooting had occurred whilst in the treatment. However, leaves of the shoots wilted, turned brown and died (Figure 4.20f and g). The rate and manner in which death occurred indicated that water stress due to lack of roots may have been the cause of death. Although the most probable cause of death would be the lack of functional roots, unfortunately the roots were not visually assessed in these treatments.

The period needed for the plantlet to be physiologically adapted to the *ex-vitro* environment may have been inadequate for sufficient rooting to occur. The leaves that were produced may not have developed sufficient cuticular waxes or stomatal function for sufficient water retention and survival in the lower relative humidity of the greenhouse.



Figure 4.20. The development of a shoot in Treatment 1 at (a) two weeks, (b) three weeks, (c) four weeks (arrow indicates cotyledon-like like leaf), (d) six weeks, (e) seven weeks, (f) eight weeks and (g) ten weeks from being planted in soil (see text Section 4.4.6 for treatment details).

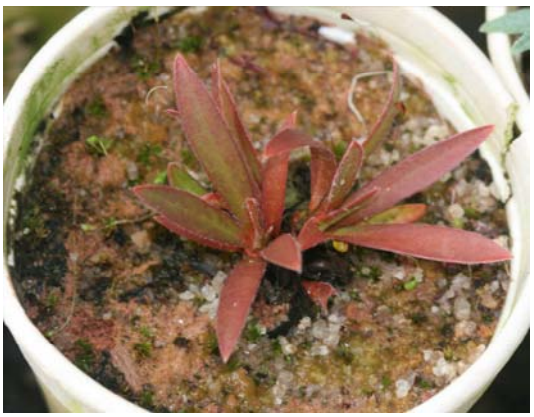


Figure 4.21. Plant propagated through rooting and hardening via Treatment 2 and maintained in the greenhouse (approximately 8 months since transfer from the *in-vitro* situation to soil, and a further 6 months surviving in the greenhouse) (see text Section 4.4.6 for treatment details).

Treatment 3

Treatment 3 was a modification of the previous two treatments. Only shoots that developed with normal leaves on Medium 1 (Figure 4.17f and g) were used and a pulse of the plant growth regulator IBA was employed to assist with root initiation (Van Staden *et al.* 1981). Furthermore the period that shoots spent in the *in-vitro* environment was extended to give these slow growing plants more time for shoots to depend on photosynthesis as a carbon source and produce functional roots. Root development was assessed when shoots were planted in the greenhouse and only 9.38% of the total number of shoots and 16% of the surviving shoots produced roots. Roots that were produced were predominantly short and thick (Figure 4.22). All shoots from the rooting and hardening Treatment 3 died after being planted in the greenhouse.

From the above rooting and hardening treatments it may be better to transfer plants to the greenhouse earlier in the plants development, hence allowing more time for development and increasing the ability of the plant to survive the harsh conditions of the greenhouse. Furthermore, greater success and survival may be achieved if somatic embryos were “germinated” directly in the greenhouse.



Figure 4.22. Roots that formed on shoots during the rooting and hardening via Treatment 3 (bar equates to approximately 5mm).

4.6. Summary and conclusion

4.6.1. Stem cuttings

The use of cuttings was advocated by Laubscher *et al.* (2009) for the propagation and conservation of rare and endangered Proteaceae on the Agulhas plain in the Western Cape. Once rooted a plant derived from cuttings will become sexually mature more rapidly and produce cones and achenes earlier than plants grown from achenes (Ben-Jaacov *et al.* 1986). However, before the use of cuttings becomes an option for *P. roupelliae* ssp. *hamiltonii,* more attention needs to be given to reducing the phenolic blackening or browning of the cuttings. Areas of progress in this regard may lie in avoiding the transportation of the cuttings over long distances to the greenhouse, optimising surface decontamination procedures (for example reducing ethanol application), optimising the antioxidant treatment, as well as determining appropriate greenhouse environmental conditions such as misting and bottom heat for the cuttings of this species (Coetzee & Littlejohn 2007). Additionally *Protea* cuttings need to be 15-20cm long terminal shoots (Coetzee & Littlejohn 2007), which is a long section of shoot tissue to remove, especially for a slow growing species on impoverished soils.

4.6.2. Zygotic embryo germination

The achenes and embryos of *P. roupelliae* ssp. *hamiltonii* germinate readily on filter paper. Germination appeared to be particularly sensitive to the pre-treatments tested, the germination process was delayed if the achenes were soaked prior to being set to germinate and germination was accelerated if the seed coat was removed. This may have been due to the alteration of oxygen availability to the embryo. Germination totality (percentage) and rates were considered to be acceptable at 25±2°C, which corresponds with the warm wet environment found in the Barberton

Montane Grassland vegetation type in November (Mucina & Rutherford 2006) when newly germinated seedlings were observed (S. Tarlton *pers. obs.* 2009).

The method used to germinate achenes with no pre-treatment was found to be adequate for the germination of *Protea roupelliae* ssp. *hamiltonii* zygotic embryos. Using no pre-treatment posed the least risk of destroying embryos through physical damage or microbial contamination. This method was therefore chosen as the protocol to be followed in all subsequent seed germination experiments.

The removal of the seed coat and other coverings may have an application in germinating achenes of *Protea* species that have low vigour as in cases where suboptimal germplasm storage was utilised. Microbial contamination however, remains a threat to the survival of the developing excised embryo and techniques should therefore be employed to suppress contamination throughout the germination process (Brown & Van Staden 1973b). Although un-tested in this study, the possibility that a cold stratification pre-treatment or incubation at low temperature may increase the oxygen solubility of the water surrounding the embryo resulting in increased germination vigour.

4.6.3. Indirect morphogenesis

The complete process from the initiation of a *P. roupelliae* ssp. *hamiltonii* culture to the reintroduction and establishment of propagules *in-situ* was not accomplished. However, there remains potential to increase the rate of multiplication and regeneration of embryos through the optimisation of media and culture techniques.

Somatic embryo culture

Employing zygotic embryo tissues as parent material substantially decreased the incidence of contamination compared with that obtained from shoot culture investigations. Zygotic embryo tissue however, does have genetic information from two parent plants and therefore if the genetics of the subsequent culture is important, controlled hand pollination would therefore be necessary.

At the onset of the study, in order to avert somaclonal variation and avoid possible genetic instability, it was attempted to avoid callus stages. However, even on a growth regulator free medium, callus was initiated, indicting the developmental processes were regulated possibly by the endogenously synthesised regulators. In this regard it may be assumed that comparably low

levels of variation arose. The production of callus and subsequent embryogenesis did have the advantage of decreasing the wounding and subsequent phenolic browning experienced by the explants that typically limits production in Proteaceae shoot cultures (Coetzee & Littlejohn 2007). Adventitious somatic embryos were not well formed, but embryo production was significantly higher from zygotic embryonic axes than from intact zygotic embryos. The media used needs to be optimised for the production of somatic embryos with normal morphology and synchronous shoot and root development.

Secondary somatic embryogenesis

Secondary embryogenesis holds the potential for the rapid multiplication of large numbers of *P. roupelliae* ssp. *hamiltonii* somatic embryos. However, in order to fully exploit this approach, stages of the process need to be better understood and controlled. For example it is necessary to maintain an embryo culture in a proliferating state until embryos are needed, at which point it is necessary to arrest the proliferation of embryos and initiate development. Cues that initiate the multiplication and the development of the embryos have not been tested. Maintaining a low pH of the medium may be beneficial for the multiplication of globular embryos and increasing the pH may stimulate the development of the embryos to the subsequent heart and torpedo stages (Smith & Krikorian 1990b). Similarly the growth regulator abscisic acid (ABA) may be effective in imposing stasis on particular embryo development stages and aid embryo development (Ammirato 1974). A period of desiccation is thought to be necessary for shoot development, which can be imposed by leaving somatic embryos on medium as it slowly desiccates over an extended period or excised embryos can be allowed to dry in the open (Figure 4.18). The reliable initiation of roots would also have to be addressed before this micropropagatory approach can be used to its full potential.

The potential for recurrent embryogenesis would allow for an unlimited number of high quality embryos to be cultured. Those somatic embryos could then be germinated under greenhouse conditions without an intervening hardening stage. The somatic embryos produced may also be amenable to cryopreservation, for the *ex-situ* conservation of this endangered species (Collin & Edwards 1998). In other *Protea* species and new cultivars of horticultural importance, where zygotic seed production is low and the genetic content thereof is unreliable, the ability to rapidly produce quantities of embryos containing a conserved genotype would be invaluable to the industry. The successful multiplication of mature zygotic embryo explants may translate into the ability to utilize immature zygotic embryo explants in embryo rescue, allowing new horticultural crosses to be cultured and rapidly multiplied.

4.6.4. Rooting and hardening of *in-vitro* derived shoots

P. roupelliae ssp. *hamiltonii* displayed a very slow rate of tissue growth *in-vitro,* a characteristic which persisted into the rooting and hardening processes. The percentage of successfully hardened off, rooted shoots was low in both treatments 1 and 2 but success did occur nonetheless (Figure 4.21). Rhizogenesis may have been more rapid with the application of IBA in Treatment 3, however there was no shoot survival in that treatment. It may be necessary to apply the phenolic compound 3,4-dihydroxybenzoic acid, which has been found to be an endogenous regulator of rooting and promotes rooting in *P. cynaroides* (Wu *et al.* 2007c), or to use commercially available IBA rooting powders which will persist for longer. Additionally the high greenhouse temperatures may have been too extreme and this experiment should be repeated at lower greenhouse temperatures to aid seedling survival. Further studies should focus on germinating somatic embryos directly in the greenhouse or *in-situ* without this intermediate hardening step.

Chapter 5

5. *Ex-situ* conservation and *in-situ* augmentation of *Protea roupelliae* ssp. *hamiltonii*

5.1 Abstract

Due to the rarity of *Protea roupelliae* ssp. *hamiltonii* and the ephemeral nature of *Protea* achenes *in-situ, ex-situ* achene storage and methods of delivering such propagules back *in-situ* were tested. An initial survey showed the storage behaviour of the achenes to be orthodox. Air dried achenes were stored under Ambient, 25°C, 4°C, -70°C and -196°C regimes. The storage treatments were evaluated based on post storage germination (vigour and viability), at periods of 6, 12 and 18 months. The efficacy of achenes from the various regimes stored for 12 months (and relatively un-stored achenes) to produce seedlings to augment the population was evaluated by comparing seedling growth at 6 and 10 weeks after germination and their survival in the field after one year. The storage of *Protea roupelliae* ssp. *hamiltonii* germplasm for 18 months proved possible in all storage treatments with varying efficacy. The mean germination percentage after 18 months of storage was 89.38% (compared with 93.67% of an un-stored treatment). The highest P.V. after 18 months of storage was achieved in the -70°C storage regime. There was a general increase in vigour (mean P.V. of 2.82 at 6 months and 5.32 at 18 months of storage) and total germination (mean germination percentage of 70.85% at 6 months and 89.37% at 18 months) over time. However, there was a decrease in vigour and total germination after 12 months of storage in the treatment exposed to ambient conditions, although the germination percentage was still high (86.81%) after 18 month of storage. A loss of vigour was also observed in the 25°C treatment. Seedlings propagated from achenes stored for 12 months of storage under the -70°C regime were initially significantly taller and the leaves significantly longer than all other treatments at 72 days from germination (however significance was lost by 139 days). Seedlings originating from all storage regimes showed a high level of survival (90.74±1.92%; ±S.E.) after one year in the field. After one year in the field seedlings originating from achenes stored at -70°C for one year appeared healthier, having more green leaves, than seedlings derived from the other storage regimes. Two methods of population augmentation into the Dr Hamilton Reserve were tested (a) the planting of achenes and (b) the planting of 76 day old *ex-situ* germinated and grown seedlings (transplants). Transplants had better survival (96.44% after 13 months and 95.37% after 25 months) compared with the total percentage of planted achenes that emerged (76.41%, 13 months after planting and 69.81%, 25 months after planting). Less than an estimated 10% of the germinable achenes that were planted emerged. The growth of transplants was significantly faster than seedlings of similar ages maintained in the greenhouse. Only the leaf production of seedlings that originated from planted achenes after a year was significantly higher than seedlings of similar ages maintained in the greenhouse, although all other measured parameters were greater in the greenhouse seedlings. These data show that the seed of *Protea roupelliae* ssp. *hamiltonii* respond well to low and ultra-low temperature storage. Seedlings originating from stored achenes at these temperatures are fit for population augmentation. Seedlings grown *ex-situ* and transplanted into the field were robust, able to establish and flourish. The success of the germplasm storage and population augmentation may also form the basis for effective *ex-situ* conservation and new population establishment that may be needed to counteract the possible extinction of the species.

Key words: cryogenic seed storage, population augmentation, seedling establishment, tested *ex-situ* conservation protocol, transplanted seedlings.

5.2. Introduction

5.2.1. *Ex-situ* conservation

Conserved genetic material can act as a safeguard against extinction in the event of environmental disasters as well as act as source material for reintroductions (Maunder 1992). By integrating both *in-situ* and *ex-situ* conservation initiatives, superior outcomes can be expected (Cochrane *et al.* 2007).

Species that produce seeds which can be stored dry and at low temperatures produce what are categorised as orthodox seeds (Roberts 1973). However, other species produce seed that do not tolerate desiccation (known as recalcitrant) or seed that may survive desiccation but are sensitive to low temperature (Ellis *et al.* 1991; Ellis *et al.* 1990; Roberts 1973). Furthermore some plant species set seed poorly, such as cassava or not at all, as in commercial banana cultivars. Germplasm conservation for most species that produce orthodox seed takes the form of seed storage (Engelmann 2000), which is estimated to cost as little as 1% of the cost that may be incurred by *in-situ* conservation (Li & Pritchard 2009). The *ex-situ* conservation of species that do not produce orthodox seed needs to be achieved though other techniques which include field gene banks, *in-vitro* storage and the cryopreservation of cell suspensions, callus, shoot buds and embryos (Engelmann 2000).

Seed storage

The continued viability of stored seeds depends on temperature, the moisture content of the seed and the length of time in storage (Ellis & Roberts 1980) however, the exact mechanisms of seed deterioration are thought to be species specific (Walters *et al.* 2005). As seeds age, the half life of molecules are reached and biological structures deteriorate within the seed and germination vigour is gradually lost (Roberts 1983). The viability of the seed is eventually lost when the seed does not have the resources or the mechanisms on imbibition to repair the damage incurred during storage (Kranner *et al.* 2010; Berjak & Villers 1972).

The desiccation of a seed is generally considered to impose some level of stress even in orthodox species (Kranner *et al.* 2010). However, once the seed tissues are dry after development and maturation, the cytoplasm of the component cell is considered to be in a vitrified state. The vitrified cytoplasm within a cell is viscous with diffusion movements substantially slowed, resulting in less deleterious reactions and a substantially decreased rate of seed deterioration (Sun

& Leopold 1997). However, viability may also be lost if seeds are desiccated beyond a point from which they are able to recover (Leprince *et al.* 1995).

The production of active/reactive oxygen species (ROS), namely $^1O_2$, O_2^-, OH^- and O_2H_2 occurs as a product of aerobic metabolism (Benson & Bremner 2004). These ROS then oxidise lipids and proteins which disrupts primary metabolic pathways, cellular membranes and compromises enzymatic antioxidant protection mechanisms (Benson & Bremner 2004). Oxidation of organic molecules can occur though enzymatic (oxidases) means or by autoxidation when molecules are exposed to oxygen (including atmospheric oxygen).

Cryogenic seed storage

In recent years concerns have been raised over the longevity of seeds stored conventionally, with ensuing viability being less than expected (Probert *et al.* 2009). In the vitreous state, although deleterious reactions are slowed they may also be temperature dependant. It is therefore recommended that ultra low storage temperatures should be adopted for the long term conservation of all orthodox seed (Li & Pritchard 2009). Seeds stored at warmer temperatures would need to be removed from storage and replaced by new acquisitions at shorter intervals as they lose viability faster, adding to the cost of the seed storage (Li & Pritchard 2009). Due to the relationship of viability with temperature, it was thought that seeds that could tolerate desiccation would maintain viability at the ultra low temperatures of liquid nitrogen at about -196°C almost indefinitely (Roberts 1983).

Physical cryoinjury can originate from the formation of ice which may further desiccate cells as intracellular water is removed and ice crystals are formed, moreover the formation of ice crystals may also physically damage cellular membranes and even cause tissue rupture (Benson 2008). A rapid change in temperature can also apply mechanical stress to tissues of a seed due to the differing coefficients of expansion and differing cooling (and warming) rates within a seed, which may lead to the cracking of the tissue (Vertucci 1989; Pritchard *et al.* 1988; Busse & Burnham 1934). The lipid content of seeds may adversely affect their longevity, especially under cold conditions (Crane *et al.* 2006; Ellis *et al.* 1990). The association between the decrease in seed viability and lipid content (and composition) is thought to be a result of the conformational changes in lipids phases (Crane *et al.* 2006). However, seed oil content has been shown to be uncorrelated to the fall of viability in 140 species stored at -20°C (Probert *et al.* 2009). The predominant type of lipid within a seed is probably more indicative of its cold sensitivity as different fatty acids change phase at different temperatures, and hence the cold sensitivity of

species is expected to vary (Li & Pritchard 2009). The relatively slow re-warming of the seed and the slow imbibition during germination are precautions that should be followed for the cryogenic storage of oil rich seed (Pritchard & Nadarajan 2008).

There is evidence that there is molecular mobility in preserved seeds at cryogenic temperatures (Walters, 2004). As more investigations are made and the seeds of a greater diversity of species are being cryopreserved, the effects of time the material is exposed to these temperatures is also being incorporated as a factor in these experiments, it is becoming clear that loss of viability can indeed occur even at -196°C (Whitaker *et al.* 2010; Varghese & Naithani 2008; Walters *et al.* 2004). This is especially true if seed deterioration has commenced prior to storage at ultra low temperatures (Varghese & Naithani 2008; Benson & Bremner 2004; Walters *et al.* 2004).

Seed storage of *Protea* species

Currently the storage of germplasm of *Protea* species is conserved in the Fynbos Genebank managed by the Agricultural Research Council (ARC). The gene bank is for the development of interspecific hybrids of ornamental potential from the Cape Fynbos and therefore consists mostly of a field gene bank, with some species conserved under seed storage (Matlhako *et al.* 2008; Littlejohn 2001).

