IN VITRO PROPAGATION STUDIES OF RARE *ARGYRODERMA* SPECIES STRICTLY ENDEMIC TO THE KNERSVLAKTE REGION OF SOUTH AFRICA

By

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DECLARATION

I, Mbulelo Ofisi 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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ABSTRACT

A study was conducted to investigate the effects of various media composition and wounding treating on the *in vitro* propagation of *Argyroderma subalbum* and *A. testiculare* explants derived from mature plants, antioxidants and plant growth regulators (PGR) concentrations.

One experiment consisted of 3 medium types including Murashige and Skoog (MS) medium strength, vitamin supplement. Fifteen replicates were used for each treatment. The shoots were then sub-cultured to ten replicate regenerated medium consisting of varying levels and combination of indole-3-acetic acid (IAA) and 10 µM 6-Benzyladenine (BA) supplements. In another experiment consisted of varying levels of auxins with MS medium strength, activated charcoal (AC) and vitamin supplements ten replicates were used for each treatment.

Results indicated the positive role of cytokinins types' 6-Benzyladenine (BA), 2-isopentyladenine (2iP) and Kinetin in inducing callus formation from wounded explants. The highest rate of friable callus formation of wounded explants was observed in media containing vitamin supplementation with BA at 10 µM. Callus formation significantly increased with the addition of vitamins at 10 µM on BA, 2iP and kinetin. With regards to the effects of various media composition and wounding explants on *in vitro* growth and regeneration of *A. subalbum* and *A. testiculare*, significant results were achieved with BA, 2iP and kinetin concentrations on explants discoloration and callus formation.

The antioxidant treatment, AC did not reduce explants discoloration, but the induction of the callus was developed furthermore, results showed that IAA with BA concentrations without addition of AC there was significantly difference on both species but *A. subalbum* dominated with browning intensity (Chapter 3). Only sub-culturing of the explants succeeded in preventing explants discoloration and subsequently increased the number of shoots. The interaction between Indole-3-acetic acid (IAA) concentrations combined with BA resulted in the most effective technique in reducing explants discoloration at the media contact point.

This study provides an insight into the contributing factor and methods of overcoming the major problem of phenolic oxidation and promoting the *in vitro* growth and regeneration of *A. subalbum* and *A. testiculare.*

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"Nothing impossible for those who believe" *Luke1: verse 37*

DEDICATION

I dedicate this thesis to my

Mother, who always inspired and encouraged me throughout my academic career and also in loving memory of my late dad.

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GLOSSARY

Terms/Acronyms/Abbreviations/Definition

NAA	α-naphthalene acetic acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
Kn	Kinetin
2, 4-D	Dichlorophenoxy acetic acid
2ip	6-(gamma, gamma-Dimethylallylamino) purine
BA	6-Benzylaminopurine
MS	Murashige and Skoog (1962) medium
PGR	plant growth regulator
Agar	a polysaccharide powder derived from algae used to gel a medium
Aseptic technique	procedures used to prevent the introduction of fungi, bacteria, viruses, mycoplasma or other microorganisms into cultures.
Autoclave	a machine capable of sterilizing wet or dry items with steam under pressure.
Auxin	a group of plant growth regulators that promotes callus growth, cell division, cell enlargement, adventitious buds, and lateral rooting.
cytokinin	a group of plant growth regulators that regulate growth and morphogenesis and stimulate cell division.

Callus	an unorganized, proliferate mass of differentiated plant cells, a wound response.
Explant	tissue taken from its original site and transferred to an artificial medium for growth or maintenance.
Micro propagation	*in vitro* Clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures.
totipotency	a cell characteristic in which the potential for forming all the cell types in the adult organism are retained.
Regeneration	In plant cultures, a morphogenetic response to a stimulus that results in the products of organs, embryos, or whole plants.
Undifferentiated	with plant cells, existing in a state of cell development characterized by isodiametric cell shape, very little or no vacuole, a large nucleus, and exemplified by cells comprising an apical meristem or embryo.
Apical meristem	undifferentiated tissue, located mostly within the shoot tip, generally appearing as a shiny dome-like structure, distal to the youngest leaf primordium and measuring less than 0.1 mm in length when excised
^0C	Degrees Celsius
dH_2O	Distilled water
'*Ex vitro*'	Cultivated in Natural conditions
In vitro	'In glass'
Min	Minutes
L Litre	

g	Gram		
HCL	Hydrochloric acid		
NaOH	Sodium Hydroxide		
µM		micromolar	10^{-3} mol/m^3

CHAPTER ONE

A review of *Argyroderma* species on an *in vitro* culture

1.1. Description of *Argyroderma* spp. of the Knersvlakte

Argyroderma is derived from a Greek word ‘argyros’, which means silver, and ‘derma’, which refers to skin, relating to its silvery greyish–green leaves (Van Jaarsveld, 2005). It is a genus that belongs to the *Aizoaceae* family, a compact dwarf, rounded cluster-forming perennial succulent plant, with growth forms which range from single stem sunken forms through branched mat-forming species (Smith *et al.*, 1998). Morphologically, *Argyroderma* spp. has a compact stone-like growth form, often comprising of single pairs of leaves and leaf pairs often fused into at the base (Van Jaarsveld, 2005).

Argyroderma comprises of 11 species, all of which are restricted to the Knersvlakte region of the Succulent Karoo biome. It is an area approximately 100 x 80 km in size (Hartmann, 1978). The genus has its species distributed on the unique quartz-gravel habitat that characterizes the Knersvlakte area (Schmiedel and Jurgens, 1999). The species are mostly quartz specialists, showing highly reduced growth forms, with one species that is highly branched and mat-forming, growing on both quartz field gravel and surrounding poor matrix habitats. Species within the genus are differentiated by very few distinct characters, with most of these being morphological traits. Divergence in morphological traits across the Knersvlakte is believed to have occurred in response to the ecological speciation driven by variation in the slope of the quartz gravel plain, pebbles density and soil salinity (Hartman, 1978).

1.2. Importance of *Argyroderma* spp.

Argyroderma spp. (*Aizoaceae)* are South African succulent plants with a high commercial value as potted flowering plants. They comprise of a high number of specialised succulents and endemic to quartz-gravel fields of the Knersvlakte region (Schmiedel *et al*., 2012). There is need to protect and conserve these commercially

viable flowering pot plants for the ornamental horticulture industry. *Argyroderma* is an indigenous succulent that is conventionally grown from seed in some nurseries of Knersvlakte. The *Argyroderma* genus has captured the attention of tourists, who keep on visiting this area of its endemism (Gosling, 2014).

The fresh leaves of *Argyroderma* spp. contain phenylpropanoids, particularly flavonoids and anthocyanins (Musil *et al*, 2005), which may be exploited for pharmaceutical purposes. Other members of the *Aizoaceae* family have already been exploited for medicinal properties and in soap making, preservatives, poultices and in preparations of psychoactive medicinal remedies. An example is *Carpobrotus* spp., where leaves are used medicinally for sore throats and treating fungal infections (Chesselet, 2004).

The unconquerable ability for survival displayed by *Argyroderma* spp. in the Knersvlakte make the area a well-known biodiversity hotspot and a fascinating area for tourists. During the spring season the concealed flora burst into bloom and display unique features that lure many tourists from all corners of the world (Lombard *et al*., 1999). The region is considered the 'spring flower show of South Africa' especially after winter rainfall when the landscape is covered with a 'flower carpet'. As a result, the Knersvlakte has been identified as a priority region for the conservation of flora (Hilton Taylor and Le Roux, 1989; Hilton-Taylor, 1994; Cowling *et al*., 1998; Lombard *et al*., 1999).

1.3. Status of some *Argyroderma* spp.

Argyroderma spp. dominate the Knersvlakte quartz-fields, a region that is vulnerable to a wide range of anthropogenic factors, which threaten the persistence of the species (Cowling *et al*., 1999). Four of the species namely *Argyroderma subalbum*, *Argyroderma framesii*, *Argyrodema theartii* and *Argyrodema testiculare* are rare; whilst the other *Argyroderma* spp. are classified as least concern (LC) plants (RDL, 2009). Such classification, however, may conceal the reported high plant mortalities expected with climatic warming (Musil *et al*., 2005). Global warming is a serious threat to biodiversity in the Knersvlakte area (Midgley *et al*., 2002; Travis, 2003; Musil *et al*.,

2005; Foden *et al*., 2007; Midgley and Thuiler 2007), where *Argyroderma* and *Conophytum* species seem to have reached their maximum tolerable temperatures, and are experiencing high plant mortalities (Musil *et al*., 2005; Musil *et al*., 2009) In addition, overgrazing, extension of communal lands (Todd and Hoffman, 1999), small scale mining ventures (Cowling *et al*., 1999) and the illegal harvesting of rare and localised endemic plants species (KD NBG, 2014) are threatening species in the Knersvlakte area. *Argyroderma* spp. are also being removed from their wild habitats due to soil erosion, especially by flush floods from ephemeral rivers (Malda *et al*., 1999). Given these threats to *Argyroderma* spp, *in vitro* propagation techniques may provide ways of *ex-situ* conservation of both rare and endangered species and their potential multiplication for commercial purposes.

1.4. Traditional propagation of *Argyroderma* spp.

Argyroderma spp. are currently propagated through seeds (Ellis and Weis, 2006). The tiny seeds are protected within capsule cavities, thus limiting their effective dispersal. In the wild *Argyroderma* spp. rely on seed dispersal by falling raindrops, which scatter the seed during heavy rain. This restricts the dispersal, germination and recruitment of the species to periods of effective winter rains. Unlike in the wild, most nurseries sow the species during autumn, mostly in loamy-soil mixed with sand and gravel quartz (Van Jaarsveld, 2005). Successful germination and emergence occurs after exposure to high soil surface temperatures for long periods of time (Daws *et al*., 2007). Germination usually occurs within 21 to 30 days from sowing. Seeds are usually sown in a shallow sand mixture covered with a thin layer of sand (2 mm) and watered through capillary action till the enough water has been absorbed (Van Jaarsveld, 2005). Despite the seed sowing of *Argyroderma* spp., there are alternative methods for regeneration of the species such as use of cuttings.

Multiplication of *Argyroderma* spp. through cuttings has been used as an alternative propagation method for conservation of its populations. Current attempts have used leaf cuttings from the base of the leaf during summer, as to accommodate rooting, that should take place within a month. Current traditional methods of

propagating *Argyroderma* spp., however, cannot meet the demand for plant material. This exerts enormous pressure on the wild populations, which is driving their over-collection and possible extinction in the future. Growth to maturity is slow with the use of seeds and the use of cuttings comes with challenges during of the rooting the leaves (Malda *et al*., 1999). *In vitro* culture is an alternative method for propagating the species, as it allows for rapid multiplication and continuous production of plants in a limited space of time. The technique has been successfully used for other members of the *Aizoaceae* family (Gratton and Fay, 1990).

1.5. Potential for *in vitro* propagation of *Argyroderma* spp.

There is no literature, to date, on tissue culture of *Argyroderma* spp. however, reports exist for other members of the *Aizoaceae* family, which suggest a potential for micropropagation of *Argyroderma* spp. For example, Cushman *et al*. (2000) developed an efficient plant regeneration procedure from hypocotyl explants of the common ice plant, *Mesembryanthemum crystallinum* (*Aizoaceae*). Somatic embryos were initiated and developed up to globular and heart stages in MS (1962) media supplemented with 3% sucrose, 0.6% bacto-agar, 80 mM NaCl, 5 mM 2, 4-D and 1mM kinetin. In addition, MS medium supplemented with 4.0 mg/L 2iP successfully regenerated shoots, whilst MS supplemented with 0.4 mg/L NAA was suitable for root induction of *Carpobrotus edulis*, with 100% root formation in response to the application of IAA, IBA and /or NAA to the culture media (Khattab and Sherif, 2011). Stem explants of *Zaleya decandra* (*Aizoaceae*) cultured on MS, B5 and Whites media containing different concentrations of auxins and cytokinins were successful in callus formation (Radfar *et al*., 2012). *Sesuvium portulacastrum* of *Aizoaceae* family was successfully propagated in *in-vitro* culture, with the highest number of shoots, successful shoot regeneration per explant were observed on MS medium supplemented with 40 µM 2-isopentenyl adenine (2iP) followed by 20 µM benzyladenine (BA). The lower concentrations (5 or 10 µM) of α-napthaleneacetic acid (NAA) proved more effective for root induction, number of roots and average root length (Lokhande *et al*., 2010).

In vitro culture might have great advantages over conventional propagation techniques in controlling some species. Rapid multiplication of plants under manageable pathogen-free conditions can be achieved, using plant growth regulators. Using this technique a greater number of shoot can be produced from small quantities of plant pieces, such as nodal, bud and meristem tissue (Fay, 1994). The use of *in vitro* propagation technique can promote enhancement in secondary metabolites production (Dornenburg and Knorr, 1995).

1.6. Environmental conditions during acclimatization

A major limitation in large scale application of tissue culture is the high mortality experienced by tissue culture raised plants when transplanted, mainly due to the huge differences between the *in vitro* and nursery environment. Cultured plants, on being transferred to harsh *ex-vitro* conditions, are exposed to abiotic factors (altered temperature, light intensity and humidity conditions) and biotic stress conditions (i.e. soil microflora), hence the need for acclimatization for successful establishment and survival of plantlets (Mathur *et al*., 2008; Deb and Imchen, 2010).

Plantlets grown from *in vitro* culture are prepared under low light intensity (1,200-3,000 lux) and temperature (25 ± 2^{o}C), therefore direct transfer to extensive sunlight (4,000-12,000 lux) and temperature (26-36^{o}C) in greenhouses might damage the leaves and cause wilting of plantlets. It is thus necessary to harden plants off to the natural conditions during a process of hardening-off or acclimatization. *In vitro* cultured plantlets can be left in a shade house for 3-6 days under disperse natural light to allow them to adjust to the conditions of the new environment and this helps in semi-hardening of the plants and leads to shoot elongation (Lavanya *et al*., 2009).

Humidity must be kept high to facilitate the development of the cuticle and functional stomatal apparatus. When exposed to low humidity, *in vitro* cultured plantlets will experience high stomatal and cuticular transpiration rates. Thus, *in vitro* cultured plantlets should be gradually transferred from high humidity to low humidity conditions (Posposilova *et al*., 1999). During acclimatization, plantlets should be transferred to pots containing a sterile soil and sand mixture and then covered with polybags for slow

acclimatization. Plantlets should also be kept in the shade house for a week or two (Short *et al*., 1987; Ritchie *et al*., 1991).

