Propagation of *Sceletium tortuosum* (L.) N. E. Br. – a South African medicinal plant

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

by

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STUDENT DECLARATION

Propagation of *Sceletium tortuosum* (L.) N. E. Br. – a South African medicinal plant

I, **Amrisha Sreekissoon**, student number: **214521705**, declare that:

1) The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg;

2) This dissertation has not been submitted for any degrees or examination at any other university;

3) This dissertation does not contain data, figures or writing, unless specifically acknowledged, copied from other researchers; and

4) Where I have produced a publication of which I am an author or co-author, I have indicated which part of the publication was contributed by me.

Signed at UKZN (Pietermaritzburg) on the 5th day of December 2019.

[redacted]

AMRISHA SREEKISSOON

DECLARATION BY SUPERVISORS

We hereby declare that we acted as supervisors of this MSc student:

Student full name: Amrisha Sreekissoon

Student number: 214521705

Dissertation title: Propagation of *Sceletium tortuosum* (L.) N. E. Br. – a South African medicinal plant

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science Higher Degrees Office for examination by the University appointed examiners.

SUPERVISOR: PROFESSOR J. VAN STADEN

CO-SUPERVISOR: PROFESSOR J. F. FINNIE

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 1 – PLAGIARISM

I, **Amrisha Sreekissoon**, student number: **214521705**, declare that:

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DECLARATION 2 – PUBLICATIONS

PUBLICATIONS FROM THIS DISSERTATION

1) **Sreekissoon, A., Finnie, J. F., Van Staden, J., 2019.** *In vitro* and *ex vivo* vegetative propagation of *Sceletium tortuosum* (L.) N. E. Br.: a South African medicinal plant. ***(In preparation).***

   Experiments, analyses and writing of the publication were done by the first author, under the supervision of the second and third authors.

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This dissertation is dedicated to my parents.

To my mum, for her undying love, support, encouragement, and assistance with my work, wherever possible. To my dad, for constantly being my chauffeur, even at the most unreasonable hours.

I would not be where I am without you both. Thank you for making this possible.

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LIST OF ABBREVIATIONS

2,4-D -	2,4-Dichlorophenoxyacetic acid
2iP -	2-isopentenyladenine
ABA -	Abscisic acid
ANOVA -	Analysis of Variance
BA -	6-Benzyladenine
cm -	Centimetres
DHZ -	Dihydrozeatin
DMRT -	Duncan's Multiple Range Test
DW -	Distilled water
ECR -	Extra Cape Subregion
g -	Gram/s
GA -	Gibberellic acid
GCFR -	Greater Cape Floristic Region
GMPs -	Greenhouse mother plants
GP -	Germination percentage
GR -	Germination rate
h -	Hour/s
HIV -	Human immunodeficiency virus
HSPs -	Hormone signalling pathways
IAA -	Indole-3-acetic acid
IBA -	Indole-3-butyric acid
KAR -	Karrikinolide
Kin -	Kinetin
L -	Litre
Mesemb -	Mesembryanthemoideae
mg -	Milligram/s
MGP -	Mean germination percentage

min - Minute/s

ml - Millilitre/s

mm - Millimetre/s

MNLP - Mean number of new leaf pairs

MRL - Mean root length

MNR - Mean number of roots

MS - Murashige and Skoog

mT - meta-Topolin

NAA - 1-Naphthaleneacetic acid

PAA - Phenylacetic acid

PAR - Photosynthetically active radiation

PGR - Plant growth regulator

PMB - Pietermaritzburg

PTC - Plant tissue culture

Rusch - Ruschiodeae

RW - Running water

SA - South Africa

SDW - Sterile distilled water

SE - Standard error

SEM - Scanning electron microscopy

SW - Smoke-water

TC - Tissue culture

TDZ - Thidiazuron

TMB - Trimethylbutenolide

VI - Vigour index

UKZN - University of KwaZulu-Natal

v/v - Volume per volume

ABSTRACT

Sceletium tortuosum (L.) N. E. Br. is a South African succulent plant widely used in traditional medicine. Overharvesting for this purpose has caused decimation of natural populations leading to its classification as a protected species. Its traditional medicinal uses as an intoxicant, stress- and anxiety-reliever and mood booster has garnered a lot of interest from the scientific community. Most research has focussed on isolating the phytochemicals responsible for the psychoactive effects and determining their pharmacological potential.

It was established that the mesembrine-type alkaloids present within *S. tortuosum* can potentially be used in the treatments of several psychological, neurological and medical conditions. Examples of these include depression, Alzheimer's and Parkinson's diseases, epilepsy, diabetes and HIV. Standardized alkaloid extracts have been produced and are incorporated in pharmaceutical treatments for stress (Elev8™).

At present, there is no commercial source of mesembrine-type alkaloids and these phytochemicals are presently extracted from fresh plant material. Commercial farming of this plant is ongoing although only a few such farms exist. Despite this, there have been no reports of these operations making efforts to replenish wild populations of *S. tortuosum*. Furthermore, no scientific reports were discovered regarding propagation methods of this species. Therefore, the current investigation aimed to propagate *Sceletium tortuosum* using different *in vitro* and *ex vivo* methods.

An *in vitro* seed germination experiment was conducted with the use of smoke-water (SW) pulse treatments. A standardized smoke extract was diluted to concentrations of 1:500, 1:1000, 1:1500, 1:2000, 1:2500 (v/v) and pulse treatment durations were for 1, 2, 8 and 24 h. The study included positive and negative control treatments. It was determined that the optimum temperature for *in vitro* germination was 20°C, and constant light was suitable. The highest mean germination percentage and rate were recorded for 1:1000 SW for 24 h (83 ± 1.9%) and 1:2000 SW for 24 h (6.35 seeds germinated/day) after 30 days of incubation. The former treatment also yielded the highest vigour index. However, seedling growth parameters were low for these significant treatments.

The *ex vivo* germination trial was carried out using only the best pulse treatments and their respective control treatments. Seeds were sown in plastic containers with a 3:2:1 mix of soil: sand: perlite. It was determined that 1:1000 SW for 24 h was the best pulse treatment with the highest mean germination percentage and rate (10% and 0.352 seedlings emerged/day) after 30 days. The lowered germination parameters may be attributed to one or more types of secondary dormancy.

A reliable sterilization protocol was developed for *S. tortuosum* for the initiation of *in vitro* cultures (pre-sterilization + 20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min) + 0.05% streptomycin sulphate (20 min) + post-sterilization). This step-wise protocol yielded one of the lowest contamination percentages during a sterilization experiment (20 ± 9.2%). However, it was concluded that 2.5% NaOCl for 10 min may be suitable for sterilization at certain times of the year, particularly when bacterial contamination is the major issue. These results suggested the presence of endophytic bacteria.

Shoot nodal explants were sterilized using the developed step-wise protocol and inoculated onto MS medium supplemented with 2.5 µM indole-3-butyric acid (IBA) for shoot multiplication. This medium allowed for root and shoot proliferation, elongation and flowering. These findings could be indicative of high endogenous cytokinin levels or a multifaceted role of IBA in controlling various plant growth responses.

In vitro-generated shoot nodal explants were used in an *in vitro* rooting study. This study made use of two auxins (IBA and indole-3-acetic acid – IAA), at different concentrations (2.5, 5.0 and 10.0 µM) and a control treatment of hormone-free MS medium. All treatments allowed for successful rooting *in vitro* after 21 days (25–55%), although the best treatment in terms of growth parameters was 10.0 µM IBA. This treatment generated the highest rooting percentage (55 ± 11.4%), highest mean number of roots (3.80 ± 0.83) and new leaf pairs (4.65 ± 0.67). It was postulated that this high concentration having yielded the best responses was due to the acclimation of shoot explants to a constant supply of 2.5 µM IBA during shoot multiplication.

In vitro-derived plantlets were potted in a 3:2:1 mix of soil: sand: perlite and placed in a mist-house for 1 week. Thereafter, they were transferred to a greenhouse. Plantlets which survived for 10 days were considered to be acclimatized. This process was very successful with 45–90% acclimatization. The control treatment was responsible for the highest acclimatization percentage (90 ± 6.9%), indicating the ability of plantlets to continue root proliferation *ex vitro*.

In comparison, an *ex vivo* study was performed on cuttings (shoot nodal segments) using the same auxins and concentrations utilized *in vitro* (IBA and IAA – 2.5, 5.0 and 10 µM). In this study, cuttings were pre-treated with auxin solutions for 10 min and watered with these solutions for the duration of the study (21 days). Control treatments consisted of untreated cuttings, and cuttings soaked in tap water for 10 minutes. It was discovered that rooting of all cuttings was extremely successful (90-100%), with 5.0 µM IBA eliciting the best rooting responses, i.e. the highest mean number of roots and mean root length (11.20 ± 1.37 and 57.18 ± 3.85 mm, respectively), and 2.5 µM IBA achieving the highest mean number of new leaf pairs (4.20 ± 0.36).

Findings from *in vitro* and *ex vivo* studies indicated that auxin treatments (more so, IBA) had profound effects on root and shoot proliferation, growth and development. Endogenous cytokinin levels may have contributed to these results, however, significant treatment effects are evident in multiple data sets.

Further investigation is necessary for this species. Seed dormancy, endophytic bacteria, endogenous hormone levels and hormone signalling pathways are some of the avenues to pursue. Additionally, the effects of these, and other *in vitro* methods on the alkaloid content of this species should be explored. This will aid in determining the most efficient method for improving alkaloid yields to generate a commercially available source of mesembrine-type alkaloids.

This investigation, in its entirety, has major implications for future *in vitro* and *ex vivo* studies by providing various methods of propagating *S. tortuosum*. This information could be vital to conservationists, commercial farms, pharmaceutical companies and future researchers who may struggle to obtain plant material quickly, easily and in sufficient quantities.

CHAPTER 1: LITERATURE REVIEW

1.1. PLANTS IN MEDICINE

Plants have been used as a source of healing since ancient times. The healing properties of numerous plant species has made them targets for drug research and development **(GURIB-FAKIM, 2006)**. Many of the drugs used today were isolated and/or developed from plant products. Common examples include morphine which is used as an analgesic (isolated from *Papaver somniferum*) **(DEVEREAUX *et al.*, 2018)**, and vincristine and vinblastine and their derivatives are used in anti-cancer therapies (isolated from *Catharanthus roseus*) **(MARTINO *et al.*, 2018)**. Apart from drugs such as these, which are critically important to medical science, various plant-based medicines and supplements have been developed to combat different ailments. These herbal products are becoming a popular choice among consumers worldwide as they are more affordable than mainstream pharmaceuticals and are considered to be safer alternatives **(BANDARANAYAKE, 2006; EKOR, 2014)**.

1.2. AFRICAN FLORAL DIVERSITY AND USE

The African continent is home to approximately 45 000 plant species **(LINDER, 2014)**. A continent with such floral diversity has a rich cultural diversity to match **(GURIB-FAKIM, 2006)**. Each culture has its own ways of practicing traditional medicine, made up of the knowledge and practices of past generations. This vast body of knowledge is used in the diagnosis, prevention, treatment and cure of illnesses and disorders **(WORLD HEALTH ORGANIZATION, 2019)**.

A large proportion of traditional medicinal customs involve the use of plants. Even with the countless advancements in pharmacology, these practices remain a way of life for many people, particularly on the African continent, as they cannot afford modern medical care or any type of over-the-counter medicine **(PHEAGE, 2017; SEN and CHAKRABORTY, 2017)**.

Many African countries reported the use of traditional medicine by 80–99% of their population **(WORLD HEALTH ORGANIZATION, 2019)**. It was estimated that 5 000 plant species are

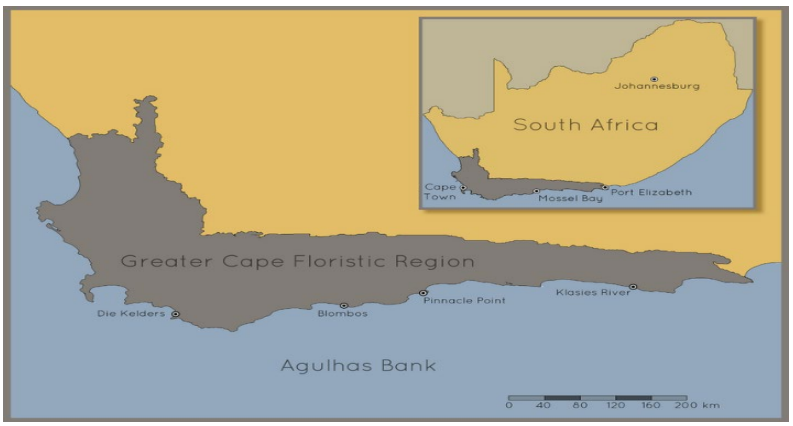
used in African traditional medicine **(MAHOMOODALLY, 2013)**, however it is difficult to determine the exact number as many species may be undocumented in scientific literature or uninvestigated by researchers **(ALLKIN, 2017)**.

A much lower number of approximately 100 African plant species are used in commercial medicine **(BRAND SOUTH AFRICA, 2008)**. Some well-known examples include *Aloe ferox*, *Agothosma betulina* and *Harpagophytum procumbens* which have been scientifically evaluated and are used to commercially produce pharmaceuticals which are exported globally **(STREET and PRINSLOO, 2012)**.

1.3. SOUTH AFRICAN BIODIVERSITY

Of the estimated 45 000 plant species on the African the continent, a hefty amount (~30 000) are found in South Africa (SA) **(LOUW *et al.*, 2002)**. This huge number is due to the fact that the country includes 3 of 36 global biodiversity hotspots – collectively known as the Greater Cape Floristic Region (GCFR) **(Figure 1.1) (CRITICAL ECOSYSTEM PARTNERSHIP FUND, 2019; SIMAIKA and SAMWAYS, 2009; SNIJMAN, 2013)**. This region is a popular area of study as well as a tourist attraction because of the extreme plant diversity observed in relatively small areas **(LOCHNER *et al.*, 2003; SNIJMAN, 2013)**.

Many plant families and their members within the GCFR are endemic or native to the area. One such family is responsible for much of the diversity in the region – the Aizoaceae **(SNIJMAN, 2013)**.



1.4. CLASSIFICATION

1.4.1. Family: Aizoaceae

This large family is also known as the ice plant family **(ENCYCLOPÆDIA BRITANNICA, 2018)** and is comprised of approximately 1 845 species contained in 142 genera **(HARTMANN and GERBAULET, 2017)**. It is the largest family of succulent plants in the world **(CHESSELET *et al.*, 2002)**, with 1 750 of its species found in the Succulent Karoo of SA **(Figure 1.2) (KLAK *et al.*, 2004)**.

The members of this angiosperm clade have vast coverage in the Extra Cape Subregion (ECR) **(Figure 1.2)** of the GCFR with 658 species located within an area of 98 869 km^2 **(SNIJMAN, 2013)**. Second only to this, is the Asteraceae with 495 species in the same area **(SNIJMAN, 2013)**. Furthermore, 38 genera are endemic to the ECR with 26 belonging to the Aizoaceae, thus this family alone is the largest contributor to the endemism of the region **(SNIJMAN, 2013)**.

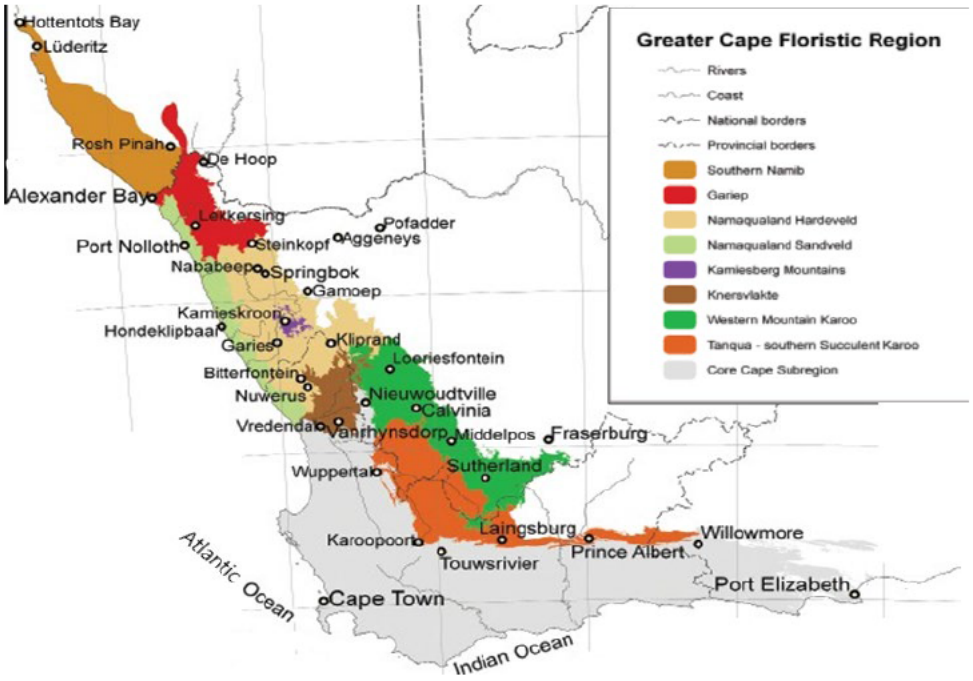


Figure 1.2: Map of Greater Cape Floristic Region of South Africa. Grey region shows Core Cape Subregion. All other coloured regions collectively show the Extra Cape Subregion **(SNIJMAN, 2013)**

The Aizoaceae has four subfamilies – Aizooideae, Sesuvioideae, Ruschioideae and Mesembryanthemoideae **(KLAK *et al.*, 2007)**. The latter two taxa are responsible for around 90% of the diversity of the entire family **(VON STADEN *et al.*, 2013)**.

The variety of growth forms observed within the Aizoaceae has created much of its aesthetic appeal. Many members are cultivated to be kept as ornamentals, e.g. *Mesembryanthemum crystallinum* (Mesembryanthemoideae) and *Lithops* spp. (Ruschioideae) **(AKBAR HUSSAIN *et al.*, 2018; KHOSHBAKHT and HAMMER, 2007)**. Some species are used in traditional medicine, e.g. *Sceletium* spp. (Mesembryanthemoideae), and some are edible, e.g. *Carpobrotus* spp. (Ruschioideae) **(GERICKE and VILJOEN, 2008; SPRINGFIELD *et al.*, 2003)**.

1.4.2. Subfamily: Mesembryanthemoideae

Within this subfamily are 15 genera composed of 103 species **(KLAK and BRUYNS, 2013)**, The myriad of growth forms within the Mesembryanthemoideae is noteworthy. Species range from annuals to perennials and includes geophytes, herbs, shrubs and shrublets **(SNIJMAN, 2013)** along with leaf- and stem-succulents **(KLAK and BRUYNS, 2013)**. Plants range in height from 20 mm to ± 1 m **(KLAK and BRUYNS, 2013; SNIJMAN, 2013)**. Further variety is observed in vegetative and reproductive morphologies **(SNIJMAN, 2013)**.

The synapomorphies used to differentiate this subfamily from its sister clades are vascular bundles in the primary cortex and koilomorphic nectaries **(GERBAULET, 2012)**.

1.4.3. Genus: *Sceletium* N. E. Br.

Sceletium comes from the Latin word for ‘skeleton’. The name was taken up due to one of the defining characteristics among the species – the skeletonized leaf venation visible on dry and withering leaves. Flowers may be white, yellow or pale pink in colour and their petals are thread-like. Plants of the genus adopt a climbing or creeping habit and are known for their succulent leaves with idioblasts (bladder cells). Species are located in the south-western regions of SA where they are well-adapted to the arid climate **(GERICKE and VILJOEN, 2008)**.

According to **WORLD FLORA ONLINE (2017)**, *Sceletium* has 20 accepted species and three others which are considered ambiguous. However, many of these species have been

reduced to synonymy. As it stands, there are eight *Sceletium* species, namely, *S. emarcidum*, *S. exalatum*, *S. rigidum*, *S. tortuosum* (syn. *Mesembryanthemum tortuosum*), *S. crassicaule*, *S. expansum*, *S. strictum* and *S. varians* **(GERBAULET, 2012)**.

These species are often split into two groups – '*emarcidum'* type (the former three species listed) and '*tortuosum'* type (the remaining five species). This division is on the basis that only the '*tortuosum'* type possess the class of secondary metabolites known as alkaloids **(PATNALA and KANFER, 2017)**. These alkaloids are responsible for the qualities ascribed to *Sceletium* **(GERICKE and VILJOEN, 2008; KRSTENANSKY, 2017)**.

1.4.4. *Sceletium tortuosum* (L.) N. E. Br.

The species' primary distinguishing features are imbricate leaves with tips incurved and clearly visible bladder cells **(GERBAULET, 2017)**. *Sceletium tortuosum* is a perennial plant, often found under bushes or in partially shaded areas. Its lifespan is roughly 3–5 years **(CHESSELET, 2005a)**.

Most growth takes place in the autumn, winter and spring months **(CHESSELET, 2005a)**, with flowering occurring between July and September **(SCOTT and SPRINGFIELD, 2004)**. Plants are insect-pollinated and fruit are hygrochastic **(CHESSELET, 2005a)**. Seeds are 1-2 mm in size, kidney-shaped and brown to black in colour **(Figure 1.3)**.

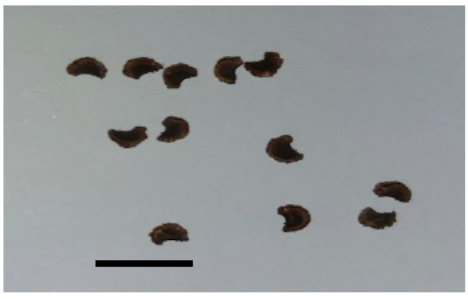


Figure 1.3: *Sceletium tortuosum* seeds. Scale bar represents 0.5 cm.

As many characteristics are shared among the genus **(GERICKE and VILJOEN, 2008)**, researchers may employ chromatographic profiling for more accurate identification **(BEZERRA *et al.*, 2018)**. The use of ultra-high performance liquid chromatography (UPLC),

hyperspectral imaging and chemometrics has been established as efficient methods of identification, specifically for differentiating *S. tortuosum* from other *Sceletium* species **(SHIKANGA *et al.*, 2013)**.

1.5. HISTORICAL AND ETHNOBOTANICAL BACKGROUND

Sceletium tortuosum has been present in SA for hundreds of years and records show that it has been utilized by indigenous people for at least three centuries **(WATERHOUSE *et al.*, 1979)**. They would use the plant to treat a variety of ailments, such as teething pain and colic in babies and toddlers **(ROOD, 1994)**, as well as diarrhoea in children **(DIGBY, 2005)**. Its calming property makes it an effective sleep aid for children and adults alike **(DIGBY, 2005; ROOD, 1994)**. Adults use it as an appetite and thirst suppressant, pain reliever and mood booster **(THUNBERG, 1795)**. Although adults would utilize it for its many medicinal capabilities, it would often be used purely for their own enjoyment **(DIGBY, 2005; THUNBERG, 1795)**, resulting in feelings of elation **(LAIDLER, 1928)**.