Most *Protea* species have canopy seed storage and do not maintain a persistent soil seed bank, with the viability of *P. repens* achenes being lost within one year of burial (Holmes & Newton 2004). Some species store achenes only for a few months after achenes mature, but most others are typically stored for over a year on the plant (Lamont *et al.* 1991; Bond 1985). *Protea neriifolia* achenes are mature by about seven months following fertilization. If achenes are harvested before the maturation period is complete, insufficient protein reserves are laid down and viability is compromised (Van Staden 1978a; Van Staden & Gilliland 1977). However, once achene maturation is complete, retaining achenes on the parent plant leads to ageing resulting in a loss of vigour and viability (Le Maitre 1990; Bond 1985; Van Staden 1978a; Van Staden & Gilliland 1977). This decline in viability highlights the importance of the development of a long-term seed storage protocol for *Protea* species and other Proteaceae, which is further emphasised by the fact that more than one third of South African Proteaceae species are listed in the Red Data Book for plants (Rebelo 2006).

Storing *Protea neriifolia* achenes at 20°C and 26°C in cotton bags for 36 months resulted in decreased germination vigour and viability (Van Staden 1978b; Van Staden & Gilliland 1977).

This loss of viability was associated with intracellular membrane damage, particularly of the spherosomes (lipid storage bodies), endoplasmic reticulum and the plastids (Van Staden 1978b). During storage in these suboptimal conditions the spherosomes coalesced, forming large "pools" of lipid on the periphery of the cell as opposed to small discreet droplets surrounding protein bodies (Van Staden 1978b). The lipid content and type is known to influence the storage of seeds (Pritchard & Nadarajan 2008). The seed oils of two *Protea* species analysed were mostly unsaturated (of the total lipid content 77.8% in *P. compacta* and 83.0% in *P. longiflora* is unsaturated), and are comprised of the relatively slow autoxidising monounsaturated oleic acid (18:1) (70.7% *P. compacta* and 73.3% *P. longiflora*) as opposed to the more reactive polyunsaturated linoleic acid (18:2) (5.7% *P. compacta* and 7.0% *P. longiflora*) (Vickery 1971). With the main energy reserves in Proteaceae embryos being in the form of lipids, with some species containing lipid contents of up to 76% of dry matter (Pate *et al.* 1986), precautions need to be made during cryogenic storage of members of the Proteaceae to avoid cryoinjury (Pritchard & Nadarajan 2008).

In *P. neriifolia* the loss of viability was not associated with the loss of water during storage (Van Staden 1978b). Van Staden (1978b) tested the storage and viability of *P. neriifolia* achenes at temperatures as low as -10°C. Therefore the extent and origin of possible cryoinjury and viability loss of *Protea* achenes stored at ultra low temperatures is unknown. Additionally the achenes of *P. neriifolia* were reported to be non-dormant and they were first sampled only after a year in storage (Van Staden 1978b).

5.2.2. Rare plant reintroductions

Reintroduction can be defined loosely to incorporate any activity that returns propagules of a species back *in-situ* within its former range (which can incorporate the establishment of new populations, the re-establishment of extinct populations and the augmentation of existing populations) (Guerrant Jr & Kay 2007; Maunder 1992). Endangered-plant reintroduction is still a relatively new field, it is based on restoration ecology and currently relies on experimentation to build necessary generalisations (Guerrant Jr & Kay 2007). It was estimated that 72% of 181 recovery plans for endangered species called for some form of reintroduction (Hoekstra *et al.* 2002). However, the practice of reintroducing endangered plants faces many challenges and consequently lead to frequent failure, many regard this method of conservation to be unreliable (Kay 2008; Fahselt 2007).

At the onset it has to be realised that the biological success of reintroductions is inherently low, with only about 11% of reintroductions considered successful, although many as yet have not been given sufficient time to succeed (Frankham *et al.* 2004). The success of rare plant reintroductions is measured biologically by the performance of individuals, populations and metapopulations (Guerrant Jr & Kay 2007). Therefore emphasis is placed on the number of reintroduced individuals or populations established; the ability of those individuals to persist through environmental perturbations and the ability of the population to become self sustaining. However, reintroduction trials also test methodological aspects such as the timing, method and location of planting, the type of propagule planted, and the post planting maintenance required for a species. Which even if biological success is not met the methodological aspects of the study provides valuable information on the species (Guerrant Jr & Kay 2007). For example planting seeds at the correct month of the year was beneficial to the four Cape serotinous Proteaceae species (*P. compacta, P. lorifolia, P. repens* and *Leucadendron rubrum*) where it was found that the monthly minimum temperature was strongly correlated to seed germination in the field (Midgley *et al.* 1988). Additionally reintroductions can rely heavily on observations for success, such as identifying a regeneration niche for propagule reintroduction (Guerrant Jr & Kay 2007; Maunder 1992; Grubb 1977).

There are two facets to the cost of a reintroduction exercise, the financial cost and the cost of each propagule (generally due to the rarity of the species). It must be realised that returning plants to their former or existing habitat is a high-risk endeavour in that high rates of propagule loss may occur (especially when using seed propagules) and it is a high-cost exercise as extensive management is required prior to and post planting (Maunder 1992; Wells *et al.* 1989; Hall *et al.* 1987). In many reintroduction situations the seeds of a rare species are so limited that the extra financial cost involved in successfully establishing transplants (out-planted greenhouse seedlings or plants) is negligible in comparison with the risk of failure in establishing seedlings *in-situ* from seed (Kay 2008; Maunder 1992). However, sometimes many seeds are available for a given species but reintroduction through seeding fails regardless of the seed numbers used, requiring the use of transplants for biological success (Guerrant Jr & Kay 2007). The lack of maintenance and monitoring is acknowledged by Hall (1987) as the most critical aspect leading to the high failure rate of transplantations. The maintenance of reintroduced propagules contributes highly to the cost of an operation. Bearing in mind that relatively basic, cheap methods can greatly influence establishment. For instance manually planting the seeds of the serotinous Proteaceae *Banksia goodii* increased the likelihood of a seed growing into a year old seedling from one in 500 to one in three (Witkowski & Lamont 1997).

The planning of a reintroduction project requires rigorous structure with discreet objectives. The objectives need to take into consideration the greatest immediate and long term threats to the population, whether it is genetic, demographic or environmental stochasticity (Clewell, 2000). The projects also require a coordinated approach, linking *ex-situ* propagation activities, germplasm collections and facilities, with *in-situ* restoration ecological management to reach their full potential (Maunder 1992). Additionally, the practice may need to be repeated at multiple times under multiple methodologies with a single species before any success is achieved (Guerrant Jr & Kay 2007)

Augmentation of the *P. roupelliae* ssp. *hamiltonii* population

The augmentation of *P. roupelliae* ssp. *hamiltonii* has been previously recommended in recovery reports (Weiersbye *et al.* 2000; Green 1995). Increasing the population numbers of *P. roupelliae* ssp. *hamiltonii* would be beneficial to the survival and genetic integrity of the species if performed properly and successfully. Rare *Protea* species require specialised propagation techniques, and the use of rooted cuttings has been advised for rare *Protea* reintroductions (Laubscher *et al.* 2009), although self-pollinating ability and inbreeding depression is of concern (Horn 1962). Reintroduction procedures typically require large numbers of propagules and germination procedures that maximise the number of seedlings produced and established per seed batch (Kay 2008). Procedures for the propagation and even the multiplication of propagules (albeit under *in-vitro* conditions) have been established for *P. roupelliae* ssp. *hamiltonii* (see Chapter 4).

Many of the genetic and provenance concerns that typically surround augmentation and reintroductions are negated by the fact there is a single population of *P. roupelliae* ssp. *hamiltonii* with few individuals. However, the polymorphism that is displayed by flower (bract) colour should be conserved. This colour polymorphism provides diversity linked to the mass and germination of achenes as well as the susceptibility of an individual plant to herbivores (Carlson & Holsinger 2010). The population is surrounded by *Pinus* species introduced from nursery propagated seedlings and to some extent the phytosanitary concerns of introducing transplants to the area (assumed to have been addressed by forestry companies prior to the establishment of the plantations) may also be relaxed.

The sporadic seed production and variable seed set is of concern when harvesting achenes from the *P. roupelliae* ssp. *hamiltonii* population (see Chapter 3), but achenes harvested for other short term experiments may be used to augment the population. Thereby valuable species information

can be collected whilst at the same time testing population augmentation procedures (Guerrant Jr & Kay 2007). Additionally the harvesting and storing achenes protects seed from the seed predators and seed ageing, that may be experienced *in-situ* and should not adversely affect a population of long lived species (over a short period) (Rebelo & Rourke 1986; Bond 1984; Van Staden 1978a).

Once a level of success is achieved in the population augmentation efforts, attempts to introduce new *P. roupelliae* ssp. *hamiltonii* populations may be initiated by building on the knowledge gained through the population augmentation experiments. The efforts may exploit the nearby conserved areas where historic populations have become extinct in the vicinity of Barberton and north-western Swaziland (Swaziland's flora data base www.sntc.org.sz/flora; Rouke 1980). Conserved areas near the Dr Hamilton reserve include the Nelshoogte Nature Reserve (about 6km away), the Glenthorpe forest (about 9km away), the Cynthna Letty and Thorncroft flora reserves (17km away) as well as the 49000ha Songimvelo Nature Reserve (about 19km away).

Managed relocation

Managed relocation (also known as assisted colonisation and assisted migration) is the translocation of species to favourable environments away from their native range to ensure their survival in the face of human induced threats (Hoegh-Guldberg *et al.* 2008). Managed relocation is a new, aggressive approach to plant conservation typically with regard to climate change. Currently, an ethical debate rages between the disadvantages of losing species that could be relocated to a more favourable environment and the possibility of the relocated species becoming invasive in their new environment and therefore negatively impacting the native species (Minteer & Collins 2010). While the impact of introduced species is thought to be inadequately understood for managed relocations to be attempted (Ricciardi & Simberloff 2009), the fact remains that a large number of indigenous nursery and garden plant species have already undergone assisted migrations (Van Der Veken *et al.* 2008). Although this study may assist in forming protocols for the establishment of *P. roupelliae* ssp. *hamiltonii* populations *de-novo,* the considerations, ramifications (such as seed transfer zones, gene flow, biotic, geographical and political boundaries as well as costs to other species) and ethics of relocating the species to new environments are beyond the scope of this study.

5.2.3. Establishing an *ex-situ* conserved species *in-situ*

At present, many species are being conserved under seed storage with the ethos of "collect and bank them now, and then plan the implementation stage when it is appropriate" (Vitt *et al.* 2010). The germplasm may be used for augmentation, reintroduction or managed relocations once the associated ethics have been resolved and the effects of climate change are realised (Vitt *et al.* 2010). However, the storage behaviour of seeds can vary (Balesevic-Tubic *et al.* 2010; Pérez-García & González-Benito 2008; Crane *et al.* 2006; Daws *et al.* 2006), and if suboptimal storage conditions are employed, the stored germplasm can be inadvertently lost as it may not be "fit-for-purpose". This could occur though the loss of viability (seed death) or seed/seedling vigour (possibly due to cryoinjury, ageing or genetic alteration) (Pérez-García & González-Benito 2008; Pollock *et al.* 1972). Laboratory viability tests, although useful indicators, do not necessarily equate to a "fit-for-purpose" recovery assessment of the stored germplasm (Benson 2008; Harding 2004), especially where established reintroduction techniques would rely heavily on the past qualities of the conserved germplasm. This is particularly true for *Protea* species where seed set is variable, dormancy breaking cues can be intricate and it may be difficult to determine whether an achene was germinable when storage commenced (Malan 1992). Standard laboratory germination tests do not necessarily correlate with the field performance of a seed lot (McDonald 1998). The rationale for the seed storage of endangered species, such as *P. roupelliae* ssp. *hamiltonii*, is for the stored seed to form the basis of a reintroduction/augmentation protocol. In terms of quality assurance of seed stored for *in-situ* conservation initiatives, it becomes necessary to not only germinate seed in the laboratory but also to ensure the seeds have the potential to become established seedlings in the field (Benson 2008).

5.3. Aims and objectives

The aim of this chapter was to develop protocols for (a) the *ex-situ* conservation of germplasm of *Protea roupelliae* ssp. *hamiltonii* and (b) the establishment of *P. roupelliae* ssp. *hamiltonii* seedlings to augment the population.

The three objectives were:

1. To test five *ex-situ* seed storage regimes.
2. To compare the use of seedlings established from achenes planted on site with transplanted seedlings for population augmentation.

3. To evaluate the efficacy of transplanted seedlings derived from seed storage regimes for population augmentation.

5.4. Materials and methods

5.4.1. Seed storage

Cones that were produced in the 2006/7 fruiting season were harvested from the Dr Hamilton Reserve on the 26th March 2008 and stored in paper bags for two months. The achenes were sorted by tactile assessment of "plumpness" (see Chapter 3) and those assessed as not "plump" were discarded. The hairs on the achenes were not removed.

Water content

The initial water content of the achenes was used to categorise the seed as either orthodox or recalcitrant, and was determined gravimetrically. A random sample of 50 achenes from various cones were each weighed to an accuracy of 0.1mg, wrapped in aluminium foil, labelled, and placed in an oven at 80°C. After 12 hours the achenes were removed from the oven, cooled in a desiccator and reweighed. This was repeated four times or until the weight of the achenes remained constant. The dry mass of the achenes was then subtracted from the original achene mass and the percentage water content calculated (wet mass basis).

Storage treatments

The initial water content (9.27±0.10%) showed that *P. roupelliae* ssp. *hamiltonii* achenes may be classified as orthodox (Roberts 1973). Therefore, storage treatments were devised accordingly.

As there were generally few "plump" achenes produced per plant each regime typically contained achenes from different plants. However, in one instance a single parent plant produced sufficient "plump" achenes that it was possible to incorporate a minimum of 15 vials per storage period (45 vials) in each of three storage regimes (25°C, 4°C and -70°C). As these achenes were from a single parent plant (and therefore of the same inflorescence morphotype (Carlson & Holsinger 2010)) less germination variation may be expected.

The cones containing achenes used in each storage regime were harvested from three different parent plants. After the assessment for "plumpness" achenes from each cone were distributed evenly between the three storage periods.

Un-stored (n=800) achenes were germinated within 3 months of harvesting (June 2008) and served as the un-stored treatment. On the 3rd June 2008, achenes (n=750 per treatment) were stored in sealed cryo-vials (five achenes per vial) in the dark under the following storage regimes:

1. Constant -196°C: Achenes were plunged into liquid nitrogen and stored.
2. Constant -70°C: Stored in a freezer at -70°C.
3. Constant 4°C Stored in a cold room at 4°C.
4. Constant 25°C Stored in a growth room at 25±2°C.
5. Ambient temperature: Ambient laboratory conditions at the University of the Witwatersrand, Johannesburg.

When vials were removed from the storage environments lower than ambient temperature they were warmed in ambient air.

Storage periods

Storage commenced on the on 3rd June 2008. The achenes were stored for 18 months and sampled every 6 months. At each sampling period 205 achenes per treatment were germinated and 45 achenes per treatment were used for water content.

Pre and post storage water content

Achene water content was determined at each sampling for each storage regime (as above) and differences were tested with an ANOVA and Tukey HSD post-hoc test.

Germination

Germination was assessed by placing the five achenes form each cryo-vial per Petri dish in germination conditions as described in Chapter 2, Section 2.4.3 for 30 days.

Germination parameters

The cumulative germination percentage of achenes filled with embryos was calculated for each treatment period. For each treatment period the following additional germination parameters were calculated:

a. The final germination percentage.
b. The peak value (P.V.) of germination (the maximum quotient derived by dividing the daily cumulative germination by the number of days taken to reach that percentage) (Czabator 1962).

c. The time taken for 50 percent of the seeds to germinate (T_{50}).

d. The peak day of germination.

The effect of the storage period and the storage regime on the germination parameters (a-d) were compared with a two-way ANOVA (without replication).

The achenes that originated from a single plant in the 25°C, 4°C and -70°C treatments had cumulative germination and the P.V. calculated for each storage period.

5.4.2. Augmentation and *in-situ* establishment

Two methods of establishing propagules *in-situ* (in the Dr Hamilton reserve) were tested, (a) achenes were planted directly *in-situ* and (b) planting germinated achenes *ex-situ* (in the greenhouse at the University of the Witwatersrand) and then transplanting the seedlings *in-situ.*

Greenhouse seedlings

Achenes that germinated in the un-stored control (see earlier) were planted into 350 ml perforated polystyrene cups filled with Dr Hamilton Reserve soil. The seedlings were maintained in the greenhouse at the University of the Witwatersrand. An automated Microjet watering system watered the seedlings three times a day for 10 minutes after which soil field capacity was attained.

Greenhouse temperatures

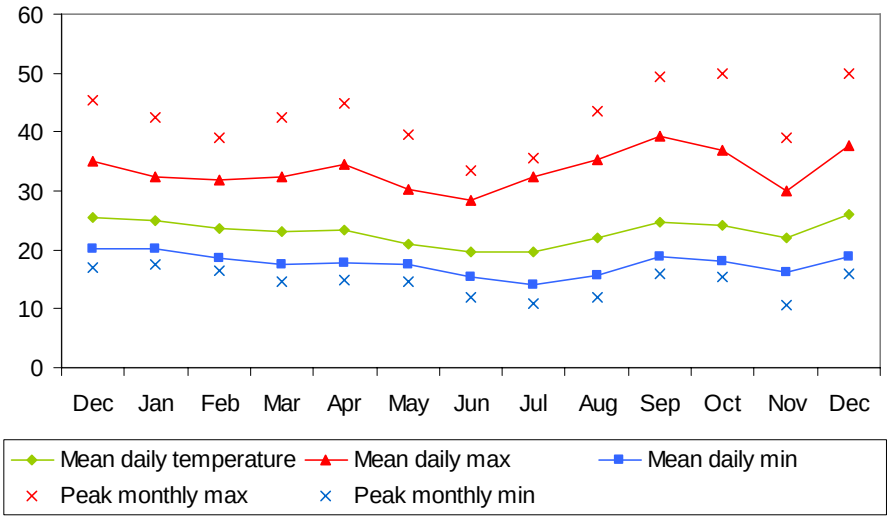


Figure 5.1: The mean daily temperature per month as well as the peak monthly maximum and minimum temperatures reached in the University of the Witwatersrand greenhouse from December 2008 to December 2009 (Helm 2011).