Another major cause of high mortality of plantlets is their unexpected exposure of the root system to microbial communities present in the soil, as they do not possess sufficient resistance against soil microflora (Hao *et al*., 2010). Requirements for acclimatization often differ with species and there are no clear generalizations (Hazarika, 2003). According to Amoo *et al*., (2009) *in vitro* propagated plants of the succulent species *Huernia hystrix* (Hook.f.) N.E.Br. (*Asclepiadaceae*) can be easily acclimatized under greenhouse conditions using a soil and sand mixture (1:1) treated with fungicides (e.g. Benlate 0.01%) at temperatures of 15^{o}C minimum and 25^{o}C maximum. Rooted plantlets of *C. edulis* (*Aizoaceae*) were successfully acclimatized in the greenhouse in pots with a moist mixture of (1:1) sand and perlite and maintained inside a plant growth chamber and irrigated with a fine mist of water for three weeks (Khattab and Sherif, 2011). Well-developed and rooted plantlets of *Sesuvium portulacastrum* were successfully hardened and established in the field with a more than 85% survival rate, after transferred to plastic pots (5×7 cm) containing sterilized sand and soil (1:1) and covered with transparent polythene bags to maintain the relative humidity. The pots were transferred to a greenhouse with a natural day/night photoperiod, 25-30^{o}C and 60 -70 % relative humidity (Lokhande *et al*., 2010).

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CHAPTER TWO

Distribution of *Argyroderma* spp. and statement of the research problem, background of research problem, project objectives, key question and significance of the study

2.1. Distribution of the *Argyroderma* spp.

Naturally, *Argyroderma* spp. are confined to quartz field habitats in the Knersvlakte area (Van Wyk and Smith, 2001), which stretches from the Matsikama Mountains in the south to Namaqualand Rocky Hills near Bitterfontein in the north. The eastern boundary is formed by the Bokkeveld Escarpment and the western part (Sandveld) is adjacent to the Atlantic Ocean (Schmiedel and Jürgens, 1999). The Knersvlakte is a winter-rainfall region characterised by exceptional endemism and high species richness in the Succulent Karoo biome of the western part of South Africa (Hilton-Taylor, 1994; Cowling *et al*, 1998). The area is associated with winter rainfall and ecologically characterised by a comparatively warm temperature regime in the Succulent Karoo biome (Cowling *et al*., 1999). The area has remarkably unique features, creating a habitat of unique vegetation, soil properties and climatic conditions.

The Knersvlakte consists of plain gravel quartz fields, with 40% of the 150 tracheophytes (vascular) plant species being endemic and a strong dominance of leaf succulent growth forms (Schmiedel, 2004). Due to its high number of endemic species, the Knersvlakte is considered as a centre for plant endemism and diversity. The area, also known as the Vanrhynsdorp Centre, supports about 138 endemic taxa. Despite the Succulent Karoo region being dominated by a variety of plant species for an arid area (Hilton-Taylor, 1996) and being registered among 25 biodiversity hot spots (Myers *et al*, 2000), the species are threatened by anthropogenic warming (Musil *et al*., 2004; 2009).

Particularly, the region comprises of specialised dwarf succulent plants, largely from the families *Aizoaceae* and *Crassulaceae* (Schmiedel and Jurgens, 1999). These species adjust themselves to grow and survive on arid regions of a special climatic condition (Schmiedel and Jurgens, 2004) and to harsh environment caused by

combination of ion toxicity and water stress (Schmiedel and Jurgens, 1999). Plants advance themselves by developing network response of physiological and biochemical protection mechanisms to defend themselves against stress.

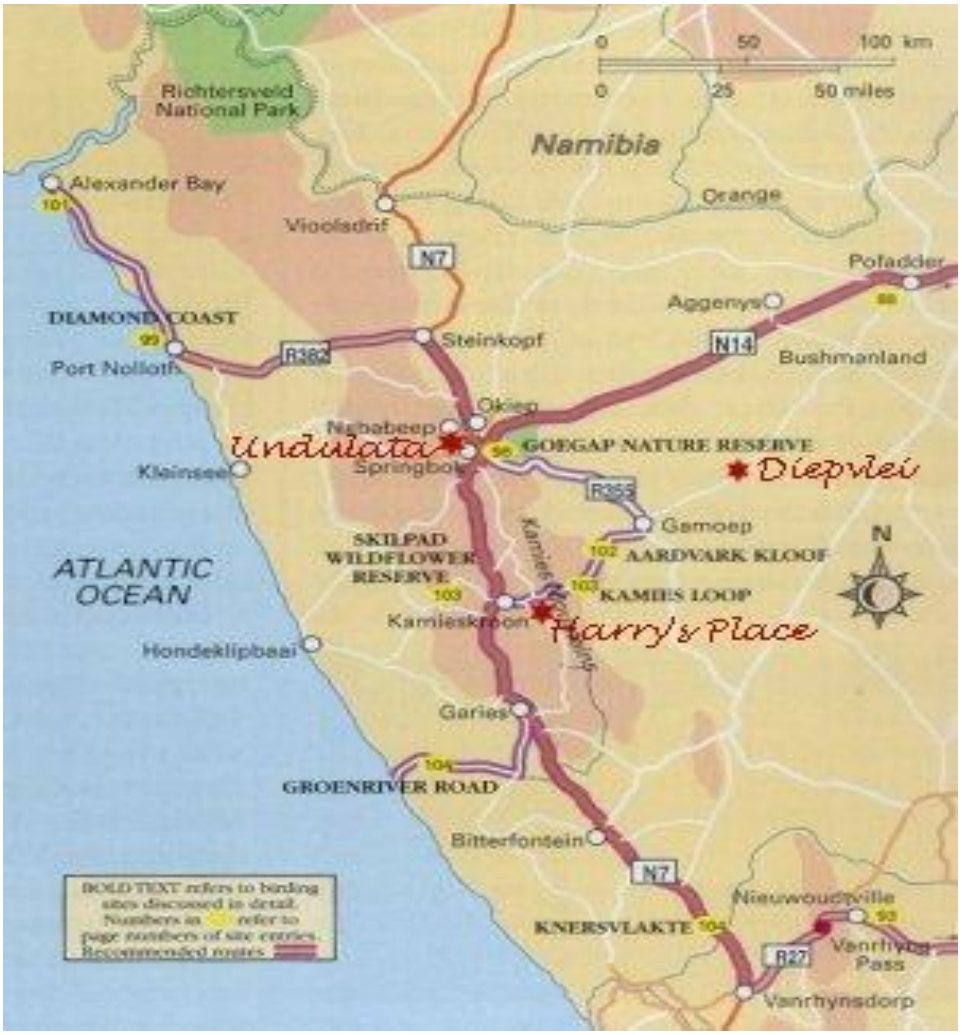

Figure 1: The above photography shows the geographical distribution of regions with frequency occurrence of quartz gravel field in southern Africa (Schmiedel, 2002).

2.2. Statement of the research problem

Argyroderma spp. are rare and minute succulents confined to the Knersvlakte's distinctive quartz-gravel habitats (Schmiedel and Jürgens, 1999). The Knersvlakte is a region in the Succulent Karoo biome, which is known for high species richness and endemism (Cowling *et al*., 1989, Hilton-Taylor, 1996; Cowling *et al*., 1998), and is

recognized as one of the world's biodiversity hotspots (Myers *et al*., 2000). Future anthropogenic global warming is, however, predicted as one of the major threats to biodiversity of flora in the Knersvlakte (Kappelle *et al*., 1999; Sala *et al*., 2000; McCarthy, 2001; Thomas *et al*., 2004; Thuiller *et al*., 2005; Parmesan, 2006). Predictions suggest an increase in aridity and number of climate incidences, which threaten the survival of floral species. Bioclimatic models have predicted a decline in species richness for the Knersvlakte (Midgley *et al*., 2002; Foden *et al*., 2007; Midgley and Thuiller 2007); with high plant mortalities recorded for the endemic *Argyroderma* (*Aizoaceae*) and *Conophytum* (*Crassulaceae*) species (Musil *et al*., 2005; 2009). The species are threatened by livestock farming (Todd and Hoffman, 1999), mining activities (Cowling *et al*., 1999) and illegal plant collection (Hilton-Taylor, 1994). Because the flora of the Knersvlakte generates income through eco-tourism, there is need for both *in-situ* and *ex-situ* conservation of the threatened species in their habitats (Hilton-Taylor and Le Roux, 1989; Hoffman, 1996). To reduce pressure of the illegal collections for commercial purposes, there is need for rapid multiplication of these species. Despite the commercial importance of *Argyroderma* spp. as potted flowers, their propagation is limited by seasonal availability of seeds. The destructive use of cuttings is unfeasible because it only produces very few plants. *In vitro* propagation of *Argyroderma* provides a novel method for rapid multiplication of disease-free nursery plants within a limited space and over a shorter period. The purpose of this study was to determine optimal concentrations of plant growth regulators, nutrients and appropriate conditions for *in vitro* shoot initiation and multiplication as well as root induction of rare *Argyroderma* spp.

2.3. Background of research problem

Argyroderma (*Aizoaceae*) originated from the Knersvlakte area of Namaqualand in the Western Cape Province of South Africa (Ellis and Weis, 2006). The Knersvlakte has recently been designated as a Nature Reserve in South Africa (Gosling, 2014) due to its distinctive endemism and high diversity of plants (Desmet *et al*., 1999). The genus grows predominantly on quartz-field habitats and has a highly compact stone-like growth form, usually without stems and often consisting of a single pair of leaves and dwarf, tufted and highly succulent leaf pairs. The blue-green leaf pairs occur at ground

level and are thumb-shaped and united at the base (Ellis *et al*., 2007). They are extremely beautiful, yet endangered ornamental plants (Struck, 1995). The *Argyroderma* genus comprises of *A*. *congretum*, *A*. *crateriforme*, *A*. *delaetii*, *A*. *fissum,A*. framesii, *A*. *framesii* subsp. *hallii*, *A*. *patens*, *A*. *pearsonii*, *A*. *ringens*, *A*. *subalbum*, and *A*. *testiculare*. Of these species, *A*. *framesii*, *A*. *subalbum*, *A*. *testiculare* and *A*. theartii are rare and endangered (Van Jaarsveld, 1997). Despite being rare, these species are now being conserved and grown as commercially viable flowering potted plants.

Populations of rare *Agyroderma* spp. in the Knersvlakte are faced with potential extinction in the near future due to a number of threats to their habitat (Musil *et al*., 2005). Some of the identified threats are erosion of soil during flush floods (Desmet *et al*., 1999), the high plant mortality due to climatic warming (Musil *et al*., 2009), overgrazing (Todd and Hoffman, 1999), clearing of land for urban development (Cowling *et al*., 1999), agriculture (Hilton-Taylor, 1994), mining (Cowling *et al*., 1999; Desmet *et al*., 1999) and the demand for succulent plants (Newton and Chan, 1998). There is need for conservation and rapid plant production of the threatened species in nurseries for both commercial trade and horticultural purposes (Van Jaarsveld, 1997).

Argyroderma spp. are propagated either from seed or vegetatively by division (Ellis and Weis, 2006). Germination of the species in their habitats is often low because of the hard sandy-loam soil that is covered by quartz-gravel substrates. Propagation of *Argyroderma* spp. through cuttings has been reported (Van Jaarsveld, 1997); however, the method provides only limited propagules from selected individuals. *In vitro* propagation generally started in the late seventies (Gupta *et al*., 1980) with more recent attempts to improve establishment and multiplication (Devi *et al*., 1994). Propagation of *Argyroderma* spp. cannot be sustained using conventional methods of seeds and cuttings alone; therefore *in vitro* propagation can provide an alternative method for sustainable production of plants for horticulture.

Conservation of *Argyroderma* spp. in the wild requires a huge financial investment, as the germplasm in the wild habitat is adversely affected by environmental conditions such as erosion, overgrazing and global warming. Thus, more research on conservation should focus on *in vitro* culture of *Argyroderma* spp. *In vitro* propagation

and conservation techniques have a potential of rejuvenating rare *Argyroderma* spp. which are adversely affected by global warming and erosion.

In vitro propagation is widely used for large scale multiplication of plants and according to Rout *et al*., (2006), it has become a major industrial technique in propagation of disease free plants (e.g. *Begonias*, *Chrysanthemum*, *Cyclamen*, *Ficus* spp.), in plant genetic improvement and production of secondary metabolites (Vanisree *et al*, 2004). Using this technique, single explants can be multiplied all year round into thousands of plants in a short period of time, under controlled conditions with and limited space. Endangered, threatened, and rare species have been successfully propagated and preserved by using *in vitro* propagation techniques, due to the high rate of multiplication and small demands on initial plant material and space.

2.4. Project objectives

The main objective of the study was to develop a protocol for rapid *in vitro* propagation of rare *A*. *subalbum*, *A*. *framesii*, *A*. *theartii* and *A*. *testiculare*.

Specific objectives of the study were:

a) To develop an *in vitro* culture protocol for initiating and multiplying shoots and roots of rare *A*. *subalbum*, *A*. *framesii*, *A*. *theartii* and *A*. *testiculare* through hypocotyl explants and leaf cultures.

b) To determine ideal combinations of auxins: cytokinins for inducing shoot and root formation in explants of rare *A*. *subalbum*, *A*. *framesii*, *A*. *theartii* and *A*. *testiculare* propagated under *in vitro* conditions.

c) To determine the ideal conditions for acclimatizing rare *A*. *subalbum*, *A*. *framesii*, *A*. *theartii* and *A*. *testiculare* propagated by *in vitro* culture.

2.5. Key question

a) Can shoot and root development in rare *A*. *subalbum*, *A*. *framesii*, *A*. *theartii* and *A*. *testiculare* be initiated under in-vitro conditions from hypocotyl and leaf tissue explants?

b) What are the ideal combinations of auxins: cytokinins for inducing shoot and root formation in rare *A*. *subalbum*, *A*. *framesii*, *A*. *theartii* and *A*. *testiculare* propagated under *in vitro* conditions?

c) What are the ideal conditions for acclimatizing rare *A*. *subalbum*, *A*. *framesii*, *A*. theartii and *A*. *testiculare* plantlets propagated through *in vitro* culture?

2.6. Significance of the study

The *Argyroderma* genus is unique in that it has some rare and minute succulents confined only to the Knersvlakte's quartz-gravel habitats (Schmiedel and Jurgens, 1999). The Knersvlakte region is known for its high species richness and endemism (Cowling *et al*., 1989, Hilton-Taylor, 1996; Cowling *et al*., 1998), and is a recognized world's biodiversity hotspot (Myers, *et al*., 2000). The species is, however, facing major threats such as high mortality rates due to climatic warming (Thuiller *et al*., 2005; Musil *et al*., 2006), livestock grazing, trampling and illegal collection for the specialist succulent horticultural trade (Raimondo *et al*., 2009). The proposed study seeks to develop a procedure for rapid multiplication of *Argyroderma* spp. Given the illegal over-collection of the species from the wild, the proposed study will enable rare species to be multiplied *in vitro* for the horticultural industry. The technique allows for the production of millions of disease-free plants in a small laboratory environment without incurring huge costs of labour. The technique provides for *in vitro* conservation of rare and endemic species and for rehabilitation of affected habitats. This study is also expected to provide recommendations on favourable and effective growing media and conditions for *Argyroderma* spp. The study has the potential to be used by commercial growers, nurseries and conservation institutions.

