Cape Peninsula
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IN-VITRO PROPAGATION STUDIES OF THE ENDANGERED SUCCULENTS

DROSANTHEMUM MICANS AND *DROSANTHEMUM HALLII* (AIZOACEAE)

by

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DECLARATION

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

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Signed   **Date**

DEDICATION

I dedicated this work to my mother Nompendulo Elda Mlungwana and my late father Siphumo Robert Mlungwana. Lala ngoxolo Mzondi, uyawuhlala uphakathi kwentliziyo zethu.

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GLOSSARY

Terms	Definition/Explanation
Karroid area	of or characteristic of the Karoo.
Anthropogenic	of, relating to, or resulting from the influence of human beings on nature.
Acclimatization	is the process in which an individual organism adjusts to a gradual change in its environment (such as a change in temperature, humidity, photoperiod, or pH), allowing it to maintain performance across a range of environmental conditions.
Perennial	living for several years.
Papillae	small fleshy projection on a plant.
Geophytes	a perennial plant, such as a crocus or tulip, propagated by buds on underground bulbs, tubers, or corms.
Aseptic	culture the growth of any type of culture on a sterile medium.
Phytotoxic	a toxic effect by a compound on plant growth.
Leaf senescence	is the cause of autumn leaf color in deciduous trees.
De-ionized water	water that has the ions removed.
Hygrochastic moisture	of or relating to the opening of a fruit or flower caused by water or

ABSTRACT

Drosanthemum micans and *Drosanthemum hallii* are endangered succulent shrubs of horticultural and medicinal value. They are restricted to the Succulent Karroo, which is one of the world's biodiversity hotspots. The species risk extinction from illegal over-harvesting for water-wise gardens, erosion by occasional flush floods from ephemeral rivers, competition from alien invasive species, overgrazing and clearing of land for agriculture and human settlement. Although seeds and cuttings may be used in propagating these species, they often require seasonal collection and planting and cuttings struggle to establish, hence the need for *in-vitro* propagation as an alternative solution. Thus, the main objective of the study was to develop a method for rapid *in-vitro* shoot and root multiplication and acclimatization of *D. micans* and *D. hallii.*

To initiate shoot formation, disinfected leaf and stem nodal explants were cultured in Murashige and Skoog (1962) media supplemented with different rates (0, 10, 20 or 30µM) of 2-isopentyladenine, 6-Benzyladenine and kinetin for *D. hallii* or 2-isopentyladenine, 6-Benzyladenine and Thiadiazuron for *D. micans.* Shoots from explants were rooted in varying rates (0, 10, 20 or 30µM) of IAA for root initiation. Three media, which were used in previous studies, were tested for acclimatization of rooted explants in i) vermiculite, ii) sand (50%): vermiculite (50%) or iii) sand (75%): perlite (25%). For quantitative evaluation of plant stress, chlorophyll fluorescence index (Fv/Fm) was measured as a proxy for plant stressf stress.

It emerged that stem nodal explants of *D. hallii* tend to produce multiple shoots whilst leaf explants tended to produce callus when cultured in full-strength Murashige and Skoog (1962). Shoot multiplication was optimal in both *D. hallii* and *D. micans* at 10 µM of kinetin. Root formation in both *D. hallii* and *D. micans* only occurred when shoots were transferred to a full-strength Murashige and Skoog (1962) media without any phytohormones added. The intensity of tissue browning increased at higher levels of cytokinins, suggesting an interaction of plant growth regulators with exudates from explants. Different acclimatization media tested showed no significant differences in the level of stress (Fv/Fm).

It is recommended that Murashige and Skoog (1962) media with10 µM kinetin be used for shoot development and multiplication, followed by transfer of the shoots to fresh full-strength Murashige and Skoog (1962) media without hormones for root development. Acclimatization of the rooted explants was possible in one of the following media: i) vermiculite, ii) sand (50%): vermiculite (50%) or iii) sand (75%): perlite (25%) and in a misted greenhouse (*ca.* 60% RH), with gradual weekly reductions in humidity by 10% over 2 weeks.

CHAPTER 1

Introduction, background to the research problem

1.1 General introduction

1.1.1 Statement of the research problem

Drosanthemum micans and *D. hallii* are restricted to the Worcester and Robertson areas of the Succulent Karroo biome of South Africa, one of the world's biodiversity hotspots (Myers et al., 2000). Currently, there are no methods of rapidly multiplying these endemic species, yet the species are seriously endangered by the current anthropogenic global warming. The species have reached their maximum tolerable temperatures (Musil et al., 2005) and are potentially at risk of extinction from erosion by occasional flush floods from ephemeral rivers, competition from alien invasive species (Klak and Raimando, 2006), overgrazing (Hendricks et al., 2005) and clearing of land for agriculture and human settlement (Klak and Riamando, 2006). Whilst seeds and cuttings may be used in propagating these species, they often require to be done only seasonally (Oliver, 2006; Riddles, 2013).

1.1.2 Background of the study

The Succulent Karoo Biome of Southern Africa is a centre of plant diversity and endemism, with remarkably unique succulent plants (Klak et al., 2004). During winter there is low rainfall and in summer there is an extreme parch in the Succulent Karoo biome. The winter rainfall ranges between 20 and 290 mm per year and a surfeit of extremely hot summer days (*ca*. 40°C). Heulwetjies, which are mounds of calcium-rich soil, are of importance in this area as they often support distinguishable plant communities in the Succulent Karoo biome. Dwarf succulent shrubs are dominant in the area and most conspicuous plants are stonecrops (Crassulaceae) and vygies (Aizoaceae) (Klak et al., 2004).

Drosanthemum hallii and *D. micans* belong to the Aizoaceae family, which consists of 127 genera with about 1700 species. Most of the species are endemic to the Succulent Karoo Biome. The Aizoaceae forms a major and distinctive component of

Southern Africa's flora and dominate the vegetation in the Succulent Karoo region. *Drosanthemum* genus comprises of over 110 species found in the arid areas of Southern Africa. The species combine specialized morphological characteristics, which enable them to adapt and survive in arid environments. The diversity of the hygrochastic fruit capsules with rain-dispersed seed are used in distinguishing different genera (Chesselet, 2004). *D. micans* and *D. hallii,* commonly known as vygies, are low growing dense and perennial shrublets. The species have been widely planted in water-wise gardens and used for ornamental purposes due to their aesthetic value (Oliver, 2006; Riddles, 2013). *D. micans* flowers have red outer petals with chrome yellow inner petals and are mainly distributed in the karroid areas in the Worcester and Robertson lowlands (Oliver, 2006). *D. hallii* has bright yellow, showy flowers and is widely distributed in the Worcester–Breede River valley (Riddles, 2013). The populations of both *D. micans* and *D. hallii* are decreasing at an alarming rate and are at a risk of extinction and have been listed as endangered in the red list of South African plants (Raimondo et al., 2009).

Alien invasion, habitat loss or destruction, land clearing for vineyards are the major threats to the species, which has led the species to be endangered (Klak and Raimando, 2006). Previous studies suggest that the species may have reached their maximum tolerance temperatures and a further rise due to the current anthropogenic warming may wipe the species (Musil et al., 2005). Therefore, rapid multiplication is required for these species before they become extinct. Multiplication will ensure that adequate planting material is produced for potential restoration of *D. micans* and *D. hallii* populations in degraded areas.

D. micans and *D. hallii* can be propagated through seeds and cuttings. Cuttings of *D. micans* should be harvested in October or early March and seeds are produced in late October and must be dry and starting to split open before collection. *D. hallii* cuttings are done in June to August and seeds are harvested from January to February and sown from March to April. Although both species can be propagated sexually and vegetatively, they require specific periods for harvesting cuttings and for sowing seeds (Oliver, 2006; Riddles, 2013).

Fig 1.1: (A) *Drosanthemum hallii* and (B) *Drosanthemum micans*

In-vitro propagation is an alternative method that can potentially be used to multiply these endangered species. Advantages of micropropagation are that it allows rapid mass production of uniform plants. It can also be an alternative method for propagating species that have extremely low germination rates, very slow growing plants and those that are difficult to propagate vegetatively. Microprogation techniques can be done throughout the year as they do not require a specific season for propagation. Large scale production through somatic embryogenesis is an advantage to nurseries for mass propagation for a number of crop plants. Micropropagation results in mass production in a short period of time, within a small space. Pathogen-free plants can be produced through meristem culture (Jha and Ghosh, 2007). Although many plants have been propagated through micropropagation, however, there is no literature to my knowledge on micropropagation of the *Drosanthemum* species.

1.2 Objectives of the study

The main objective of this study was to develop a method for rapid *in-vitro* multiplication of *D. micans* and *D. hallii*. Specific objectives of the study were to:

1. Develop an ideal medium for inducing *in-vitro* shoot development in *D. micans* and *D. hallii*.
2. Develop an ideal medium for inducing *in-vitro* root development in *D. micans* and *D. hallii*.
3. Evaluate survival of *in-vitro* propagated *D. micans* and *D. hallii* when exposed to varying acclimatization conditions in the greenhouse.
4. Evaluate different root media for acclimatization of *D. hallii* and *D. micans*.

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CHAPTER 2

Is *in-vitro* propagation of endangered *Drosanthemum* species endemic to the Succulent Karoo possible?

2.1 Introduction

The Succulent Karoo Biome of Southern Africa is a global center of plant diversity and endemism, with remarkably unique succulent plants (Klak et al., 2004). Approximately 10 000 succulent species are known worldwide, of which one-third is harbored by the Succulent Karoo region. Consequently, the Succulent Karoo Biome was crowned as being the richest in succulent flora in the world (Cowling, 2016). The biome is home to over 1940 endemic plant species, of which *ca.* 674 species are either threatened or extinct (Brooks et al., 2002). Diverse reptiles and many invertebrate taxa, especially monkey beetles, are also endemic in this ecoregion, and are regarded as the most biodiversity arid area in the world (Le Maitre et al., 2009). However, only <3 % of all this diversified ecoregion is conserved in ten statutory reserves (Cowling, 2016).

The biome is home to over 1,700 species that are leaf succulents, 700 of which are stone plants and their allies (eg. *Conophytum, Lithops*) (Hilton-Taylor, 1996). Crassulaceae (stonecrops), Aloaceae and Aizoaceae (vygies) are major contributors of diversity in the Succulent Karoo. The Aizoaceae forms a major and distinctive component of Southern Africa's flora and dominate the vegetation in the Succulent Karoo region (Chesselet, 2004).

The main threats to the Succulent Karoo biodiversity are urban development, farming, forestry, mining, overgrazing of natural vegetation (Hendricks et al., 2005), land degradation, limited water, alien invasive species and illegal over collection for medicinal and horticultural purposes (Klak and Raimando, 2006). Due to the current anthropogenic global warming, the species have also reached their maximum tolerance temperatures (Musil et al., 2005).

Currently, some Aizoaceae species are critically endangered, this is compounded by lack of information on how to multiply or conserve them *in-vitro*. *In-vitro* propagation, also known as tissue culture, is propagation of plants in controlled, artificial environment in vessels or jars with defined growing medium (Smith, 2013).

In this chapter, previous work on *in-vitro* propagation of Aizoaceae is reviewed. Focus was thrust on the endangered Aizoaceae succulents that have medicinal and commercial value that have been propagated *in-vitro* for conservation purposes. The review also focused on the potential of *in-vitro* propagation of *Drosanthemum* genus.

2.2   Succulent plants and their importance in South Africa

South Africa is abundantly rich in succulent plants of which some species are endemic and belong to the prominent vygies (Aizoaceae), plakkies (Crassulaceae), milkweed family (Asclepiadaceae) and the noors family (Euphorbiaceae). Whilst succulents are found in all South African biomes, they are more abundant and diverse in the dry regions, particularly the Succulent Karoo. The tourism sector in South Africa has displayed high growth rates, consistently outperforming all the other sectors of the economy .Succulent Karoo is of international importance and a tourist attraction owing to its exceptionally unique diversity of succulents and geophytes (Khavhagali, 2010).

Richness of succulent plant diversity is related to the extensive and complex array of climate, terrain and soil (Nchai, 2008). Unique plant community structures overlay an unusual habitat islands, comprising of mosaics of quartz-fields and heulwetjies in most regions. Due to micro-habits presents, some succulent lineages in the Aizoaceae have undergone diversification which has resulted in the fine-scale demarcation of subtle edaphic gradients within the saline quartz-patch habitats. Further, the reliable seasonal winter rainfall and reduced thermal stress from quartz have also played a role in the evolution of quartz patch specialists (Schmedel and Jürgens, 1999). This has enabled the plants to survive extreme drought with limited water. Some species have a short life span and they store long-lived seeds underground when the plants die.

The main threats to the Succulent Karoo biodiversity are urban development, farming, forestry, mining, overgrazing of natural vegetation, land degradation, alien invasive species and illegal collection for medicinal and horticultural purposes and limited water (Khavhagali, 2010). There is an increase in the harvesting and collection of plant materials due to growth of population, more access and economic demand and that cause conservation problem. In the national and international markets, the horticultural, ornamental and medicinal plants, particularly succulents and bulbous taxa are of great importance and are highly in demand which led them being threatened and in need of more protection (Donaldson and Raimondo, 2000). Approximately 10% of plant materials traded in South Africa is succulents and 20% are geophytes (Le Maitre et al., 2009). In 1985, US$2,488 million was made from imports of cutflowers, cut foliage and plants (Oldfield, 1992). Internally, South Africa has a growing trade in succulent plants which is largely based on plants that are artificially propagated by commercial nurseries. The bulk of the propagated succulents are exported. During the 1940's and 1950's, there was an escalation of interest in collection, propagation and selling of succulent plants (Newton and Chan, 1998). South Africa exports succulent plants mainly to Europe, the United States and several far Eastern countries. From 1981 to March 1995, approximately 791,741 succulent plant specimens comprising of dry flower, herbarium specimens, packets of seeds, plants, plant cuttings, seed capsules and seedlings were exported to potentially importing countries (Newton and Chan, 1998).