The temperature within the greenhouse at the University of the Witwatersrand attains peak temperatures as high as 50°C and as low as 11°C (Figure 5.1), with the mean daily temperature of 23.03°C (Helm 2011).

Achenes planted directly *in-situ* (Planted achenes)

In spring (13th October 2008) the adult plants within the Dr Hamilton reserve were divided into four sites based on their locality within the reserve (Figure 5.2), eight "wet" cones (bracts surrounding the base of the cones were still green and achenes were tightly held in the cones) and eight "dry" cones (bracts were dead and the achenes were being released) were harvested from plants within each site. The wet cones were left for 24 hours in ambient conditions for the cones to open and release the achenes.

Eight randomly placed 1m by 1m quadrats were identified per site. The soil within the quadrats was scoured with furrows to a depth of 10mm. On 14th October 2008 all the achenes from either two wet cones or two dry cones were then scattered in each quadrat before the furrows were smoothed over with soil. Each quadrat was fitted/covered with an herbivore exclosure constructed from wire (chicken) mesh (apertures of 12-13mm).

Each site therefore had four quadrats containing seeds from wet cones (2007/8 fruiting season) and four quadrats containing seeds from dry cones (2006/7 fruiting season). Previous experiments within this study showed that over a range of plant sizes, 36.61±3.21 (mean±S.E.) germinable achenes are produced per cone (see Chapter 2, Section 2.5.3). Therefore it was expected that about 73 germinable achenes were planted per quadrat.

After 34 days (17th November 2008) from planting, the quadrats were checked for germination. Laboratory experiments showed it takes at least seven days for the first achenes to germinate. A midpoint between day seven and day 34 was taken as the day of emergence for these seedlings and which was therefore calculated to be approximately 13 days. The quadrats were then rechecked when the age of the seedlings were calculated (as above) to be 77 days and again at 141 days from germination. At 77 and 141 days it was assumed that the seedlings that had produced the most leaves had germinated by 34 days from planting. When the seedlings that had emerged by 34 days from planting were calculated to be 141days old, they were marked with labelled wooden chopsticks which were pushed into the ground. At 381 days (approximately after one year) and 730 days (approximately after two years) they were re-measured and their survival was noted.

Seedlings planted *ex-situ* and transplanted *in-situ* (transplants)

Achenes that had germinated from the un-stored control of the seed storage experiment were maintained under the same conditions as the greenhouse seedlings (described above). In spring, after two and a half months (85% of seedlings were 76±7 days) in the greenhouse the seedlings were transported to and carefully planted in the Dr Hamilton Reserve on the 14-16th October 2008. Individual planting sites for seedlings were identified as bare patches of soil between grass tufts, no growing tufts were removed. Dead organic matter (surface litter) was pushed aside to facilitate planting. After planting the seedlings were watered-in and shaded (with pine branches) until rain fell on the 17th October 2008, after which the pine branches were removed and no additional post-planting maintenance was provided. The number of transplants planted into the Dr Hamilton Reserve at three different sites (Figure 5.2) totalled 281 seedlings. At 241 days, the seedlings were marked with labelled chopsticks and survival was assessed at 477 (one year and three months) and 835 days (two years and three months).

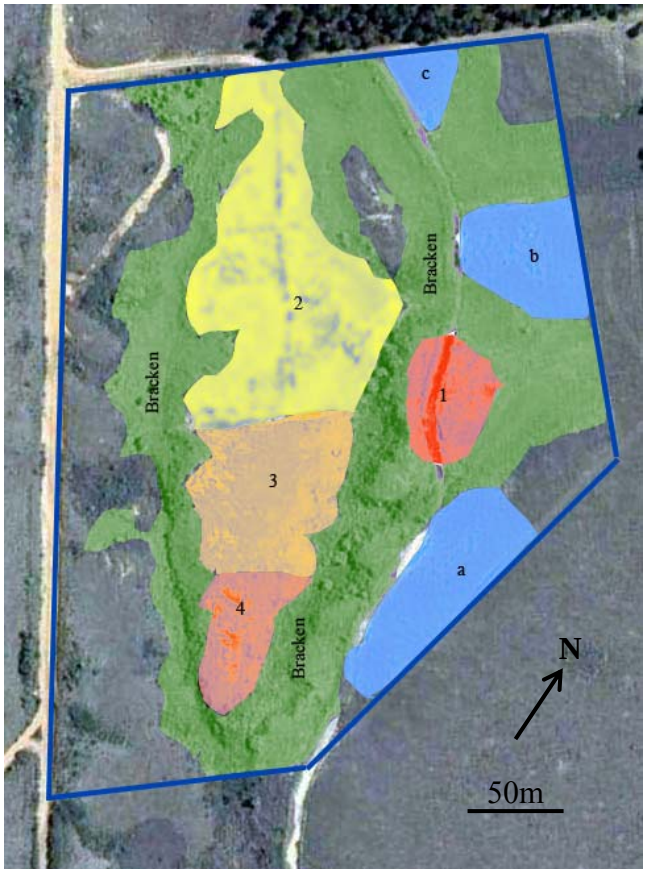


Figure 5.2. The areas where achenes and transplants were planted within the fenced area of the Dr Hamilton Reserve. The perimeter fence of the reserve is represented by the blue line. Shaded areas 1-4 are the four sites where the achenes were planted *in-situ* and areas labelled a-c are areas where the transplants were planted.

Measurements and data analysis

The stem diameter of the seedlings was measured directly below the point of attachment of the epigeal cotyledons. It was noted whether the cotyledons were alive and attached (green) or dead (dried up, brown) and/or shed. To ensure that the seedlings were not damaged by the vernier callipers, only the condition of the cotyledons, the number of leaves and the height of the seedlings were assessed during the first two and a half months of growth.

The total number of leaves was counted, including those of axillary buds and branches. Once seedlings were over a year old and it was estimated that there were over 50 leaves on a seedling, the number of leaves per seedling was estimated. In these cases to ensure that the estimate was accurate, the number of leaves on the largest branch of the seedling was counted and a sample of 10 seedlings per planted site were counted exactly (leaf estimates were only taken from transplants).

Seedlings were considered to be alive if any green leaves were present, if the seedling had no green leaves a small section of the stem was carefully removed and if the underlying tissue was green the seedling was categorised as alive. Seedling establishment *in-situ* was considered successful once seedlings had survived a full year *in-situ* and seedling survival was monitored for two years from planting.

The following measurements were taken:

a. The height of the seedling.
b. The length of the longest leaf.
c. The stem diameter.
d. A count of the leaves.
e. A count of the axillary buds.

The measurements ‘a’ to ‘c’ taken from seedlings that originated from planted achenes and transplants were compared with those of seedlings in the greenhouse using t-tests. Measurements ‘d’ and ‘e’ taken from seedlings that originated from planted achenes and transplants were compared with those of seedlings in the greenhouse using Mann-Whitney U tests.

Seedlings originating from planted achenes

Only the seedlings of which the age could be calculated (those germinating within 34 days from planting) and those that germinated from “wet” cones were used in comparisons. Measurements

taken from seedlings originating from planted achenes at 141 and 372 days were compared with measurements from greenhouse seedlings at 139 and 376 days respectively. Emergence was noted and leaf counts were taken at 13 and 77 days.

Transplants

Transplanted seedling measurements taken at 241 and 477 days were compared with measurements taken from greenhouse seedlings at 247 and 480 days respectively.

5.4.3. Post storage growth and survival

Achenes that were stored for one year in each of the five storage regimes were set to germinate as described (above Section 5.4.1 and Chapter 2 Section 2.4.3) on 3rd June 2009. An un-stored treatment consisting of achenes (n=100) harvested in March 2009 from the 2007/8 fruiting season was set to germinate on the same day. The seedlings which germinated from each regime and the un-stored treatment were planted into 350ml volume perforated polystyrene cups filled with soil collected from the Dr Hamilton Reserve. The seedlings were maintained in the greenhouse at the University of the Witwatersrand (as above).

Ex-situ growth

A random sample of 30 seedlings from each treatment were measured at two and a half months and four months. The following measurements were taken:

a. The height of the seedling.
b. The length of the longest leaf.
c. The stem diameter.
d. A count of the leaves
e. A count of the axillary buds

The measurements ‘a’ to ‘c’ were compared across storage treatments using an ANOVA and a post-hock Tukey HSD and measurements ‘d’ and ‘e’ were compared using a Kruskal–Wallis test and post-hoc Mann-Whitney U tests.

In-situ survival and condition

After four months in the Greenhouse (21st November 2009), seedlings from each storage regime and the un-stored treatment were planted out in the 4 sites within the Dr Hamilton Reserve. Each site contained 24 seedlings from each storage regime. One year after planting (12th November

2010), the seedling survival was assessed and the condition of each seedling was categorised with regard to:

a. Most of the leaves were dead and/or leaves were stunted and small relative to other seedlings.
b. Dead leaves were present but new leaves were also present and appeared healthy.
c. All leaves were green and appeared healthy.
d. Leaves were large, numerous and growth seemed vigorous relative to other seedlings.

Seedling survival and condition were compared between storage regimes and the mean percentage of seedlings in each of the seedling condition categories were compared using Kruskal–Wallis test.

5.5. Results

5.5.1. Seed storage

In all storage regimes the achenes sampled at six months germinated more slowly than achenes sampled at 12 and 18 months of storage (Figure 5.3). Additionally achenes sampled after 18 months of storage germinated earlier and more rapidly than other samples (Figure 5.3), with the exception of the Ambient storage regime, where the 18 month stored sample commenced germination later than achenes sampled at six months (Figure 5.3a).

After six months of storage, the -196°C regime commenced germination after all other regimes (Figure 5.4a). At 12 months of storage the Ambient storage regime germinated the slowest however, the cumulative germination did eventually surpass the 4°C regime after 15 days (Figure 5.4b). Additionally after 18 months of storage the achenes sampled from the Ambient storage regime commenced germination the latest of all storage regimes (Figure 5.4c). After 18 months of storage the -70°C regime commenced germination earlier than the other storage regimes (Figure 5.4c).

In general the germination percentage of each storage regime increased with the period of storage, this was especially true in the 4°C regime, which increased gradually with each sampling period (Figure 5.5a and b). However, achenes from the 25°C storage regime increased only slightly over 18 months of storage (Figure 5.5a). The germination percentage of achenes sampled from the Ambient storage regime was erratic compared with the other storage regimes, with the achenes

sampled after 12 months of storage providing the highest final germination percentage and highest P.V. compared with other sampling periods (Figure 5.5a and b).

The P.V. increased in all storage regimes with the period that achenes were stored, except in achenes from the Ambient storage regime (Figure 5.5b). The P.V. of achenes from the Ambient storage regime decreased between the 12 and 18 months of storage, whereas the achenes sampled from the -196°C storage regime increased only slightly over that period (Figure 5.5b). After 12 months of storage the T_{50} of all regimes were between 12 and 13 days, which decreased to between 11 and 12 days after 18 months of storage, but the T_{50} of the Ambient storage regime did not decrease below 14 days (Figure 5.5c). The peak day of germination was earlier (or the same) after 18 month of storage than after six months of storage (Figure 5.5d). However, in the Ambient, 25°C and -70°C regimes, the peak day of germination was at least one day later after 12 months of storage than after six months of storage (Figure 5.5d).

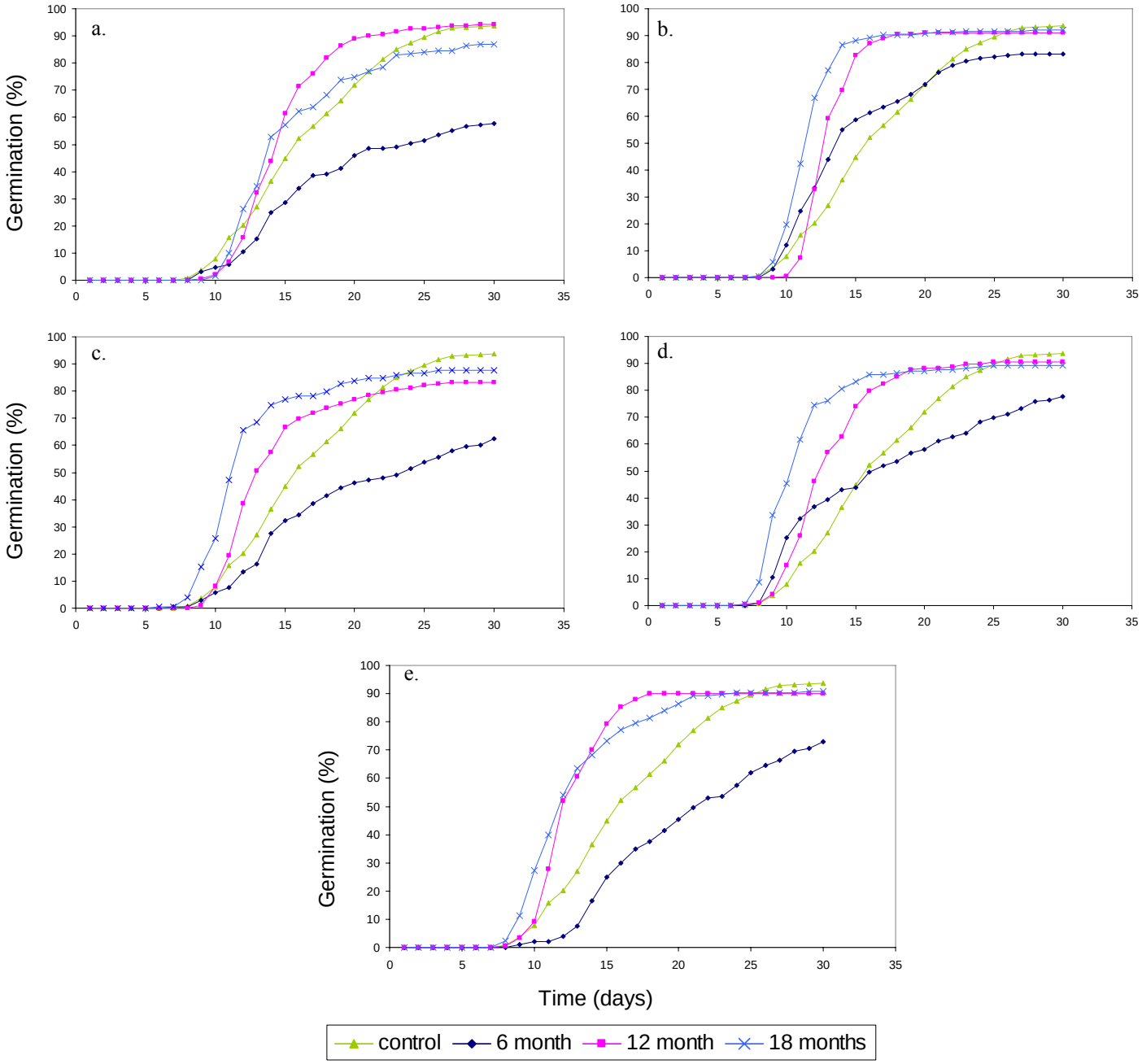


Figure 5.3. Cumulative germination for each of the storage regimes, (a) Ambient temperature, (b) 25°C, (c) 4°C, (d) -70°C and (e) -196°C, over the full test period.

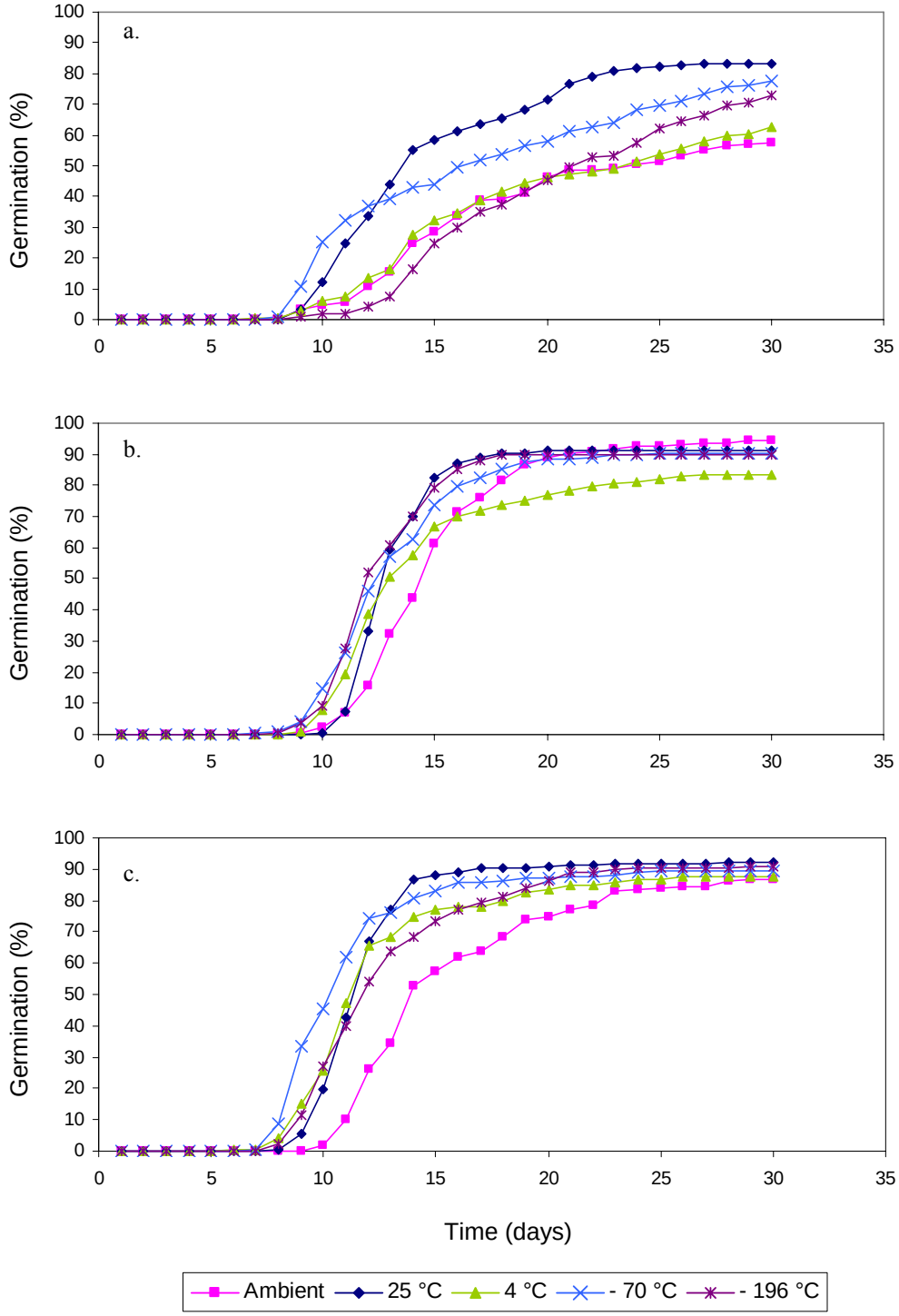


Figure 5.4. Cumulative germination percentage for the storage regimes at each sampling period; (a) 6 months, (b) 12 months and (c) 18 months.