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CHAPTER THREE

In vitro propagation studies of rare *Argyroderma subalbum* and *Agyroderma testiculare (*Aizoaceae) endemic to the Knersvlakte Region of South Africa

3.1. Abstract

In vitro propagation of *Argyroderma subalbum* and *Argyroderma testiculare* (*Aizoaceae*) is not known, yet the species are rare and endangered gems of high commercial value as potted flowering plants. We tested varying concentrations (0, 10, 20 and 30 µM) of the cytokinins 6-Benzyladenine (BA), 2-isopentyladenine (2iP) and kinetin (Kn) in Murashige and Skoog (MS) (1962) medium containing 3% sucrose for callus, shoot and root induction for potted-flower production. Leaf explants of *A. subalbum* and *A. testiculare* produced friable calluses on MS medium supplemented with 3% sucrose and 10 µM of BA and Kn. Unlike in *A. subalbum, A. testiculare* callused with 2iP.BA at 10 µM was the most effective cytokinin for both callus and multiple shoot induction in *A. testiculare*. The produced shoots, however, did not root and may require experimentation with varying auxin concentrations for root induction. The study provides a basis for multiplying material for cryopreservation protocols for *A. subalbum* and *A. testiculare* in the future.

Keywords: *Argyroderma* spp., Conservation, 6-Benzylaminopurine (BA), 6-(gamma,gamma-Dimethylallylamino) purine (2ip), kinetin (Kn),*In vitro* culture, Shoots, Callus.

3.2. Introduction

Argyroderma subalbum and *A. testiculare* are rare and minute succulents confined to the Knersvlakte's distinctive quartz-field habitats (Schmiedel and Jurgens, 1999). The Knersvlakte, a region in the Succulent Karoo biome, is known for high species richness and endemism (Cowling *et al.*, 1989, Hilton-Taylor, 1996; Cowling *et al*., 1998), and is recognized as one of the world's biodiversity hotspots (Myers *et al*., 2000). Knersvlaktesoils comprise of heterogeneous sediments of the Nama Group, which are dominated by shale, phyllite and limestone, with intrusions of numerous

quartz veins (Watkeys, 1999), with a gravel layer originating from the quartz veins (Schmiedel and Jurgens 1999).Future anthropogenic global warming is, however, predicted as one of the major threats to biodiversity of flora in the Knersvlakte (Kappelle *et al*., 1999; Sala *et al*., 2000; McCarthy, 2001; Thomas *et al*., 2004; Thuiller *et al*., 2005; Parmesan, 2006).

Populations of rare *Agyroderma* spp. in the Knersvlakte, for example, are faced with potential extinction in the near future due to a number of threats to their habitat (Musil *et al*., 2005). Predictions suggest an increase in aridity and number of climate incidences, which threaten the survival of floral species. Bioclimatic models have predicted a decline in species richness for the Knersvlakte (Midgley *et al*., 2002; Midgley and Thuiller 2007; Foden *et al*., 2007); with high plant mortalities recorded for the endemic *Argyroderma* (*Aizoaceae*) and *Conophytum* (*Crassulaceae*) species (Musil *et al.*, 2005; 2009). The species are threatened by livestock farming (Todd and Hoffman, 1999), mining activities (Cowling *et al.*, 1999) and illegal plant collection (Hilton-Taylor, 1994). Some of the identified threats are erosion of soil during flush floods (Desmet *and* Cowling, 1999), clearing of land for urban development (Cowling *et al*., 1999) and the demand for succulent plants (Newton and Chan, 1998).

Because the flora of the Knersvlakte generates income through eco-tourism, there is need for both *in-situ* and *ex-situ* conservation of the threatened species (Hilton-Taylor and Le Roux, 1989; Hoffman, 1996), the latter demanding rapid multiplication and possibly cryopreservation techniques. *Argyroderma* spp. are currently propagated either sexually from seed or vegetatively by division (Ellis and Weis, 2006). Germination of the species in their habitats is often hampered by the shallow and hard sandy-loam soil overlying the quartz-gravel substrates. Propagation of *Argyroderma* spp. through division has been reported (Van Jaarsveld, 1997); however, the method is not sustainable as it provides only limited propagules from the dwindling numbers of selected individuals.

In vitro propagation started in the late seventies (Gupta et al., 1980), but has not been reported for *A. subalbum* and *A. testiculare.* Given that propagation of *Argyroderma* spp. cannot be sustained by the current conventional methods of seeds

and division alone, *in vitro* propagation can provide an alternative method for sustainable multiplication of the species. The method will not only supply material for *ex-situ* conservation of selected ecotypes, but will also reduce pressure from the illegal collections for commercial purposes. Despite the commercial importance of *Argyroderma* spp. as potted flowers, and as a source of secondary phenolic compounds their nursery multiplication is constrained by lack of propagation material and technical skills. *In vitro* propagation potentially provides a novel method for rapid multiplication and assurance of disease-free nursery plants within a limited space and over a shorter period.

This study explored the optimal concentrations of cytokinins for *in vitro* callusing, shoot and root development of rare *A. subalbum* and *A. testiculare* for use in cryopreservation studies and potted flower production. Currently, there is no literature to our knowledge on *in vitro* propagation of *A. subalbum* and *A. testiculare.*

3.3. Material and Methods

3.3.1. Preparation of media

Media for shoot induction comprised of a full strength Murashige and Skoog (1962) (MS), 30 g L^{-1} sucrose, supplemented with varying concentrations of different cytokinins and solidified with 7.5 g L^{-1}agar. The cytokinin concentrations were 0, 10, 20 or 30 µM of 6-Benzyladenine (BA), 2-isopentyladenine (2iP) or kinetin (Kn). Media for root induction experiments comprised of MS, 30 g L^{-1} sucrose, supplemented with varying concentrations of naphthalene acetic acid (NAA) or indole acetic acid (IAA) at concentrations of 0, 10, 20 or 30 µM. The media were solidified with 7.5 g L^{-1}agar, but before the addition of agar, the pH was adjusted to 5.7 and then heated after adding agar, with constant stirring until attaining a temperature of 96^{o}C. Each medium had 15 replicates of 40 mls made in 250 ml glass-vessels (Consol, Stellenbosch, RSA) and autoclaved for 20 min at 121°C.

3.3.2. Preparation of explants

A. subalbum and *A. testiculare* collected from Kokkerboom Kwekery in Knersvlakte (Latitude -31.611074, Longitude 18.746165), had their youngest leaves excised and washed with running tap water, rinsed with distilled water then surface sterilized with 70% ethanol for 30s and rinsed in 3% sodium hypochlorite (NaOCl) with 2 drops of 0.1% tween-20 added. Ultraviolet (UV) light of the laminar flow hood was switched on for 15 minutes to kill bacteria before surface-sterilization with 70% ethanol. Leaves were then rinsed three times with sterile distilled water under a laminar flow hood for 20 minutes each rinse to remove traces of disinfectants. Leaves were cut to small cubes (*ca.* ± 1cm) and aseptically plated on the different sterilized shoot induction media. For the root induction experiment, multiple shoots produced from the shoot induction study were cultured onto the prepared root induction media. Cultured explants were incubated in the growth room at 25±2^{o}C with a day/night cycle of 16/8 hours. The mean photosynthetic photon flux density (PPFD) in the growth room was 950 µmol $m^{-2}s^{-1}$.

3.3.3. Data collection and analysis

Counts of shoots, callused explants and browning explants were done at 14 day intervals over 23 weeks and photos taken for visual observations. After a log (x+1) transformation of the data for normality, a factorial two-way analysis of variance (ANOVA) was done using Statistica 12.0 (Dell Inc., USA). A Fischer's least significant difference (LSD) test was used for separation of significantly different means.

3.4. Results

3.4.1. Callusing response of *A. testiculare* and *A. subalbum*

A. testiculare produced varying responses to different cytokinin levels of 2iP, BA and Kn (Fig 3.1). Leaf explants of *A. testiculare* had the highest number of friable callus across all cytokinins at 10 µM (Fig 3.1 & Fig 3.2 B-D). However, *A. testiculare* had

puzzling results with BA, as it formed callus also at 30 µM but not at 20 µM. Callusing only happened for *A. subalbum* at 10 µM of BA and kinetin (Fig 3.2 E & F).

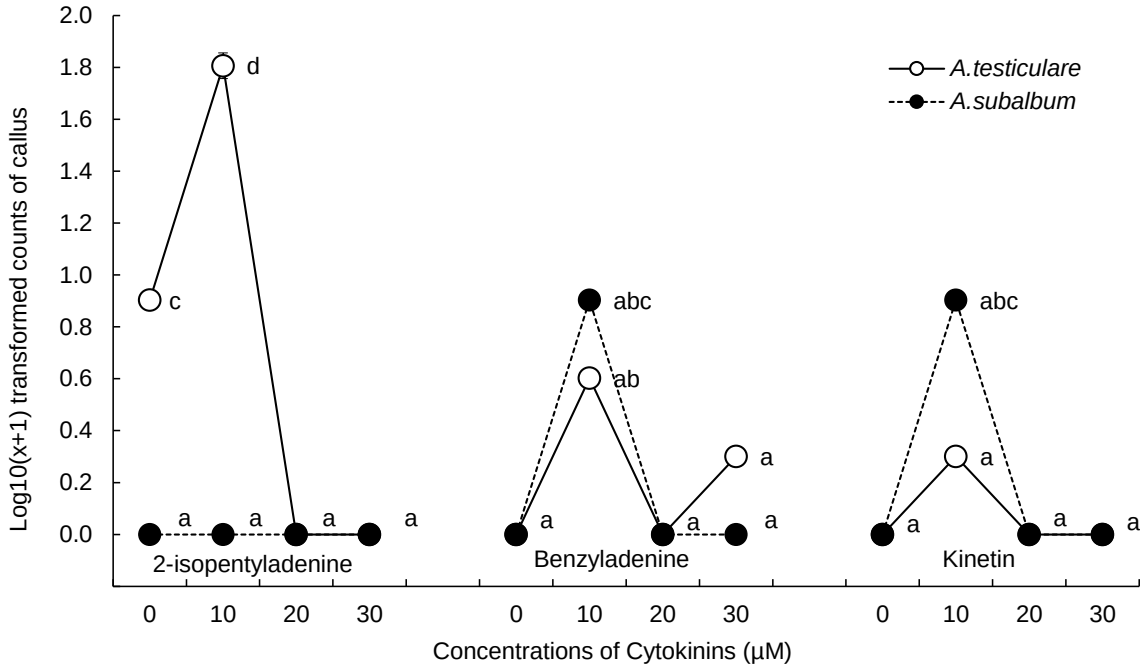

Fig.3.1. Callusing of *A. testiculare* and *A. subalbum* in response to increasing levels (0, 10, 20 and 30 µM) of 2-isopentyladenine (2iP), 6-Benzyladenine (BA) and Kinetin (Kn). Values are mean ±se of the $\log_{10}(x+1)$ transformed counts of callusing specimens. Values with different letters are significantly different at p=0.05.

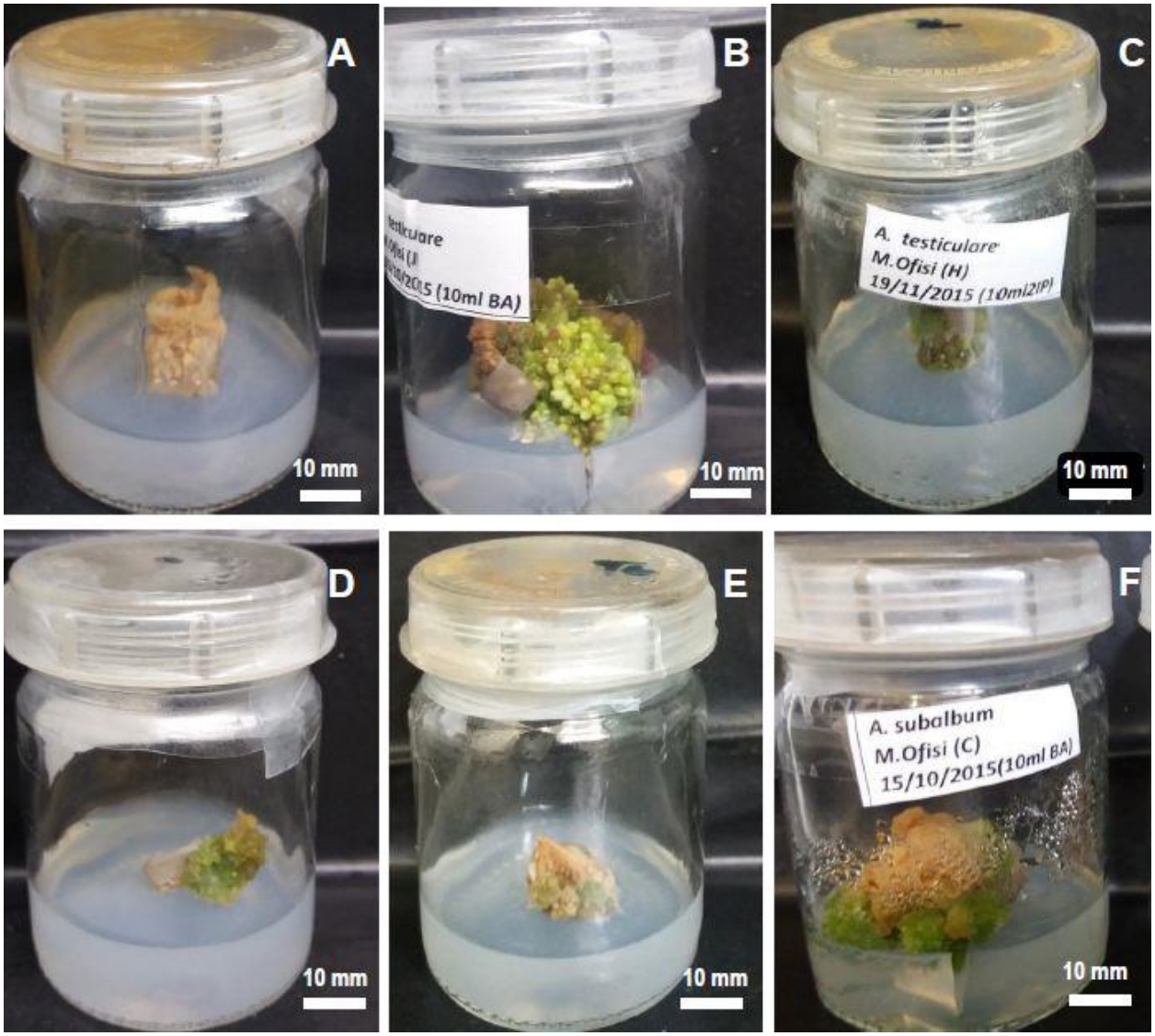

Fig. 3.2. (A) Callus formation in *A. testiculare* in Murashige and Skoog (1962) media control, (B) Callus formation in *A. testiculare* at 10 µM BA, (C) Callus formation in *A. testiculare* at 10 µM of 2iP, (D) Callus formation in *A. testiculare* at 30 µM BA, (E) Callus formation in *A. subalbum* at 10 µM kinetin, (F) Callus formation in *A. subalbum* at 10 µM of BA.

3.4.2. Callus area of *A. testiculare* and *A. subalbum*

A. subalbum and A. testiculare produced varying callusing responses to 10 µM of the cytokinins 2iP, BA and Kn (Fig 3.3). Leaf explants of *A. subalbum* and *A. testiculare* both produced friable callus with all cytokinins at 10 µM, with the exception of *A.*

subalbum, which did not callus with 10 µM of 2iP (Fig 3.3). However, *A. subalbum* had the biggest size of friable callus at 10 µM of BA (Fig 3.3).

