



***In vitro* conservation of selected endangered plant species indigenous to the
Cape Floristic Region, South Africa**

by

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Thesis submitted in partial fulfilment of the requirements for the degree of

MAGISTER TECHNOLOGIAE: HORTICULTURE

in the

FACULTY OF APPLIED SCIENCES

at the

CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

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CAPE TOWN

2016

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DECLARATION

I, **Bonolo Mosime**, declare that the contents of this thesis represent my own work, and that the thesis has not previously been submitted for academic examination towards any other qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

This study focused on optimising four types of *in-vitro* conservation methods, namely: 1), micropropagation, 2) *in-vitro* slow growth, 3) seed germination and 4) cryopreservation for selected endangered plant species indigenous to the Cape Floristic Region. It is one of the targets set by United Nations millennium development goals, to integrate different conservation measures in order to preserve plant diversity and mitigate losses of genetic diversity. Therefore this study uses *Phalaenopsis* hybrids as a trial species that can be studied for the conservation of endangered *Disa* and *Eulophia* species through micropropagation and *in vitro* slow growth. Also conservation attempts on *Leucadendron* and *Mimetes* species that occur in the Cape Floristic Region were attempted to increase population densities by increasing germination percentages using smoke. Furthermore, the study attempted to store seeds by assessing different cooling rates for optimising cryopreservation measures for effective conservation.

The use of tissue culture to increase propagules especially critically endangered species in South African has proven to be feasible. For the trial hybrids, shoot and protocorm explants of *Phalaenopsis Psychosis Pink X P. No. 1*; *P. Large white X P. Large pink*; *P. No. 1 X P. Large pink*; *P. Mini pink X Brighton belle*; and the *P. aphrodite* formed clusters of protocorms and shoots when cultured on ½ strength MS media supplemented with 10, 20 and 30gL⁻¹ banana extract or ½ strength Murashige and Skoog, (1962) (MS) media supplemented with peptone. Continuous protocorms formation could therefore be obtained by culturing endangered *Disa* and *Eulophia* shoots and protocorms on banana containing media. Plantlet conversion from somatic embryos produced on 10gL⁻¹ banana extract enriched media was successfully achieved on ½ strength MS supplemented with 20gL⁻¹ sucrose and no plant growth regulators in the medium. However, optimum rooting was achieved on ½ strength MS supplemented with 30gL⁻¹ of banana extract and this medium yielded the highest survival percentages for plantlet acclimatisation. Furthermore, ½ strength MS supplemented with 1gL⁻¹ of peptone served as a stimulant for shoot development and protocorm formation. When coupled with banana extract at all stages of development, regeneration and rooting were enhanced.

For *In vitro* banking, investigations were undertaken to determine the effect of mannitol concentration on the *Phalaenopsis Psychosis Pink X P. No. 1*; *P. Large white X Large pink*; *P. No. 1 X Large pink*; *P. Mini pink X Brighton belle*; and the *P. aphrodite*. Shoot and protocorm explants conserved in semi-solid ½ strength MS media enriched with 10, 20 and 30gL⁻¹ mannitol in the absence of sucrose for up to fifteen months. *Phalaenopsis* hybrids

were tolerant to osmotic stress as they exhibited sufficient growth and regeneration whilst in storage. However, the conserved plantlets of all *Phalaenopsis* tested regained their ability to generate once cultured in a multiplication medium, with 95% survival percentages for *P. Large white* X *P. Large pink* when stored in ½ MS supplemented 10 gL⁻¹ mannitol, 100% for *P. Large white* X *P. Large pink*, *P. aphrodite* and *P. Mini pink* X *P. Brighton belle* shoots when stored in ½ MS supplemented with 20gL⁻¹ of mannitol. Protocorms of *P. Large white* X *P. Large pink*, *P. aphrodite* and *P. Mini pink* X *Brighton belle* also displayed 100% survival post storage when conserved in ½ MS supplemented with 30gL⁻¹. This conservation measure proves viable to store regenerated *Disa* and *Eulophia* endangered species.

The germination of endangered *Proteaceae* species in the Cape Floristic Region, research was undertaken to determine effective sterilization and germination protocols by varying concentrations of aqueous smoke, incubation temperature, including the effect of decoating seeds and the influence of nutrition on germination of *Leucadendron salignum*, *L. cryptocephalum*, *L. argenteum*, *L. chamalaea* and *Mimetes chrysanthus*. Germination percentages, rate of germination, mean germination time varied across the species studied. Exposure to sulphuric acid for three minutes resulted in no germination across all species. Aqueous smoke increased germination percentages and reduced the culture time required for the germination of *L. salignum*, *L. cryptocephalum*. The removal of seed coat also resulted in increased germination percentages and reduced germination time of *M. chrysanthus* and *L. argenteum*, especially on medium supplemented with 6 mL⁻¹ of aqueous smoke.

To establish a protocol for cryopreservation of endangered *Proteaceae* seeds, mature seeds of *L. salignum*, *L. cryptocephalum*, *L. argenteum*, *L. chamalaea* and *M. chrysanthus* were frozen at different cooling rates and stored in liquid nitrogen. After storage, the seeds were cultured on a semi-solid medium containing 7gL⁻¹ agar to assess the germinability, germination rate and the duration for germination. Cryopreservation increased germination percentages of mature seeds of *L. argenteum* and minimised the duration for germination of *L. argenteum*, *M. chrysanthus*. Successful cryopreservation was achieved for *L. salignum*, *L. cryptocephalum* when cooled at 80° C for 60 minutes prior too freezing in liquid nitrogen.

In conclusion, the methods developed in this study can be used for the conservation of endangered plant species for sustainability and protection of plant diversity.

DEDICATION

I dedicate this thesis to my family, especially my mother, Maelagongwe R. Dintwe, who has inspired, supported and encouraged me during the quest for my degree.

ACKNOWLEDGEMENTS

I wish to thank the following:

- Prof Joseph Kioko and Prof Charles Laubscher, for their guidance and support throughout the project.
- Family and friends for supporting and encouraging me throughout my studies.

BIOGRAPHICAL SKETCH

Bonolo Mosime was born in Mochudi: Botswana on 13 March 1987. She attended Primary School in Botswana and later attended High School in Ledumang Senior Secondary School, where she completed her O-level in 2005. In 2007 she enrolled at Cape Peninsula University of Technology for a National Diploma in Horticulture. In 2010 she completed her B Tech degree in Horticulture at the same University. Her research was based on promoting the use of biotechnological tools for conservation of both indigenous endangered species that are potentially collected for floriculture and the dominant Orchidaceae in the floriculture industry. She previously worked as a plant Science/tissue culture laboratory assistant and is currently working at as a teaching assistant at CPUT.

LIST OF OUTPUTS

The following outputs are contributions by the candidate to scientific knowledge and development:

Manuscripts submitted for publication still under review:

Mosime, B., Kioko, J.I. & Laubscher C. 2016. Potential production and conservation of germplasm of commercially important species of the Cape Floristic Region via plant biotechnology: A Review (*Submitted to Applied Ecology and Environmental Research, February 2016*)

Conference list: Poster presentations

- **Mosime, B.** & Kioko, J.I. 2014. *In vitro* germplasm conservation of *Phalaenopsis* spp and hybrids under slow growth conditions. U6 international conference, 6-10 September 2014, Cape Town.
- **Mosime, B.**, Kioko, J.I. & Laubscher C. 2012. Enhancing *in vitro* asymbiotic seed germination of *Disa* and *Phalaenopsis* hybrids by altering medium components and illumination. The Joint International conference on Science and Technology for development in Africa, 26-28 June 2012, Cape Town (South Africa).
- **Mosime, B.**, Kioko, J.I. & Laubscher C. 2012. Plant biotechnology: A review on potential production and conservation of germplasm in the floriculture market. The Joint International conference on Science and Technology for development in Africa, 26-28 June 2012, Cape Town (South Africa).

Conference list: Poster presentations

Mosime, B. & Kioko, J.I. 2012. Sustenance of *Leucadendron* spp. using micropropagation to enhance economic growth in the Cape Floral Kingdom. The National Conference on Global Change from 26-28th November 2012, Johannesburg (South Africa).

Preface

The overall aim of the study was to use biotechnological tools for propagation and conservation of endangered species and highly valuable floricultural plants. The references are listed at the end as a separate chapter in accordance with the Harvard method of referencing.

The thesis is subdivided into the following chapters:

- **Chapter 1:** The literature review chapter provides insight on the application of the technological tools on various species, which could be adopted for preservation of the selected species in this study. It highlights seed germination, tissue culture, slow growth and cryopreservation.
- **Chapter 2:** It presents research questions to be addressed by the study, lists the overall objectives and clearly states topics that were not covered by the study.
- **Chapter 3:** Investigations on optimization of *in vitro* regeneration of *Phalaenopsis* hybrids using banana extracts and optimizing *in vitro* storage using mannitol are elaborated in this chapter as an example of treatments that can be adopted in conservation of endangered species.
- **Chapter 4:** In this chapter, a) investigations on optimization of *Leucadendron* species and *Mimetes chrysanthus* (dwindling species specific to the Cape Floral Kingdom) under two incubation temperatures, and b) optimizing the cooling rate for cryostorage of the selected seeds.
- **Chapter 5:** Overall discussion and conclusion. In this chapter, each of the research questions listed in Chapter 2 are answered also listing recommendations for future research, are listed.
- **Chapter 6:** Contains the references, used for the study.

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GLOSSARY

Abbreviations	Explanation/Definition
MS	Murashige and Skoog (1962) medium
MR	Mean germination rate
MT	Mean germination time
ABA	Abscisic acid
AC	Activated charcoal
BAP	6-Benzylaminopurine
IBA	Indole butyric acid
IAA	Indolyl-3-acetic acid
NAA	α -Naphthalene acetic Acid
PGR	Plant growth regulator
PBLs	Protocorm-like bodies
ROS	Reactive oxygen species
TDZ	Thidiazuron
TTC	2, 3, 5-Triphenyltetrazolium chloride
LN	Liquid nitrogen
ANOVA	Analysis of variance
CV	Coefficient of variation of germination time
Protea atlas	Protea Atlas Project

CHAPTER ONE

Potential production and conservation of germplasm of commercially important species of the Cape Floristic Region via plant biotechnology: A Review

1.1 Introduction

The major biomes which are found in southern African occupy an estimated area of 2.5 million square kilometres. Some of these areas can be recognised as the Afromontane region, Cape Floristic Region, Kalahari-Highveld transition zone, Karoo-Namib region, Tongaland-Pondoland region and the Zambebian region (Goldblatt, 1978). Most of these biomes are recognised for their exceptionally rich terrestrial biodiversity (van Wyk & Viljoen, 2011). The flora found within these biomes consists of 1930 genera, comprising 185,000 species (Goldblatt, 1978). This rich biodiversity continues to be threatened by human activities such as urbanization, agriculture, consumption, deforestation and alien invasive species (Goldblatt, 1978; Chugh et al., 2009; Cheeseman et al., 2010).

The biodiversity of the Western Cape in South Africa is the backbone of the country's indigenous floriculture industry, which is also one of the fastest growing sector (Netnou-Nkoana & Eloff, 2012) and a tourist attraction. More than 70% of the flower stems presented for trade are directly harvested from the wild within the Cape Floristic Region (Petersen et al., 2012). South Africa generated on average 507 million US\$ between 2006 and 2011 on floriculture products (Maleka et al., 2013), and this sector employs more than 17500 people (Department of Agriculture, Forestry and Fisheries, 2013). Despite certain geophytes being recorded as endangered, some products are continuously made available to the trade (Winter & Botha, 1994). The continuous overharvesting of flowers prevents reproductive cycles to replace species in the wild is a further cause of threat to these wild populations.

Apart from harvesting in the wild, there has been an intensive hybridisation of many indigenous commercial flower genera, including *Disa*, *Erica*, *Lachenalia*, *Leucadendron*, *Leucospermum*, *Serruria*, and *Strelitzia*, which dominate the international floriculture industry (Bester et al., 2009). Such hybridization can accelerate the loss of native genotypes and hence further

threaten biodiversity. Other taxa sought out for their characteristics of being good ornamental plants include *Brunia*, *Kniphofia* and *Rhodocoma* (Reinten et al., 2011).

Based on cultural beliefs, the majority of the rural South African population has been reported to use plants for medicinal purposes and basic nutrition, amongst other several uses (Goldblatt, 1978; Cunningham, 1995; Bodeker, 2006; Moyo et al., 2011). Williams (1996) reported that 99% of the 550 medicinal plants species used in South Africa are directly harvested from the wild. Human populations in Southern Africa mainly rely on indigenous plants, resulting in intensive exploitation of these native species. It is significant, however, that the rate of depletion of these natural resources due to consumption is not accompanied by an appropriate replacement in nature.

Besides direct extraction of species from the wild for trade or medicinal use, the human impact on native plant species also includes the introduction and advancement of alien invasive plants. These have serious and detrimental ecological effects, including suppression of indigenous vegetation growth (van Wilgen, 2010) and impact on water availability (Le Maitre et al., 2002; Richardson & van Wilgen, 2004; Chamier et al., 2012). Due to the fact that higher densities of invaders occur along riparian ecosystems (Richardson & van Wilgen, 2004) these species have the ability to consume excess amounts of available water. According to Enright (2000), the Western Cape alien invasive species account for approximately 16% loss of run-off water and result in elevated evaporation rates from the canopies, while changes in biomass accumulation patterns alter the natural element availability in ground water (Chamier et al., 2012). This in turn negatively affects the indigenous plants dependent on ground water. Alien plants account for at least 14% of the total flora within the city of Cape Town, and their proliferation is further fuelled by the increasing human population (Rebelo et al., 2011).

Human population growth is a driver of urban development and the transformation of natural ecosystems. Within the Cape Floristic Region, the population of Cape Town has increased from 2.56 million people in 2006 to 3.74 million in 2011 (Allsopp et al., 2014). This process of urbanisation has resulted in the transformation (extinction) of various vegetation types. In a study conducted by Rebelo et al., (2011), the renosterveld vegetation, which occurs mostly in fertile soils, has been transformed in the range of 74-100% by agriculture. It was further established that up to 94% Alluvium Fynbos have been transformed and urbanisation process and deforestation has led to extinction (100% transformation) of at least four out of 56

vegetation subtypes, with up to 19% of the remaining sub types being critically endangered within the Western Cape (Rebello et al., 2011).

The overall impact of the various ecological threats, such as those illustrated above, continue to exert pressure on the flora of southern Africa. Of the plants indigenous to South Africa, there are at least 32 taxa listed as extinct, 1153 as critically endangered, 1295 as vulnerable and 375 as near-threatened (Raimondo, 2009). This highlights the need for conservation of the many valuable species which are of serious conservation concern.

Among the options available for conservation is legal protection. Globally, about 60% of the world flora is estimated to be under protection according to the Global Strategy for Plant Conservation (Coates & Dixon, 2007). Pursuant to article 6 of the Convention in Biological Diversity (CBD), national institutions (such as the Botswana National Botanic Garden (NBG) and the South African National Biodiversity Institute [SANBI]) emerged to facilitate the preservation of plant diversity via in situ or ex situ means. In situ conservation is the conservation of germplasm in its natural habitat (Maxted et al., 1997; Schmelzer et al., 2010), usually under legislative control. However, maintaining plant diversity in natural fragmented ecosystems is often ineffective as the conserved taxa remain vulnerable to threats such as the instability of habitats, pollution, the decline of pollinators, or inadequate legal protection against the harvesting of plants (Withers, 1991; Engelmann, 2004; Swarts & Dixon, 2009; Moyo et al., 2011). Despite in situ conservation in Botswana, the heavily exploited *Adenium boehmianum* was recorded as endangered in 2009, and is currently almost extinct with only one remaining plant left in the country (Tema & Chiyapo (pers. comm. 2012). Globally, Jantz et al., (2015) estimate a 26-58% reduction in the vegetation of hot spots. This illustrates the necessity for complementing in situ conservation with appropriate ex situ conservation – i.e. the conservation of components of biodiversity outside their natural habitat (Schmelzer et al., 2010) in order to achieve effective conservation.

One approach to *ex situ* conservation is the use of the tools of plant biotechnology. This combines techniques that use plant DNA, cells, tissues and organs, to provide viable options for ex situ conservation of plant germplasm (Chen & Chen, 2007; Parawira & Khosa, 2009). In particular, plant biotechnology can be used to rapidly multiply and increase populations of threatened species through micropropagation (Hamza et al., 2013); to provide means of *in vitro* storage of germplasm via slow growth cultures or cryopreservation (Berjak et al., 2011; de

Carvalho et al., 2014); to assess the maintenance of genetic diversity and integrity of conserved genotypes (Reed et al., 2013); to produce virus-free stock from infected plants (Awodoyin et al., 2015); or to provide means of accurate molecular-based disease and pathogen assessment (Ncube et al., 2012).

1.2 *In vitro* propagation

Plant tissue culture refers to the growing of plantlets, plant tissues or plant cells (e.g. callus, meristems, somatic embryos, or shoots) on artificial growth media under aseptic conditions (Paunescu, 2009). The starting material for tissue culture (the explant) can be any part of the parent plant, usually either shoot tips, nodal segments, part of the leaf, floral parts, immature embryos, excised zygotic embryos/ seeds, cotyledons, or single cells (Mccartan & van Staden, 2003; Kaliamoorthy et al., 2008; Suárez et al., 2010; Gutiérrez et al., 2011).

Successful tissue culture depends on the optimization of aspects such as the culture medium and environment, as well as desired morphogenetic routes. The next section will cover those factors systematically:

a) Culture medium

The culture medium has to avail plant mineral elements and be able to physically support the explants whilst in culture. The macro nutrients N, P, K, Ca, Mg, and S, the micro nutrients Fe, Mn, Zn, B, Cu, Cl, Mo, and Ni are all essential to facilitate plant growth and cell division (Gautheret 1955; White & Brown 2010). Murashige & Skoog MS (1962), woody plant medium WPM (Lloyd & McCown, 1980), White's medium (White, 1943) Gamborg medium (Gamborg et al., 1968); Nitsch and Nitsch (NN) medium (Nitsch & Nitsch, 1969) are some examples of well devised and documented formulations of mineral elements suitable for various plant species. Additionally, vitamins are often added to the medium, such as Thiamine (B1), Nicotinic acid (B3) and Pyridoxin (B6), which assist in the development of cells (Abrahamian & Kantharajah 2011). A source of carbon is used supplement the medium, as most *in vitro* cultures are not autotrophic at initiation (Kozai, 1990; Yaseen et al., 2013). For this purpose, sucrose is often incorporated at quantities of 2-3% in the medium (Benson, 2000; Yaseen et al., 2013). However, different forms of carbohydrates may be used, such as glucose (Yu & Reed, 1993), lactose, maltose (Cheeseman et al., 2010), galactose and starch (Rout et al., 2000).

To provide physical support, the nutrient medium is solidified by different gelling agents, such as agar (Kumar et al., 2012), gelrite (McCartan & Van Staden 1998), or alginate (Gombotz & Wee, 2012). These are used at different strengths depending on the desired viscosity. Ivanova & van Staden (2011) point out that the gelling agent may have significant impact on the rate of multiplication and hyperhydricity of shoots. Finally, the culture medium must be at a pH that will facilitate cell division, regulation of cytoplasmic activities and the uptake of nutrients by explants from the medium (Ostrolucká et al., 2010). The optimum pH range that has proven to be ideal for growth ranges between 5.5 and 6 (Ostrolucká et al., 2010; Poorter et al., 2012).

The growth or morphogenesis of cultured explants is dependent on the interaction of plant growth regulators (PGRs) in the medium and within the explant (Thomas & Jiménez 2005). The interaction of plant growth regulators in micropropagation varies with each plant species. High levels of auxins promote root development, whereas low concentrations of auxins and higher cytokinins promote shoot development (Werner et al., 2001). Supplementing the tissue culture medium with auxins can also stimulate the development of callus (George et al., 2008a).

Plant growth regulators are used to mediate biochemical pathways which explants undergo during development. These induce a series of cell divisions that result in the regeneration of cells; which is either direct or indirect morphogenesis. The morphogenetic route taken by explants is dependent on the relative concentrations of different types of plant growth regulators. Generally, a high ratio of cytokinins: auxins promotes adventitious shoot formation (Murashige, 1962); while the converse induces the development of unorganised mass of tissue known as callus (Murashige, 1962; Smith, 2013). Thus, the medium onto which explants are cultured should be carefully and deliberately designed to achieve the desired morphogenesis.

When all the variables for successful *in vitro* propagation are considered, the optimum conditions are often species- or even genotype-specific.

I. Possible micropropagation routes

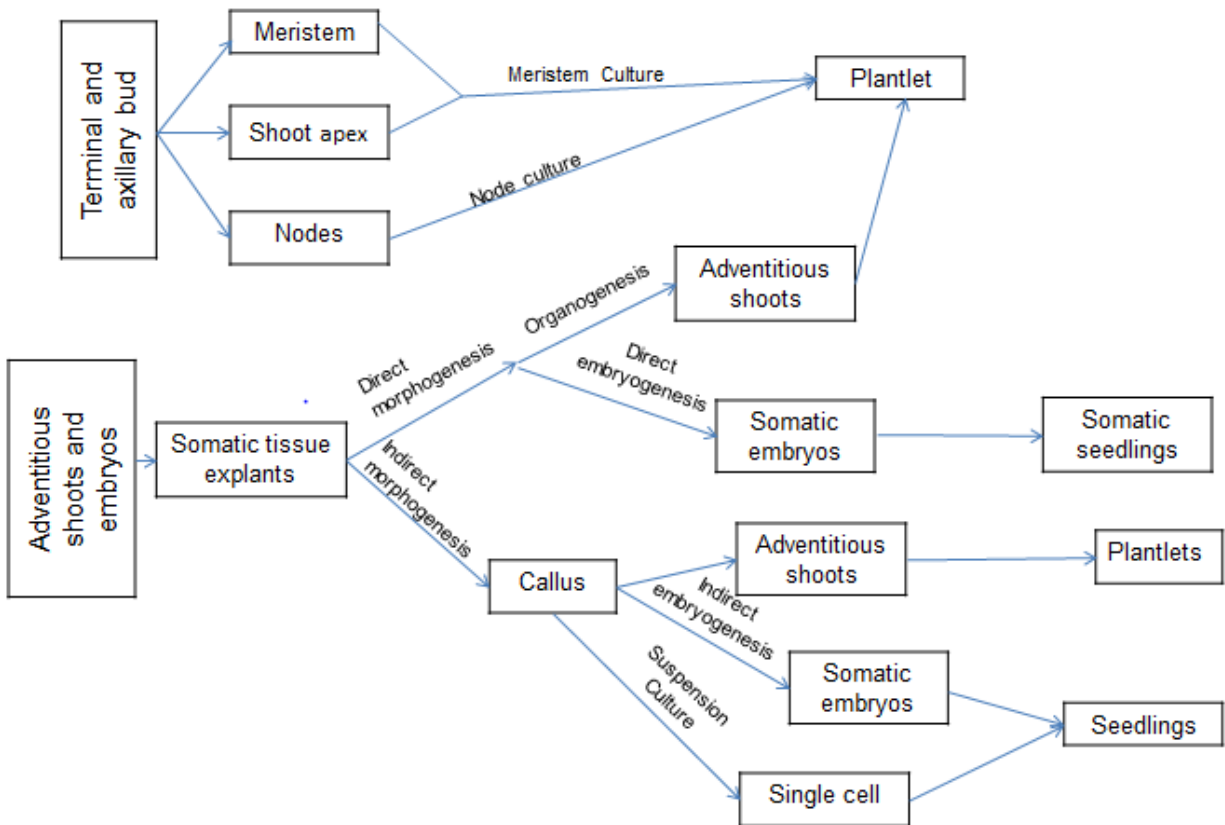


Figure 1.1: Micro-propagation routes of adventitious shoots and embryos to grow into plantlets and seedlings and terminal and axillary buds to grow into plantlets.

Incubation conditions

a) Temperature

One of the major environmental factors in micropropagation is temperature. It influences the morphogenetic response and metabolic processes in plants and an optimum temperature is ideal for growth and development of the plants. Low temperatures may influence the tissues to be dormant, whereas high temperature may be lethal to the tissues (Atkin & Tjoelker, 2003). Findings have however, clearly indicated the species-specificity of temperature requirements in plants; and therefore optimum growth temperatures vary from one species to another (Galmes et al., 2005).

For *Arachis hypogea*, 35 ± 5 °C was reported to be the optimal temperature for direct organogenesis from both cotyledonary and leaf tissues (Pestana et al., 1999). Furthermore, a study on *Asparagus officinalis* L. cultures by Li & Wolyn (1996) indicated that a temperature regime for maximum bipolar embryo production from the callus was 27 °C and the least globular embryos produced from the callus was achieved at 24 °C. Furthermore, an optimum temperature has proven to be essential for the conversion of somatic embryos to plantlets. A similar observation was made by Huang et al., (1991), for *Brassica napus*, in a study in which when embryos were incubated at 25 °C, they underwent somatic embryogenesis, whereas embryos cultured at 20 °C transformed to plantlets.

Although a constant temperature during incubation of explants may be adequate for cultures, this method fails to accommodate the temperature variation between day and night temperatures, under which plants undergo with *ex vitro* conditions. The interactive effect of temperature and CO₂ absorption is illustrated by Nievola et al., (2005), indicating that *Ananas comosus in vitro* plantlets display CAM-type photosynthesis when incubated 28 °C light and 15 °C dark, but display C3 carbon fixation when incubated continuously 28 °C.

b)Light

Light is an essential factor for the development of plants as it influences the photosynthetic ability and rate of plants. Carbon absorption and fixation are major metrics of photosynthesis and are dependent on the quality of light and duration of exposure (Buchanan & Balmer, 2005; Liu et al., 2007). At low irradiance, the growth rate of plants significantly slows down (Zivcak et al., 2015), indicating that the assimilation of CO₂ is reduced at low light intensities. To maximise carbon dioxide absorption capacity by the explant and seedlings, Guo et al., (2012) suggested increasing the photosynthetic photon flux to about $200\mu\text{mol}\cdot\text{m}^{-2}\text{s}^{-1}$. In a study on *Phalaenopsis amabilis* var. Formosa, Guo & Lee (2006) further highlighted that the carbon dioxide absorption in the leaves increases with but declines after an optimum age (240 days, in that study). The duration of light exposure also, plays an important role in the survival of explants. This is highlighted from a study by Velez-Ramirez et al., (2011), showing the adverse effects of continuous light, which was found to be detrimental to plants. A study by Moyo et al., (2014) suggested that by exposing plants to a 16 hour photoperiod, maximum stomata opening could be reached, which is ideal for increasing CO₂ absorption.