The plant could be "consumed" in several ways. Most often it would be chewed on, sometimes in its entirety, or simply on the leaves and stems **(SMITH *et al.*, 1996; WATERHOUSE *et al.*, 1979)**. Otherwise, it would be taken as a tincture, used as a tea, smoked or inhaled as snuff **(PAPPE, 1868; SMITH *et al.*, 1996)**. Some chose to exploit the plant in its raw state, others chose to "bake" it, or dry and/or ferment it **(GERICKE and VILJOEN, 2008)**. Baking requires approximately 60 minutes whereas fermentation requires little more than a week for the process to be complete. The specifics of these preparation methods are well-reported in a review by **SMITH *et al.* (1996)**.

The KhoiSan termed the plant "Kanna" or "Channa", but these names were initially used to describe more than one species of the '*tortuosum'* type **(LEWIN, 1998)**, likely due to their similar properties and difficulty in distinguishing between species. Nowadays, the common name Kanna is still used but only in reference to *Sceletium tortuosum* **(GERICKE and VILJOEN, 2008)**.

The alternative common name "Kougoed" – meaning "something to chew" – arose from the Dutch settlers when they arrived in the 16th century **(WATERHOUSE *et al.*, 1979)**. They learned of the true power – medicinal and otherwise – of *S. tortuosum* from the KhoiSan and utilized these plants in the same way – by chewing on them **(SMITH, 1966)**.

1.6. MODERN MEDICINE AND PHARMACOLOGY

Even though *Sceletium tortuosum* was most popularly used for intoxication, this species captured the attention of many in the scientific community because of its anxiolytic properties **(GERICKE and VILJOEN, 2008; KRSTENANSKY, 2017; ROOD, 1994)**. It was determined that there is an assortment of alkaloids found in *S. tortuosum* belonging to five distinct classes, namely, mesembrine-type, tortuosamine-type, joubertiamine-type, channaine and *Sceletium* A-4 **(Figure 1.4) (JEFFS *et al.*, 1969, 1982; MAKOLO *et al.*, 2019; POPELAK and LETTENBAUER, 1967; VEALE *et al.*, 2018; YIN *et al.*, 2019)**.

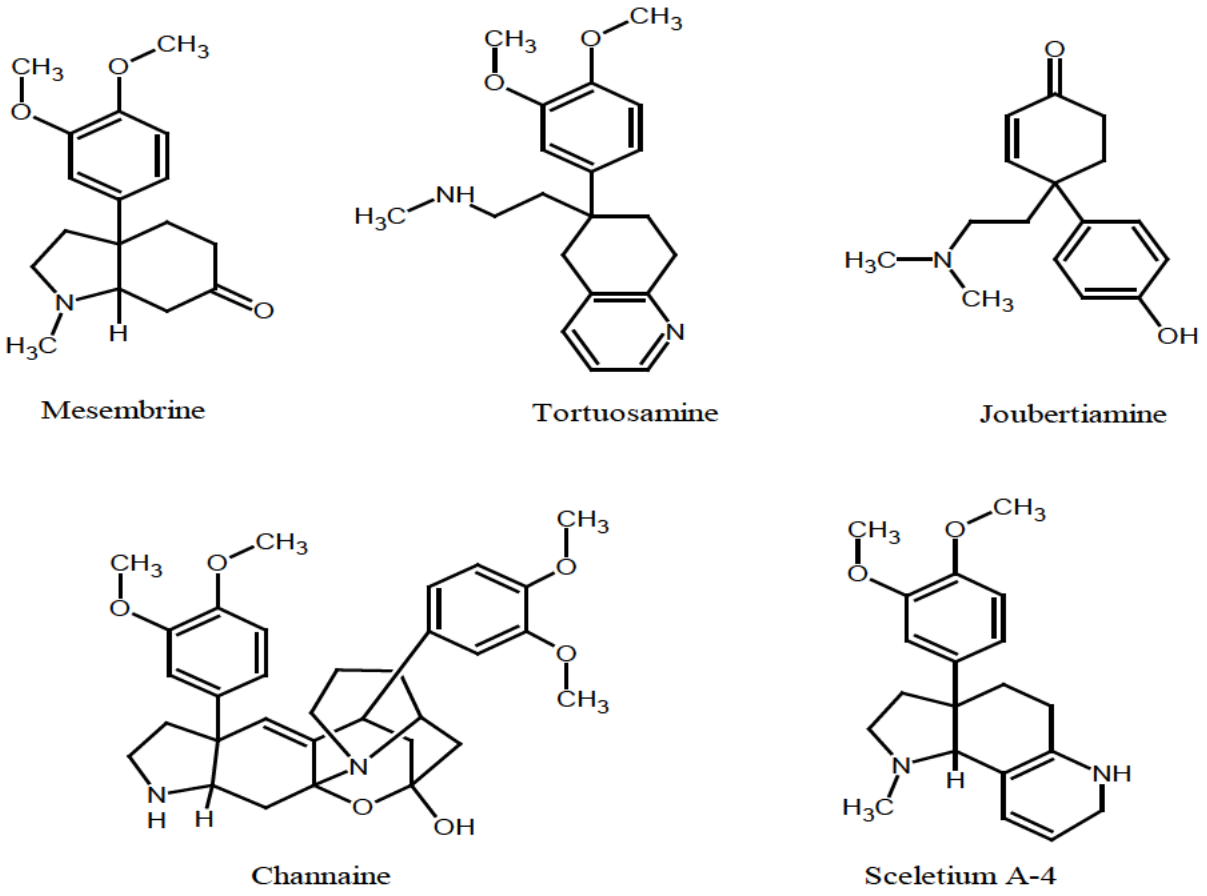


Figure 1.4: Chemical structures of five types of *Sceletium tortuosum* alkaloids (adapted from **MAKOLO *et al.*, 2019**)

The mesembrine-type class consists of approximately 15 alkaloids with a characteristic 3a-aryl-cis-octahydroindole skeleton **(Figures 1.4 and 1.5) (PATNALA and KANFER, 2017)**. The most well-studied alkaloids of this class are mesembrine, mesembrenone, mesembranol and mesembrenol **(Figure 1.5) (GERICKE and VILJOEN, 2008; SMITH *et al.*, 1996)**, as they have been shown to be responsible for the psychotropic effects of the plant **(GERICKE and VAN WYK, 2001)**.

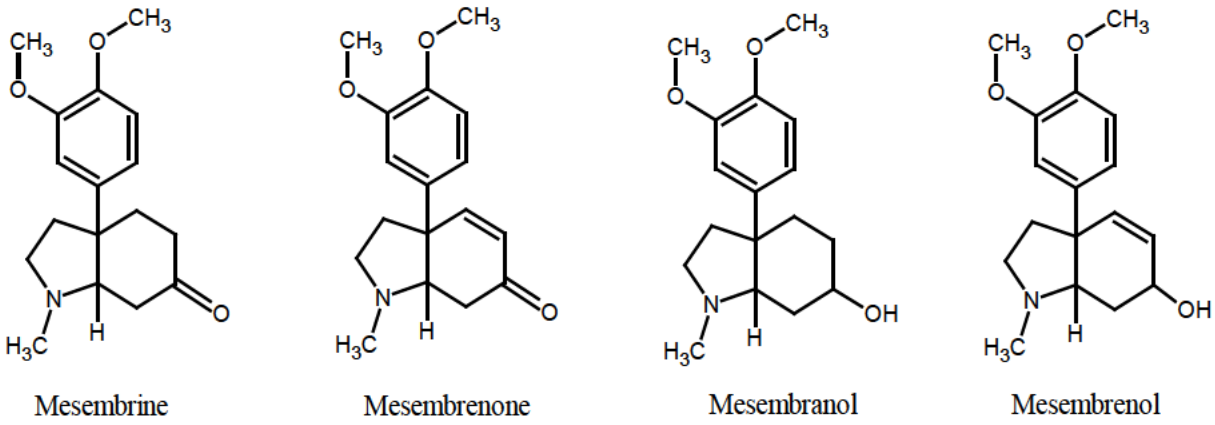


Figure 1.5: Principal mesembrine-type alkaloids (adapted from **TERBURG *et al.*, 2013**)

Research into the effects of this plant was sparked by historical accounts and anecdotal evidence boasting its benefits **(GERICKE and VILJOEN, 2008; LAIDLER, 1928; ROOD, 1994; SMITH *et al.*, 1996; THUNBERG, 1795)**. Animal case studies and human case studies were then conducted **(GERICKE, 2001; GERICKE and VILJOEN, 2008; HIRABAYASHI *et al.*, 2002, 2004)**. All the results were positive showing a reduction or cessation of symptoms of dementia **(HIRABAYASHI *et al.*, 2002, 2004)**, depression and anxiety **(GERICKE, 2001; GERICKE and VILJOEN, 2008)**.

The historical accounts, anecdotes and case studies were more than adequate to warrant the interest of pharmaceutical companies. HG&H Pharmaceuticals (SA) secured an integrated export and bioprospecting permit in 2009 **(PATNALA and KANFER, 2009)**. This permit allows for research and development, as well as sales and exports of a standardized *Sceletium tortuosum* extract. This company produced and patented the first standardized extract from the plant – Zembrin® **(PATNALA and KANFER, 2009; ZEMBRIN®, 2018)**. The process of producing Zembrin® is detailed in **MURBACH *et al.*'s (2014)** publication.

The alkaloid content of this extract is 0.35–0.45% w/w, with mesembrenone and mesembrenol contributing more than 70% and mesembrine contributing less than 20% **(HARVEY *et al.*, 2011; MURBACH *et al.*, 2014)**. Zembrin® is registered in SA and is used to produce tablets which are marketed under the name Elev8™. These pills are sold online and over-the-counter for stress and anxiety relief and mood enhancement **(HEALTH24, 2017; WILD, 2015)**.

Trimesemine™ is another standardized extract of *S. tortuosum* with a patent pending **(SWART and SMITH, 2016)**. Its alkaloid content is 3% with mesembrine contributing approximately 80%, and mesembrenone and Δ^7 – mesembrenone making up the remaining 20% **(COETZEE *et al.*, 2016; SWART and SMITH, 2016)**.

Standardized extracts, direct plant extracts and dried, milled plant material have been used by researchers to investigate the pharmacological actions, effects, side effects and toxicity of *S. tortuosum*'s principal alkaloids **(BENNETT *et al.*, 2018; DIMPFEL *et al.*, 2018; HARVEY *et al.*, 2011; NELL *et al.*, 2013)**.

For the purpose of this dissertation, *Sceletium* extract refers to an extract from *S. tortuosum* only.

Table 1.1: Potential uses for *Sceletium* extracts and individual mesembrine-type alkaloids and their modes of action (if established)

Treatment	**Mode of action**	**Alkaloid source**	**Reference**
Asthma and other inflammatory disorders	Phosphodiesterase-4 (PDE4) inhibition	Mesembrine-HCl	**NAPOLETANO *et al.* (2001)**
Depression, anxiety, schizophrenia	Mesembrine – Serotonin (5-HT) transport inhibition Mesembrenone – 5-HT inhibition and PDE4 inhibition	Zembrin®	**HARVEY *et al.* (2011)**
Anxiety	5-HT transport inhibition PDE4 inhibition	Zembrin®	**TERBURG *et al.* (2013)**
Alzheimer's disease	PDE4 inhibition	Zembrin®	**CHIU *et al.* (2014)**
Depression, Alzheimer's disease, attention deficit disorders	Monoamine releasing agent	Trimesemine™	**COETZEE *et al.* (2016)**
Parkinson's disease, addictions to cocaine and alcohol	Increases vesicular monoamine transporter (VMAT-2) expression	Trimesemine™	**COETZEE *et al.* (2016)**
Parkinson's disease	Anti-oxidant activity	*Sceletium* extract (high Δ^7- mesembrenone)	**BENNETT *et al.* (2018)**

Diabetes, obesity	Anti-inflammatory activity	*Sceletium* extract (high mesembrine)	**BENNETT *et al.* (2018)**
Depression, arthritis, Alzheimer's disease	Anti-inflammatory activity	Trimesemine™	**BENNETT and SMITH (2018)**
HIV	Inhibition of HIV-1 reverse transcriptase (RT) and HIV-1 protease (PR) Free radical scavenging activity	*Sceletium* extract	**KAPEWANGOLO *et al.* (2016)**
Epilepsy	Decreases α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor mediated transmission	Zembrin®, and mesembrenol and mesembranol separately	**DIMPFEL *et al.* (2018)**
Stress	-	Zembrin®	**NELL *et al.* (2013)**
Stress, anxiety	-	*Sceletium* extract containing mesembrine	**SMITH (2011)**
Depression, pain	-	*Sceletium* extract with mesembrine as major compound	**LORIA *et al.* (2014)**
Stress, anxiety	-	Zembrin®	**MURBACH *et al.* (2014)**
Depression	-	*Sceletium* extract containing all four principal alkaloids	**SCHELL (2014)**

Sceletium extracts and alkaloids are suitable for use in treatments of a broad spectrum of illnesses, ranging from psychological disorders and neurological diseases to inflammatory diseases and human immunodeficiency virus (HIV) **(Table 1.1)**. Other than the potential uses tabulated above, the modes of action of these alkaloids have implications for treatments of other conditions, e.g. schizophrenia **(KANES *et al.*, 2007)**, bulimia nervosa **(GERICKE and VAN WYK, 2001)**, chronic obstructive pulmonary disease, allergic dermatitis and psoriasis among others **(DYKE and MONTANA, 1999; HOUSLAY *et al.*, 2005)**.

In terms of adverse effects of *S. tortuosum*'s alkaloids, there has been no conclusive evidence of this **(NELL *et al.*, 2013)**. However, anecdotal evidence provided from a psychiatrist and psychopharmacologist suggest that side effects are dose-dependent, as are withdrawal symptoms **(GERICKE *et al.*, 2017)**.

1.7. PROBLEM STATEMENT

Sceletium tortuosum is a protected species as its natural populations have severely declined in recent decades **(ELEV8™, 2017; GERICKE and VILJOEN, 2008)**. This was likely caused by unsustainable harvesting for traditional medicinal purposes and local trade, along with plant diseases and poor environmental practices and management **(GERICKE and VILJOEN, 2008)**.

This plant has tremendous value to the nation in terms of biodiversity, culture and economy. It is a source of ground-breaking pharmacological research which could effect a positive change on medicine on a global scale. Such a species should be thoroughly investigated and documented starting at the "root" level – propagation. However, there is a severe lack of scientific literature regarding the propagation of *S. tortuosum*.

1.8. AIMS AND OBJECTIVES

1.8.1. Aims

To propagate *Sceletium tortuosum* using different methods in order to determine the most efficient approach to produce healthy self-sustaining plants.

1.8.2. Objectives

1) Perform an *in vitro* germination study using appropriate methods and biostimulants
2) Evaluate germination and growth parameters
3) Conduct *ex vivo* germination study using selected treatments from (1)
4) Compare *in vitro* and *ex vivo* germination
5) Develop a sterilization protocol for initiation of plant tissue cultures
6) Generate sufficient quantities of *in vitro* plant material for an *in vitro* rooting study
7) Conduct *in vitro* rooting experiment using plant rooting hormones
8) Acclimatize *in vitro*-generated plants to greenhouse conditions
9) Propagate plants *ex vivo* using conventional cutting methodology in combination with plant rooting hormones
10) Compare growth parameters of *in vitro*-derived and *ex vivo*-derived plants.

1.9. STUDY SIGNIFICANCE

Sceletium tortuosum has shown remarkable potential in pharmacology **(Table 1.1) (BENNETT and SMITH, 2018; GERICKE and VAN WYK, 2001; HARVEY *et al.*, 2011)** and has been well-investigated in this respect. However, no scientific literature was found documenting other aspects of the plant. This could be fundamentally important especially since it is a protected species **(ELEV8™, 2017)**.

The plant is commercially farmed in and around SA **(KAPEWANGOLO *et al.*, 2016; WILD, 2015)**, however the primary purpose of these farms is to supply pharmaceutical companies with plant material for producing standardized extracts, pharmaceuticals, and for international exports **(ELEV8™, 2017; WILD, 2015)**. Thus, it can be quite difficult for researchers to obtain the plant material necessary for experimentation.

Despite the existence of commercial farms, no evidence was found to suggest that they are being utilized to re-establish natural populations. Furthermore, there were no scientific reports found on methods of propagation of this plant. It has been mentioned that these farms use seeds for propagation but the way in which this is done was not declared **(ELEV8™, 2017)**.

Propagation is the key to ensuring that *S. tortuosum* does not become extinct in its natural environment, as well as ensuring that there is no shortage of plant material for further investigation and pharmaceutical production.

The intention of this study is to explore the propagation aspect of this medicinal plant. This research will provide alternate propagation methods which will benefit researchers, commercial farms and in turn, pharmaceutical companies. It could provide a foundation for the development of conservation programs to aid in the replenishment of wild populations. Additionally, it may well provide easier and more effective propagation methods which could pave the way towards commercial production of mesembrine-type alkaloids in their pure form, which is unavailable at present **(KRSTENANSKY, 2017)**.

CHAPTER 2: SEED GERMINATION

2.1. INTRODUCTION

2.1.1. Seed-bearing plants

Plants are classified into two distinct categories – gymnosperms (seeds borne on the surface of cones) and angiosperms (seeds borne within floral ovaries which later develop into fruit). Between gymnosperms and angiosperms, the latter has greater value in terms of global food supplies and pharmacological significance **(GURIB-FAKIM, 2006)**. Although, the fundamental composition of all seeds is the same. Mature seeds are composed of a fertilized ovule containing an embryo; nutritive tissue providing a food source for the embryo; and a seed coat (testa) **(FENNER and THOMPSON, 2005)**.

2.1.2. Importance of seeds

Plant propagation using seeds was the first and only method used to cultivate plants for millennia **(CURWEN and GUDMUND, 1961)**. Many essential food crops continue to be cultivated in this way, e.g. maize, wheat and rice **(ROY'S FARM, 2019a, b, c)**. These monocotyledonous plants are among the most commercially important, but many dicotyledonous plants also have substantial impacts as food crops (e.g. beans, potatoes and tomatoes) **(IANNOTTI, 2018)**.

Propagation of plants from seeds serves several other purposes, e.g. cultivation of ornamentals for commercial sale, medicinal plants for traditional use; as well as for further research on seed and plant physiology. Seed-propagated plants are also utilized in pharmacological studies to determine the potential uses of their phytochemicals in medicine **(CUSHMAN *et al.*, 2000; GURIB-FAKIM, 2006; LIGHT *et al.*, 2002; SANTOS-HERNÁNDEZ *et al.*, 2005; SARASAN *et al.*, 2011; SINGH, 2019)**.

2.1.3. Factors influencing seed formation

It is well established that trade-offs exist in nature for plants and animals alike. Such mechanisms exist for the prime purpose of ensuring the survival of the species. Organisms often have to make a choice between survival and reproduction, particularly when environmental conditions are harsh or unfavourable **(CRAWLEY, 1997; FENNER and THOMPSON, 2005)**.

The choices and sacrifices made are dependent on the plant itself (genetics), its life cycle and life span. Annual plants can afford to devote a substantial amount of energy and nutritional resources to seed production because reproduction must occur irrespective of environmental conditions – this often signals the end of the plants' life **(FENNER and THOMPSON, 2005)**. Perennial plants have longer life spans and will produce seeds more than once in their lifetime. In this case, if conditions are harsh during the flowering season, the plant must "decide" to either divert all resources towards reproduction, thereafter dying; or survive and only allow a small amount of resources to be invested in reproduction **(CRAWLEY, 1997; FENNER and THOMPSON, 2005)**.

Reproduction refers not only to ovule, pollen and flower production, but also to seed formation following pollination and fertilization Although seed composition is essentially the same in angiosperms, the biochemical make-up of seeds among and within species may differ **(FENNER and THOMPSON, 2005)**.

Environmental factors, particularly water and nutrient availability affect when seed formation occurs, the nutritional content of seed tissues (proteins, lipids and carbohydrates), and the number of seeds produced by plants **(BEWLEY and BLACK, 1994; FENNER, 1991; FENNER and THOMPSON, 2005; GUTTERMAN, 1994)**. The conditions experienced by the mother plant also have a significant effect on seed coat and embryo characteristics **(EVENARI *et al.*, 1966; FENNER, 1991; GUTTERMAN, 2000)**

Once seeds have been produced, i.e. an ovule has been fertilized and is mature, the ovary will develop into a capsule or fruit. This structure serves as a storage and dispersal vessel **(FENNER, 1985)**. Following seed dispersal, the next logical physiological response is seed germination. However, this process is much more complex than it initially sounds. Various factors are involved which must come together in perfect harmony before this crucial step can

take place **(BEWLEY, 1997)**. First and foremost, before germination can occur, seed dormancy (if present) must be broken.

2.1.4. Seed dormancy

Dormancy is best defined as the "*failure of seeds to germinate although environmental conditions are favourable for germination*" **(SOLTANI *et al.*, 2019)**. The phenomenon encompasses numerous broad categories which often overlap. The initial division is primary and secondary dormancy **(GENEVE, 1998)**. Primary dormancy is acquired during seed development, usually while the seed is on the mother plant. It prevents germination before the seed has been shed and dispersed **(BEWLEY, 1997; CHAHTANE *et al.*, 2017)**.

Secondary dormancy is an imposed dormancy. It occurs when conditions are unfavourable for germination, but may persist for an indefinite period of time, irrespective of whether conditions become favourable or not **(BASKIN and BASKIN, 1998; GENEVE, 1998)**. A prime example of this is the exposure of seeds to low temperatures. *Coreopsis lanceolata* seeds exhibited secondary dormancy which remained after this unfavourable condition was no longer present **(BANOVETZ and SCHEINER, 1994)**.

2.1.4.1. Types of primary dormancy

Primary dormancy may also be known as organic dormancy **(GENEVE, 1998)**. Organic dormancy may first be divided into endogenous and exogenous dormancy **(NIKOLAEVA, 1969, 1977)** . The difference between the two is that endogenous dormancy is caused by some characteristic of the embryo which prevents germination, while exogenous dormancy refers to other characters of the seed (apart from the embryo) which hinder germination **(NIKOLAEVA, 1969, 1977)**. There are five classes within these categories which will be discussed further.

2.1.4.1.1. Exogenous dormancy

2.1.4.1.1.1. Physical vs. mechanical dormancy

Physical dormancy occurs when seeds are unable to imbibe water, i.e. the seed coat is impermeable. This not only affects water uptake but may also prevent oxygen uptake. This can be overcome by removing the seed coat entirely or by scarification. Mechanical dormancy is similar in that it is caused by the embryo's surrounding structures (endosperm, seed coat or endocarp) which may be hard and resistant to embryo growth and subsequent germination, however, this structure is water-permeable. Mechanical dormancy requires cold or warm stratification to be broken **(BASKIN and BASKIN, 1998; NIKOLAEVA, 1969, 1977, 2001)**.