Species from the Aizoaceae stabilize soil, their blossoms year round cater to various insects, their leaves are vital as fodder and their fruits nourish rodents hence they are an important group of plants. Garden planting is the main use of Aizoaceae plants as they produce a magnificent display in spring and early summer. The Aizoaceae species' also have medicinal uses that are widely well known. The juice from *Carpobrotus* leaves is highly astringent and used for sore throat, fungal infection as a traditional remedy (Omoruyi et al., 2012). *Sceletium* species have been used to combat fatigue, the relief of thirst and hunger, and as a stimulant known as *kougoed* (Gericke and Viljoen, 2008). Most Aizoaceae species are easily propagated from seeds or

cuttings and are cultivated in different seasons throughout the year depending on the species' requirement and where they occur (Smith et al., 1998).

2.3   Diversity of the Aizoaceae family

Aizoaceae, also known as the ice plant family, is an extremely well developed family ranging from annuals to perennial herbs, subshrubs, trailing woody plants, succulents and even small trees in Southern Africa (Klak et al., 2004; Klak and Bruyns, 2013). The Aizoaceae (formerly Mesembryanthemaceae) consists of 127 genera with over 1700 species, of which mostly are endemic to Southern Africa (Chesselet, 2004). Fruits have been extensively used in defining genera and groups of the Aizoaceae, with some recent consideration of their leaves and flowers (Hartmann, 1988; 1991 cited by Klak et al., 2013). The subfamily Ruschiodeae, is by far the largest group of plants in the Succulent Karoo, which consists of about 1,563 species in 101 genera (February et al., 2013). The extremely diverse Aizoaceae forms a distinctive and major constituent of southern Africa's arid land vegetation. The family is characterized by succulent leaves, bright shiny-petalled flowers and hygrochastic fruit as they display features not seen elsewhere in the plant kingdom. Landscapes, where vygies mostly occur, range from afromontane, karroid and arid to subtropical coastal belts and grasslands (Oliver, 2006).

The family mostly occurs in the southwestern regions of Africa, from Angola down to the Western Cape province of South Africa, expanding well into the east over the central plateau of South Africa and reaching into Zimbabwe and Botswana as well. Amongst the 25 globally recognized, Succulent Karoo Region is the world's only arid biodiversity 'hotspot (Cowling et al., 1998; Myers et al., 2000). About 20 Aizoaceae species occur naturally elsewhere, the rest is entirely endemic to southern Africa. Some *Delosperma* species are found from northern Tanzania up to the Yemen and also in the Arabian Peninsula and Madagascar, while a few of *Mesembryanthemum* occur around the Mediterranean Sea, and most species of the genus *Disphyma* are found in Australia, New Zealand and the adjacent islands (Barnard et al., 1998; Myers et al., 2000). *Sarcozona* species and some of *Carpobrotus* are restricted to Australia, but species of *Ruschia* and *Lampranthus* may not be indigenous to Australia. It is also not

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clear whether species of *Malephora* and *Carpobrotus* are recent introductions to South America, and the populations of *Carpobrotus* found along the west coast of North America were certainly introduced by man. *Drosanthemum* species are endemic to the winter-rainfall regions of southern Africa (Cowling, 2016).

2.4   *Drosanthemum* species

Drosanthemum is one of the genera that belongs to the Aizoaceae family and has more than 100 species described, of which some of them occur in Angolan border north through the western parts of Namibia and the rest in southwestern South Africa (Fig 2.1) (Court, 2010). The genus is widely distributed in the western parts of southern Africa; however, there are records of species in Lesotho (Smith et al., 1998). *Drosanthemum* species, also known as vygies, are prized by gardeners as one of the brilliant, vibrant, stunning genus from the Aizoaceae family. *Drosanthemum* species are sometimes called dewflowers due to their small succulent leaves that are covered with little crystalline blisters which make them glitter in the sun (Hartmann, 2011). Their stems are maroon to ochre with or without short or long hairs. Mostly, the leaf tips are rounded with rarely fused bases. The flowers are yellow, white, orange purple or scarlet and occur in clusters or solitarily with filamentous staminodes present or absent. The staminodes are pink, white or deep black. Long erect stalks bear fruit capsules and the fruits are light-colored and short-lived. Mostly spring and summer are flowering time and flowers are open during midday and closed by evening (Hartmann, 2001). *Drosanthemum* species grow easily from seeds and cuttings. Seeds are sown during autumn or in summer and cuttings are best taken after fruiting from midsummer to autumn (Smith et al., 1998).

Abbreviation depicts: WC- Western Cape; EC- Eastern Cape; KZN- KwaZulu-Natal; MP- Mpumalanga; FS-Free State; GP- Gauteng; LP- Limpopo; NW-North West; NC- Northern Cape
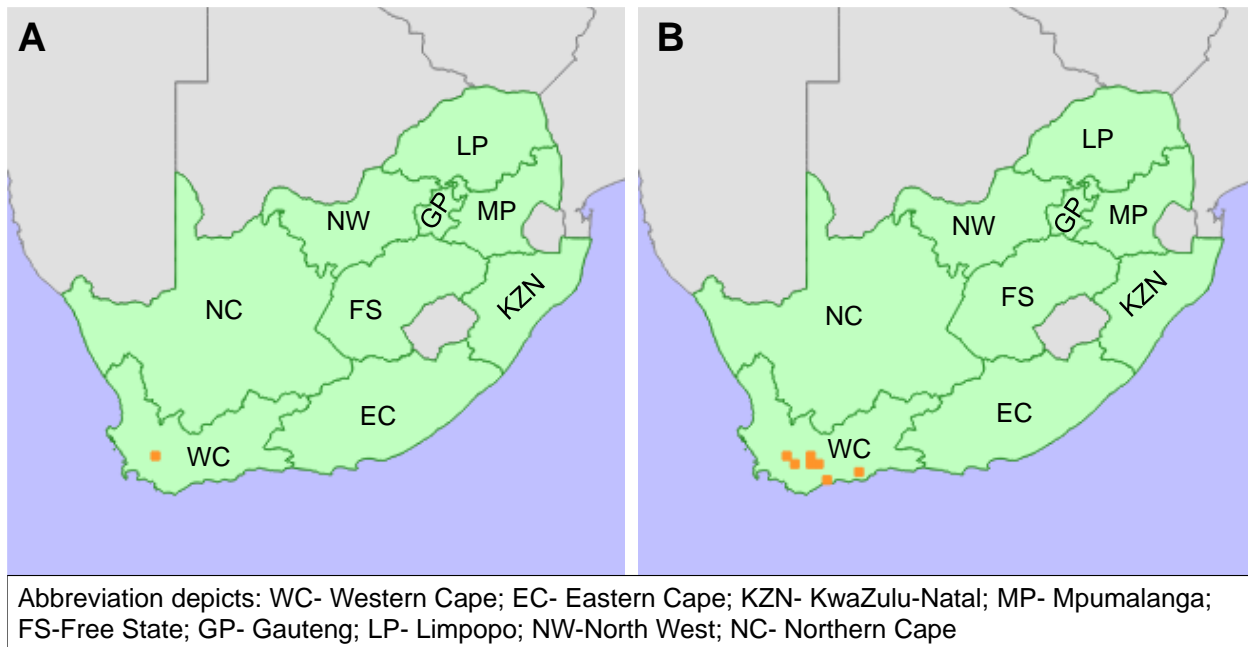
Fig 2.1: (A) Geographical distribution of *D. hallii* in the Worcester lowlands (Klak and Raimando, 2006) and (B) geographical distribution of D. micans in Montagu and Worcester to Swellendam (Klak and Raimando, 2006).

2.5 Taxonomy and conservation of the Aizoaceae family

It is a consequential challenge to effectively conserve South Africa's indigenous plants (von Staden et al., 2013). The world's richest temperature flora is mostly found in South Africa (Germishuizen et al.*,* 2006). In addition, South Africa is one of the only two countries in the world whose borders contain three globally recognized hotspots of biodiversity, namely Maputaland Pondoland Albany, Cape Floristic Region and Succulent Karoo (Mittermeier et al., 2005). These biodiversity hotspots have exceptional high levels of species diversity and endemism which are also under significant threat from human impact on the environment. Limited resources are allocated to conservation, which is why it is indispensable to develop effective conservation methods. Red Lists are primarily used to provide information in a wide range of conservation initiatives in South Africa, and because they are reliant on adequate taxonomic treatments, a sound taxonomic baseline is needed to avoid the misdirection of scarce resources resulting from a lack of knowledge of species, their distributions and habitat requirements.

Just over 50% of the Aizoaceae taxa are in need of revision as it is listed as top priority for taxonomic research. Eighty percent of the top 20 highest priority genera belong in the Aizoaceae and 81.3% of the 16 South African plant genera without any revision are also in the Aizoaceae. The Aizoaceae is on top on the list of South Africa's 20 largest plant families in need of revision, whilst *Drosanthemum* is in third place amongst the top 20 genera (containing more than 10 indigenous species) that are of priority in taxonomy research (von Staden et al., 2013). The South African National Biodiversity Institute (2015) indicated that more than 90% of *Drosanthemum* species are endemic to the Western Cape of South Africa, of which some are either threatened, rare or endangered (Table 2.1).

Table 2.1: Status of *Drosanthemum* species that are of concern and their distribution in
South African provinces

Species	Status	Provincial Distribution	References
D. autumnale	Threatened	WC	SANBI (2015a)
D. bellum	Endangered	WC	Klak & Raimondo (2006c)
D. chrysum	Rare	WC	Raimondo et al. (2008a)
D. crissum	Threatened	WC	SANBI (2015b)
D. dipageae	Threatened	EC	SANBI (2015f)
D. flavum	Endangered	WC	Helme et al. (2012)
D. giffenii	Threatened	WC	SANBI (2015c)
D. hallii	Endangered	WC	Klak & Raimondo (2006d)
D. lavisii	Endangered	WC	Klak et al. (2006)
D. leptum	Rare	WC	Raimondo et al. (2008b)
D. micans	Endangered	WC	Klak & Raimondo (2006a)
D. pulverulentum	Threatened	WC	SANBI (2015d)
D. quadratum	Endangered	WC	Klak & Raimondo (2006e)
D. ramosissimum	Rare	WC	Raimondo et al. (2008c)
D. thudichumii	Endangered	WC	Klak & Raimondo (2006b)
D. tuberculiferum	Threatened	WC	SANBI (2015g)
D. worcesterense	Threatened	WC	SANBI (2015e)
D. zygophylloides	Endangered	WC	Klak & Raimondo (2006f)

Abbreviation depicts: WC-Western Cape; EC- Eastern Cape

2.6 Cryopreservation of endangered Aizoaceae species

The plant biology community has resorted into looking for alternatives to *in situ* conservation due to the increasing loss of plant biodiversity both in nature and within agricultural systems. The storage of tissues, organs, organisms and viable cells at extremely low temperatures is called cryopreservation. Cryopreservation is usually done in liquid nitrogen to a minimum temperature of −196°C (Benson, 2008; Prudente and Paiva, 2017). For long-term storage, cryopreservation is an essential tool that can be used for future generation (Dhungana et al., 2017; Matsumoto, 2017). According to Engelmann (2004), cryopreservation can also be used for cryoselection, which is the selection through freezing of samples with special properties, and cryotherapy which is the elimination of viruses from infected plants through apex cryopreservation. Long-term storage of plant genetic resources requires a minimum space and maintenance only (Matsumoto, 2017). There is an increase in endangerment of species and also an interest in genetic engineering of plants. Therefore, preservation of cultured cells and somatic embryos with unique attributes is of greater significance (Sakai et al., 2008).

Cryopreservation was recently reported to be promising for intensifying preservation of endangered and rare plants (Touchell, 1995; Touchell and Dixon 1996). Storage can be in form of either primary or secondary. Embryogenic cultures can lose their capacity formation with time, but primary storage is provided by cryopreservation to revive culture and use them to produce more embryos at a later day (Kaviani, 2011). Secondary storage form is when a secure backup for living collections is designed for conservation of plant resources. The storage form depends on the reason for preserving plant collection and can either be seed, pollen, shoot apices, dormant buds, excised embryonic axes, zygotic or somatic embryos, callus or cell cultures depending on the species involved (Kalaiselvi et al., 2017). Cryopreservation is a useful tool for long-term maintenance of select plant germplasm, however, it is not a panpharmacon for global loss of biodiversity. Although there are many techniques/protocols available in literature, they are often difficult to interpret for everyday use (Towill, 2002).

The commonly used cryopreservation techniques are controlled rate cooling, vitrification, encapsulation dehydration and dormant bud preservation (Reed, 2001; 2017). Endangered species or those susceptible to diseases, climatic conditions, insects or other environmental conditions that lead to death of the plant, should be a top priority when cryopreserving (Reed, 2008). Cryopreservation is labour intensive and requires unlimited financial and human resources for the initial implementation. However, there are common techniques that do not require special equipment, fast and easy procedures and keep cryoprotectants non-toxic (Reed, 2001).

Although there is no literature on the cryopreservation of either endangered or least concern *Drosanthemum* species, cryopreservation has been done to an Aizoaceae species namely *Gunniopsis gabra* (Touchell and Dixon, 1993). Other taxa such as Musaceae (Panis et al*.,* 2005), Liliaceae (Chen et al., 2011) and more than 20 other families have also been cryopreserved (Touchell and Dixon, 1993).

2.7 *In-vitro* propagation of Aizoaceae plants

Modern propagation techniques can potentially meet the growing needs for plants in the horticulture industry. *In-vitro* propagation is one such technique that is used by growers to meet the demands of the industry. Gottlieb Haberlandt was the first to attempt culturing isolated single palisade cells from leaves in knop's salt solution enriched with sucrose but was unsuccessful (Hussain et al*.,* 2012). Several types of high-yielding tissue culture systems such as specific organ cultures, suspension cultured cells selected for high productivity on production media and high-density culture have been developed (Raveh et al., 1973; Liu and Staba, 1979; Watanabe et al., 1982; Zieg et al., 1983 cited by Dicosmo and Misawa, 1995).

The purpose of *in-vitro* propagation is to provide a viable alternative method (Waseem et al., 2008) to propagate plants that are not easily propagated vegetatively or by seeds. *In-vitro* culture also helps in propagating plants whose population are heavily declining due to lack of rapid multiplication propagation methods, to be re-established on their natural habitats. *In-vitro* micropropagation has enabled endangered, threatened

and rare species to be successfully multiplied, grown and conserved (Hussain et al., 2012). The advantages of *in-vitro* propagation are that mass clones are produced from a single explant or seed within a short time span, producing disease free plants, rapid propagation for seeds that do not readily germinate (Ikenganyia et al., 2017). The process of *in-vitro* propagation involves the establishment of the aseptic culture, multiplication of explants and preparation for acclimatization of plants in soil (Murashige, 1974a; 1974b).