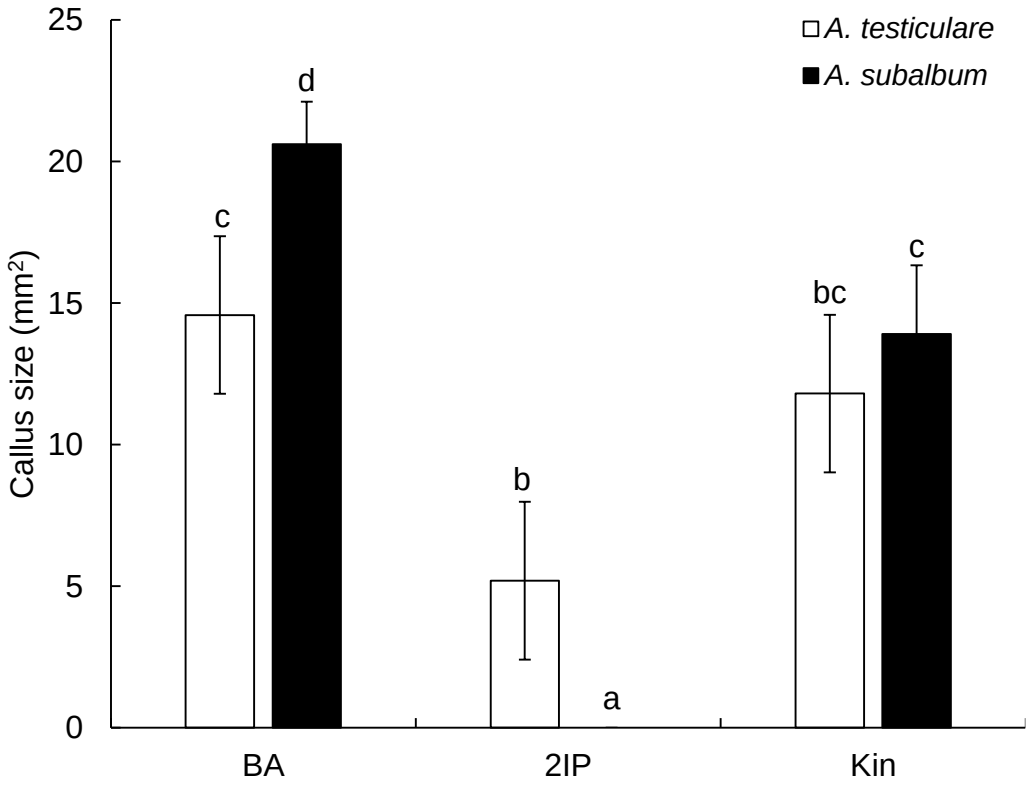

Fig. 3.3. Callus size of *A. testiculare* and *A. subalbum* in Murashige and Skoog (1962) media, supplemented with 10 µM of 2-isopentyladenine (2iP), 6-Benzyladenine (BA) and Kinetin (Kn). Bars represent mean ±se of callus area, determined images using Image-J software (National Institute of Health, USA).

3.4.3. Shoots response of *A. testiculare* and *A. subalbum*

A. testiculare and *A. subalbum* produced varying differences in response to different cytokinins levels 2iP, BA and Kinetin (Fig 3.4). *A. testiculare* had the highest number of shoots at 10 µM of BA (Fig 3.4 & Fig 3.5C). However, *A. testiculare* also produced shoots with BA at 30 µM but not at 20 µM. Subsequently, shoots were also produced on *A. testiculare* at 10 µM in BA or 2iP and also without addition of Kinetin

(Fig 3.4).On *A. subalbum* shoots were only produced at 10 µM of BA and kinetin (Fig 3.5 E & F).

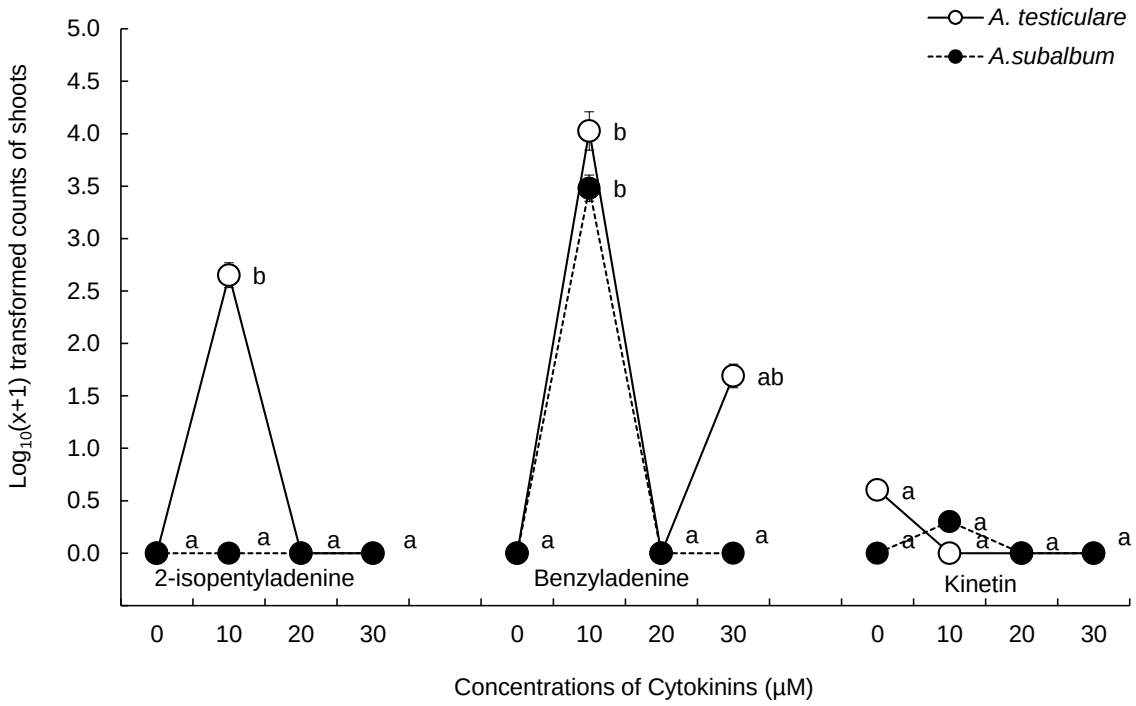

Fig. 3.4 Shoots formation of *A. testiculare* and *A. subalbum* in Murashige and Skoog (1962) media supplemented with different levels (0, 10, 20 and 30 µM) of 2-isopentyladenine (2iP), 6-Benzyladenine (BA) and Kinetin (Kn). Circles and bars represent mean ±se of $log_{10}(x+1)$ transformed counts of shoots specimens.

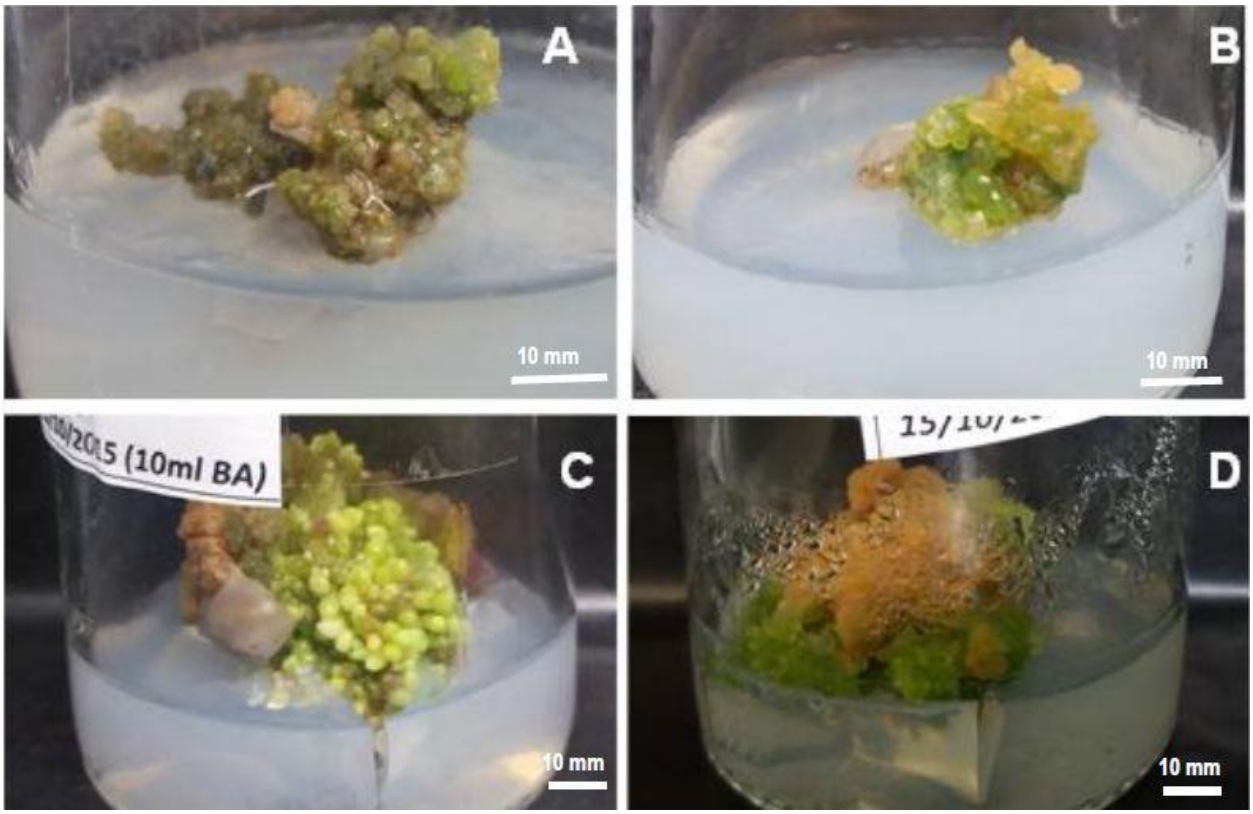

Fig 3.5 Shoots formation of *A. testiculare* in Murashige and Skoog (1962) supplemented with 10 µM of (A) 2iP, (B) 30 µM BA, (C) 10 µM BA and (D) Shoots formation in *A. subalbum* at 10 µM BA. [White scale bars = 10mm.]

3.4.4. Browning of explants in *A. testiculare* and *A. subalbum*

Phenolic compounds occur in many plant species, which results in browning of their cultured explants, as was observed in this study. Browning was associated with failure of callus induction, as was noted in this study. Different levels of cytokinins had varying degrees of browning of explants in 2iP, BA and Kinetin (Fig 3.6). Definite patterns emerged with increasing cytokinin concentrations for both *A. testiculare* and *A. subalbum.* When supplemented with 2iP and BA, the callus were characterised by an initial increase in browning followed by a general decline at the highest concentration (Fig 3.6). *A. testiculare* and *A. subalbum* had similar trends, with the highest numbers of explants browning with low levels of cytokinin (Fig 3.6) and the lowest numbers occurring at 30 µM (Fig 3.6).

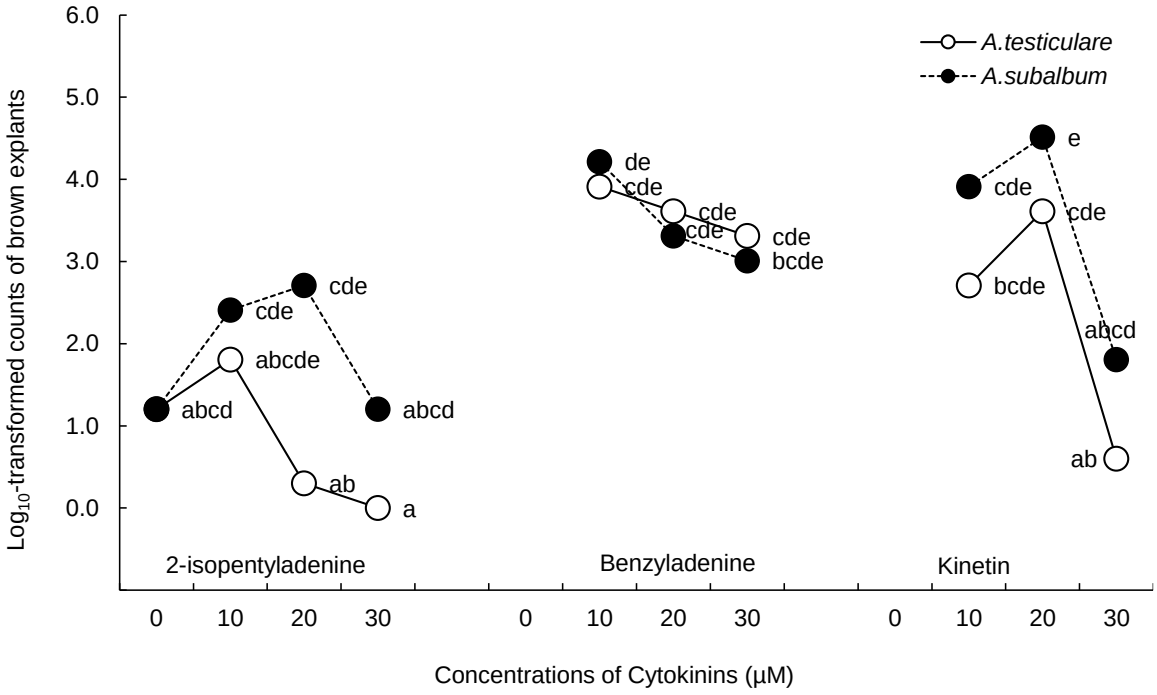

Fig 3.6. Presence of phenolic compounds on explants browning of wounded tissues, of *A. testiculare* and *A. subalbum* in response to increasing levels (0, 10, 20 and 30 µM) of 2-isopentenyladenine (2iP), 6-Benzyladenine (BA) and Kinetin. Values represent $log_{10}(x+1)$ transformed counts of shoots specimens.