2.1.4.1.1.2. Chemical dormancy

It is caused by chemical compounds which either inhibit embryo growth or seed germination. These chemicals may be present within components of the seed or its surrounding tissue (fruit) **(NIKOLAEVA, 1969, 1977)**. Abscisic acid (ABA) is implicated in dormancy induction and in many dormant seeds an accumulation of this hormone is observed. In order for germination to occur, the amount of ABA must decrease. Leaching of seeds or fruits is usually effective in reducing the amount of germination-inhibiting substances **(BASKIN and BASKIN, 1998, 2014)**

Stratification methods may also be effective in breaking chemical dormancy. They may decrease the level of germination inhibitors (e.g. ABA); increase the level of germination stimulants (e.g. gibberellic acid – GA); or increase the tolerance of the embryo to inhibitors, such that dormancy is broken and germination occurs **(BASKIN and BASKIN, 1998, 2014)**

2.1.4.1.2. Endogenous dormancy

Three types of endogenous dormancy have been described by **NIKOLAEVA (1969, 1977)** and **BASKIN and BASKIN (1998, 2014)**, namely morphological, physiological and morphophysiological. Morphological dormancy is caused by the embryo being underdeveloped which in turn delays germination. Conditions which allow for the development, differentiation or maturation of the embryo are necessary to break this dormancy.

Physiological dormancy is due to a physiological inhibiting mechanism of the embryo that prevents germination **(BASKIN and BASKIN, 2014; NIKOLAEVA, 1969, 1977)**.

Lastly, morphophysiological dormancy is a combination of the aforementioned types. Physiological and morphophysiological dormancy can usually be broken by warm or cold stratification. It should be noted that all types of endogenous dormancy have different levels or sub-divisions and the dormancy-breaking treatment (type, lengths or combinations) required depends on the specific level **(BASKIN and BASKIN, 2014; CROCKER, 1948; NIKOLAEVA, 1969, 1977)**.

2.1.4.2. Dormancy in desert plants

In the harsh conditions of deserts, dormancy is a crucial mechanism for controlling germination **(HUANG *et al.*, 2004)**. Seeds must only germinate when conditions are the most favourable in order to ensure survival of the species **(BASKIN *et al.*, 1993; FREAS and KEMP, 1983; GUTTERMAN, 2000)**, thus different dormancy mechanisms have been observed. The most common types found in seeds of desert plants are physiological and physical **(BASKIN and BASKIN, 2014; ERICKSON *et al.*, 2016)**, but dormancy cycles and dormant soil seed banks have also been observed **(BASKIN *et al.*, 1993; CAO *et al.*, 2012)**.

Dormancy cycling essentially means that seed germination follows a rhythm, usually brought about by seasonal conditions **(BASKIN *et al.*, 1993; HYATT *et al.*, 2000)**. Most seeds of a given seed lot will germinate in one season and afterwards, germination will be lower or absent altogether, until the favourable season is experienced once again. This phenomenon may be observed over a number of years **(BASKIN *et al.*, 1993)**. So far, it has been documented for *Eriogonum abertianum* and *Eriastrum diffusum* (annuals) **(BASKIN *et al.*, 1993; CAO *et al.*, 2012)** and *Kalidium gracile* (perennial) **(CAO *et al.*, 2013)**. In these studies, seeds were extracted from soil seed banks (i.e. an accumulation of seeds buried in the soil or remaining on the dead mother plant **(FENNER, 1985)**), or buried in the ground until commencement of the study. Although a study on *Mesembryanthemum nodiflorum* (Mesemb) showed that seeds stored under laboratory conditions at ambient temperature also exhibit a dormancy cycle **(GUTTERMAN, 1980)**.

2.1.5. Seed germination

Seed germination begins with the uptake of water (imbibition) causing active embryo growth. The process is complete once the radicle has protruded from the seed coat **(BEWLEY and BLACK, 1994; COPELAND and MCDONALD, 2001)**. Environmental conditions play a significant role in the occurrence of this physiological process **(GUTTERMAN, 1994; KOORNNEEF *et al.*, 2002)**. As with dormancy-breaking, a specific set of conditions are required for seeds to germinate. In some instances, the conditions required to break seed dormancy may also be ideal for germination **(BASKIN and BASKIN, 2014; MERRITT *et al.*, 2007)**. A guide to determining the optimum conditions may be found by taking into account the plants' natural environment **(BASKIN and BASKIN, 2014)**.

2.1.5.1. Factors influencing seed germination

2.1.5.1.1. Water

Water content of the soil or growth medium is a major factor in seed germination **(TAYLOR *et al.*, 1992)**. A certain amount of water in the growth medium is necessary to allow for seed imbibition **(RAHMAN *et al.*, 2011)**. However, germination trials typically involve a seed imbibition period prior to seed sowing **(RODRIGUEZ-PÉREZ *et al.*, 2007; SPARG *et al.*, 2005)**. This period can become problematic even under controlled circumstances. It has been reported that imbibition injury may occur during water uptake **(POWELL and MATTHEWS, 1978)**. Seeds are more prone to this type of injury if they have been completely dried out (e.g. during storage) or the seed coat has been damaged, resulting in rapid water uptake. This ultimately causes damage to seed tissues thus reducing germination or preventing it altogether **(POWELL and MATTHEWS, 1978; RAHMAN *et al.*, 2011)**.

In some crop species, imbibition rate can be controlled by temperature **(CAPTSO *et al.*, 2008; KADER and JUTZI, 2002)**. Similarly, in four desert Malvaceae species, seed imbibition was difficult without hot water **(COMMANDER *et al.*, 2017)**. The period of imbibition was also found to affect species from arid areas. **MOTT (1974)** showed a negative effect of a 16 h imbibition period on seed germinability of *Aristida contorta*, but this result was not repeated in *Helipterum craspedioides* and *Helichrysum cassinianum*.

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2.1.5.1.2. Temperature and light

Seeds of numerous plant species rely on temperature cues to signal the onset of germination **(GUTTERMAN, 1994)**. Several germination investigations employ a constant temperature of 25°C or 30°C **(BHATTACHARYA and KHUSPE, 2001; COLOMBO *et al.*, 2015; OBENDORF *et al.*, 1983; SPARG *et al.*, 2006)**, which works well for a multitude of plant species. However, some species have different temperature requirements, particularly desert species **(GUTTERMAN, 1994)**. Arid environments often experience hot, dry days with cold nights, thus warm, cold or a combination of temperatures may be necessary for germination **(ROJAS-ARÉCHIGA *et al.*, 1997)**. For instance, temperatures of 20°C and 25°C were suitable for germination of *Lespedeza davurica* and *Artemisia sphaerocephala* seeds **(HUANG and GUTTERMAN, 1999; TOBE *et al.*, 2001)**, while other species were capable of germinating over a range of temperatures (e.g. *Acacia colei* and *Hakea lorea*) **(COMMANDER *et al.*, 2017)**. Certain alternating temperature regimes improved germination percentages and rates for *Artemisia halodendron* and *Medicago sativa*, among others **(LAI *et al.*, 2016; WANG *et al.*, 2019)**.

Light conditions provide an additional cue for germination. Although seeds are covered by soil, light penetrates through soil allowing for irradiation of seeds. The soil type can alter the photon flux and light quality experienced by seeds **(ROJAS-ARÉCHIGA *et al.*, 1997)**. Additionally, specific wavelengths of light can signal germination. For example, red and far-red light (as well as white light) were suitable for germination in *Ferocactus robustus*, *F. recurvus* and *F. flavovirens*, but a lack of light inhibited germination completely **(ROJAS-ARÉCHIGA *et al.*, 1997)**. Whereas the seeds of the desert shrubs *Artemisia ordosica* and *Artemisia sphaerocephala* only germinated in darkness **(LAI *et al.*, 2016)**.

As with temperature, different light regimes may be necessary. The ideal conditions for germination are species-specific and can only be determined through trial and error **(KIGEL, 2017)**.

2.1.5.1.3. Growth medium and pH

The growth medium or soil substrate must also be considered as a germination requirement. Desert species usually require sandy soils for healthy growth, and this often extends to their seeds **(HUANG and GUTTERMAN, 1999)**. For example, sand generally gains and loses heat

much faster than darker soils **(FREE, 1911)**, this heating and cooling effect may influence germination **(KIGEL, 2017)**. Additionally, its coarse nature may act as a scarifying agent which could further affect germination **(BASKIN and BASKIN, 2014)**. Light penetration and subsequent exposure of the seed is also influenced by the soil substrate **(see 2.1.5.1.2)**.

Growth media are responsible for supplying water and nutrients to plants, but their pH level must also be suitable for germination and seedling survival **(HACKETT, 1964; KABOUW *et al.*, 2010)**. Certain plants may require acidic pH substrates, others may prefer the substrate to be basic or neutral **(KABOUW *et al.*, 2010)**. In the Fynbos region of the ECR **(Figure 1.2)**, soils have a pH of 4–7 **(RICHARDS *et al.*, 1997)**, however in the Succulent Karoo, soil pH is approximately 8.0 **(BEUKES and ELLIS, 2003)**. Many members of the Mesembryanthemoideae reside in both these regions **(KLAK *et al.*, 2004; MAGER and HUI, 2012; SNIJMAN, 2013)**, thus pH requirements are surely species-dependant within the Aizoaceae.

Although the growing medium can be mimicked in germination trials, most *in vitro* studies do not follow this approach. They usually involve sowing seeds on moistened filter paper **(HUANG and GUTTERMAN, 1999; TOBE *et al.*, 2001)** or agar-containing medium **(CUSHMAN *et al.*, 2000; DELGADO-SÁNCHEZ *et al.*, 2010)**. Nevertheless, even with completely different conditions compared to the external environment, dormancy-breaking is possible and *in vitro* germination trials can be extremely successful **(BAIRU *et al.*, 2009; VENDRAME *et al.*, 2007)**.

2.1.5.1.4. Smoke

Countless plant species are native to fire-prone regions, yet they are known to germinate well and thrive after fires **(VAN STADEN *et al.*, 2000)**. This knowledge led to germination trials to determine whether it was the heat from the fire or the smoke produced by it which prompted germination **(DE LANGE and BOUCHER, 1990)**. It was established that smoke acts as a germination cue, much like the previously mentioned factors **(PIERCE *et al.*, 1995)**.

Furthermore, it was discovered that aerosolized and liquid smoke (derived from burning vegetation and channelling smoke through water **(BROWN and VAN STADEN, 1997)**) promote germination in species from a variety of plant families, even if they were not native to fire-frequented habitats **(BROWN, 1993; DE LANGE and BOUCHER, 1990; DIXON,**

1995; PIERCE *et al.*, 1995). Conversely, some species from such habitats were not significantly affected by smoke, thus it should not be regarded as a "fool-proof" method for germination-induction **(BROWN, 1993; PIERCE *et al.*, 1995)**.

Nonetheless, smoke and more commonly, smoke-water (SW) has been an effective germination-stimulator in Fynbos **(BROWN, 1993)** and Succulent Karoo plant species **(PIERCE *et al.*, 1995)**. Concentrations of SW frequently used range from 1 ml in 10 ml (1:10) to 1:5000 dilutions **(ARRUDA *et al.*, 2012; SINGH *et al.*, 2014)**. Often, SW is used in seed-priming or pre-sowing ("pulse") treatments. Alternatively, seeds are germinated on a SW-containing medium **(VAN STADEN *et al.*, 2000)**.

In addition to having a stimulatory effect on seed germination, SW has also been shown to improve seedling vigour in forest, desert and food crop species **(ARRUDA *et al.*, 2012; KANDARI *et al.*, 2012; KULKARNI *et al.*, 2007; SINGH *et al.*, 2014; SPARG *et al.*, 2005)**.

2.1.6. Dormancy and germination in the Aizoaceae

Dormancy in *Mesembryanthemum crystallinum* (Mesemb) is said to last 1–30 days **(ADAMS *et al.*, 1998)**. Their germination is dependent on the position of individual seeds within the capsule, with larger seeds germinating first **(ADAMS *et al.*, 1998; GUTTERMAN, 1980)**. The same seed position effects were reported in *Mesembryanthemum nodiflorum* (Mesemb) **(GUTTERMAN, 2000)**, which likely indicates that morphological dormancy is exhibited by both species **(BASKIN and BASKIN, 2014)**. Later, **GUTTERMAN (2002)** reported that *M. nodiflorum* seeds can be stored for years and will germinate at 25°C in light after being kept moist for approximately two weeks.

Mesembryanthemumum crystallinum (Mesemb) seeds also have seed coat-imposed dormancy (physiological dormancy). **VISSCHER *et al.* (2018)** successfully mitigated this by exposing seeds to dry heat. Despite the two types of dormancy described for this species, **CUSHMAN *et al.* (2000)** did not report any difficulty germinating seeds *in vitro* on **MURASHIGE and SKOOG (1962)** (MS) medium. However, theirs was not a germination trial hence the proportion of seeds germinated was not specified.

Seed coat-imposed dormancy was also reported by **SILVERTOWN (1984)** for the Aizoaceae family. Apart from the Mesembryanthemoideae exhibiting this characteristic, *Zaleya pentandra* (Sesuvioideae) seeds showed this to be true when germination was only successful

after mechanical and acid scarification treatments **(MUNAWAR *et al.*, 2015)**. Furthermore, the application of dormancy-breaking or germination-stimulating chemicals (e.g. GA and kinetin) were ineffective in lifting dormancy of *Aizoon canariense* (Aizooideae) seeds **(EL-KEBLAWY and GAIROLA, 2017)**.

Carpobrotus modestus (Ruschiodeae) was considered to be dormant after germination trials encompassing different temperatures and light conditions, as well as high heat exposure, did not result in any germination whatsoever **(PARSONS, 1997)**. *Carpobrotus acinaciformis* and *C. edulis* (Rusch) were successfully germinated in sand *in vitro* with incubation at 18–20°C **(SUEHS *et al.*, 2004)**. However, **PIERCE *et al.* (1995)** documented that SW was an effective germination stimulant for various Ruschioideae species from the Succulent Karoo and Fynbos regions.

No scientific reports of germination investigations involving *Sceletium tortuosum* were found, although it was mentioned that seeds are used by commercial farms for cultivation **(ELEV8™, 2017)**.

2.1.7. Objectives

1) Use different seed pre-sowing treatments to discern which have the most positive effects on *in vitro* germination;

2) Conduct thorough germination study using the most effective pre-sowing agent at different concentrations for different time periods;

3) Compare germination percentages and rates between pre-sowing treatments and calculate vigour index for the most significant treatments; and

4) Repeat pre-sowing treatments which achieved the most positive results by seed sowing *ex vivo*.

2.2. MATERIALS AND METHODS

2.2.1. Source of seeds

Seed capsules were donated by a personal nursery housing *S. tortuosum* plants. Seeds remained in the capsules, at room temperature until harvest. Thereafter, they were stored in dry glass bottles at 10°C in a freezer.

2.2.2. Seed viability and imbibition tests

Seeds were opened using a dissecting tool and their viability was determined by the presence of white tissue/s. Seed coat colour differences were observed for viable and non-viable seeds. Based on these differences, screening for viable seeds was done manually (by eye) prior to use in the succeeding investigations.

Three replicates of 10 seeds were used for imbibition tests. Low replicate number was due to limited seed availability for germination studies. Seeds were soaked in distilled water for 8 h. Weight of the seeds was recorded prior to soaking and hourly thereafter. Seeds were lightly blotted with tissue paper before weight was measured.

2.2.3. Scanning electron microscopy (SEM) sample preparation and imaging

SEM work was carried out at the Microscopy and Microanalysis Unit (MMU) at UKZN (PMB). Seed samples were already dry due to freezer storage, thus no additional preparation was required. Seeds were mounted on SEM stubs and transferred to a sputter coater (Quorum Q150R ES). The sample was gold-coated and viewed under high vacuum using a ZEISS EVO LS15 Scanning Electron Microscope.

2.2.4. *In vitro* germination studies

2.2.4.1. Seed surface sterilization

Seeds were rinsed with tap water, then soaked in 2.5% NaOCl (sodium hypochlorite) for 10 min on a laminar flow bench. This was followed by three rinses with sterile distilled water (SDW). Seeds were then sown on agar-containing media in 65 mm Petri dishes (10 seeds per Petri dish). Petri dishes were sealed with parafilm and placed in transparent plastic bags for incubation in growth rooms. This sterilization protocol was followed for all germination trials and experiments.

2.2.4.2. Preliminary germination trials

A smoke extract was prepared in March 2018 as described by **GUPTA *et al.* (2019)**. The constituents of this extract and their concentrations were determined using the methods described by **HRDLIČKA *et al.* (2019)**. The standardized smoke extract was used in the preparation of all SW dilutions (v/v = SW/DW).

Fifty seeds were used per treatment (= 10 seeds per replicate). Seeds were pulsed in a 450 mg/L promalin solution containing 0.5% 1:1000 SW for 24 h. Alternatively, seeds were rinsed under running water (RW) for 2 days then placed in 2.8 ml/L ethrel (Ethephon) for 4 h. Seeds were sown on $1/10^{th}$ strength **MURASHIGE and SKOOG (1962)** (MS) medium. Incubation took place under constant light (PAR 9.07 x 10 $\mu mol.m^{-2}.s^{-1}$) at 20°C and 25°C (± 2°C).

The subsequent trial was conducted based on results and inferences made from the above experiment. Thirty seeds were used per treatment (= 10 seeds per replicate). Treatments used were: soak in distilled water for 1 and 2 days, rinse under RW for 2 days, rinse under RW (1 day) + pulse with 2.8 ml/L ethrel for 4 h, pulse with 2.8 ml ethrel for 2, 6 and 24 h, and pulse with SW (1:500 and 1:2000) for 2, 6 and 24 h. Seeds were then rinsed with distilled water, surface-sterilized and sown on water agar (6 g/L) and incubated at 20 ± 2°C in a growth room with constant light (PAR 9.07 x 10 $\mu mol.m^{-2}.s^{-1}$). Germination was recorded after 2 weeks for each trial.

2.2.4.3. Smoke-water germination study

A total of 5 000 seeds were used (4 replicates of 50 seeds per treatment). Seeds were pulsed with different concentrations of SW (v/v) (1:500, 1:1000, 1:1500. 1:2000, 1:2500) for 1, 2, 8 and 24 h. For positive controls, seeds were soaked with distilled water for each time period and the negative control was untreated seeds.

Upon completion of pulse treatments, seeds were rinsed with distilled water and sterilized as stated in **2.2.4.1** and sown on water agar (6 g/L). Seeds were incubated at 20 ± 2°C under constant light (PAR 9.07 x 10 µmol.m^{-2}.s^{-1}) for 30 days. Germination was recorded daily. A seed was considered to have germinated when protrusion of the radicle was ± 1 mm.

2.2.4.4. Calculations and measurements

Germination percentage was calculated using: $GP = \frac{number\ of\ seeds\ germinated}{total\ number\ of\ seeds\ sown}$ x 100. Mean germination percentage (MGP) was calculated for the four replicates. Germination rate (GR) was determined graphically.

Shoot and root length (mm) were measured using digital calipers. These measurements were recorded for treatments with the highest and lowest germination percentages (1:2500 24 h and 1:2500 1 h respectively), fastest and slowest germination rates (1:2500 24 h and 1:2000 2 h respectively), and the respective positive controls and the negative control. Vigour index was calculated using measured lengths (converted to cm) and final germination percentage: $VI = (shoot\ length + root\ length)$ x GP.

2.2.5. *Ex vivo* germination experiment

Treatments were selected based on results from the SW germination study **(2.2.4.2)**. Four replicates of 10 seeds were used per treatment. Seeds were pulsed in 1:1000 and 1:2500 SW for 24 h. As a positive control seeds were soaked in distilled water for 24 h while the negative control consisted of untreated seeds. Seeds were sown in a 3:2:1 mix of soil: sand: perlite in rectangular plastic containers (22.5 cm x 15.5 cm, depth 3.5 cm) with 10 seeds per container. Containers were placed in full-sun with watering carried out by automated sprinklers (twice a

day for 6 min each). Emergence was recorded daily for 30 days and percentage (mean) was calculated as per **2.2.4.4**.

2.2.6. Statistical and graphical analyses

Germination data from the preliminary trial **(2.2.4.2)** were analysed using two-sample t-tests ($P \leq 0.05$). Data from the remaining germination experiments were arcsine transformed and analysed with one-way ANOVAs using Genstat 18.0. Duncan's Multiple Range Tests (DMRTs) were used to specify significant differences between treatments at the $P \leq 0.05$ level.

Germination rates were determined using Microsoft Excel 2016. A line of best-fit was added to the data and the rate was taken as the slope of the line. All graphs were generated using GraphPad Prism 8.0.

2.3. RESULTS AND DISCUSSION

2.3.1. Seed viability and imbibition

Dark brown seeds contained visible white tissues (perisperm + endosperm) and were considered to be viable. Non-viable seeds were light brown in colour and devoid of inner white tissues (empty). These colour differences allowed for the selection of only viable seeds for experiments.

Seeds successfully imbibed water as determined by the increase in weight of seed batches after the 8 h imbibition period (results not shown). This result indicated that seeds did not exhibit physical dormancy, eliminating the need for physical or chemical scarification as dormancy-breaking treatments. Successful imbibition encouraged investigations with germination stimulating treatments **(2.3.3)**.

2.3.2. Scanning electron microscopic imaging

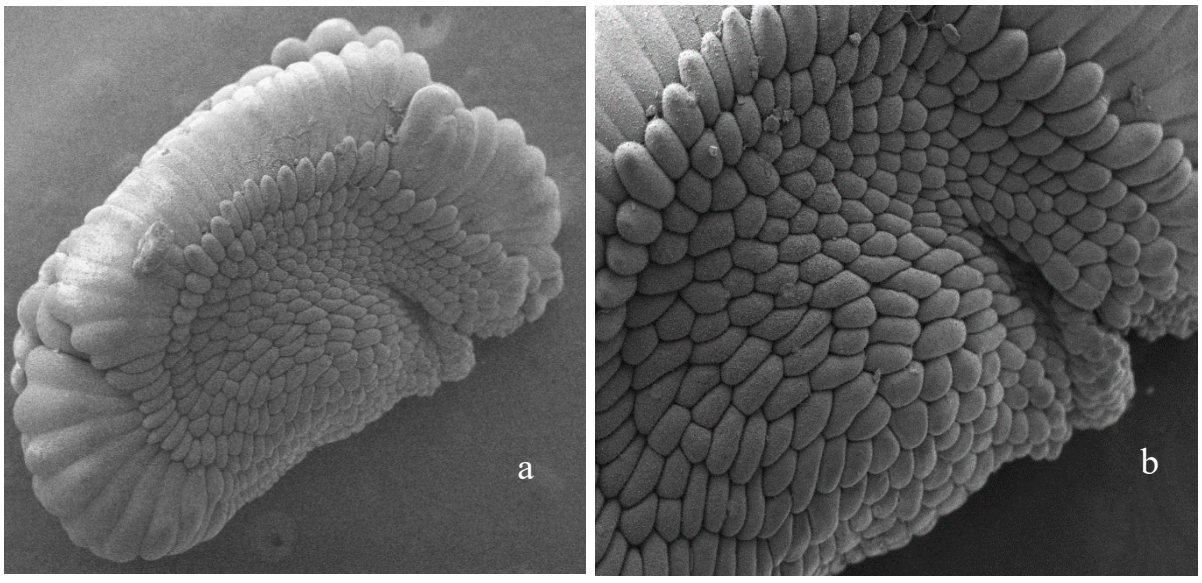


Figure 2.1: SEM micrograph of a *Sceletium tortuosum* seed at 100x **(a)** and 214x **(b)**

Figure 2.1 shows the seed coat of a *S. tortuosum* seed which exhibits a centrospermoid cell arrangement. This term refers to the specific arrangement of small and large cells and has been previously documented in the Aizoaceae, Caryophyllaceae and Portulacaceae **(SINGH, 2019)**. This image also appeared in the **BARTHLOTT (1984)** publication, with the species name

given as *Sceletium campactum* and publications ever since have presented the same name. However, it is now known that this species was reduced to synonymy as *S. tortuosum* **(WORLD FLORA ONLINE, 2017)**. **Figure 2.1** can be regarded as further evidence in support of this taxonomic alteration.