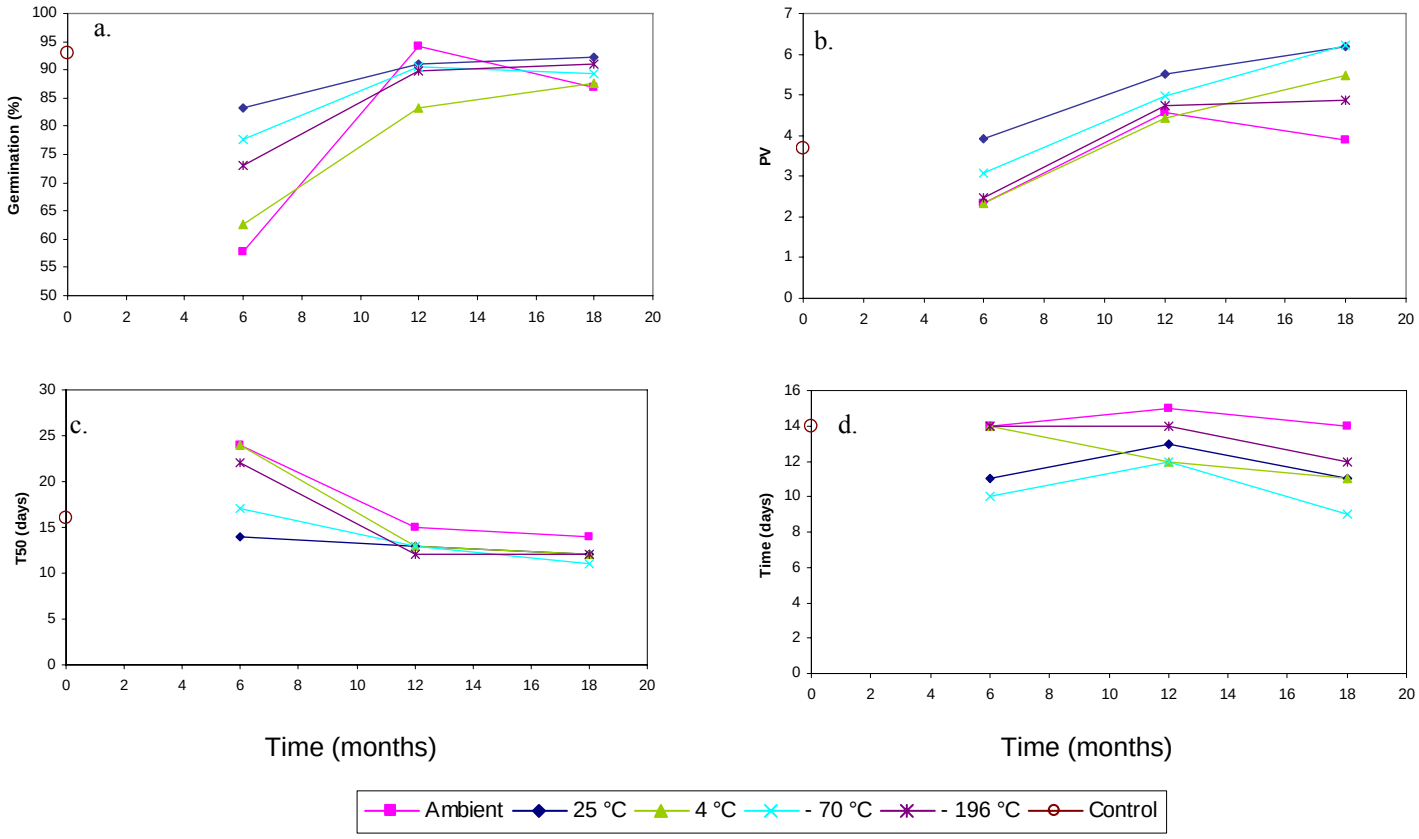


Figure 5.5. Germination parameters within storage regimes at each sampling period; (a) the final germination percentage, (b) the P.V., (c) the T_{50} and (d) the peak day of germination.

Storage period and regime

The two-way ANOVA shows that both the storage period and the storage regime had a significant effect on the P.V and peak day of germination of stored achenes (Table 5.1). However, the storage regime did not have a significant effect on the germination percentage or the T_{50}, where as the storage period did have a significant effect within each of the measured parameters ($P<0.05$).

Table 5.1. Two-way ANOVA without replication (hence without interaction) comparing the effect of the period of storage and the storage regime on the germination of stored achenes (final germination percentage, P.V, T_{50} and peak day of germination).

Variable	Storage period	Storage regime
Germination percentage	$F_{2,8}=16.698$; $P=0.001$	$F_{4,8}=1.763$; $P=0.229$
P.V.	$F_{2,8}=48.043$; $P<0.0001$	$F_{4,8}=6.852$; $P=0.011$
T_{50}	$F_{2,8}=16.286$; $P=0.002$	$F_{4,8}=1.874$; $P=0.208$
Peak day of germination	$F_{2,8}=4.846$; $P=0.042$	$F_{4,8}=8.154$; $P=0.006$

Storage of achenes harvested from a single parent plant

The plotted cumulative germination percentage after six months of storage in the 25°C storage regime had a more sigmoidal shape than achenes sampled after six months in the 4°C and -70°C storage regimes (Figure 5.6a-c). Achenes sampled from the 25°C regime after 6 months of storage germinated more rapidly than those from the 4°C and -70°C storage regimes, however after 12

and 18 months of storage, the achenes under the 25°C storage regime commenced germination later than the 4°C and -70°C regimes (Figure 5.6d-f).

The P.V. of the 25°C storage treatment decreased after 18 months of storage from the peak P.V. attained after 12 months of storage, whereas in the 4°C and -70°C regimes the P.V. maintained the increasing trend over this period (Figure 5.7).

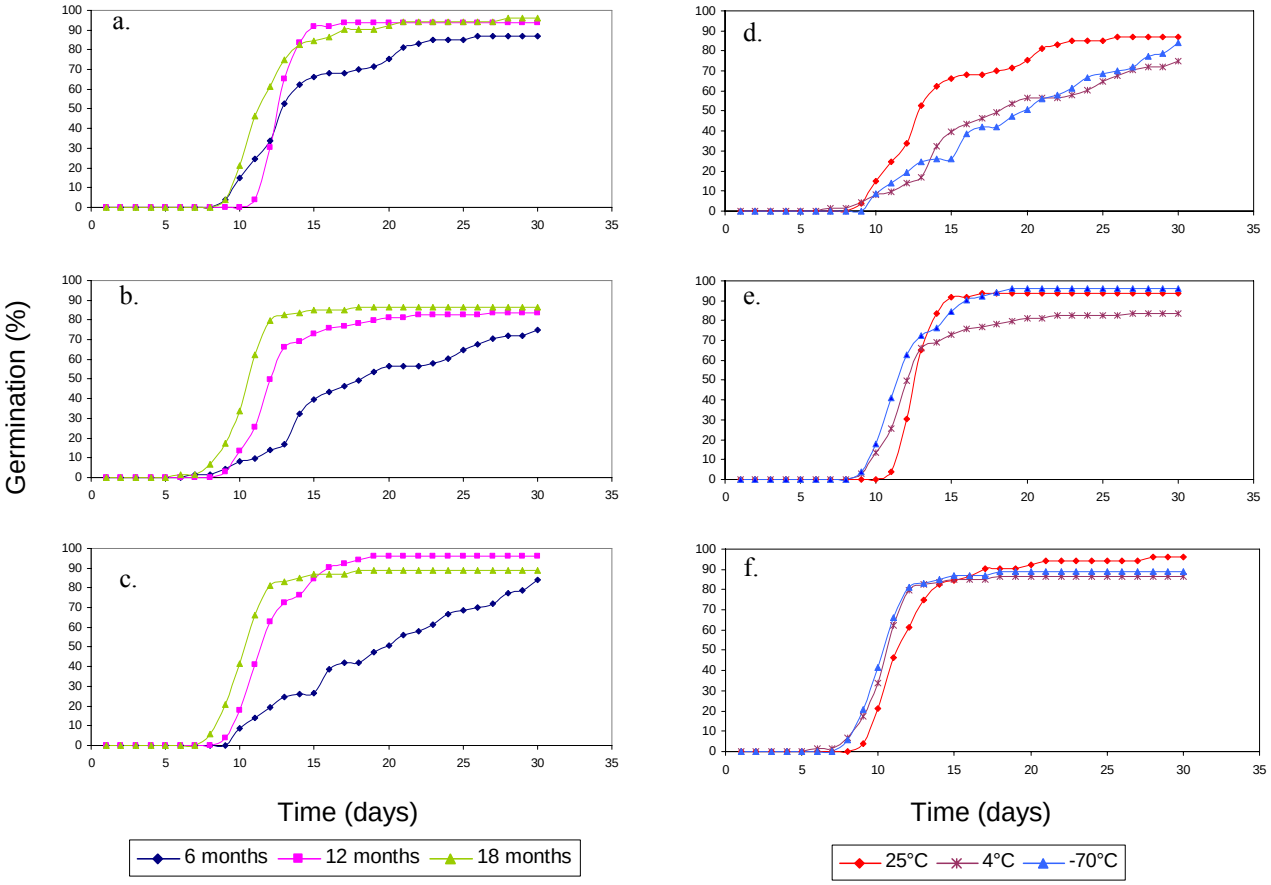


Figure 5.6. Cumulative germination of achenes sampled from a single parent plant stored at (a) 25°C, (b) 4°C and (c) -70°C as well as the cumulative germination at sampling periods of (d) 6 months, (e) 12 months and (f) 18 months of storage.

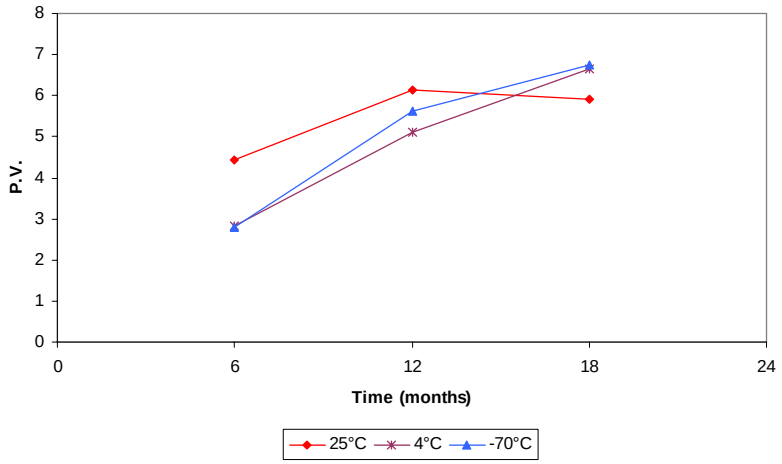


Figure 5.7. The P.V. of achenes harvested from a single parent plant at each sampling period for three storage regimes.

Achene water content

The mean (±S.E.) measured water content over all storage regimes after storage was 8.01±0.09% (w/w, wet mass) and ranged from 5.74% to 12.52% (Figure 5.8a). At six months of storage no significant differences were found between all storage regimes (Figure 5.8a). At 12 months of storage, the highest recorded water content (9.40±0.52%; mean±S.E.) was sampled from achenes in the -196°C storage regime (Figure 5.8a), this water content was significantly higher ($P<0.05$) than the water content of achenes sampled from the Ambient storage regime at 12 months (Figure 5.8a). Achenes sampled from the Ambient and 25°C storage regimes at 18 months had the lowest water content: the Ambient storage regime was significantly lower ($P<0.05$) than all other storage regimes, except the 25°C regime and the 25°C was significantly lower than the -196°C and 4°C regimes ($P<0.05$) (Figure 5.8a).

For the entire storage period the percentage of water lost by achenes sampled from the Ambient storage regime was significantly higher ($P<0.05$) than all other regimes except the 25°C storage regime at each of the six month sampling periods (Figure 5.8b). The percentage of water lost by the sampled achenes in the 25°C regime was significantly higher ($P<0.05$) than the 4°C storage regime at six months of storage and significantly higher than all other regimes, except the Ambient regime, at the other two, six month sampling intervals (Figure 5.8b).

The coefficient of variation of the water content was higher in each storage treatment before the achenes were stored than after storage (Figure 5.9). Therefore the variation in achene water content (Figure 5.8a) prior to storage may be a more important factor affecting post storage variation in water content than the storage environment itself.

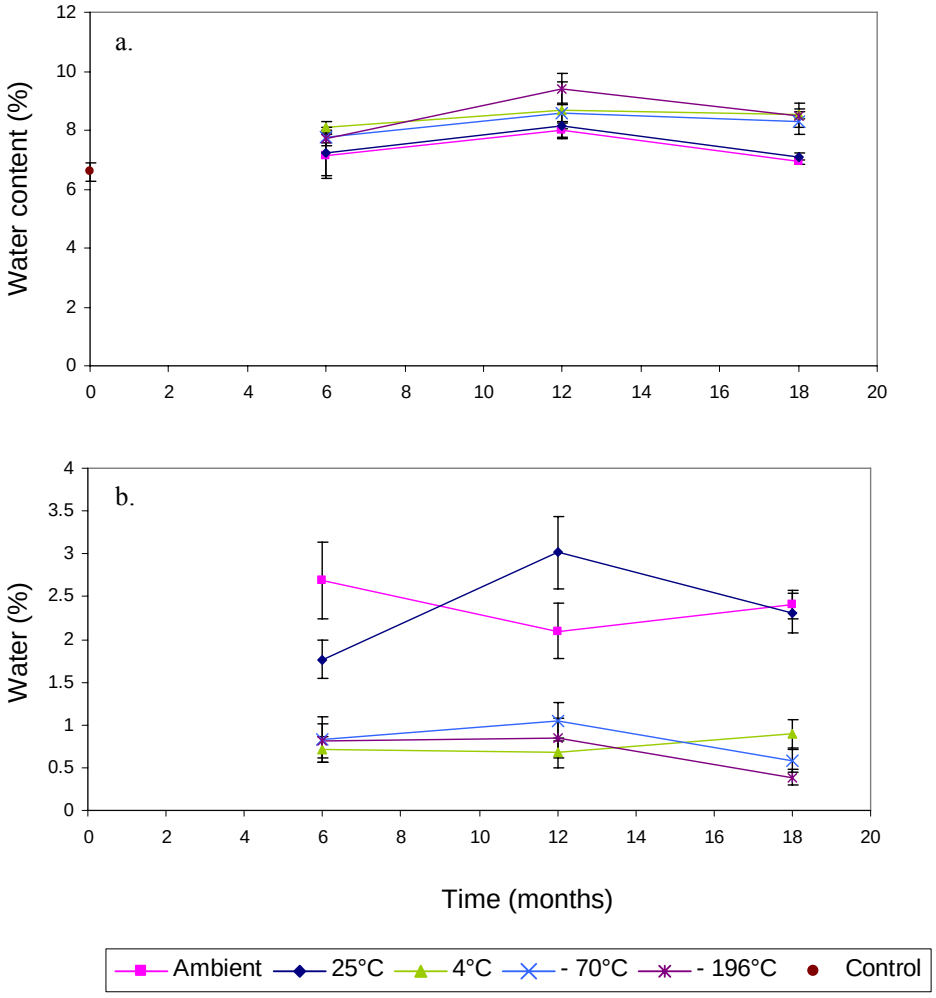


Figure 5.8. (a) The water content of achenes (wet mass) post storage and (b) the percentage water lost during storage.

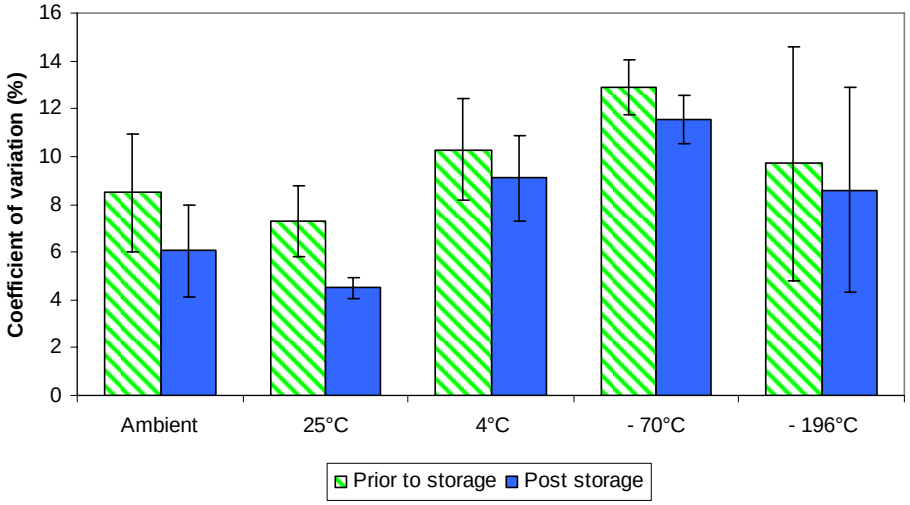


Figure 5.9. Coefficient of variation of the water content of achenes within each storage regimes prior to and post storage.