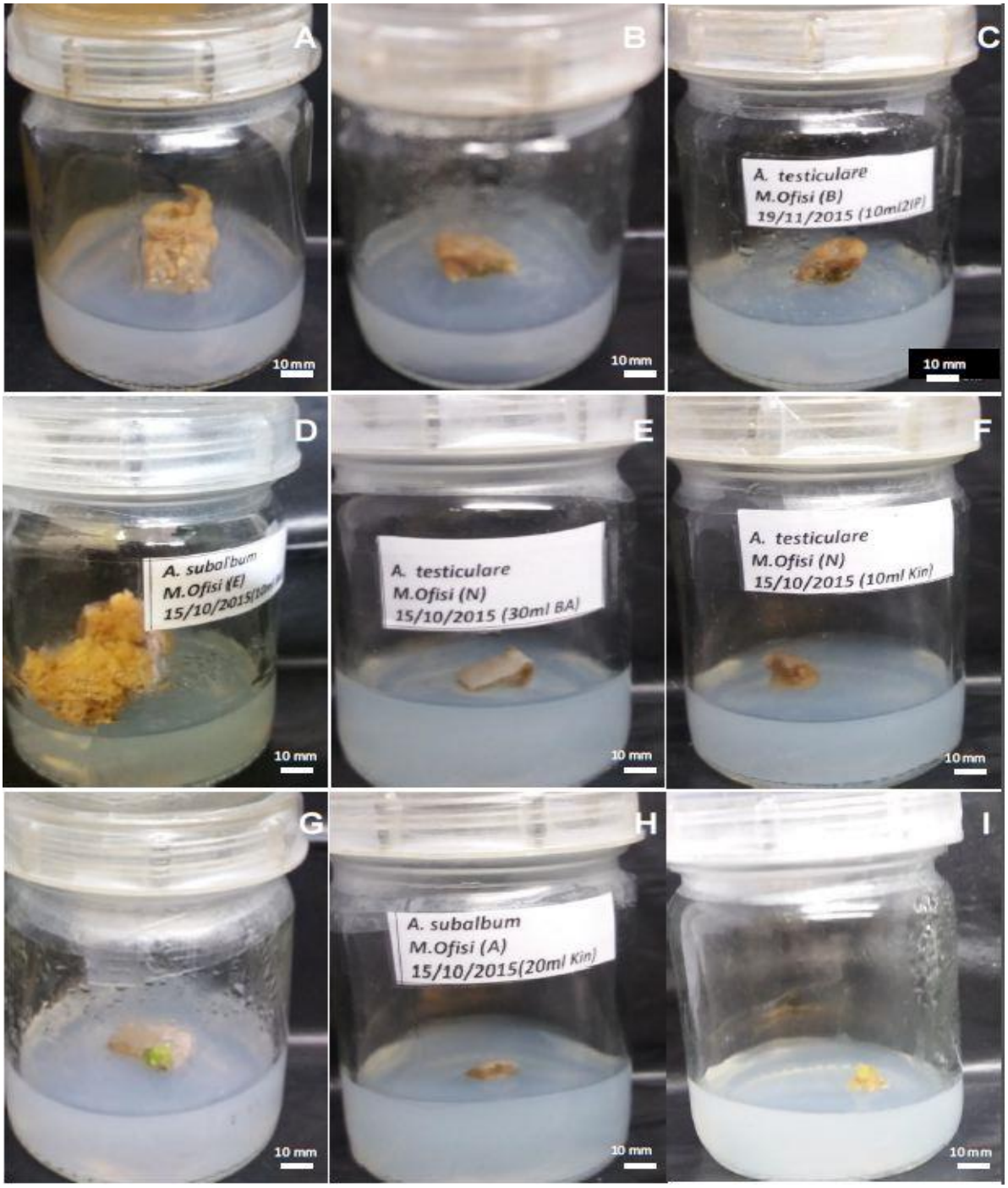

Fig. 3.7. (A) Occurrence of explants browning in *A. testiculare* in Murashige and Skoog (1962) media control, (B) control *A. subalbum,* (C) *A. testiculare* at 10 µM of 2iP,

(D) *A. subalbum* at 10 µM of BA, (E) *A. testiculare* at 30 µM BA, (F) *A. testiculare* at 10 µM of kinetin, (G) *A. testiculare* at 10 µM of BA, (H) *A. subalbum* at 20 µM of 2iP, (I) *A. subalbum* at 20 µM of Kinetin

3.4.5. Rooting response of *A. testiculare* and *A. subalbum*

Despite the successes in callusing and multiple shoots formation, none of the tested concentrations (0, 10, 20 or 30 µM) of naphthalene acetic acid (NAA) or indole acetic acid (IAA) induced rooting in both *A. testiculare* and *A. subalbum*.

3.5. Discussion

It was demonstrated in this study, for the first time that callusing and shoots formation are inducible in both *A. subalbum* and *A. testiculare* when cultured in full-strength Murashige and Skoog (1962) supplemented with 2iP, BA or Kn. Evidence of callusing was apparent at 10 µM concentration, despite the browning that occurred due to the potential accumulation of polyphenols. Browning is associated with a reduced number of callused explants, as was noted in the study.

Unlike in *A. subalbum* which only callused in 10 µM of BA or kinetin, callusing in *A. testiculare* was induced in the presence of 10 µM of 2iP, BA or Kn. The leaf explants developed a friable callus after three weeks, which differentiated into multiple shoots. As was observed in this study, previous work has demonstrated that 2iP can induce plant regeneration in *C. edulis,* an *Aizoaceae* spp. at 20 µM 2iP (Khattab and EI Sherif, 2011). Similarly, it was possible to induce callus formation with 10 µM Kn in both *A. testiculare* and *A. subalbum*. These results concur with previous studies that callus formation is achievable in an *Aizoaceae* spp. (*Zaleya decandra*) with kinetin supplementation (Radfar *et al*., 2012).

The observed difference in sizes of friable calluses from the leaf explants in all the cytokinins at 10 µM (Figure 3.3) demonstrated the varying potencies of the cytokinins. BA was the most effective hormone to induce optimum callus growth (Fig. 3.3). The calluses obtained in this study were relatively compact and pale-green in

colour. Similar observations of BA induced callusing have been reported in some members of the *Aizoaceae*, *Agavaceae* and *Crassulaceae* (Gratton and Fay, 1990).

The fact that multiple shoots of *A. subalbum* and *A. testiculare* were obtained at 10 µM 2iP and BA demonstrated that these species can be rapidly propagated from leaf explants *in vitro*. As roots were not observed from both the cytokinin levels and auxin levels tested, it is recommended that other varying exogenous auxins be tested on the multiple shoots that emerged from explants for purposes of inducing root formation. Current studies (not reported here) evaluated varying levels of 2.4D on root development. Feasibility studies of cryopreservation of *A. subalbum* and *A. testiculare* are a priority, given the threat of the species from illegal collections, mining, trampling by livestock and anthropogenic warming of their endemic to the Knersvlakte (Musil *et al*., 2006).

The high rate of *in vitro* callusing of *Aizoaceae* species, which are enriched with bioactive principles, can commercially be exploited for selective secondary metabolite production, particularly for production of medicinally important compounds. Leaves of *Argyroderma* spp. are known to contain phenylpropanoids, particularly flavonoids and anthocyanins (Musil, 2005), which can be exploited for pharmaceutical purposes. As in *Carpobrotus* spp. (*Aizoaceae*), these secondary metabolites can potentially be exploited from the produced calluses for medicinal properties (sore throats and treating fungal infections), in soap making, preservatives, poultices and in preparations of psychoactive medicinal remedies (Bouftira *et al*., 2009).

In conclusion, *A. subalbum* and *A. testiculare* were induced *in vitro* to produce calluses and multiple shoots from 2iP, BA and kinetin. Although root development from the shoots remains unresolved, both species form callus at 10 µM concentrations of the tested cytokinins, but to varying degrees. BA was the most efficient in inducing callusing. Given that *in vitro* callusing and shoot multiplication were achievable, further studies can focus on root induction, cryopreservation of the multiple shoots and/or callus of these rare and endangered species.

3.6 Conclusion

A. subalbum and *A. testiculare* can be multiplied *in vitro* as was demonstrated in the current study. Whilst root induction remains unresolved, it can be concluded that both species can be induced to form callus at 10 µM concentrations of the tested cytokinins, although to varying degrees. In particular, BA was the most efficient in terms of callus induction. Further studies would be focused at increasing the rate of shoots regeneration from callus in efforts to initiate root formation in *Argyroderma spp.*

3.7 Acknowledgements

We would like to express tremendous gratitude to DST-NRF for funding this project and the Department of Horticultural Sciences, Cape Peninsula University of Technology for the use of research facilities. Lastly, we are very thankful to Kokerboom Nursery/Kwekery for their consistence assistance of plant material.

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CHAPTER FOUR

Role of indole-3-acetic acid (IAA) and activated charcoal on *in vitro* root initiation of *A. subalbum* and *Argyroderma testiculare*.

4.1. Abstract

In vitro rooting of *Argyrodeema subalbum* and *A. testiculare* explants is inhibited by polyphenolic exudates, which lead to 'browning' of cultures. Activated charcoal was tested on the two species as an anti-oxidant against browning, together with four concentrations of auxins for promoting callusing, shoot and root initiation. Leaf explants were cultured on Murashige and Skoog (MS) (1962) basal medium containing 7.5 $g.l^{-1}$sucrose, supplemented with varying concentrations (0, 10, 20 and 30 µM) of indole-3-acetic acid (IAA) with or without 2 $g\ L^{-1}$ activated charcoal. IAA at 20 µM was effective in callus induction in *A. testiculare* with no activated charcoal. Absence of activated charcoal resulted in intense browning of *A. subalbum* cultures, whilst media with activated charcoal had the least brown cultures in both species. Activated charcoal suppressed callus formation. Indole-3-acetic acid had no significant effect on callus induction. Contrary to previous studies, activated charcoal was not beneficial to *A. subalbum* and *A. testiculare* explants, but had inhibitory effects on callusing and root development. It can be concluded that using activated charcoal for reducing browning intensity in *A. subalbum* and *A. testiculare* explants does not guarantee better callusing and rooting. Alternative antioxidants are therefore recommended in place of activated charcoal.

Keywords: Explants browning, MS media, Media composition, Vitamins, *In vitro culture*, Phenolic compounds.

Abbreviations: MS- Murashige and Skoog (1962), AC-activated charcoal.

4.2. Introduction

Exogenous auxins such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are generally credited with enhanced adventitious root formation (Ludwig-Müller,

2000), particularly for species that release tolerable quantities of phenolic compounds. Like most members of the *Aizoaceae*, however, the genus *Argryderma* produces high levels of phenolics (Musil *et al*., 2005), which often accumulate in tissue media, resulting in 'browning' of *in vitro* cultures. Browning of cultured explants is a serious challenge in *in vitro* propagation of plants (North *et al*., 2010), including *A. subalbum* and *A. testiculare* releasing excessive phenolics from the cut edges of their explants. Polyphenols can be phytotoxic, inhibiting callus formation, shoot and root initiation (Pierik, 1987) and necrosis and death of explants (Laukkanen *et al*, 1999). Ascorbic acid (Ndakidemi *et al.,* 2014), citric acid (Wang *et al*., 2005), activated charcoal (Pan and van Staden, 1998) and sub-culturing of explants are potential remedies for reducing oxidation of polyphenols in propagation media.

Addition of activated charcoal is commonly used in tissue culture media to promote or inhibit *in vitro* growth, depending on the species and tissues used. Activated charcoal functions by establishing a darkened environment; adsorbing the undesirable/inhibitory polyphenols (Thomas, 2008); adsorption of growth regulators and other organic compounds, or the release of growth promoting substances present in or adsorbed by activated charcoal (Pan and van Staden, 1998). Activated charcoal is a porous and tasteless material distinguished from elementary carbon by removal of all non-carbon impurities and the oxidation of carbon surface. Despite its important adsorbent properties in sequestering toxic substances, activated charcoal is often used to improve cell growth, somatic embryogenesis and promoting growth alteration or darkening of culture media (Thomas, 2008).

The main objective of this study was to overcome 'browning' of *in vitro* cultures so as to initiate root development in *A. subalbum* and *A. testiculare.* For root initiation, different levels of an auxin IAA were tested, combined with or without activated charcoal, which is an antioxidant for reducing effects of phenolic exudates.

4.3. Material and Methods

4.3.1. Preparation of media

Media for callus induction comprised of half-strength MS, 7.5 g L^{-1} sucrose, supplemented with 10 µM BA and one of varying concentrations (0, 10, 20 or 30 µM) of IAA. The media had either 2 g L^{-1} of either activated charcoal or 2 g L^{-1} dH_2O, thus giving a 2x4 factorial experiment. The media were solidified with 1,875 g L^{-1} of agar, but before the addition of agar, the pH was adjusted to 5.7 using drops of NaOH or HCl before and heated after adding agar, with constant stirring until attaining a temperature of 96°C. Each medium had 10 replicates of 25 mls made in 250 glass-vessels (Consol, Stellenbosch, RSA) and autoclaved at 121°C for 20 min.

4.3.2. Preparation of explants

A. subalbum and *A. testiculare* collected from Kokkerboom Kwekery (Latitude-31.611074, Longitude 18.746165), had their youngest leaves excised and washed with running tap water, rinsed with distilled water then surface sterilized with 70% ethanol solution for 30s and rinsed in 3% sodium hypochlorite (NaOCl) with 2 drops of 0.1% tween-20 added. Ultraviolet (UV) light of the laminar flow hood was switched on for 15 minutes to kill bacteria before surface-sterilization with 70% ethanol. Leaves were then rinsed three times with sterile distilled water under laminar flow hood for 20 minutes each rinse to remove all the traces of disinfectants. Leaves were cut to small cubes (*ca.* ± 1cm) and aseptically plated on the different sterilized induction media. Cultured explants were incubated in the growth room at 25±2^{o}C temperature with a day/night cycle of 16/8 hours. The mean photosynthetic photon flux density (PPFD) in the growth room was 950 µmol $m^{-2}s^{-1}$.

4.3.3. Data collection and analysis

Counts of callused explants, browning explants were done in14d intervals and over 23 weeks and photos taken on visual observations. After a $\log_{10}$ (x+1) transformation of the data for normality, Browning of explants was quantified using Basic Intensity Quantification with Image-J software (National Institute of Health, USA), a factorial two-way analysis of variance (ANOVA) was done using Statistical 12.0. (Dell Inc., USA). A Fischer's least significant difference (LSD) test was used for separation of significantly different means.

4.4. Results

4.4.1. Response of *A. subalbum* and *A. testiculare* with and without activated charcoal

All explants showed oxidative browning, with the intensity of browning reduced with activated charcoal (Fig 4.1). Although activated charcoal reduced the intensity of lethal browning (Fig 4.1), it did not significantly enhance the callusing of leaf explants. Browning intensity was highest on media free of activated charcoal. Callusing increased with IAA supplements on both *A. subalbum* and *A. testiculare.* Callus proliferation was relatively low and all explants exhibited browning regardless of the presence or absence of activated charcoal (Fig. 4.2).