1.2.1 Procedures for the application of *in vitro* propagation of plants

In order to develop a protocol for micropropagation, which may be species- genotype- or even explant-specific, there are several general steps that must be optimized. These steps are outlined below. The outline deals with only multiplication from shoot-tips, seeds, and somatic embryos as they are the most widely-used micropropagation explants in the floriculture industry in South Africa:

a) Decontamination

Microorganisms including fungi, algae, and bacteria are often sheltered by the plant material. These microorganisms could either be pathogenic or non-pathogenic (Rout et al., 2000). They should be eliminated from the plant material before culture as the establishment of microbial growth in the tissue culture medium hampers the establishment of explants (Withers, 1991). Microbial growth may overgrow and limit both nutrient uptake by the explant or may outcompete the explant denying it access to light (Rout et al., 2000).

Decontamination is made more imperative by the fact that the basic tissue culture medium is a conducive environment for microbial growth. Research has indicated that the most common decontamination process involves soaking explants in 70% ethanol succeeded by soaking the explants in 5-10% sodium hypochlorite (NaOCl) (Pence et al., 2007) or calcium hypochlorite. Alternatively, 0.01-0.1% (w/v) mercuric chloride is also commonly used for surface-sterilisation of tissue culture lines (Rout et al., 2000). Pence (2005) indicated the necessity of incorporating antibiotics or fungicides in the sterilising procedure in order to reduce contamination levels. In conducting surface-sterilization treatments, explants may be exposed to the process for different periods resulting in different outcomes.

In instances where the microorganisms are tissue-borne and not just on the surface of the explants, surface-sterilization is not sufficient (Luna et al., 2013). Ajayi et al., (2006b) indicate that incorporating systemic fungicides and in the culture media significantly reduces tissue-borne microorganisms, however, this may result in delayed growth.

North et al., (2010), working on *Strelitzia reginae*, highlighted the lack of studies in decontamination of Cape floral species and the challenges that faces propagation of this important flora.

1.3 Conservation via rapid multiplication

For species that are endangered or on the brink of extinction, ex-situ multiplication is crucial in increasing population sizes. The most rapid method of propagation is tissue culture which can be successfully used to combat extinction of plant species (Hannweg et al., 1996; Pence et al., 2007; Moyo et al., 2011). This technique uses small quantities of plant tissue to regenerate growth and produce multiple plants under sterile conditions (Smith, 2013; George et al., 2008b). It hinges on the fact that plant cells are totipotent, and therefore each cell can be induced to form a new plant under the right culture and conditions (George et al., 2008b). A further advantage of micropropagation is that there is no need to wait for plants to mature and produce seeds for propagation; it is applicable even when no fertile seeds are produced, and viable populations can be regenerated from a single individual or explants (Smith 2013; George et al., 2008b).

Also, a major contribution of *in vitro* propagation to the floriculture industry is mass propagation of selected genotypes, especially when vegetative explants are used instead of seeds (Chugh et al., 2009). Some of the approaches that can be used for this purpose include somatic embryogenesis and shoot tip culture via organogenesis, as presented in Figure 1.1.

b)The initiation stage:

In somatic embryo induction, a few primary cells are receptive to hormone induction. Kutschera (1994) described the mechanism where auxins regulate growth at a concentration of 1 to 10 μM .

c)Growth or Multiplication

During growth and multiplication, photosynthetic activities are very important for successful shoot development. Therefore, incubation and lighting become critical factors. Once the explants initiate the desired growth, they are transferred into a multiplication medium (which may differ from the initiation medium), containing both cytokinins and auxins which facilitate

multiplication and proliferation (Murashige, 1962; Kanchanapoom et al., 2010). This is done to increase the number of propagules and the multiplication medium is used to facilitate growth and maturation of somatic embryos, generate multiple shoot development and promote shoot elongation (George et al., 2008a). Multiplication has been used very successful for many important commercial species, for example many orchid species from the Cape Floral Region can benefit from this technique (Thompson, 2007).

d)Rooting/ germination of somatic embryos

This stage allows the plantlets to become self-sufficient. Often, elongated shoots are transferred to media that generally contain high ratios of auxins to cytokinins or omission of cytokinins in order to facilitate rooting (Smith, 2013). Activated charcoal can also be added to the rooting medium to enhance root growth (Kanchanapoom et al., 2010). Forced ventilation (increasing the CO₂ concentration in roots) has the capacity to reduce wilting of plants, and hence improve transplantation (Pospóšilová et al., 1999). Forced ventilation has proved to be essential in improving the rate of survival during acclimatisation in *Bauhinia cheilantha* (Gutierrez et al., 2011). This method may be adopted for other species to successfully grow strong rooted plants. Additionally, low dosages of plant growth regulators enhance maturation of somatic embryos.

e)Acclimatising rooted plantlets to field conditions

The process of tissue culture is incomplete without successfully getting the plants to adapt to the field environment. *In vitro* propagated plants are cultured under conditions of high humidity and low light intensity, which limits the deposition of epicuticular wax on the leaves and the leaves develop high densities of stomata that are open continuously (Sáez et al., 2012). During the transitional period of adapting to *ex vitro* conditions, *in vitro* plantlets lose a great deal of water, owing to their under-developed physiological attributes. As a result of high evapotranspiration and the photosynthetic potential of the plantlets are reduced, leading to high mortality rates (Pospóšilová et al., 1999; Sáez et al., 2015).

Thus, the main aim of the acclimatisation phase is to limit water stress for the plantlets to ensure survival. In some instances, abscisic acid is administered on the leaves to reduce transpiration (Dias et al., 2014). Other studies indicate that carbon dioxide enrichment and increasing illumination during acclimatization improves the photosynthetic capacity of plantlets (Laforge et

al., 1991; Yue et al., 1992; Shin et al., 2013), thereby increasing survival. Many woody species from the Cape Floral Region are susceptible to phytophthora root rot infection (Nagel et al., 2013). Trail methods for adaption to transplanting and acclimatising to field conditions can greatly benefit future successful production of these species.

i. Regeneration through Somatic embryogenesis

Somatic embryogenesis involves the formation of embryo-like structures from somatic cells. The somatic embryos go through the process of embryogeny without fusion of gametes, and are then converted to produce to new plants (Zimmerman 1993; Fehér, 2015). Despite this method offering the highest rates of plant formation compared with other methods of micropropagation (Smith, 2013), it is rarely used for commercial purposes owing to high somatic variations (Neumann et al., 2009).

However, somatic embryogenesis has been applied to various orchid species, including *Oncidium* 'Gower Ramsey', *Phalaeonopsis*, and *Cymbidium* (Ishii et al., 1998; Chen & Chang, 2001; Tokuhara & Mii, 2003; Gow et al., 2010). Also, somatic embryogenesis has been demonstrated in highly utilized species such as *Phoenix dactylifera* (Othmani et al., 2009), and *Acacia mangium* (Xie & Hong, 2001). Various studies have attempted to increase the yield of plantlets from somatic embryogenesis, achieving different outcomes. For example, Junaid et al., (2006) noted that somatic embryo induction was highest when using hypocotyls as explants for *Catharanthus roseus*. In the case of indirect somatic embryogenesis, Othmani et al., (2009) recommended partially desiccating the calli to increase the surface area for absorption to stimulate rapid maturation of embryos while Krishna & Singh (2007) and Litz & Gomez-Lim (2005) recommend the use of higher concentrations of sucrose (up to 6%) to aid the maturation of embryos. Additionally, the use of abscisic acid (ABA) in Murashige & Skoog (MS) medium supplemented with 5% sucrose gave high maturation percentages (100%) in *Copiapoa tenuissima* (Lema-Rumińska et al., 2013). It has generally been shown that Thidiazuron (TDZ) is the most effective cytokinin for inducing somatic embryos in numerous species. The compound can enhance shoot and embryo induction and proliferation without any alteration to the plant genetic makeup (Chen & Chang, 2001; Kishor & Devi, 2009).

Incubation conditions are also key to the induction and proliferation of somatic embryos. Yang et al., (2013) recommended increasing temperatures to 30°C for induction of secondary somatic

embryos and reducing the temperature to 20°C for the development of embryos and further conversion to plantlets. Too high temperatures, however, could cause the embryogenic processes to be suppressed. Regeneration through somatic embryogenesis holds unlocked potential for important species especially orchid species from the Cape Floristic Region in advancing their commercial production and conservation status.

i. Regeneration through organogenesis

Organogenesis is achieved when explants are cultured in appropriate nutrient media to induce the production of organs (shoots or roots) on the explant at optimum incubation conditions (Narayanaswamy, 1977; Van & Trinh, 1990). Studies by Hicks (1980) and Thorpe (1990) indicated that the parenchyma cells of the explant form meristemoid cells which ultimately can give rise to either a shoot or root primordia. Organogenesis hinges on the development of pre-formed axillary buds which, upon interaction with the medium, may promote elongation of the buds, or form adventitious shoots or roots; leading to development of new plantlets in culture (Debergh et al., 1990; Smith & Drew, 1990).

A number of researchers report the use of shoot organogenesis for the micropropagation of some orchid species (Kalimuthu et al., 2006). Additionally, as a conservation strategy, *in vitro* propagation using organogenesis has been achieved on *Maytenus senegalensis*, *Savia africana-lutea* using nodal explants (Matu et al., 2006; Makunga & van Staden, 2008), *Huernia hystrix* using stem explants (Amoo et al., 2009), *Thapsia garganica*, using petiole and leaf explants (Makunga et al., 2005).

ii. Regeneration through shoot tip culture

Shoot tip culture refers to the use of shoot-bearing meristems as explants with the aim to increase the number of secondary axillary branching of explants. Under appropriate conditions, Neumann et al., (2009) indicated that from one shoot apex in bananas, up to 30 plants can be produced. Shoot-tip cultures can be used as a technique to eliminate virus infection in plants, as the shoot meristems are usually free of the infection (Wang et al., 2009). For this purpose, the excised shoot explants must be kept small, with sizes of 0.2-0.5 mm recommended (Grout 1999; Wang et al., 2009). However, smaller explants increase the chances of wounding the explant, which results in the reduced regenerative ability (Nhut et al., 2007). Thus, for the

successful rapid regeneration via shoot tip culture of plants that do not require virus elimination, it is recommended to use explants of 1-2 mm in length (Grout, 1999; George et al., 2008b).

Plants in the horticulture and agricultural sectors are faced with epidemic viral diseases that have substantial influence on the quality of growth and regeneration. Shoot tip culture is extensively utilised for clonal propagation and as a virus or disease eliminating technique from the genome. For example, shoot tip culture is used to rid endogenous bacteria strains that are common in *Gerbera* genotypes (Cardoso & Teixeira da Silva, 2013). Several studies have shown that shoot tip culture can be achieved by isolating meristem tips of *Aloe barbadensis* (Natali et al., 1990) and axillary buds of *Spilanthes mauritiana* (Bais et al., 2002) for subsequent *in vitro* culture.

iii. *In vitro* generation using seeds or their component tissue as explants

Sexual propagation is considered one of the most efficient means of increasing diversity in the genetic pool of any species, including plants (Reed et al., 2011). In tissue culture, regeneration from zygotic embryos may involve the use, as explants, of hypocotyl segments (Junaid et al., 2006), cotyledonary nodes (Alam et al., 2010), nodal segments (Purkayastha et al., 2008) or shoot buds (Wu & du Toit, 2012). However, this method has its own limitations such as unavailability of adequate seeds in the case of endangered species or species with sterile seeds. Even so, this technique remains valuable in advancing commercialization of unknown orchid species of the Cape Floral Region and to highlight their importance in conservation (Thompson et al., 2011).

1.4 *In vitro* banking/ storage

1.4.1 Artificial seeds

An advantage of using somatic embryos for propagation is that they can be used for the production of synthetic seeds (Liu et al., 1992; Ipekci & Gozukirmizi, 2003). This process involves the encapsulation of somatic embryos with hydrogels like sodium alginate to form an 'artificial seed' (Sharma et al., 2013). The general procedure of producing artificial seeds includes an initial step of producing embryos via tissue culture and allowing them to mature to the torpedo stage. Embryos are then separated and placed into drops of sodium alginate to encapsulate them and the transferred into a solution of calcium chloride for the alginate to

solidify into a capsule, with the embryo/s inside (Patel et al., 2000; Saiprasad, 2001; Kanchanapoom & Promsorn, 2012). Plant growth regulators, nutrients, pesticides, antibiotics and fungicides can be incorporated as a part of the alginate capsule (Liu et al., 1992; Standardi & Piccioni, 1998; Gangopadhyay et al., 2011). Artificial seeds are storable for long periods (Ara et al., 2000), which allows for mass production of elite plant varieties since they are vegetatively produced (Sharma et al., 2013), and also eliminates the last stage of micropropagation – i.e. acclimatization, which can be unsuccessful. Successful use of artificial seeds of vanilla species was achieved with 80% germination success after storage in distilled water for a period of 10 months indicating viability of synthetic seeds (Divakaran et al., 2006).

1.4.2 *In vitro* slow growth

As a means of conserving species (especially those which cannot be stored as true seeds), *in vitro* slow growth cultures can be used (Fay, 1994). This is a method of tissue banking which allows for medium term storage by restricting morphogenic growth and development of clonal material by modifying environmental conditions and/or the culture medium (Withers, 1991; Sarkar & Naik, 1998; Engelmann, 2011).

The most widely used techniques for *in vitro* slow growth of explants is the alteration of incubation conditions, namely the temperature and light intensity. The optimum incubation temperature for cold tolerant plants can range from 0-10°C. Iriondo & Perez (1996) showed that shoots of *Centarium rugalii* could be stored for up to three years at a temperature of 5°C, with a survival rate of 90%. On the other hand, tropical plants can tolerate temperatures ranging between 15-20°C (Withers, 1991; Rao, 2004; Engelmann, 2011), and cultures of *Spilanthes acmella* have been maintained for eight months at 15±2°C with a viability of 52.6% (Joshi & Jadhav, 2014). However, it is strongly recommended to compliment the reduction in temperature with decreased the light intensity (Sarkar & Naik, 1998; Rao, 2004).

Delaying intervals for subculturing is another method of minimizing the development of clonal material as nutrient availability is limited during the lengthened culture intervals (Bertrand-Desbrunais et al., 1992). Divakaran et al., (2006) explored the potential of maintaining cultures of coffee and vanilla for a year without subculturing. However, Sarkar & Naik (1998) concluded that in order to achieve storage of micro-plants for up to 30 months without subculturing, the use of mannitol and manipulating the photoperiod are recommended.

Alteration of the basal medium content, which includes reduced concentrations of nutrients in the culture medium or using lower dosages of sucrose, decreases the rate of development. Reduction of sucrose content is mostly used in combination with other factors to effectively conserve germplasm under slow growth conditions (Watt et al., 2000).

The application of osmotic stress by the use of osmoregulatory compounds such as mannitol and sorbitol to induce slow growth in plant cultures has been widely investigated. Mannitol has been reported to increase survival rates during storage (Sarkar & Naik, 1998; Negash et al., 2001). Successful *in vitro* conservation of vanilla species was achieved by the use of mannitol enrichment in MS medium and cultures were maintained for up to seven years without reduction of vigour once the plants were subcultured (Divakaran et al., 2006). Furthermore, Joshi & Jadhav (2014) indicated that high concentrations of mannitol (4%) and sorbitol (15%) are effective growth inhibitors of explants for storage up to seven months and eight months, respectively. Additionally, growth retardants or inhibitors such as abscisic acid (ABA) have successfully been used to reduce development of germplasm *in vitro*. In the preservation of *Eucalyptus grandis* shoots, Watt et al., (2000) achieved successful storage for up to 10 months.

1.4.3 Cryopreservation

This is a novel technique applied to the ex-situ conservation of plant germplasm - the storage of living biological tissue at a temperature at extremely low temperatures, typically in liquid nitrogen at -196°C (Mycock et al., 1995; Engelmann, 2011). At this temperature, all metabolic and biochemical processes are arrested without alteration of the germplasm genetic makeup (Engelmann, 2004), and the material can therefore be stored theoretically indefinitely (Benelli et al., 2013). Cryopreservation is currently considered to be the only feasible means of long-term storage for species which produce recalcitrant seeds or which reproduce primarily vegetatively (Ajayi et al., 2006; Berjak et al., 2011; Kioko et al., 2006).

Cryostorage of explants which are hydrated and metabolically active puts plant cells at risk of desiccation and freezing injury (Wesley-Smith et al., 1992; 2004). Owing to the intricacies of propagatory units used for germplasm conservation, protocols designed for specific explants or genotypes have no guarantee to succeed on other explants or genotypes (Reed, 2001), hence different techniques have to be developed and optimised. Several cryopreservation approaches

for conservation of plant apices, such as encapsulation, dehydration and vitrification, have been explored (reviewed by González-Benito et al., 2004). However, vitrification has proven to be a more suitable means for cryopreservation of shoot tips (Matsumoto et al., 2001; Sharaf et al., 2011). Furthermore, meristematic tissue, generally considered to be pathogen-free can be excised and preserved in liquid nitrogen for future usage (Wang et al., 2009). In the recovery medium, Engelmann et al., (2004) highlighted that the use of gibberellic acid (GA3) at a rate of 100mgL^{-1} increases the rates of survival of previously stored immature embryos. Furthermore, Tarre et al., (2007) indicated that cryopreservation of *Encholirium* and *Dyckia* seeds improved their germinability. Table 1 illustrates the number of different plant tissues that can be used and post-freezing survival rates achieved for selected species. Seed from endangered *Leucadendron elimense* subsp. *Elimense* which does not propagate vegetatively and other Proteaceae species of the Cape Floristic Region can benefit greatly from cryopreservation and cryostorage to ensure future conservation.

Table 1.1 Tabulation of different explants used in cryobanking for plant germplasm

Germplasm	Cryopreservation Protocol	Success Rates
Zygotic axes	Flash drying with glycerol cryoprotectant of the zygotic embryos of <i>Amaryllis belladonna</i> .	70% success (Sershen, 2012)
	Flash drying naked zygotic embryos over silica gel of <i>Ekebergia capensis</i> for 20mins	80% survival was attained (Perán et al., 2006)
Shoot tips	Dehydration with PVS2 for 20 minutes; followed by plunging into LN.	Regrowth was attained within twenty one days with an average shoot formation of 89.8% (Matsumoto et al., 2001).
	Preculture of shoot tips of <i>Trichilia emetica</i> in 0.3 M sucrose + 0.5 M glycerol; followed by cooling 1 °C min ⁻¹ in PVS2	Successful recovery post cryostorage was reflected from the 71% shooting sprouts (Varghese et al., 2009).
Somatic embryos	Pregrowth of somatic embryos of citrus in 0.75M sugar, air dried for desiccation, followed by rapid freezing.	A survival rate of 86% was reached (González-Arno et al., 2003).
Pollen	Cooling rate of 200°C/minute followed by shifting the vials to -170 to -180°C.	The fluorochromatic reaction test indicates retention of viability (Chaudhury et al., 2010).

1.5 Re-introduction of species

An objective of multiplying wild species which are endangered or declining must be to re-introduce them back into the wild in order to facilitate population viability and growth. Successful re-introduction includes key steps such as extensively studying the species biology, taking into account the phenology (including flowering and fruiting) and long term monitoring after planting in the wild (Godefroid et al., 2011).

Additionally, an analysis of conditions that led to the risk of extinction in the first place has to be conducted. In this way, the reintroduced plants can be secure as the threat can be minimised or eliminated. To optimise the success of reintroduction of species in fragmented and degraded landscapes, Questad et al., (2014) classified high suitability areas for planting to be topographically protected from prevailing winds. For re-introduction, it is generally recommended to germinate the seeds in order to increase the heterozygosity of the population (Holmes et al., 2005; Godefroid et al., 2011). It is this variation in the genetic makeup of the species which increases the survival rates of species in the ecosystem (Holmes et al., 2005; Montalvo et al., 1997).

The success of the reintroduction of species should be determined by the survival percentages of plants in the wild, their flowering, fruiting and seed bearing capacities (Godefroid & Vanderborght, 2011). Also, the ability of the reintroduced species to augment and minimise the ecological degradation is crucial to assess. The success of saving almost extinct *Erica verticillata* through successful propagation and re-introduction into the wild is just one example of applying advanced biotechnology in conservation of species of the Cape Floristic Region (Maschinski and Duquesnel, 2007).

1.6 Conclusion and future prospects

This review has highlighted the different aspects of plant biotechnology suitable for the production and conservation of plant biodiversity within the Cape Floristic Region of southern Africa. It is anticipated that if these techniques could be used to manage populations of ecologically and economically important species the survival of threatened species will be enhanced and ecological and anthropogenic pressures bearing on them will be released. The

efficient production of high-value species, like medicinal plants, floricultural plants, and otherwise ecologically important species through micro-propagation can help to sustain livelihoods and maintain economic growth.

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CHAPTER TWO

PROBLEM STATEMENT, HYPOTHESIS AND OBJECTIVES

2.1 Research Problem

According to Kaviani (2011), advances in biotechnology provide some critical tools for the improved production, conservation and management of species. Furthermore, there is an increase in studies indicating how these biotechnological developments provide several possibilities for the production and storage of species such as *Gladiolus*, *Freesia*, *Strelitzia* and *Protea* (Reinten & Coetzee, 2002; Fennell et al., 2004). However, despite this progress, the optimum protocols for biotechnological processes such as *in vitro* propagation for many taxa of conservation concern in the CFR, such as *Disa*, *Leucadendron* and *Mimetes* remain unknown. This lack of knowledge is exacerbated by the fact that the relevant biotechnological processes are usually species specific.

2.2 Research Questions

Based on the above, the research was guided by the following primary research question: Under what parameters would the *in vitro* conservation of selected *Disa*, *Eulophia*, *Leucadendron* and *Mimetes* species be optimum?

Sub-questions:

- How do varied concentrations of banana extract incorporated into the media affect the *in vitro* development and regeneration capacity of selected *Disa* and *Eulophia* species?
- What are the levels of plant growth regulators that will ensure the optimum development and regeneration of *Disa* and *Eulophia* species in the micropropagation processes?
- How does aqueous smoke affect the germination percentages of *Leucadendron* and *Mimetes* seeds?
- How does the cryopreservation cooling rates affect the germination percentages and survival of *Leucadendron* and *Mimetes* seeds?

2.3 Research Aim and Objectives

The primary aim of this study was to determine the optimum conditions under which Orchidaceae and Proteaceae can be germinated, stored and regenerated using selected biotechnological processes.

Sub-objectives: To achieve the primary research aim, the study also seeks to achieve the following;

- i. To establish how the concentration of banana extract and peptone affect growth and regeneration of *Disa* and *Eulophia* species;
- ii. To establish how variation of parameters (smoke, temperature, nutrients) affect the germination of *Leucadendron* and *Mimetes* species.
- iii. To determine the influence of mannitol concentration and storage time on the in vitro slow growth of *Disa* and *Eulophia*;
- iv. To determine the cooling rates which are ideal for the preservation of *Leucadendron* and *Mimetes* seeds;
- v. To regenerate preserved shoots and protocorms of *Disa* and *Eulophia* species;
- vi. To regenerate preserved seeds using synthetic medium to compare the germination of preserved to non-preserved seeds when such media is used.

2.4 Significance of the study

This study complements existing studies on the use of biotechnological tools in the production, storage and regeneration of *Disa*, *Eulophia*, *Leucadendron* and *Mimetes* species. From a practical perspective, the study can be used as a guideline during micropropagation activities to approximate the conditions for germinating, preserving and re-germinating preserved orchidaceae seeds. By establishing optimum conditions for achieving maximum germination, preservation and regeneration of seeds, the study has practical and theoretical significance. Also, the study adds on to the scholarly body of knowledge by confirming and criticising prior results in other studies, providing an extension of explanations on critical areas and providing new perspectives especially on the *Disa*, *Eulophia*, *Leucadendron* and *Mimetes* species preservation methods. It is hoped that this study can also contribute to the on-going discussions in the field.

2.5 Delineation

This study only focused on a selected number of endangered Proteaceae species and *Disa* and *Eulophia*. *Phalaenopsis* hybrids were selected as a trial for orchid species from the Cape Floral Region due to the scarcity and unavailability of material. The use of plant growth regulators on *in-vitro* regeneration of *Phalaenopsis* hybrids was limited to predetermined concentrations of thidiazuron & 6-benzylaminopurine supplemented with α -naphthalene acetic acid, from previous literature to confirm or challenge the results.

2.6 References

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CHAPTER 3

MICROPROPAGATION OF POTENTIAL ORCHID SPECIES: PHALAENOPSIS AS A TRIAL SPECIES.

3.1 Introduction

Floriculture has taken a toll on ornamental indigenous plant species, including Orchidaceae in Southern Africa. The most traded species in South Africa floriculture include the *Disa*, *Eulophia* etc. (Thompson et al., 2007). Orchids tend to have low and irregular fruit set, often resulting from pollinator limitation, which has frequently been recorded to cause poor reproductive success (John van Wyhe, 2002; Johnson & Hobbhahn, 2010) and the disruption of plant–pollinator interactions has been identified as a cause of decline in some species (Chugh et al., 2009). Furthermore, orchid seeds are also minute, have limited nutrient reserves and the seedlings often require a fungal symbiont for successful establishment. When the seeds are shed, in most instances, the embryos are morphologically immature and therefore impose morphophysiological dormancy on the orchid seeds (Thompson et al., 2007). Also, because of their small size, many seeds could be dispersed to sites unsuitable for germination or destroyed before introduction *in situ* (Rasmussen, 1995). The orchids are therefore extremely susceptible to extinction should conditions not be conducive for germination.

In the case of terrestrial orchids, the numerous small seeds are in most cases structurally adapted for wind dispersal, yet little is known about their fate after dispersal. Some studies of seed viability *in situ* indicate survival for up to two years in temperate orchid specie (Thompson et al., 2007). A major consideration when planning measures for the conservation of endangered plant species which are susceptibility to rapid decline is effective management of viable populations is required to minimize potential extinction. One of the large orchid genus that occur in southern Africa is the *Disa* (Bytebier et al., 2007), which includes up to 170 species (World Checklist of Monocots, 2004). This genus is more predominant in the Cape Floristic Region and has up to 78 species endemic to this region (Goldblatt & Manning, 2002), where it is under threat of extinction, and is currently legally protected. The traded species include the *Disa*, *Eulophia* etc. (Thompson et al., 2007; Reinten et al., 2011). More pressure is imposed on the *Disa* germplasm from its endangered state and harvesting for ornamental usage. Thus

efforts to curb extinction of the germplasm and retain economic benefits of ornamental geophytes, *ex situ* conservation are required.