5.5.2. Population augmentation

Growth of greenhouse seedlings

Greenhouse seedlings grew vigorously until about 189 days, after which the growth rate began to slow (Figure 5.10). Most seedlings retained their cotyledons until 139 days and only 83.3% of seedlings had lost their cotyledons by 480 days (Figure 5.10e).

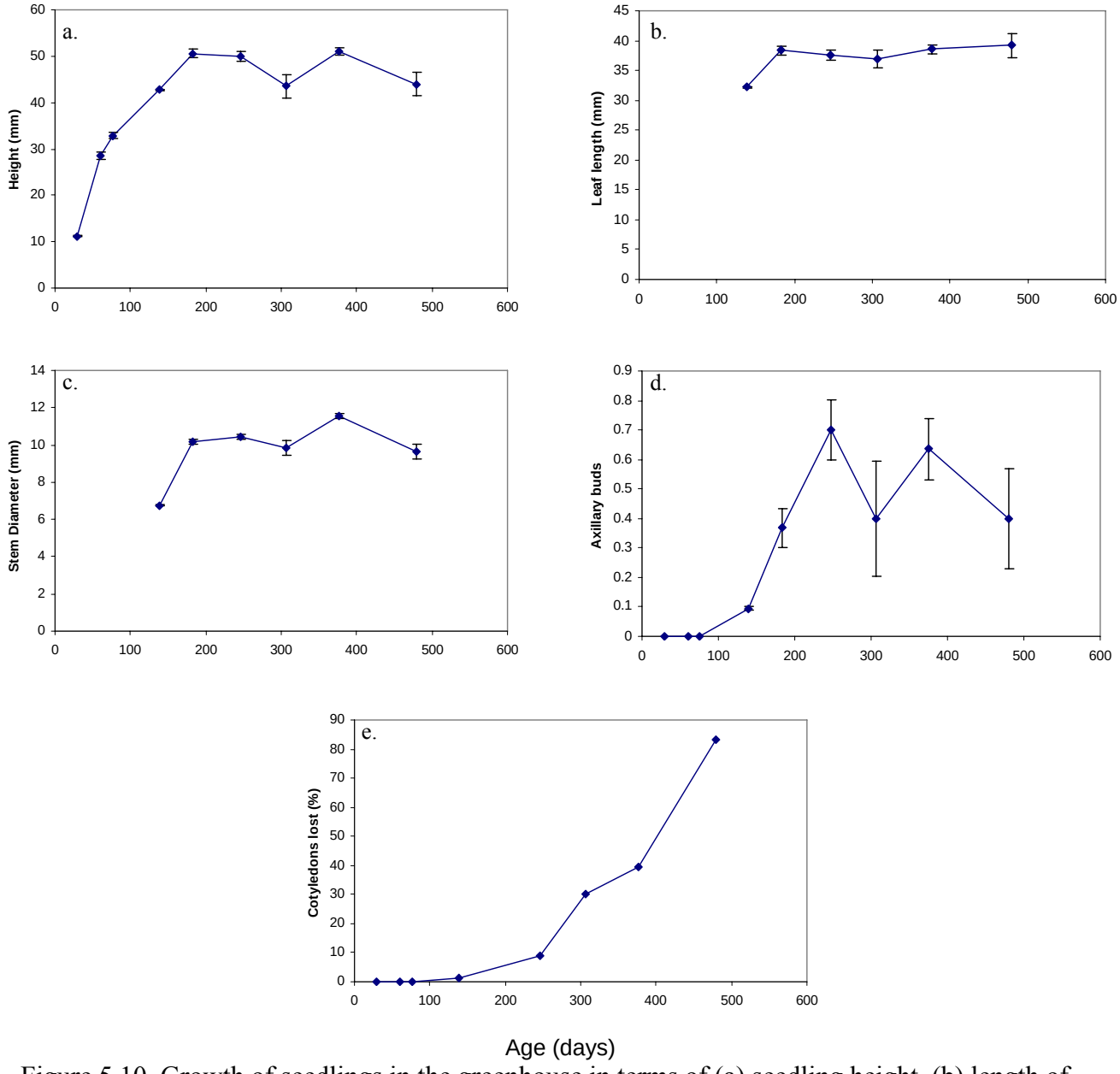


Figure 5.10. Growth of seedlings in the greenhouse in terms of (a) seedling height, (b) length of the longest leaf, (c) the stem diameter, (d) the mean number of axillary buds produced and (e) the percentage of seedlings that had lost cotyledons.

Growth of seedlings originating from planted achenes and transplants

Seedlings originating from planted achenes

The mean number of achenes that emerged per quadrat from the planted achenes was low. The quadrats that had achenes harvested from wet cones had significantly higher emergence than quadrats with achenes harvested from dry cones (Figure 5.11). Most of the seedling emergence that did occur took place within 34 days of planting however, newly emerged seedlings were also observed 162 days from planting (Figure 5.11).

At 141 days after the planted achenes were calculated to have germinated, the growth of seedlings originating from planted achenes was significantly smaller in terms of height, leaf length, stem width and number of leaves produced than the seedlings maintained in the greenhouse (Figure 5.12a). This was also true for seedlings at 372 days after germination, except that the mean number of leaves produced was significantly higher in the planted achenes than those in the greenhouse (Figure 5.12b).

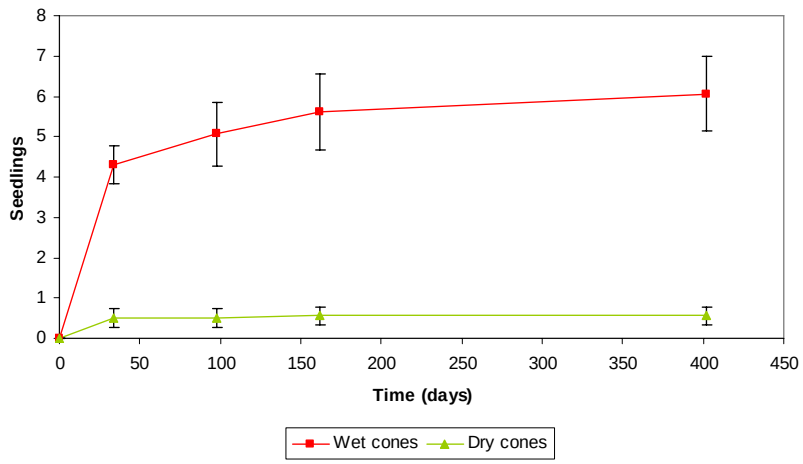
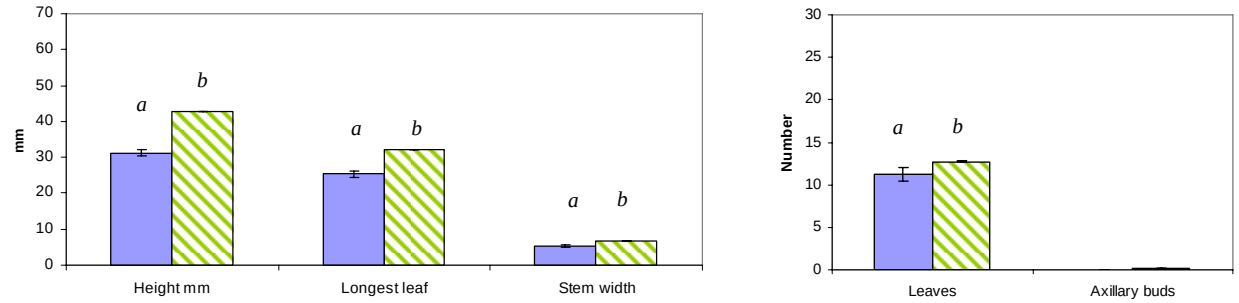


Figure 5.11. Mean (±S.E.) cumulative seedling emergence from an estimated 73 achenes sown/quadrat originating from either "wet" (2007/8 fruiting season) or "dry" (2006/7 fruiting season) cones. At all periods (taken from day of planting) there were significantly more germinants from "wet" than from "dry" cones ($P<0.05$).

a.



b.

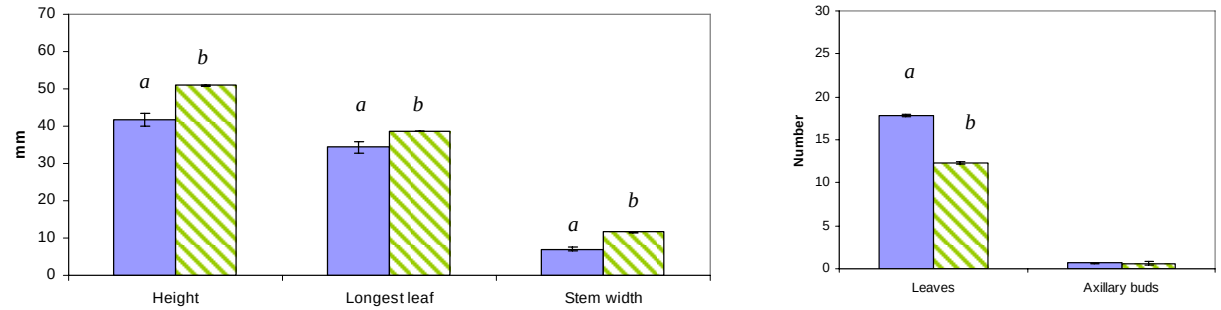


Figure 5.12. Dimensions of seedlings (mean±S.E.) that originated from achenes planted *in-situ* compared with those maintained in the greenhouse, calculated to be (a) 141 and 139 days respectively and (b) 372 and 376 days old respectively. Differing letters indicate a significant differences ($P<0.05$).

Transplants

Transplants that were 241 days old had significantly more and longer leaves and more axillary buds than seedlings grown in the greenhouse (Figure 5.13a). Transplants that were 477 days old were significantly larger than greenhouse seedlings in all measured parameters (Figure 5.13b).

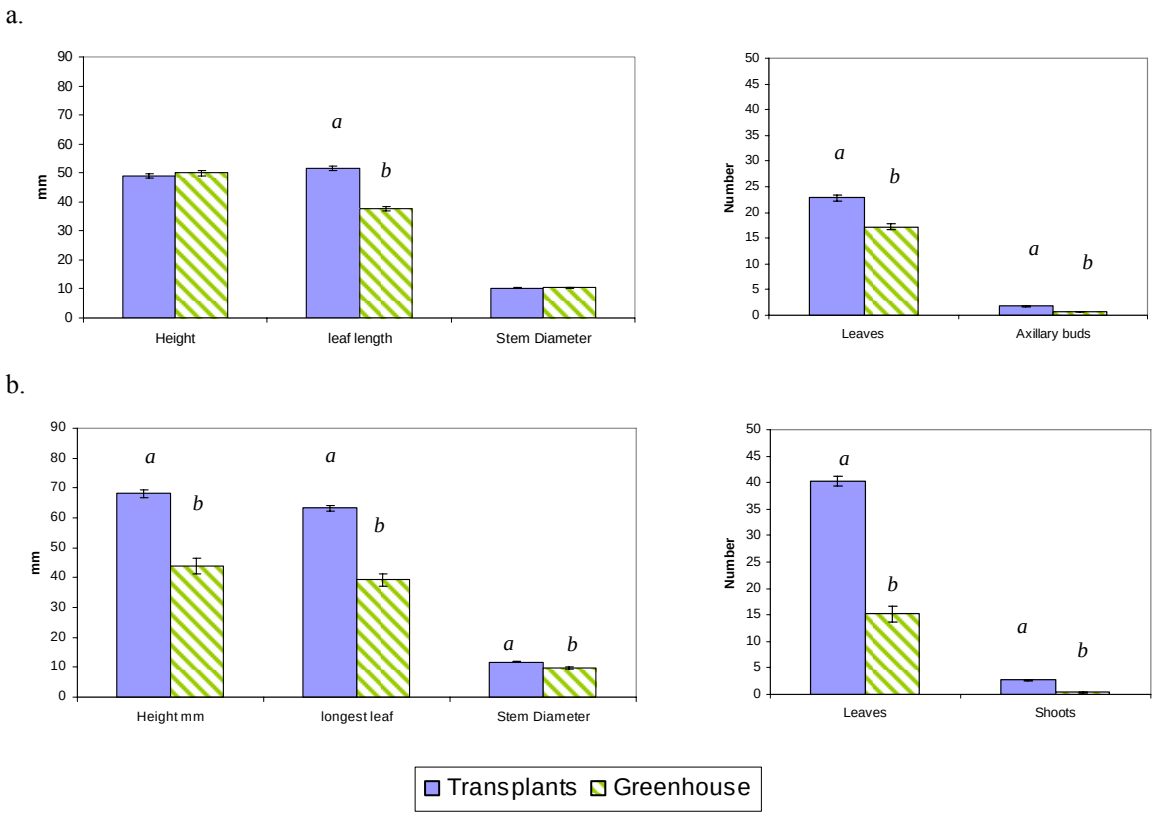


Figure 5.13. Dimensions (mean±S.E.)of transplants planted *in-situ* compared with those maintained in the greenhouse calculated to be (a) 241 and 247 days respectively and (b) 477 and 480 days old respectively. Differing letters indicate a significant differences ($P<0.05$).

Growth of seedlings *in-situ*

At 372 days, 82.33% of the seedlings that originated from planted achenes had lost their cotyledons while 88.5% of transplants had lost their cotyledons by 477 days. The rate of leaf production in transplants was much higher than that of seedlings that germinated from planted achenes (Figure 5.14a). The growth rate of the other measured parameters differed to a lesser extent between the two methods of population augmentation (Figure 5.14b-f). However, the effect of the higher rate of leaf production on the size of transplanted seedlings resulted in a large difference in overall seedling size (Figure 5.15f and 5.16)

Transplanted seedlings experienced low levels of herbivory when evaluated in March and November 2009, however in November 2010, 21.6±0.116 % (mean±S.E.) of each seedling's foliage had been removed by herbivores (part of the fence had been removed at this time). In contrast each of the seedlings that originated from planted achenes experienced a small degree of herbivory (0.98±0.28%), possibly from insects that could fit through the mesh of the exclosures.

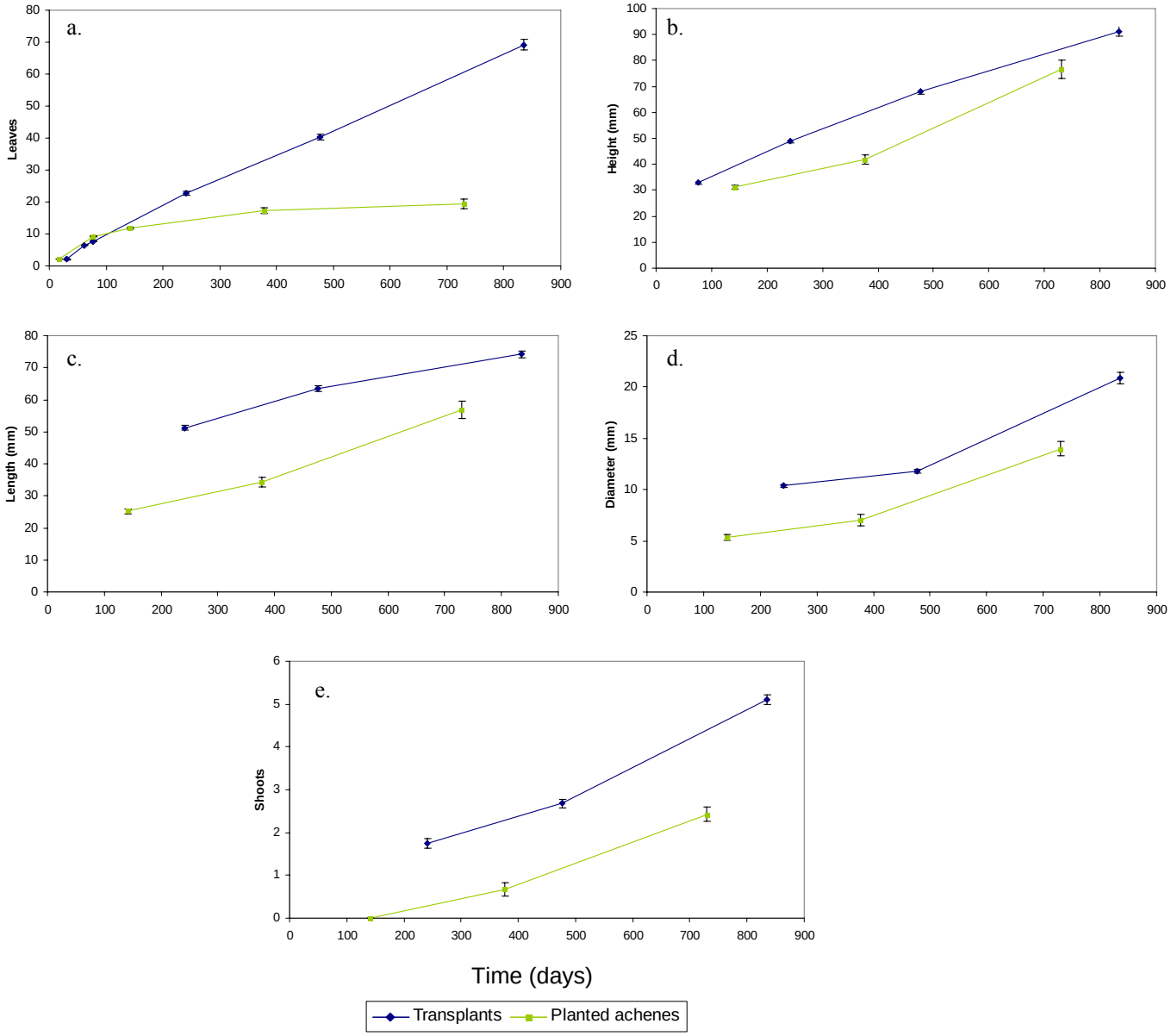


Figure 5.14. The growth *in-situ* of transplants and seedlings (mean±S.E.) that originated from planted achenes in terms of (a) leaf production, (b) seedling height, (c) leaf length, (d) stem diameter and (f) terminal shoots.