Although there is no literature on the *in-vitro* propagation of *Drosanthemum* species, there are species from the Aizoaceae family that have been successfully propagated by means of *in-vitro* tissue culture (Table 2.2) (Sunangwa et al., 2007; Lokhande et al., 2010; Khattab and Sherif, 2011; Radfar et al., 2012).

Table 2.2: Species from the Aizoaceae family that have been cultured *in-vitro*, type of explants and plant growth regulators used in the MS media

Species	Explant	Plant Growth Regulator used for inducing			Sources
		Callus	Shoot	Root	
Carpobrotus edulis	leaf	TDZ	BA, Kin or 2iP	IAA, IBA or NAA	Khattab and Sherif (2011)
Sesuvium portulacastrum	axillary shoot, stem	-	Kinetin, TDZ or 2iP	IAA or NAA	Lokhande et al. (2010)
Zaleyade candra	callus	BAP or IBA+Kin	-	-	Radfar et al. (2012)
Mesembryanthemum chrystallinum	cotyledon hypocotyl	-	Kin, Zeatin, CPPU or TDZ+NAA	No PGR	Sunagawa et al. (2007)

Symbols/abbreviation depicts: (-) no induction experiment was conducted, no pgr- only media without plant growth regulator was used, TDZ: Thidiazuron, BA: 6-Benzyladenine, BAP: Benzylaminopurine, 2iP: 2-isopentyladenine, IAA: Indole-3-acetic acid; NAA-1-Naphthaleneacetic acid; IBA: Indole-3-butyric acid.

2.8 Growth regulators for *in-vitro* propagation of Aizoaceae

Plant growth regulators play critical roles in physiological and morphological processes, germination of seeds and growth of plants (Rahdari et al., 2013). Plant growth regulators such as auxins and cytokinins can inhibit factors such as growth of pathogens, phytotoxic metabolites and pathogen life cycle when added to the micropropagation medium (Helgeson et al., 1972 cited by King and Morehart, 1987).

Cytokinins play a role in developmental processes such as shoot growth and branching, control of apical dominance in the shoot, inducing plant cell division, chloroplast development, and leaf senescence (Mok, 1994; Werner et al., 2001). Cytokinins have the capability to induce the formation of shoots from unorganized callus tissue (Skoog and Miller, 1957), retard the rate of leaf senescence (Richmond and Lang, 1957), to stimulate pigment accumulation (Bamberger and Mayer, 1960), and support plastid development (Stetler and Laetsch, 1965). An example is benzyladenine (BA), which is a synthetic cytokinin-type of plant hormone (Rahdari and Sharifzadeh, 2012). The addition of BA, a synthetic cytokinin, increases the ratio of cytokinin to auxin in the plant, disrupting apical dominance (Cline, 1991). Generally, BA stimulates auxiliary and adventitious shoot proliferation, regulates cell cycle and cell division, regulates differentiation and inhibits root formation (Taiz and Zeiger, 2002).

Auxins play key roles in cell elongation, cell division, vascular tissue, differentiation, root initiation, apical dominance, leaf senescence, leaf and fruit abscission, fruit setting and flowering (Davies, 1987; Raoofi et al., 2014). Naphthalene acetic acid (NAA) is a synthetic plant hormone in the auxin family, which is a rooting agent that has been used in rooting horticultural plants (mostly for tissue culture purposes) and agricultural products (Cerveny and Gibson, 2005). NAA has also been indicated to be a potential antifungal agent in some investigations (Nakamura et al., 1978; Tomita et al., 1984; Michniewicz and Rozej, 1988 cited by Raoofi et al., 2014).

2.8.1 6-Benzyladenine (BA), 2-isopentenyladenine (2iP), kinetin and thidiazuron (TDZ) as shoot inducing hormones

Several scientific publications indicate that the combination of BA and NAA plant hormones is the best for shoot induction *in-vitro* culture (Saniewski et al.*,* 1974; Ault, 1995; 1996; Yadav et al., 2012). However, Lokhande et al. (2010) indicated that 40 µM of 2iP followed by 20 µM of BA proved to be more effective for shoot induction in *in-vitro* culture of *Sesuvium portulacastrum.* A rate of 4.7 µM of kinetin was effective in inducing shoots of *Carpobrotus edulis* (Khatttab and Sherif, 2011). Whilst 11.4 µM of TDZ was 85.7 % effective on adventitious shoot formation of *Mesembryanthemum chrystallinum* (Sunagawa et al., 2007). The rates of BA, 2iP, kinetin and TDZ that can be used, however, vary depending on the requirements of the species cultured.

2.8.2 Indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA) as root induction phytohormones

Although *ca.* 15 types of auxins have been isolated for potential use in plant tissue culture protocols (Sigma-Aldrich, 2015), the commonly used ones are indole-3-butyric acid (IBA), indole-3-acetic acid and 1-naphthalene acetic acid. IBA is thought to be a precursor of indole-3-acetic acid (IAA), the most abundant and the basic auxin naturally occurring and functioning in plants. IAA generates the majority of auxin effects in intact plants, and is the most potent natural auxin. NAA is a synthetic phytohormone auxin that is added to cell culture media for root initiation (Sigma-Aldrich, 2015). Although there is no evidence for use of auxins in rooting *Drosanthemum* cultures, the phytohormones has successfully been used in other Aizoaceae species such as *Carpobrotus edulis* and *Sesuvium portulacastrum* (Table 2.2).

2.9 Conclusion

South African plants continuously contribute substantially to the world trade of the horticulture industry (Reinten et al., 2011). Tissue culture is one of the investigated propagation methods to understand the potential of indigenous flora. In the absence of viable conventional propagation methods such as cuttings and seeds, *in-vitro* has

enabled recalcitrant and threatened plant species to be micropropagated. As more species face risk of extinction, in recent years, *in vitro* techniques have increasingly conserved threatened plants. In conclusion, evidences of micropropagated Aizoaceae species endorse that there is a potential to conserve *Drosanthemum* species via *in-vitro*. Conservation of threatened or endangered *Drosanthemum* species can benefit from *in-vitro* multiplication and acclimatization protocols that focus on propagating *Drosanthemum* species for reintroduction to its habitat.

2.10   References

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CHAPTER 3

In-vitro propagation studies of the endangered succulent
Drosanthemum hallii (Aizoaceae)

3.1 Introduction

Drosanthemum hallii (Aizoaceae) is a low growing, dense and perennial shrublet, which flowers during early spring in the Succulent Karoo biome of Southern Africa. The biome is a global centre of plant diversity and endemism (Myers et al., 2000; Klak et al., 2004), with remarkably unique succulent flora, such as the Aizoaceae (ice plants). Aizoaceae plants dominate the biome in terms of species numbers (1,750 species in 127 genera) and density of coverage (Ihlenfeldt, 1994; Low and Rebelo, 1996; Chesselet, 2004). Whilst some clades of the Aizoaceae that are almost exclusively endemic to southern Africa have diversified very recently and very rapidly, some species such as *D. hallii* are threatened with extinction. *D. hallii* has leaves that are oblong to roundish in shape with shimmering papillae and bright yellow showy flowers (Jacobsen, 1970). The fibrous root system of *D. hallii* is adapted to quickly absorb the often patchy soil moisture (Schwantes, 1957). The centre of the flower comprises of numerous filamentous white or pink, with a few black staminodes arranged in a cone. The species, which is endemic in the Worcester area, has now been widely planted in water-wise gardens and used for ornamental purposes owing to its aesthetic value (Herre, 1971; Riddles, 2013).

The population of *D. hallii* is decreasing at an alarming rate and the species was listed as endangered in the red list of South African plants (Raimondo et al., 2009). Invasion by alien invasive species, habitat loss or destruction and expansion of vineyards are some of the major threats to the species (Klak and Raimando, 2008). Most Aizoaceae species have reached their maximum tolerable temperatures and current anthropogenic global warming may further drive their rate of loss (Musil et al., 2005). Rapid multiplication is therefore needed for the species to build up current populations before they become extinct. Rapid multiplication methods will ensure that adequate planting material is produced for water-wise gardens thus minimizing

overharvesting of wild populations. Multiplication protocols developed can be adopted for bulking-up nursery populations for commercial use and reclamation of degraded areas.

Currently, *D. hallii* can be propagated through seeds and stem cuttings. Stem cuttings of *D. hallii* are done from June to August and seeds are harvested between January and February, with sowing being done from March to April. Availability of seed is seasonal although these species can be propagated sexually or vegetatively (Oliver, 2006; Riddles, 2013).

In-vitro propagation provides an alternative method that can potentially be used to multiply these endangered species. Unlike seeds and cuttings, *in-vitro* propagation allows rapid mass-production of uniform plants, which is an advantage for commercial nurseries. It is also an alternative method for propagating species that have extremely low germination rates, very slow growing plants and those that are difficult to propagate vegetatively. *In-vitro* propagation techniques can supply seedlings throughout the year as they do not require a specific season for propagation. *In-vitro* propagation through meristem culture results in mass production of pathogen-free plants in a short period of time, within a small space (Jha and Ghosh, 2007). Although many plants have been successfully propagated through *in-vitro* methods, literature on micropropagation of *D. hallii* is scarce.

The objectives of this study were: i) to determine optimal cytokinin concentrations for shoot initiation and ii) optimal auxin concentration IAA for root initiation when using either leaf or stem nodal explants.

3.2 Materials and methods
3.2.1 Explant source

Potted and disease-free *D. hallii* plants were collected from Karoo Desert Botanical Garden, Worcester, South Africa (19°27'01.7" East 33°37'00.2" South and Longitudes 19.44976 Latitudes -33.61217). After 2 weeks of acclimatization under

greenhouse conditions at Cape Peninsula University of Technology, Bellville Campus, leaf and stem nodal explants were excised for *in-vitro* propagation.

3.2.2 Sterilization method

Excised explants (*ca.* 30 mm long, 3 mm diameter), with a node intact, were washed under running tap water to remove soil and loose debris and then rinsed with deionized water. The explants were surface sterilized with 70% ethanol solution for 30 sec then rinsed with deionized water. Explants were then sterilized with 3.5 % v/v sodium hypochlorite (NaOCl) in deionized water with the addition of two drops of 0.1% Tween-20 for 20 min and then rinsed three times for 20 minutes each interval with sterile distilled water under laminar flow hood.

3.2.3 Culture medium and conditions

The sterilized explants were cultured in Murashige and Skoog medium (Murashige and Skoog, 1962) containing basic salts and vitamins, supplemented with 3% (w/v) sucrose, 7.5 g L^{-1} agar and varying concentrations/combinations of 0, 10, 20 or 30 µM of 6-Benzyladenine or 2-isopentyladenine or Kinetin. For root induction the MS medium was supplemented with 10 µM of Kinetin (based on this study) and then different rates (0, 10, 20 or 30 µM) of IAA were tested. The pH of the medium was adjusted to 5.7 prior autoclaving at 121°C for 20 min. Media were sterilized by autoclaving at 1.05 kg cm^{-2} for 20min, and 25 mL of each medium placed in 250-mL containers and placed in a fridge (4°C). After 7 d, the shoots were transferred to a culture medium and the culture jars sealed with parafilm after explant implantation. The cultures were then incubated under growth room conditions (25±2°C), with a photoperiod of 16 hour light 8 dark photoperiod and light intensity of 4000 kLux provided florescence lamps (Philips TLM 40W/33RS).

3.2.4 Acclimatization

Rooted shoots from the growth room were taken to the preparation room and the agar gently washed-off under running tap water. The roots were covered in a

moist sterile cloth to avoid desiccation during planting. Shoots were planted in 36-cell polystyrene plug trays, each cell containing 160 cm^3 of one of the media namely i) 3 sand: 1 peat ii) vermiculite or iii) 1 sand: 1 vermiculite. The trays were then transferred to the greenhouse set at 30-35°C temperature and 65-75% relative humidity for acclimatization. To minimize the stress of the plants, wire hoops were placed above the trays and used to hold plastics covering the plug trays. Seedlings height, leaf numbers, root length and chlorophyll fluorescence index (Fv/Fm) measurements were measured on the acclimatizing seedlings. Fv/Fm is a normalized ratio created by dividing variable chlorophyll fluorescence (Fv) by maximum chlorophyll fluorescence (Fm), which indicates the maximum potential efficiency of Photosystem II in plants if all capable reaction centres were open. Practically, an Fv/Fm value in the range of 0.79 to 0.84 is the approximate optimal value for many plant species, with lowered values indicating plant stress (Maxwell and Johnson, 2000).

3.2.5 Data collection and statistical analysis

Due to limited plant material only ten cultures were used for each treatment. Counts of callused explants, shooted explants, rooted explants and browning were recorded every fortnight and the data were transformed using log (x+1) and a one-way analysis of variance (ANOVA) done using STASTICA 12 Software. Fisher's least significant difference (LSD) was used to separate the means at P ≤ 0.05 level of significance.

3.3 Results

3.3.1 Callus induction in D. hallii

Callus, which is a mass of undifferentiated cells, was noted only at two concentration levels of 2-isopentyladenine. In each case, a compact callus of cells that were tightly joined forming a solid mass of tissue was produced. Callus grew from stem explants grown in full-strength Murashige and Skoog (1962) medium supplemented with 20 µM whilst leaf explants callused when supplemented with 30 µM of 2-isopentyladenine (Fig. 3.1, Fig. 3.2 A-D). Callus formation increased with the

higher level of 2-isopentyladenine of 30 µM (Figure 3.1, Fig. 3.2 A-D). Of the two callusing treatments, leaf explants in 30 µM of 2-isopentyladenine had a two-fold significantly (P<0.05) higher percentage of callused explants. On the contrary, no callus formed with both 6-Benzyladenine and Kinetin on all the tested rates (0, 10, 20 and 30 µM). Callused explants produced indirect or *de novo* shoots.