Phalaenopsis species are epiphytes that belong to the Orchidaceae family, subfamily Epidendroideae of Vandeeae (Dressler, 1993). Members of this genus have a short stem, with fleshy and leathery leaves, typically with three to six leaves per plant (Batchelor, 1982). Their natural habitat has been identified in tropical regions of India, south eastern Asia and the Philippines (Sweet, 1980). This study initially assessed the potential *in vitro* germination conditions for the recruitment of mature *Disa oreophilla*, *D. scullyii*, *D. fragrans*, *D. uniflora* and *Eulophia coddii* seeds. A study by Thompson et al., (2001) have demonstrated that on average seed germination success of *Disa* seeds is low and that subsequent growth is thought to be highly specific in some species. However, due to limited plant material of these species, the study focused on using exotic *Phalaenopsis* hybrids; worldwide trading orchids, to act as a suggestion of trials to undertake on indigenous Orchidaceae in Southern Africa.

Under conventional propagation methods, *Phalaenopsis* yields are poor and it has proven to be slow and difficult to propagate (Ishii et al., 1998; Košir et al., 2004). Seed germination is the most predominant method of propagation used for rapid multiplication of *Phalaenopsis*. It has however been proven that germination is difficult and yields lower quantities due to necrosis. The difficulty in germination and high necrosis susceptibility influenced the choice of Orchids (*Phalaenopsis*) as a trial study that could be used for endangered *Disa* cultivation.

Previous studies have suggested the use of complex organic additives to the germination and growth media as they offer a modulated approach to propagation as response impeccable and economic as compared with the use of artificial plant growth hormones. For industrial production, the use of peptone, banana extract and coconut water, to mention a few, have been suggested as essential tissue culture components of high yields of various germplasm. It is thus empirical to use other methods *in vitro* to enhance the quantities and qualities of the species.

3.2 Materials and methods

Micropropagation of *Phalaenopsis* hybrids

3.2.1 Genotypes: five *Phalaenopsis* hybrids

In vitro seed germination of *Phalaenopsis aphrodite* and four hand-pollinated *Phalaenopsis* hybrids i.e. *P. Psychosis Pink* X *P. No. 1*; *P. Large white* X *P. Large pink*; *P. No. 1* X *P. Large pink* and *P. Mini pink* X *P. Brighton belle*, was carried out. Seeds were cultured on ½ MS medium which contained 1mgL⁻¹ 6-Benzylaminopurine, 20 gL⁻¹ sucrose and 7 gL⁻¹ Agar bacteriological (Biolab) at a pH of 5.8. These were germinated in the dark for eight weeks and transferred to light until they reached the protocorm stage. The protocorms and shoots of *Phalaenopsis* hybrids were used in the procedures for *in vitro* multiplication to determine the optimum concentration of banana extract on development of the hybrids.

3.2.2 Screening multiplication media

The protocorms were then cultured on ½ MS medium supplemented with 1.0 mgL⁻¹BAP, 1.0 mgL⁻¹ TDZ or 10-30 gL⁻¹ banana extracts either with or without 1.0 gL⁻¹ peptone. Each treatment consisted of thirty replicates. Cultures were then incubated at 25±2 °C for a 16 hour photoperiod of 25 µmolm⁻²s⁻¹.

3.2.3 *In vitro* rooting and elongation

The root development and elongation medium comprised of ½ MS salts supplemented with banana extract and indole butyric acid (IBA). IBA, which served as a root inducer, was assessed at two concentrations: 0.1 and 0.2 mgL⁻¹.

3.2.4 Slow-growth storage

3.2.4.1 Osmotic treatment

In vitro storage was investigated for the *Phalaenopsis* hybrids to optimise conservation procedures using mannitol to regulate the quantity of plant production. Protocorms or shoots (with two or three leaves) were obtained from 10-12 week old axenic protocorm-like bodies were used for slow-growth storage (Figure 3.1). They were cultured on ½ MS medium different concentrations of mannitol to achieve different osmotic conditions (Table 3.1). The culture tubes were sealed with parafilm and incubated for a period of up to 12 months under 16 hour photoperiod with a light intensity of 125 µmolm⁻²s⁻¹ at 24±2 °C. Cultures were observed for *P. aphrodite*, *P. No. 1* X *P. Large pink*, *P. Mini Pink* X *P. Brighton Belle*, *P. Psychosis Pink* X *P. No. 1* and *P. Large white* X *P. Large pink* during storage and cultured on the shoot multiplication medium at the end of the storage period.

Table 3.1: Slow-growth media composition

Species/ Variety	Conservation treatments: MS media with	
	Sucrose (gL ⁻¹)	Mannitol (gL ⁻¹)
<i>P. aphrodite</i>	0	0; 10; 20; 30
<i>P. No. 1</i> X <i>P. Large pink</i>	0	0; 10; 20; 30
<i>P. Mini Pink</i> X <i>P. Brighton Belle</i>	0	0; 20; 30
<i>P. Psychosis Pink</i> X <i>P. No. 1</i>	0	10; 20; 30
<i>P. Large white</i> X <i>P. Large pink</i>	0	10; 20



Figure 3.1: Regenerated shoots of *Phalaenopsis* hybrids in the *in vitro* storage media.

3.2.5 Recovery/Regrowth and establishment of plantlets

The regeneration capacity of protocorms and plantlet cultures were assessed on a basic multiplication medium incubated at 25 ± 2 °C. The number of new buds and shoots induced on the multiplication medium were observed thirty days after culture.

Environmental conditions for *ex vitro* growth differ substantially from *in vitro* conditions; hence acclimatisation is a crucial stage of tissue cultured plants (Osório et al., 2012). For acclimatisation, 30 plantlets with at least two leaves and three roots from each treatment were acclimatised under greenhouse conditions in coco peat, Styrofoam and silica (6:2:1) in seedling trays (Figure 3.2). The roots of hybrids cultured on various MS media were thoroughly washed with distilled water to remove any remnants of agar. Plants were then maintained in humidity chambers for 14 days, with the chambers being gradually opened over this period. Thereafter, the plants were grown for 50 more days in the greenhouse.



Figure 3.2: Plastic domes used for acclimatising *in vitro* plantlets

3.2.6 Data collection

Data collection was taken at the end of predetermined storage duration. The percentage microplant survival, microplant growth rate, regeneration and quality were determined by counting the number of leaves, roots, and the microplant condition determined on a visual scale of leaf color. A scale of 1 to 5 was used to rate the color of the leaves; 5 being the green leaves, with 1 being used for severe chlorotic leaves. The root length was also as a measure to determine the growth and development of roots. The regeneration ability was determined by counting the number of plantlets and ability to produce protocorms.

3.2.7 Statistical analysis

For data analysis, statistica software 8 was used, and ANOVA test was used for parametric variables. The differences in mean values were compared by Turkey's test ($P \leq 0.05$). Significance was set at ($P \leq 0.05$).

3.3 Results and discussions

3.3.1 *In vitro* growth of *Phalaenopsis* hybrids

Seed derived protocorms were initiated on $\frac{1}{2}$ MS media supplemented with peptone, BAP and banana extract to examine and quantify the development of *Phalaenopsis* hybrids. Multiple shoots were formed at the basal tissue of the protocorms. The average number of leaves in most cases was not greatly influenced by the addition of supplements.

3.3.1.1 Influence of the Banana extract (BE)

Of particular interest in this study was the influence of the banana extract on the growth and regeneration of the selected *Phalaenopsis* hybrid protocorms. The stimulatory effect of banana extract on *in vitro* cultures is well established for shoot development, regenerative capacity and rooting. The highest mean number of leaves was obtained in the 20 gL^{-1} BE for *P. Mini pink* X *P. Brighton Belle* (Table 3.3) and *P. psychosis* Pink X *P. No. 1* (Table 3.6). Furthermore, supplementing $\frac{1}{2}$ MS media with 30 gL^{-1} banana extract yielded the longest leaves and the highest leaf span observed for *P. aphrodite* (Table 3.7), *P. Psychosis* Pink X *P. No. 1* (Table 3.6) and *P. No1* X *P. Large pink* (Table 3.8). It could be hypothesized that BE is rich in

compounds such as cytokinins, which may induce cell development and proliferation of shoots. Studies by van Staden & Stewart (1975) confirmed that banana fruits contain endogenous zeatin, zeatin riboside and 2Ip, which display cytokinin characteristics and are well known for their improvement in shoot development and multiplication (Werner et al., 2001). Concentrations exceeding 20 gL⁻¹ however, imposed an inhibitory influence on leaf elongation (Table 3.6 and Table 3.7). Culture on medium containing a high concentration (≤ 60 gL⁻¹) of banana extract resulted in reduced overall growth of *P. No1 X P. Large pink* (Table 3.8). Kaur & Bhutani (2012) indicated that at high concentrations banana extract was lethal to cultures of *Cymbidium pendulum* as seen in the current study with *P. No1 X P. Large pink*.

i. Regeneration

In an attempt to determine the influence banana extract has on the regeneration capacity of selected Phalaenopsis hybrids, the culture medium supplemented with 10; 20; 30gL⁻¹ banana extract were used. Dominguez-Puigjaner et al., (1992) observed an increase in polypeptides as the banana pulp ripens, leading to the accumulation of proteins. The banana extract is derived from this rich protein feed. Additionally, Vyas et al., (2009) observed the stimulatory ability of banana in the regeneration ability of cultures. All the hybrids exhibited regeneration ability in the banana extract treated experiments. Of the positively responding hybrids, *P. Psychosis Pink X P. No. 1* attained the highest regeneration. Banana extract at lower concentrations (≥ 20 gL⁻¹) was effective in regeneration rate of microplants of the hybrids at 25 \pm 2 °C. In comparison, banana extract was effective even at low concentration (10 gL⁻¹) in improving regeneration especially when supplementing the medium with 1 gL⁻¹ peptone. Culture media supplemented with 30gL⁻¹, yielded the least microshoots and protocorms. Regeneration for *P. No1 X P. Large Pink* yielded the most plantlets in the control ($\frac{1}{2}$ MS supplemented with 1mgL⁻¹ BAP + 0.1mgL⁻¹ NAA). It was also observed that the 1mgL⁻¹ BAP + 0.1mgL⁻¹ NAA enriched medium produced more secondary protocorms instead of further development into shoot. Further to this, our study shows that when using microshoots are less responsive are less regenerative compared to using protocorms.

ii. Rooting

The addition of banana extract to the culture medium increased the root development of the Phalaenopsis hybrids in this study. At a concentration of 10gL⁻¹ banana extract, root induction

was highest for *P. Mini pink* X *P. Brighton Belle* and *P. Psychosis Pink* X *P. No. 1* as demonstrated in Table 3.3 and Table 3.6 respectively. However, root development and elongation was more pronounced in the ½ MS media supplemented with 30gL⁻¹ banana extract for *P. No1* X *P. Large pink* (Table 3.8), *P. Psychosis Pink* X *P. No. 1* (Table 3.7) and *P. aphrodite* (Table 3.6). Lower concentrations in the study yielded shorter and rather fewer number of roots. Khalifah (1966b) observed that there was a high concentration of Indolyl-3-acetic acid in the banana pulp, when the sample was analysed using chromatography. In a separate study, Khalifah (1966a) further elaborated on content analysis of banana fruits and concluded that gibberilic acid; G4 and G7 were also present in the crude extracts. Deducing that the presence of this compound can influence root multiplication. Similar results were obtained by Chen et al., (2011). Auxins play a role in cell elongation and proliferation, shoot development (George et al., 2008a) and stimulate the formation of root primordia. Hence, the positive response of the hybrids to improved root systems when cultured in banana enriched media.

3.3.1.2 Peptone

Peptone had the highest mean number of leaves (4.1), with the least mean length (7.5 mm) and the mean leave span (17.5 mm) for *P. aphrodite* (Table 3.6). The results of this study also indicated that peptone significantly ($P \leq 0.001$) increased the mean number of leaves and the leave length and of the *P. Mini pink* X *P. Brighton Belle* (Table 3.3). Peptone without BE however, yielded the shortest span (18.3 mm). Furthermore, the culture media supplemented with 10 gL⁻¹ BE indicated the highest yield in stem diameter (3.2 mm), which was statistically similar to that of microshoot stems cultured in ½ MS supplemented with peptone only (Table 3.3). Supplementation with 1 gL⁻¹ peptone, indicated reduced mean leaf length (14.1 mm), mean leaf span (29.2 mm) and a mean stem diameter of 3.7 mm for *P. Large white* X *P. Large pink* microplantlets (Table 3.5).

According to Kraiser et al., (2011) peptone is a compound comprising of organic nitrogen (14 % w/w) and other amino nitrogen (2.6 %w/w). Organic nitrogen and amino nitrogen have been documented to improve the root development (Kraiser et al., 2011; Schwambach et al., 2015). This study revealed that supplying peptone to the medium stimulated the development of leaves, the formation of protocorms and enhanced shoot induction in *Phalaenopsis* hybrids. Additionally; several studies ascribe a stimulatory role of peptone in diverse developmental processes during plant growth.

The combination of banana extract and 1 gL^{-1} peptone, further positively influenced protocorm regeneration and shoot development. Thus it was concluded that the addition of peptone to the culture medium further promoted shoot elongation at the expense of bud development. Kraiser et al., (2011) highlighted that auxin which is found in the BE; improves the modulation of roots, resulting in an increased nitrogen absorption from the peptone.

Table 3.2: Effect of banana extract in combination with IBA on the induction and microplant growth of *P. Mini pink X P. Brighton Belle* cultures at 25±2 °C.

Treatment	mean no. of Leaves (SE)	mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
10 gL ⁻¹ BE	3.6±0.3ab**	3.4±0.3b***	11.7±0.6a***	0.4±0.1ab***	20.7±2.0a***	26.3±2.0a**	3.2±0.2a
10 gL ⁻¹ BE +0.1 mgL ⁻¹ IBA	4.3±0.3a**	3.4±0.3b***	8.8±0.3b***	0.2±0.01b***	14.7±1.1b***	19.7±0.7b**	3.2±0.2a
10 gL ⁻¹ BE +0.2 mgL ⁻¹ IBA	3.2±0.2b**	5.1±0.4a***	8.4±0.5b***	0.6±0.1a***	26.3±2.2a***	20.3±1.7b**	3.3±0.1a

Values presented are means ± SE, n = 30. **, *** = significant at P≤0.01, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.3: Effect of banana extract in combination with peptone on the induction and microplant growth of *P. Mini pink X P. Brighton Belle* cultures at 25±2°C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
1 gL ⁻¹ peptone	3.7±0.3a***	4.1±0.5b***	7.9±0.5c***	0.4±0.1b***	16.3±1.7b***	18.3±1.8b***	2.7±0.4a***
10 gL ⁻¹ BE + 1 gL ⁻¹ peptone	2.1±0.1b***	9.0±0.5a***	24.3±1.1a***	1.5±0.1a***	35.1±3.0a***	45.1±2.2a***	3.2±0.2a***
20 gL ⁻¹ BE + 1 gL ⁻¹ peptone	3.9±0.4a***	5.6±0.7a***	19.1±1.7b***	1.8±0.2a***	43.6±3.3a***	43.1±3.7a***	2.0±1.2b***

Values presented are means ± SE, n = 30. *** = significant at P≤0.001, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.4: Effect of banana extract in combination with peptone on the induction and microplant growth of *P. aphrodite* cultures at 25±2°C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
30 gL ⁻¹ BE+1 gL ⁻¹ Peptone	2.9±0.2b***	6.6±0.4a	17.5±1.1a***	1.3±0.2a***	34.0±3.0a***	36.4±2.6a***	2.1±0.1a
30 gL ⁻¹ BE	1.9±0.1c***	5.6±0.4a	14.1±0.9b***	0.6±0.1b***	22.9±1.7b***	26.2±1.7b***	1.8±0.1a
1 gL ⁻¹ peptone	4.1±0.2a***	5.5±0.2a	7.5±0.2c***	0.4±0.04b***	10.8±0.7c***	17.5±0.7c***	2.3±0.2a

Values presented are means ± SE, n = 30. *** = significant at P≤0.001, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.5: Effect of banana extract in combination with peptone on the induction and microplant growth of *P. Large white* X *P. Large pink* cultures at 25±2 °C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
30 gL ⁻¹ BE	3.1±0.2a	4.6±0.3a	19.8±1.1a***	1.3±0.2a***	29.9±2.0a***	46.1±3.1a***	4.0±0.2ab*
30 gL ⁻¹ BE+ 1 gL ⁻¹ peptone	2.7±0.2a	4.4±0.3a	14.1±1.0b***	0.6±0.1b***	20.9±1.2b***	29.2±2.1b***	3.7±0.2b*
1 gL ⁻¹ peptone	2.8±0.1a	4.7±0.2a	22.3±1.5a***	1.6±0.2a***	34.8±2.3a***	46.5±2.4a***	4.5±0.2a*

Values presented are means ± SE, n = 30. *, *** = significant at P≤0.05, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.6: Effect of banana extract in combination with peptone on the induction and microplant growth of *P. Psychosis Pink* X *P. No. 1* cultures at 25±2 °C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
10 gL ⁻¹ BE+ 1 gL ⁻¹ peptone	2.1±0.2a	6.7±0.4a	13.5±1.2ab***	1.5±0.1a*	26.2±1.5b***	25.2±2.3b***	2.6±0.2a*
20 gL ⁻¹ BE+ 1 gL ⁻¹ peptone	2.6±0.3a	5.7±0.6a	10.6±0.8b***	1.0±0.1a*	23.4±1.8b***	20.3±2.0b***	1.8±0.1b*
30 gL ⁻¹ BE+ 1 gL ⁻¹ peptone	2.0±0.1a	6.5±0.4a	16.1±1.1a***	1.4±0.2a*	32.7±1.9a***	32.5±2.5a***	2.0±0.1b*

Values presented are means ± SE, n = 30. *, *** = significant at P≤0.05, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.7: Effect of banana extract in combination with peptone on the induction and microplant growth of *P. aphrodite* cultures at 25±2 °C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
10 gL ⁻¹ BE+ 1 gL ⁻¹ peptone	2.9±0.2a	5.7±0.5a	12.9±1.2b***	1.2±0.2a	25.9±1.5b*	30.0±2.8ab***	3.1±0.3a***
20 gL ⁻¹ BE+ 1 gL ⁻¹ peptone	2.5±0.2a	6.7±0.6a	11.6±0.8b***	0.7±0.1a	23.9±1.4b*	22.2±1.4b***	1.6±0.1b***
30 gL ⁻¹ BE+ 1 gL ⁻¹ peptone	2.9±0.2a	6.6±0.4a	17.5±1.1a***	1.3±0.2a	34.0±3.0a*	36.4±2.6a***	2.1±0.1b***

Values presented are means ± SE, n = 30. *, *** = significant at P≤0.05, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.8: Effect of banana extract in combination with peptone on the induction and microplant growth of *P.No1 X P. Large White* cultures at 25±2 °C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
10 gL ⁻¹ BE	1.8±0.1b***	6.0a±0.3a***	17.3±1.0a***	0.6±0.1b***	19.0±1.1b***	29.2±2.1a***	2.0±2.1b**
30 gL ⁻¹ BE	2.6±0.1a***	6.6±0.4a***	18.3±1.5a***	1.5±0.2a***	27.3±0.2a***	35.7±2.6a***	3.2±0.2a**
60 gL ⁻¹ BE	1.8±0.1b***	3.0±0.2b***	11.5±0.9b***	0.4±0.1b***	16.1±1.0b***	20.8±2.1b***	2.8±0.3ab**

Values presented are means ± SE, n = 30. **, *** = significant at P≤0.01, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

3.3.1.3 Thidiazuron supplementation

The predetermined plant regulator concentrations studied on other Orchidaceae confirm that the hybrids generated are able to reproduce and develop into plantlets. Mok et al., (1982) and Visser et al., (1992) reported on the presence of cytokinin activity in TDZ supplemented cultures. Since then, further studies have indicated varying responses from plant tissue material to thidiazuron as it displays a range of responses normally seen from exogenous application of both auxin and cytokinin (Murthy et al., 1998; Guo et al., 2011). The synergistic approach from this compound thus supersedes other auxin and cytokinin plant regulators when used individually, hence increased production. In this study, $\frac{1}{2}$ MS media supplemented with 1 mgL^{-1} TDZ was successfully used to induce protocorm formation from shoots of *Phalaenopsis* hybrids.

In the $\frac{1}{2}$ MS media supplemented with 2 mgL^{-1} BAP and 1 gL^{-1} peptone, there was a high significance in the number of leaves produced (Table 3.10). Shoots of *P. aphrodite* induced in 1 mgL^{-1} TDZ produced leaf lengths and leaf widths similar to those grown on 1 gL^{-1} BAP medium supplemented with 1 mgL^{-1} peptone (Table 3.10).

3.3.1.4 Acclimatization to environmental conditions

After six months of acclimatization, the percentage survival of plantlets in greenhouse conditions was higher for plantlets which had been cultured on banana extract regardless of the *in vitro* conditions used on the hybrids. This increase could be a result of numerous and elongated roots that were developed *in vitro* creating a more efficient rooting system. Also, the overall size of the plantlet had an influence on the survival of microplant establishment *ex vitro*. The plantlets from the BAP alone had the lowest acclimatisation rates when directly planted, without induction of the rooting stage.

Table 3.9: Comparison between TDZ and BA in combination with peptone on the induction and microplant growth of *P. Mini pink X P. Brighton Belle* cultures at 25±2 °C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
1 mgL ⁻¹ TDZ	4.0±0.3a	2.1±0.1b***	11.6±0.7b***	0.4±0.04b***	15.6±1.2b***	26.5±1.2b***	4.1±0.3a***
1.0mgL ⁻¹ BAP +1 gL ⁻¹ PEP	4.1±0.3a	6.9±0.6a***	19.7±1.4a***	1.7±0.2a***	24.2±1.5a***	45.5±3.3a***	2.1±0.2b***
1.5 mgL ⁻¹ BAP +1 gL ⁻¹ PEP	3.6±0.3	5.7±0.3a***	17.4±1.7a***	0.8±0.1b***	22.8±1.8a***	37.3±3.1a***	2.9±0.3b***

Values presented are means ± SE, n = 30. *** = significant at P≤0.001, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.10: Comparison between TDZ and BA in combination with peptone on the induction and microplant growth of *P. aphrodite* cultures at 25±2 °C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
0.5mgL ⁻¹ BAP +1gL ⁻¹ Peptone	4.1±0.2a***	4.9±0.3a	7.2±0.6b***	0.5±0.1b***	15.8±1.9c***	17.9±1.3b***	2.7±0.1a***
1mgL ⁻¹ BAP +1gL ⁻¹ Peptone	2.1±0.1c***	5.6±0.3a	15.6±0.7a***	1.0±0.1a***	29.7±2.1a***	23.7±1.8a***	1.8±0.1bc***
2mgL ⁻¹ BAP +1gL ⁻¹ Peptone	3.3±0.2b***	5.4±0.4a	8.7±0.6b***	0.5±0.1b***	22.1±2.2bc***	18.1±1.0b***	2.2±0.1ab***
1mgL ⁻¹ TDZ	2.0±0.1c***	4.4±0.3a	13.5±0.6a***	0.4±0.04b***	27.1±1.6ab***	27.1±1.5a***	1.5±0.1c***

Values presented are means ± SE, n = 30. *** = significant at P≤0.001, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.11: Comparison between TDZ and BA in combination with NAA on the induction and microplant growth of *P.No1X* *P. Large Pink* cultures at 25±2 °C.

Treatment	Mean no. of Leaves (SE)	Mean no. of roots (SE)	Mean leaf length (SE)	Mean wet weight (SE)	Mean root length (SE)	Mean leaf span (SE)	Mean stem radius (SE)
1 mgL ⁻¹ TDZ	4.4±0.4a***	4.5±0.5b**	10.9±0.5b***	0.8±0.1a	24.5±2.0a	24.5±1.4a	4.0±0.2a***
1 mgL ⁻¹ BA+ 0.1 mgL ⁻¹ NAA	2.1±0.1b***	5.9±0.4a**	14.9±1.0a***	0.7±0.1a	25.4±3.2a	27.6±2.5a	2.3±0.1b***

Values presented are means ± SE, n = 30. **, *** = significant at P≤0.01, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

3.3.2 *In vitro* slow growth

Carbon sources regulate cellular growth by affecting water potential of the tissue culture medium. Carbon in the medium determines the water and nutrient absorptive capacity by plant tissues. There are different types of carbon that can be used in tissue culture. For preservation purposes, sugar alcohols are often used. The sugar-alcohols induce osmotic pressure in the culture media; thereby creating stress on the tissue by bringing the cell cycle progression to a halt, yet allowing for recovery once conditions are ideal (Lata et al., 2010; Skiryecz et al., 2011). In this study, osmotic stress was achieved by addition of mannitol (a sugar alcohol) to the conservation media. The results revealed that there was successful conservation of both protocorms and shoots in mannitol enriched media. The results of the study indicated that there is a reduction in growth of various *Phalaenopsis* cultivars when cultures are stored in 20 to 30 gL⁻¹ mannitol. Lower concentrations of mannitol however indicated that there was higher regeneration ability during storage, with more plantlets produced at 10gL⁻¹ than at higher concentrations.

Using the analysis of variance, the suppressed growth of protocorms and plantlets using mannitol and delaying subculturing had a significant ($p \leq 0.05$ to $p \leq 0.001$) impact on all parameters with regards to the effect of mannitol. This suggested that the hybrid interaction with mannitol was not uniform for plant growth over the various concentrations incorporated in the media for all parameters.

3.3.2.1 *The effect of culture time (months) on plantlet yield*

During plant development, it is essential to regulate sugar levels in processes such as seedling development, root and leaf differentiation (Gibson, 2005). For this study the only carbon source was mannitol, which was also intended to be the conserving compound in the preservation media. The study revealed that increasing the mannitol concentration in ½ MS medium without sucrose increased survival of protocorms at 24±1 °C, for up to fourteen months for *P. Mini Pink* X *P. Brighton Belle* (Table 3.12), fifteen months for *P. No. 1* X *P. Large pink* (Table 3.13) without subculturing. Conservation of shoots however yielded low survival rates with the increase in mannitol without sucrose at 24±1 °C. At lower concentrations, 10 gL⁻¹ mannitol was effective in increasing microplant survival at 24±1 °C of the *P. Psychosis Pink* X *P. No. 1*, (100 %) (Table

3.16) of conserved which microplants survived for thirteen months of storage *in vitro*. The results obtained also showed that the survival of *Enset* plantlets was reduced when shoots were stored for longer than 6 months, with fifteen months yielding the least number of surviving conserved shoots (Negash et al., 2001).