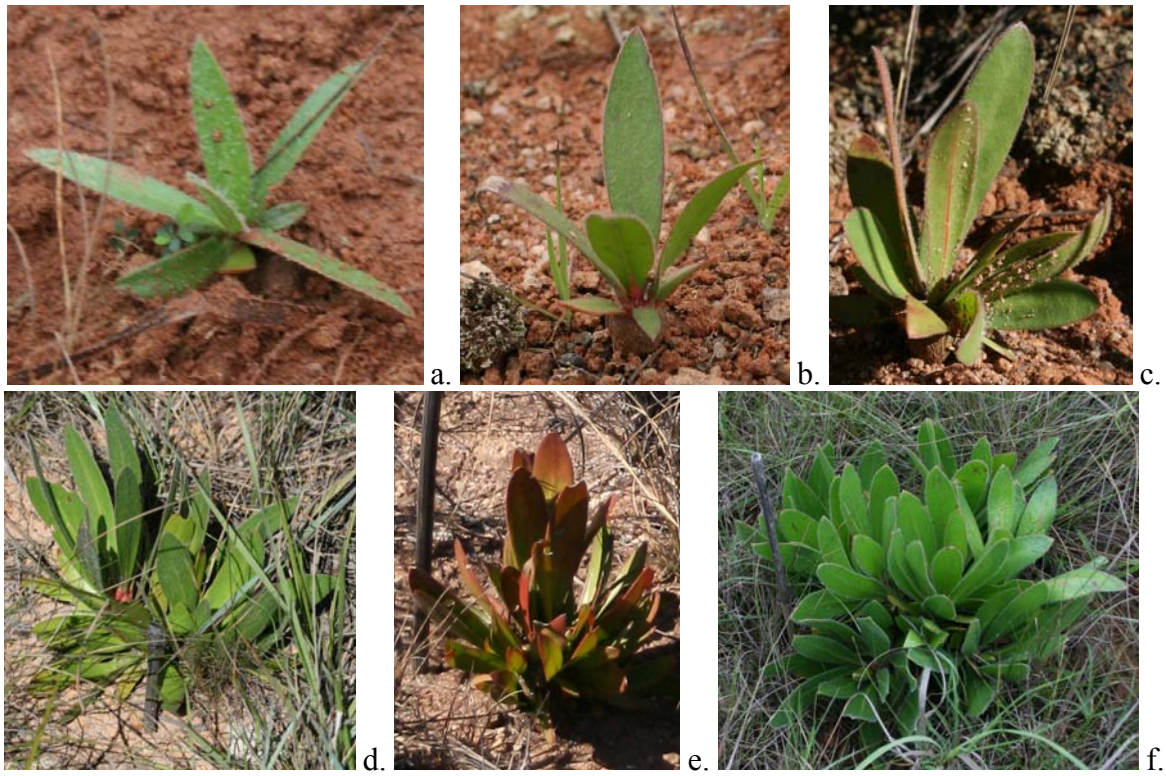


Figure 5.15. The growth of transplanted *P. roupelliae* ssp. *hamiltonii* seedlings. Transplants were planted at (a) October 2008, photographs were taken at (b) November 2008, (c) January 2009, (d) November 2009 (477 days of age), (e) August 2010 (end of the dry season) and (f) November 2010 (835 days of age).



Figure 5.16. Seedlings originating from planted achenes under mesh exclosures at 730 days of age (November 2010).

Survival and establishment

The survival of the seedlings that emerged from planted achenes was low with 23.6% of the seedlings that had emerged by March 2009 subsequently dying by November 2009. This mortality rose to 30.2% by November 2010. Therefore 76.41% of the seedlings that emerged from planted achenes, a mean±S.E. of 2.81±0.58 seedlings/quadrat were considered to have established *in-situ*

after the first year (November 2009) and survival dropped to 69.81% (2.62±0.54 seedlings/quadrat) a year later (November 2010).

In contrast there was low mortality in the transplants. In November 2009 there were five confirmed dead plants and a further five transplants (and their markers) that were not located. Therefore 96.44% of the transplants were considered to have established by November 2009. In November 2010 more seedlings were located (one seedling was still missing), and 12 seedlings were confirmed dead, hence decreasing the percentage of surviving seedlings to 95.37%. Apart from those confirmed dead, five seedlings that were completely defoliated to bare stems but these were considered alive as resprouting may still have been possible (Figure 5.17).


a.

b.

c.

Figure 5.17. Transplanted seedling stems remaining (a) alive and (b and c) resprouting after defoliation by herbivores (bar equates to approximately 7mm).

5.5.3. Quality assurance of *ex-situ* stored seed

Growth *ex-situ* post storage

Seedlings originating from achenes that had been stored for 12 months in the 4°C and -70°C storage regimes at two and a half months of age were significantly taller than seedlings that developed from all other storage regimes, including the un-stored treatment (Table 5.2). Seedlings from the -70°C storage regime had the longest mean leaf length of all measured seedlings. Seedlings originating from the -70°C, Ambient, and 25°C storage treatments had the broadest mean stem diameter (Table 5.2). The seedlings with the largest mean number of leaves originated from the Ambient and 25°C achene storage treatments as well as the un-stored treatment (Table 5.2). By four months after germination many of the significant differences present at two and a half months from germination between the achene storage treatments were no longer evident (Table 5.2). However, seedlings stored at ambient temperatures still had a significantly greater stem diameter than the un-stored treatment (Table 5.2).

Table 5.2. The size (mean±S.E.) of seedlings with a mean age of two and a half months that originated from achenes stored for 12 months in various storage treatments. Different letters indicate a significant differences between storage regimes (P<0.05).

	Storage treatment					
Measurement	Control	Ambient	25°C	4°C	-70°C	-196°C
Two and a half months						
Seedling height (mm)	35.66 ± 1.17^{b}	36.19 ± 0.97^{b}	36.44 ± 1.12^{b}	38.08 ± 0.87^{ab}	41.83 ± 0.85^{a}	37.15 ± 1.29^{b}
Length of longest leaf (mm)	29.87 ± 1.95^{b}	27.16 ± 0.70^{b}	29.02 ± 0.54^{b}	29.27 ± 0.67^{b}	31.45 ± 0.69^{a}	29.23 ± 0.95^{b}
Stem diameter (mm)	3.37 ± 0.09^{c}	4.55 ± 0.14^{a}	4.39 ± 0.15^{ab}	3.89 ± 0.10^{b}	4.07 ± 0.12^{ab}	3.83 ± 0.14^{bc}
Number of leaves	10.97 ± 0.19^{a}	11.2 ± 0.19^{a}	10.93 ± 0.22^{ab}	10.17 ± 0.19^{b}	10.27 ± 0.20^{ab}	9.9 ± 0.18^{b}
Four months						
Seedling height (mm)	43.96 ± 1.55	40.91 ± 1.56	42.07 ± 1.10	42.81 ± 0.89	44.29 ± 1.68	42.51 ± 1.32
Length of longest leaf (mm)	32.60 ± 1.09	30.26 ± 1.24	33.52 ± 0.80	31.87 ± 0.71	33.28 ± 1.42	31.52 ± 1.04
Stem diameter (mm)	6.01 ± 0.27^{b}	7.32 ± 0.20^{a}	6.82 ± 0.17^{ab}	6.79 ± 0.18^{ab}	6.78 ± 0.18^{ab}	6.77 ± 0.19^{ab}
Number of leaves	13.13 ± 0.55	12.5 ± 0.48	12.87 ± 0.41	12.13 ± 0.46	12.3 ± 0.34	13.4 ± 0.34
Axillary buds/shoots	0.07 ± 0.07	0.13 ± 0.08	0.17 ± 0.10	0.07 ± 0.05	0.07 ± 0.07	0.07 ± 0.07

Survival and establishment *in-situ* post storage

One of the four sites in which seedlings from the various storage regimes were planted had a very low mean survival (Table 5.3) decreasing the mean survival across all storage regimes to 72.91±6.93% (±S.E.). As this uncharacteristically low survival of 19.44±9.17% (mean±S.E.) was restricted to a single planting site (Table 5.3), it was clear that the survival and condition of the seedlings from this particular unfavourable site would be highly influenced by site rather than seed storage conditions. From observation this site had more litter and grass cover than the other three sites and it seemed as though the loss of seedlings was due to herbivore activity. This site was the closest site to the length of fencing which was stolen during August and September 2010 (R. Green *pers. comms.* 2010), however, most identified seedling damage and therefore seedling loss appeared to be the result of evident rodent activity. Hence this site was excluded from the statistical analyses, and only data from the other three sites were used to compare *in-situ* survival and seedling condition between storage regimes. The percentage of planted seedlings that survived one year *in-situ* increased to 90.74±1.92% (mean±S.E.) when the data from the site with excessively high herbivory was excluded.

No significant differences were found in the number of seedlings that survived to establishment and all treatments had a high percentage of survival (Figure 5.18). Additionally there were no significant differences in the percentage of seedlings within each condition category between seedlings that originated from the storage regimes and the un-stored treatment (Figure 5.19). Although not significant, seedlings that originated from the -70°C storage regime had notably more seedlings classified as growing vigorously and less seedlings classified as having mostly dead leaves (Figure 5.19).

Table 5.3. Percentage of transplanted seedlings that originated from achenes stored for one year and survived one year *in-situ* (n=25/treatment/site).

	Site			
	1	2	3	4
Un-stored	37.50	75.00	91.60	83.30
Ambient	0	83.33	83.33	83.30
25°C	54.16	100	100	95.8
4°C	4.16	95.83	95.83	79.16
-70°C	0	95.83	91.16	100
-196°C	20.83	95.83	83.33	100
Mean±S.E.	19.44±9.17	90.97±3.94	90.97±2.73	90.28±3.83

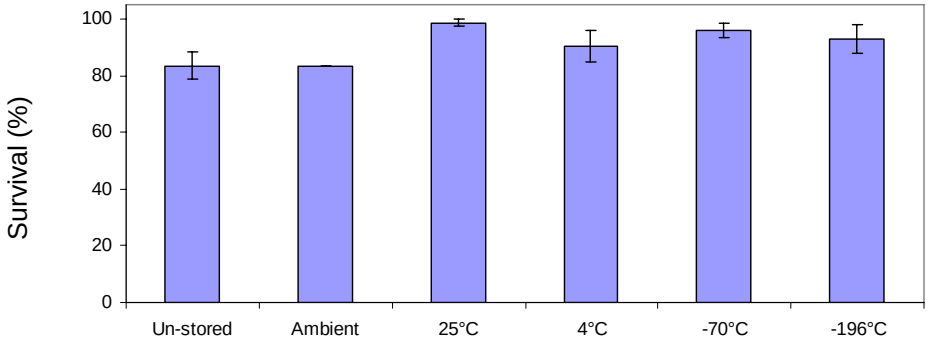


Figure 5.18. Percentage (mean±S.E.) of seedlings planted from each storage regime that survived a year *in-situ.* There were no significant differences between storage treatments (P<0.05).

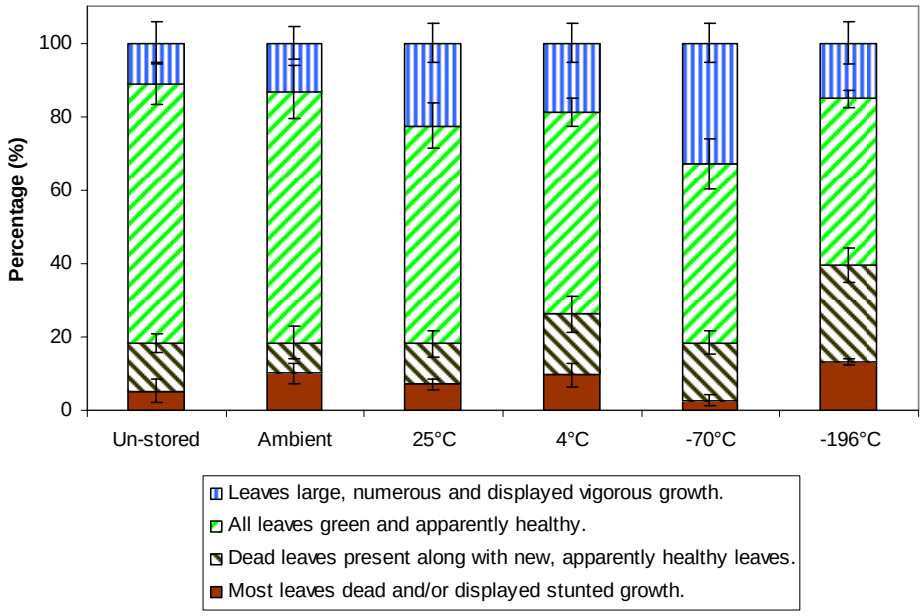


Figure 5.19. Percentage (mean±S.E.) of seedlings from each storage regime in each seedling condition category. No significant differences were found between the same seedling categories across storage regimes.

5.6. Discussion

5.6.1. Possible variation in germination

The impact of the seed storage regimes were tested through the germination response of the *P. roupelliae* ssp. *hamiltonii* achenes after the various periods in storage. Dormancy within a seed sample can render negative results, affecting the subsequent interpretation of the efficacy of seed storage for a species (Pérez-García *et al.* 2007). Therefore factors that could influence germination of *P. roupelliae* ssp. *hamiltonii* achenes, had to be identified

Variation in the germination of domestic populations such as lettuce, rye and wheat can be high (Niedzielski *et al.* 2009), and the variation in wild populations with dormancy requirements may be considered to be even higher (Qu *et al.* 2005; Walters *et al.* 2004). The achenes of *Protea* species reportedly have a high variation of germination (Deall & Brown 1981; Van Staden 1966), which can be attributed to many variables such as seed age, dormancy mechanisms, germination temperature, moisture availability, atmospheric oxygen concentration, pollination vector, flower colour, planting depth and available soil nutrients (Carlson & Holsinger 2010; Hargreaves *et al.* 2004; Le Maitre 1990; Stock *et al.* 1990; Brown & Van Staden 1973b). Although particular attention was given to ensure that *P. roupelliae* ssp. *hamiltonii* achenes from each cone were distributed between the three sampling periods of a storage regime, natural variation from the development, maturation and ageing (during canopy seed storage) of the achenes may have manifested in the germination behaviour. Furthermore, due to the rarity of *P. roupelliae* ssp. *hamiltonii* the seed sample size per storage period was limited.

When provided with suitable conditions, the seeds of Proteaceae are thought to germinate within eight weeks of being released from the parent plant (Rebelo & Rourke 1986). Seeds of *Protea* species that do not germinate in the first rainy season are probably lost to predation (Rebelo & Rourke 1986). Therefore, it is speculated that there is no reason for *Protea* achenes to be long lived once released (Rebelo & Rourke 1986), and indeed *P. repens* does not form a persistent soil seed bank (Holmes & Newton 2004). It has been reported that achenes from *P. neriifolia* lost viability within three years when stored at 20°C in cotton bags (Van Staden, 1978a). Furthermore, the viability of *Protea* achenes reportedly decrease whist still attached to the parent plant (Van Staden 1978a; Van Staden & Gilliland 1977). The *Protea roupelliae* ssp. *hamiltonii* achenes were harvested from cones on the plants from the previous fruiting season (stored for at least one year on the plant) and therefore the process of ageing and deterioration had commenced (Le Maitre 1990; Van Staden 1978a). Both the germination percentage and vigour are reported to decrease

while achenes are held on the plant, however the rate of deterioration whilst on the plant may be contentious (Le Maitre 1990; Van Staden 1978a). A decrease in the germination percentage of *P. neriifloia* has been reported seven months after fertilisation (Van Staden 1978a; Van Staden & Gilliland 1977), whilst a decrease in germination vigour alone was found in achenes of *P. neriifloia* that had remained on the parent plant for over two years with no significant decrease in the final germination percentage (Le Maitre 1990). Therefore the rate of achene deterioration whilst on the plant may be variable. The relatively un-stored achenes of *P. roupelliae* ssp. *hamiltonii* did however reach a high germination percentage (93%) and therefore the level of deterioration reached when achenes were harvested was not severe (Figure 5.5a).

5.6.2. Storage of *Protea roupelliae* ssp. *hamiltonii* achenes

Many of the differences observed between and within storage regimes may have been due to the inherent variation in germination of a wild *Protea* population as described above.

Period and temperature of storage

The most striking aspect of stored seeds of *P. roupelliae* ssp. *hamiltonii* was the increase in the germination percentage and the P.V. in most of the storage regimes with the length of time achenes spent in storage (Figure 5.3, 5.5a and b). Cumulative germination curves generally become steeper or germination commenced earlier with each six month period that samples were taken, except in the case of the Ambient storage regime (Figure 5.4). This increase in germination with storage period also occurred in both cryogenic storage regimes (-70°C and -196°C) at temperatures at which little biological activity is expected to occur (Benson 2008).

The period of storage had a significant effect on all the germination parameters measured the storage temperature did not (Table 5.1). This was unanticipated given that there was a large range of temperatures spanned by the storage regimes (221°C excluding temperatures reached by the Ambient regime) and achenes were stored for a relatively short maximum period (18 months).

Storage commenced in June 2008. Therefore, during the first three months of the six month period sample the Ambient storage regime was predominantly during a Johannesburg winter, making the 25°C regime the warmest regime over this six month period. However, over the next six months (achenes sampled at 12 months) achenes stored in the Ambient regime would have experienced a complete Johannesburg summer, with fluctuating temperatures peaking higher than all other regimes. The 25°C storage regime was the warmest storage regime over the first six months of

storage and consequently this regime had the highest germination percentage (Figure 5.4a and 5.5a). Achenes sampled at 12 months from the Ambient regime, when this regime was the warmest, showed a marked increase in the final germination percentage (Figure 5.5a)

The period of storage negatively effected the germination of achenes stored at higher temperatures. Achenes stored for 12 and 18 months under the Ambient storage regime commenced germination later than the achenes stored for six months (Figure 5.3a) and a decrease in germination vigour was observed after 18 months of storage (Figure 5.5b). Achenes sampled from a single parent plant stored at 25°C also displayed similar deterioration with the period of storage (Figure 5.6a and 5.7). The longevity of seeds stored at higher temperatures was expected to be shorter than those stored at lower temperatures (Dickie *et al.* 1990). The decreasing vigour of *P. roupelliae* ssp. *hamiltonii* achenes stored at higher temperatures (Figure 5.5b and 5.7) indicates that seed ageing had occurred in these achenes during the final six months in these storage regimes (Walters *et al.* 2004; Roberts 1983; Berjak & Villers 1972).