Fig 3.1: Percentage on counts callused explants of *D. hallii* on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of 2-isopentyladenine (2iP), 6-Benzyladenine and Kinetin after 3 months in culture
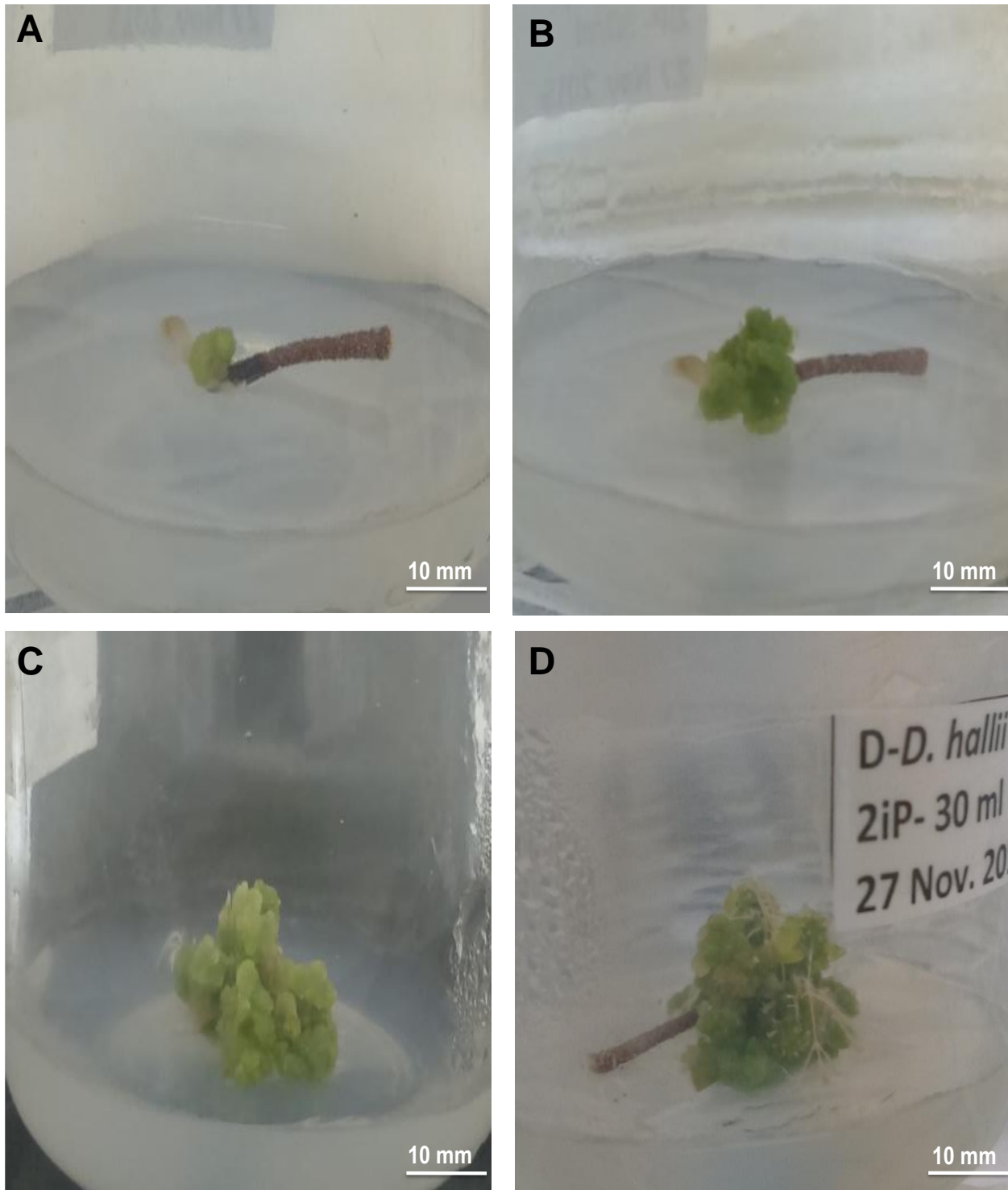
Fig 3.2: Callus formation of *D. hallii* stem nodal explants after (A) 5 weeks, (B) 11 weeks (C) 15 weeks and (D) 18 weeks of culturing on full-strength Murashige and Skoog (1962) supplemented with30 µM of 2-isopentyladenine

3.3.2 Shoot formation of D. hallii

Most stem nodal explants produced shoots directly from their internodes without callus formation. The different rates (0, 10, 20 and 30 µM) of 2-isopentyladenine, 6-Benzyladenine and Kinetin had significantly (P< 0.001) different proportions of shooted explants (Fig. 3.3; Fig. 3.4). The highest number of shoots from stem nodal explants emerged when cytokinins were not supplemented (i.e. 0 µM) and declined at higher levels. Thus, supplementing with 10-20 µM of 2-isopentyladenine led to a five-fold decrease in number of shooted stem nodal explants compared to control plants without cytokinins, whilst a higher rate of 30 µM inhibited shoot formation. Likewise, shoot production in stem nodal explants significantly (P< 0.001) declined with higher levels of either 6-Benzyladenine or kinetin. However, supplementing with higher rates of Kinetin from 0-30µM, resulted in slightly lower shooted explants (viz. 60%) compared to 6-Benzyladenine which had 20%. Stem nodal explants produced more new shoots than leaf explants. Unlike stem nodal explants, the leaf explants had more shoot forming at higher levels of 6-Benzyladenine.
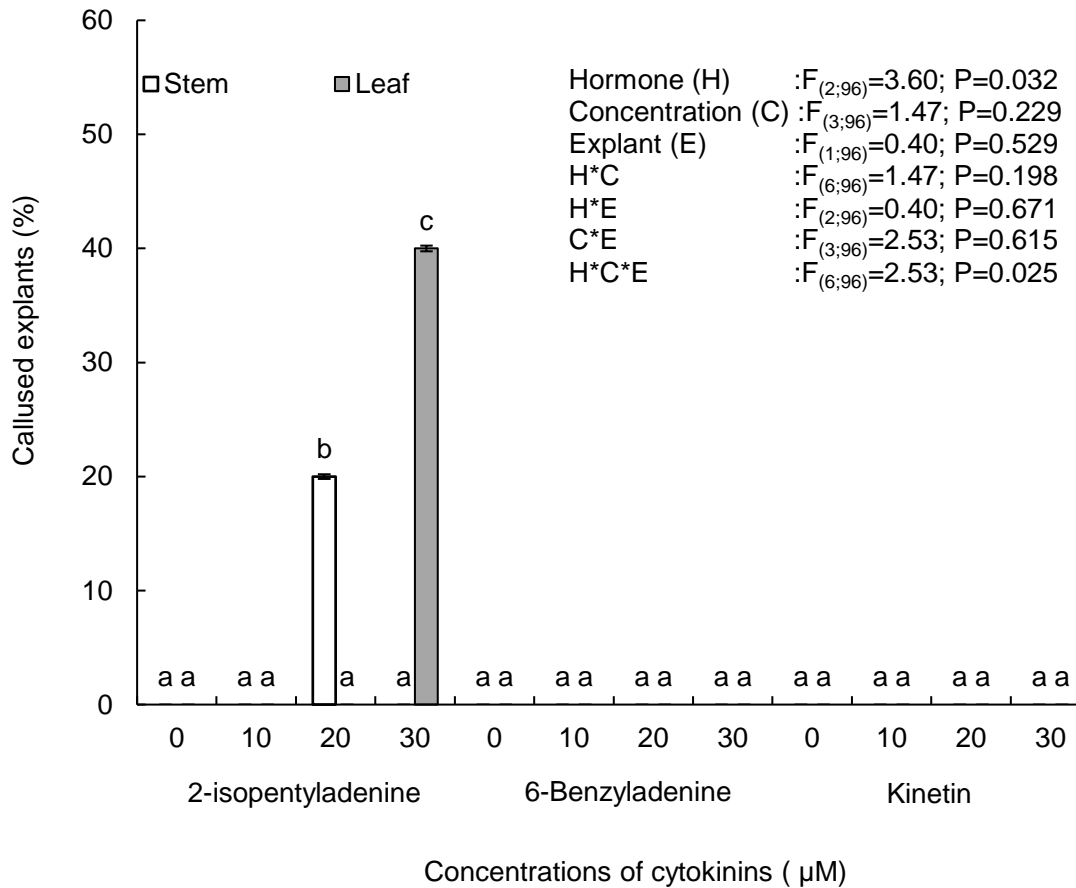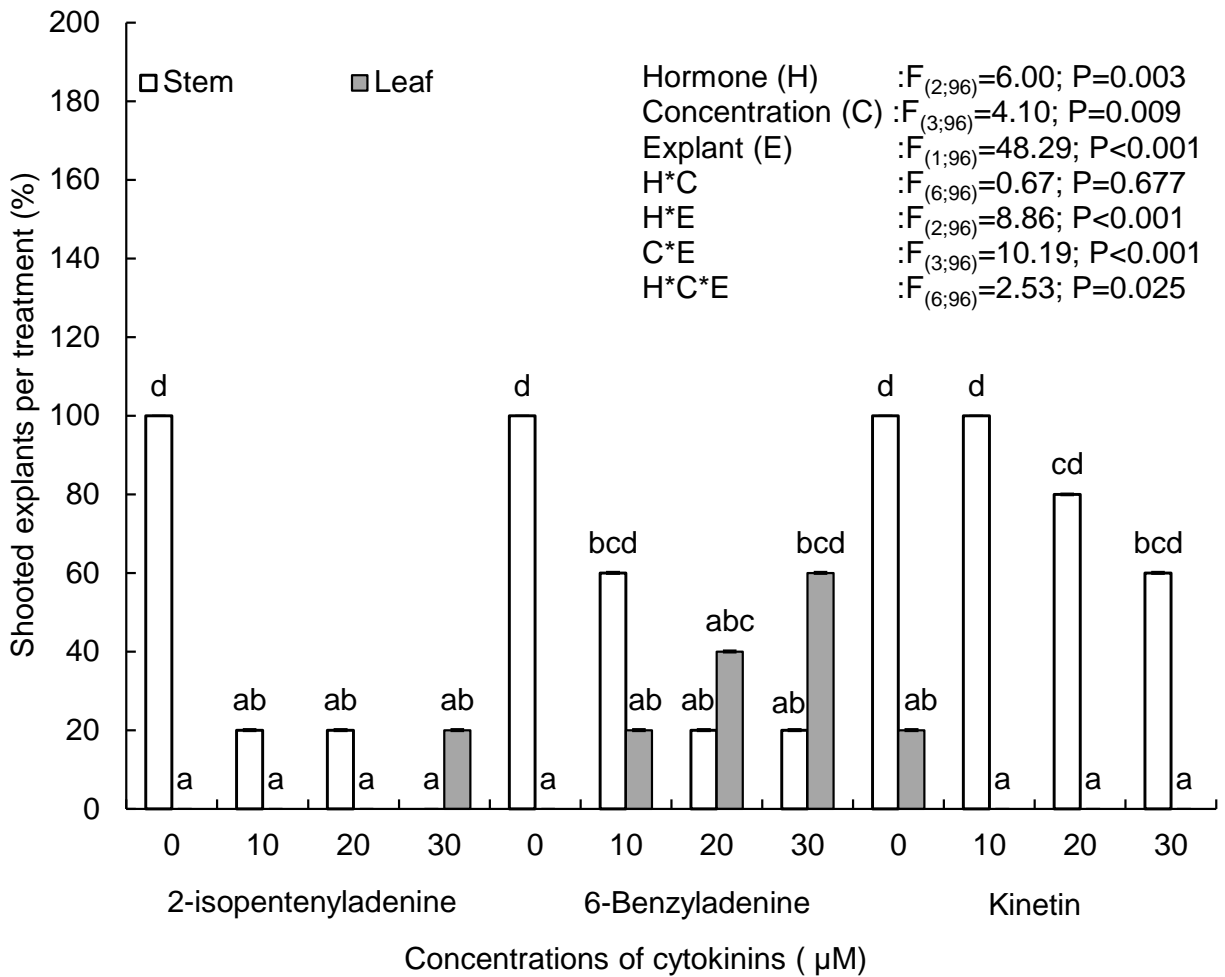
Fig 3.3: Proportion of shooted explants and multiple shoots (per explant) of *D. hallii* on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of 2-isopentyladenine (2iP), 6-Benzyladenine and Kinetin after 3 months in culture. Different letters denote significantly different means after a Fischer LSD test.
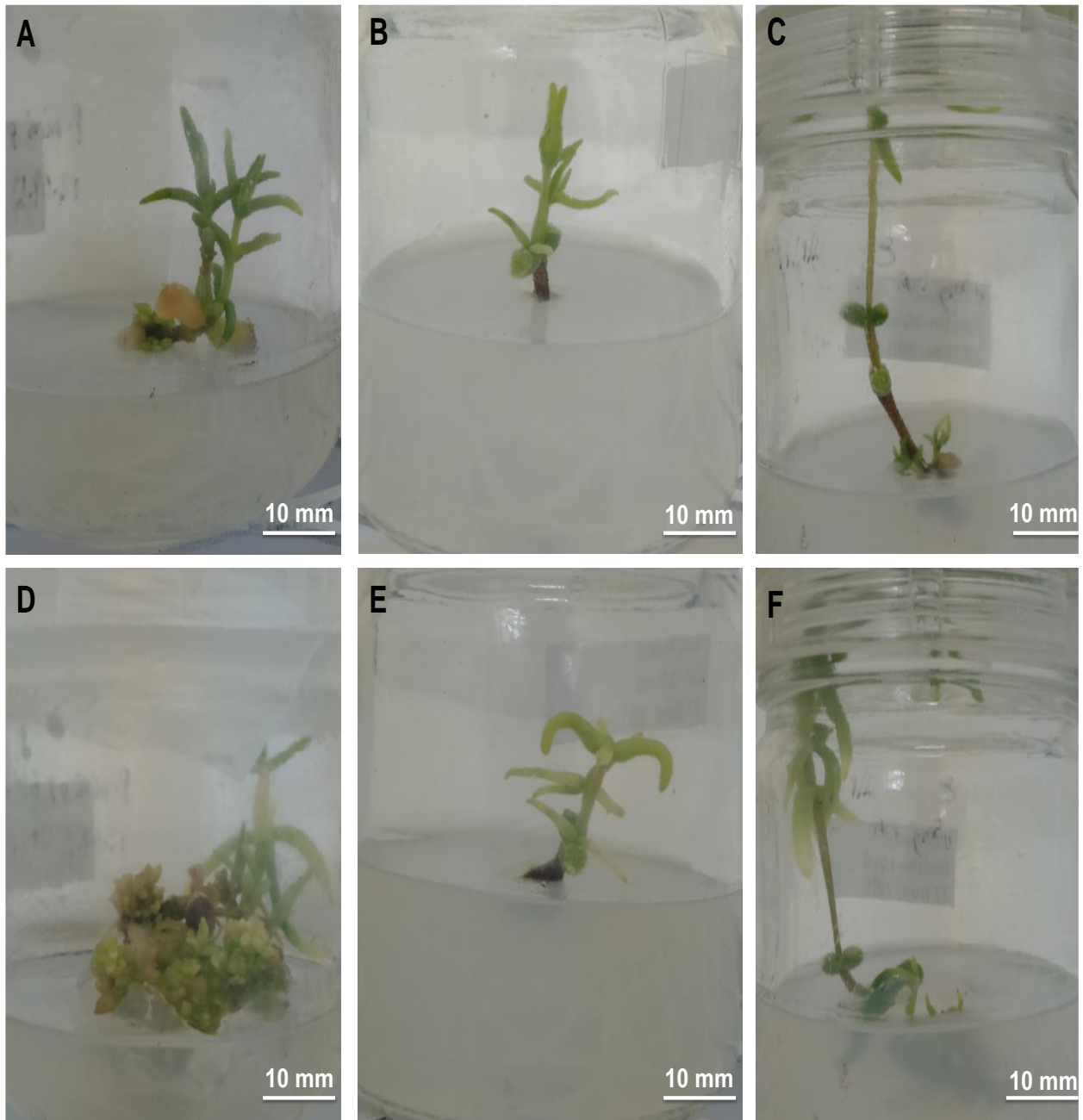
Fig 3.4: Shoot proliferation of *D. hallii* on full MS medium containing (A) 20 µM 2-isopentyladenine, (B) 10 µM of 6-Benzyladenine phytohormone and (C) 10 µM of Kinetin after 3 weeks of culturing and (D) 20 µM 2-isopentyladenine, (E) 10 µM of 6-Benzyladenine phytohormone and (F) 10 µM of Kinetin after 9 weeks of culturing