Additionally for this study, micro-shoots were able to be stored for up to eleven months on MS medium without any supplements. Shoots of *P. aphrodite* were stored for a period of 54 weeks. The study further compared the storage of shoots of *P. No. 1 X P. Large pink* for (12 and 15 months), *P. aphrodite* (six and twelve months), *P. Psychosis Pink X P. No.1* and *P. Mini pink X Brighton Belle* for (eleven and fourteen months) to determine growth patterns between the duration of storage. Conserving the *P. No. 1 X P. Large pink* (Figure 3.3), *P. aphrodite* (Figure 3.4) and *P. Psychosis Pink X P. No. 1* (Figure 3.5) hybrids for prolonged periods on $\frac{1}{2}$ MS (no sucrose) supplemented with 10 gL^{-1} mannitol at $23 \pm 2 \text{ }^\circ\text{C}$ resulted in an increase in leaf numbers, root length and number of plantlets. However, storage of *P. Mini pink X P. Brighton Belle* in $\frac{1}{2}$ MS (no sucrose) supplemented with 20 gL^{-1} mannitol produced shorter roots (Figure 3.6).

3.3.2.2 The influence of mannitol concentration on the number of leaves

The degree to which stomata open is greatly reduced as osmotic pressure induced by exogenous mannitol application is increased (Seki et al., 2007). It is this effect that limits exchange of carbon dioxide for photosynthesis to take place, hence limit the overall leaf development and growth (Pinheiro & Chaves, 2010). This was depicted in this study indicating that increasing the mannitol gradient in the medium improved the reduction in leaf number. It however became more apparent on 20 and 30 gL^{-1} when compared with lower dosages. Skiryicz et al., (2011) also reports on mannitol as an effective compound in reducing the leave size by up to $\approx 50 \%$. With extended exposure to the mannitol, the leaves tend to be shorter compared to those cultured on mannitol for a shorter period.

During *in vitro* conservation of microshoots of *P. Mini pink X P. Brighton Belle*, the storage media comprising $\frac{1}{2}$ MS (with no sucrose) only or $\frac{1}{2}$ MS (with no sucrose) supplemented with, 20 gL^{-1} and 30 gL^{-1} mannitol, displayed a significant ($p \leq 0.05$) increase in the leaf number (Table 3.12). The most leaves per plantlet occurred on the microshoots cultured in $\frac{1}{2}$ MS supplemented with, 30 gL^{-1} mannitol (2.7) compared with the control (2.6) while 20 gL^{-1} showed

the least number of leaves (2.1). *P. No. 1* X *P. Large pink* hybrid cultured on either ½ MS (with no sucrose) or ½ MS (with no sucrose) supplemented with 10 gL⁻¹, 20 gL⁻¹ and 30 gL⁻¹ mannitol showed a highly significant ($p \leq 0.001$) response between the treatments. Similar to the *P. Mini pink* X *P. Brighton Belle*, 30 gL⁻¹ mannitol had the most leaves (3.5) per microshoot. There was a significant ($P \leq 0.01$) increase in leaf number per micro plantlet of *P. aphrodite* when stored in ½ MS (with no sucrose) or ½ MS (with no sucrose) supplemented with 10 gL⁻¹ (3.4), 20 gL⁻¹ (3.8). Increasing the concentrations to 30 gL⁻¹ mannitol reduced the leaf number (2.9), which was statistically similar to the control. There was a significant difference between the influence of 10 and 20 gL⁻¹ mannitol for *P. Large white* X *P. Large pink* (Table 3.12). ½ MS supplemented with 10gL⁻¹ mannitol and 30 gL⁻¹. *P. Psychosis Pink* X *P. No.1* also displays similar results to that of *P. Large white* X *P. Large pink*; increasing osmotic pressure reduced the number of leaves per plantlets during storage in ½ MS supplemented with mannitol (Table 3.15). Overall, 10 gL⁻¹ yielded most leaves (3.9), 20 gL⁻¹ produced an average of (3.5) leaves and 30 gL⁻¹ had the least number of leaves (2.3).

3.3.2.3 Regeneration of cultivars

Exogenous applied mannitol induced the cultivars to reproduce (Pimsen & Kanchanapoom, 2011). In this study, embryonic induction was more responsive to higher levels of mannitol, with quantitative differences being observed amongst the plants studied relative to the control. Microplant regeneration of *P. No. 1* X *P. Large pink* and *P. Mini pink* X *P. Brighton Belle* plantlets was significantly ($P \leq 0.001$) increased with higher applications of mannitol (0-30 gL⁻¹) in ½ MS at 24±1 °C (Table 3.13, Table 3.12 and Table 3.14). The control and ½ MS supplemented with 30 gL⁻¹ mannitol replicates of *P. aphrodite* plantlets were not significantly different ($p \leq 0.05$), despite that 30 gL⁻¹ showed the highest regeneration during conservation (Table 3.14). Additionally, *P. Psychosis Pink* X *P. No. 1* displayed the least number of plantlets in the ½ MS supplemented with 30 gL⁻¹ mannitol media. Both the 10 and 20 gL⁻¹ yielded the same number of plantlets (0.6), which in this case was the highest for *P. Psychosis Pink* X *P. No. 1* (Table 3.16). The lowest dosage (10 gL⁻¹) of mannitol yielded the least number of plantlets for *P. aphrodite* (0.5) (Table 3.14), *P. Large white* X *P. Large pink* (0.5) (Table 3.15). ½ MS without supplements had no regeneration for *P. No.1* X *P. Large pink* and *P. aphrodite*. However, the *P. Mini pink* X *P. Brighton Belle* had 0.7 mean number of plantlets per microshoot conserved (Table 3.12). This response was highly prompted by elevating osmotic stress in the medium. Also, storage for longer on ½ MS medium supplemented with mannitol yielded more plantlets and protocorms.

Further to this, minute protocorms that didn't germinate were observed. It is depicted from the results that the effect of $\frac{1}{2}$ MS supplemented with mannitol had varying confidence levels with the different levels (10, 20 and 30 gL⁻¹) tested in this experiment. Data in Table 3.13 indicate a significant ($P \leq 0.05$) improvement in protocorm formation when cultures were grown at 24 ± 1 °C in the presence of the osmotic stress modifying agent. The medium supplemented with the highest (30 gL⁻¹) mannitol revealed the lowest microplant regeneration capacity of *P. Mini pink X P. Brighton Belle* (0.2) (Table 3.12), *P. aphrodite* (0.1) (Table 3.14), *P. Large white X P. Large pink* (0.1 for 20 gL⁻¹) (Table 3.15) and *P. Psychosis Pink X P. No. 1* (0.1) (Table 3.16). However, *P. Mini pink X P. Brighton belle*, *P. Large white X P. Large pink* and *P. Psychosis Pink X P. No. 1* displayed no statistical difference compared to the control.

3.3.2.4 Number of roots generated

It has been found that the root lengths are increased by raising the osmolarity of the culture medium by addition of mannitol. A gradual significant increase ($P \leq 0.01$ for *P. Mini pink X P. Brighton Belle* and *P. Psychosis Pink X P. No. 1*) to $P \leq 0.001$ for *P. No. 1 X P. Large pink*, *P. aphrodite* and *P. Large white X P. Large pink*] in the number of microplant roots was observed with increasing the concentration of mannitol at 24 ± 1 °C. The highest number of roots occurred in $\frac{1}{2}$ MS supplemented with 20 gL⁻¹ mannitol on the following hybrids; *P. No. 1 X P. Large pink* (5.0) (Table 3.13), *P. Large white X P. Large pink* (3.8) (Table 3.15), *P. Psychosis Pink X P. No. 1* (5.5) (Table 3.16). Thereafter, the root numbers were reduced with further increase in mannitol concentration to 30 gL⁻¹ and 20 gL⁻¹ mannitol for *P. Psychosis Pink X P. No. 1*; with *P. No. 1 X P. Large pink* hybrids showing no further significant increase or reduction (Table 3.16 and Table 3.13). Contrary to the above mentioned results, *P. Brighton Belle* (Table 3.12) and *P. aphrodite* (Table 3.14) revealed that the number of roots was highest (2.9 and 5.3, respectively) on $\frac{1}{2}$ MS supplemented with 30 gL⁻¹ mannitol. As a response to less water in the media, plants tend to develop long roots in search for water (Pawlicki & Welander, 1995; Glyn Bengough et al., 2011). Furthermore, when cultures were stored for longer, the root count was reduced as compared to when stored for twelve months across the hybrids. Visual observation of the plantlets stored longer, indicated more wilting than when stored for shorter duration.

3.3.2.5 Color of leaves: a measure of the quality of plants

Visual assessment of leaf colour on cultures of *P. Mini pink* X *P. Brighton Belle*; *P. No. 1* X *P. Large pink* and *P. aphrodite* indicate a significant ($P \leq 0.01$ to $P \leq 0.001$) increase in the leaf colour strength after twelve months of storage, respectively. Mannitol only at the highest concentration (30gL^{-1}) was effective in maintaining the highest intensity of green in the leaves. However, *P. Large white* X *P. Large pink* and *P. Psychosis Pink* X *P. No. 1* displayed no statistical difference compared with the control, with the darkest leaf scoring 3.9 at 10 and 20gL^{-1} for *P. Large white* X *P. Large pink* (Table 3.13) and 4.0 at 30gL^{-1} *P. Psychosis Pink* X *P. No. 1* (Table 3.16) at the end of the storage. Shoot and protocorm explants were not fully autotrophic; mannitol was the only carbon source to sustain the tissue (Muller et al., 2011). Sugar alcohols such as mannitol in the media have been documented to be used as a substitute for glucose to facilitate plant tissue regeneration (Yaseen et al., 2012). This supports the findings of this experiment which showed that a spike in mannitol concentration yielded better leaf colour than in the absence of exogenous mannitol. Providing mannitol avails carbon, hence an increase in leaf color. Despite this, the hybrids produced a decline in the normal dark green leaves across all treatments. *P. Mini pink* X *P. Brighton Belle* (Table 3.12) and *P. aphrodite* (Table 3.14); however, attained a lighter green at the end of their storage period. Molassiotis et al., (2006) determined the effect of osmolarity on the chlorophyll content, which supports the findings of this experiment. Also, a study by Cha-Um & Kirdmanee (2010) indicated degradation of chlorophyll in cultures exposed to mannitol.

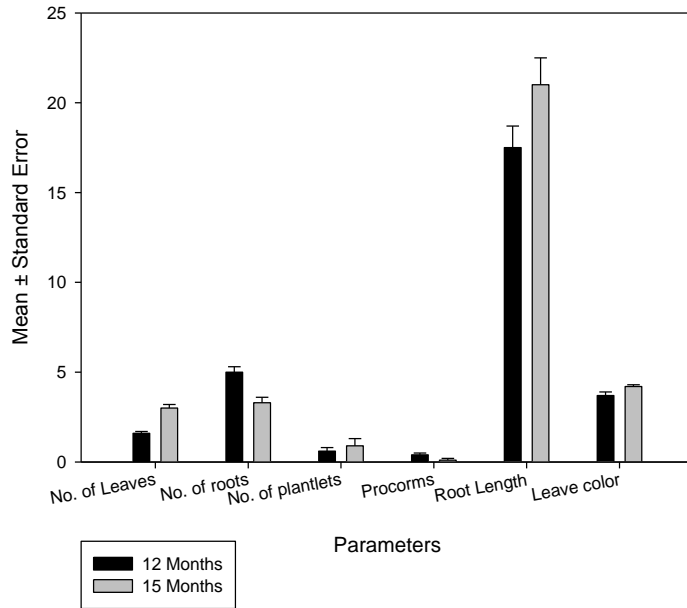


Figure 3.3: Effect of *in vitro* storage duration on *P. No. 1 X P. Large pink shoots* cultured on $\frac{1}{2}$ MS supplemented with 10 gL^{-1} mannitol with an incubation temperature of $23 \pm 2 \text{ }^\circ\text{C}$, at the end of twelve and fifteen months without subculturing, followed by plating on multiplication media. Values presented are means \pm SE, $n = 21$, at $P=0.05$.

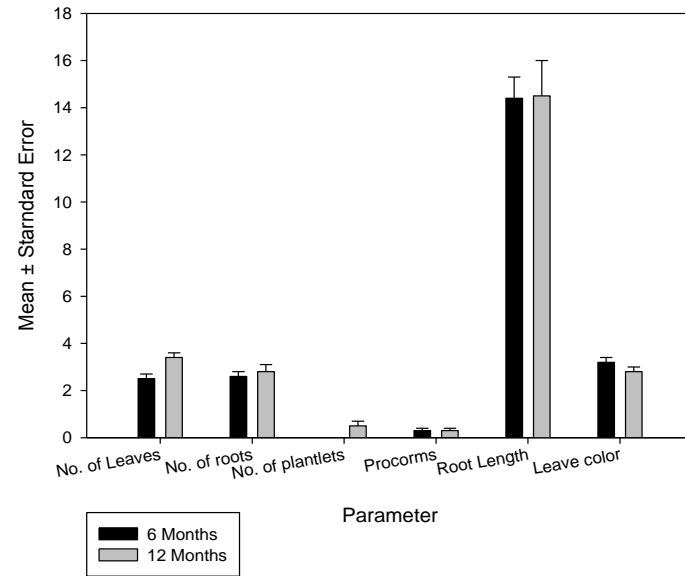


Figure 3.4: Effect of *in vitro* storage duration on *P. aphrodite* shoots cultured on $\frac{1}{2}$ MS supplemented with 10 gL^{-1} mannitol with an incubation temperature of $23 \pm 2 \text{ }^\circ\text{C}$, at the end of six and twelve months without subculturing, followed by plating on multiplication media. Values presented are means \pm SE, $n = 21$, at $P=0.05$.

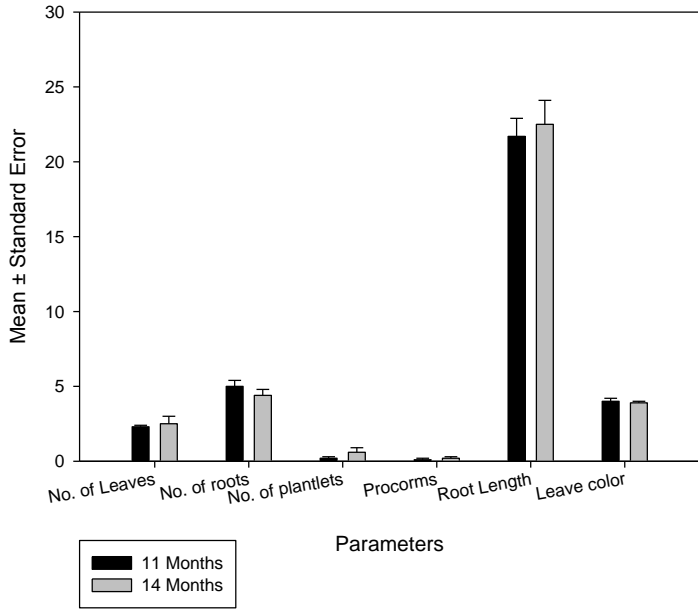


Figure 3.5: Effect of *in vitro* storage duration on *P. Psychosis Pink X P. No. 1* shoots cultured on $\frac{1}{2}$ MS supplemented with 30 gL^{-1} mannitol with an incubation temperature of $23 \pm 2 \text{ }^\circ\text{C}$, at the end of eleven and fourteen months without subculturing, followed by plating on multiplication media. Values presented are means \pm SE, $n = 21$, at $P=0.05$.

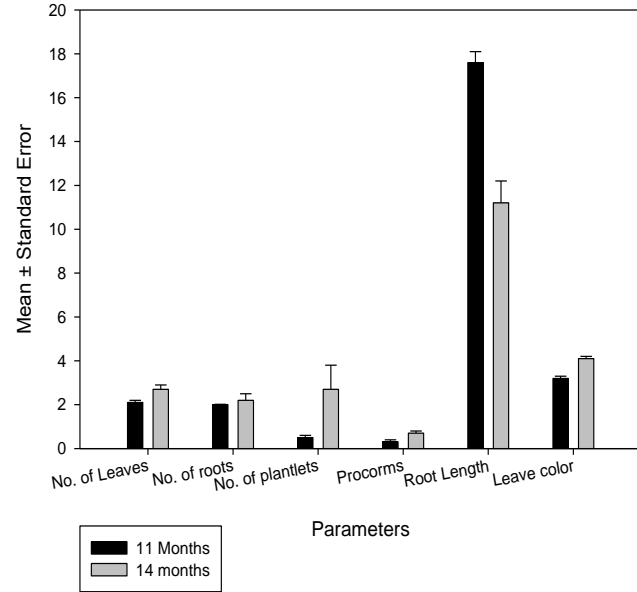


Figure 3.6: Effect of *in vitro* storage duration on *P. Mini pink X P. Brighton Belle* shoots cultured on $\frac{1}{2}$ MS supplemented with 20 gL^{-1} mannitol with an incubation temperature of $23 \pm 2 \text{ }^\circ\text{C}$, at the end of eleven and fourteen months without subculturing, followed by plating on multiplication media.

Table 3.12: Effect of mannitol on induction and microplant growth during minimal growth conservation *in vitro* on *Phalaenopsis Mini Pink X P. Brighton Belle* cultures at 23±2 °C, at the end of fourteen months without subculturing, followed by plating on multiplication media.

Treatment	mean no. of leaves (SE)	mean no. of roots (SE)	mean no. of plantlets (SE)	mean root length (SE)	Mean no. of protocorms (SE)	Mean leaf color (SE)
½ MS without mannitol	2.6±0.2a*	3.0±0.1b**	0.7±0.2a***	12.6±0.6a***	0.4±0.1a	3.2±0.1a**
½ MS+20 mannitol gL ⁻¹	2.1±0.1a*	2.0±0.15a**	0.5±0.1a***	17.6±0.5a***	0.6±0.1b	3.2±0.1a**
½ MS+30 mannitol gL ⁻¹	2.7±0.2a*	2.9±0.2b**	2.2±0.4b***	12.7±0.9a***	0.2±0.1a	3.6±0.1b**

Values presented are means ± SE, n = 21. *, **, *** = significant at P≤0.05, P≤0.01, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.13: Effect of mannitol on induction and microplant growth during minimal growth conservation *in vitro* on *Phalaenopsis No1 X P. Large Pink* cultures at 23±2 °C, at the end of fifteen months without subculturing, followed by plating on multiplication media.

Treatment	mean no. of leaves (SE)	mean no. of roots (SE)	mean no. of plantlets (SE)	mean root length (SE)	Mean no. of protocorms (SE)	Mean leaf color (SE)
½ MS without Mannitol	1.0±0.4a***	1.0±0.4a***	0.0±0.0a***	2.2±0.9a***	0.5±0.1ab*	1.5±0.3a***
½ MS+10 mannitol gL ⁻¹	3.4±0.3b***	3.3±0.3ab***	1.0±0.4a***	11.9±0.5b***	0.2±0.1a*	3.8±0.1b***
½MS+20 mannitol gL ⁻¹	1.6±0.1a***	5.0±0.3c***	0.6±0.1a***	17.5±1.2c***	0.4±0.1ab*	3.7±0.2b***
½ MS+ 30gL ⁻¹ mannitol	3.5±0.2b***	4.7±0.4c***	5.1±0.9b***	15.8±0.8c***	0.7±0.1b*	3.8±0.1b***

Values presented are means ± SE, n = 21. *, *** = significant at P≤0.05, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.14: Effect of mannitol on induction and microplant growth during minimal growth conservation *in vitro* on *Phalaenopsis aphrodite* cultures at 23±2 °C, at the end of fifteen months without subculturing, followed by plating on multiplication media.

Treatment	mean no. of leaves (SE)	mean no. of roots (SE)	mean no. of plantlets (SE)	mean root length (SE)	Mean no. of protocorms (SE)	Mean leaf color (SE)
½ MS without Mannitol	2.7±0.2a**	0.7±0.2a***	0.05±0.05	3.1±0.8a***	0.7±0.1b***	1.3±0.1a***
½ MS+10 gL ⁻¹ mannitol	3.4±0.2ab**	2.8±0.3ab***	0.5±0.2	14.0±1.5b***	0.3±0.1a***	2.8±0.2b***
½ MS+20 gL ⁻¹ mannitol	3.8±0.3b**	3.0±0.3ab***	0.6±0.3	18.3±1.4bc***	0.2±0.1a***	3.7±0.1c***
½ MS+30 gL ⁻¹ mannitol	2.9±0.2a**	5.3±0.5c***	0.9±0.6	19.2±1.3c***	0.1±0.1a***	3.8±0.1c***

Values presented are means ± SE, n = 21. **, *** = significant at P≤0.01, P≤0.001 respectively, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.15: Effect of mannitol on induction and microplant growth during minimal growth conservation *in vitro* on *Phalaenopsis Large white X P. Large Pink* cultures at 23±2 °C, at the end of fifteen months without subculturing, followed by plating on multiplication media.

Treatment	mean no. of leaves (SE)	mean no. of roots (SE)	mean no. of plantlets (SE)	mean root length (SE)	Mean no. of protocorms (SE)	Mean leaf color (SE)
½MS+10 mannitol gL ⁻¹	4.3±0.3b***	2.5±0.21a***	0.5±0.3ns	7.7±0.7a***	0.2±0.1	3.9±0.1
½MS+20 mannitol gL ⁻¹	2.6±0.1ba***	3.8±0.3b***	1.5±1.1ns	19.7±0.9b***	0.1±0.1	3.95±0.2

Values presented are means ± SE, n = 21. *** = significant at P≤0.001, at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

Table 3.16: Effect of mannitol on induction and microplant growth during minimal growth conservation *in vitro* on *Phalaenopsis P. Psychosis Pink X P. No. 1* cultures at 23±2 °C, at the end of fifteen months without subculturing, followed by plating on multiplication media.

Treatment	mean no. of leaves (SE)	mean no. of roots (SE)	mean no. of plantlets (SE)	mean root length (SE)	Mean no. of protocorms (SE)	Mean leaf color (SE)
½ MS+10 mannitol gL ⁻¹	3.9±0.2b***	3.7±0.4a**	0.6±0.2	15.8±0.1a***	0.1±0.7	4.0±0.1
½ MS+20 mannitol gL ⁻¹	3.5±0.2b***	5.5±0.3b**	0.6±0.3	21.0±1.1b***	0.1±0.8	4.0±0.1
½ MS+30 mannitol gL ⁻¹	2.3±0.1a***	5.0±0.3b**	0.2±0.1	21.7±1.2b***	0.1±0.8	4.05±0.2

Values presented are means ± SE, n = 21. **, *** = significant at P≤0.01, P≤0.001 respectively, NS = not significant at P=0.05, SE = standard error. Means followed by dissimilar letters in a column are significantly different from each other at P=0.05 according to Turkey least significance difference.

3.3.2.6 Percentage survival of plantlets in storage

The highest survival percentage was observed when explants were cultured on mannitol supplemented medium, while explants cultured on MS with no supplements yielded the lowest survival percentage. There was an increase in the percentage of protocorm survival, in relation to the increase in mannitol concentration during *in vitro* storage. When stored in ½ MS (no sucrose) supplemented with 10 gL⁻¹ mannitol at 23±2 °C, the percentage survival attained was 81 % for protocorms and 76 % shoots of *P. aphrodite* (twelve months) (Table 3.17). Percentage of survival of *P. Mini pink X P. Brighton belle* protocorms was lower (81 %) than survival of shoots (100 %) when stored *in vitro* in ½ MS supplemented with 20 gL⁻¹ of mannitol for fourteen months without subculturing (Table 3.17).

Storage of *P. Psychosis Pink X P. No. 1*, for twelve months and *P. Large white X P. Large pink* for fourteen months displayed the lowest survival when stored in the ½ MS media supplemented with 30 gL⁻¹ of mannitol (62 %) (Table 3.17). The negative responses to high concentrations of mannitol have been reported previously to reduce survival especially for prolonged storage periods (Charoensub & Phansiri, (2004).

3.3.3 Repeated experiments

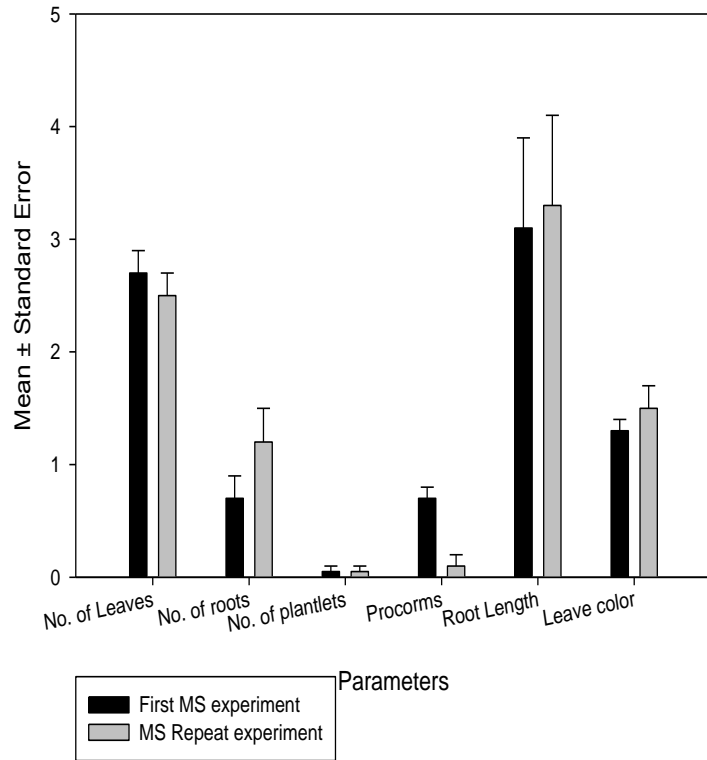


Figure 3.7: A comparison of inducing *P. aphrodite* cultures on 1/2 MS media with no supplements at 23±2 °C, at the end of six months without subculturing, followed by plating on multiplication media.

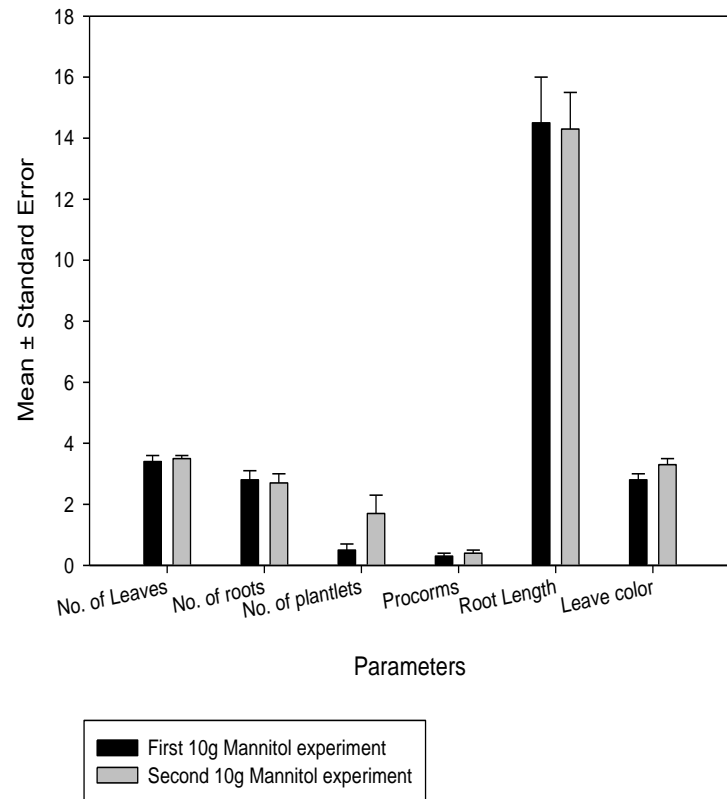


Figure 3.8: A comparison of inducing *P. aphrodite* cultures on 1/2 MS supplemented with 10 gL⁻¹ mannitol media with no sucrose at 23±2 °C, at the end of twelve months without subculturing, followed by plating on multiplication media.