Consequences of the centrospermoid arrangement of seed coats on germination were not found in the literature. However, thickness of the seed coat may have major implications for germination of this species. It was recently reported that during storage, flavonoids may be produced within seeds, leading to increased seed coat thickness thus imposing dormancy **(DEBEAUJON *et al.*, 2007)**.

Further implications for germination may stem from the inner seed tissues. Aizoaceae seeds contain perisperm in addition to endosperm **(ECKARDT, 1976; MANNING, 2019; PRAKASH, 1967)**. The function of this additional layer is for its nutrients to be absorbed by the endosperm, then supplied to the embryo for growth **(BITTRICH, 1993; FOTEDAR, 2017)**. Perisperm has been found to influence germination in other species **(WELBAUM and BRADFORD, 1990)**, although the specific implications of this tissue layer on *S. tortuosum* germination were not found in the literature.

2.3.3. Preliminary germination trials

Between two treatments (promalin + SW – 24 h and RW (2 days) + ethrel 4 h), the treatment containing ethrel achieved the higher mean germination percentages (MGPs) for both temperatures tested (46 ± 10.3% and 90 ± 12.3% for 25°C and 20°C respectively). This treatment also produced a significantly higher MGP than promalin + SW at the optimal temperature (20°C). The promalin + SW treatment resulted in low MGPs at 25°C and 20°C (22 ± 4.9% and 58 ± 7.4% respectively) **(Figure 2.2)**.

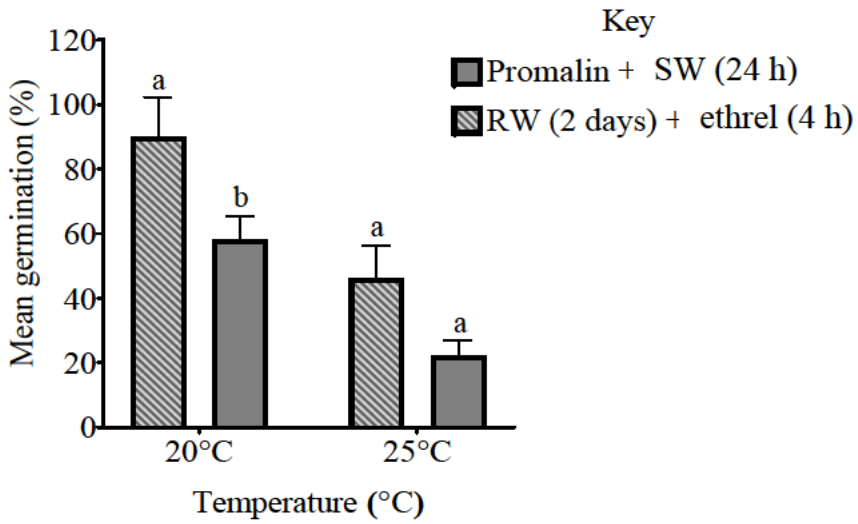


Figure 2.2: Mean germination percentages of seeds from different treatments when incubated at 20 and 25°C. Error bars show SE of the mean. Letters above bars indicate significant differences between treatments ($P \leq 0.05$).

The effect of the treatment incorporating ethrel could be caused by running water first allowing germination inhibitors to leach out of seeds, followed by ethrel which is a germination stimulant **(BASKIN and BASKIN, 2014; BIAŁECKA and KĘPCZYŃSKI, 2003; CORBINEAU *et al.*, 2014)**. Low MGPs stemming from the promalin + SW treatment could be due to the fact that promalin contains 2 types of GA and benzyladenine (BA), and the final solution used also contained SW. Thus, the combination of all 4 germination-promoting chemicals may have had a detrimental effect on the delicate hormone balance which controls germination **(CORBINEAU *et al.*, 2014; GINZBURG, 1974)**.

The effects of both treatments were improved when temperature was lowered. These results indicated that 20°C was the more suitable temperature for germination of *S. tortuosum* seeds. Studies on other desert species (e.g. *Streptoglossa macrocephala, Zygophyllum xanthoxylon*, *Atraphaxis bracteata*) have previously arrived at the same conclusion **(COMMANDER *et al.*, 2017; TOBE *et al.*, 2001; YOUSSEF, 2009)**. This response is indicative of a specific, constant temperature requirement of *S. tortuosum* seeds. Additionally, constant light appears to be conducive to germination, hence both these conditions were unchanged for subsequent experiments.

All mean germination percentages were 70% and above, with the highest stemming from SW (1:2000) pulses for 2 h (93.3 ± 3.3%), 6 h (90 ± 0.0%) and 24 h (90 ± 5.8%). Following this, was a 2 h ethrel pulse, and RW for 2 days (86.7%). However, only the SW treatment was statistically significantly different from the lowest GP achieved (70 ± 10%) **(Table 2.1)**.

Table 2.1: Mean germination percentages achieved from different seed pre-sowing treatments after 2 weeks of incubation at 20°C

Treatment	**Mean GP ± SE (%)**
Soak in distilled water for 1 day	70 ± 10^{b}
Soak in distilled water for 2 days	76.7 ± 3.3^{ab}
Running water (1 day) + ethrel (4 h)	80 ± 10^{ab}
Running water (2 days)	86.7 ± 8.8^{ab}
Ethrel for 2 h	86.7 ± 3.3^{ab}
Ethrel for 6 h	80 ± 0.0^{ab}
Ethrel for 24 h	80 ± 5.8^{ab}
Smoke-water (1:500) for 2 h	83.3 ± 6.7^{ab}
Smoke-water (1:500) for 6 h	83.3 ± 6.7^{ab}
Smoke-water (1:500) for 24 h	76.7 ± 6.7^{ab}
Smoke-water (1:2000) for 2 h	93.3 ± 3.3^{a}
Smoke-water (1:2000) for 6 h	90 ± 0.0^{ab}
Smoke-water (1:2000) for 24 h	90 ± 5.8^{ab}

Letter superscripts indicate significant differences as per DMRT ($P \leq 0.05$).

It was clear from these results that SW had the most remarkable effects on germination, thus it was decided to further investigate seed germination of *S. tortuosum* with the use of SW pulse treatments. Additionally, the use of water agar instead of $1/10^{th}$ strength MS medium did not appear to have significantly impacted seed germination **(Figure 2.2, Table 2.1)**. This is not surprising, since the majority of germination trials are conducted on filter paper or a soil substrate. In both cases, the substrate is moistened with water and/or a germination stimulant – not nutrient solutions **(DREWES *et al.*, 1995; LIGHT *et al.*, 2002)**.

2.3.4. *In vitro* smoke-water germination study

The effects of various SW and control treatments on mean germination percentages and rates can be seen in **Table 2.2**. There was a large significant difference between the highest and lowest MGPs – originating from 1:1000 SW for 24 h (83 ± 1.9%) and 1:2500 SW for 1 h (59.5 ± 3.1%). The 1:1000 SW 24 h treatment was also significantly higher than the negative control (65 ± 3.5%). Although this percentage was not statistically significantly different from the positive control (24 h), it was 11% higher than this value (72 ± 2.6%).

Almost all SW treatments appear to follow a trend for MGP, i.e. with increasing pulse-time, germination percentage also increased. Only the 1:1500 SW treatment had a higher MGP for 8 h than for 24 h (75 ± 4.4% and 74 ± 2.5% respectively), but not surprisingly, there was no statistically significant difference between these values **(Table 2.2)**. Additionally, only the 8- and 24 h treatments (for all SW concentrations) achieved higher percentages than their positive (62 ± 7.0% and 72 ± 2.6% respectively) and negative controls (65 ± 3.5%) **(Table 2.2)**.

The highest MGPs were from treatments with average (1:2000) to moderately high (1:1000) SW concentrations pulsed for the longest time period, whereas the lowest percentages originated from 1 h SW treatments **(Table 2.2)**. There were no significant differences between the 1 h pulses and either control treatment. This indicates that treating seeds for 1 h with any concentration of SW (or with DW) is essentially the same as not treating seeds at all **(Table 2.2)**.

Similar to the trend observed for MGPs, increases in germination rates of SW treatments correlated with increases in pulse time. Only the 1:2000 SW treatment resulted in a higher GR for the 1 h pulse as compared to the 2 h pulse **(Table 2.2)**. However, this SW concentration was responsible for the highest GR (6.35 seeds/day) when seeds were pulsed for 24 h. Additionally, all 24 h pulse treatments achieved higher GRs than both control treatments **(Table 2.2)**. The slowest GR of all treatments – including controls – originated from the 1:2500 SW solution for 1 h (4.80 seeds/day) **(Table 2.2)**.

Table 2.2: Mean germination percentages and rates (seeds germinated/day) from smoke-water (SW) and control treatments after 30 days *in vitro*

Treatment	**1 h**		**2 h**		**8 h**		**24 h**	
	Mean GP ± SE (%)	**Rate**	**Mean GP ± SE (%)**	**Rate**	**Mean GP ± SE (%)**	**Rate**	**Mean GP ± SE (%)**	**Rate**
Control	63.5 ± 4.7^{cd}	5.45	69.5 ± 6.4^{bcd}	5.49	62 ± 7.0^{cd}	4.95	72 ± 2.6^{abcd}	5.75
1:500 SW	67.5 ± 3.9^{cd}	5.23	71.4 ± 3.9^{abcd}	5.42	72.5 ± 1.7^{abcd}	5.83	75 ± 5.1^{abc}	5.91
1:1000 SW	67 ± 6.0^{cd}	5.18	72 ± 3.4^{abcd}	5.23	74.5 ± 3.3^{abc}	5.87	**83 ± 1.9^{a}**	6.05
1:1500 SW	69 ± 4.0^{bcd}	5.37	70.5 ± 1.5^{bcd}	5.47	75 ± 4.4^{abc}	5.71	74 ± 2.5^{abc}	5.90
1:2000 SW	66 ± 2.9^{cd}	5.12	66.1 ± 2.9^{cd}	5.04	71 ± 4.5^{abcd}	5.62	80.5 ± 3.8^{ab}	**6.35**
1:2500 SW	$*59.5 \pm 3.1^{d}$	*4.80	67.9 ± 2.8^{cd}	5.06	71.9 ± 1.3^{abcd}	5.71	72.5 ± 3.3^{abcd}	6.07
Control 0 h	65 ± 3.51^{cd}	5.10						

Letter superscripts show statistically significant differences at $P \leq 0.05$ as per DMRT. Values in bold and with asterisks indicate the highest and lowest values respectively.

Before relating any results to observations by other researchers, it should be acknowledged that despite the use of SW in several studies, a standardized smoke extract only appeared recently in the literature **(GUPTA *et al.*, 2019; HRDLIČKA *et al.*, 2019)**. Thus, for previously utilized SW, its constituents and their concentrations are unknown and likely differed among researchers. Variations in the chemical composition of SW are due to the type of plant material used and the technique of preparing smoke extracts **(GUPTA *et al.*, 2019)**.

With that said, the findings in the present study mirror those discovered by **LIGHT *et al.* (2002)** and **DREWES *et al.* (1995)**. The combination of mean germination percentages with germination rates, indicate that pulse time has a profound effect on germination **(Table 2.2)**. **LIGHT *et al.* (2002)** determined that that 1 h pulses in SW did not have a significant impact on germination in lettuce seeds. Germination percentages increased with 2 h pulses and longer. Additionally, their highest GPs were also produced from a 1:1000 SW pulse treatment when compared to more concentrated SW solutions. Further support can be found in the **DREWES *et al.* (1995)** publication. Their investigations with lettuce seeds showed that a 24 h treatment with 1:1000 SW was the most effective at inducing germination, more so, at a temperature of 20°C. A GP of 90% was achieved **(DREWES *et al.*, 1995)**, which is strikingly similar to the 83% achieved from our study using the same conditions **(Table 2.2)**.

BROWN *et al.* (1998) primed *Syncarpha vestita* and *Rhodocoma gigantea* seeds (species from the GCFR) for 24 h in SW and germinated them successfully months later, further supporting the use of 24 h pre-sowing SW treatments. This is also consistent with a recommendation from **BROWN *et al.* (2004)** stating that Aizoaceae species (Mesembryanthemaceae at the time) should be exposed to liquid smoke solutions for 24 h.

It is noteworthy that the effects of the 1:2000 SW treatments are significantly lower than the percentages obtained from preliminary testing **(Table 2.1 and 2.2)**. The largest difference is found in the 2 h treatment where it was initially 93.3% **(Table 2.1)**, and in final experiment was 66.1 ± 2.9% **(Table 2.2)**. In fact, all germination percentages for all SW treatments were lower than the percentages obtained in the pre-trial, but 1:2000 SW showed the most significant decreases. This can be attributed to a number of factors. First, the age of the seeds **(BUCHVAROV and GANTCHEFF, 1984)**. Preliminary testing was done 3–9 weeks prior to the final experiment. Although seeds were stored at 10°C, they must still age with time **(BUCHVAROV and GANTCHEFF, 1984; SUNG, 1996)**, accounting for decreased germination percentages even with an experiment twice as long.

Another possible explanation is secondary dormancy. **BANOVETZ and SCHEINER (1994)** documented that *Coreopsis lanceolata* seeds which were stored at cold temperatures became dormant and remained so, even after removal from storage; coinciding with a report by **DEBEAUJON *et al.* (2007)** proving that seed coat thickness can increase while seeds are stored. **KHAN (1980)** reported that dormancy could be induced and broken in lettuce seeds, depending on how deep it was, i.e. how long seeds were exposed to the unfavourable condition. This would certainly explain the increased amount of time for mean germination percentages to be almost as high as the previous trial **(Table 2.1** and **2.2)**.

Nevertheless, the use of SW in pre-sowing treatments was undoubtedly effective in promoting germination compared to control treatments **(Table 2.2)**. This may be viewed as odd because *S. tortuosum* is primarily found in a non-fire prone area (Succulent Karoo) **(CHESSELET, 2005b; PIERCE *et al.*, 1995)**. However, it must be considered that the Fynbos and Succulent Karoo biomes are in close proximity to each other and smoke is not a stagnant entity **(ESLER *et al.*, 2015; KEELEY and FOTHERINGHAM, 1997)**. Fires in the GCFR can burn for days, thus it is more than likely that the aerosol smoke resulting from fires in the Fynbos, is experienced in the Succulent Karoo as well **(KEELEY and FOTHERINGHAM, 1997)**. In this way, smoke may signal germination in Succulent Karoo species without seeds having been exposed to the fire itself or the chemicals left behind in the ground after burning. Support for this notion comes from **ROCHE *et al.* (1998)** who applied SW to unburnt areas which were adjacent to burnt areas and found it to induce seeding emergence. **LLOYD *et al.* (2000)** also reported that aerosol smoke stimulated seedling emergence 10-fold as compared to SW, with a study conducted in the same area as **ROCHE *et al.* (1998)**.

It has previously been reported that seeds of non-fire prone species from the Succulent Karoo and Fynbos regions were better influenced by smoke than fire-prone species. This was evident in the Ruschiodeae **(PIERCE *et al.*, 1995)**. Not only does the present study agree with this information, but it indicates that germination of Mesembryanthemoideae species is also positively affected by smoke.

The germination-promoting ability of smoke is clear from historical and recent findings. The fact that smoke does not discriminate between fire- and non-fire-prone species has led to extensive research to determine why. It was initially hypothesized that smoke had a counteractive effect on a germination inhibitor found in seeds **(PIERCE *et al.*, 1995)**. This was evidenced by lettuce and *Nicotiana attenuata* seeds, where GA levels increased and ABA

levels decreased after the application of a smoke extract **(GARDNER *et al.*, 2001; SCHWACHTJE and BALDWIN, 2004)**.

More recently though, it was discovered that chemicals contained in smoke – karrikinolides (3-methyl-2H-furo[2,3-c]pyran-2-one or KARs) – are responsible for its germination-stimulating property. However, the exact mechanism via which these chemicals act is still under investigation **(CONN and NELSON, 2016; FLEMATTI *et al.*, 2004; WATERS *et al.*, 2014)**. There are six type of karrikinolides found in smoke (KAR_1, KAR_2, KAR_3, KAR_4, KAR_5, KAR_6), along with trimethylbutenolide (TMB) **(GUPTA *et al.*, 2019; HRDLIČKA *et al.*, 2019)**. Karrikinolides are stimulatory to seed germination while TMB is inhibitory **(CHIWOCHA *et al.*, 2009; FLEMATTI *et al.*, 2004; GUPTA *et al.*, 2019)**.

In this study, the smoke extract used for dilutions contained a higher concentration of TMB than KAR_1 and KAR_2 (individually) **(GUPTA *et al.*, 2019)**. **GUPTA *et al.* (2019)** used this extract and various dilutions to evaluate their effects on the germination of lettuce seeds. Based on their findings, they postulated that certain dilutions of SW cause the TMB level to decrease. This results in a reduction in its inhibitory effect, while allowing KAR_1 and KAR_2 to stimulate germination **(GUPTA *et al.*, 2019)**. This reasoning would explain the negative effect of concentrated SW solutions (1:500) as compared to more dilute solutions (1:1000) **(Table 2.2)**.

Based on the vigour indices (VIs) obtained from four SW and four control treatments, the most active seeds lots were as a result of 1:1000 SW (24 h) pulse treatment (191.69 ± 7.82) and untreated seeds (187.43 ± 8.19), whilst the lowest VI was from the 1:2500 SW (1 h) treatment (153.89 ± 7.47). It can be seen that there is no significant difference between the best treatment and the control (0 h) **(Figure 2.3d)**. However, upon closer inspection of individual growth parameters, the results are quite variable. Surprisingly, the longest mean root length was obtained for the negative control (24.90 ± 1.22 mm) followed by the 1 h control (22.44 ± 1.02 mm), then 1:2500 SW (1 h) (21.47 ± 1.22 mm) **(Figure 2.3a)**. The longest mean shoot length was also reached by the aforementioned SW treatment (4.19 ± 0.15 mm) **(Figure 2.3b)**. This makes 1:2500 SW (1 h) the best SW treatment in terms of obtaining the largest seedlings. However, no statistically significant differences were found between the seedling size from this treatment and the rest of the treatments **(Figure 2.3c)**.

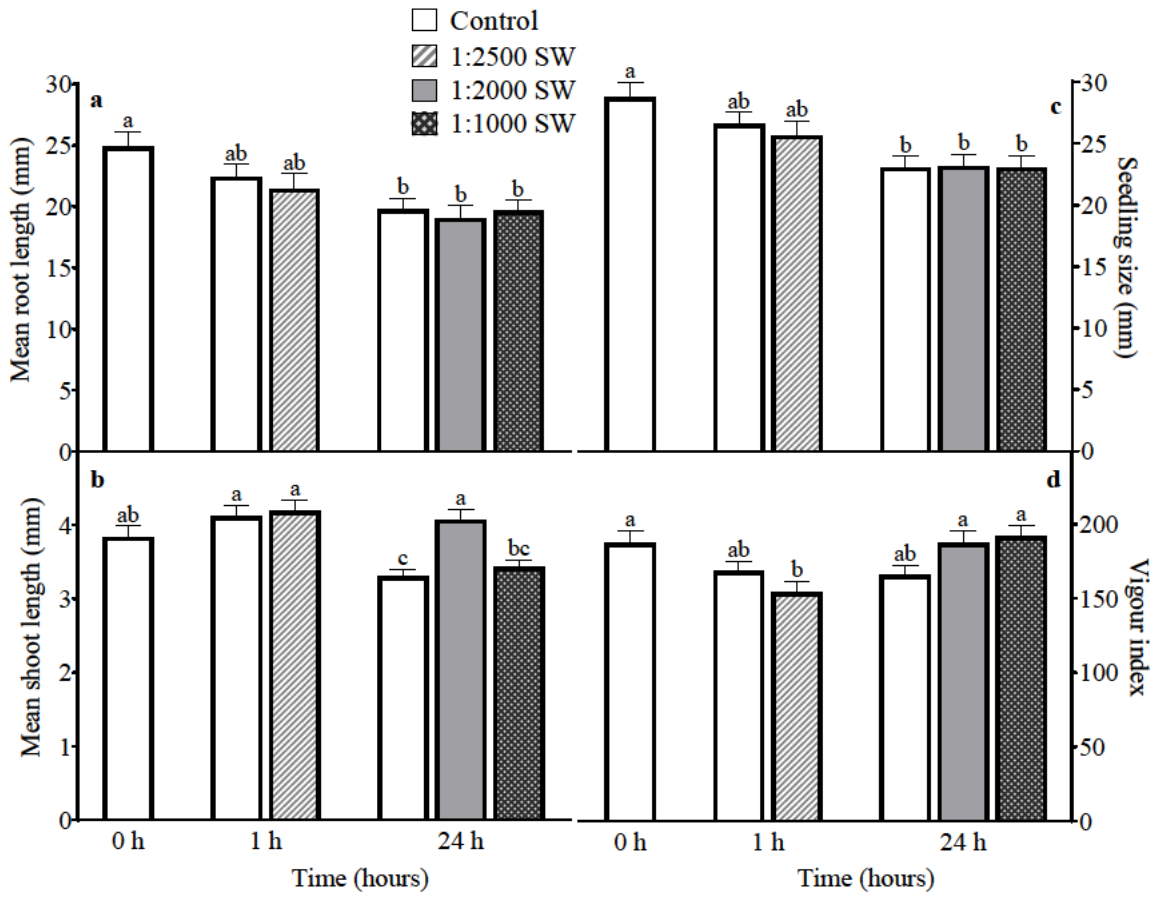


Figure 2.3: Effects of smoke water (SW) and control treatments on mean root length **(a)**, mean shoot length **(b)**, mean seedling size **(c)**, and vigour index **(d)** of *Sceletium tortuosum* seedlings. Error bars show SE of the mean. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$).