3.3.3 Browning of D. hallii

Drosanthemum plants are naturally rich in polyphenols, which may be released as polyphenols oxidize when they are dissected resulting in browning of tissues and poor callus growth. After 2 weeks of culturing browning was observed on some leaf and stem explants of Kinetin (0, 20 µM), 2-isopentyladenine and 6-Benzyladenine (0, 10, 20 and 30 µM) which led to death of some explants cultured (Fig. 3.5). Stem nodal explants cultures without cytokinins had the least proportion of brown cultures, the frequency increasing at higher cytokinin levels. In contrast, all leaf explants cultures had significantly (P< 0.001) higher incidences of browning (40 – 100%), the levels somewhat lower at the highest concentration of cytokinins. The 2-isopentyladenine had the highest proportion of brown explants at 0, 10 and 20 µM for leaf explants and 30 µM for stem nodal explants whilst the lowest number of browning occurred at 30 µM of leaf explants cultures. With 6-Benzyladenine, the more added the lower the number of brown leaf explants and browning of stem nodal explants was consistency at 20 and 30 µM. Kinetin had the lowest number of leaf explant browning at 10 µM and an increase of the cytokinin resulted in more tissue deaths of both leaf and stem nodal explants.
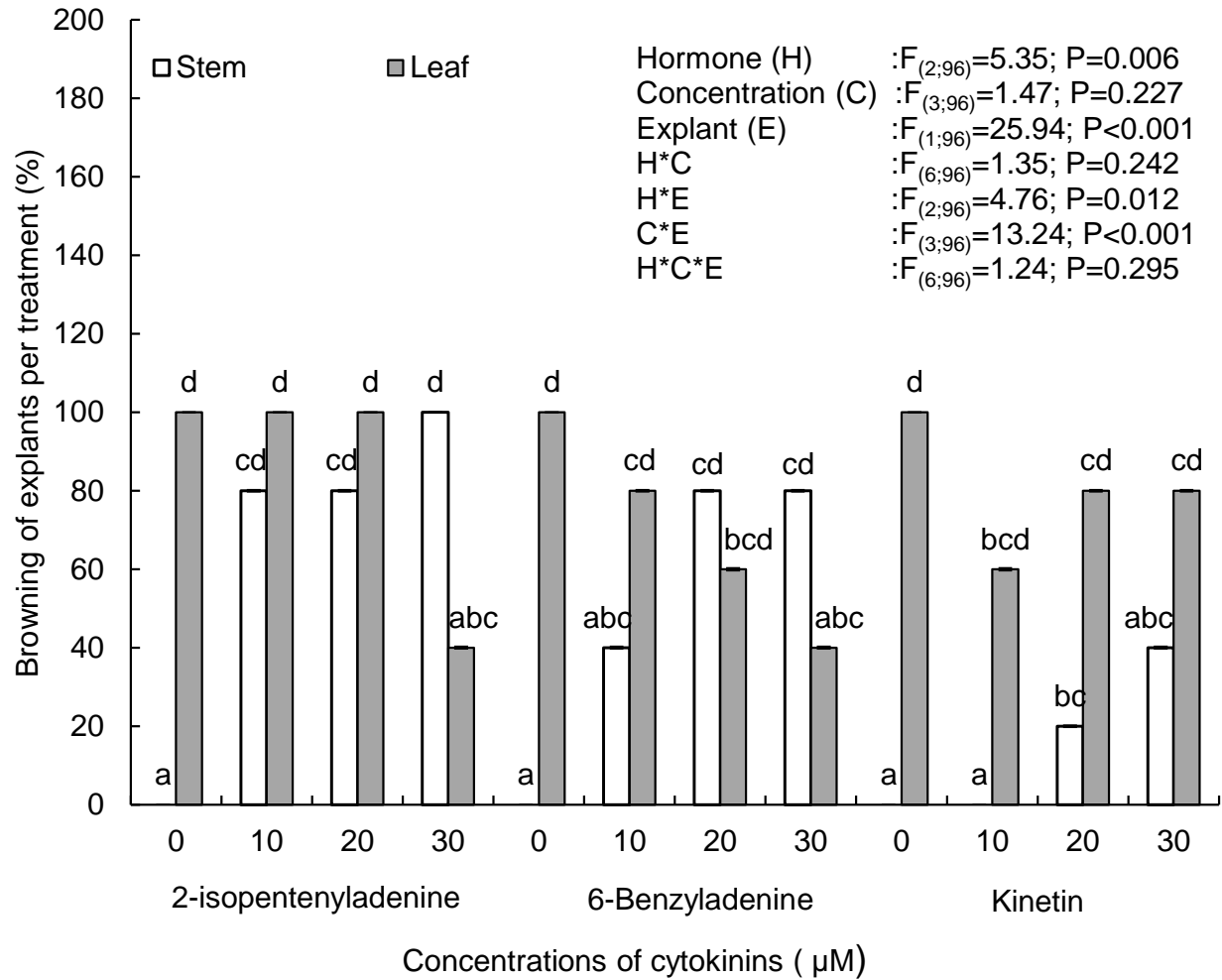
Fig 3.5: Percentage data on counts brown explants of *D. hallii* on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of 2-isopentyladenine (2iP), 6-Benzyladenine and Kinetin after 3 months in culture. Different letters denote significantly different means after a Fischer LSD test
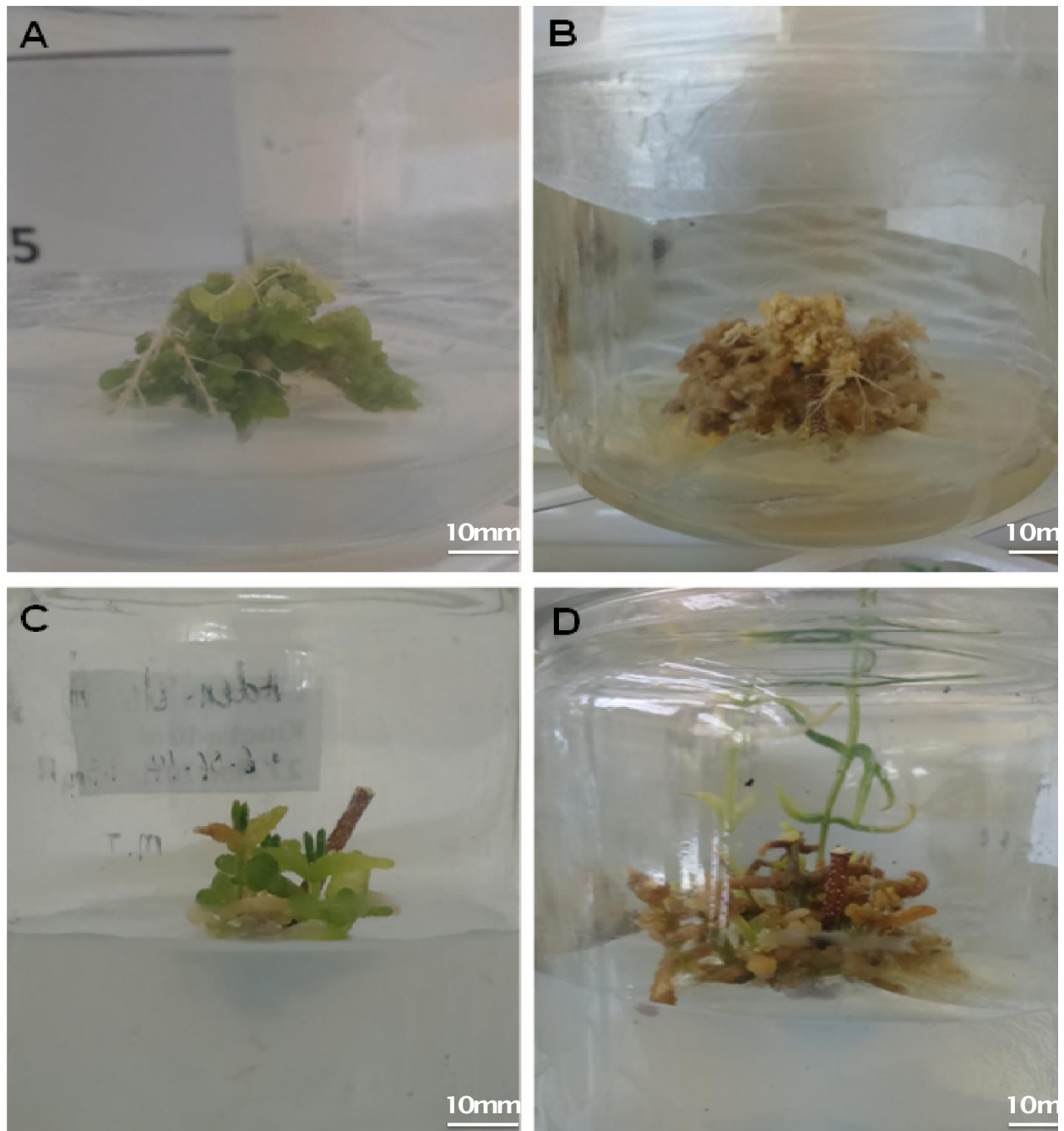
Fig 3.6: Callus induction and rooting of *D. hallii* after (A) 18 weeks, (B) occurrence of callus browning after 26 weeks of culturing on full MS medium containing 30 µM 2-isopentyladenine (2iP) phytohormone, (C) shoot regeneration of *D. hallii* after 12 weeks, (D) browning of regenerated shoots of *D. hallii* after 24 weeks of culturing on full MS medium containing 10 µM Kinetin

3.3.4 Rooting of D. hallii

There were no significant differences in the counts of rooted cultures in cultures of both leaf and stem nodal explants in response to indole-3-acetic acid. Therefore, image-J was used to estimate root density from the area (mm^2) of root images (Table 3.1). Although the counts of rooted cultures displayed no differences, the leaf explants had less roots, producing roots mostly in medium without indole-3-acetic acid. Stem nodal explants rooted across all levels of indole-3-acetic acid, with more roots developing in the untreated control. Stem nodal explants significantly ($P<0.05$) produced the longest roots on media without Indole-3-acetic acid.