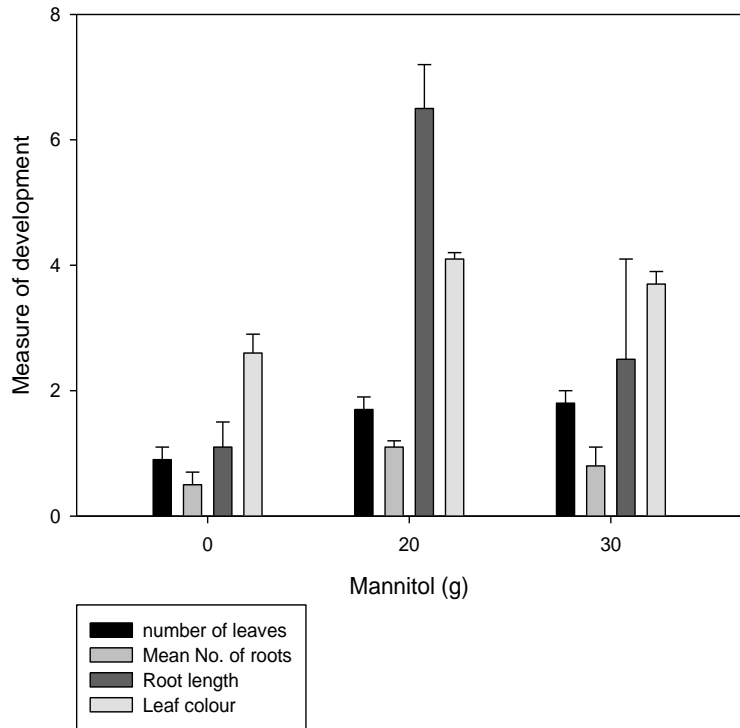


Figure 3.9: The effect of supplementing mannitol to ½ MS media with no sucrose on the regenerative ability *P. Mini Pink X P. Brighton Belle* cultures whilst in storage at 23±2 °C, at the end of twelve months without subculturing.

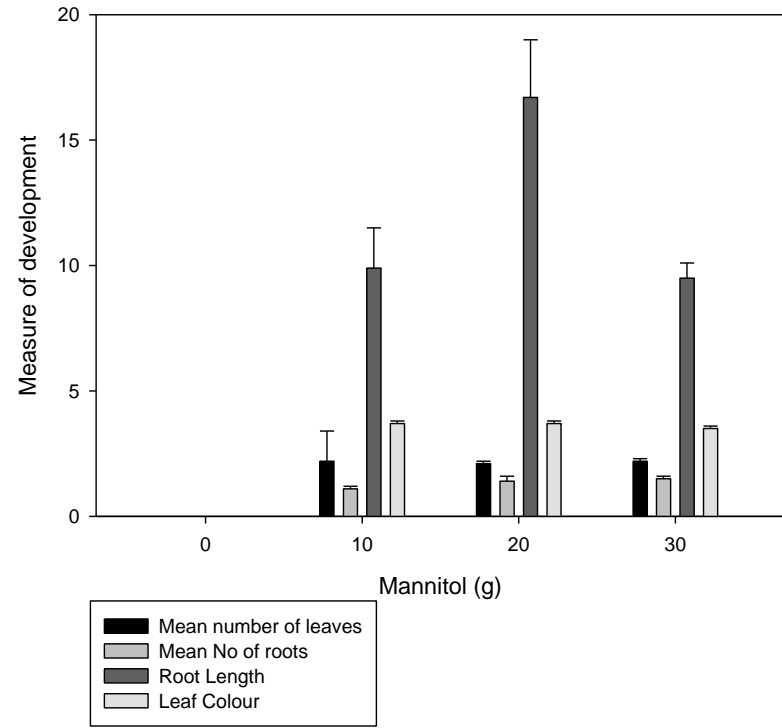


Figure 3.10: The effect of supplementing mannitol to ½ MS media with no sucrose on the regenerative ability *P. No1 X P. Large pink* cultures whilst in storage at 23±2 °C, at the end of twelve months without subculturing.

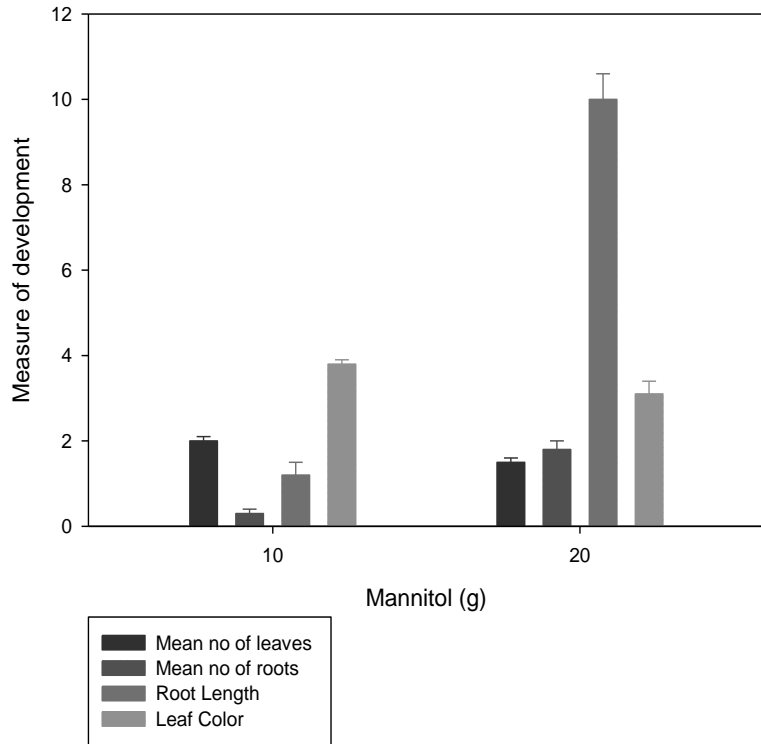


Figure 3.11: The effect of supplementing mannitol to ½ MS media with no sucrose on the regenerative ability *P. Large white X P. Large pink* cultures whilst in storage at 23±2 °C, at the end of twelve months without subculturing.

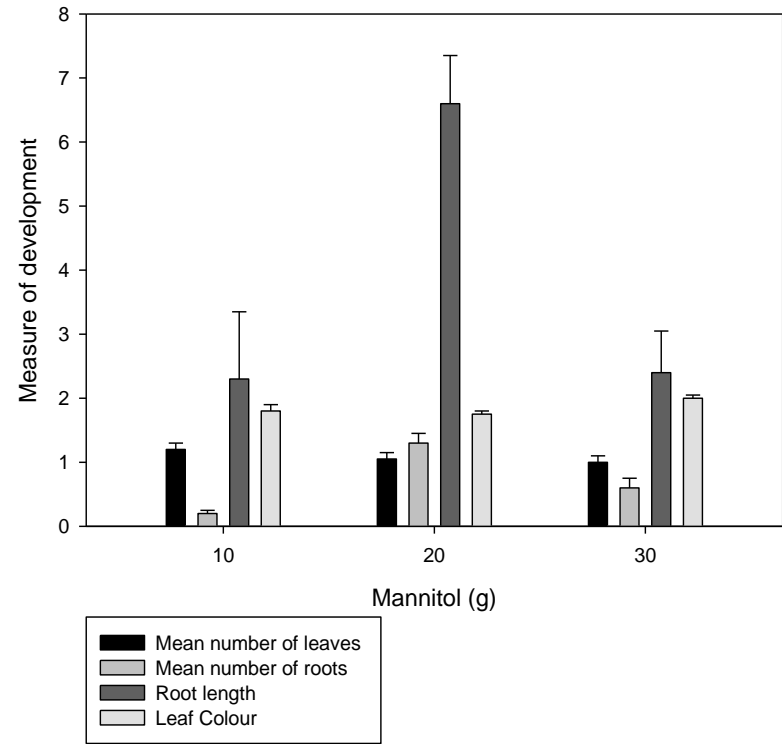


Figure 3.12: The effect of supplementing mannitol to ½ MS media with no sucrose on the regenerative ability *P. Psychosis Pink X P. No. 1* cultures whilst in storage at 23±2 °C, at the end of twelve months without subculturing.

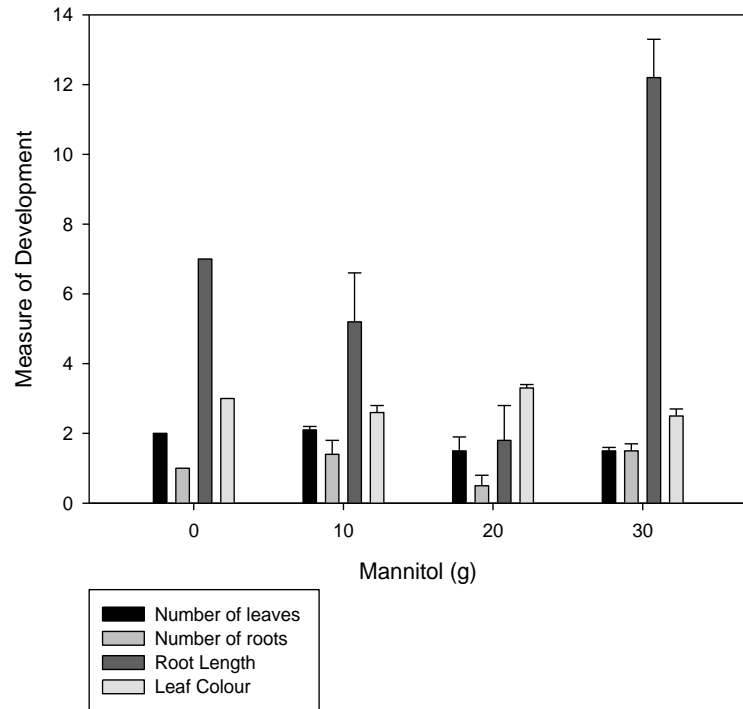


Figure 3.13: The effect of supplementing mannitol to $\frac{1}{2}$ MS media with no sucrose on the regenerative ability *P. aphrodite* cultures whilst in storage at 23 ± 2 °C, at the end of twelve months without subculturing.

Table 3.17: Effect of mannitol on post storage survival and reproducibility of hybrids in the multiplication media

Storage conditions	Species	Type of explant	Duration of storage	Survival during storage %	Protocorm formation	Survival (Post storage) %
½ MS	<i>P. aphrodite</i>	shoot	10	71 %	Y	80 %
	<i>P. aphrodite</i>	Protocorms	10	81 %	Y	88 %
	<i>P. Mini Pink X P. Brighton Belle</i>	Protocorms	11	33 %	Y	43 %
	<i>P. No. 1 X P. Large pink</i>	Shoots	11	76 %	Y	81 %
	<i>P. Psychosis Pink X P. No. 1</i>	Shoots	10	0 %	NT	NT
	<i>P. Large white X P. Large pink</i>	Shoots	10	0 %	NT	NT
½ MS+10 gL ⁻¹ Mannitol	<i>P. aphrodite</i>	protocorm	12	76 %	Y	94 %
	<i>P. aphrodite</i>	Shoot	15	100 %	Y	90 %
	<i>P. aphrodite</i>	Shoot	12	81 %	Y	88 %
	<i>P. No. 1 X P. Large pink</i>	shoots	12	90 %	Y	89 %
	<i>P. Psychosis Pink X P. No. 1</i>	shoots	13	100 %	Y	95 %
	<i>P. Large white X P. Large pink</i>	shoots	14	100 %	Y	95 %

Cont: 3.18: Effect of mannitol on post storage survival and reproducibility of hybrids in the multiplication media

½ MS+20 gL ⁻¹ Mannitol	<i>P. aphrodite</i>	shoots	12	76 %	Y	100 %
	<i>P. No. 1 X P. Large pink</i>	shoots	12	86 %	Y	94 %
	<i>P. No. 1 X P. Large pink</i>	shoots	14	100 %	Y	95 %
	<i>P. Mini Pink X P. Brighton Belle</i>	protocorms	14	81 %	Y	88 %
	<i>P. Mini Pink X P. Brighton Belle</i>	shoots	14	100 %	Y	100 %
	<i>P. Psychosis Pink X P. No. 1</i>	shoots	16	100 %	Y	95 %
	<i>P. Large white X P. Large pink</i>	shoots	16	100 %	Y	100 %
½ MS+30 gL ⁻¹ Mannitol	<i>P. aphrodite</i>	protocorms	12	100 %	Y	100 %
	<i>P. No. 1 X P. Large pink</i>	protocorms	15	100 %	Y	100 %
	<i>P. Psychosis Pink X P. No. 1</i>	protocorms	12	62 %	Y	92 %
	<i>P. Psychosis Pink X P. No. 1</i>	shoots	10	100 %	Y	95 %
	<i>P. Mini Pink X P. Brighton Belle</i>	protocorms	14	86 %	Y	100 %

3.3.3.1 Survival post storage

After storage in either mannitol or MS medium, survived shoots and protocorms were subsequently transferred to ½ MS medium supplemented with 1 mgL⁻¹ BA and 1 gL⁻¹ peptone. Data collected reflected the ability of *Phalaenopsis* hybrids to produce protocorms and shoots after storage under the studied supplementation conditions. On this multiplication media, micro plantlets previously stored in higher concentrations of mannitol (20 and 30gL⁻¹) yielded the highest survival percentages as indicated in Table 3.17. To further illustrate this, *P. aphrodite* shoots previously stored in ½ MS supplemented with 30 gL⁻¹ mannitol, demonstrated a 100 % survival compared to the shoots previously stored in 10 gL⁻¹ mannitol, which had 88 % survival when cultured in the multiplication media. Furthermore, *P. No. 1 X P. Large pink* survival post storage was better on ½ MS supplemented with 20 gL⁻¹ mannitol (100 %) than on ½ MS supplemented with 10 gL⁻¹ mannitol (95 %). The concentration of mannitol on ½ MS in the *in vitro* storage media had no influence on the microshoot survival of *P. Psychosis Pink X P. No. 1* and *P. Mini Pink X P. Brighton Belle*, which the percentage survival was the same 95 % and 100 %, respectively, across all the mannitol supplemented media tested (Table 3.17).

3.3.3.2 Regeneration post storage

Reproducibility post storage of *Phalaenopsis aphrodite*, *P. Psychosis Pink X P. No. 1*, *P. Large white X P. Large pink*, *P. No. 1 X P. Large pink* and *P. Mini pink X P. Brighton Belle* in ½ MS supplemented with mannitol was feasible Table 3.17. This renders the ability of the studied species for *in vitro* conservation.

3.4 Conclusion

In South Africa, plant degradation the floriculture industry plays an important role in the economic development of the country. Endangered *Disa* and *Eulophia* species, like other endangered species require constant reproduction to retain adequate population size in the wild. Due to low recruitment in the wild, *ex vitro* methods of production and conservation become important in the survival of the germplasm. Micropropagation is a possible technique for

regeneration and *in vitro* slow growth as a technique for preservation were examined by using Phalaenopsis hybrids as trial for there was limited plant materials of the endangered species. Research in this study showed that banana extract, peptone and thidiazuron could favourably be used for effective regeneration of protocorms, shoot development and efficient rooting for *Phalaenopsis* Psychosis Pink X *P.* No. 1; *P.* Large white X *P.* Large pink; *P.* No. 1 X *P.* Large pink; *P.* Mini pink X *P.* Brighton Belle; and the *P. aphrodite*. The beneficial effect of banana extract was not only observed in improving microshoot performance *in vitro*, but also was effective in increased microplant survival *ex vitro* during acclimatisation in the greenhouse. The positive influence on the overall reproductive rates was not as pronounced as on other growth features.

Micropropagated plants showed good growth and uniformity when cultured in banana extract, with 10 gL⁻¹ banana extract yielding the most somatic embryos. ½ MS supplemented with 20 gL⁻¹ banana extract promoted somatic embryo germination and leave growth, yet sustaining protocorm regeneration. Banana extract at a concentration of 30 gL⁻¹ banana extract induced the most roots and played a role in increasing the root length.

Phalaenopsis hybrids were able to translocate mannitol and supported their growth and embryogenic callus regeneration. For conservation of generated protocorms and shoots, mannitol was added into the culture medium to decrease the water osmotic potential, reducing the uptake of nutrients in the cells, hence limiting the plant growth of *in vitro* cultures for the preservation of *Phalaenopsis* Psychosis Pink X *P.* No. 1; *P.* Large white X *P.* Large pink; *P.* No. 1 X *P.* No. 3; *P.* No. 1 X *P.* Large pink; *P.* Mini pink X *P.* Brighton belle; and the *P. aphrodite*. A 20gL⁻¹ banana extract media tends to reduce in plant growth of *Phalaenopsis* hybrids studied. From the success of the treatments of both the banana extract and peptone in regeneration of Phalaenopsis, authors suggest their use in micropropagation of endangered *Disa* and *Eulophia* especially with its extreme low availability to increase propagules.

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CHAPTER 4

Cryopreservation of Native *Leucadendron* and *Mimetes* species seeds in the Cape Floristic Kingdom

4.1 Introduction

4.1.1 Cape floral Region

The Cape Floristic Region (CFR) occurs in a Mediterranean climate area on the southern tip of Africa; approximately 31°30'S-34°50'S, 18°00'E-25°30'E (Quinn & Woodward, 2015). The type of vegetation occurring in this region is prone to fires and is dependent on fire for regenerative purposes (Stock & Allsopp, 2015; van Wilgen et al., 1996). However, as indicated by Mustart et al., (2012), these fires can be detrimental to the survival of the adult plant especially the sprouting Fynbos, whilst other proteoids are highly dependent on veld fires for the success of seed germination. For example, *Leucadendron elimense* sub *elimense* which has been classified ranging from endangered to critically endangered (Tansley & Brown, 2000) can potentially become extinct as these are non-sprouting plants. Cabral & Schurr, (2010), further indicated that sprouters tend to have limited sexual reproduction, also affirming the potential of reduced populations should fires be too persistent. Despite seed germination and seed emergence being a major contributor to plant ecological distribution, proteoids are dependent on highly specialized germination conditions which are not always ideal in nature (Brown & van Staden, 1975; Mustart et al., 2012), making fynbos susceptible to population decline. Furthermore, fires cause a reduction of specialist pollinator visitation in burnt areas, resulting in a major decline in the number of seed that is set (Geerts et al., 2012). Although fires are highly crucial for the survival of fynbos, they may negate the survival of ecosystems if they are too frequent.

At the heart of the CFR is the Cape Peninsula, which is by far one of the fastest growing regions in terms of immigration from other areas of Southern Africa (Richardson et al., 1996), and is therefore prone to high risks of ecological degradation. Agricultural activities have been noted by Turpie et al., (2003) as major contributors to fynbos degradation. Numerous fynbos species have dwindling populations as a result of human population growth. The current efforts in conservation are not suffice, with the depletion rates of plant diversity being observed (van

Wilgen, 1996; Rebelo et al., 2011). Therefore, newer approaches are required. Cowling et al., (2003) suggested that the area currently under protection in the CFR be supplemented by an additional area equivalent to 42% of the total available area, in order to achieve the retention of target species in an area.

In order to promote the persistence of ecological processes, the main objective for the conservation of the fynbos biome is to improve the current biodiversity status and maintain ecological thresholds indefinitely. Fragmentation or the decline of one species could negatively impact on the survival of other living organisms and significantly alter ecological functions and services (Bond, 1994). For example, Pauw & Hawkins (2011) suggested that the decline in pollination by the oil-collecting bee, *Rediviva peringueyi* is a contributing factor to the decline in the *Coryciinae* population around the Signal hill area. This depicts the synergistic interactions of multiple species in natural systems. Besides the ecological consequences of extinction, Higgins et al., (1997) also highlighted that, severe economic repercussions can emanate from the extinction of a single species. For example, according to Turpie et al., (2003), thatching reed contributes about R6.3 million in economic trade, while Buchu from both cultivated and veld plants can generate an estimated R13.5million with the honey bush generating about R375000, annually. Within a hypothetical 4 km² of mountain fynbos ecosystem, Higgins et al., (1997) estimated an average annual income of between R19 million that can be generated on a neglected area and R300 million under good management. Conserving this biodiversity therefore contributes to securing ecological safety and improving economic growth. Apart from this, the mountain fynbos ecosystem indirectly plays a major role in the fruit production industry, bee keeping, water purification, biogeographical patterns, ecotourism, stabilizes soil to prevent erosion and also serves as a genebank (Higgins et al., 1997). Thus, the extinction of any one species results in the loss of other species which could cause ecological degradation, with significant economic impact.

The main floricultural product from the fynbos is the Proteaceae, the commercialization of which has been rapidly growing over many years. The main commercial genera of this family include *Protea*, *Leucospermum* and *Leucadendron*. At present in South Africa, the dry floral industry and the fresh cut flower industry directly harvests the flower stalks from the wild (Coetzee & Middelman, 1997; Littlejohn & Robyn, 2000), with up to 70% flower stems harvested directly from their natural habitats (Petersen et al., 2012). This jeopardizes the existence of certain species and has considerable impact on the quality and quantity of flower production for export

(Higgins et al., 1997; Bomhard et al., 2005). With harvesting directly from the wild, Privett et al., (2014) established that close to 100% of plants that are cut, die, and this therefore reducing re-seeders and resprouters after harvesting. On the current Proteaceae taxa, Bomhard et al., (2005) predict a further 9% decline owing to land use and climate change.

Among the Proteaceae, Leucadendrons have been reported to be the most sought-after flowers, accounting for roughly half of the overall sales of Proteaceous cut stems worldwide (Ben-Jaacov & Silber, 2006). The foliage, striking flowers and high post-harvest durability have made Leucadendrons popular as cut flowers for export. The L.'Safari Sunset', is the most desired Leucadendron worldwide, accounting for an estimated 90% of the total produce of Proteas in Israel. Littlejohn (2001) further estimated that this hybrid accounts for 25 million stems, while Ben-Jaacov & Silber (2006) assert that the demand exceeds 40 million stems per annum.

Heavy exploitation of the *L. platyspermum* R.Br. female cones for the dry flower industry has led to their depletion in natural reserves (Rebelo et al., 2010). A significant loss has been incurred of this species as the main reproductive organs (flowers fruits and seeds) are harvested and no form of replacement of this species is practised; hence population decline has been observed. Other species such as the *Erica turgida*, wild populations had been almost completely wiped out in the wild, but as a result of *ex-situ* conservation efforts, the plant was able to be re cultivated and its re-introduced into the wild (Oliver et al., 2007). Furthermore, the fynbos species support the existence of arthropods and pollinators which heavily rely on the abundance of species diversity. As predicted by Wright & Samways (1999), reduction in plant population sizes may result in the demise of insect biota in co-existence with the Proteaceae.

4.1.2 Challenges in seed germination

4.1.2.1 Seed Dormancy mechanism

The absence of appropriate and adequate conditions poses the greatest difficulty in seed germination of Proteaceae, mainly as a result of the dormancy of the seeds. This is due to germination inhibitors thought to occur within the seeds (Arnolds et al., 2015), which results in low germination percentages and prolonged the germination period. These may include:

4.1.2.2 Physical dormancy

The seed coat poses a challenge for the seed to absorb water readily and for the radicle to penetrate the testa. As a method of overcoming this dormancy, (Deall & Brown, 1981; Brown & Dix, 1985) advice that the seed coat be removed. Removal of seed coat however, could result in leakage of solutes (sugar and proteins) and electrolytes, which extensively damage the cotyledons (Bewley et al., 2012) if the cotyledons are injured during the removal of the testa. Also, Brown & Van Staden, 1973, reported that there is an increase of microorganism attack of the embryo, which leads to the deterioration of the seed. Hence the testa is important contributor to successful germination. On the contrary, excised embryos of *L. cordifolium* (Brown & Van Staden, 1973), *P. magnifica* (Deall and Brown, 1981), *Leucadendron tinctum* (Brown & Dix, 1985) and *P. cynaroides* (Wu & du Toit, 2010) improved the germination rates.

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The conservation status of the *Leucadendron* in the Cape Floral Region (CFR) is alarming. This illustrates the importance of human intervention in reducing the degradation of biological systems and integrating economic services with conservation efforts. In order to increase the number of stems and financial turnover in the *Leucadendron* flower industry, whilst contributing to the attainment of South Africa's conservation goals (e.g. goal B-target 5 and C- target 13 of the Global Strategy for Plant Conservation)¹, it is necessary to prioritize the propagation of critically endangered species. Propagation can be achieved by either seed or vegetative means. However, successful vegetative propagation of endangered Proteaceae is severely lacking (Hartmann et al., 2002; Wu et al., 2007).

4.2 Materials and methods

4.2.1 Species under study

The species for investigation in this study were selected on the basis of endemism, degree of endangerment and wide exploitation in the floriculture industry. Four *Leucadendron* species (*L. chamelaea*, *L. argenteum*, *L. cryptocephalum* and *L. salignum*) and *Mimetes chrysanthus* were selected for their endangerment.

4.2.1.1 *Leucadendron* spp.

The genus *Leucadendron* belongs to the Proteaceae family. Leucadendrons are predominant along the east coast within the Cape Floral Region (Paterson-Jones, 2000). In total, there are about 85 recorded species (Baker et al., 2004), of which about 81% are on the IUCN red data list (Sanbi, 2014) with their conservation status; ranging from near threatened to critically endangered.

4.2.1.2 *Leucadendron argenteum*: Silver cone bush

In its natural habitat, this species grows along the northern part of the Cape Peninsula, Paarl mountain, Tygerberg, Simonsberg and the Helderberg (Rebelo et al., 2006a). The tree can

Global Strategy for Plant Conservation, Strategic Plan for Biodiversity 2011-2020, including Aichi Biodiversity Targets viewed from July 2013

<https://www.cbd.int/doc/decisions/cop-11/full/cop-11-dec-en.pdf>¹

produce flowers within 2 years of germination and flowering can be delayed to a period of 8 years. The species is endangered, but there are significant efforts towards *in-situ* conservation in protected areas (The Protea atlas, 2008). Internationally, the species is grown in New Zealand, California and Australia for the floriculture industry.