Overall, all 24 h treatments (including the control) generated seedlings with the shortest mean root and shoot lengths, thus the smallest seedlings **(Figure 2.3a–c)**. It appears that a short (1 h) pulse in the most dilute concentration (1:2500) of SW is most suitable for the enhancement of growth parameters. These data are somewhat conflicting because they imply that seeds should have a short exposure to SW to improve seedling growth, however from the germination results, this is not plausible due to negative impacts on GP and GR. A similar negative response in terms of seedling vigour was documented by **SPARG *et al.* (2006)**. Their observation was also noted after exposure to smoke for long periods of time.

Interestingly, the treatments with the highest GP (1:1000 SW 24 h) and GR (1:2000 24 h) had the smallest seedlings from the SW treatments **(Figure 2.3c)**. An explanation for this may be

found in general seed physiology and germination traits. **BASKIN and BASKIN (2014)** mentioned that germination relies on the embryo and its ability to grow and push through the surrounding tissues resulting in radicle emergence. This process can be sped up by germination stimulants **(BROWN, 1993; CORBINEAU *et al.*, 2014; RODRIGUEZ-PÉREZ *et al.*, 2007)**. Therefore, results showing that treatments with high GRs (1:2000 and 1:1000 SW for 24 h) caused decreased seedling sizes **(Figure 2.3c)** may be explained by the stimulatory effect of SW itself. Perhaps, it caused embryos to push through the tissues faster than they were able to fully mobilize food reserves contained by the perisperm, endosperm and cotyledons **(ECKARDT, 1976; FOTEDAR, 2017; MANNING, 2019; MCALISTER and KROBER, 1951)** This would also account for the larger seedling size reached by the remaining SW treatment which had a slower GR (1:2500 SW 1 h) **(Figure 2.3a–c, Table 2.2)**.

Alternatives to enhancing growth parameters should be investigated. For instance, **BAIRU *et al.* (2009)** showed that growth parameters can be enhanced by the introduction of nutrients to the germination medium (e.g., using MS medium instead of water agar). Alternatively, newly-germinated seedlings can be transplanted onto MS medium after establishment (e.g. 30 days) **(VENDRAME *et al.*, 2007)**.

2.3.5. *Ex vivo* germination experiment

Seedling emergence was first observed after 21 days, thus emergence percentages for all *ex vivo* treatments were extremely low with no significant differences between them. Only the 1:1000 SW (24 h) pulse and the corresponding positive control reached the maximum of 10% **(Table 2.3)**. This has some correlation with the *in vitro* experiment because the very same SW treatment obtained the highest final GP **(Table 2.2**). Unexpectedly, the treatment responsible for the highest GR *in vitro* (1:2000 SW for 24 h), showed the lowest emergence percentage and rate *ex vivo* (5 ± 3.0% and 0.242 seedlings/day). The fastest emergence rates originated from 1:1000 SW (24 h) and control (0 h) treatments **(Table 2.3)**.

Table 2.3: Mean emergence percentages and rates of seedling emergence from seeds sown *ex vivo*. Treatments are after smoke-water (SW) pulse treatments which yielded highest germination percentage and rates *in vitro* and the positive and negative controls

Treatment	Mean emergence ± SE (%)	Rate of emergence (seedlings/day)
Control 0 hrs	7.5 ± 2.5^{a}	0.352
Control 24 hrs	10 ± 0.0^{a}	0.055
1:1000 SW 24 hrs	10 ± 0.0^{a}	0.352
1:2000 SW 24 hrs	5.0 ± 3.0^{a}	0.242

Letter superscripts indicate significant differences as per DMRT ($P \leq 0.05$).

When considering these results, it must be acknowledged that germination and emergence are very different. Especially when conducting studies using soil substrates, it cannot be seen when germination has occurred and the emergence of the seedling may only occur some days after the germination event **(BENVENUTI *et al.*, 2001)**. With that said, there is still a large difference between the percentages obtained from *in vitro* and *ex vivo* experiments, despite the use of the same pulse treatments **(Table 2.2** and **2.3)**.

Naturally, the conditions *in vitro* and *ex vivo* are totally different. Temperature could not be kept constant *ex vivo* and the fluctuations experienced in PMB could have posed a problem for seed germination, i.e. temperatures were in the range of 7–43°C at the time of the experiment. Such temperature fluctuations may be experienced in the plants' natural environment as well, and in many cases does not create an issue for germination studies **(BAIRU *et al.*, 2009; RODRIGUEZ-PÉREZ *et al.*, 2007)**, however the soil substrate must be considered together with temperature. **FREE (1911)** reported that lightly-coloured sand gains and loses heat much faster than darkly-coloured soils. As the growth medium used was not pure sand and was quite dark in colour, this could have resulted in much warmer or colder soil temperatures than were conducive to germination.

Seeds sown *ex vivo* received less light due to natural light conditions (8–10 h of sunlight), and sowing seeds in the soil mixture. Seeds simply may not have had enough light or the right quality of light to allow for germination **(ROJAS-ARÉCHIGA *et al.*, 1997)**. Also, soil pH may have been unsuitable for germination **(BEUKES and ELLIS, 2003; HACKETT, 1964)**.

Apart from secondary dormancy imposed by environmental and experimental conditions, other dormancies are certainly possible. As previously mentioned, dormancy could have been caused by storing seeds at a low temperature **(BANOVETZ and SCHEINER, 1994)**. Given that the seeds used in the *ex vivo* trial were stored for a longer period than those used for *in vitro* studies, this could have imposed a deeper dormancy **(KHAN, 1980)**. Perhaps, a dormancy-breaking treatment (stratification or chemical treatment) is necessary after keeping seeds in cold storage for ± 6 months, prior to application of SW **(BANOVETZ and SCHEINER, 1994; BASKIN and BASKIN, 2014; CHEN, 1968; KHAN, 1980)**.

2.4. CONCLUSIONS

Germination of *Sceletium tortuosum* seeds was optimal at 20°C under constant light. Smoke-water was remarkably effective in promoting germination, with the highest *in vitro* germination percentage and rate produced by 1:1000 SW (24 h) and 1:2000 SW (24 h) pulse treatments, respectively. The former treatment also resulted in the highest vigour index achieved. Preliminary trials indicated that a soak in ethrel (2.8 ml/L) for 2 h was another cost-effective, efficient method of inducing germination, although further investigation is necessary to determine if this treatment consistently generates high MGPs.

Despite the repetition of these significant SW treatments in an *ex vivo* investigation, seedling emergence percentages peaked at 10%. Only the 24 h control and 1:1000 SW treatments yielded this percentage after 30 days. The highest seedling emergence rate was achieved by 1:1000 SW and untreated seeds.

Initially, the best SW treatment was 1:2000 for 2 h. However, the high MGP achieved by this treatment in the pre-trial was not maintained in the final *in vitro* experiment. Based on the *in vitro* and *ex vivo* experiments, a 24 h pulse treatment in 1:1000 SW was the best treatment for germination of *S. tortusoum* seeds. The only downfall of this and other 24 h SW treatments is reduced seedling size, thus the introduction of nutrients to the germination medium is recommended.

Secondary dormancy and aging may be factors in the reduction of germination over the course of these investigations. A storage-imposed or seed-coat dormancy may have been acquired by *S. tortuosum* seeds while in cold storage. Further electron microscopy work is necessary to determine the exact characteristics of *S. tortuosum* seeds and if seed coat thickness increases

with storage condition and time. This type of work will also allow for the evaluation of changes within seeds during different stages of germination to determine the influence of the perisperm. These studies will be crucial in determining the ideal storage-setting, the type of dormancy exhibited, as well as providing fundamental information for future germination studies with this species.

CHAPTER 3: VEGETATIVE PROPAGATION

3.1. INTRODUCTION

Various plants reproduce asexually via vegetative propagules. Plantlets form on structures such as stolons, bulbs, corms and rhizomes. *Ranunculus repens*, *Lilium* spp. and *Iris* spp. are some of the many plants capable of reproducing in this way. The new plantlets produced will have the same genetic material as the parent plant **(FENNER and THOMPSON, 2005)**. This statement can be regarded as the fundamental principle of vegetative propagation.

It has long been known that plants can be propagated using somatic cells, tissues and organs, and in this way, genetic diversity remains relatively unchanged within populations or species **(IMANI *et al.*, 2009; LEAKEY *et al.*, 1994)**. This is fundamentally important for a number of reasons. Food crops are globally important and genetic diversity must be maintained to ensure high crop yields **(GÜREL and GÜLŞEN, 1998a)**. Medicinal plants are essential in pharmacology and chemotypes are selected based on their yield of phytochemicals **(ELEV8™, 2017)**. Genetic variations can also lead to varying stress tolerances within populations which can increase plant losses **(GÜREL and GÜLŞEN, 1998a)**.

Some plant species have long life spans and take months or years to set seed **(CRAWLEY, 1997; FENNER and THOMPSON, 2005)**. Thereafter, when seeds are sown, germination, growth and maturation can result in long waiting periods for farmers, florists, researchers and pharmaceutical companies. This is simply not feasible in terms of business and research **(REINHARDT *et al.*, 2018; RIAHI *et al.*, 2017)**. Thus, vegetative propagation methods are used to significantly reduce waiting-times.

Additionally, some seeds produced by inter-generic crosses will not produce fertile seeds, others may not retain the characteristics of one or both parents, and certain seeds may only be viable for a short period of time. This is evident in several commercially important species **(CHAMBERS *et al.*, 1991; IANNOTTI, 2019b; NAGDIR *et al.*, 1984; RAHAMAN *et al.*, 2006)**, exemplifying the necessity for vegetative propagation strategies.

3.1.1. Non-aseptic techniques

3.1.1.1. Grafting, budding and layering

Grafting can be done using one or two species and is commonly used for propagating tree and ornamental species, as well as succulents **(GINOVA *et al.*, 2012; REMA *et al.*, 1997; SANOU *et al.*, 2004)**. It is usually carried out with a rootstock (bottom of one plant) and a scion (top of another plant). Basically, the scion is inserted into or onto the root stock, such that there is contact of vascular tissues or cambial layers. In the case of trees and roses, the bark is peeled away slightly for the scion to be grafted onto the branch or stem.

Budding grafts are done the same way, the only difference is that the scion piece must have a developed bud **(BILDERBACK *et al.*, 2014)**. The patches of peeled away bark can be used for patch budding by adhering this patch to another tree where a similar sized piece has been removed **(WILLEY, 2016)**. These are popular methods for the propagation of fruit trees **(GEORGE *et al.*, 2008; WILLEY, 2016)**.

Layering may also be used for trees and other plant species that have aerial stolons. The organ used for layering should not be detached from the mother plant **(EVANS and BLAZICH, 1999)**. For tree species, the bark is peeled off below a node and the inner tissue lightly scarified. A plastic container or bag is attached around this point and soil is added. Over time, roots will form at this region and the new plant can be removed from the mother plant thereafter **(BRENNAN and MUDGE, 1998; KATHIRESAN and RAVIKUMAR, 1995)**. Plants with stolons can be propagated by simply covering a node with soil until roots grow **(RELF and BALL, 2009)**.

3.1.1.2. Cuttings

This is a common practice with many plant species, particularly ornamentals **(ABBAS *et al.*, 2006)**. It is used by home-growers, florists, farmers, commercial growers as well as researchers **(KROIN, 1992)**. Effectively, the process involves taking a cutting of a stem, shoot, root or a leaf and inducing root or shoot proliferation **(RELF and BALL, 2009)**.

3.1.1.2.1. Stem and shoot cuttings

Stem cuttings are probably the most employed cutting-strategy due to its simplicity. A piece of stem or shoot is cleanly excised from the mother plant and placed in an appropriate soil substrate for rooting to occur **(IANNOTTI, 2019a)**. Stem and shoot cuttings are sometimes left in water or an auxin solution for root proliferation to occur before potting the new plant in soil **(VAN BRAGT *et al.*, 1976)**. Alternatively, cuttings may be treated/pulsed with an auxin solution prior to planting **(BLYTHE *et al.*, 2004)**.

Rooting powders (containing IBA) have become quite popular but successes vary depending on the species **(IANNOTTI, 2019a)**. For example, **VAN BRAGT *et al.* (1976)** showed that auxin solutions (IBA and IAA) were more effective than rooting powders for stimulating root production in *Berberis* spp. and *Pyracantha* spp., while rooting powder was more successful for *Rosa damascena* and *R. centifolia* **(ABBAS *et al.*, 2006)**.

3.1.1.2.2. Leaf cuttings

This type of cutting is useful for succulent and semi-succulent species which have leaves that are easy to twist off cleanly (without damage to leaf tissue) **(GORELICK, 2015; JACOBSEN *et al.*, 1960)**. Leaves are left to callus over at the wounded site, then placed onto a moist soil medium until roots and new plantlets form. Leaf cuttings are possible for species in a number of plant families, including the Crassulaceae, Brassicaceae and Aizoaceae **(JACOBSEN *et al.*, 1960; KERNER and OLIVER, 1902; RAJU and MANN, 1971)**.

3.1.1.2.3. Root cuttings

Certain species are only propagatable by roots. Root cuttings are done by placing root pieces in soil with the upper root portion only lightly covered **(RABO, 2019)**. *Armoracia rusticana, Talinum triangulare* and *Robinia pseudoacacia* were successfully propagated using this methodology **(DORE, 1953; KESERŰ *et al.*, 2019; RABO, 2019)**.

3.1.2. Aseptic techniques

3.1.2.1. Plant tissue culture

Plant tissue culture (PTC) is the broad term used to refer to all cell, tissue and organ cultures **(GEORGE, 1993)**, but for the purpose of this dissertation, it will be used to refer to tissue and organ cultures only. The technique is based on the concept of totipotency, i.e. that every cell has the genetic ability to develop into a new individual plant **(HABERLANDT, 1902)**. Plant tissue culture is a versatile technique for the propagation of disease-free plants which can be implemented for large scale production **(HUSSAIN *et al.*, 2012)**. It is used for the propagation of rare and endangered species, as well as medicinal plants, ornamentals and crops **(BAIRU *et al.*, 2007; TORRES, 1989; ZIMMERMAN *et al.*, 1986)**. Plant tissue culture techniques allow for the development of genetically modified plants which is invaluable in the agricultural industry, especially with global climates rapidly changing **(HUSSAIN *et al.*, 2012)**.

The process has become more sophisticated over the years with just about every plant tissue and organ being utilized in some culture scheme, i.e. shoots, roots, embryos, pollen, anthers petals and bark **(AYGUN and DUMANOGLU, 2015; CHOVEAUX and VAN STADEN, 1981; GOWDA *et al.*, 2019)**. The basics of the technique involve inoculating the plant tissue/organ on a suitable medium for cell differentiation (or de-differentiation) to occur **(SKOOG and MILLER, 1957)**. Thus, before commencement of tissue culture experiments, one must decide what type of culture is required. Callus cultures can be initiated from various plant tissues and induced to proliferate plant organs (indirect organogenesis), or to produce embryos (somatic embryogenesis). Alternatively, larger parts of organs may be cultured (pieces of shoots or roots) and growth of the rest of plant is induced (e.g. root proliferation is induced from shoot explants) (direct morphogenesis) **(HUSSAIN *et al.*, 2012; ILIEV *et al.*, 2010)**.

3.1.2.1.1. Stages of plant tissue culture

3.1.2.1.1.1. Stage 0: Selection of mother plant

The mother plant must be selected and well-maintained until the *in vitro* work begins. The plant must be healthy and disease-free. It has been suggested that mother plants undergo disease-screening before use so that a pre-treatment can be determined if necessary **(DEBERGH and MAENE, 1981; GEORGE *et al.*, 2008)**.

3.1.2.1.1.2. Stage 1: Establishing sterile cultures

By far, the most fundamental stage in tissue culture is surface-sterilizing plant material prior to inoculation on tissue culture (TC) media **(GEORGE *et al.*, 2008)**. Numerous factors can make this process difficult. For instance, seasonal and weather conditions experienced when plant material is harvested has a major influence on epiphytic contaminants. Different explant types may also be affected by different contaminants **(DA SILVA *et al.*, 2015; HOHTOLA, 1988; MEHTA *et al.*, 2011)**. Additionally, some plants possess endophytic contaminants – bacteria and/or fungi **(BUCKLEY and REED, 1994; RAY and ALI, 2017)**.

Various chemicals are used in the surface-sterilization process. The most popular sterilants are calcium hypochlorite (CaOCl) and sodium hypochlorite (NaOCl), followed by mercuric chloride ($HgCl_2$) and hydrogen peroxide (H_2O_2) **(DA SILVA *et al.*, 2015; JAHAN *et al.*, 2009; TIWARI *et al.*, 2012)**. Alcohol (70–100%) can be used alone, but most protocols incorporate alcohol before or after administering the sterilant to the plant material **(BELLO *et al.*, 2018; JAHAN *et al.*, 2009)**. The material is usually rinsed prior to immersion in the sterilant solution (pre-sterilization), and thoroughly washed with SDW after exposure to the sterilant **(DA SILVA *et al.*, 2015)**.

Sterilization protocols vary in the types of sterilants used, their concentrations and time periods of immersion. Often, the use of one sterilant (± alcohol) is not sufficient to reduce contamination, therefore some protocols incorporate more than one sterilant in a step-wise fashion. Alternatively, a mixture of two sterilants is used **(CHEN *et al.*, 2017; MAHMOUD and AL-ANI, 2016)**. Researchers may also utilize fungicides and antibiotics as part of surface-sterilization or include these in TC media **(RAY and ALI, 2017; TIWARI *et al.*, 2012)**.

3.1.2.1.1.3. Stage 2: Multiplication of propagules

Explants must be inoculated on TC media which provide a source of nutrients. Numerous recipes exist for creating nutrient media, e.g. B5, White's medium, Murashige and Skoog (MS) medium. MS is regarded as the most versatile medium to use for most culture types **(SAAD and ELSHAHED, 2012)**.

Plant growth regulators (PGRs) are key factors in controlling growth and development *in vitro* **(FEHER et al., 2003)**. These hormones are added to TC media to elicit specific responses namely, shoot or root proliferation, callus induction and somatic embryo formation (see

3.1.2.1.2) **(ILIEV *et al.*, 2010; SAAD and ELSHAHED, 2012)**. Typically, shoot multiplication is induced first. Shoots are sub-cultured numerous times onto new media to generate a sufficient quantity of new shoots and new *in-vitro* plants **(HUSSAIN *et al.*, 2012; ILIEV *et al.*, 2010)**.

3.1.2.1.1.4. Stage 3: Rooting of plantlets

When a suitable number of healthy shoots have proliferated, these are sub-cultured onto new media for root proliferation and elongation. In some cases, spontaneous rooting occurs, eliminating the need for further sub-culture **(BAIRU *et al.*, 2007; HUSSAIN *et al.*, 2012)**.

3.1.2.1.1.5. Stage 4: Acclimatization

The final goal is to acclimatize *in vitro* plantlets to greenhouse or field conditions. Once plantlets have well-developed shoots and roots, they can be transplanted to an *ex vitro* environment **(GEORGE *et al.*, 2008; HUSSAIN *et al.*, 2012)**. However, the morphological characteristics acquired *in vitro* make this a tricky transfer **(MATHUR *et al.*, 2008)**. As plantlets are acclimated to high humidity *in vitro*, it is recommended that they are exposed to gradual decreases in humidity **(HUSSAIN *et al.*, 2012; SHARMA *et al.*, 2015)**. Usually, plantlets are first transplanted to a mist-house, followed by a greenhouse, and finally to the field/open air if necessary **(GEORGE *et al.*, 2008; HAZARIKA, 2003)**.

Another challenge that acclimatization brings, is the possibility of plant loss due to microbial contaminants. Plantlets likely have little to no tolerance to air or soil microbes and can easily succumb to infections **(CHANDRA *et al.*, 2010)**. Air contaminants are unavoidable, however, soil contaminants can be reduced by soil fumigants, e.g. methyl bromide **(LEELAVATHY and SANKAR, 2016; SINGH, 2018)**. Autoclaving the soil substrate is sometimes substituted for this **(ASLAM *et al.*, 2013; GALUS *et al.*, 2019)**.

3.1.2.1.2. Role of PGRs in tissue culture

3.1.2.1.2.1. Auxins

Various auxins are produced naturally by plants, such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), phenylacetic acid (PAA) and 4-chloro-IAA **(GEORGE *et al.*, 2008)**, The most commonly used auxins in PTC are IAA, IBA, 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) **(Figure 3.1)**.

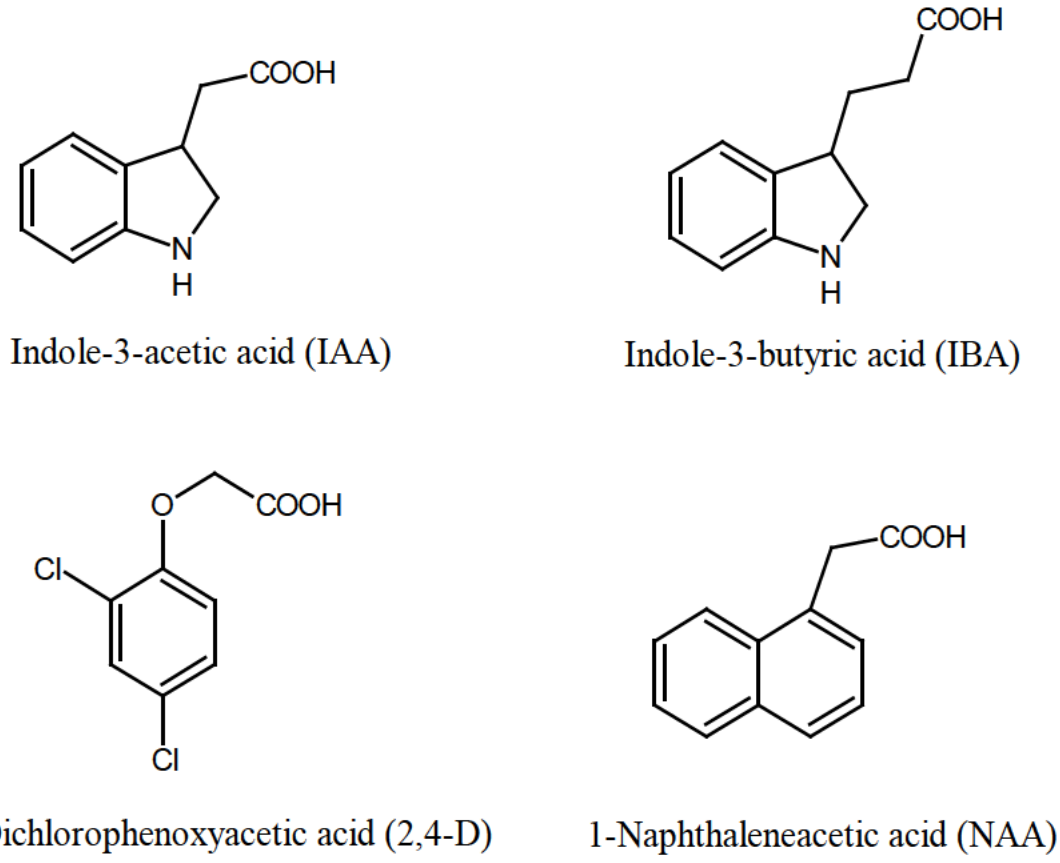


Figure 3.1: Auxins commonly used in plant tissue culture

Auxins have several roles in plants including root induction, shoot elongation, flower formation and controlling apical dominance **(ALONI *et al.*, 2006; OVERVOORDE *et al.*, 2010)**. They are produced in shoots, particularly in young leaves, as well as in roots. It has been inferred that IBA (and/or its derivatives) are synthesized, stored and transported to be converted to IAA. IAA is the primary hormone involved in root induction. Hormone signalling pathways (HSPs)

are used to direct the flow or storage of auxins **(GEORGE *et al.*, 2008; LJUNG *et al.*, 2005)**. Despite this knowledge, IAA is used slightly less frequently in PTC due to its photodegradative property **(GEORGE *et al.*, 2008; QUAMBUSCH *et al.*, 2017)**

Although one of the primary roles of auxins in PTC is for adventitious root formation, this PGR is often essential to initiate shoot proliferation as well **(ALONI, 2004)**. Both IBA and IAA are known to powerfully induce root proliferation and elongation in a number of species, e.g. *Fragaria vesca* and *Prunus avium* **(QUAMBUSCH *et al.*, 2017; YILDIRIM and TURKER, 2014)**.