Table 3.1: Root area (mm^2) of stem and leaf explants cultured on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of indole-3-acetic acid and Kinetin 10 µM after 3 months of culturing. ($F_{(3, 127)}$ = 1.009; P=0.039)

Indole-acetic acid concentrations (µM)	Mean root area using image-J analysis (mm^2)	
	Stem nodal explants (mm^2)	Leaf explants (mm^2)
0	683,4 a	36.3 b
10	55,7 b	0 c
20	22,1 b	0 c
30	34,7 b	0 c
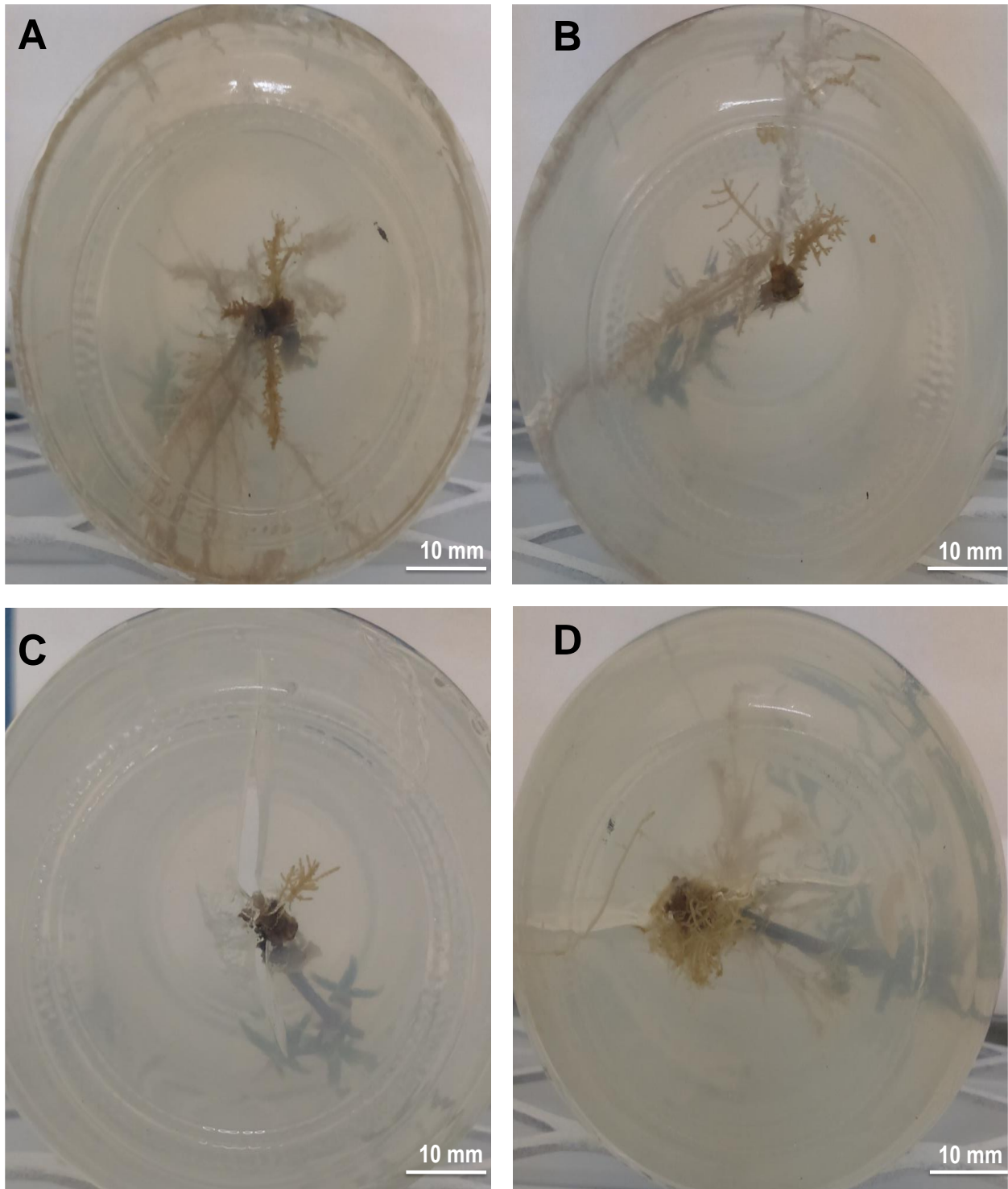
Fig 3.7: Root formation on *D. hallii* stem nodal explants cultured in full-strength Murashige and Skoog (1962) supplemented with 10 µM Kinetin and (A) 0 µM Indole-3-acetic acid,(B)10 µM Indole-3-acetic acid (C) 20 µM Indole-3-acetic acid and (D) 30 µM Indole-3-acetic acid after 3 months of culturing

3.3.5 Acclimatization of D. hallii

There were no significant differences in height, number of leaflets, root length or Fv/Fm values of seedlings from *in-vitro* propagation that were potted in the different plant media (Table 3.2). Acclimatizing seedlings had values lower than the optimal range of 0.79-0.84, which is recommended for stress-free terrestrial plants.

Table 3.2: Height (mm), leaflet numbers, chlorophyll fluorescence parameter (Fv/Fm) and root length (mm) of *in-vitro* propagated *D. hallii* seedlings transplanted to different media types for acclimatization.

Plant medium	Sample size (n)	Height (mm)	Leaflet numbers	Fv/Fm	Root length (mm)
Vermiculite (100%)	7	71.6	28.3	0.613	37.3
Sand (50%): Vermiculite (50%)	7	64.6	20.9	0.557	8.9
Sand (75%): Peat (25%)	7	60.0	31.0	0.645	36.0
$F_{(2,18)}$ statistic		0.399	0.313	0.737	0.745
P-value		0.677NS	0.735NS	0.492NS	0.488NS

3.4 Discussion and conclusion

For the first time to my knowledge, this study has demonstrated the *in-vitro* propagation of *D. hallii* as an alternative to the conventional use of seeds and rooting of cuttings. The *in-vitro* technique allows for a year-round source of plant material for large-scale rehabilitation projects in destroyed habitats, as well as a foundation for *in-vitro* cryopreservation and germplasm conservation of this endangered endemic species. The aim of this study was to determine the most effective concentration (0, 10, 20 or 30 µM) of cytokinins (6-Benzyladenine (BA), 2-isopentyladenine (2iP) or Kinetin) for callus and shoot production. Shoots previously

established *in-vitro* were transferred to MS culture media supplemented with different concentrations (0, 10, 20 or 30 µM) of a rooting hormone, indole-3-acetic acid. *De novo* root regeneration, a process of root organogenesis for recovery from damage (Xu, 2018), was apparent from both the callusing leaf explants and stem nodal explants, this demonstrating that supplemental auxins for root induction were unnecessary as dense rooting was obtained without of auxin supplements.

The compact and pale green callus observed on cultured leaf explants of *D. hallii* in full-strength MS medium with 30 µM of 2-isopentyladenine, but not on stem nodal explants, suggested key differences in apical dominance and distribution of meristematic regions. Unlike stem-nodal explants with axillary buds which develop new shoots, leaf explants lack distinct buds or dominant meristems. Therefore, when MS media was supplemented with 2-isopentyladenine, a cytokinin, apical dominance was broken in stem nodal explants resulting in shoot emergence without callus formation. Apical dominance is suppressed under the effect of high cytokinin concentration and permits the development of axillary buds (Bhatia and Sharma, 2015). In previous studies that compared stem nodal explants to leaf explants (Bhattacharya et al., 1990; Subbaiah and Minocha, 1990; Sen et al., 2014), callussing was also higher in leaf explants than stem nodal and apical shoot explants. The fastest induction of callus was observed at 20 µM of 2-isopentyladenine from leaf explants (Shadparvar, 2012). My data exhibited that 6- Benzyladenine and Kinetin did not exhibit any callusing but produced direct shoots.

The observed higher counts of shooted stem nodal explants compared to leaf explants demonstrated that stem nodal explants are the ideal starting material for *in-vitro* propagation of *D. hallii*. In this study, kinetin proved to be the best cytokinin for shoot induction of *D. hallii* from stem nodal explants. The highest number of shooted explants and multiple shoots was observed when stem nodal explants cultured in MS media were supplemented with 10 µM kinetin, compared to other rates of 2-isopentyladenine and 6-benzyladenine. This study demonstrated that cytokinins can induce multiple shoot formation and internode lengthening in *D. hallii.* The results are supported by previous work done by Van Staden (2008), Chawla

(2009), Jafari Najaf-Abadi and Hamidoghli (2009), Tornero (2009), Gomes et al., (2010), Hashemabadi and Kaviani, (2010), Rahman et al. (2010) and Hesar et al. (2011). When stem nodal and leaf explants grown *in-vitro* were cultured on different media in order to induce shoot formation, explants turned brown. Leaf explants cultured in MS media with 2-isopentyladenine had 100% necrosis at 0, 10 and 20 μM rates. In stem explants, the more phytohormone added, the higher the necrosis of explants. Browning and necrosis decreased the number of explants available shooted before they could even exhibit rooting.

Whilst leaf explants only produced rooting from the callus at 0 μM, stem nodal explants produced roots on all rates of indole-3-acetic acid (combined with 10 μM kinetin), with 0 μM producing explants with the highest root area (mm^2). Several studies have discussed the effects of combinations of cytokinin and auxin group of hormones on rooting and plant development. Although the auxin–cytokinin interaction is known to regulate *in vitro* organogenesis, the molecular mechanism of such auxin-cytokinin interaction is mostly unknown. Cytokinin and auxins may regulate events of major cell specification during embryogenesis (Müller and Sheen, 2008; Möller and Weijers, 2009), thereby controlling shoot meristem development (Ying-Hua et al., 2011). Recent advances in understanding interactions between auxin-cytokinin show a crosstalk in the specification of cell types. The multistep nature of specification processes may be against a single morphogenetic signal (Chandler and Werr, 2015). This may explain the observed differences in shoot and root formation in response to cytokinins that was observed between leaf explants and stem nodal explant of *D. hallii.* Previous studies where IAA and kinetin were used for shoot and root development on a different species yielded contrasting results. Early work by Meredith et al., (1970) analyzed the effectiveness of IAA and kinetin on *Feijoa sellowiana* and found that both kinetin and IAA promoted rooting of one clone but had no effect on the other clones.

The auxin group of hormones has generally been credited with root development (Overvoorde et al., 2010; Lavy and Estelle, 2016). In *D. hallii*, however, explants with supplemental indole-3-acetic acid, an auxin, had less root density,

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possibly suggesting sufficient endogenous auxins for root development. Endogenous auxins present in the explants were not quantified. Nevertheless, the natural levels of endogenous auxins may possibly explain why *D. hallii* has been previously rooted from stem cuttings, albeit with difficulty.

The insignificant differences amongst seedlings acclimatized with vermiculite, sand (50%): vermiculite (50%) or sand (75%): perlite (25%) suggests that any of the media can be used for acclimatization. The observed Fv/Fm values, which were lower than the recommended range of 0.79-0.84 indicates that the seedlings were stressed. This may be related to the acclimatization of the leaves from using root-supplied carbon to building their photosystem II apparatus. Whilst no seedlings were lost during acclimatization, different growing conditions can be explored to minimize the observed plant stress.

The present study gave an efficient protocol for *in-vitro* regeneration of *D. hallii* as an alternative method for vegetative propagation. An effective and reproducible convention for callus, shoot and root regeneration from nodal stem cuttings in *D. halllii* has been developed. I recommend use of stem nodal explants cultured in MS culture media, supplemented with kinetin at 10 μM for the highest percentage of regeneration. No need for transfer to rooting media. The ability to regenerate shoots *in vitro* is directly useful for research in genetic transformation and plant improvement via somaclonal variation. Conservation of threatened or endangered *Drosanthemum* species can benefit from *in-vitro* multiplication for re-introduction to its habitat or commercial nurseries. This study thus serves as a basis from which to develop future protocols for *Drosanthemum* species.

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CHAPTER 4

Micropropagation studies of an endemic and endangered succulent *Drosanthemum micans* (Aizoaceae)

4.1 Introduction

Drosanthemum micans is a low growing, dense and perennial shrublet, with flowers that have red outer petals with chrome yellow inner petals and mainly distributed in the karroid areas in the Worcester and Robertson lowlands (Germishuizen et al., 2006). *D. micans* belongs to the family Aizoaceae and is an endemic succulent listed as an endangered species on the Red List of South African Plants. Both seeds and cuttings can be used for propagation of *D. micans,* however, seed production is seasonal (late October) and the cuttings should be harvested in October or early March. Thus, conventional propagation methods limit the multiplication of the species to particular periods of the year, thus reducing the availability of the plant in nurseries. Micropropagation is an alternative method that can potentially be used to propagate these endangered species. Micropropagation techniques are advantageous in that they do not require specific seasons for propagation and can be done throughout the year. Although many plants have been propagated through micropropagation methods, literature on *in-vitro* propagation of the *D. micans* is scarce.

Therefore, the objectives of this study were to determine optimal cytokinin concentrations for shoot initiation and root initiation when using stem nodal.

4.2 Materials and methods

4.2.1 Sourcing of explant

Potted and disease-free *D. micans* plants were bought from New Plant Nursery, George, South Africa (33.9881° S, 22.4530° E). After 4 weeks of adaptation under greenhouse conditions at Cape Peninsula University of Technology, Bellville Campus, stem nodal explants were excised for *in-vitro* propagation.

4.2.2 Sterilization

Excised stem explants (ca. 30mm long, 3mm diameter), with a node intact, were washed under running tap water to remove loose debris and soil and were then rinsed with deionized water. For 30 sec, 70% ethanol solution was used to surface-sterilize the explants then rinsed with deionized water. Explants were then sterilized with 3.5% v/v sodium hypochlorite (NaOCl) in deionized water with the addition of two drops of 0.1% Tween-20 for 20 min and then rinsed three times for 20 minutes each interval with sterile distilled water under laminar flow hood.

4.2.3 Culture medium and conditions

The sterilized explants were then cultured in Murashige and Skoog medium (Murashige and Skoog, 1962) containing basic salts and vitamins, supplemented with 3% (w/v) sucrose, 7.5 g L^{-1} agar and varying concentrations of 0, 10, 20 or 30 µM of 6-Benzyladenine or Kinetin or Thidiazuron. The pH of the medium was then adjusted to 5.7 using either HCl or NaOH prior to autoclaving at 121°C for 20 min. Media were sterilized by autoclaving at 1.05 kg cm^{-2} for 20 min, and 25 mL of each medium placed in 250-mL containers and placed in a fridge (4°C). After 7 d, the shoots were transferred to a culture medium and the culture jars sealed with parafilm after explant implantation. The cultures were then incubated under growth room conditions (25±2°C), with a photoperiod of 16 hour light 8 dark photoperiod and maximum light intensity of 1000 µmol m^{-2} s^{-1}.

4.2.4 Observation of cultures and statistical analysis

Only 15 cultures were used for each treatment. Counts of shooted and multi-shooted explants, rooted explants and browning were recorded every fortnight and the data were transformed using log (x+1) and a one way analysis of variance (ANOVA) done using STASTICA 12 Software Programme. Image-J software (National Institute of Mental Health, Maryland,USA) was used to calculate the root area (mm^2) of the rooted explants Fisher's least significant difference (LSD) were used to separate the means at P = 0.05 level of significance.