4.2.1.3 *Leucadendron chamelaea*: Crown cone bush

Endemic to the CFR, *L. chamelaea* is critically endangered and is currently almost confined to the Koue Bokkeveld and the Franschoek valley (Rebelo et al., 2011a), which is an agricultural area. The plant can flower in the first year of growth, but in most cases flowers fully develop in the fourth to fifth year. This species is not conserved *in-situ*, and therefore it is more susceptible to extinction (the Protea atlas, 2008).

4.2.1.4 *Leucadendron cryptocephalum*

This species is endemic to South Africa and it is critically endangered (Rebelo et al., 2006c), but not currently conserved in any Nature Reserve (the Protea Atlas, 2008). This underlines the need for the conservation of this species. This is aggravated by the long period from germination to flower production, as the plants first develop flowers after 8 years of development (the Protea atlas, 2008).

4.2.1.5 *Leucadendron salignum*: Sunshine cone bush

This species is one of the few *Leucadendron* species that tolerates and survives a wide range of habitats (Paterson-Jones, 2000). Because of its adaptability it is currently considered 'of least concern' (Rebelo et al., 2006b) and is found in nature reserves where it is conserved *in situ*. Furthermore, its adaptability has made *L. salignum* a popular species for extensive hybridization, leading to development of the most predominant hybrid *L. Safari Sunset*, which is extensively traded in the European floriculture cut flower market (Littlejohn, 2001).

4.2.1.6 *Mimetes chrysanthus*

In its natural habitat, this species occurs on the South facing slope of Gamkaberg, on the northern foothills of the Outeniqua Mountains, where it is vulnerable to extinction (Paterson-Jones, 2000). The Protea atlas (2008) reports that, the plant produces flowers after two years of growth, and there are no records of cultivation towards its preservation.

4.2.2 Proteaceae seed germination

Brown & van Staden, 1973; highlight that Proteaceous seed germination can be improved by using gibberellic acid or cytokinins to substitute chilling requirements of this genus. Brits et al., (1995) confirms this by indicating the elevation of endogenous gibberellic acid and cytokinins levels in the seeds of *Leucospermum* spp. after hydration of seeds. Aqueous smoke or smoke has been proved to also trigger and enhance the seed germination of the proteaceae taxa (Bond, 1984, Keeley & Bond, 1997).

4.2.2.1 Acquisition of seeds

Seeds used for this study were purchased from the Kirstenbosch seed room of the South African National Biodiversity Institute (SANBI) and Silverhill seeds in Cape Town, South Africa.

4.2.2.2 Seed size and seed mass

The thickness of the cotyledons was measured using a vernier caliper placed midway across the seed for all the species under study. Below (Table 4.1) is table illustrating seed mass and size.

4.2.2.3 Pre-treatment methods

Different scarification treatments were tested on mature seeds of *L. argenteum*, *L. chamalaea* and *M. chrysanthus* were used. They were soaked for 24 hours in 1% hydrogen peroxide at room temperature or 96% sulphuric acid for 1-3 minutes (*L. chamalaea* and *M. chrysanthus*) or 3-5 minutes (*L. argenteum*). Each pre-treatment method was followed by three rinses with sterile distilled water.

Table 4.1: Seed size and seed mass of the proteaceae species used in the present investigation

Species	Seed size (mm)	Seed mass (mg)
<i>L. argenteum</i>	8.4	241
<i>L. chamalaea</i>	3.8	21.8
<i>L. cryptocephalum</i>	5.8	22.3
<i>L. salignum</i>	3.5	8.3
<i>M. chrysanthus</i>	4.7	108

4.2.2.4 Surface sterilization of seeds

It has been shown that many seeds harbour a wide spectrum of microflora, which leads to contamination (Rout et al., 2000). In preparation for *in vitro* seed germination studies, the seed testa of *L. salignum*, *L. argenteum*, *L. chamalaea* and *M. chrysanthus* were either first removed and the seeds were soaked in a solution of 70% ethanol for 3 minutes, followed by soaking in 1.75% sodium hypochlorite solution containing 0.02% Tween 20. They were then rinsed three times with sterile distilled water.

4.2.2.5 Screening of *in vitro* germination media

Different germination media were tested for their effectiveness in promoting germination of seeds or embryos. The basic medium was ½ strength Murashige & Skoog (1962) i.e. MS media containing 2% sucrose (except for seeds of *L. chamalaea*, *L. salignum* and *L. cryptocephalum*, for which the medium contained no MS and no sucrose) and solidified with 0.8 % agar [Agar bacteriological (Biolab)]. The pH was adjusted to 5.6 using HCl/ NaOH prior to autoclaving at 117.7 kPa for 20 minutes at 121 °C.

For smoke treatment, media were supplemented with aqueous smoke (commercially prepared by Kirstenbosch Botanical gardens) at concentration of 2; 4; 6; 8 and 10 mL⁻¹ of aqueous smoke per litre of medium. Seeds were cultured onto the germination media contained in test tubes (*L. argenteum* and *Mimetes chrysanthus*) or 90 mm Petridishes (*L. chamalaea*, *L. salignum* and *L. cryptocephalum*). Seeds of *L. chamalaea*, *L. salignum* and *L. cryptocephalum* were plated on 0.8 % solidified agar media supplemented with 2-10 mL⁻¹ aqueous smoke to determine the optimum concentration at the given temperature regime. The test tubes/Petridishes were then sealed with Parafilm ® and incubated under a 16/8h light/dark photoperiod at two temperature regimes: either at 10 °C or 25 °C.

Germination was assessed periodically, with the assessment period depending on the natural germination rate for each species: *L. chamalaea*, *L. argenteum* and *Mimetes chrysanthus* assessment was fortnightly; for *L. cryptocephalum* it was weekly, and for *L. salignum*, germination was assessed every second day. Seeds were considered germinated when the radicle emerged and reached at least 5 mm in length. Based on the responses, germination percentage (GP) was used as an estimate of the viability, which was calculated as:

$$\text{GP} = \text{Number of seeds germinated} \div \text{Total number of seeds} \times 100$$

The rate of germination was calculated either as the velocity of germination or mean germination time, as follows: using the formula:

$$\text{Velocity of germination} = (\sum_{i=1}^k fi / \sum_{i=1}^k fixi) 100$$

Where: *fi*: number of seeds newly germinated on day *i*; *xi*: number of days from sowing; *k*: last day of germination (Ranal & Santana, 2006).

$$\text{Mean Germination Time} = \sum_{i=1}^k niti / \sum_{i=1}^k ni$$

Where: *ni*: number of seeds germinated in the *i*th time; *ti*: time from the start of the experiment to the *i*th observation; *k* last day of germination (Ranal et al., 2009).

4.2.2.6 Biochemical viability test

In order to gauge the viability of seeds may failed to germinate under the experimental conditions assessed, despite being viable, seeds of *Leucadendron* spp and *Mimetes chrysanthus* were immersed in 2, 3, 5-Triphenyl tetrazolium chloride (TTC) for a biochemical viability assay as used by Porter et al., (1946).

Three sets of 10 seeds each were used. De-coated seeds were pre-soaked in distilled water at 30 °C for 12 hours for imbibition. They were then incubated in a 1 % (w/v) solution of TTC, in the dark at room temperature for 48 hours. The seeds were removed from the TTC solution and rinsed with sterilized distilled water. The mean percentage of embryos staining red was calculated and recorded as viable. A scale of one to four was used to rate the colour of the stain on the seeds; 1= colourless, 2= pale pink, 3= pink and 4= red.

4.2.3 Proteaceae seed Cryopreservation procedure

To determine the effect of the cooling rate on the viability of seeds, thirty seeds per treatment were subjected to 3 different cooling rates namely:

4.2.3.1 Rapid cooling

Seeds (in batches of 5) were enclosed in an envelope made from one layer aluminium foil. The envelope was just large enough to contain the seeds in one layer. The envelope was then quickly plunged into liquid nitrogen and maintained there for an hour Figure 4.1. This method of cryopreservation has been shown to achieve cooling rates of about $1000^{\circ}\text{C}^{-1}$ (Wesley-Smith et al., 2004).



Figure 4.1: Inserting wrapped seeds contained in cryocanes in liquid nitrogen.

4.2.3.2 “Less Rapid” cooling

Seeds (4-5) were enclosed in 2mL polypropylene cryotubes and submerged directly into liquid nitrogen and kept for a minimum of 60 minutes Figure 4.3. This method of cryopreservation has been shown to achieve cooling rates of about 200 °C/ min (Walters et al., 2008).

4.2.3.3 Two-stage cooling

Seeds (4-5 seeds) were placed in 2 mL polypropylene cryotubes, which were then placed in to a Mr Frosty® (Nalgene, USA) container (Figure 4.2). The container was filled with iso-propanol and placed in a -80 °C ultra-deep freezer for 60 minutes. The cryotubes were then submerged in to liquid nitrogen for an hour. This method of freezing achieves cooling at a rate of 1 °C min⁻¹ (López Lastra et al., 2001).



Figure 4.2: Freezing container; Mr Frosty™ freezing container used

4.2.3.4 Storage

After cooling, the ampules were placed in a Dewar that was undisturbed, and the specimens were kept for an hour (Figure 4.3).



Figure 4.3: Inserting samples in the Dewar for storage

4.2.3.5 Rewarming

Thawing was achieved by immersing samples (still in cryotubes or aluminium envelopes) in a water bath at 40 °C for two minutes (Figure 4.4).

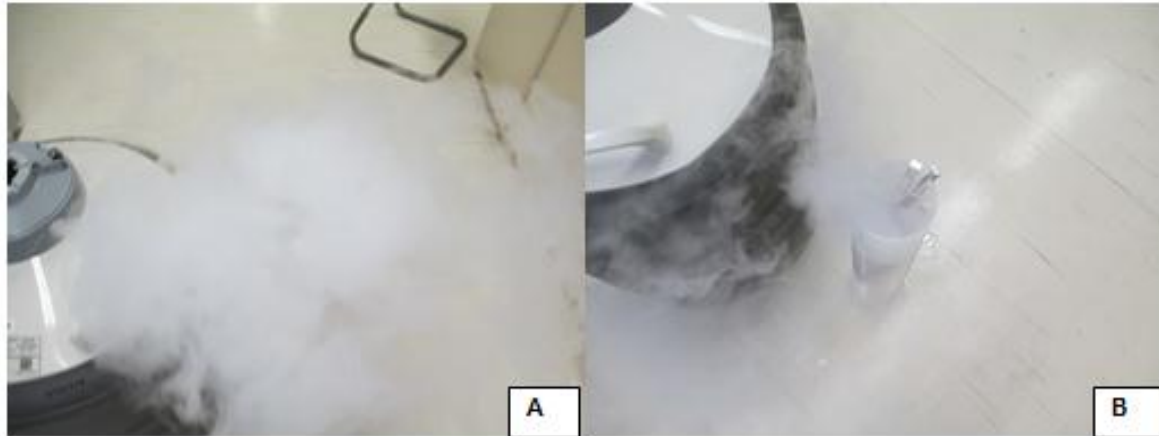


Figure 4.4: a) Rapidly transferring cryocanes into the water bath; b) Rewarming seeds in the water bath.

4.2.3.6 Plant recovery

After thawing, seeds were sterilized by soaking in 70 % ethanol for 3 minutes followed by soaking in 1 %, 1.75 % and 4 % NaOCl for 20 minutes with subsequent rinsing (thrice) with sterile distilled water. The seeds were then transferred to the *in vitro* germination medium without MS and sucrose, as outlined in section 4.2.2.5 above. Seeds were assessed for germination for up to six weeks.

4.3 Results and Discussion

4.3.1 Decontamination

Leucadendron argenteum, *L. chamalaea* and *Mimetes chrysanthus* seeds showed no sign of germination when sterilization with ethanol and sodium hypochlorite preceded the pre-treatment with hydrogen peroxide and sulphuric acid. However, radicle growth was observed when seeds were not pre-treated with hydrogen peroxide and sulphuric acid. Therefore, pre-treatment with hydrogen peroxide and sulphuric acid was not performed in subsequent tests, and *in vitro* germination was studied for seeds which were sterilised whether contaminated and/or clean cultures. Additionally, pre-soaking seeds in aqueous smoke and smoke disks displayed high contamination on the cultures as the smoke disks weren't sterile. The germination results displayed for this study were generated from bioassays that were cultured in aqueous smoke.

4.3.2 Effect of smoke treatment on germination

The germination percentages of *Leucadendron argenteum*, *L. salignum*, *L. chamalaea*, *L. cryptocephalum* and *Mimetes chrysanthus* in response to different concentrations of aqueous smoke are presented in Figure 4.5 to Figure 4.16. Once seeds are imbibed, germination is underpinned by a series of metabolic processes such as dormancy breaking signals and degradation of structures surrounding the embryo followed by the loosening of cell walls until the emergence of embryonic axis (Bewley, 1997). The success of germination however has been indicated to be dependent on ideal environmental conditions such as dormancy breakers, temperature, and nutrition (Baskin et al., 2000; Wada et al., 2011). This current study focused on optimisation of *in vitro* germination conditions, incubation temperatures and nutrition on the selected Proteaceae species.

i. Germination percentage

From the experiment conducted, germination of the seeds of four of the five studied species positively responded to aqueous smoke (*Leucadendron argenteum*, *L. salignum*, *L. chamalaea* and *Mimetes chrysanthus*) as indicated by increased germination percentages when culture media were supplemented with aqueous smoke Figure 4.12, Figure 4.8, Figure 4.16 and Table

4.6. Changes in germination percentages were also observed by Keeley & Bond, (1997), van Staden et al., (2000) and Wu & du Toit (2010), in studies performed on Proteaceae. However, the highest germination percentage obtained for *L. cryptocephalum* on smoke enriched media 97 % at 8 and 10 mL⁻¹. It was further observed that seeds on the media without smoke (Figure 4.5) retained high germination percentages. On the other hand, the germination percentages of *Mimetes chrysanthus* were reduced at higher concentrations of aqueous smoke. A decrease in germination percentages from 29 % on media with 6 mL⁻¹ smoke to 19 % on media containing 10 mL⁻¹ aqueous smoke was observed. On the whole, the highest germination percentages achieved for *Leucadendron argenteum* and *Mimetes chrysanthus* was (29 %), when cultured on 6 mL⁻¹ smoke as indicated in Figure 4.12 and Figure 4.11 respectively. When the germination medium was supplemented with 6 or 8 mL⁻¹ aqueous smoke, for *L. salignum* seeds, a maximum germination percentage of 100 % was observed (Figure 4.8).

ii. Germination rates

In this study, germination rate of the seeds of two species positively responded to the addition of aqueous smoke, namely *L. argenteum* (Table 4.5) and *L. chamalaea* (Table 4.7). However, at high concentrations of aqueous smoke, seeds of *L. salignum* (Table 4.3) and *M. chrysanthus* (Table 4.4) negatively responded, displaying a decline in germination rate at 10 mL⁻¹ and 8 mL⁻¹ for *L. cryptocephalum* as demonstrated in Table 4.2. This study displayed the highest mean germination rate at 6 mL⁻¹ smoke for *L. cryptocephalum*, *M. chrysanthus*, 8 mL⁻¹ aqueous smoke for *L. salignum* and 10mL⁻¹ aqueous smoke for *L. argenteum*.

iii. Mean germination time

Increasing the concentration of smoke in the germination media reduced the mean germination time for seeds of *Leucadendron argenteum*, *L. salignum*, *L. chamalaea*, *L. cryptocephalum*. The addition of 2 mL⁻¹ aqueous smoke reduced the germination time for *L. cryptocephalum*. However, increased concentrations of the aqueous smoke increased the mean germination time by 25 days for seeds germinated on media containing 4 mL⁻¹ aqueous smoke (Table 4.2). Also, an increase in smoke concentration increased the mean germination time for *M. chrysanthus*. When germinated on a medium containing 4 mL⁻¹ aqueous smoke, the mean germination time for *M. chrysanthus* was lower (96 days) compared with 116 days when germinated on a medium containing 10 mL⁻¹ smoke (Table 4.9). Additionally, *L. salignum* displayed a negative response in the germination time to the addition of smoke at

concentrations exceeding 6 mL⁻¹ in the germination media (Table 4.7). The positive response to the gradual increase of smoke in the media was observed on *Leucadendron argenteum*, and the results in Table 4.10 shows that media supplemented with 10 mL⁻¹ aqueous smoke achieved the least germination time (90 days) compared with the lower concentrations of smoke (150 days).

Plant-derived smoke plays an important role as an environmental cue in promoting the seed germination as it acts as a strong germination promoter (Light et al., 2010). Plant bioactive compounds known as karrikins, especially the karrikinolide (Merritt et al., 2007; Waters et al., 2013), with 3-methyl-2H-furo[2,3-c]pyran-2-one (1) as the main compounds responsible for improved germination (Flematti et al., 2004; van Staden et al., 2004). Like other plant growth regulators, if applied at high concentration, development of plant tissues may be hampered. Furthermore, the species further display specificity with regards to the reaction to smoke concentrations.

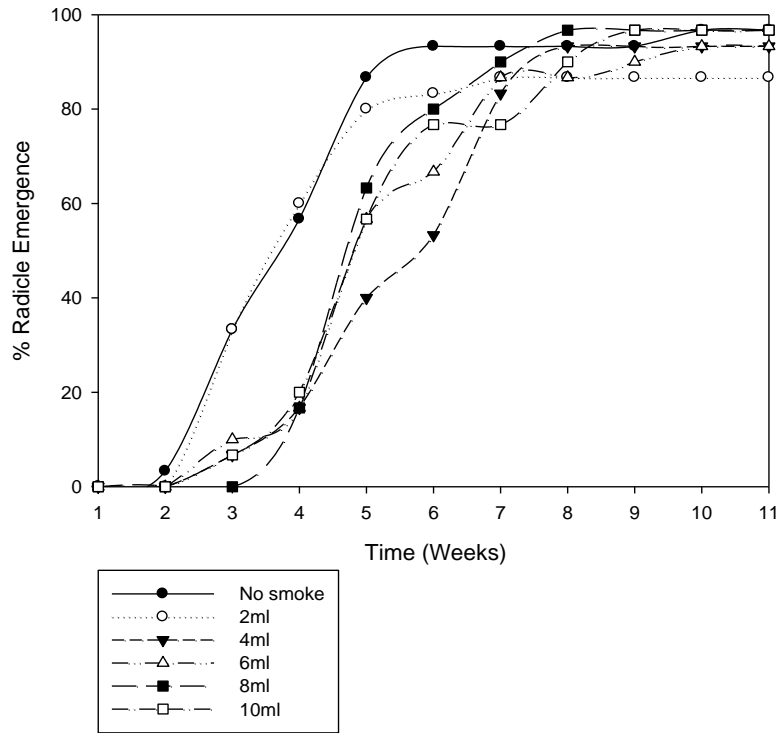


Figure 4.5: Percentage radicle emergence of *L. cryptocephalum* in different treatments (no smoke, 2; 4; 6; 8; and 10 mL⁻¹ aqueous smoke) on water agar media.

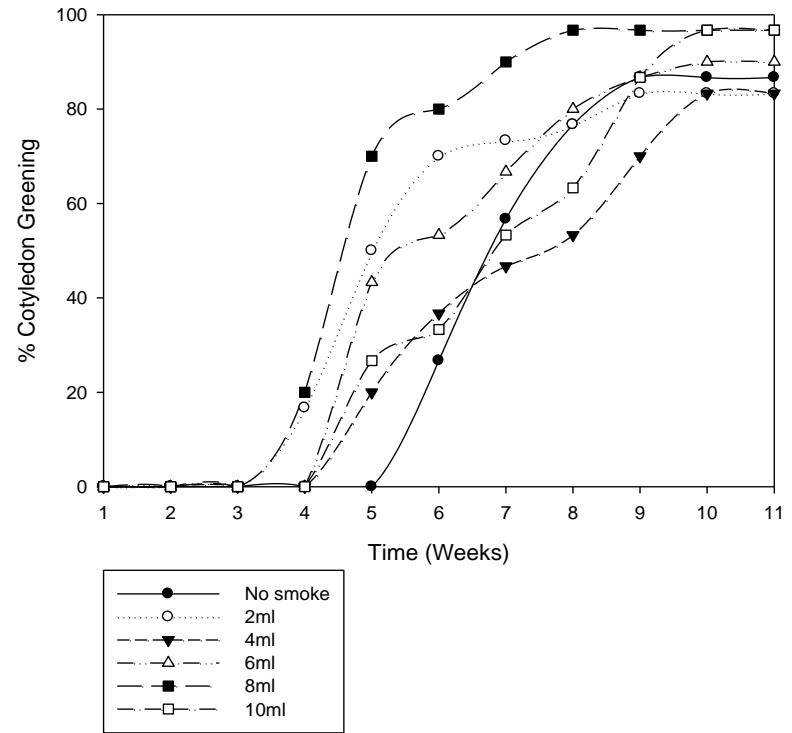


Figure 4.6: Percentage greening of cotyledons in *L. cryptocephalum* for different treatments (no smoke, 2; 4; 6; 8; and 10 mL⁻¹ aqueous smoke) on water agar media.

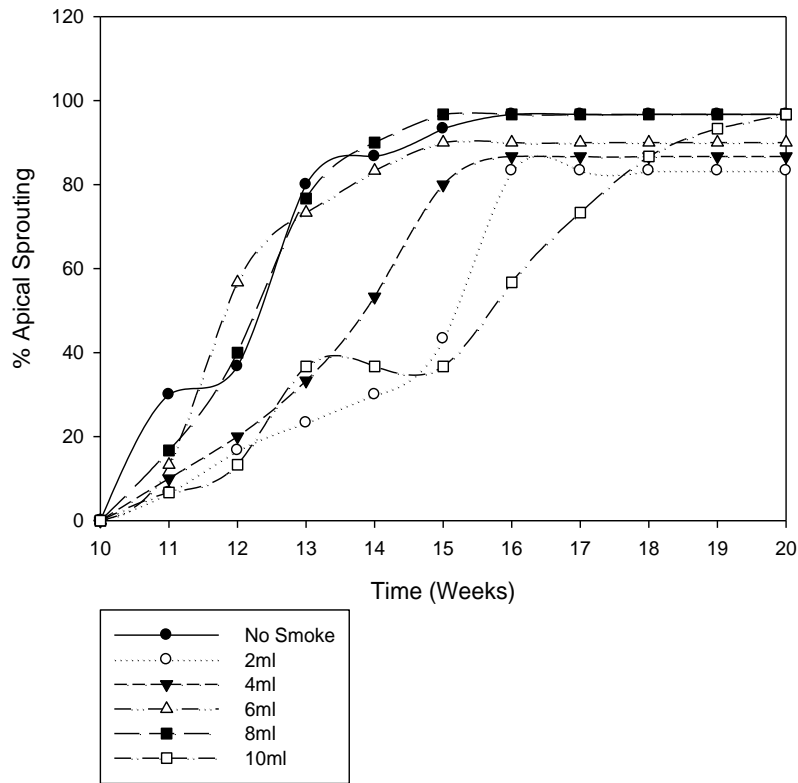


Figure 4.7: Percentage apical sprouting of *L. cryptocephalum* in response to different treatments (no smoke, 2; 4; 6; 8; and 10 mL⁻¹ aqueous smoke) on water agar media.

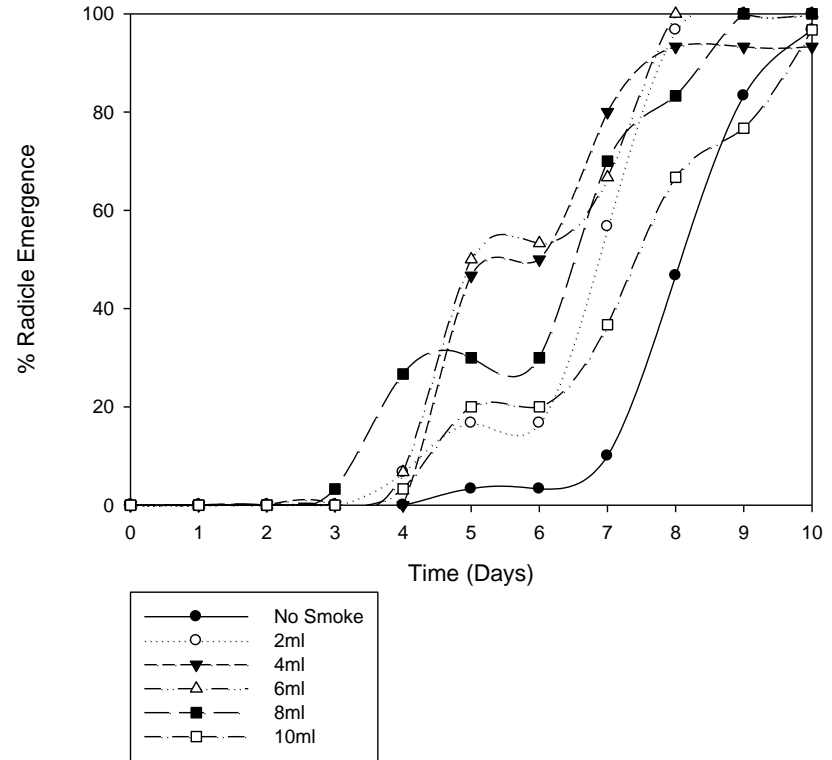


Figure 4.8: Percentages of radicle emergence of *L. salignum*, for different treatments (no smoke, 2; 4; 6; 8; and 10 mL⁻¹ aqueous smoke) on water agar media.

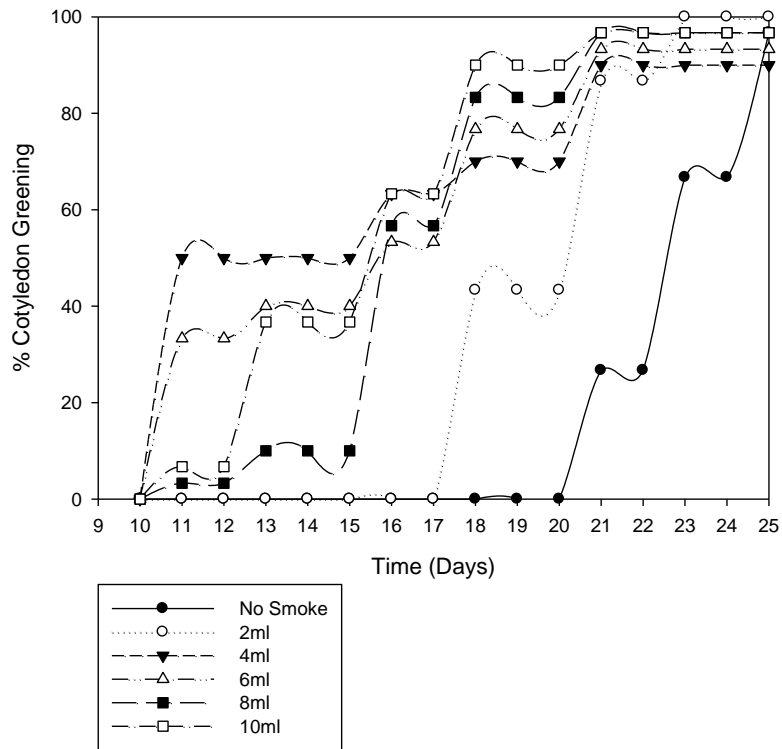


Figure 4.9: Percentage greening of cotyledons of *L. salignum*, for different treatments (no smoke, 2; 4; 6; 8; and 10 mL⁻¹ aqueous smoke) on water agar media.