Naturally, the specific auxin and its concentration are crucial for achieving the desired effects in PTC. One auxin might be successful in root induction in one species but ineffective in another. For instance, in *Pyrus elaeagrifolia*, IBA induced rooting while NAA did not **(AYGUN and DUMANOGLU, 2015)**. Conversely, *Rotula aquatica* rooted better in NAA compared to the two natural auxins **(MARTIN, 2003)**, while IAA resulted in better rooting and acclimatization percentages in *Eulophia streptopetala* **(KHUMALO *et al.*, 2017)**.

Although 2,4-D is considered as an auxin, it is not regularly used for *in vitro* rooting. Predominantly, it is used for callus induction, either for the production of somatic embryos or indirect morphogenesis **(AGARWAL and KAMAL, 2004; ISLAM *et al.*, 2005; PÉREZ-MOLPHE-BALCH *et al.*, 2015)**.

3.1.2.1.2.2. Cytokinins

Many cytokinins occur naturally in plants, including zeatin, isopentenyladenine (2-iP), dihydrozeatin (DHZ**)** and meta-topolin (mT) as well as many derivatives of these compounds **(GEORGE *et al.*, 2008; STRNAD *et al.*, 1997)**. Kinetin (Kin) and 6-benzyladenine (BA) are the most commonly used in PTC **(Figure 3.2)**. However, mT (and its derivatives) are becoming increasingly popular **(AKBAS *et al.*, 2009; AMOO and VAN STADEN, 2013; HERRERA *et al.*, 1990; WILLIAMS and TAJI, 1991)**.

Cytokinins are largely synthesized in plant roots and transported to other organs for use or storage **(ENTSCH *et al.*, 1980)**. These compounds are responsible for shoot proliferation and elongation, and flower induction in nature and in PTC. Often, shoot formation can only be induced with the addition of a cytokinin to TC media **(D'ALOIA *et al.*, 2011; GEORGE *et al.*, 2008; WANG *et al.*, 1997)**.

As with auxins, the most effective cytokinin differs among species. For example, callus cultures of *Anthurium andraeanum* produced multiple shoots in the presence of BA **(JAHAN *et al.*, 2009)**, while in *Spathiphyllum floribundum*, this was possible in the presence of mT **(WERBROUCK *et al.*, 1996)**. **WOJTANIA (2010)** documented mT to be more favourable than BA in various *Pelargonium* hybrid cultivars by inducing shoot multiplication, improving shoot quality as well as inducing root proliferation. Flower induction and development are also possible in PTC with the addition of cytokinins. BA is reportedly the most effective inducer of these reproductive organs **(FAISAL *et al.*, 2018; TAYLOR *et al.*, 2005; WANG *et al.*, 1997)**.

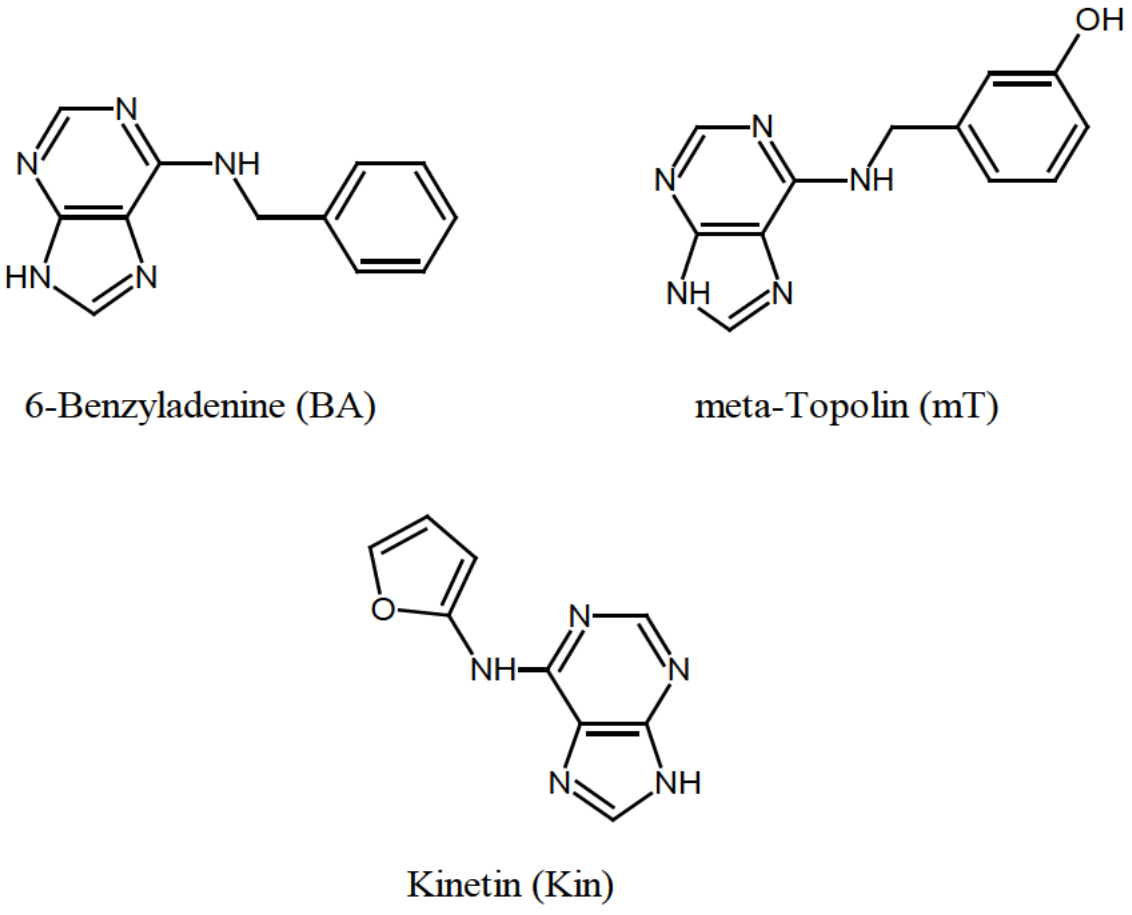


Figure 3.2: Cytokinins commonly used in plant tissue culture

3.1.2.1.2.3. Auxin-cytokinin interaction

It is well established that the key to inducing differentiation and/or de-differentiation of explants *in vitro* lies with the ratio of auxin: cytokinin **(SKOOG and MILLER, 1957)**. Growth

media with a high auxin: cytokinin ratio typically produce roots; and high cytokinin: auxin ratios usually produce shoots. An intermediate concentration of both PGRs induces callus formation **(SKOOG and MILLER, 1957)**. Naturally, optimal concentrations, ratios and specific PGRs are dependent on the species, the conditions experienced by the mother plant and *in vitro* conditions **(CASSELLS, 1979; GEORGE *et al.*, 2008; PAVLOVÁ and KREKULE, 1984)**. In some instances, the addition of both PGRs is necessary to initiate and enhance shoot and/or root proliferation and flower development **(GEORGE *et al.*, 2008; RINGE and NITSCH, 1968)**.

However, the PGRs used to supplement TC media can affect the endogenous levels of auxins **(NEGRUTIU *et al.*, 1979; ZAŽIMALOVÁ *et al.*, 1995)** and cytokinins **(CHOVEAUX and VAN STADEN, 1981; MERCIER *et al.*, 2003)**. In some instances, the interaction of these two PGRs result in the suppression of one of their effects **(HANSEN *et al.*, 1985; HARRIS and HART, 1964)**

3.1.2.1.2.4. Gibberellins, abscisic acid and ethylene

A primary function of GAs is the promotion of shoot elongation **(GEORGE *et al.*, 2008)**, however effects on shoot proliferation and growth are rarely observed in PTC. GA_3 and GA_4+ GA_7 are used in PTC, although much less frequently than other PGRs because they often suppress morphogenesis of shoots, roots and somatic embryos **(GASPAR *et al.*, 1996; MURASHIGE, 1964; SANKHLA *et al.*, 1994)**.

In some instances, gibberellins behave in a similar manner as auxins **(MURASHIGE, 1964)**. They may induce and promote callus formation. However, this effect is normally observed when an auxin and cytokinin are also present in the TC medium **(DALESSANDRO, 1973; ENGELKE *et al.*, 1973)**. To prevent the suppression of morphogenesis in tissue cultures, researchers sometimes sub-culture plantlets onto gibberellin-supplemented medium for shoot elongation, after shoot proliferation has occurred. This was effective for *Ficus benjamina* and *Acacia sinuata* **(DEL AMO-MARCO and PICAZO, 1994; VENGADESAN *et al.*, 2003)**.

In nature, when plants experience unfavourable environmental conditions, abscisic acid (ABA) levels increase **(MONGRAND *et al.*, 2003)**. This PGR is responsible for controlling water and ion uptake, among other crucial functions **(GEORGE *et al.*, 2008)**. *In vitro*, ABA supresses callus growth however, it effectively induces somatic embryogenesis and results in high quality

embryos by increasing their stress tolerance **(RAI *et al.*, 2011)**. Abscisic acid promotes shoot generation when used together with other PGRs **(MAGGON and SINGH, 1995)** and induces flowering when used singly **(SAXENA *et al.*, 2008; TANIMOTO *et al.*, 1985)**.

Ethylene is most noted for its role in seed dormancy and seedling establishment **(CORBINEAU *et al.*, 2014)**, but it is involved in other developmental process such as senescence and fruit ripening **(ABELES *et al.*, 2012)**. Ethylene production increases when a plant is wounded, i.e. when an explant is excised from the mother plant, ethylene will be produced by the explant **(GEORGE *et al.*, 2008)**. Once explants are placed *in vitro*, ethylene will continue to be produced in the culture vessel **(BIDDINGTON, 1992)**.

Ethylene produced or applied *in vitro* has various effects in PTC. Some researchers documented the inhibition of callus formation in the presence of exogenously applied ethylene **(BOLTON and FREEBAIRN, 1975; ZOBEL and ROBERTS, 1978)**, while others observed the promotion of callus formation **(BRADLEY and DAHMEN, 1971; WILLIAMS *et al.*, 1990)**. Such variations were also reported for morphogenesis. For example, an increase in shoot proliferation was observed in *Oryza sativa*, but not in a *Begonia* hybrid species **(CORNEJO-MARTIN *et al.*, 1979; RINGE, 1972)**. Ethylene effects on the remaining morphogenetic traits (root proliferation, embryonic callus induction, somatic embryo formation) varies according to the stage which it is applied, along with concentration and the species **(COLEMAN *et al.*, 1980; ISHIZAKI *et al.*, 2000; KOCHBA *et al.*, 1978; PEREZ-BERMUDEZ *et al.*, 1985)**.

3.1.3. Vegetative propagation of the Aizoaceae

Delosperma cooperi (Rusch) and two *Lampranthus* (Rusch) species cuttings were rooted *ex vivo* and *in vitro*. *Ex vivo* cuttings were placed in 3:1 peat: sand mixture. *In vitro* cultures made use of hormone-free MS medium **(BRAUN and WINKELMANN, 2015)**. *Carpobrotus edulis* (Rusch) leaf explants produced callus after two weeks on MS medium supplemented with 2.3 µM 2,4-D **(DIADEMA *et al.*, 2003)**. However, neither of these studies were focussed on the propagation of these species, thus the exact success rates are unclear.

Tissue culture has been successful with *Mesembryanthemum crystallinum* (Mesemb). **CUSHMAN *et al.* (2000)** made use of hypocotyl explants from *in vitro* germinated seeds and induced embryonic callus formation on MS medium supplemented with 80 mM NaCl + 5 µM 2,4-D + 1 µM Kin. The somatic embryos obtained regenerated well when places on hormone-

free MS medium. *Drosanthemum micans* (Rusch) and *Drosanthemum hallii* (Rusch) were also cultured *in vitro* using stem nodal explants **(MLUNGWANA, 2018)**. It was reported that shoot multiplication was optimized with 10 µM Kin, while root proliferation took place on hormone-free MS medium **(MLUNGWANA, 2018)**.

There are many online reports stating that Aizoaceae species are easily propagated by vegetative cuttings. Species propagatable in this manner include *S. tortuosum* (Mesemb), *Delosperma abbottii*, *D. saxicola* (Rusch) and *Prenia vanrensburgii* (Mesemb) **(CHESSELET, 2005a; VAN JAARSVELD, 2019a**, **2019b; VAN JAARSVELD, 2016)** among others. However, no scientific publications were found pertaining to the vegetative propagation of *S. tortuosum* by any means.

3.1.4. Objectives

1) Use various concentrations of sterilant/s, fungicide/s and antibiotic/s for different time periods to develop a reliable tissue culture sterilization protocol;

2) Use different plant organs as explants, along with different combinations and concentrations of PGRs to determine regenerative capabilities;

3) Initiate tissue cultures with ideal explant type and PGRs to multiply *in vitro* plant material;

4) Use explants from multiplication cultures for *in vitro* rooting investigation incorporating different concentrations of PGRs;

5) Record growth parameters of *in vitro*-generated plantlets prior to transplantation to *ex vitro* environment;

6) Acclimatize plantlets to greenhouse conditions;

7) Conduct *ex vivo* cuttings trial with the same concentrations of PGRs used in the *in vitro* rooting investigation; and

8) Record growth parameters of cuttings after growth for the same period allowed for (4).

3.2. MATERIALS AND METHODS

3.2.1. Source of plant material

Sceletium tortuosum plants were collected near Oudtshoorn (S 33° 40.883' E 22° 09.749'). A voucher specimen was deposited in the Bews herbarium (NU0089203) at the University of KwaZulu-Natal (UKZN). Plants were potted in a 1:1 mixture of vermiculite and soil and maintained in a greenhouse in the botanical garden at UKZN (PMB). Material obtained from these plants was used for *in vitro* and *ex vivo* investigations.

3.2.2. General *in vitro* preparations and conditions

Media used for all TC studies was standard **MURASHIGE and SKOOG (1962)** (MS) medium (3% sucrose, pH 5.8, 10 g/L OXOID agar no. 3). For the development of a sterilization protocol, hormone-free MS medium was used. For all further TC studies, PGRs were used to supplement the MS media.

Media were dispensed into TC vessels (10 ml each) and autoclaved (121°C, 15 psi) for 15 min along with all glassware, instruments and distilled water. All cultures were incubated at 25 ± 2°C under constant light (PAR 9.07 x 10 $\mu mol.m^{-2}.s^{-1}$).

3.2.3. Development of a sterilization protocol

For simplicity, pre-sterilization refers to a rinse of plant material under running tap water for 1–2 min. Post-sterilization was the rinse of plant material in 70% ethanol for 2 min (after removal from the sterilant), followed by three rinses with SDW. Only shoots were used for development of the protocol, where nodal segments were intact for sterilization and leaves were sliced into 0.5–1.0 cm explants for inoculation onto TC media.

Following pre-sterilization, plant material was transferred to sterile beakers containing the sterilant + Tween 20 (2 drops per 100 ml). Sodium hypochlorite (NaOCl) (1.5%, 2.5%), mercuric chloride ($HgCl_2$) (0.1%, 0.2%) and hydrogen peroxide (H_2O_2) (10%, 20%) were used to sterilize plant material for 5, 10 and 20 min. Based on these investigations, 20% H_2O_2 (20 min) was selected for further use.

3.2.3.1. Introduction of fungicide

Benomyl (1 g/L) was added to the 20% H_2O_2 protocol. In these cases, following pre-sterilization, plant material was placed in a sterile beaker containing SDW + Tween 20 (2 drops per 100 ml) and shaken with an orbital shaker (150–170 rpm) for 20 min. Thereafter, the Tween 20 solution was thoroughly rinsed off using distilled water. Benomyl (1 g/L) was poured into the sterile beaker and it was re-placed on the orbital shaker for 20 or 40 min. Following this, Benomyl was removed and plant material was transferred to the laminar flow bench where the sterilant was added. Plant material was immersed for 20 min before following the post-sterilization method. Immersion in Benomyl for 40 min was selected for further use.

3.2.3.2. Introduction of antibiotic

The addition of streptomycin sulphate to the sterilization protocol was similar to **3.2.3.1**. Pre-sterilization was followed by orbital shaking in Tween 20 solution for 20 min, then orbital shaking in Benomyl (1 g/L) for 40 min. After removal of the Benomyl solution, 0.01% or 0.05% streptomycin sulphate was poured into the sterile beaker. It was placed on the orbital shaker for 20 min. The beaker was then moved to the laminar flow bench where the antibiotic was removed and the sterilant was introduced. Plant material was immersed in H_2O_2 for 20 min followed by the post-sterilization method.

3.2.4. Sterilization experiment

Due to variations in contamination throughout the year, the developed sterilization protocol was re-evaluated in Spring. Sterilants, concentrations and time periods of immersion were as above **(3.2.3)** with the exception that 5 min was excluded for $HgCl_2$ and H_2O_2. Step-wise protocols were also repeated as per sections **3.2.3.1** and **3.2.3.2**. Twenty replicates were used for each treatment.

3.2.5. Selection of explant type for further study

Leaf, internode, shoot (nodal segment) and woody stem explants were tested for regenerative capability. Plant material was sterilized using: pre-sterilization + Tween 20 (20 min) + 1 g/L Benomyl (40 min) + 0.05% streptomycin sulphate (20 min) + 20% H_2O_2 (20 min) + post-

sterilization. Explants were cut into 0.5–1.0 cm pieces and inoculated onto MS medium supplemented with 2.5 µM IBA or 2.5 µM TDZ + 2.5 µM BA.

3.2.6. Selection of PGRs for *in vitro* multiplication

Shoot explants sterilized as above **(3.2.5)** were inoculated on 20 ml MS medium supplemented with 2.5 µM mT, 2.5 µM mT + 2.5 µM IBA or 2.5 µM IBA. Photographs were taken to document the growth of the explants on different media. Following this, 2.5 µM IBA was used to generate shoots for the *in vitro* rooting study.

3.2.7. *In vitro* rooting study

Twenty replicates were used per treatment. IBA and IAA were used to supplement MS media with concentrations of 2.5, 5.0 and 10.0 µM (no combination treatments were used). The control treatment was standard MS medium. Tissue culture vessels contained 20 ml of the growth medium. Shoot explants taken from *in vitro* bulked-up plants were used to inoculate the TC media. Shoot explants were taken only from plants which had not flowered. Cultures were incubated at 25°C under constant light (PAR 9.07 x 10 $\mu mol.m^{-2}.s^{-1}$) for 21 days.

3.2.7.1. Measurements and statistical analyses

Root length was measured using digital Vernier calipers and the number of roots and new leaf pairs was recorded. Data were analysed using Genstat 18.0. One-way ANOVAs with DMRTs were used for all data. An unbalanced regression analysis was used to determine if treatment and the type of rooting observed had an effect on the number of new leaf pairs that emerged.

3.2.7.2. Acclimatization

All *in vitro*-generated plantlets were potted in plastic pots (diameter 75 mm, depth 45 mm) containing a 3:2:1 mixture of soil: sand: perlite. Soil and sand were autoclaved prior to addition of sterile perlite. Plantlets were placed in a mist-house for 1 week where they were watered every 6 min for 10–12 h (during the day) and once for 5 min at midnight. Thereafter, they were

moved to a greenhouse with watering once a day. Plantlets that survived for 10 days under greenhouse conditions were considered to be acclimatized.

3.2.8. *Ex vivo* cuttings

Shoot pieces (4–7 cm) were cut from mature greenhouse-maintained plants. Shoots with 1-2 nodes were used. Shoots with small leaf pairs that had newly emerged were used. Flowers were also removed. The cuttings (cut-ends) were pre-treated with different auxin solutions for 10 min prior to potting. Treatments applied were 2.5, 5.0 and 10.0 µM IBA or IAA. The positive control was a pulse in tap water for 10 min while the negative control was not pulsed in any solution.

Cuttings (10 replicates per treatment) were planted in plastic pots (diameter 75 mm, depth 45 mm) containing a 3:2:1 mix of soil: sand: perlite and watered with the respective auxin solutions. Both controls were watered with tap water. All cuttings were placed in a greenhouse partially covered with a mesh shade cloth where they were watered 2–3 times a week (when drying was observed) with the treatment solutions. Temperature ranged from 7–40°C. Growth was allowed for 21 days.

3.2.8.1. Measurements and statistical analyses

Root length (mm) was measured using digital Vernier calipers, and the number of roots and new leaf pairs were recorded. Data were analysed using Genstat 18.0. One-way ANOVAs were performed with DMRTs. All graphs were generated used GraphPad Prism 8.0.

3.3. RESULTS AND DISCUSSION

3.3.1. Development of sterilization protocol

Preliminary trials to establish a suitable sterilization protocol for *S. tortuosum* explants showed high incidences of contamination for leaf explants sterilized with 1.5% and 2.5% NaOCl (5, 10 min), as well as 0.1% and 0.2% $HgCl_2$ (5, 10, 20 min) (results not shown). The lowest contamination was observed with 10% and 20% H_2O_2 for 20 min. However, this protocol was established in Autumn. As the year progressed, fungal contamination became more frequent, thus it was decided to alter the initial protocol by adding 1 g/L Benomyl, this was tested for 20 and 40 min. Based on these observations, 40 min was selected for further use (results not shown).

With an additional time lapse (end of Winter – beginning of Spring), bacterial contamination was more prevalent. Therefore, streptomycin sulphate (0.01% and 0.05%) was tested, where the latter concentration more effectively eradicated contamination (results not shown). The final protocol was established as 20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min) + 0.05% streptomycin sulphate (20 min). However, many of the aforementioned treatments were repeated at the same time of year (Spring) to validate the established protocol.