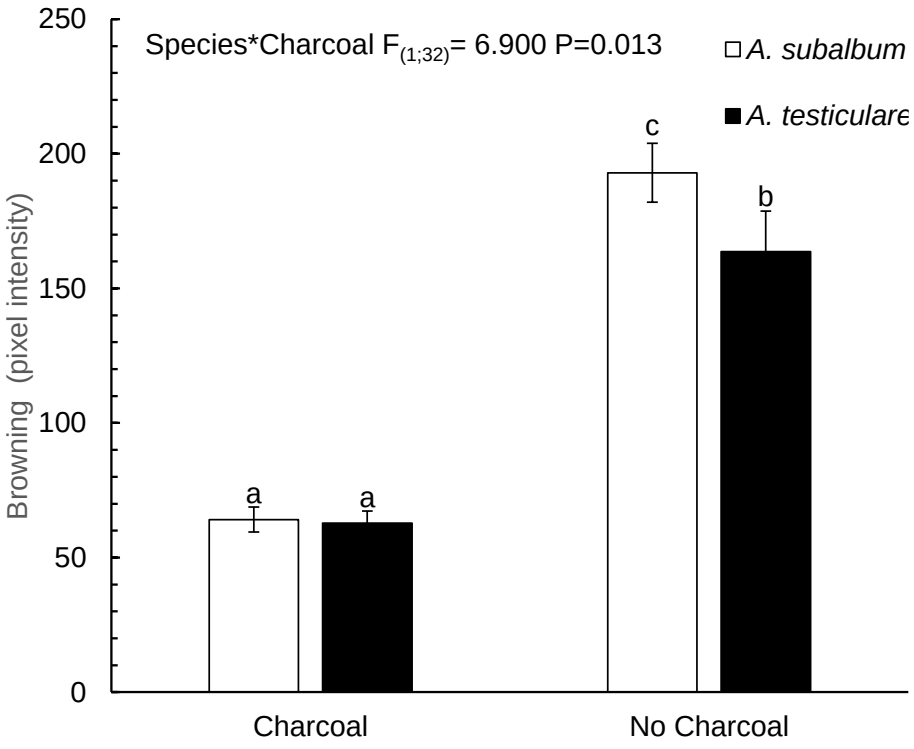

Fig. 4.1. Response of *A. subalbum* and *A. testiculare* in increasing levels (0, 10, 20 and 30 µM) of IAA incorporated with and without Activated charcoal to overcome oxidative browning of explants, Bars represent means± se brown intensity explants, determined images using Image-J software (National Institute of Health, USA).

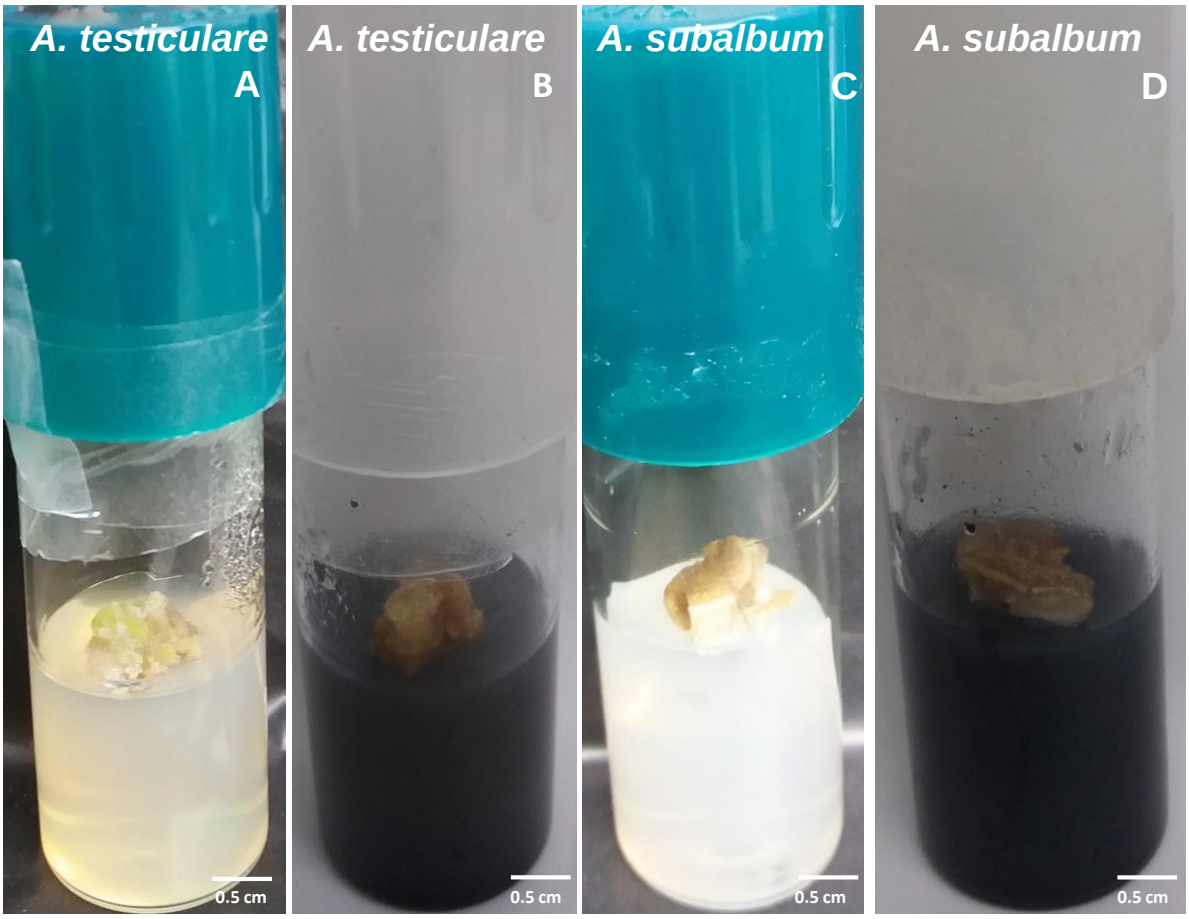

Fig.4.2. Response of *A. testiculare* to 20 µM of indole-3-acetic acid and A) without activated charcoal and B) with activated charcoal. Response of *A. subalbum* to 20 µM of indole-3-acetic acid and C) without activated charcoal and D) with activated charcoal. [White scale bars =0.5 cm].

4.4.2 Callusing response of *A. testiculare* and *A. subulbam* with and without activated charcoal

Callus formation was achieved from matured leaf explants on *A. testiculare* species, but not on *A. subalbum*. *A. testiculare* explants produced friable callus at 20 µM indole acetic acid (Fig 4.3). Callus development occurred only at 20 µM of IAA, with an addition of activated charcoal (Fig 4.3).

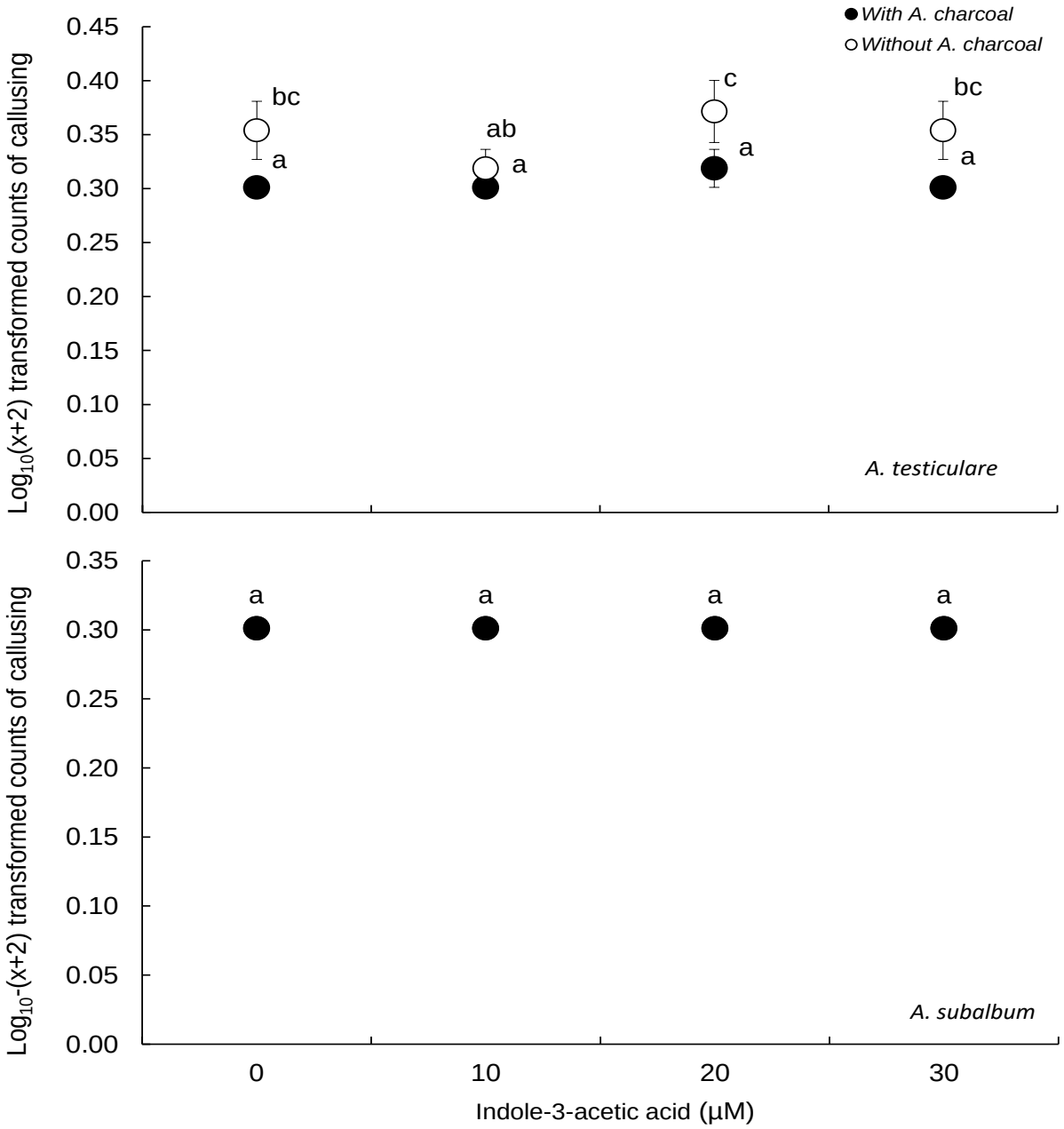

Fig. 4.3. Callus formation in *A. testiculare* in response to increasing levels (0, 10, 20 and 30 µM) of IAA with and without addition of activated charcoal. Values are means ±se of the $\log_{10}(x+1)$ transformed counts of callusing specimens. Values with different letters are significantly different at p=0.05.

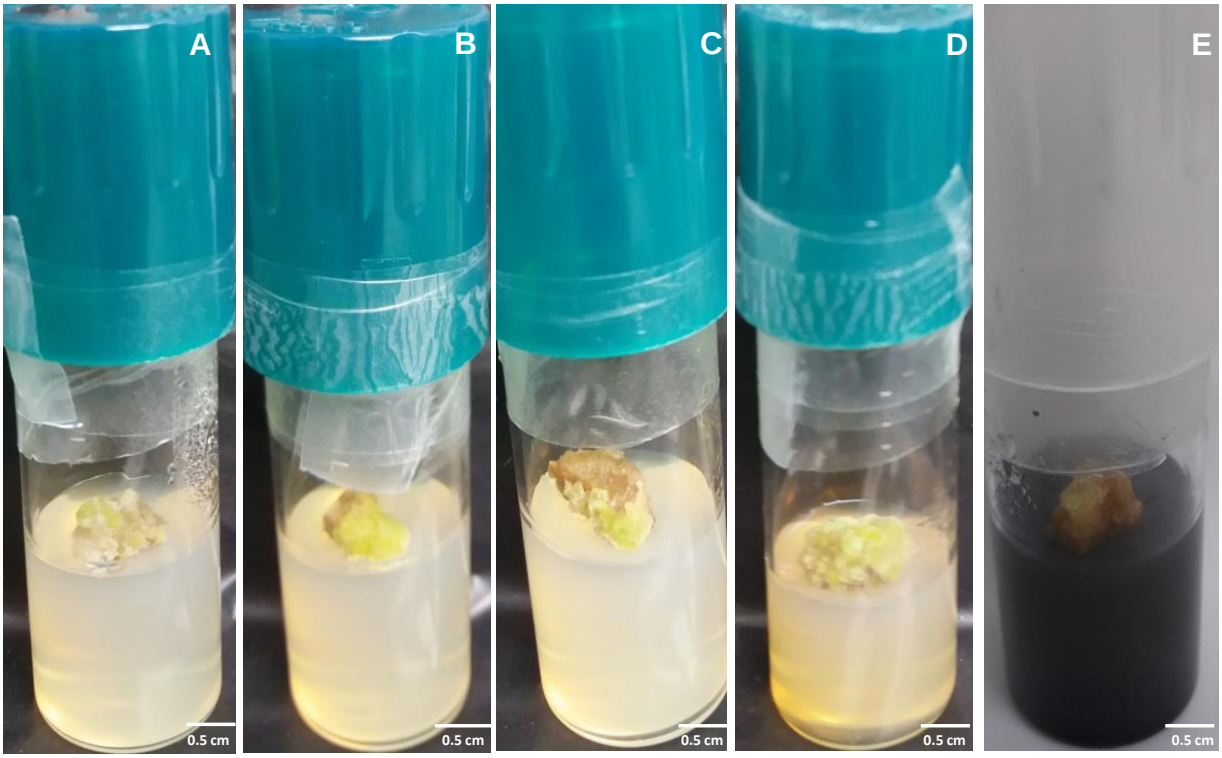

Fig. 4.4. (A) Callus formation of *A. testiculare* in Murashige and Skoog (1961) medium, (B) Callus formation of *A. testiculare* at 10 µM of IAA,(C) Callus formation of *A. testiculare* at 20 µM of IAA, (D) Callus formation of *A. testiculare* at 30 µM of IAA and (E) Callus formation of *A. testiculare* at 20 µM of IAA with charcoal. [White scale bars =0.5 cm

4.5 Discussion

Argyroderma spp. accumulate polyphenolic substances (Musil *et al*., 2005), which led to the observed 'browning' of *A. subalbum* and *A. testiculare* tissue cultures. Whilst activated charcoal is commonly used as an anti-oxidant against browning, it had not been tested on *A. subalbum* and *A. testiculare*, until now, for promoting callus initiation and rooting of *in vitro* cultures. To achieve optimum rooting, activated charcoal was tested with or without a root inducing hormone, IAA.

Although activated charcoal reduced the intensity of browning, its presence appeared to inhibit rooting and callusing. Firstly, it was demonstrated that callus was inducible in *A. testiculare* when propagated in half-strength MS with or without activated charcoal. Contrary to previous studies (Thomas, 2008), activated charcoal neither enhance callusing nor root initiation. Instead, *A. testiculare* cultured at 20 µM of IAA had significantly higher levels of callus induction without activated charcoal. It emerged that callusing was dominant in explants without activated charcoal, which had a high intensity browning.

Supplementing activated charcoal for reducing 'browning' of *in vitro* cultures of *A. subalbum* and *A. testiculare* could not be supported by the current study. Activated charcoal comprises of carbon, arranged in a quasi-graphitic form, with a very fine network of pores with large inner surface area on which many substances can be adsorbed (Thomas, 2008). Activated charcoal has various essential components of *in vitro* culture media, which prevent explants from browning of cultured tissues and media by adsorption of toxic substances like polyphenols released by cultured tissues (Thomas, 2008). Since the scope of the study did not allow for measurement of ingredients available to plants after the experiment, it remains unknown whether essential ingredients of the media got adsorbed onto the activated charcoal of *A. subalbum* and *A. testiculare* explants. Activated charcoal may adsorb plant growth regulators, hence reducing their availability to the explants (Zhou *et al*., 2010).