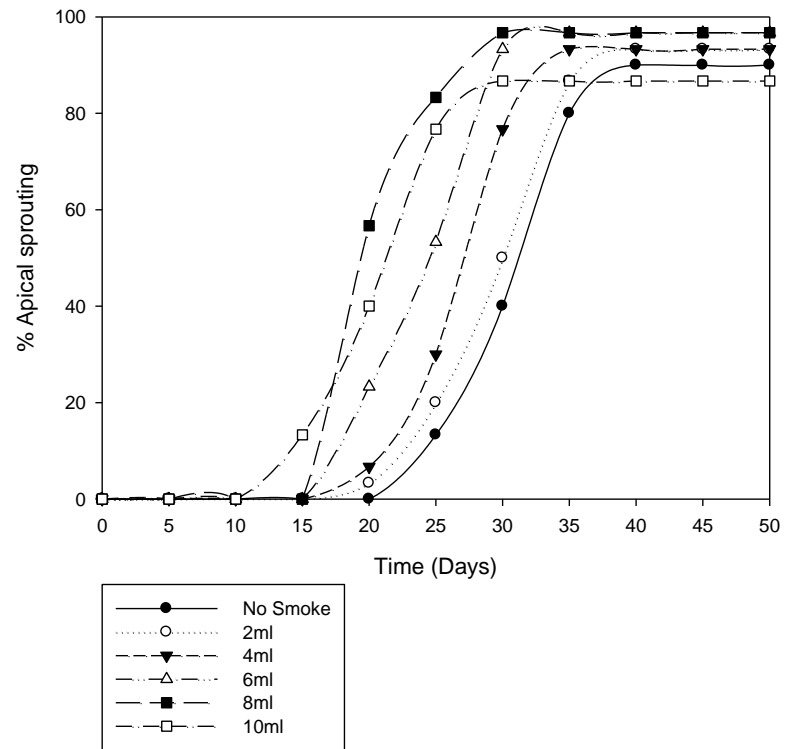


Figure 4.10: Percentage of apical sprout of *L. salignum*, for different treatments (no smoke, 2; 4; 6; 8; and 10 mL⁻¹ aqueous smoke) on water agar media.

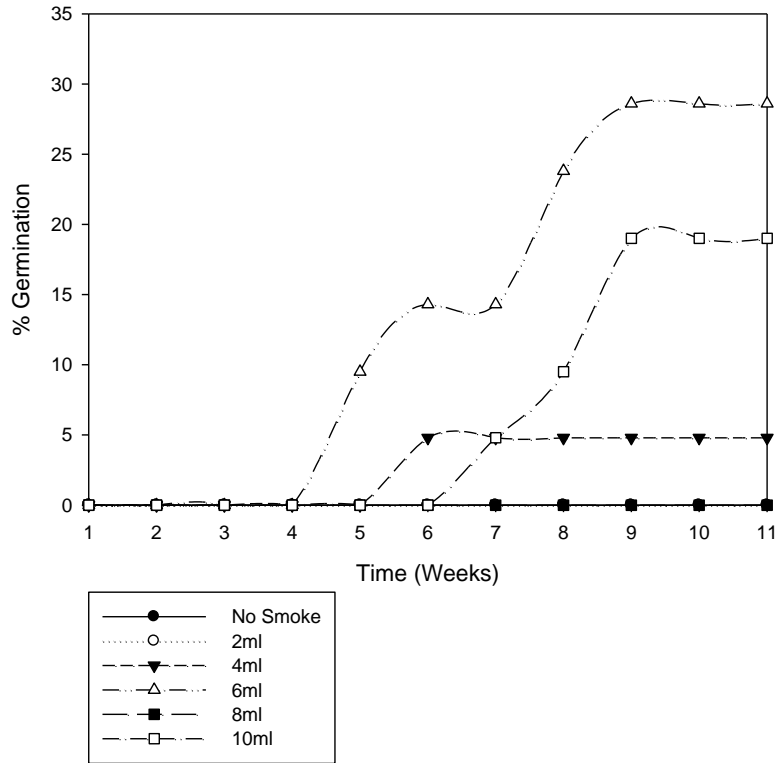


Figure 4.11: Percentage germination of *M. chrysanthus*, for different treatments (no smoke, 2; 4; 6; 8; and 10 mL⁻¹ aqueous smoke) on water agar media.

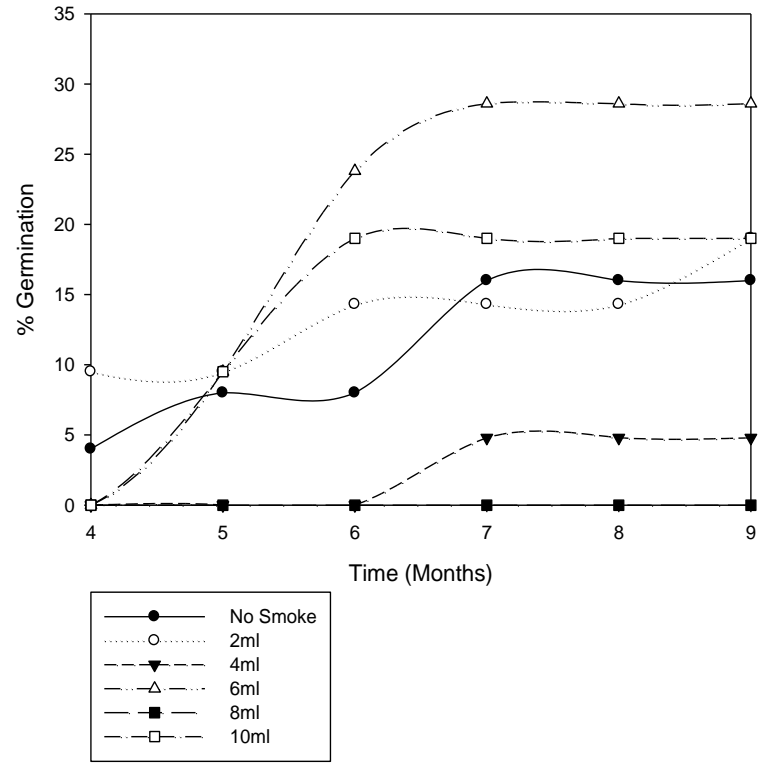


Figure 4.12: Percentage germination of *L. argenteum*, for different treatments (no smoke, 2; 4; 6; 8; and 10 mL⁻¹ aqueous smoke) on water agar media.

Table 4.2 Effect of different smoke concentration on the mean germination time, rate of germination and mean germination time of *L. cryptocephalum* seeds cultured on water agar with an incubation period of 11 weeks

Species	Parameters	No smoke	2 mLL ⁻¹ Smoke	4 mLL ⁻¹ Smoke	6 mLL ⁻¹ Smoke	8 mLL ⁻¹ Smoke	10 mLL ⁻¹ Smoke
<i>L. cryptocephalum</i>	Mean germination time (days)	59.9	56.5	82	78	75.8	78.9
	Rate of germination (%)	26.8	19.7	24.5	30	17.7	25.3
	Mean germination rate (day ⁻¹)	0.02	0.02	0.01	0.01	0.01	0.01

Table 4.3: Effect of different smoke concentration on the mean germination time, rate of germination and mean germination time of *L. salignum* seeds cultured on water agar with an incubation period of 10 days

Species	Parameters	No smoke	2 mLL ⁻¹ Smoke	4 mLL ⁻¹ Smoke	6 mLL ⁻¹ Smoke	8 mLL ⁻¹ Smoke	10 mLL ⁻¹ Smoke
<i>L. salignum</i>	Mean germination time (days)	20	16.1	14.2	14.5	15.1	17.4
	Rate of germination (%)	4.8	6.2	6.2	7.6	9.5	8.5
	Mean germination rate (day ⁻¹)	0.05	0.06	0.07	0.07	0.07	0.06

Table 4.4: Effect of different smoke concentration on the mean germination time, rate of germination and mean germination time of *M. chrysanthus* seeds cultured on water agar with an incubation period of 11 weeks

Species	Parameters	No smoke	2 mLL ⁻¹ Smoke	4 mLL ⁻¹ Smoke	6 mLL ⁻¹ Smoke	8 mLL ⁻¹ Smoke	10 mLL ⁻¹ Smoke
<i>M. chrysanthus</i>	Mean germination time (days)	-	-	84	95.7	-	115.5
	Rate of germination (%)	-	-	0	22.5	-	10.8
	Mean germination rate (day ⁻¹)	-	-	0.01	0.01	-	0.01

Table 4.5: Effect of different smoke concentration on the mean germination time, rate of germination and mean germination time of *L. argenteum* seeds cultured on water agar with an incubation period of 9 months

		No smoke	2 mLL ⁻¹ Smoke	4 mLL ⁻¹ Smoke	6 mLL ⁻¹ Smoke	8 mLL ⁻¹ Smoke	10 mLL ⁻¹ Smoke
<i>L. argenteum</i>	Mean germination time (days)	0	0	150	110	0	90
	Rate of germination (%)	0	0	0	27	0	31.6
	Mean germination rate (day ⁻¹)	0	0	0.01	0.01	0	0.01

4.3.3 The effect of temperature different regimes on germination rates

Proteaceae seeds germinate during the cold seasons (Bell, 1994). In correspondence to this ecological condition, an attempt to establish protocols for achieving high germination rates for the selected species was made, using two temperature regimes (10 ± 3 °C and 25 ± 2 °C) for *in vitro* germination. The winter effect was achieved by adjusting incubation temperature to 10 °C for *L. cryptocephalum*, *L. salignum* and *L. chamalaea*. Additionally, the germination responses of *L. chamalaea* were assessed against incubation at 25 °C to determine which temperature favored their germination. The results are outlined below:

4.3.3.1 Germination percentages, rates and time

Seeds of *L. chamelaea* had a gradual increase in germination percentages on all treatments in response to lower temperatures (Table 4.6). However, *L. chamalaea* displayed nearly two-fold greater germination percentages when aqueous smoke was incorporated in the germination media than in the control. The highest percentage of germinating seeds was 97 % in the media supplemented with 2 mL⁻¹ or 10 mL⁻¹ aqueous smoke germinated at 10 ± 3 °C. Hence, the study indicated that lower temperature coupled with aqueous smoke was beneficial for germination. Without smoke, germination percentages were 47 % at 10 ± 3 °C and 43 % at 25 ± 2 °C (Table 4.7; Table 4.6). There are various reports about the effect of temperature on the germination rate of *Leucadendron* species (Thuiller et al., 2004). Higher mean germination rate were achieved when species were exposed to lower temperatures compared with when incubated at 25 °C. Additionally, the mean germination time ranged from 40-48 days at 10 ± 3 °C and 80 %; with a mean germination time of range of 57-82 days at 25 ± 2 °C, (97%) being achieved (Table 4.7). The enhancement of the germination of *L. chamelaea* by temperature reduction and aqueous smoke mimics the field situation for this species in which germination occurs post fire with subsequent rainfall that occurs in winter.

4.3.4 The effect of Removal of seed coat on germination

According to Rebelo (1995) and Baker et al., (2004), the differences in viability may be explained by the different types of fruits yielded by *Leucadendron* species. In turn, the type of fruits produced by the different species becomes a major determinant of the seed coat quality. The latter may extensively have an influence on varying degrees of testa permeability and the regenerative capacity of the species. It can be inferred from the above results that the removal of seed coat increased germinability in *L. chamalaea*, *L. argenteum* and *M. chrysanthus*. This is consistent with studies by Wu & du Toit (2010), who demonstrated increased germination percentages when *Protea cynaroides* seed coats were removed compared to when they were not removed. Furthermore, Guo et al., (2012) report the positive influence of removing the seed testa in increased germination percentages and reduced germination time of *Grevillia* hybrids. However, in this study, the germination was not as high as in other studies. In $\frac{1}{2}$ MS supplemented media, there was a decline in germination of the decoated seeds. These findings support findings by Stock et al., (1990) in a study in which seeds of *Leucadendron* species germinate and thrived better in media deprived of essential nutrients.

The decoated seeds also had high levels of exudation which when they were imbibed and was likely to have also resulted from high injury of cotyledons during decoating. This has been proposed to cause seeds to abort or have reduced germination (Zhang et al., 2011).

4.3.4.1 Germination percentages, rates and time

There is evidence from the present study that the germinability increased with the removal of testa as indicated by the germination percentages of *L. argenteum* (20 %) (Figure 4.14) and *M. chrysanthus* (50 %) (Figure 4.13). *L. argenteum* yielded no germination for the control and 2 ml smoke; 10 % for 6 mL⁻¹ smoke; 6.7 % for both 4 and 8 mL⁻¹ smoke and 13 % at 10 mL⁻¹ of smoke (Figure 4.15). Addition of aqueous smoke to the germination media negatively affected the germination rate of decoated seeds of *L. argenteum*, exhibiting the highest germination rate on germination media without smoke (Table 4.10). The mean germination rate of decoated seeds of *L. argenteum* (Table 4.10) and *M. chrysanthus* (Table 4.8) was the same 0.01 day⁻¹. The mean germination rate was also higher when seeds were decoated compared with seeds with their testa, yielding minimal germination rates. When $\frac{1}{2}$ MS medium was used on the decoated seeds, there was a decline in germination percentages and *M. chrysanthus* displayed

the longest germination time compared to control experiments (Table 4.8), whilst *L. argenteum* positively responded to the ½ MS media and displayed a greater germination rate (Table 4.9).

Table 4.6: Germination percentages of *L. chamaleae* cultured on water agar with an incubation period of 11 weeks at 25±2 °C and 12 weeks at 10±3 °C.

Incubation temperature	No smoke	2 mLL ⁻¹ Smoke	4 mLL ⁻¹ Smoke	6 mLL ⁻¹ Smoke	8 mLL ⁻¹ Smoke	10 mLL ⁻¹ Smoke
10±3 °C	46.7	96.7	80.0	73.3	90	96.7
25±3 °C	42.9	23.8	76.7	83.3	80.0	-

Table 4.7: Effect of different smoke concentration on the mean germination time, rate of germination and mean germination time of *L. chamaleae* seeds cultured on water agar with an incubation period of eleven weeks.

		No smoke	2 mLL ⁻¹ Smoke	4 mLL ⁻¹ Smoke	6 mLL ⁻¹ Smoke	8 mLL ⁻¹ Smoke	10 mLL ⁻¹ Smoke
<i>L. chamaleae</i>	Mean germination time (days)	40.4	44.8	46.9	41.4	47.8	48.3
	Rate of germination (%)	21.9	30.7	40	26.4	22.6	25.7
	Mean germination rate (day ⁻¹)	0.02	0.02	0.02	0.02	0.02	0.02

Table 4.8: Effect of different smoke concentration on the mean germination time, rate of germination and mean germination time of *M. chrysanthus* seeds. cultured on water agar with an incubation period of twenty weeks.

<i>M. chrysanthus</i>		No smoke	1 mLL ⁻¹ smoke	½ MS+1 mLL ⁻¹ smoke
without testa	Mean germination time	80.5	86.8	102.4
	Rate of germination	46.6	40.4	37.5
	Mean germination time	0.01	0.01	0.01

Table 4.9: Effect of different smoke concentration on the mean germination time, rate of germination and mean germination time of *L. argenteum* seeds cultured on water agar with an incubation period of eight months.

		No smoke	½ MS+1 mLL ⁻¹ smoke
<i>L. argenteum</i>	Mean germination time	156	157.5
	Rate of germination	28	59.5
	Mean germination time	0.01	0.01

Table 4.10: Effect of different smoke concentration on the mean germination time, rate of germination and mean germination time of *L. argenteum* seeds cultured on water agar with an incubation period of six months.

		No smoke	4 mLL ⁻¹ Smoke	6 mLL ⁻¹ Smoke	10 mLL ⁻¹ Smoke
<i>L. argenteum</i>	Mean germination time	172.5	210	175	165
	Rate of germination	30	0	15.6	11.7
	Mean germination time	0.01	0.005	0.01	0.01

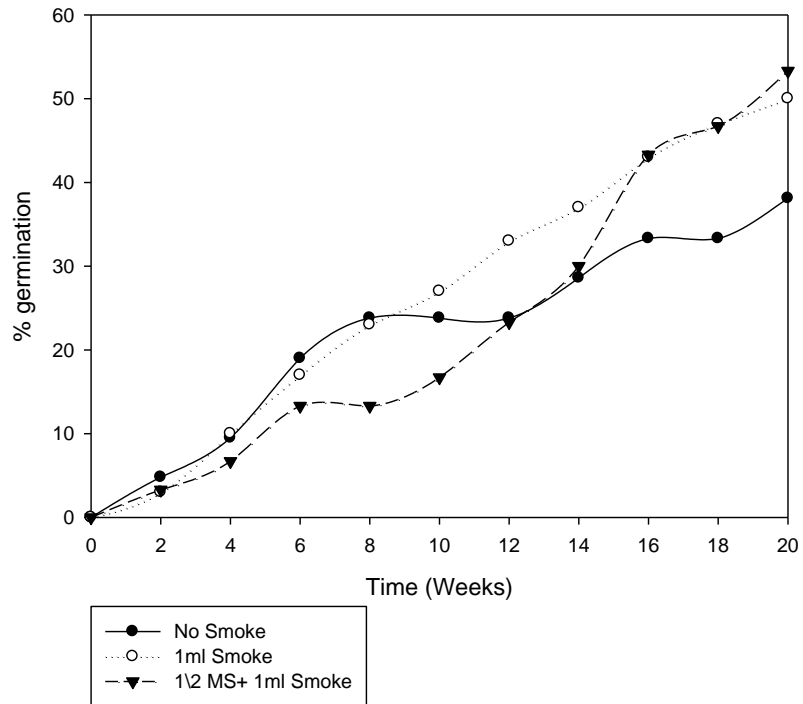


Figure 4.13: Effect nutrient enrichment on the germinability of decoated *M. chrysanthus* seeds cultured on $\frac{1}{2}$ MS supplemented with 1 mL^{-1} aqueous smoke with an incubation period of 12 weeks.

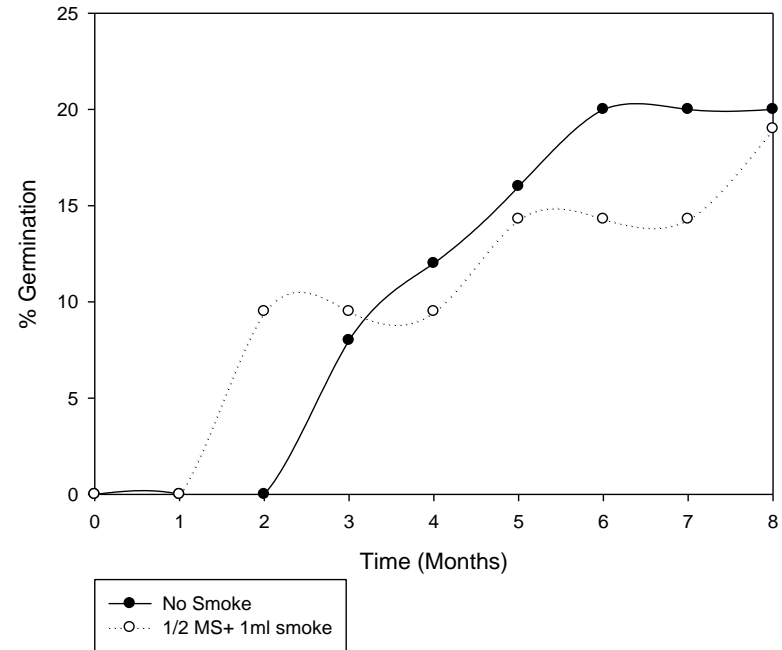


Figure 4.14: Percentage germination of *L. argenteum*, on nutrient enriched media, supplemented with 1 mL^{-1} aqueous smoke, incubated at $25 \pm 1 \text{ }^\circ\text{C}$; calculated as percentage values of all tested smoke concentrations.

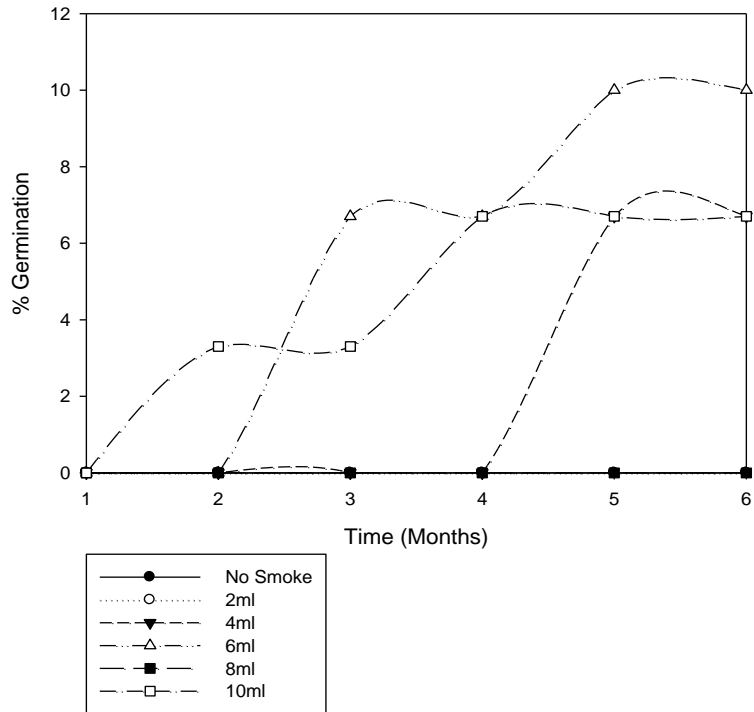


Figure 4.15: Percentage germination of *L. argenteum*, on nutrient enriched media, supplemented with different concentrations of aqueous smoke (no smoke, 2; 4; 6; 8 and 10 mL⁻¹) aqueous smoke, incubated at 10±3 °C; calculated as percentage values of all tested smoke concentrations.

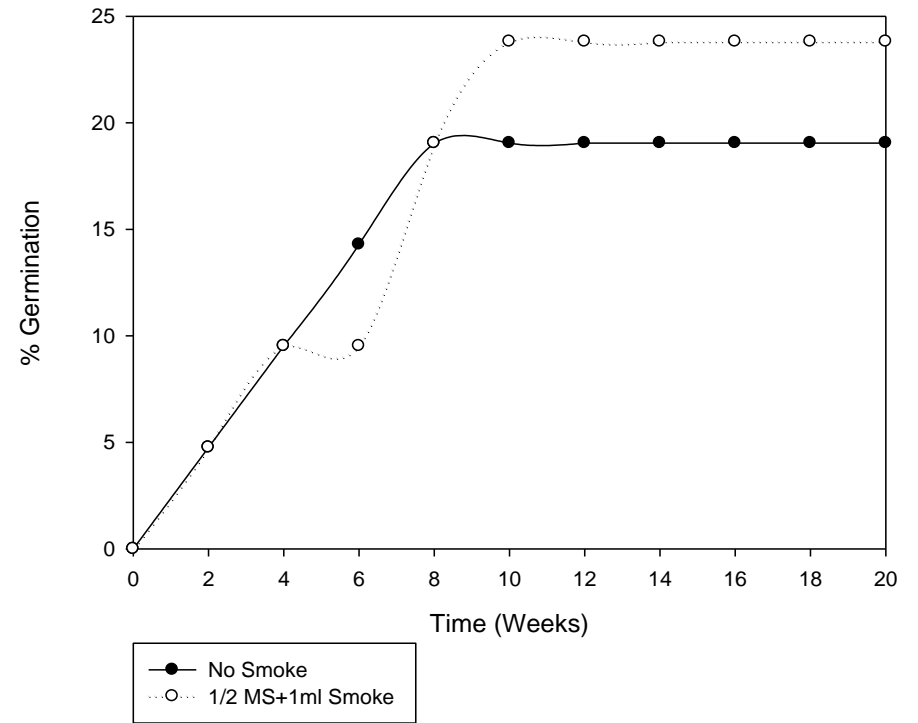


Figure 4.16: Percentage germination of *L. chamalaea*, on nutrient enriched media, supplemented with 1 mL⁻¹ aqueous smoke, incubated at 25±1 °C; calculated as percentage values of all tested smoke concentrations.

4.3.5 Viability test with 2, 3, 5-Triphenyl tetrazolium chloride (TTC)

To determine the condition of the seed lot, viability tests were conducted using TTC and the results in Table 4.11 show that for all populations, staining of the seeds was observed; and the highest percentage was recorded for *Leucadenron salignum*. The maximum viability of seeds was as follows:

Table 4.11: Degree of red staining on Proteaceae seeds in 2, 3, 5-Triphenyl tetrazolium chloride.

Species	Mean number of viable seeds			
	No change in colour	Pale Pink	Pink	Red
<i>L. argenteum</i>	13 %	10 %	13 %	64 %
<i>L. chamalaea</i>	7 %	12 %	15 %	66 %
<i>L. cryptocephalum</i>	10 %	10 %	17 %	63 %
<i>L. salignum</i>	5 %	8 %	10 %	77 %
<i>M. chrysanthus</i>	7 %	16%	10 %	67 %

Depending on the degree of staining of the cotyledon color, the viability test determined using TTC indicated that a greater percentage of seeds were not viable, whereas the *ex situ* germination rates were displayed a greater germination for the *L. cryptocephalum*, *L. salignum* and *L. chamalaea* (Table 4.11). Previously, Nikishina et al., (2007) has reported that seeds may tend to not reflect the real population viability when using TTC. This indicates that the TTC technique underestimates the seed viability compared with the *ex situ* germination. The TTC estimates of viability rates were higher for smaller seeds than for bigger ones, which were also observed in other experiments that smaller seeds were more responsive to germination treatments. The study also reveals a greater percentage of viable seeds for *L. argenteum* and

M. chrysanthus which was not reflected using the germination procedures. The mean percentage of embryos staining red was therefore does a reflection of the true viability of these species.