When dry, the cytoplasm of an orthodox seed (having vitrified) is in a glassy state and it is the physio-chemical stability of these intracellular glasses which allows for the long-term survival of orthodox seeds (Buitink & Leprince 2008). It is recommended that dried seed is stored at -70°C below the glass transition temperature (Tg; the temperature at which a solution undergoes vitrification) which is calculated to a temperature of about 10°C for orthodox seed (Pritchard & Nadarajan 2008). However, the properties of water differ considerably at temperatures below 10°C. In most biological systems the water may be supercooled to the region of -40°C (below which ice is formed), additionally the Tg for aqueous solutions is -130°C to -137°C (Benson 2008). The storage of seed at different temperatures (below their Tg) may highly influence the longevity of stored seed (Walters *et al.* 2004; Pritchard & Seaton 1993). This was observed in *P. roupelliae* ssp. *hamiltonii* in the differences in germination between the 4°C, -70°C and -196°C storage regimes.

Dormancy and storage of *Protea roupelliae* ssp. *hamiltonii* achenes

As with other *Protea* species, removal of the seed coat has been found to increase the rate of germination of excised *P. roupelliae* ssp. *hamiltonii* embryos and soaking the achenes decreased both the rate and germination percentage (see Chapter 4). Therefore the same dormancy mechanism present in the Proteaceae studied by Brown and Van Staden (1973 a & b), Deall & Brown (1981) and Brown & Dix (1985) may be considered to be present in *P. roupelliae* ssp. *hamiltonii.*

A period of stratification (30 days) has been reported to improve the germination of both *P. compacta* and *Leucadendron daphnoides* and lift dormancy (Brown & van Staden 1973c). However, the germination (percentage and rate) of achenes stored under sub-zero temperatures increased along with those stored at higher temperature until the effects of seed ageing were identified (Figure 5.4).

Lifting of physical dormancy during storage

Exposure to liquid nitrogen can reduce the physical impermeability of a seed coat (Pritchard & Nadarajan 2008). If a physical reaction of *P. roupelliae* ssp. *hamiltonii* achenes to the temperature of storage regimes occurred, the response would be most noticeable at six months compared with the un-stored treatment. However, the greatest difference seemed to be between six and twelve months of storage (Figure 5.3a-c) and cumulative germination curves at six months of storage for most of the storage regimes (including the -196°C regime) were below that of the un-stored control (Figure 5.3). It would also be expected that the extent that physical dormancy was lifted would be temperature related with a greater change at temperature extremes, however achenes stored for 6 months at -196°C commenced germination after other regimes and those stored at -70°C commenced before other storage regimes (Figure 5.4a).

The seed coat of *Leucospermum* species with nut like seeds are thought to be impermeable to oxygen (Van Staden & Brown 1973), allowing the fruits to remain viable for 200 years under suboptimal storage (Daws *et al.* 2007). In *Leucospermum cordifolium* cycles of desiccation and wetting breaks the oxygen impermeable exo- and endotesta lifting physical dormancy (Brits *et al.* 1993). The degree of endotesta breakage was significantly related to the period the fruits were desiccated although the experiment was terminated after 40 hours (Brits *et al.* 1993). At sub-zero temperatures, freezing causes dehydration of cells and repositioning water within tissues (Benson 2008; Muldrew & Acker 2004). Additionally the achenes stored at higher temperatures also lost water (Figure 5.7). The movement of water was expected to have occurred in all regimes during storage, via freezing or through evaporation, and may have influenced the seed coat physically. Although the seed coat of *P. roupelliae* ssp. *hamiltonii* is fundamentally different from that of the fruits of *Leucospermum* species, a physical change in the seed coat over time may have aided the diffusion of oxygen on imbibition, thereby influencing germination.

Chemical and physiochemical germination inhibitors during storage

If germination of *Protea* species is inhibited by a chemical, it has not been fully identified, however based on Van Staden and Brown (1972), it can be assumed that it is not the plant growth regulator ABA, but of phenolic origin. In all likelihood it may be coumarin or a coumarin like compound present in the seed coat and or in the embryo (Van Staden & Brown 1972). The observed increase in germination (percentage and P.V.) over time may therefore be attributed to the progressive degradation of germination inhibiting compounds (Oracz *et al.* 2007; Bailly 2004). Similarly compounds within the embryo may degrade singly or in tandem with those in the seed coat, enhancing the effect on the germination rate.

Phenolic compounds in the seed coat which previously maintained the embryo in anoxic conditions may have degraded during storage (Debeaujon *et al.* 2007). This degradation would free oxygen diffusion through the seed coat, releasing the achenes from inhibited germination (Coumans *et al.* 1976), but would also have had to occur at all tested storage temperatures at a similar rate for the period of storage to have an effect on germination (Table 5.1).

Coumarin, ROS and peroxidase during storage

The reaction of the coumarin scopoletin with hydrogen peroxide catalyzed by peroxidase (intermediate compound II) is an efficient reaction (Andreae 1955; Boveris *et al.* 1977). However, it is not the only enzyme that uses H_2O_2 as a substrate and peroxidases are not specific to scopoletin therefore, this reaction cannot be used as a direct measurement of H_2O_2 in a biological system (Boveris *et al.* 1977). Furthermore the controlling effect of germination inhibitors in Proteaceae is contentious (Brown & Van Staden 1975).

The 4°C storage regime displayed an increase of the final germination percentage and the rate of germination at each consecutive sampling period (Figure 5.3c, 5.5a and b) and increased more slowly than other regimes (Figure 5.4). At a constant 4°C, the achenes were exposed to neither high temperatures that would accelerate the rate of lipid peroxidation (Van Staden & Gilliland 1977), nor to low enough temperatures to allow the phase change of lipids or cryoinjury, leading to cellular disruption and oil-body coalescence (Pritchard & Nadarajan 2008; Volk *et al.* 2006; Walters *et al.* 2004; Benson 1992). The cellular disruption from peroxidation or cryoinjury, which may have been experienced by achenes stored under other storage regimes, may have resulted in an increased production of ROS on imbibition (Kranner *et al.* 2010). An increased production of ROS in the other storage regimes would lead to increased oxidation of coumarin or other

germination inhibiting phenolics by peroxidases or autoxidation, resulting in increased germination vigour (Boveris *et al.* 1977).

Therefore in the low and ultra low temperature storage regimes, for germination to increase over the period of storage, autoxidation or ROS production would have to have occurred not only during the moment of freezing, the moment of thawing and on imbibition (as this would have been the same for all sample periods) but also over the period achenes spent in storage. The effects of oxygen and ROS cannot be ignored at cryo-temperatures (Benson & Bremner 2004), but ROS accumulation from aerobic activity in achenes stored at low and ultra low temperatures for 18 months is doubtful. Cryopreservation does substantially increase the longevity of seeds but deterioration and therefore the production of radicals does occur at ultra low temperatures (Whitaker *et al.* 2010; Varghese & Naithani 2008; Walters *et al.* 2004). There is also evidence that molecular mobility is possible in cryogenically stored seeds (Walters 2004). The oxidation of lipids, proteins and phenolics may therefore have occurred though autoxidation, with the atmospheric oxygen present in cryovials and may have been accelerated by the ROS (that accumulated over the year of canopy seed storage on the plant) already within the achene prior to storage (Varghese & Naithani 2008; Walters *et al.* 2004). However, the germination biology of *Protea* species is not adequately understood with regard to germination inhibitors to draw conclusions concerning their activity during storage, or their role in the germination of stored *P. roupelliae* ssp. *hamiltonii* achenes.

5.6.3. Population augmentation

Ex-situ seedling production

The mean daily temperature of the greenhouse did not drop below 19.7°C for any month of the year and the mean annual temperature was 21.5 °C (Figure 5.1). These temperatures were much higher than what is expected *in-situ* (the Barberton Montane Grassland), with a mean annual temperature of 16.7°C (Mucina & Rutherford 2006). The high greenhouse temperatures may have preconditioned greenhouse seedlings to heat stress that is often detrimental to seedlings that have not been "hardened" to the harsh *in-situ* conditions (Hartmann *et al.* 2002).

The growth rate of seedlings maintained in the greenhouse decreased from 189 days after they were planted, and there was little further growth until the study was concluded, 500 days from planting (Figure 5.10 a-d). The decrease in the growth rate occurred after the epigeal cotyledons were shed (Figure 5.10e), indicating that the decreased growth rate may coincide with the

depletion of the nutrient reserves within the seed. The cotyledons of Proteaceae fruit and therefore Proteaceae seedlings contain high concentrations of phosphorous and nitrogen relative to other species (Groom & Lamont 2010) and are known to survive 300 days in nutrient free sand (Stock *et al.* 1990). However, the growth rate of *Protea* seedlings may also increase with increased exogenous nutrient levels (Witkowski 1991). It can therefore be assumed that the nutrients within the small volume of soil (<340ml) that greenhouse seedlings were planted in had been depleted by that time, possibly by leaching. The high greenhouse temperatures may have also had a negative effect on the seedling growth rate, as heat stress inhibits plant growth (Levitt 1980). A combination of nutrient and heat stress may have resulted in the stunted growth of the seedlings maintained in the greenhouse.

In-situ seedling emergence

Proteaceae seeds dispersed *in-situ* are at high risk from herbivory; small mammals are capable of significantly reducing seed reserves (by an estimated 72.6%) before germination (Bond 1984). Germination was poor in all quadrats with regard to the expected number of germinable achenes that were planted. The mean number of germinable achenes expected per "wet" cone was 36.61±3.21 (mean±S.E.) (see Chapter 2, Section 2.5.3), and therefore an expected number of about 73 germinable achenes were planted per quadrat from the two "wet" cones. This provided a mean estimated emergence of less than 10% of the germinable achenes planted per "wet" cone quadrat, although this may be an underestimate of actual emergence, as some germinants may have emerged, died and disappeared between observations. This value was substantially lower than the mean germination percentage (23.7%) attained in Cape Proteaceae planted *in-situ* (Midgley *et al.* 1988). *Protea* achenes that do not germinate during the first season are expected to lose viability or to be consumed by herbivores (Holmes & Newton 2004; Rebelo & Rourke 1986; Bond 1984). Rain followed the planting of the achenes within three days and the majority of the seedlings that did emerge, emerged within 34 days of planting (Figure 5.11). The commencement of the second wet season did coincide with further germination but this was limited and no further germination was recorded in the second year (Figure 5.11). As there was an absence of mammalian herbivores in the exclosures, these results corroborate reports that achenes are short lived *in-situ* and *Protea* species do not maintain a persistent soil seed bank (Holmes & Newton 2004). Serotinous achenes germinate optimally *in-situ* when lying on the soil surface (Rebelo & Rourke 1986), however in order to stop achenes being blown or washed away they needed to be covered with a thin layer of soil, which may have influenced the recorded *in-situ* germination rate of *P. roupelliae* ssp. *hamiltonii* (Le Maitre 1990). Higher emergence of Proteaceae has also been related to soils with higher soil moisture contents (Mustart & Cowling 1993).

It is common practice to harvest seed from plants only once seeds are being dispersed from the mother plant to ensure that only mature seeds are harvested (Hong & Ellis 1996). Achenes harvested from "dry" cones displayed significantly lower emergence compared with that of "wet" achenes (Figure 5.12). The achenes from "dry" cones may have been exposed to herbivores which selected the achenes filled with an embryo or were exposed to adverse conditions to a greater degree (for example temperature extremes and fluctuations as in the Ambient storage regime), decreasing seed vigour and viability resulting in decreased emergence (Van Staden 1978a). Achenes that had decreased vigour from "dry" cones may not have been able to emerge *in-situ* after burial as *Protea* achenes have decreased emergence when covered with soil (Le Maitre 1990).

In-situ and _ex-situ_ seedling growth

The growth rate of the greenhouse seedlings decreased after 198 days, this was possibly due to the extreme temperatures and the small containers in which the greenhouse seedlings were grown. A comparison of greenhouse seedlings with seedlings established *in-situ* may therefore be considered to be flawed. However, seedlings originating from achenes planted *in-situ* were significantly smaller than the greenhouse seedlings at 140 and 374 days of age, except with regard to the number of leaves produced (Figure 5.12a and b).

The superior growth of the transplants was seen from 244 days, when the mean leaf length, number of leaves produced and the mean number of axillary buds per seedling were significantly higher than seedlings maintained in the greenhouse (Figure 5.13a). By 478 days all measurements that were taken from the transplants were significantly higher than for the greenhouse seedlings (Figure 5.13b).

Due to the slow growth rate of seedlings originating from achenes planted *in-situ*, it may be concluded that there are stresses *in-situ* comparable with those experienced by the seedlings maintained in the greenhouse. However, only the above ground growth was measured and no destructive methods (digging up seedlings) were used in the comparison due to the rare status of the species. *Protea* species are known to have vast, variable root systems (Watt & Evens 1999; Dinkelaker *et al.* 1995; Richards *et al.* 1995). It is therefore possible that there may have been differences in the above and below ground biomass of the seedlings.

The superior growth rate of *P. roupelliae* ssp. *hamiltonii* transplants was emphasised in the comparison of leaf production (Figure 5.14a), even in the face of high levels of herbivory. Transplants had received an extra three months (100 days in the greenhouse), where generally higher temperatures and constantly available water allowed for additional period of growth before the onset of winter. This additional growth in the first season may have provided transplants with a further advantage (size and establishment ability) over those that germinated from planted achenes (Galen & Stanton 2008). Although the actual size of transplants and seedlings from planted achenes differed, the rate of seedling growth seemed to be similar in all other measured aspects apart from leaf production (Figure 5.14b-e). However, the difference in the size of the seedlings due to the extra time (age and exposure to a growth promoting environment) and the effect of planting was noticeable after two years (Figure 5.15f and 5.16).

Survival and establishment *in-situ*

The seedlings that germinated *in-situ* had a low survival compared with transplants, 76.41% of *in-situ* germinated seedlings survived a full year *in-situ* and 69.81% survived a further year. The cause of the death of these seedlings could not be determined as the seedlings had decomposed by the time the subsequent observations were made. These seedlings were protected by herbivore exclosures, therefore herbivory, if it did occur, was expected to occur exclusively from insects. Edaphic factors may have played a role in seedling establishment. The surface soil in the Dr Hamilton Reserve was very hard especially when dry (S. Tarlton, *pres. obs.* 2009), and seedlings' roots would have needed to penetrate this surface in order to withstand the seasonal drought. It has been reported that if roots of Proteaceae seedling encounter stones near the surface and are not able to penetrate the soil surface they die due to drought stress (Midgley 1988; Bond 1984). The act of planting transplants into well dug holes would have assisted root penetration and the subsequent access to water.

Transplants are expected to have better growth and survival than seeds planted and germinated *in-situ* (Wallin *et al.* 2009; Guerrant Jr & Kay 2007), however reintroduction/augmentation by transplantation is not always successful (Maunder 1992). Maunder (1992) identified that the majority of reintroductions by transplantation, fail due to a combination of poor horticultural practice, insufficient ecological understanding and a lack of post planting maintenance. The only post planting maintenance *P. roupelliae* ssp. *hamiltonii* seedlings received was water and shade on the days of planting (14-16th October), however rains started soon after planting was complete. The *P. roupelliae* ssp. *hamiltonii* transplants had 96.4% establishment after a full year *in-situ*.

This was higher than that of other Cape *Protea* species planted at their "home sites" and at other sites (Latimer *et al.* 2009).

The survival and establishment of *Protea* seedlings *in-situ* is reportedly restricted to a greater extent by environmental conditions as opposed to competitive interactions (Mustart and Cowling 1993; Midgley 1988; Bond 1984). However, interspecific competition with the alien *Acacia saligna* has been shown to decrease the growth rate of *Protea* seedlings in an experimental pot trial and concurs with field observation (Witkowski 1991). Although drought stress is thought to not affect seedling mortality of Cape Proteaceae (Midgley *et al.* 1988; Midgley 1988), 25.2% of *P. lorifolia* seedling mortality *in-situ* could be explained by drought (Midgley 1988). Additionally water availability reportedly effects the survival of transplanted seedlings of four *Protea* species, as the greatest seedling mortality was experienced at the driest sites (Latimer *et al.* 2009). Although germination *in-situ* of Cape *Protea* species is reported to be cued by low temperatures rather than rainfall, seedling death has been associated with drought (Bond 1984). *P. roupelliae* ssp. *hamiltonii* seedlings that originated from planted achenes also experienced most of the mortality during the dry season (between sampling in March and November 2008). This highlights the necessity of planting *Protea* achenes and seedlings just before or early in the wet season as performed in this experiment, thereby allowing the seedlings to reach sufficient size to withstand seasonal drought.

Substantial post germination *Protea* seedling loss can be attributed to herbivores in Cape Fynbos, with an estimated 67.2% loss of seedlings in recently burned sites and 61% in mature vegetation due to herbivory (Bond 1984). Very little herbivory was observed on the transplants after one year *in-situ,* however in the second year (2010) higher levels were observed. This change in herbivory from one year to the other may be due to burning of adjacent areas (winter 2010) forcing animals to forage closer to the transplants or due to the higher visibility of the larger seedlings. Additionally a portion of the herbivory experienced is expected to have originated from large herbivores that entered the Dr Hamilton reserve during the period between the fence being stolen in September 2010 and its replacement a few weeks later (R. Green, *pers. comm.* 2010). A substantial portion of herbivore damage was performed by rodents which favoured the stems and new leaves of the seedlings as opposed to older foliage (Figure 5.20). Continued high levels of herbivory may further decrease seedling survival (Bond 1984), possibly requiring the implementation of post planting maintenance, two years after planting *in-situ.*

When seedlings were planted they were planted slightly lower than the surrounding soil forming a depression in the soil surface allowing surrounding water to flow into and pool at the base of the seedling instead of flowing away from the seedling. This effectively created a microweir which has been observed to aid successful establishment in other *Protea* seedlings (Midgley 1988).



Figure 5.20. Transplanted seedling withstanding herbivory from rodents which have targeted new leaves and stems (photograph taken November 2010).