Comparing *A. subalbum* to *A. testiculare* revealed that *A. testiculare* was more potent, callusing readily even with activated charcoal, particularly at 20 µM. Despite having a lower browning intensity than *A. testiculare*, that neither improved callusing nor increased rooting of explants. Poor callusing with activated charcoal (Fig.4.1-4.2), has been reported on other *Aizoaceae* spp. (Zuraida *et al*., 2015). Addition of activated charcoal prevented the embryo from producing callus formation (Priya *et al*, 2015). Activated charcoal does not only adsorb toxic substances, but also nutrients in media (Nisyawati, 2013). Activated charcoal was not an ideal absorbent to reduce browning on both species.

Although tissues comprising of high concentrations of phenolic compounds are difficult to grow on *in vitro* culture (Scalbert *et al*., 1990; Dodds and Roberts, 1987; Khatri et al., 1997; Zweldu and Ludders, 1998; Abeyaratne and Lathiff, 2002; Titov *et al*., 2006), there are alternative antioxidants that reduce browning. To reduce this problem, modified medium can be supplemented with ascorbic acid and citric acid (Wang *et al*., 2005). Transfer of cultures to new media can reduce the accumulation of phytotoxins in tissue cultures.

Callusing in *A. testiculare* can be induced in the presence of IAA. This was demonstrated in this study by observing callus, which was significantly higher at 20 µM. Despite 20 µM being the highest on callusing without addition of activated charcoal, friable callus on *A. testiculare* was also produced at 10 µM and 30 µM. The previous studies (Shrivastava and Rajani, 1999) supported that the presence of IAA in the medium was found to be the most effective to induce callus induction. Evidence from this study, showed that callus can be induced in *A. testiculare* with addition of activated charcoal without controlling oxidative browning of explants cultured. Previous studies demonstrated that the addition of the activated charcoal on *in vitro* culture media can be beneficiary or adverse to explants growth.

4.6. Conclusion

Contrary to previous studies, activated charcoal was not beneficial to *A. subalbum* and *A. testiculare* explants, but inhibited callusing and root development. Although roots were not obtained, callusing was optimum with 20 µM of IAA. Therefore, using activated charcoal for reducing browning intensity in *A. subalbum* and *A. testiculare* explants does not guarantee better callusing and rooting. Alternative antioxidants such as ascorbic and citric acid should be pursued in place of activated charcoal.

4.7. Acknowledgements

We would like to express tremendous gratitude to DST-NRF for funding this project and the Department of Horticultural Sciences, Cape Peninsula University of Technology for the use of research facilities. Lastly, we are very thankful to Kokerboom Nursery/Kwekery for their consistence assistance of plant material.

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CHAPTER FIVE

In vitro propagation of shoots through somatic embryogenesis from matured leaf explants of *Argyroderm testiculare*

5.1 Abstract

Knowledge concerning *in vitro* propagation and developmental responses of *Argyroderma testiculare* is known. A rapid method of *in vitro* shoots multiplication was tested. This study was aimed at determining the role of sub-culturing for root regeneration, plant growth regulators (PGRs) and exudation browning of explants *in vitro* culture involving *A. testiculare*. An efficient and rapid method for *A. testiculare* was developed, resulting in shoots multiplication after 3 weeks of sub-culturing. Transferred shoots were regenerated from friable callus cultured on Murashige and Skoog (MS) (1962) medium containing 3% sucrose and supplemented with varying concentrations of 6- Indole-3-acetic acid (IAA) (0, 10, 20 and 30 µM). Results showed that various 6-Indole-3-acetic acid (IAA) and 6-benzylaminopurine (BA) concentrations significantly affected phenolic exudation. MS medium supplemented with 10 µM BA proved to be the most effective treatment in shoots multiplication on *A. testiculare*. Sub-culturing of shoots improved *in vitro* shoots quality and increased survival after evaluated, resulted in sub-culturing significantly reduced phenolic compounds content in all varying concentrations of auxins in sub-cultured shoots.

Keywords: Phenol exudation, browning, 6- Indole-3-acetic acid (IAA), transferred shoots.

5.2 Introduction

Argyroderma testiculare (*Aizoaceae*) is one of significant South African succulent plants with a high commercial value as potted flowering plants. The plant species originate from South Africa and is endemic to quartz-gravel fields of the Knersvlakte region (Schmiedel *et al.*, 2012). Its success is limited by the slow conventional methods of propagation (Malda *et al*., 1999). Despite its conventional methods of propagation being slow *A. testiculare* captured the attention of tourists, who keep on visiting the area

of its endemism (Gosling, 2014). Due to these constraints on propagating; there is need for the development of reliable *in vitro* culture techniques for this plant. However *A. testiculare* has proved to be having more phenolic compound *in vitro* culture. *In vitro* propagation attempts of this species have showed limited success due to the high oxidative of explants browning as this was noted on previous experiment.

This critical problem was also witnessed by genera related species to *Strelitzia*, namely *Musa* and *Ensete* (Zeweldu and Ludders, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004; Titov *et al*., 2006; Martin *et al*., 2007; Ko *et al*., 2009) and *Musa acuminata* (Nisyawati, 2013) but not Aizoaceae family. Acccording to Ozyigit (2008), the occurrence of oxidative browning and death of cultured explants is a major problem *in vitro* culture that is always dependent on the phenolic compounds and the total quantity of phenols. Phenolic compounds take place as secondary metabolites in all plant species (Antolovich *et al*., 2000) and polyphenols are synthesized by the plant or excreted and then oxidized (Ibdah *et al*., 2002). In tissue culture studies, phenolic substances, especially oxidized phenols, generally affect *in vitro* development negatively (Chatris *et al*., 2001). Oxidized phenolic compounds may inhibit enzyme activity and result in the darkening of the culture medium and successive toxic browning of explants (Laukkanen *et al*., 1999). Activated charcoal is commonly used in an *in vitro* culture media to improve cell growth and prevent oxidative browning of explants (Pan and Van Staden, 1998; Thomas, 2008), to enhancing somatic embryogenesis, shoots formation, plant recovery and rooting (Buter *et al*., 1993; Mathews *et al*., 1993). The beneficial effects of activated charcoal may be recognized due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially reduce the poisonous metabolites, phenolic exudation and exudate accumulation (Fridborg *et al*., 1978; Thomas, 2008). This high adsorptive capacity is due to the structure of activated charcoal. It has a very fine network of pores with a large inner surface area on which many substances can be adsorbed (Pan and van Staden, 1998; Thomas, 2008). During *in vitro* propagation the exudation of phenols is very common and it always influences the results. It was reported on previous studies by Soniya and Sujitha (2006) that leaching of polyphenols affected the shoot growth in *in vitro* culture, though an addition

of activated charcoal in the medium as well as improved frequency sub-culturing reduced this problem.

In view of the above, this study was aimed at determining the optimal antioxidant, plant growth regulator concentration and oxidative browning treatment in efforts to regenerate roots formation from established shoots and overcome the crucial problem of phenolic oxidation for the successful *in vitro* regeneration of *A. testiculare*. This will provide insight into the processes contributing to the exudation of phenolic compounds and also can be minimized as it is crucial for successful *in vitro* culture of *A. testiculare*.

5.3 Material and Methods

5.3.1. Preparation of media

Propagation media comprised of a full strength Murashige and Skoog (1962) (MS), 30 g l^{-1} sucrose, supplemented with varying concentrations of different cytokinins and solidified with 7.5 g l^{-1}agar. The cytokinins concentrations were 0, 10, 20 or 30 µM of 6-Benzylaminopurine (BA). Before the addition of agar, the pH was adjusted to 5.7 followed by autoclaving at 121°C for 20 min. Each medium had 15 replicates.

5.3.2. Preparation of explants

A. testiculare collected from Kokkerboom Kwekery, Knersvlakte (Latitude -31.611074, Longitude 18.746165), had their youngest leaves excised and washed with running tap water, rinsed with distilled water then were surface sterilized with 70% ethanol solution for 30 s and rinsed in 3% sodium hypochlorite (NaOCl) with 2 drops of 0.1% Tween-20 added. Leaves were then rinsed three times with sterile distilled water under laminar flow hood for 20 minutes each rinse to remove all the traces of hypochlorite (NaOCl). Ultraviolet (UV) light of the laminar flow hood was switched on for 15 minutes to kill bacteria before surface-sterilization with 70% ethanol. Leaves were cut to small cubes (*ca.* ± 1cm) and aseptically plated on various sterilized induction

media. Explants cultured were incubated in the growth room at 25±2ºC temperature with a day/night cycle of 16/8 hours.

Transferred shoots

Shoots were aseptically transferred to culture bottles, 30 ml containing half-strength MS nutrient medium supplemented with 7.5 g l^{-1} sucrose and solidified with 1.875 g l^{-1}agar. The pH was adjusted to 5.7 prior to autoclaving at 121°C for 20 min. Shoots were placed in a 30 ml bottle five with shoots in each. Shoots were inoculated onto media in a completely randomized fashion and were incubated in growth room at 25±2ºC temperature with a 16 h light and 8 h dark cycle. Therefore, in this experiment shoots were chosen for use to initiate root induction and overcome explants browning.

5.3.3. Data collection and analysis

Counts of shoots, callused explants and explants browning were recorded every 14d intervals and photos on visual observations were on the same day. After a log (x+1) transformation of the data for normality, a factorial-way analysis of variance (ANOVA) Statistical 12.0. Fischer's least significant difference (LSD) was used for separation of significantly different means.

5.4 Results

5.4.1. Transferred material of *A. testiculare*

A. testiculare shoots were transferred in varying levels of IAA (0, 10, 20 and 30 µM) of IAA with combination of 10 µM of BA to reduce explants browning for root induction. At these concentrations, ratios multiplication of shoots occurred in all concentrations. When the medium was supplemented with 10 µM IAA + 10 µM BA (Fig 3.1.1 & Fig 3.1.2 B), the highest number of shoots was observed. However, *A. testiculare* had also produced results at 20, 30 µM and with no addition of IAA (Fig 3.1.1 & Fig 3.1.2 A, C & D). Pink pigmentation of shoots occurred in all treatments in this study, as it was noted

in a previous experiment. Using sterile and transferred shoots to overcome explants browning for root induction to this experiment, the results obtained suggested that through this procedure many shoots could be produced and remain unchanged than become brown.

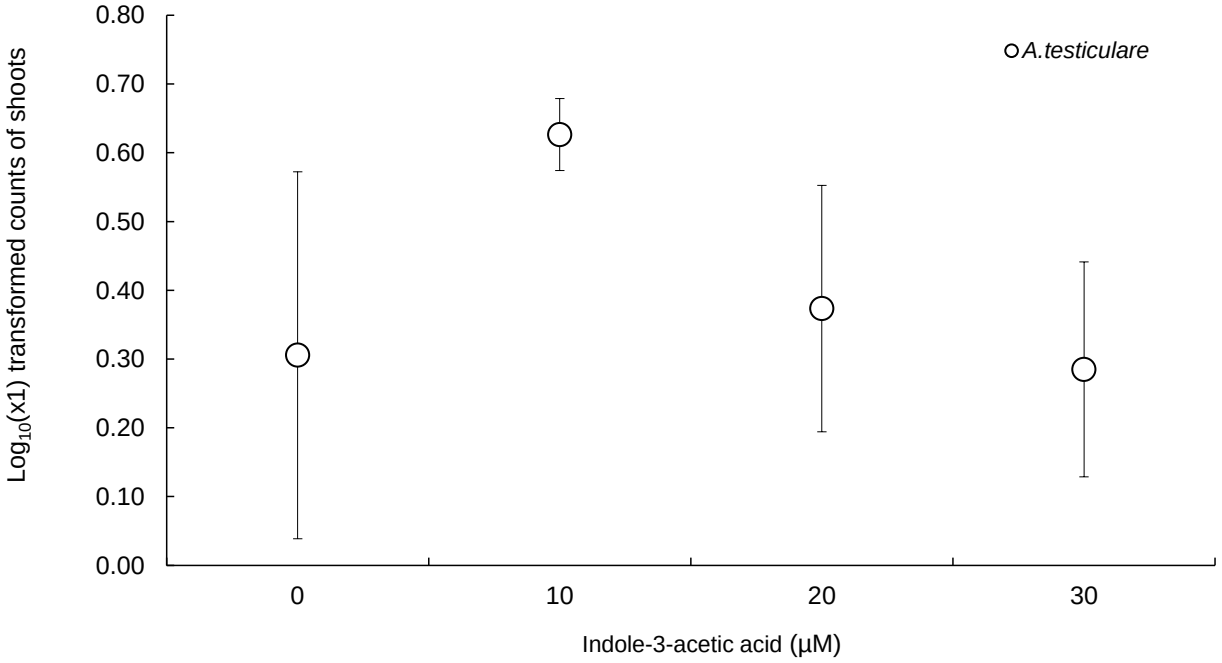

Fig. 5.1. Shoots formation of *A. testiculare* in response to increasing levels (0, 10, 20 and 30 µM) of Indole-3-acetic acid (IAA). Values represent $log_{10}(x+1)$ transformed counts of shoots specimens.

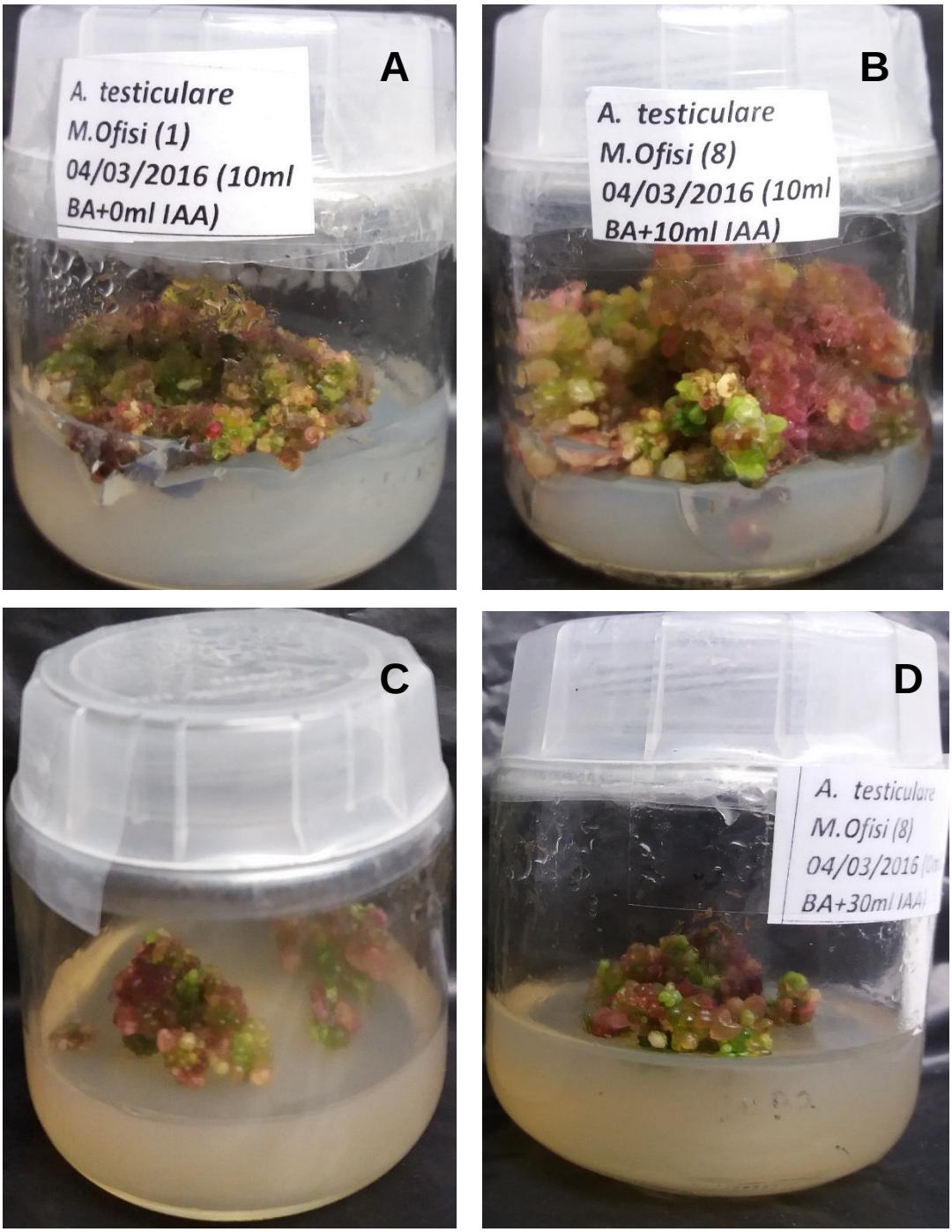

Fig. 5.2. Shoots formation in *A. testiculare* with no addition of IAA (A), Shoots formation in *A. testiculare* at 10 µM IAA (B), Shoots formation in *A. testiculare* at 20 µM IAA (C), Shoots formation in *A. subalbum* at 30 µM IAA (D).