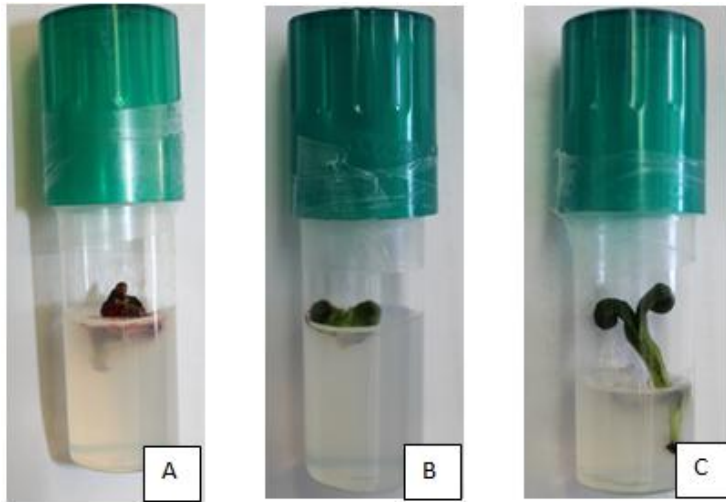


Figure 4.3: An outline of physiological changes on the *L. argenteum* seeds during germination. a) Radicle elongation of *L. argenteum* seeds; b) splitting of cotyledons; c) elongated seedling

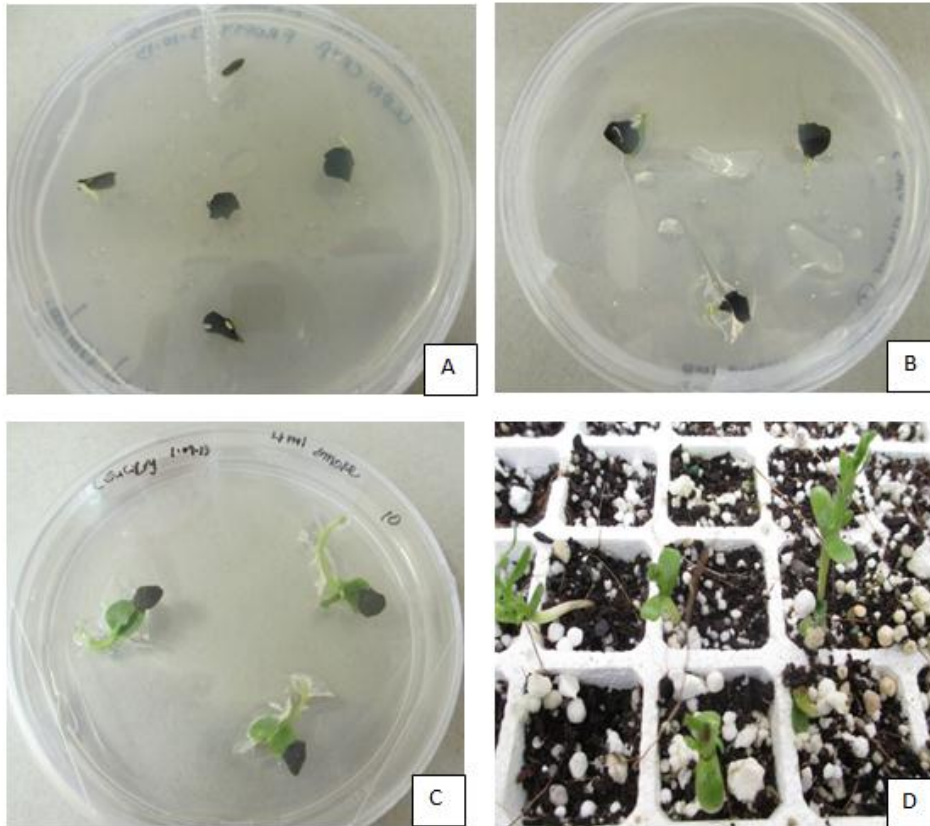


Figure 4.3: An outline of physiological changes on the *L. cryptocephalum* seeds during the germination process.

In this study, the germination achieved was not the same across the studied species. The possible reason for this could be a significant variation in size and the type of seed. The average seed mass of 108 to 241mg which in this study were considered to be larger seeds yielded the least germination as compared to the smaller seed with an average mass of 8.3 to 22.3mg. The circumference of the seeds however ranged between 4.7 mm to 8.4 mm for larger seeds and 3.5 to 3.8 mm for smaller seeds. It was noted that *L. cryptocephalum* despite having less mass, the circumference was greater as the seeds were flat. Germination was generally higher for smaller seeds, with a mean germination time lower than that of larger seeds. The absorptive rate of smaller seeds is higher; hence faster metabolic reactions are triggered compared with the larger seeds. The larger seeds also were characterised by thicker seed coats which tend to reduce the absorptive rate of this seeds. This was also observed with the TTC staining which restricted absorption. The *L. argenteum* and *M. chrysanthus* tend to have the

thicker seed coats and had the lowest germination success and staining with TTC. The *L. chamalaea* however also displayed a slightly thicker seed than the *L. cryptocephalum*, *L. salignum* as indicated with the prolonged germination time for the *L. chamalaea*.

4.3.6 Cryopreservation

Cryopreservation provides a cost effective means for the long-term *ex situ* conservation of plant germplasm, as has been shown with the successful cryopreservation of the seeds and embryos of many endangered plants (Touchell & Dixon (1993), Gonçalves & Romano (2009); Jitsopakul et al., 2012). Among the factors which are critical for successful cryopreservation is the rate of cooling (Reed 2001; Wesley-Smith et al., 2004), and this study investigated the effect of three cooling rates on successful cryopreservation of *L. chamalaea*, *L. cryptocephalum* *L. salignum*, *L. argenteum* and *M. chrysanthus*.

4.3.6.1 Germination percentages

In the current study, freezing the seeds in their most native state, covered with one layer of foil yielded the highest germination percentage in *L. argenteum* (73 %); *M. chrysanthus* (20 %); *L. salignum* (Figure 4.17, Figure 4.21 and Figure 4.18). On the contrary, this procedure yielded lower germination percentages in *L. chamalaea* (70 %) and *L. cryptocephalum* (73 %) compared with non-cryopreserved seeds as shown in Figure 4.20 and Figure 4.19. However, the use of the two staged cooling for *L. salignum* and *L. cryptocephalum* prior to cryo-storage increased their germination percentages which rendered as a better cooling rate compared to the others as indicated in Figure 4.18 and Figure 4.19. A decline in germination occurred on *L. argenteum* when seeds were exposed to the two staged cooling in the Mr Frosty container as Figure 4.17 shows that the lowest germination percentages of the cryopreserved seeds. Seed tolerance to storage in liquid nitrogen was efficacious when seeds of all the species tested were precooled in cryovials; however other precooling procedures yielded better germination percentages (Figure 4.17. to Figure 4.21)

4.3.6.2 Germination rate

The research in cryopreservation of seeds has been prolific, although with few applications on *in vitro* conservation of these endangered indigenous species. Touchell & Dixon (1993) have demonstrated that cryopreservation of seeds increases their germination percentages. An increase in the cooling rate promoted the germination rates of *L. salignum* and *L. argenteum* as indicated in Table 4.12, however, the other species displayed decline in the germination rate.

The rate of germination was considerably faster for seeds pre cooled in vials prior to freezing in liquid nitrogen for *L. cryptocephalum*, with a coefficient of variation of the germination time of 38.5% and mean germination rate of 0.03 day⁻¹ (Table 4.12). The efficacy of the rapid cooling procedure was demonstrated for the *M. chrysanthus* seeds, which displayed a coefficient of variation of the germination time of 24 % and mean germination rate of 0.02 day⁻¹ (Table 4.12). Furthermore, as depicted by Table 4.12, the coefficient of variation of the germination time of all species remained lower than the other cooling rates and the mean germination rates achieved for the seeds were relatively the same.

4.3.6.3 Mean germination time

Various studies have reported about the positive influence of cryo-storage on reducing the germination time of seeds. There is evidence from the present study that the mean germination time was reduced post cryo-storage. Increasing the cooling rate prior to storage in liquid nitrogen also reduced germination time compared with the other lower cooling rates for *L. salignum*, *L. chamalaea*, *L. argenteum* and *M. chrysanthus* (Table 4.12). The *M. chrysanthus* however displayed no germination at the slowest cooling rate (Mr Frosty).

4.3.6.4 Rewarming seeds

During rewarming, it was noted that 10 % ("less rapid" cooling); 3.3 % (rapid cooling) of *M. chrysanthus* and 16.7 % ("less rapid" cooling); 20 % (rapid cooling) *L. chamalaea*'s testa ruptured. The other species had their testa intact post rewarming. Reduced germination percentages after cryo-exposure was probably due to the increased exudation of cotyledon content observed in *M. chrysanthus*, *L. chamalaea* and *L. salignum* seeds. The adversity of seeds testa rupturing and exudation of electrolytes from the seeds could have had an injurious impact on the cotyledon, which could be associated with low germinability (Verleysen et al., 2004). Also, the study by Vendrame et al., (2007) confirmed observation for this study as seeds exposed to liquid nitrogen yielded reduced germination for *Dendrobium candidum* seeds.

Table 4.12: The effect of cryopreservation on MT (Mean Germination Time), CV (Coefficient of variation of germination time) and MR (Mean Germination Rate) of *L. salignum*, *L. cryptocephalum*, *L. chamalaea*, *L. argenteum* and *M. chrysanthus*

L. salignum

Treatment	MT	CV	MR
Control	15.4	13.7	0.06
“Less Rapid” Cooling	11.2	9.3	0.09
Rapid cooling	10.6	22.8	0.09
Mr Frosty	13.4	18	0.07

L. cryptocephalum

Treatment	MT	CV	MR
Control	61.4	33.6	0.02
“Less Rapid” Cooling	42.6	38.5	0.02
Rapid cooling	35	35.3	0.03
Mr Frosty	35.5	33.1	0.03

L. chamalaea

Treatment	MT	CV	MR
Control	53	33.2	0.02
“Less Rapid” cooling	47.2	32	0.02
Rapid cooling	43.3	30.5	0.02
Mr Frosty	47.7	28.1	0.02

M. chrysanthus

Treatment	MT	CV	MR
Control	0	0	0
“Less Rapid” cooling	65.3	24.7	0.02
Rapid cooling	67.7	15.6	0.01
Mr Frosty	0	0	0

L. argenteum

Treatment	MT	CV	MR
Control	84	0	0.01
“ Less Rapid” cooling	89.8	15.7	0.01
Rapid cooling	84.6	20.7	0.01
Mr Frosty	100.3	13.1	0.01

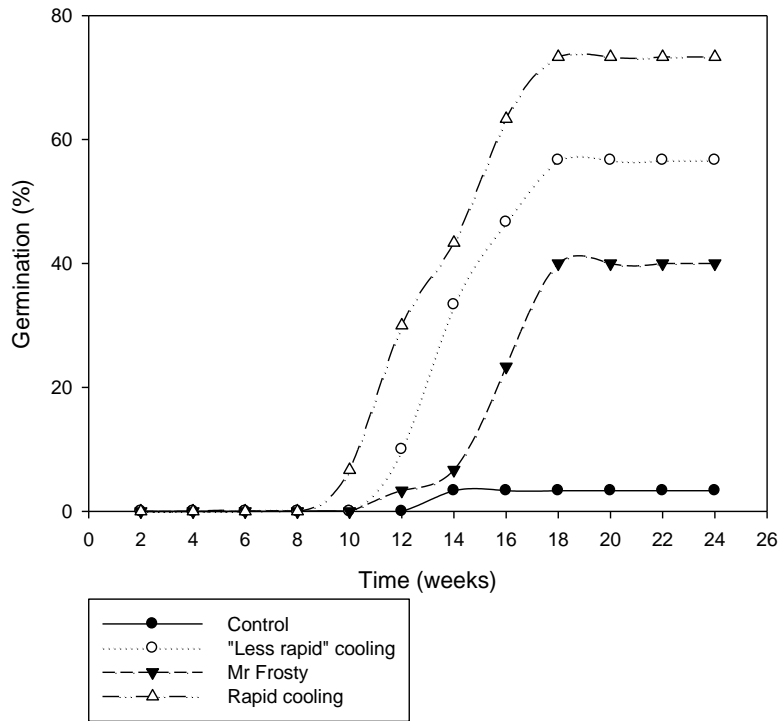


Figure 4.17: The effect of cooling rate on the survival and germinability of *L. argenteum* seeds post storage at -196 °C.

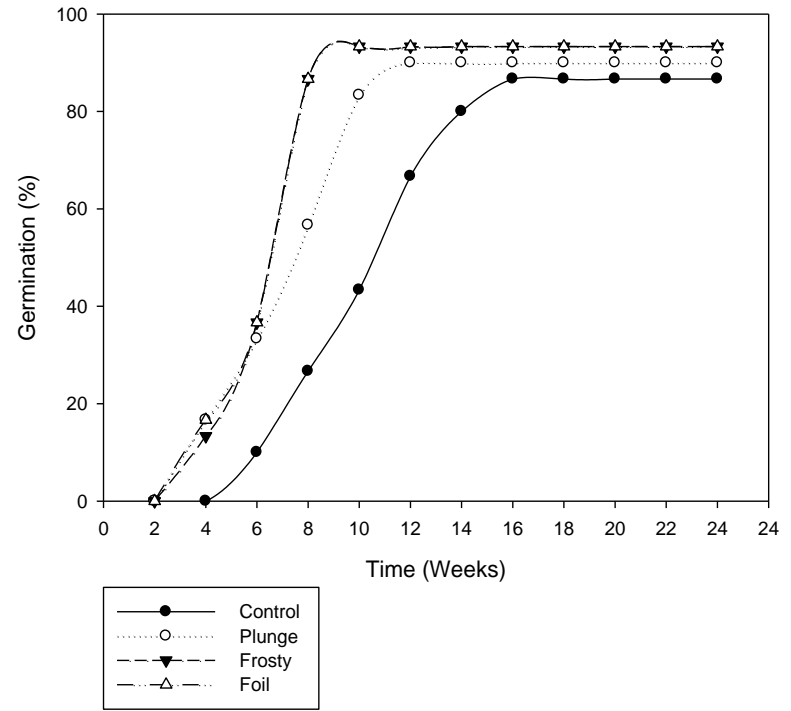


Figure 4.18: The effect of cooling rate on the survival and germinability of *L. salignum* seeds post storage at -196 °C.

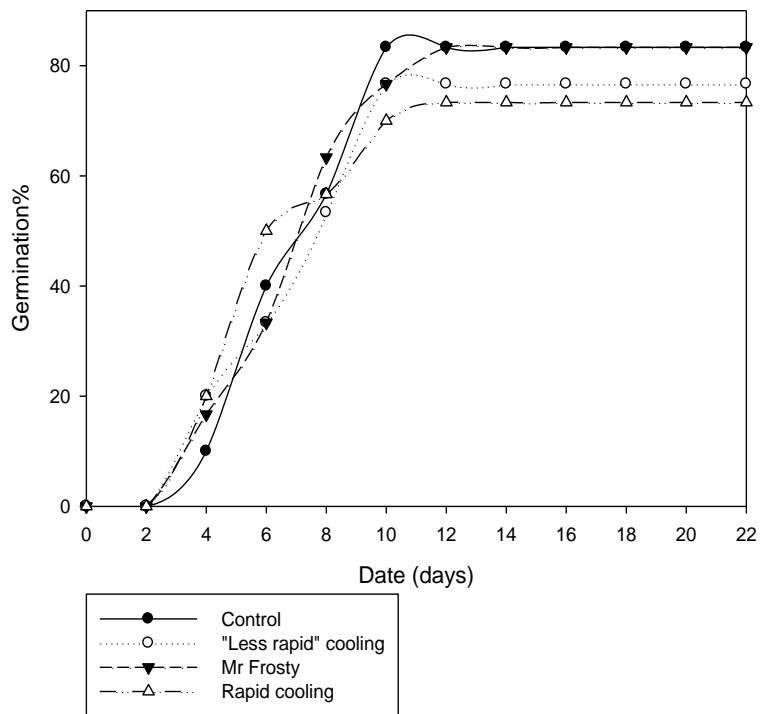


Figure 4.19: The effect of cooling rate on the survival and germinability of *L. cryptocephalum* seeds post storage at -196 °C.

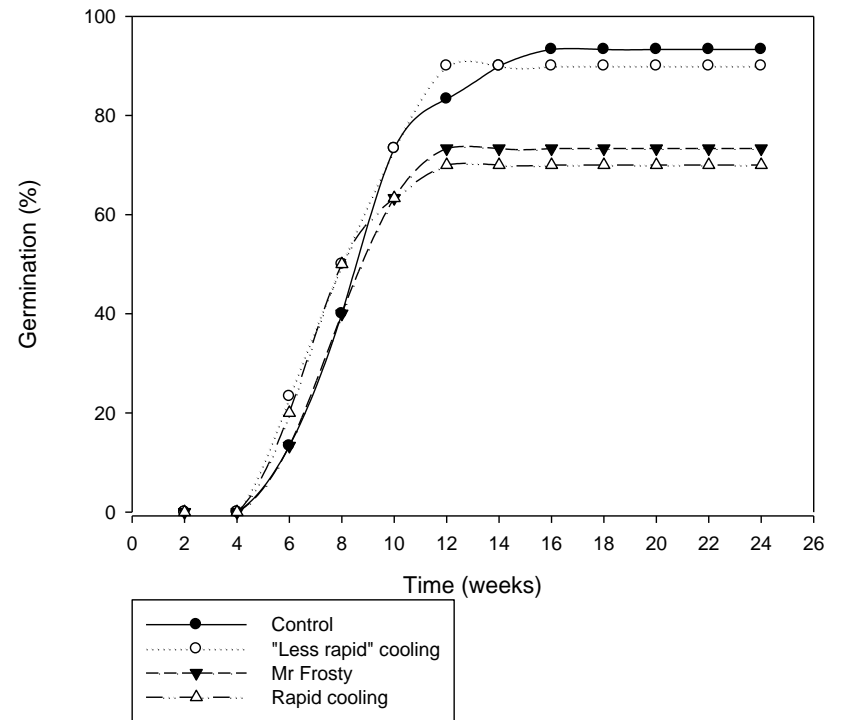


Figure 4.20: The effect of cooling rate on the survival and germinability of *L. chamalaea* seeds post storage at -196 °C.

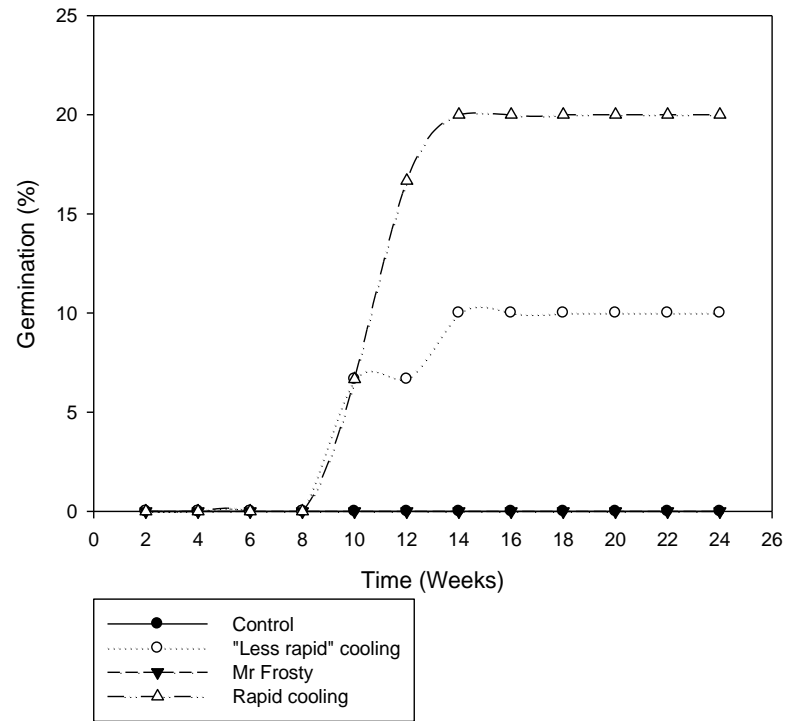


Figure 4.21: The effect of cooling rate on the survival and germinability of *M. chrysanthus* seeds post storage at -196 °C.

L. argenteum and *M. chrysanthus* seeds which were impervious to water and oxygen due to their thick seed coats displayed low germination in their most native state, despite the use of smoke as a germination cue. After storage in liquid nitrogen, there was tremendous increase in germination percentages especially for *L. argenteum*. The study therefore assumes that exposure to liquid nitrogen could act as scarification method, as this was displayed with other seeds rupturing during thawing. The study also assumes that liquid nitrogen acts as a stratification tool owing to the seed dependence on low temperatures to germination. Larger seeds tend to tolerate faster cooling rates prior to cryostorage. However, the smaller seeds display increased germination when slower cooling rates are used. The negative impact of fast cooling rates on smaller seeds could arise from an increased ratio of freeze damage relative to the size compared to the larger seeds.

4.4 Conclusion

As a result of ecological degradation on the natural environment, plant populations, such as other indigenous species tend to be subjected to decline. However, the *in-situ* conditions are not always ideal to curb against endangerment. This study seeks to promote conservation of endangered Proteaceae by improving *in vitro* seed germination and cryopreservation of the seeds. The study showed positive results for the use of *ex situ* germination as an alternative to TTC viability seed viability test for Proteaceae seed in efforts to determine population viability towards conservation. Staining using TTC proved unsuitable for this purpose, since as it is biased with respect to seed cotyledon colour. When using *ex situ* germination, it was observed that germinating *L. argenteum*, *L. chamelaea* and *M. chrysanthus* seed in aqueous smoke increased the germination rate, percentage germination and reduced the mean germination time. Removing the seed coat increased the germination percentages and reduced the mean germination time for *L. argenteum* and *M. chrysanthus*. Another method that has been identified to improve germination is reducing incubation temperatures to 10 ± 3 °C. The study further demonstrated that inoculation of decoated seeds in ½-strength MS media decreased the germination percentages of *M. chrysanthus* and the mean germination time remained the same. The optimum concentration for germination derived from this study was observed in agar media supplemented with 6 mL^{-1} smoke water at a constant temperature of 10 ± 3 °C.

For long term storage, cryopreservation of the Proteaceae seeds was conducted. In successfully storing seeds in liquid nitrogen, it is critical to ensure that there is minimal ice

forming crystallisation of cell contents; which is lethal to the seeds. Three cooling rates namely; plunging, rapid cooling and two-staged cooling were undertaken. The study determined that cryopreservation significantly increased germination percentages and germination rates for the Proteaceae seeds. Mean germination times were reduced by 5 days for *L. salignum*, 26 days for *L. cryptocephalum*, 10 days for *L. chamalaea*, as compared with the control experiment. The lowest mean germination time for *M. chrysanthus* was achieved when rapid cooling was used. To this end, it can be concluded that cryopreservation could be adopted as a tool for the preservation *Leucadendron* and *Mimetes* seeds for their utilization in reintroduction programmes. There was a positive effect for cryogenic exposure in the species seeds which was attributed to liquid nitrogen acting as a stratification tool to enhance germination.

4.5 References

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CHAPTER 5

4.6 Summary and concluding remarks

4.6.1 Overall discussion

Indigenous plants play an important role in ecological processes. In this regard, the Orchidaceae and Proteaceae are threatened with extinction by fragmentation within the Cape Floristic Region. This fragmentation could ultimately negatively impact the survival of other living organisms and significantly alter ecological functions and services. Also, the selected indigenous germplasm are economically and socially important, with *Disa*, *Leucadendron* and *Mimetes* ranking as the most traded plants in the floriculture industry within South Africa despite their ecological status. The main aim of this study was to facilitate conservation by determining optimum *in vitro* regeneration and long term storage protocols for indigenous endangered *Disa*, *Eulophia*, *Leucadendron* and *Mimetes* to prevent indefinite loss of these species.

Literature, promotes the use of organic substrates for *in vitro* propagation to replace the artificial plant growth regulators. This study aimed to determine the optimum multiple regeneration, growth and shoot elongation, rooting and increasing the adaptability to greenhouse conditions for endangered *Disa* and *Eulophia*, by using *Phalaenopsis* as a trial species because of the limited availability of the selected endangered seeds. The study revealed that ½ strength MS supplemented with 10gL⁻¹ banana extract induced somatic embryo development. However, there were limitations in the somatic embryo germinating and secondary protocorm regeneration was observed when this medium was used. To germinate the somatic embryos, ½ MS supplemented with 20gL⁻¹ banana extract resulted in the highest percentage growth. Roots were observed in all the banana extract media tested; however, the most roots occurred in the media supplemented with 30 gL⁻¹ banana extract. Supplementing the ½ MS media with 30 gL⁻¹ and 60 gL⁻¹ banana extract negatively impacted on the regenerative ability of the *Phalaenopsis* hybrids, yielding less protocorms and shoots. These two concentrations also resulted in stunted growth. Peptone at 1 gL⁻¹ enhanced the regeneration of somatic embryos and shoot development.

When peptone was combined with banana extract, there was increased rapid regeneration and shoot development.

Attempts to conserve the regenerated hybrids, revealed that mannitol caused growth retardation and shoots were able to be stored for up to fifteen months without subculturing. However, the study also shows that the *Phalaenopsis* hybrids were able to assimilate mannitol as regeneration and the leaf colour were normal and did not show signs of osmotic stress.

Seeds stored in liquid nitrogen exhibited improved seed germination, often retrieval with an increased mean germination rate. However, on thinner testa seeds, rapid cooling resulted in high exudation of cotyledon content and lower seed germination. Cryopreservation in general or rapid cooling as a long term storage strategy may be applicable to other South Africa's rare and endangered Proteaceae species, for successful germplasm conservation by using different freezing regimes. With a wide taxonomic range of Proteaceae taxa, further studies must be undertaken to investigate more parameters affected by the cooling rate. According to the literature review, the current conservation measures in the wild do not suffice as the threats still persist *in-situ*, resulting in the depletion rate being higher. Subsequently, these species may become extinct if they are not stored *in-situ*. The results obtained in this study show that germination can be hastened and increased by addition of aqueous smoke in the germination media coupled with lowering incubation temperature to 10° C for the selected species.

4.7 Recommendations

This research study reported on the influence of Banana extract on the regenerative ability and development and maturation of the selected *Phalaenopsis* hybrids. Further to this, the possibility of *in vitro* storage

However, the following aspects still need to be addressed:

- Determining the effect of the treatments at molecular level; transcriptional and genomic responses.
- Determine the effect of seed size on the germinability of seeds within the same species.

- Determine the cause of low germination for different types of Proteaceae.
- Micropropagation of the *Leucadendron* species and *Mimetes chrysanthus*.
- For cryopreservation, only the cooling rates were assessed. To enhance conservation further, the impact of cryopreservation should be determined *ex vitro*; in the wild, to determine if the stored seedlings can withstand the natural environment.

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