5.6.4. Quality assurance of *ex-situ* stored seed

The link between *ex-situ* and *in-situ* conservation needs to be complementary (Volis & Blecher 2010), with a simultaneous failure in both conservation methods resulting in the extinction of the species. Unfortunately an example of the failure of *ex-situ* conservation can be taken from *P. roupelliae* ssp. *hamiltonii*. *P. roupelliae* ssp. *hamiltonii* plants maintained in the Kirstenbosch Botanical Garden, were reported to be growing vigorously (Rourke 1980), but these plants have subsequently "died or disappeared" (Weiersbye *et al.* 2000). If *in-situ* conservation had failed during this period, extinction of the species would have been total, except for a small population at the Nelshoogte Forestry Station garden of questionable genetic origin (Weiersbye *et al.* 2000).

Seed storage is cheaper than maintaining growing plants (*in-vitro* or in botanic gardens) and there is also substantially less risk of the acquisitions having "died or disappeared" if preserved correctly (Li & Pritchard 2009). Paramount with this approach is the purpose of seed storage, which in most cases concerning endangered species is the eventual reintroduction of the species *in-situ*.

Post storage seedling growth

Seedlings originating from achenes stored in the -70°C storage regime showed early growth that was either significantly greater than other regimes (tallest seedlings and longest leaf) or not significantly different from the largest seedlings (stem diameter and number of leaves) (Table 5.2). This increased early growth rate may translate into an increased ability of the achenes in the -70°C storage regime to become established seedlings (Perry 1970). However, at four months of age most of the significant differences in the size of the seedlings had dissipated, except for stem diameter (Table 5.2). The measurable effect of the different seed storage regimes may have dissipated, indicating that the regime in which *P. roupelliae* ssp. *hamiltonii* achenes are stored for 12 months does not effect the consequent seedling growth under greenhouse conditions.

Post storage capacity for transplant establishment

The seedlings planted *in-situ* that originated from achenes stored for one year in the various storage regimes had good survival after one year *in-situ* (Figure 5.18). There were no significant differences in the seedling survival or the categories of seedling condition between storage regimes after one year (or the un-stored treatment). However, it is necessary to state that good site selection is imperative for transplant seedling survival, as Site 1 had substantially lower survival than the other three (Table 5.3). This site with the lowest survival had more litter and grass cover, which would have provided protection for small mammalian herbivores and the grass possibly competed with the *P. roupelliae* ssp. *hamiltonii* seedling for nutrients, water and light (Kerley 1992; Witkowski 1991).

The lowest mean survival percentage was found in seedlings originating from the Ambient storage regime (Figure 5.18), which corresponded with the decrease in germination vigour seen at 18 month of storage in this regime. The highest mean percentage of surviving seedlings was from achenes stored at 25°C (98.61±1.39%; mean±S.E.) (Figure 5.18). Transplants which germinated from achenes stored at -70°C had the highest percentage of seedlings which were categorised be to in a superlative condition (Figure 5.19) and also had relatively high survival (95.83±2.41%; mean±S.E.) (Figure 5.18). Interestingly both the 25°C and the -70°C storage regimes had high germination vigour after 18 months of storage compared with other regimes (Figure 5.5b).

Storage may affect the ability of the seedlings to persist *in-situ* (Benson 2008). This may have been more noticeable if the experiment was performed with achenes that had been stored for longer and commenced at a point where a measurable, altered germination response was displayed

by different storage regimes. Unfortunately a genetic component cannot be ignored and achenes in the 25°C and the -70°C storage regimes may have both had higher genetic fitness.

5.7. Summary and conclusion

5.7.1. Seed storage

The achenes of *P. roupelliae* ssp. *hamiltonii* can withstand storage at cryogenic temperatures. The achenes maintained viability in all storage regimes for 18 months however, at higher temperatures signs of decreased vigour were evident (Figure 5.5b and 5.7). The most detrimental seed storage regimes were associated with fluctuating ambient conditions and warm temperatures. During achene storage it was assumed that dormancy mechanisms were partially lifted progressively over time, allowing increased germination vigour to occur with an increased storage period (except the Ambient storage regime).

The experiments performed in this study were based on storage over a short term (18 months) and provide insights into the storage behaviour of *P. roupelliae* ssp. *hamiltonii* achenes. A temperature of 25°C is considered high for seed storage and may induce rapid seed ageing compared with cryogenic temperatures (Pritchard and Nadarajan 2008; Walters 2004) and therefore not conducive to long term seed storage. The achenes stored for 12 months under the -70°C storage regime displayed rapid early growth rates *ex-situ* (Table 5.2) and superior seedling condition after one year *in-situ* (Figure 5.19), which was coupled with high germination vigour after 18 months of storage (Figure 5.5b). This indicated that the -70°C storage regime was possibly the most favourable regime for the long term storage of *P. roupelliae* ssp. *hamiltonii* achenes for the purpose of augmenting the population. However, these results were acquired over a short period of storage, the germination percentage and vigour was still increasing with the period the achenes spent in storage, and hence may not have reached a peak by the time the experiment was completed. In this experiment there was insufficient time for the effects of seed ageing to be elucidated from storage regimes as high as 4°C, in addition to the inherent germination variation associated with *Protea* species (Deall & Brown 1981). It would therefore take considerably longer to definitively distinguish between the storage behaviour of achenes stored in the -70°C regime from those at -196°C.

5.7.2. Population augmentation

Although the number of truly germinable achenes planted were only an estimate, the reintroduction of *P. roupelliae* ssp. *hamiltonii* transplants was more conservative to the number of

achenes utilised per established plant. This conservation of seed resources would be necessary if restoration was required from a limited stock of seed bank stored achenes.

Harvesting seeds and planting them under enclosures was not time consuming and it did not require additional facilities (greenhouses, irrigation and planting containers) and labour (planting in cups, transport of seedlings and soil as well as planting *in-situ*). However, a greater percentage of transplants survived to establishment and leaf production of transplants *in-situ* was higher, providing a more expedient evaluation of success. Utilising transplants for the augmentation and possible reintroduction of *P. roupelliae* ssp. *hamiltonii* would therefore reduce the need for the less effective reintroduction via planting of seed which, although less costly in the short term would require repeated attempts and evaluations. Due to the higher rate of leaf production in transplants, the use of transplants would also expedite the effective population augmentation or reintroduction of *P. roupelliae* ssp. *hamiltonii*.

Chapter 6
6. Conclusion

6.1. The *Protea roupelliae* ssp. *hamiltonii* population as a direct result of this study

The study as a whole was designed to use the least amount of destructive sampling. Achenes that were harvested and used in experiments were, when possible, returned to the environment as seedlings. This experimental protocol resulted in an increase in the population numbers. Apart from the 281 transplants returned during the population augmentation trial (October 2008) and the 576 transplants used to test the quality of stored achenes along with a further 564 seedlings were planted in November 2009 and in November 2010, 283 seedlings were planted. Therefore a total of 1707 transplants were planted in the Dr Hamilton Reserve through this study period, without taking into account the seedlings that arose from planted achenes, there has been a considerable increase in the *Protea roupelliae* ssp. *hamiltonii* population size *in-situ*. A complete census of the population is needed and the number of planted seedlings that reach reproductive age/size in the coming years would be interesting. If the population was left to its own devices, the increase in population size would have taken much longer if at all, possibly keeping the species at higher risk of extinction for a considerably longer period (Pimm *et al.* 1988).

According to the IUCN Standards and Petitions Subcommittee (2010), only mature individuals (individuals capable of reproduction), are considered and therefore the increase in population numbers (due to population augmentation) does not reflect on the IUCN category of the species until they are reproductive. The population reduction has been observed, the reduction is clearly reversible but in order for the IUCN category to be altered from A2 to A1 the reduction has to have ceased. Eight individuals died between 2009 and 2010 and there has been a reduction in the number of reproductive individuals from 2008 to 2010 (Chapter 2, Section 2.5.1 and 2.5.5), therefore the population decline has not ceased. In this regard, the IUCN category of *Protea roupelliae* ssp. *hamiltonii* remains critically endangered: A2ac, B1ab+2ab, C2a (see Chapter 1; Rebelo 2006) until more individuals are reproductive, the range of the species is increased and additional populations are established.

Apart from being a testament to the efficacy of intervention as a means for conserving rare plant species *in-situ*, achenes were harvested by S. Tarlton and E. T. F. Witkowski in November 2009 and delivered to SANBI. Although this sample of achenes was small, it was the first accession of

Protea roupelliae ssp. *hamiltonii* in a seed bank (Millennium Seed Bank with the assistance of E. Van Wyk, SANBI). This simple act, in essence established an *ex-situ* population, greatly enhancing the conservation of the species.

Along with the direct increase in population numbers conserved *in-situ* and *ex-situ*, the available information surrounding the propagation and regeneration of the species has been greatly enhanced, specifically in the four objectives outlined in Chapter 1 of the study:

6.1.1. Risk of extinction that *Protea roupelliae* ssp. *hamiltonii* faces *in-situ* (Chapter 2).

The plant-animal interactions associated with *Protea* species are generally restricted to pollination, seed predation and insect herbivory (Bond 1994). In the case of *Protea roupelliae* ssp. *hamiltonii* mammalian herbivores (large and small) seemed to have an overriding effect on the seed production and seedling establishment and the decline of the species. Extensive herbivory decreases the size of adult plants forcing them into a functionally juvenile state. Plants in this juvenile state (either imposed by herbivory or being of a young age) produce inferior progeny in terms of quality and numbers. Large plants are invaluable for seedling recruitment, additional consideration should be made to increase and conserve the size of the plants.

6.1.2. Achene mass and germination (Chapter 3).

Individual plants seem to produce achenes of similar mass which relates to the size of the plant and possibly the phenotype (Carlson & Holsinger 2010). The mass of an individual achene does not necessarily predict the quality of that achene (in terms of germination vigour) but the mean achene mass from a cone may predict the germination of the filled achenes within that cone. Utilizing achene mass as a method of selecting viable, embryo filled achenes on a plant by plant basis for use in *ex-situ* conservation is effective and may reduce artificial selective pressures.

6.1.3. Propagation *Protea roupelliae* ssp. *hamiltonii* (Chapter 4).

Propagation by seed/achene/zygotic embryo remains a superior method of propagation for the conservation of species due to the enhanced genetic diversity of the offspring. However, it is possible that dormancy mechanisms do exist in *Protea roupelliae* ssp. *hamiltonii* achenes. By removing the seed coat the germination percentage and germination rate was greatly accelerated whilst soaking achenes in water prior to being set to germinate had the opposite effect on achene germination. Both these responses are indicative of dormancy control.

Protea species require prolonged periods from germination to seed production and the seed produced is of variable quality and quantity which diminishes the feasibility of *ex-situ* breeding programs (Rebelo & Rourke 1986). However, there is potential to further develop the established micro-propagatory techniques into *in-vitro* derived plantlets or synthetic seed technology or for mass propagation to be utilised for the conservation or the horticultural trade of *Protea* species (Manjkhola *et al.* 2005).

6.1.4. *Ex-situ* conservation and population augmentation of *Protea roupelliae* ssp. *hamiltonii* (Chapter 5).

a. The achenes of *Protea roupelliae* ssp. *hamiltonii* may be stored successfully for 18 months at fluctuating ambient temperatures and constant temperatures of +25°C, +4°C, -70°C and -196°C (liquid nitrogen). Seed ageing (loss of germination vigour) was associated with fluctuating and warm (+25°C) temperatures. In general the period of storage at colder temperatures caused an increase in the germination vigour and germination percentage, although much of these changes may have been due to germination variation. There is a possibility that storage lifts dormancy mechanisms in relation to the storage period. The quality of achenes after one year of storage was high at all tested temperatures including cryogenic temperatures as achenes had sufficient vigour to grow *ex-situ* (greenhouse) and establish as transplants *in-situ* (the Dr Hamilton Reserve).
b. The survival and leaf production rate of transplants was higher than that of seedlings derived from planted achenes. Both methods of augmentation were successful but the utilisation of one over the other depends on the resources and the period available for the conservation effort. The transplant approach was aggressive. It required the maintenance of seedlings, growing facilities, transport and logistics to ensure seedlings arrived on site and were planted at the correct time of year in optimal condition. After planting was completed however, seedling survival and growth was good. On the other hand the planting of achenes required less than one full day with the construction of herbivore exclosures requiring the greatest time and financial investment. However, survival was poor and seedlings did not thrive compared with the transplants. In order to reach the surviving number of transplanted seedlings that has been achieved in this project it is expected the experiment involving the planting of achenes would have to be repeated many times, over successive years. This would be possible provided high achene production is maintained by the population every year.

6.1.5. Additional species information.

The following additional information from *Protea roupelliae* ssp. *hamiltonii* was also gathered mainly by observation:

a. Flowering time

Flowering may be staggered but has been observed to occur between October and December and *en masse* in late November (R. Green *pers. comm.* 2011; S. Tarlton *pers. obs*. 2008; Figure 6.1a and b) and not February to April as indicated in some field guides (Rebelo 1995).

b. Pollinators

Gurneys sugarbird (*Promperops gurneyi*), the Amethyst sunbird (*Chalcomita amethystina*) and the Greater double-collared sunbird (*Cynnyris afer*) have been observed in the Dr Hamilton reserve. The sugarbird was observed feeding on *Leucospermum gerrardii* as the *Protea roupelliae* ssp. *hamiltonii* were not in flower at that time. The sunbirds were frequently observed feeding on *Protea roupelliae* ssp. *hamiltonii* (Figure 6.1c and d).

c. Survival after fire (seedlings)

A sample of 24 transplanted seedlings (less than one year old) was inadvertently burnt during a controlled burn of the bracken filled valley of the Dr Hamilton Reserve during winter 2010. These seedlings appeared scorched in August 2010 and were expected to die. But when sampling occurred in November 2010, only two individuals from the sample were confirmed dead and the others showed remarkable recovery and were almost indistinguishable from other (un-burnt) seedlings planted at the same time. This observation may provide insight into the ability of this species to survive fire.


a.


b.


c.

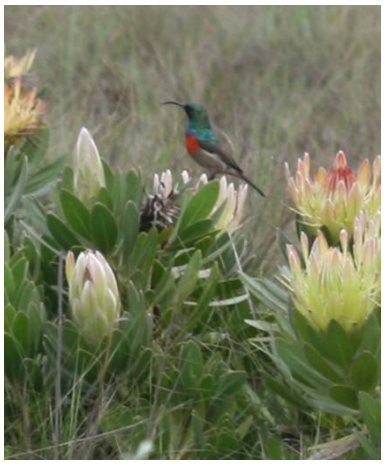

d.

Figure 6.1. *Protea roupelliae* ssp. *hamiltonii* (a and b) flowering on 17 November 2008 and the avian pollinators (c) the Amethyst sunbird (*Chalcomita amethystina*) and the (d) Greater double collard sunbird (*Cynnyris afer*) in the Dr Hamilton Reserve.

6.2. Recommendations

6.2.1. Management recommendations

- It is unfortunate that *Protea roupelliae* ssp. *hamiltonii* population numbers declined to the point of near extinction before the greatest threat to its survival was identified and

removed. In this case, the species experienced a possible genetic bottle neck before the influence of large herbivores was excluded. Now however, small mammalian herbivores pose the greatest threat to this species (Chapter 2). Plant ecologists and zoologists need to devise and implement strategies such as fire regimes to depress the small mammal population within the Dr Hamilton reserve without decreasing the canopy size of the plants.

- The *Protea roupelliae* ssp. *hamiltonii* population density within the fenced area of the Dr Hamilton Reserve is now considerably higher than it was at the beginning of this study. The fenced area of the Dr Hamilton Reserve should be increased to allow the population to continue its growth. Now that this higher density has been reached it may now be necessary to reintroduce individuals of *Protea roupelliae* ssp. *hamiltonii* to sites where populations have become extinct or to establish new populations in the nearby conserved areas.

- The floral display of the two endangered species, *Leucospermum gerrardii* and *Protea roupelliae* ssp. *hamiltonii*, are unique. It could become an eco-tourist attraction, and the increased appreciation by a wider audience could create public awareness and the possibility of fund raising for endangered plant conservation. However, a fine line is drawn between creating public awareness and fuelling the endangered species trade.

- Many chronic problems may be cheaper, easier and quicker to solve than to manage (Gladwell 2010). This present study presents tested methods by which the problems (i.e. the risk of extinction due to low population numbers and a small range (Pimm *et al.* 1988)) faced by *Protea roupelliae* ssp. *hamiltonii* can be solved instead of managed as they have been for several decades. The additional management requirements for endangered species are known to be costly and extend over an indefinite period (Wilcove & Chen 1998). The cost of solving the problem (removing *Protea roupelliae* ssp. *hamiltonii* from the Red Data List) through augmentation and reintroductions should be compared with the cost of managing this endangered species through its slow, natural regeneration in conjunction with the risk of the species going extinct in the process.

6.2.2. Research recommendations

- It should be established whether a significant mass difference between achenes filled with an embryo and achenes that are not filled is present in all *Protea* species and whether this mass difference should be exploited for seed selection in the conservation of all *Protea* species.

- The transplants from all the storage regimes at a single site showed poor establishment compared with the other three sites after one year *in-situ* (Chapter 5), therefore it is clear that successful establishment of transplants may be site specific. In order to optimise the future survival of propagules (transplants and/or planted achenes), environmental factors that influence the establishment of transplants and planted achenes should be quantifiably identified.

- The *in-vitro* multiplication of *Protea roupelliae* ssp. *hamiltonii* somatic embryos has been achieved via secondary somatic embryogenesis (Chapter 4) however, in order for the potential of this technology to be fully realised the following still has to be achieved:
    - The control of somatic embryo development into a plantlet (with roots and shoots).
    - The rooting of *in-vitro* derived shoots.
    - The "hardening" of *in-vitro* derived plantlets into greenhouse conditions.
    - The quantification of genetic instability brought about by indirect embryogenesis.

- The germination of stored achenes seemed to improve (in terms of germination vigour and percentage) with the period of storage, similarly germination was increased with the removal of the seed coat. Interestingly the maximum P.V. attainted after 18 months of storage (-70°C and 25°C regimes which attained a P.V of 6.20 and 6.18 respectively) of *P. roupelliae* ssp. *hamiltonii* achenes was similar to that reached by un-stored *P. roupelliae* ssp. *hamiltonii* achenes that had their pericarps removed (P.V. of 6.03) (Chapters 4 and 5). This relationship should be tested further and may be found in other *Protea* species, if this relationship is substantiated, the mechanisms involved should be identified.

7. References

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