3.3.2. Sterilization experiment

Contamination observed after various sterilization treatments are shown in **Table 3.1**. It can be seen that 2.5% NaOCl for 10 min resulted in the lowest incidence of contamination, followed by 20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min) + 0.05% streptomycin sulphate (10 ± 6.9% and 20 ± 9.2% respectively), although these two treatments were not significantly different from each other. It should be noted that 100% of contamination observed across all treatments was due to bacteria (results not shown). The addition of 0.05% streptomycin sulphate reduced contamination significantly compared to 20% H_2O_2 (20 min) alone (20 ± 9.2% compared to 95 ± 5.0%), and 20% H_2O_2 + Benomyl for 20 and 40 min (85 ± 8.2% and 55 ± 11.4% respectively) **(Table 3.1)**.

For both concentrations of $HgCl_2$, contamination increased with increasing immersion time **(Table 3.1)**. This was also apparent for 20% H_2O_2. However, a 10% solution of H_2O_2 for the shorter time period (10 min) resulted in a higher percentage of contamination than for 20 min.

A similar result was observed for NaOCl. At 1.5%, the longer time period was less effective in sterilizing explants (30 ± 10.5%), but at 2.5%, the converse was true (10 ± 6.9%) **(Table 3.1)**.

Oddly, based on preliminary findings NaOCl was by far the most ineffective in reducing bacterial contamination, whilst 20% H_2O_2 was the most effective. However, in this experiment, the opposite was evident, with the highest percentage contamination reached by 20% H_2O_2 for 20 min (95 ± 5.0%) and the lowest obtained from NaOCl for 10 min (10 ± 6.9%) **(Table 3.1)**.

Table 3.1: Response of leaf explants to different sterilization treatments in Spring

Treatment	**Percentage contaminated (%) ± SE**
1.5% NaOCl (5 min)	30 ± 10.5^{fghi}
1.5% NaOCl (10 min)	35 ± 10.9^{fghi}
2.5% NaOCl (5 min)	50 ± 11.5^{efgh}
2.5% NaOCl (10 min)	10 ± 6.9^{i}
0.1% $HgCl_2$ (10 min)	55 ± 11.4^{defg}
0.1% $HgCl_2$ (20 min)	60 ± 11.2^{bdef}
0.2% $HgCl_2$ (10 min)	45 ± 11.4^{efgh}
0.2% $HgCl_2$ (20 min)	90 ± 6.9^{abc}
10% H_2O_2 (10 min)	90 ± 6.9^{ab}
10% H_2O_2 (20 min)	75 ± 9.9^{abcde}
20% H_2O_2 (10 min)	40 ± 11.2^{fghi}
20% H_2O_2 (20 min)	95 ± 5.0^{a}
20% H_2O_2 (20 min) + 1 g/L Benomyl (20 min)	85 ± 8.2^{abcd}
20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min)	55 ± 11.4^{efg}
20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min) + 0.01% streptomycin sulphate (20 min)	25 ± 9.9^{ghi}
20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min) + 0.05% streptomycin sulphate (20 min)	20 ± 9.2^{hi}

Different letters indicate significant differences as per DMRT ($P \leq 0.05$).

It has been established that contamination is massively affected by seasonal and weather conditions **(HOHTOLA, 1988; RAY and ALI, 2017)**. This is certainly evident in this sterilization investigation. Owing to preliminary findings, it was observed that contamination incidence varied at different times of the year, as did type of contamination. At the time of the final investigation, NaOCl appeared to be the best treatment for reducing contamination **(Table 3.1)**. Thus, implying that the specific types of bacteria and fungi present on/in plant material were successfully eradicated by this solution, whereas H_2O_2 was almost completely unsuccessful in this task **(Table 3.1)**.

The addition of an antibiotic to the sterilization protocol reduced contamination significantly at both H_2O_2 concentrations tested **(Table 3.1)**. This supports the notion that bacterial contamination was the primary issue during this season (Spring). The sum total of these findings indicated that contamination increased significantly at this time of year, despite the dry conditions experienced in Pietermaritzburg. This information contradicts various publications showing that contamination increases during rainy seasons **(DEVI and SHARMA, 2009; MEHTA *et al.*, 2011; RAY and ALI, 2017)**.

MEHTA *et al.* (2011) determined that endophytic contamination increased during the rainy season, and recently **MANGANYI *et al.* (2018)** found endophytic fungi to be present in *S. tortuosum*. Given the current findings, two hypotheses were formulated: 1) although endophytic bacteria have not been isolated from *S. tortuosum*, this does not prove that there are none. Endophytic bacteria may only colonize plant tissues at certain times of the year, e.g. during dry periods. 2) *Penicillium*, *Aspergillus* and *Fusarium* species were among the 60 species isolated from the plant, of which some fungal extracts showed antibacterial activity **(MANGANYI *et al.*, 2018, 2019)**. It is possible that during the dry season, the amount of endophytic fungi decreased, thereby reducing the production and effects of antibacterials produced by them **(BRADER *et al.*, 2014; PATEL *et al.*, 2018)**, allowing bacterial colonization. Either of these hypotheses would account for the high incidence of bacterial contamination and the radical reduction observed with the addition of an antibiotic **(Table 3.1)**.

Bacterial contamination was best removed by $HgCl_2$, followed by NaOCl and H_2O_2 **(TYAGI *et al.*, 2011)**. Even though $HgCl_2$ was not the best sterilant of the three used, it was a better sterilant than H_2O_2 (alone) **(Table 3.1)**. Moderate to high percentages of contamination reached with $HgCl_2$ could be due to the concentration used – 3% solutions were used in the study by **TYAGI *et al.* (2011)**. This could indicate that a higher concentration of $HgCl_2$ may have been

more effective. **TYAGI *et al.* (2011)** also showed that NaOCl effectively eradicated a number of bacteria (e.g. *Micrococcus* sp, *Staphylococcus aureus*, *Escherichia coli*), which supports the antibacterial effects observed **(Table 3.1).**

Conversely, a 3% H_2O_2 solution was found to be effective against these bacteria **(TYAGI *et al.*, 2011)**, but the higher concentrations utilized in this study were not. This could mean that additional bacteria were found on/in *S. tortuosum*, which could not be eliminated with either concentration or time period of immersion in H_2O_2 **(Table 3.1)**.

Despite the fact that NaOCl proved to be the most effective sterilant for the decontamination in early Spring, it should be noted that the step-wise decontamination protocol with the highest concentrations and longest time periods (20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min) + 0.05% streptomycin sulphate (20 min)) was consistently more suitable for sterilization of explants for a large part of the year (5–6 months). Other *in vitro* researchers have employed step-wise sterilization protocols with much success **(CHEN *et al.*, 2017; DAUD *et al.*, 2012)**. Therefore, this should be regarded as a more reliable protocol than 2.5% NaOCl for 10 min. However, the NaOCl treatment may be suitable at certain times of the year and it is worth testing before employing the step-wise protocol described.

3.3.3. Regenerative capabilities of explants

Woody stem explants were able to form callus and roots proliferated after 2–4 months on MS medium supplemented with 2.5 µM IBA (results not shown). However, the use of stem explants was not feasible for these investigations due to lack of plant material and the lengthy period required for root induction.

Leaf explants were regenerative in some cases. With explants inoculated on 2.5 µM TDZ + 2.5 µM BA, leaves proliferated after 3-5 weeks, but without shoot elongation and root proliferation. As a result, 4–6 weeks later, explants did not survive (results not shown).

Shoot (nodal) explants were the most regenerative in the shortest amount of time. Roots proliferated 10–14 days after culture initiation on MS medium supplemented with 2.5 µM IBA (results not shown).

3.3.4. Selection of PGRs for *in vitro* multiplication

Explants placed on 2.5 µM mT exhibited shoot proliferation with callus proliferation and growth and thickening of tissue **(Figure 3.3a)**. Explants inoculated on MS medium supplemented with 2.5 µM mT + 2.5 µM IBA showed improved shoot proliferation. However, some leaves showed signs of senescence within the first month **(Figure 3.3b)**. Callus growth was similar to 2.5 µM mT alone **(Figure 3.3a and b)**.

After 3 months *in vitro* on 2.5 µM mT + 2.5 µM IBA, excessive callus growth was evident, along with extensive shoot proliferation. Shoot proliferation and growth were almost entirely compact, with new leaves emanating from different areas of callus. The majority of shoots were hyperhydric with internodes small to absent **(Figure 3.4a)**. Thus, explants could not be taken from this treatment for the rooting investigation.

Healthy *in vitro* plants were obtained from MS media supplemented 2.5 µM IBA. Shoots proliferated well with visible internodes which could easily be excised for use in the subsequent study. Roots also proliferated within the first month of culture initiation **(Figure 3.3c, 3.4c and d)**. Overall plant growth with 2.5 µM IBA was much healthier and larger than the treatments containing mT. Root proliferation was observed by an extremely small proportion of cultures from 2.5 µM mT + 2.5 µM IBA **(Figure 3.4b)**. However, after 3 months *in vitro*, root growth was still not comparable to root generation from 2.5 µM IBA only **(Figure 3.4)**.

Figure 3.3: *Sceletium tortuosum* shoot nodal explants placed on 2.5 µM mT **(a)**, 2.5 µM mT + 2.5 µM IBA **(b)**, and 2.5 µM IBA **(c)** after 1 month *in vitro*. Scale bar represents 1.0 cm.

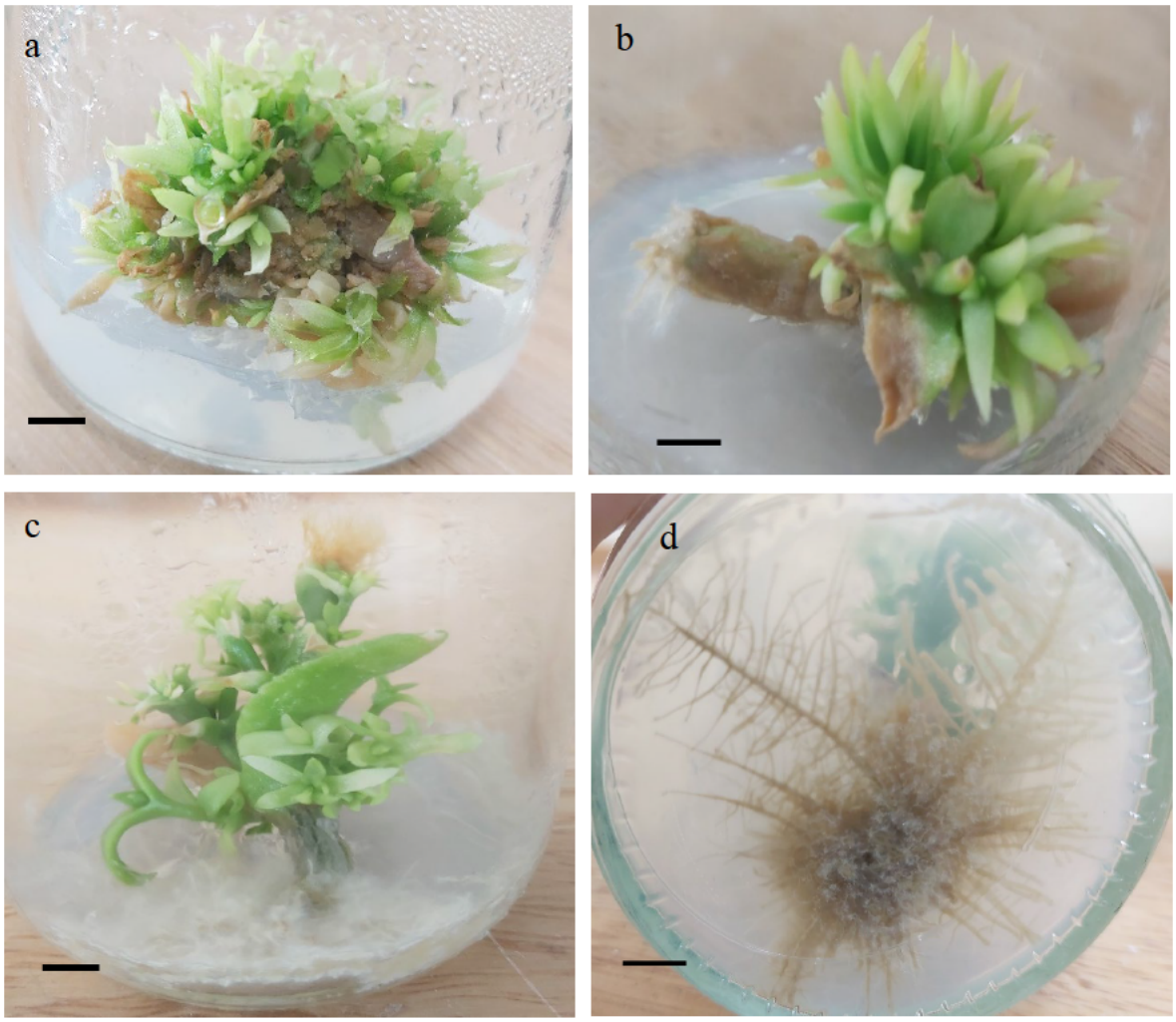


Figure 3.4: Shoot nodal explants of *Sceletium tortuosum* placed on 2.5 µM mT + 2.5 µM IBA **(a)** and **(b)**, and 2.5 µM IBA **(c)** and **(d)** after 3 months *in vitro*. Scale bar represents 1.0 cm.

Meta-topolin (mT) is known for favouring the production of multiple shoots and this was certainly evident here **(WERBROUCK *et al.*, 1996)**. This hormone is often used to substitute for other cytokinins (e.g. BA, zeatin), and in some cases, produces better results **(KUBALAKOVA and STRNAD, 1992)**. Although the results have no discord with the multiple investigations proving that mT improves shoot proliferation **(AREMU *et al.*, 2012; KAMINEK *et al.*, 1987; KUBALAKOVA and STRNAD, 1992; ROSALES *et al.*, 2008)**, there is some disagreement with studies documenting overall healthy plantlets together with increased shoot multiplication and spontaneous rooting **(AMOO *et al.*, 2011; BAIRU *et al.*, 2007; WOJTANIA, 2010)**.

The lack of normal shoot proliferation and healthy plantlets due to mT treatments may be caused by an incorrect ratio of auxin: cytokinin **(SKOOG and MILLER, 1957)**. **SHAHZAD**

***et al.* (2011)** noted that a combination of IBA and BA decreased shoot number, length and the percentage of plantlets producing shoots for *Veronica anagallis-aquatica*. These researchers postulated that the negative reaction was caused by an interaction between endogenous and exogenous PGR levels.

However, given that 2.5 µM mT used alone also resulted in callus formation that thickened over time, it is possible that the concentration of this PGR was inappropriate. **GEORGE *et al.* (2008)** stated that in cultures with high cytokinin levels, multiple shoots proliferate that do not elongate. They also stated that such a condition could produce hyperhydric shoots. Furthermore, it has been documented that high cytokinin concentrations (2.07 – 41.5 µM) suppress the root proliferation-effect of auxins **(BEN-JAACOV *et al.*, 1991; SCHRAUDOLF and REINERT, 1959)**.

Nevertheless, the most significant discovery of these investigations was that the introduction of a cytokinin is unnecessary for shoot proliferation and elongation **(Figure 3.3 and 3.4)**. This finding contrasts with numerous publications showing that shoot proliferation is only possible with cytokinins or a combination of cytokinin and auxin **(BABAEI *et al.*, 2014; GÜREL and GÜLŞEN, 1998b; LING *et al.*, 2013)**.

3.3.5. *In vitro* flowering

Shoot explants inoculated on 2.5 µM IBA or 2.5 µM mT + 2.5 µM IBA flowered in culture. Both treatments produced flowers after 1 or 2 months **(Figure 3.5)**. **Figure 3.5c** shows flowering which occurred 1–2 months before greenhouse mother plants (GMPs) began flowering. Flowers seen in **Figure 3.5 a and b** developed while GMPs were flowering. The flowers produced from MS medium supplemented with 2.5 µM mT were more short-lived and overall plant growth was much less healthy than media containing 2.5 µM IBA only **(Figure 3.5)**.

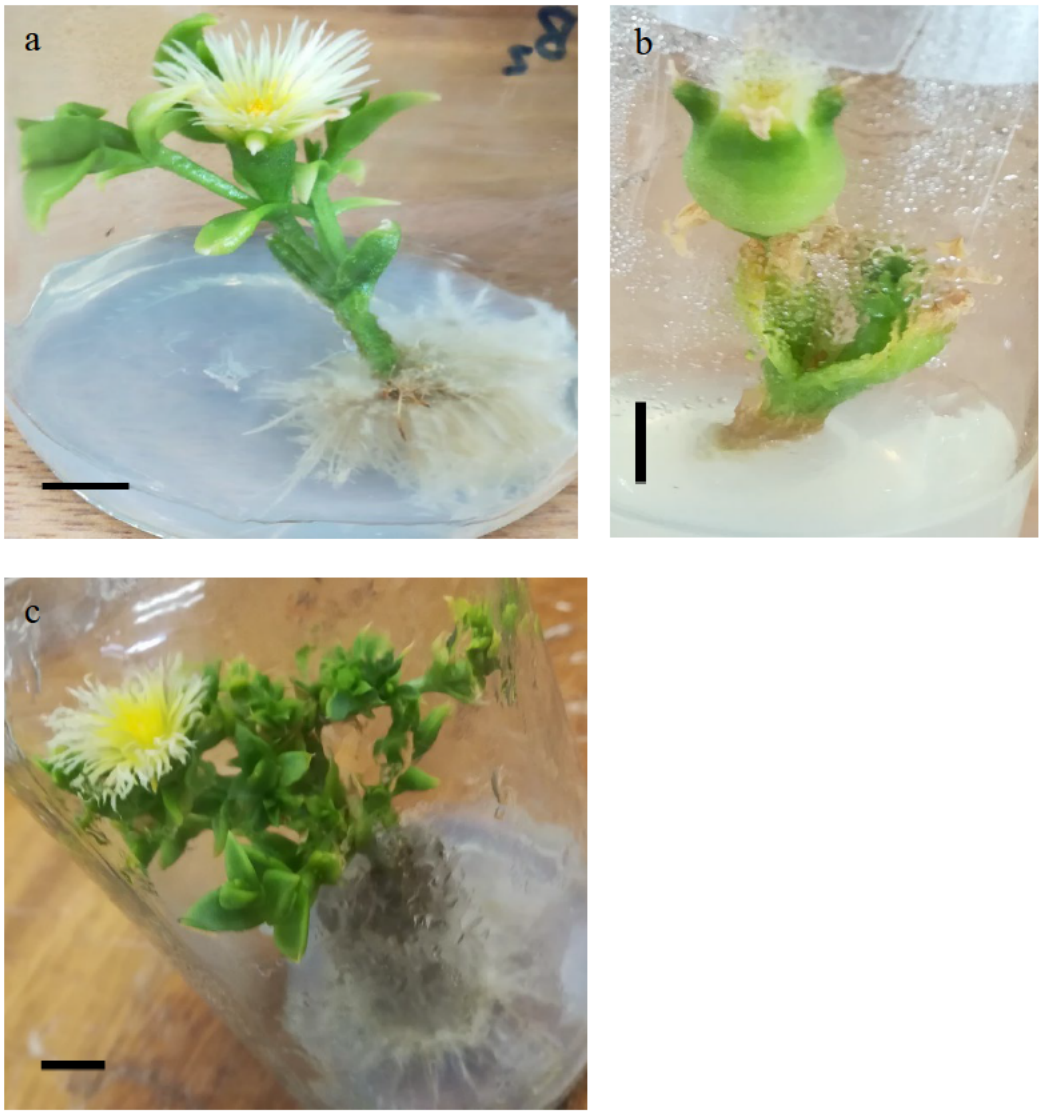


Figure 3.5: *Sceletium tortuosum* flowering plantlets obtained from **(a)** 2.5 µM IBA after 1 month *in vitro*, **(b)** 2.5 µM mT + 2.5 µM IBA after 1 month *in vitro*, **(c)** and 2.5 µM IBA after 2 months *in vitro*. Scale bar represents 1.0 cm.

The occurrence of flowering *in vitro* particularly on auxin-containing medium raises various distinct possibilities. There could be high levels of endogenous cytokinins present within shoot explants (when excised from GMPs), or cytokinins were produced in roots **(GOH and YANG, 1978; MERCIER *et al.*, 2003; VAN TRAN THANH, 1973)**. Due to the vegetative propagation of these plantlets, shoot explants would have retained the genetics of mother plants and were induced to flower at a set time/season of the year, i.e. when flowering occurred with GMPs **(BERNIER *et al.*, 1993; SCIENCEDAILY, 2009)**.

Alternatively, shoot explants that flowered earlier than GMPs may have been genetically programmed to do so once provided nutrients or when cytokinin levels were suitable **(BERNIER *et al.*, 1993; CHAMBERS *et al.*, 1991; DIELEN *et al.*, 2001)**. Temperature, photoperiod, irradiance and water availability also provide cues for the induction of flowering in nature. These conditions may have been ideal *in vitro* thus stimulating flower formation and development **(BERNIER *et al.*, 1993)**.

The hypothesis that cytokinin levels were appropriate to induce flower production would explain why some plantlets placed on mT-containing medium flowered although they had not rooted **(Figure 3.5b)**. Support for this finding comes from **TAYLOR *et al.* (2005)**, who reported that root proliferation was unnecessary for flower production *in vitro*. However, these, and other authors also reported that auxin alone was inhibitory to flower formation, which was induced by the addition of cytokinins to TC media **(GOH and YANG, 1978; WANG *et al.*, 1997)**. Contrary to these reports, the current findings proved that media containing auxin alone was conducive to flower development *in vitro*, and that the addition of a cytokinin was detrimental to plant health and growth. These results **(Figure 3.3c, 3.4c and 3.5a and c)** suggest that *S. tortuosum* has high endogenous cytokinin levels allowing for not only shoot proliferation, but shoot elongation and flower formation as well. However, this needs investigation.

3.3.6. *In vitro* rooting

There were no significant differences between treatments with regards to mean rooting percentage, with 25–55% of shoot explants producing functional roots after 21 days. Interestingly, the control treatment achieved the same rooting percentage as two IAA treatments (40 ± 11.2%) **(Figure 3.6a)**. The mean number of roots (MNR) produced was heavily influenced by hormone treatments, with the lowest and highest MNR produced from the control (0.95 ± 0.26) and 10.0 µM IBA (3.80 ± 0.83) respectively **(Figure 3.6b)**. Significant differences were observed for mean root length (MRL) and mean number of new leaf pairs (MNLP) as well **(Figure 3.6a and b)**. Surprisingly, MS medium supplemented with 5.0 µM IAA produced the longest MRL (10.04 ± 3.13 mm), yet 10.0 µM IAA yielding the shortest MRL (1.97 ± 1.16 mm) of all treatments, including the control (6.56 ± 1.68 mm) **(Figure 3.6a)**.