4.3 Results

4.3.1 Shoot proliferation of D. micans

Drosanthemum micans shooted directly without initially producing any callus. No noticeable callus was formed on all stem nodal explants cultured in varying concentrations (0, 10, 20 and 30 µM) of 6-Benzyladenine, Kinetin and Thidiazuron. There were highly significant ($p<0.001$) differences in the numbers of shooted explants in response to the varying rates of cytokinins used. The highest numbers of shooted explants were recorded when cytokinin levels were not higher than 10 µM. Higher rates led to fewer shooted explants. Significant ($p=0.036$) differences in the numbers of shooted explants were evident in response to the type of cytokinin used. Overall, Kinetin had the highest numbers of shooted explants followed by 2-isopntyladenine and lastly thiadiazuron (Fig. 4.1a, Fig. 4.2). Similarly, significant ($p=0.022$) differences were also noted in the numbers of explants that produced multiple shoots in response to different levels of cytokinins. The highest number of multiple shooted explants was at 10 µM (Fig. 4.1b, Fig. 4.2).
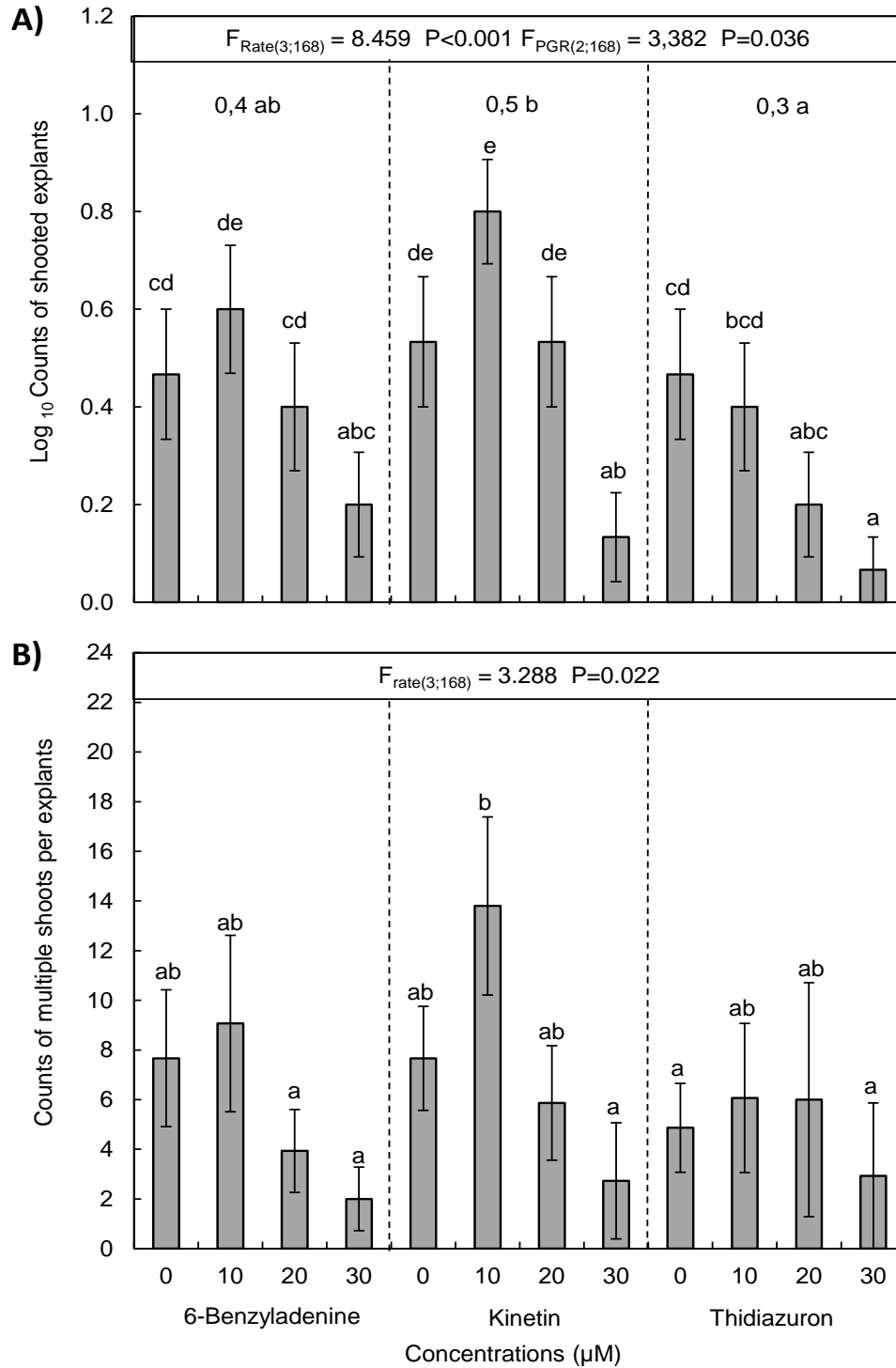
Fig 4.1: A) Log_{10}-transformed counts of shooted explants and B) multiple shoots per culture of *D. micans* on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of, 6-Benzyladenine, Kinetin or Thidiazuron after 2 months in culture.

Fig 4.2: Shoot proliferation of *D. micans* on full MS medium containing (A) 0 µM 6-Benzyladenine, (B) 0 µM of Kinetin,(C) 0 µM of Thidiazuron, (D) 10 µM 6-Benzyladenine, (E) 10 µM of Kinetin, (F) 10 µM of Thidiazuron after 2 months of culturing.

4.3.2 Explants browning of D. micans

Browning of explants and nutrient media, which occurs mostly in response to oxidation of polyphenols, was noted after 3 weeks from culturing in explants treated with 0, 10, 20, 30 µM of Kinetin, 6-Benzyladenine and Thidiazuron. Control treatments, without cytokinins, significantly (P<0.001) exhibited the lowest incidences of browning whilst explants with higher levels of cytokinin had more brown explants (Fig. 4.3). Browning was linked to death of some explants cultured (Fig. 4.4).
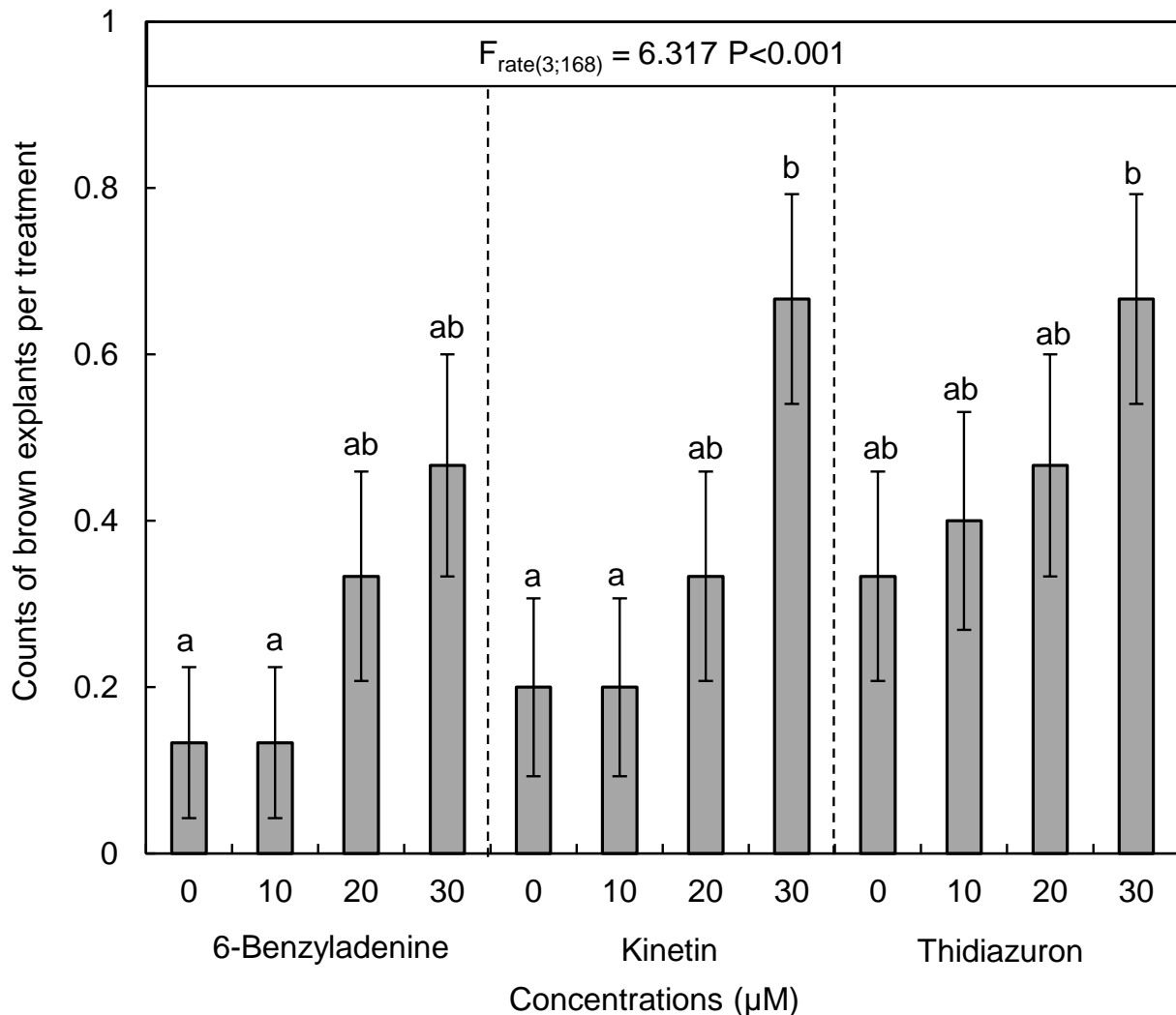
Fig 4.3: Decimal percentage of counts of brown explants of *D. micans* on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of, 6-Benzyladenine, Kinetin or Thidiazuron after 2 months in culture
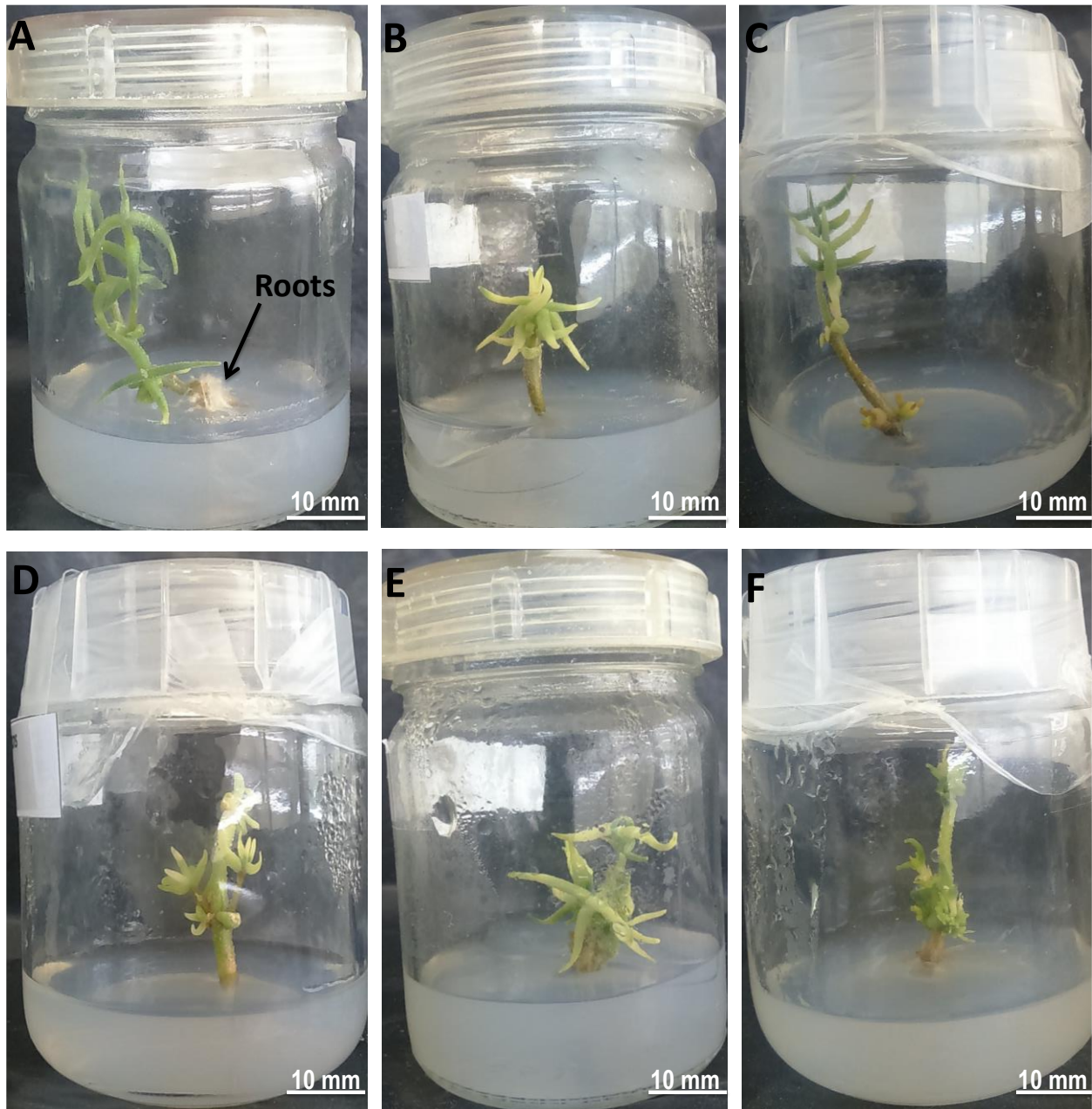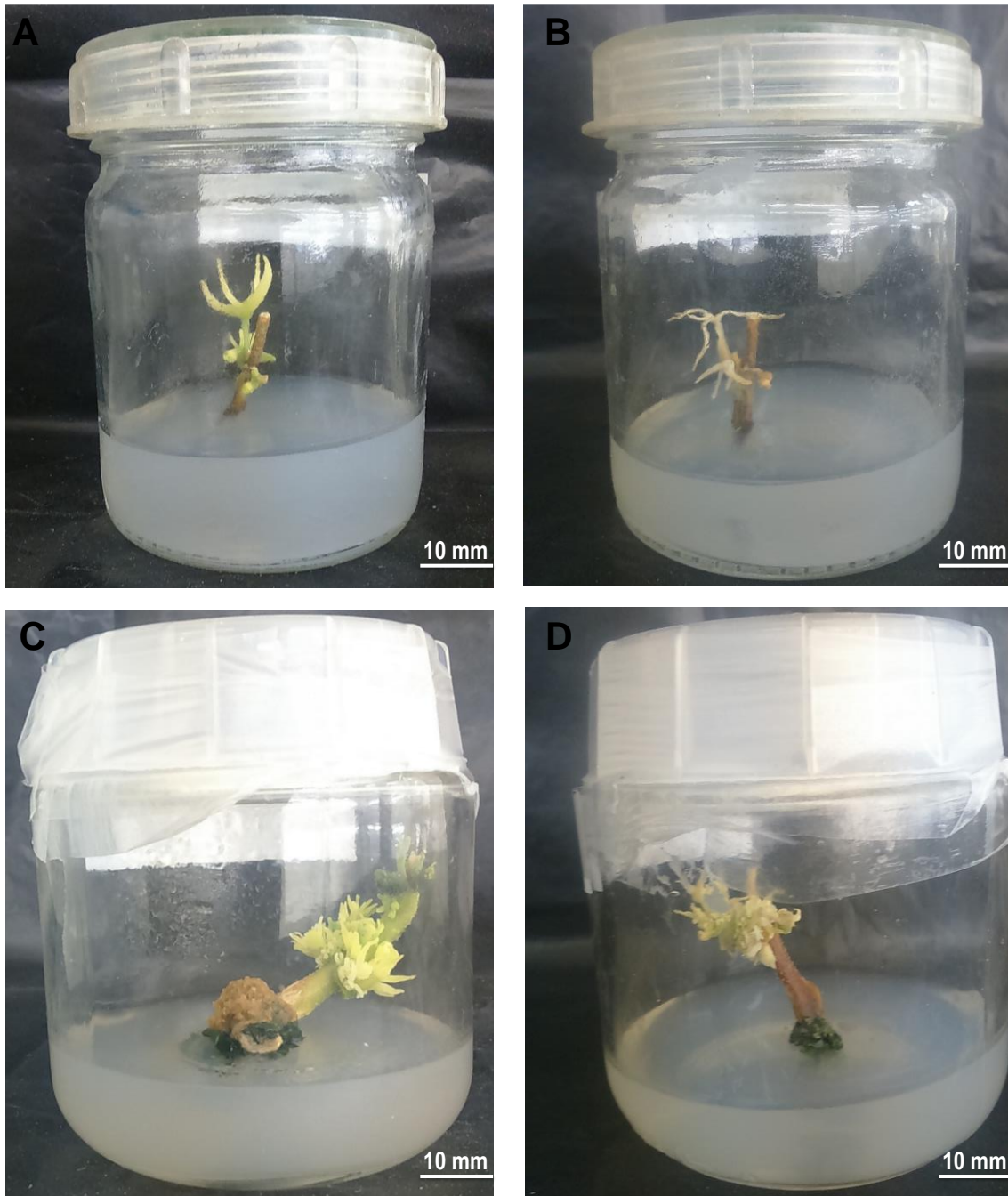
Fig 4.4: Shoot proliferation of *D. micans* after (A) 5 weeks (prior to browning), (B) browning of regenerated after 14 weeks of culturing on full MS medium containing 30 µM Kinetin phytohormone, (C) shoot regeneration of *D. micans* after 5 weeks, (D) occurrence of explant browning and contamination of *D. micans* after 14 weeks of culturing on full MS medium containing 30 µM Thidiazuron