Table1. Response of *A. testiculare* in varying concentrations of IAA+10µM of BA hormone combinations. A response was only recorded on this species provided more than 60% of explants reacted uniformly on *in vitro* culture.

	BA (µM)	**IAA (µM)**	**Initial shoots transferred**	**Multiple shoots per medium**	**Shoots multiplication rate (-times)**
	10	0	25	119	4.8
	10	10	25	395	15.8
	10	20	25	69	2.8
	10	30	25	45	1.8

*Values represent the total number of regenerated shoots scored per treatment cultured on MS basal medium.

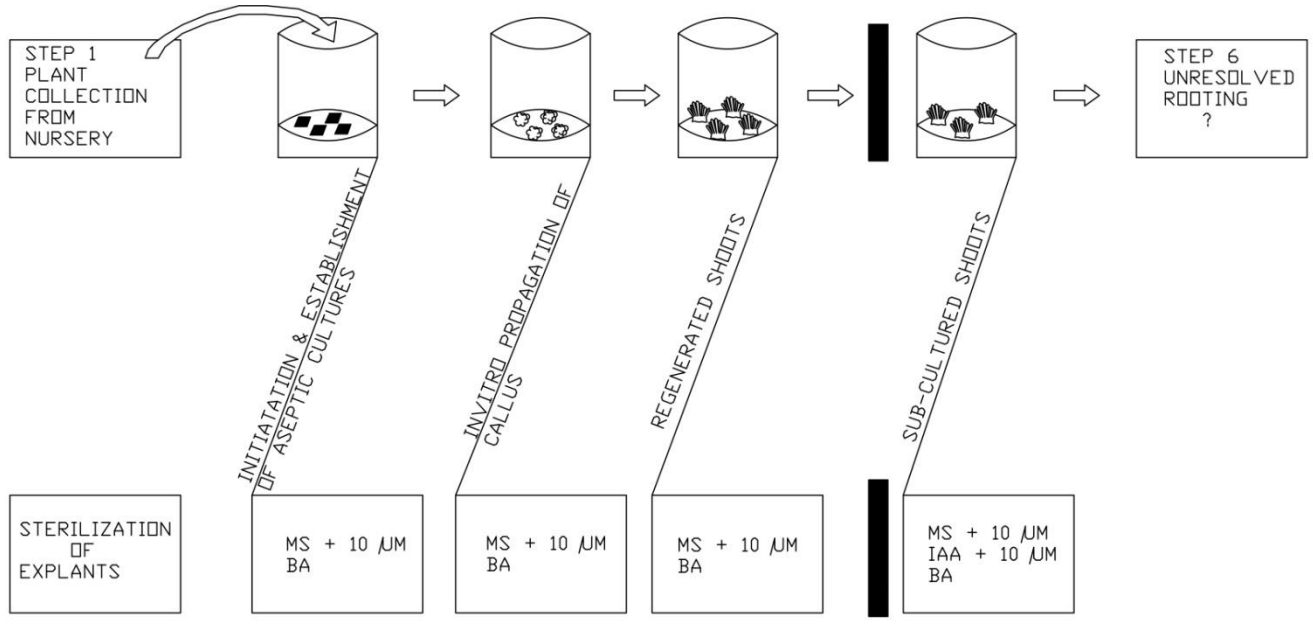

Diagram 1. A schematic representation of the successive stages of somatic embryogenesis of Argyroderma testiculare

5.5 Discussion

This study demonstrated that an *in vitro* propagation of shoots derived from callus oxidative browning of explants can be controlled through sub-culturing to strive for root induction. *A. testiculare* was cultured in half-strength MS basal media supplemented with varying concentrations of IAA (0, 10, 20 and 30 µM). Evidence obtained from this study displayed that shoots dominated with pigmented multiple shoots without observation of oxidative browning of explants. The sub-cultured shoots were successfully developed and multiplied at 10 µM of IAA as was noted in this study.

It is necessary to carry out an *in vitro* propagation technique to obtain the necessary plant material, due to limited, seasonal availability of seeds and low germination rate of *Argyroderma* spp. This study has highlighted the least concern and endangered plant species of *Argyroderma* spp. which need further attention. General aseptic techniques regarding *in vitro* culture of shoots were followed on this experiment when the MS supplemented with varying concentrations of IAA (Table1). *A. testiculare* shoots sub-cultured in varying concentrations IAA (0, 10, 20 and 30 µM of IAA), on MS basal medium displayed shoots. It was observed that at these concentrations shoots were able to multiply them without changing the plant growth regulator. Data presented in (Table1) and (Figure 3.1.1.) showed that *in vitro* shoots multiplication of *A. testiculare* occurred in all IAA concentrations tested. The control treatment without having auxin in the medium was able to regenerate an optimum number of (4.8) shoots observed in 10 µM (BA). All concentrations of IAA facilitated shoots differentiation, although 10 µM+10µM of (BA+IAA) supplemented with MS medium being the most effective in terms of shoots numbers produced per treatment. Amongst the different concentrations of IAA tested shoots numbers increased with (15.8) shoots as indicated on (Table 1). These results confirm that there was a positive effect of IAA on the regeneration capacity to induce shoots/multiply (Table1) and (Figure 3.1.1). IAA at the highest concentration (10 µM+30 µM (BA + IAA) regenerated the lowest number of shoots, (1.8) shoots multiplication produced in the treatment (Table 1). In this study the number of shoots was found to be very low with the increased levels of IAA concentration. The

positive role of IAA with BA combination on the capacity to induce shoots regeneration has been reported previously for *L. mutabilis* (Mulin and Bellio-Spataru, 2000). Vengadesan *et al.* (2000) reported that maximum callus with average shoots was found in *Acacia sinuate* on different concentrations of IAA with BA combination, although these species were not an Aizoaceae family. These results suggest that shoots multiplication on *A. testiculare* can be achievable when cultured in a medium containing IAA with BA combination. It was also mentioned on a study that medium containing IAA has an influence to induce shoots plant species (Shrivastava and Rajani, 1999).

During *in vitro* propagation, the exudation of phenols is very common and it always influences the results. Phenolic concentration is often affected by several internal and external factors (Zapprometov, 1989). Some nutrients (Lux-Endrich *et al*., 2000) and some stress factors like drought, water, radiation and pathogen infection from injured surfaces affect concentrations of the phenolics in plants (Zapprometov, 1989; Kefeli *et al*., 2003). The various plant growth regulators concentrations may affect pohenolic exudation as phenols are reactive compounds (Lux-Endrich *et al*., 2000). Explant browning is a serious problem in *in vitro* culture. To reduce this problem modified MS medium need to be provided with AC, ascorbic acid and citric acid (Wang *et al*, 2005).

Despite these additives being added to control explants browning in *in vitro* culture, the results confirmed that sub-culturing of shoots to fresh medium observed to be the best technique to overcome explants browning. The interaction between plant growth regulator and sub-cultured shoots incubated with varying concentrations of IAA significantly reduced phenolic compound content culture media in this study. It was reported on previous studies by Soniya and Sujitha (2006) that leaching of polyphenols in *Aristolochia indica* affected the shoots growth *in vitro* culture; however it was not an Aizoaceae family. Furthermore an increased frequency of sub-culturing in the medium reduced this problem. Similarly, it was also observed that complete plantlets developed when sub-cultured on MS medium supplemented with 0.01g/l activated charcoal, IAA and BA combination (Khalil *et al.,* 2002). These results concur with current results obtained in this study that sub-culturing of shoots for rooting as well as to overcome

oxidation of explants browning can be achieved. Notwithstanding the occurrence of shoots multiplication on this study on *A. testiculare*, well developed carotenoids pigmentation was observed. The significant interactive role of sub-culturing together with the PGR as treatment on exudation of phenolic compound from shoots to culture medium is indicated in Table 1 and Figure 3.1.1. Sub-culturing to culture medium resulted in a significantly reduced phenolic content in all concentrations of auxins, as compared to activated charcoal supplemented treatments, as it was observed in the previous experiment.

5.6 Conclusion

An efficient alternative protocol for micro propagation of *A. testiculare* was established for callus and shoots multiplication. *A. testiculare* can be multiplied *in vitro* as was demonstrated in this study. Whilst root induction remains unresolved, it can be concluded that *A. testiculare* can be induced to form multiple shoots at 10 µM+10 µM (BA+IAA) concentrations of the tested auxin. Further studies would be focused at increasing the rate of shoots regeneration from callus in efforts to initiate root formation in *Argyroderma* spp.

5.7 Acknowledgements

We would like to express tremendous gratitude to DST-NRF for funding this project and the Department of Horticultural Sciences, Cape Peninsula University of Technology for the use of research facilities. Lastly, we are very thankful to Kokerboom Nursery/Kwekery for their consistence assistance of plant material.

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CHAPTER SIX

6.1 GENERAL DISCUSSION

The introductory chapter indicated the rationale for this study with a review of literature, highlighting the significance of commercial value, and high levels of polyphenols containing phenylpropanoids, particularly flavonoids and anthocyanins (Musil, 2005) on *Argyroderma subalbum* and *Argyroderma testiculare*. Despite the high demand, it is not widely spread due to the constraints on its production. The slow and unfeasible conventional methods of propagation limit its success. This study identified the need for an *in vitro* propagation method of these plant species. The development of a reliable technique and advanced propagation methods would overcome the constraints that the species face in their natural habitat and enable the large scale, commercial production of *A. subalbum* and *A. testiculare.*

Despite high demand for these plant species, a reliable method of their *in vitro* propagation has not yet been developed. The limited success in the application techniques of *in vitro* culture is mainly accredited to the oxidative explants browning. This study was investigated through the use of mature leaf explants as the starting experimental material.

In an effort to initiate callus induction, callus derived from leaf explants were subjected to various media. Different types of cytokinins 6-Benzylaminopurine (BA), 6-(gamma, gamma-Dimethylallylamino) purine (2ip) or kinetin (Kn) concentrations significantly affected callus induction. The proportions of these plant growth regulators were manipulated to enhance callus induction from the leaf explants cultured, also in efforts to promote shoots development. A comparison amongst hormones was carried out to determine the most effective ratios/concentrations in inducing callus and shoots induction. The insight was gained into the effects of the plant growth regulators (BA, 2ip and Kn) on *in vitro* growth and explants discoloration. This study found phenolic compounds more effective on discoloration of the entire explants at highest levels of concentrations. It is at the point of contact between the explants and the medium that

oxidative browning was exaggerated. The levels of hormones in culture medium significantly affected entire explants discoloration on *A. subalbum* and *A. testiculare*, with the increasing levels of concentrations 10 µM of 2ip and 10 µM BA) being the most effective. The discoloration was also observed in the control treatment without addition of hormones. Callus formation was formed in BA, 2ip and kinetin treatments. The highest levels of callus formation formed in 10 µM of 2ip and BA. However, there was no callus formed in control (concentration without BA, 2ip and Kinetin). The BA in the culture media prompted shoots formation. The shoots developed in BA treatments at 10 µM were 15.8, and amongst the total number of shoots found in all treatments BA at 10 µM was found to be the most efficient in shoots multiplication. Plant regenerations through explants were not significantly different. However, to improve *in vitro* propagation of *Argyroderma* spp. for future studies, the focus should be on increasing the number of shoot multiplication through *in vitro* culture with the aim of rooting development on *Argyroderma* spp.

Oxidative browning has been identified as a major problem in developing an efficient protocol for the *in vitro* propagation of *A. subalbum* and *A. testiculare*. The study was carried out to identify and prevent factors contributing to reduced explants browning on media composition. The phenolic compounds were found to have a reactive response to media composition and on wounded tissues. The Lowest concentration of 2ip, BA and Kinetin resulted in the increased highest phenol content recorded in the culture media. Wounding of explants significantly increased phenol exudation. In a comparative study, changing of media/transfer of explants over time was found to be effective in reducing explants browning than activated charcoal. It can be concluded that changing or transferring of explants into new media in order to reduce explants browning and enable successful *in vitro* culture was found to be more effective on *A. subalbum* and *A. testiculare*.

6.2 CONCLUSION

This thesis explores and confirms that *A. subalbum* and *A. testiculare* were induced in an *in vitro* culture, callus was produced and multiple shoots from 2iP, BA and kinetin. Although root development from the shoots remains unresolved, both species form callus at 10 µM concentrations of the tested cytokinins, particularly in this study 10 µM of BA was found to be the most efficient on initiating callus induction (Chapter three). In this study, activated charcoal was used to reduce explants browning and enabled successful *in vitro* culture of explants; however activated charcoal was not beneficial to *A*. *subalbum* and *A*. *testiculare* to cultured explants, but inhibited callusing and root development. Although roots were not obtained, callusing was optimum with 20 µM of indole-3-acetic acid. Therefore, using activated charcoal for reducing browning intensity in *A*. *subalbum* and *A*. *testiculare* explants does not guarantee better callusing and rooting. Alternative antioxidants such as ascorbic and citric acids should be pursued in place of activated charcoal (Chapter 4).

6.3 RECOMMENDATIONS

Further studies must focus on optimizing the multiplication rate of *A. subalbum* and *A. testiculare* propagules *in vitro* culture. Attention must be paid to inducing shoots and root formation via the regenerated callus formation shoots for rooting of *A. subalbum* and *A. testiculare in vitro* culture.

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6.5 Appendices to Chapter 3

Appendex 3.1.Preparation of Sterilization, medium for leaf explants of *Argyroderma subalbum* and *Argyroderma testiculare* in tissue culture laboratory.