4.3.3 Root formation in D. micans

After 4 weeks of culturing *D. micans* on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of 6-Benzyladenine, Kinetin or Thidiazuron, roots started developing. Roots only developed on control (0 µM) media without cytokinins (Table 4.1). Root density was significantly higher in control media, without cytokinins (Figure 4.5) (Table 4.2).

Fig 4.5: Root formation in *D. micans* on full MS medium containing (A) 0 µM 6-Benzyladenine and (B) 0 µM of Kinetin after 2 months of culturing.

Table 4.1: Rooted explants ($F_{(6;168)}$ = 3.093; P=0.007) of *D. micans* on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of, 6-Benzyladenine, Kinetin and Thidiazuron after 2 months in culture
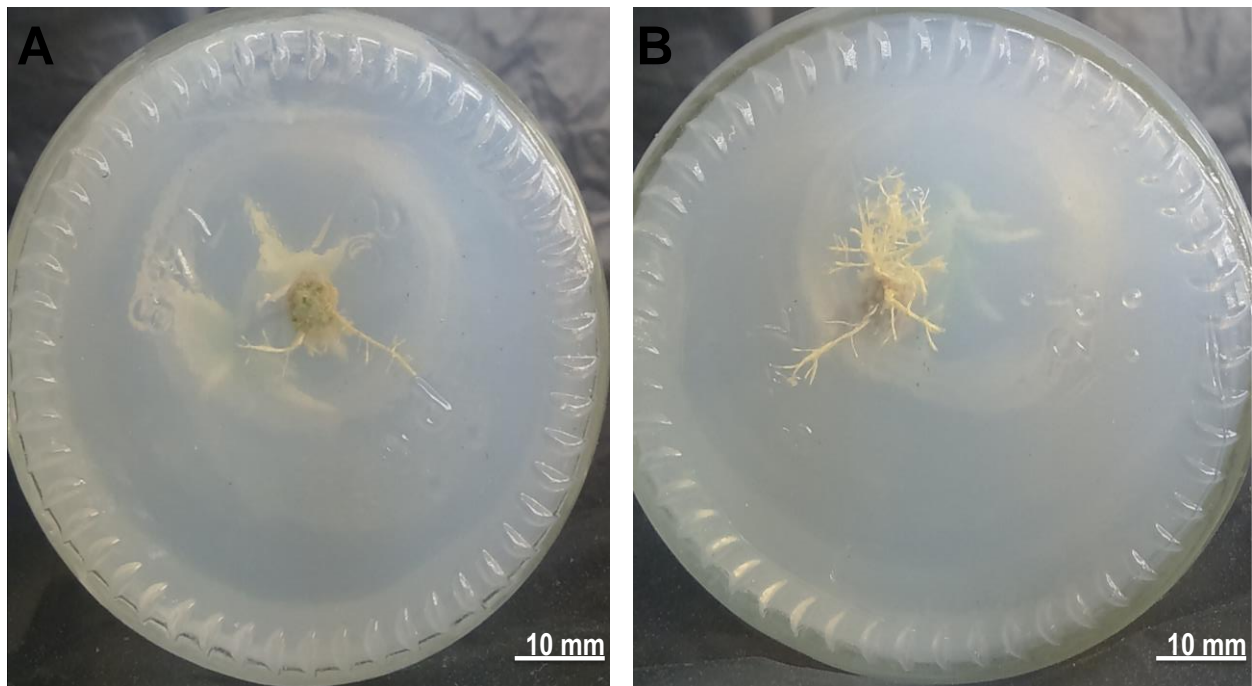
Concentrations (µM)	Mean of rooted explants		
	6-Benzyladenine	Kinetin	Thidiazuron
0	0.3 c	0.2 b	0 a
10	0 a	0 a	0 a
20	0 a	0 a	0 a
30	0 a	0 a	0 a

Table 4.2: Area of rooted explants (mm^2) ($F_{(3;168)}$ = 3.604; P=0.015) of *D. micans* on full-strength Murashige and Skoog (1962) supplemented with 0, 10, 20 or 30 µM of, 6-Benzyladenine, Kinetin and Thidiazuron after 2 months in culture

Concentrations (µM)	Mean of root area using image-J analysis (mm^2)		
	6-Benzyladenine	Kinetin	Thidiazuron
0	168.3 b	46.2 ab	0 a
10	0 a	0 a	0 a
20	0 a	0 a	0 a
30	0 a	0 a	0 a

4.4 Discussion and Conclusion

It has been demonstrated in this study that *in-vitro* propagation of *D. micans* is possible as an alternative to the conventional use of seeds and stem cuttings (Hartmann, 2011). The *in-vitro* propagation technique allows for a year-round source of plant material for large-scale nurseries, and provides a foundation for *in-vitro* cryopreservation and germplasm conservation of this endangered endemic species.

The observed initiation of shoots from stem nodal explants of *D. micans* without noticeable callus formation is consistent with results from previous studies. Initiation of

axillary meristems, which produce new shoots and leaves, is associated with a low auxin environment and a cytokinin signaling pulse (Wang et al., 2010). Genetic analyses done suggest that cytokinin perception and signaling are both required for axillary meristem initiation. After initiation, axillary buds may either continue to develop into branches (i.e., undergo outgrowth) or may remain dormant for different periods of time (Stirnberg et al., 2002). The observed significant differences in numbers of shoots formed by *D. micans* when treated with different cytokinin levels points to the role of cytokinins in shoot formation. Auxin and cytokinin have antagonistic functions in the control of axillary meristem outgrowth because auxin inhibits the outgrowth of axillary meristems, whereas cytokinins antagonize auxins by promoting activation of buds (Wang et al., 2010). Indeed, the highest numbers of shoots produced with 10 µM of kinetin displays the role of cytokinins in promoting the activation of buds. Thus, it can be recommended that for shoot multiplication in *D. micans,* 10 µM of kinetin should be applied.

The observed highest numbers of shooted explants with the use of kinetin, followed by 2-isopntyladenine and lastly thiadiazuron has not been previously reported for *D. micans*. Thus, kinetin had the highest potency in activating buds to shoot and develop from stem nodal explants compared to 2-isopentyladenine and thiadiazuron. Despite thiadiazuron being the least potent of the cytokinins tested, regeneration of African violet (*Saintpaulia ionantha* Wendl.) via shoot organogenesis and somatic embryogenesis was observed from thidiazuron (TDZ) treated leaf and stem-nodal (petiole) explants of greenhouse and *in-vitro* grown plants (Mithila et al., 2003). The response of cultures to other growth regulators over a range of 0.5 µM to 10 µM was 50% less than that observed with TDZ. TDZ displayed simultaneous shoot organogenesis and somatic embryogenesis in *S. ionantha* at low (<10 µM) and high (>10 µM) levels respectively.

Whilst the concentration-dependent shoot proliferation of *D. micans* at 10 µM of Kinetin was repeatable, it must be noted that the concentration may produce a different outcomes depending on endogenous levels of other plant growth regulators present in the used explants. Two or more hormones can interact synergistically or

antagonistically. Similarly, any given plant growth regulator may affect the biosynthesis or metabolism of another, consequently affecting endogenous levels (Van Staden, 2008; Tornero, 2009). Environmental factors such as light, water status, wounding and pathogens may modify responses and indeed the levels of plant growth regulators, which further complicates the overall plant response. As growth regulators and environmental factors interact to produce an integrated response, it is difficult to predict how any growth regulator will affect any given plant system.

The observed browning of explants must have resulted from oxidation of phenolic compounds by polyphenol oxidase (PPO). The challenge can be corrected by pre-treating the explants with polyvinylpyrolidone (PVP) and antioxidants such as ascorbic and citric acids or by reducing their accumulation in tissue culture media (Krishna et al., 2008). High temperatures and light can increase the activity of phenoloxidation enzymes, which escalates the rate of browning (Dobránszki and Teixeira, 2010). The concentration-dependant severity of browning on *D. micans* could not be explained using previous results.

Root initiation that occurred *ca.* 30 days after transferring the stem nodal explants to full strength MS media without supplental plant growth regulators (0 µM) suggests that the stem nodal explants had a balanced concentration of phytohomones for rooting. The intensity of rooting, estimated from root area (mm^2) using Image J revealed insignificant differences, thus suggesting that the explants transplanted must have had somewhat similar levels of endogenous phytohomes.

In conclusion, the successful *in-vitro* propagation of *D. micans* provides an effective means for the rapid multiplication and *in-vitro* conservation of the endangered *D. micans*. It can be recommended that 10 µM Kinetin be used for shoot multiplication, followed by a transfer to full-strength Murashige and Skoog (1962) media for root development. In the absence of kinetin, 6-benzyladenine and thidiazuron may be used at 10 µM for shoot development, albeit with relatively lower success rates when compared to kinetin.

4.5 References

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CHAPTER 5

Conclusions and recommendations

5.1 Main Conclusions

An effective *in -vitro* propagation protocol was developed for the endangered and succulents, *D. hallii and D. micans* endemic to the Succullent karoo biome of South Africa. Although the species have been propagated from seeds and cuttings in the past, their planting material is harvested and sown seasonally (Oliver, 2006; Riddles, 2013), hence, the is a need for an alternative propagation method. *In-vitro* propagation can be done throughout the year as it does not require a specific season for planting. The main conclusions of the study were as follows:

1. Stem nodal explants *D. hallii* produced multiple shoots whilst leaf explants tended to produce callus when cultured in full-strength Murashige and Skoog (1962) media supplemented with 2-isopentyladenine, 6-benzyladenine and kinetin (*ca*. 0 - 30 μM range).
2. Shoot multiplication in both *D. hallii* and *D. micans* was optimal when full-strength Murashige and Skoog (1962) media was supplemented with 10 μM of kinetin.
3. Root formation in both *D. hallii* and *D. micans* only occurred when shoots were transferred to a full-strength Murashige and Skoog (1962) media without any phytohormones added.
4. Browning of culture media increased with higher levels of cytokinins, suggesting an interaction of plant growth regulators with exudates from explants to increase the intensity of browning.
5. There were no significant differences in the level of stress in rooted explants of *D. hallii* and *D. micans* planted in i) vermiculite, ii) sand (50%): vermiculite (50%) or iii) sand (75%): perlite (25%) and maintained in a misted greenhouse (60% RH), followed by weekly reduction in relative humidity by 10% over a fortnight.

5.2 Recommendations

This study confirms that *in-vitro* propagation of *D. micans* and *D. hallii* can be accomplished by following the suggested recommendations:

1 Excise stem nodal explants (*ca*. 30mm long, 3mm diameter) with intact nodes and wash under running tap water to remove loose debris and soil and were then rinsed with deionized water.

2 Surface-sterilize the explants for 30 sec with 70% ethanol solution and then rinse with deionized water. Sterilize with 3.5% v/v sodium hypochlorite (NaOCl) in deionized water with two drops of 0.1% Tween-20 for 20 min and then rinsed three times for 20 minutes each interval with sterile distilled water under laminar flow hood.

3 Transfer the stem nodal explants to full-strength Murashige and Skoog (1962) media supplemented with 10µM kinetin for shoot development and multiplication.

4 Transfer the shoots to fresh full-strength Murashige and Skoog (1962) media without supplemental hormones for root development

5 Acclimatize the rooted explants to one of the following media: i) vermiculite, ii) sand (50%): vermiculite (50%) or iii) sand (75%): perlite (25%) and maintain them in a misted greenhouse (60% RH), reducing the humidity by 10% every week, over 2 weeks.

5.3 References

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