Figure 3.6 indicates that overall, MS medium supplemented with 10.0 µM IBA was the optimal treatment, with 3 of 4 measured parameters reaching the highest of all seven treatments. It yielded the highest percentage of rooted plantlets (55 ± 11.4%), as well as the highest MNR (3.80 ± 0.83) and MNLP (4.65 ± 0.67). For MRL, 10.0 µM IBA was only surpassed by 5.0 µM IAA (7.98 ± 2.32 mm and 10.04 ± 3.13 mm respectively).

The treatment which had the most negative impact on shoot explants is more difficult to discern as there is no single treatment in which all growth parameters were low. In terms of rooting percentage, IBA (5.0 µM) resulted in only 25% of shoot explants growing functional roots, whilst the control treatment produced the lowest MNR (0.95 ± 0.26), and 10.0 µM IAA produced the shortest MRL (1.97 ± 1.16 mm) and lowest MNLP (1.85 ± 0.41) **(Figure 3.6)**.

However, when considering the hormones individually, it can be seen that 10.0 µM was the optimal concentration for IBA, resulting in the highest values for all parameters, exceeding the control parameters as well **(Figure 3.6)**. For IAA, 5.0 µM may be seen as the optimal concentration, with 3 of 4 of the highest parameters compared to the remaining concentrations, with only a low MNR (1.55 ± 0.50). This was somewhat compensated for by MRL (10.04 ± 3.13 mm). Also, 5.0 µM IAA exceeded all control parameters, with the exception of mean number of new leaf pairs (3.70 ± 0.55 compared to 4.00 ± 0.79 (control)) **(Figure 3.6)**.

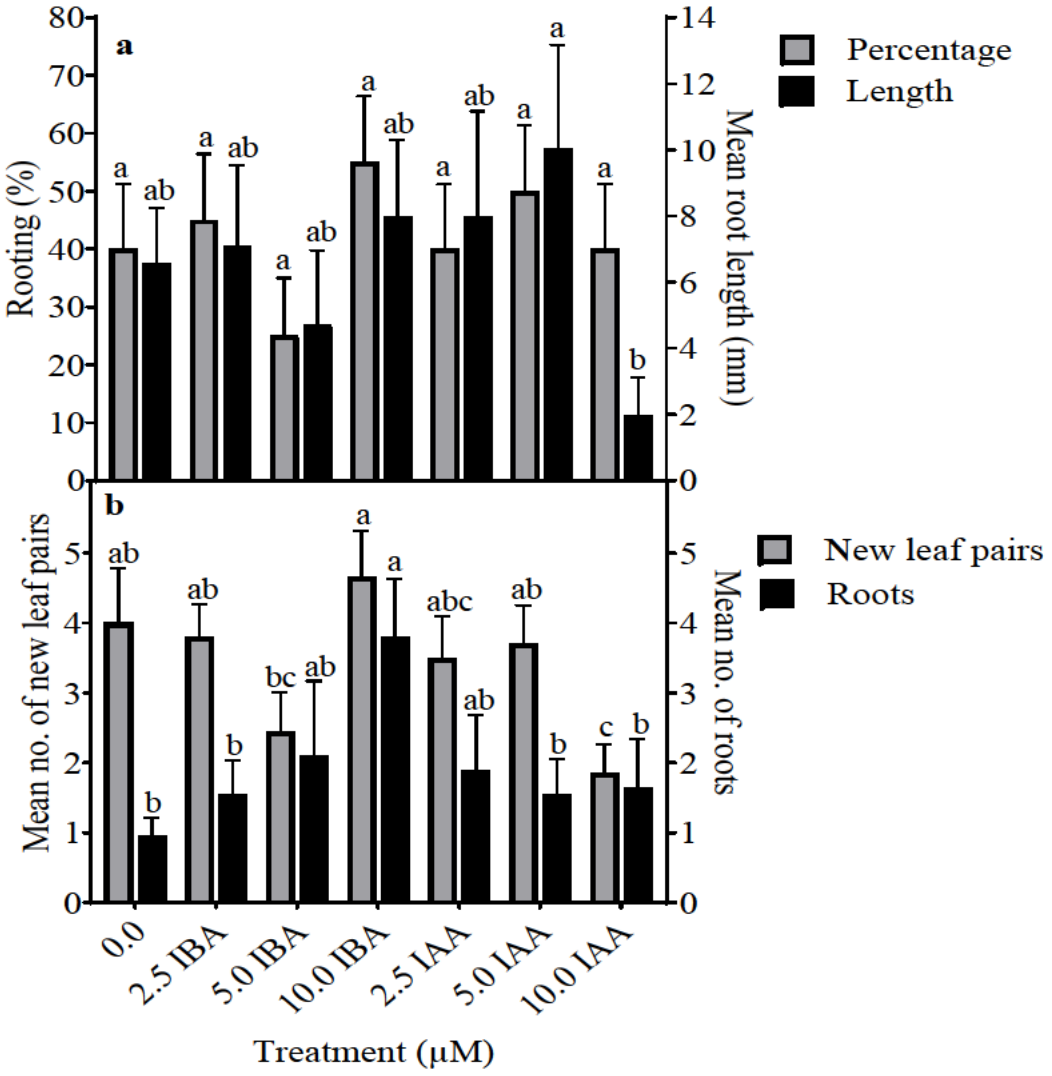


Figure 3.6: Percentage of *Sceletium tortuosum* plantlets with functional roots and their mean root length **(a)** and mean number of roots and new leaf pairs **(b)** from different treatments after 21 days *in vitro*. Error bars show SE of the mean. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$).

It was determined that MNLP was affected by treatment ($P = 0.003$), and by functionality of roots ($P < 0.001$) **(Table 3.2)**. However, the interaction between treatment and functional roots did not have a significant effect on the number of new leaf pairs produced ($P = 0.608$). Simply put, **Table 3.2** shows that plantlets with functional roots have more new leaf pairs than those with non-functional roots.

Table 3.2: Effect of root type on the mean number of new leaf pairs produced

Type of roots	Mean no. of new leaf pairs ± SE
Functional	5.17 ± 0.42^{a}
Non-functional	2.80 ± 0.20^{b}

Letters superscripts indicate significant differences at the $P \leq 0.05$ significance level as per accumulated ANOVA.

Although there were no treatments with cytokinins used in the rooting study, all auxin treatments successfully generated plantlets with a significant number of new leaf pairs in just 21 days **(Figure 3.6b)**. This phenomenon of shoot proliferation as a result of rooting hormones is not well documented. Studies involving shoot proliferation in PTC are usually conducted with cytokinins alone, or in combination with an auxin. However, a study by **GÜREL and GÜLŞEN (1998b)** documented that a low concentration of IBA (0.5 µM) improved shoot growth in two cultivars of *Amygdalus communis*. Furthermore, **DAHAB *et al.* (2005)** stated that the number of leaves produced by *Ruscus hypoglossum* were improved by half strength MS medium supplemented with 3.0 mg/L IBA (≈ 15.0 µM IBA). These reports support the use of auxins for enhancing shoot proliferation and growth. Additionally, **DAHAB *et al.* (2005)** and **GÜREL and GÜLŞEN (1998b)** showed that both these parameters were also improved on hormone-free MS media, which supports the high MNLP observed in the control treatment **(Figure 3.6b)**.

The ability of auxins to induce rooting *in vitro* **(AGARWAL and KAMAL, 2004; ISLAM *et al.*, 2005; JAHAN *et al.*, 2009; YILDIRIM and TURKER, 2014)** was supported by findings of this study. Seeing that IBA and IAA are produced naturally in plants, these hormones did not appear to present any problems for uptake and rooting *in vitro*. However, the results indicated that IBA was the more appropriate auxin for rooting explants of *S. tortuosum* **(Figure 3.6)**. This is consistent with a number of investigations concluding that IBA has more positive effects compared to IAA **(ISLAM *et al.*, 2005; ŠTEFANČIČ *et al.*, 2005)**.

Recently, **FATTORINI *et al.* (2017)** discovered that IBA controls adventitious rooting in *Arabidopsis thaliana*. This may well be the case in *S. tortuosum* because the best response was observed with an IBA treatment **(Figure 3.6)**. Further demonstration of the enhanced effect of IBA was found in a publication by **FATTORINI *et al.* (2017)**. Their results showed that 10.0

µM IBA had a stronger effect on root induction than 10.0 µM IAA. This was also consistent with the current findings **(Figure 3.6)**.

Additional support for the effects of IBA on *in vitro* rooting was found in the **BABAEI *et al.* (2014)** publication. These authors documented an increase in lateral roots produced from adventitious roots with the use of IBA in *Curculigo latifolia* cultures. This was clearly observed with all IBA concentrations tested **(Figure 3.7)**.

Apart from these studies documenting overall stronger effects of IBA, there may be another reason for the pronounced effects of this particular concentration (10.0 µM). IBA (2.5 µM) was exogenously supplied *in vitro*, and plantlets grew on this medium for 1–6 months before excision of explants for use in the rooting study. This could mean that multiplication plantlets were affected by a "carry-over" effect of IBA. Given the long period of growth on this medium, it is possible that plantlets became "accustomed" to the uptake of this concentration of IBA, thus allowing them to tolerate and make full use of an even higher concentration afterwards (10.0 µM).

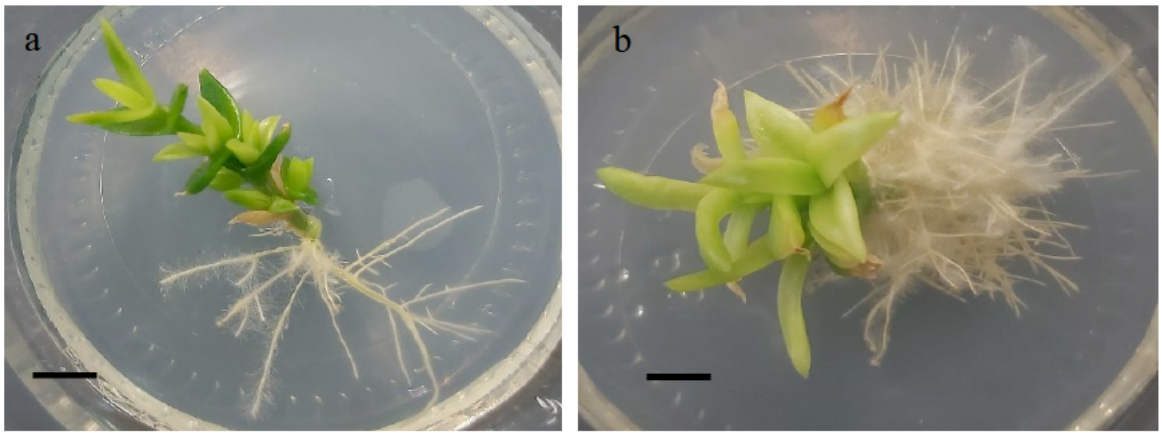
In the multiplication cultures, plantlets would have had to convert the IBA supplied to IAA **(NORMANLY *et al.*, 2010; OVERVOORDE *et al.*, 2010)**. However, these plantlets had never before experienced an exogenous supply of IAA. Therefore, shoot explants may have had to acclimate to the exogenous supply of IAA. This could have induced signalling for storage of some IAA in addition to signalling for root proliferation **(KORASICK *et al.*, 2013; LJUNG *et al.*, 2005)**. It is possible that the combination of both types of signalling resulted in slower mobilization of IAA for root proliferation and elongation **(Figure 3.7g)**.

With the exception of 10.0 µM IBA, rooting capabilities of all auxin treatments were quite variable, with different concentration eliciting different responses **(Figure 3.6)**. This may best be explained by the endogenous auxin content of the explants at each stage of the TC process. Naturally, IBA and IAA could be present in various amounts in GMPs. This would have been influenced by their age, the time of day, and the seasonal and weather conditions experienced when explants were excised **(CASSELLS, 1979; GEORGE *et al.*, 2008; PAVLOVÁ and KREKULE, 1984)**. Multiplication cultures were initiated at different times of the year due to lack of plant material, thus the levels of endogenous auxins likely varied among these cultures. Also, multiplication plantlets were maintained on 2.5 µM IBA for different time periods, further altering endogenous auxin levels. The combination of these variations may have affected the response of explants to auxin treatments. This argument may account for the low

growth parameters found in different treatments, as well as the variations in rooting parameters of the IAA treatments **(Figure 3.6)**. It is possible that conditioning with 2.5 µM IBA allowed a higher concentration of IBA (10.0 µM) to overcome the variations among explants and produce the most positive result **(Figure 3.6)**.

Alternatively, 10.0 µM may have simply been the optimum concentration of IBA allowing for rapid uptake and conversion to IAA. Thus, enhancing rooting parameters. While 5.0 µM was optimal for IAA, with a lower concentration (2.5 µM) being insufficient for good rooting responses, and a higher concentration (10.0 µM) too strong for explants, resulting in a disruption in HSPs **(LI *et al.*, 2009; OVERVOORDE *et al.*, 2010; SKOOG and MILLER, 1957)**. This proposed disruption or imbalance may not have been as severe as observed with 2.5 µM mT + 2.5 µM IBA **(Figure 3.4a and b)**, but would require some time for regulation of hormones and HSPs to occur. This could also explain the slow root growth observed in 10.0 µM IAA **(Figure 3.6 and 3.7g)**.

Surprisingly, the control treatment did not yield the most negative results in terms of rooting **(Figure 3.6 and 3.7a)**. However, this result is supported by reports from various authors indicating that rooting occurs well on hormone-free MS medium **(BABAEI *et al.*, 2014; ISLAM *et al.*, 2005; SHAHZAD *et al.*, 2011)**. In fact, the control results closely correlate with findings from **AGARWAL and KAMAL (2004)**. These researchers found that root and shoot proliferation were successfully induced on hormone-free MS medium.



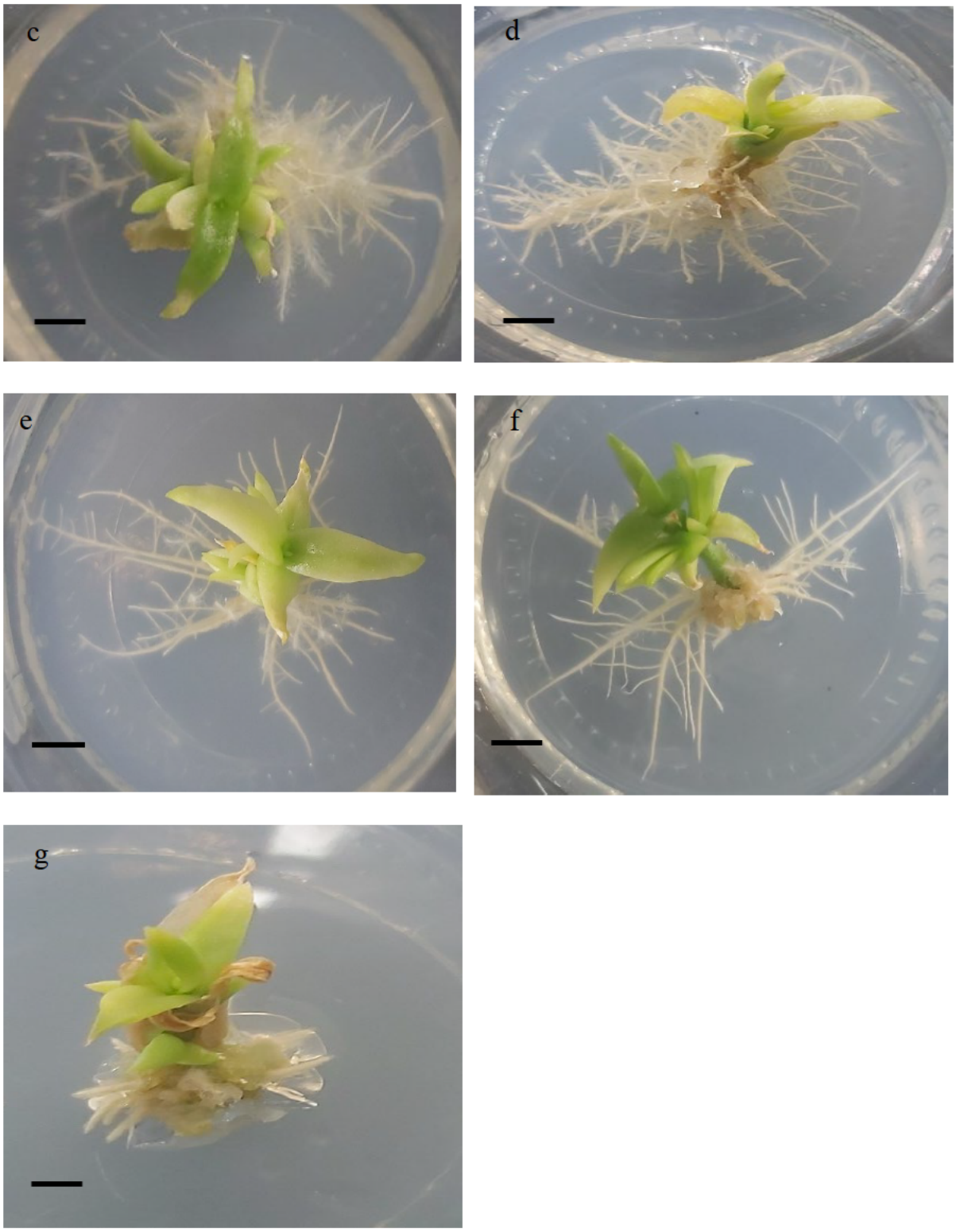


Figure 3.7: *Sceletium tortuosum* plantlets obtained from different treatments after 21 days *in vitro*. Treatments correspond to: **(a)** Control (MS), **(b)** 2.5 µM IBA, **(c)** 5.0 µM IBA, **(d)** 10.0 µM IBA, **(e)** 2.5 µM IAA, **(f)** 5 µM IAA and **(g)** 10.0 µM IAA. Scale bar represents 1.0 cm.

3.3.6.1. Acclimatization

Acclimatization was successful for 45-90% of *in vitro*-generated plantlets. The highest percentage of plantlets acclimatized came from the control treatment (90 ± 6.9%), followed by 2.5 and 5.0 µM IAA (75 ± 9.9 %). The best overall treatment for improving growth parameters *in vitro* (10.0 µM IBA) resulted in 50% of plantlets successfully acclimatized after 10 days **(Figure 3.8)**.

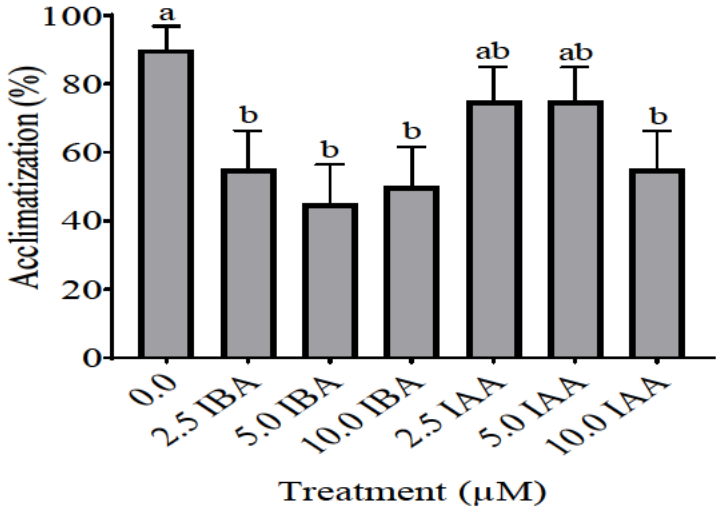


Figure 3.8: Acclimatization percentages of *in vitro*-derived plantlets after transfer to greenhouse conditions. Error bars show SE of the mean. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$).

Figure 3.9: *In vitro*-derived plantlets acclimatized to greenhouse conditions. Scale bar represents 1.0 cm.

All but one treatment (10.0 µM IBA) had a greater percentage acclimatized than the percentage of plantlets with functional roots **(Figure 3.6 and 3.8)**. The major discovery here was that plantlets devoid of active roots were able to acclimatize to mist-house and greenhouse conditions and grow well **(Figure 3.9)** This indicates the ability of plantlets even with non-functional roots (or no roots at all) to acclimatize successfully, suggesting that root proliferation occurred *ex vitro* without the supply of nutrients and hormones. This is not a surprising development as some researchers induce rooting *ex vitro* instead of *in vitro* **(BENMAHIOUL *et al.*, 2012; MARTIN, 2003)**.

Surprisingly, the control treatment gave the highest percentage of successful acclimatization **(Figure 3.8)**. This is an indication that at least 50% of surviving plantlets had non-functional roots when transplanted to *ex vitro* conditions **(Figure 3.6 and 3.8)**. This provides support for the notion that root proliferation continued to occur *ex vitro*. This is consistent with *in vitro* findings, i.e. despite the low values obtained for rooting parameters **(Figure 3.6)**, adventitious root proliferation was achieved in shoot explants without an exogenous supply of auxin **(Figure 3.7a)**. Thus, plantlets generated from the control treatment may have had more of an inherent ability to continue root proliferation *ex vitro*.

Plantlet losses observed after the acclimatization period were likely due to the risks associated with the process itself **(CHANDRA *et al.*, 2010; HUSSAIN *et al.*, 2012; ILIEV *et al.*, 2010)**. Conditions in mist-houses and greenhouses are totally different from *in vitro* conditions. Young plantlets were acclimated to *in vitro* conditions with their anatomical parameters adapted accordingly **(ILIEV *et al.*, 2010)**. The high humidity and low light intensity may have altered the amount or composition of leaf epicuticular wax and the structure and functionality of stomata **(GEORGE *et al.*, 2008; ILIEV *et al.*, 2010)**. These structural abnormalities may have resulted in rapid water loss when plantlets were exposed to lower humidity and higher light intensity *ex vitro* **(GEORGE *et al.*, 2008)**. Additionally, plantlets were not reliant on their own photosynthesis *in vitro*, as sucrose was supplied in the TC media. Plantlets would have had to adapt to be completely self-reliant *ex vitro*. Any inability to do this would mean they were unable to meet their own photosynthetic requirements resulting in plant mortality **(GEORGE *et al.*, 2008)**.

Furthermore, bombardment by bacteria and fungi likely posed a problem for these young plantlets **(CHANDRA *et al.*, 2010; LEELAVATHY and SANKAR, 2016)**. The soil substrate was not fumigated would have allowed various soil microbes to rapidly colonise the soil. These

microbes may have harmed young plant tissues and organs caused the variation among acclimatization percentages **(SINGH, 2018)**.

3.3.7. *Ex vivo* cuttings

All treatments and controls resulted in rooted cuttings within 21 days. All but one treatment (10.0 µM IAA) gave 100% rooting. IBA (5.0 µM) proved to be the best treatment of all eight treatments tested. Contrary to the *in vitro* study, 5.0 µM IBA proved to the best treatment for rooting *ex vivo* cuttings. This treatment achieved the highest MNR and MRL (11.20 ± 1.37 and 57.18 ± 3.85 mm respectively). The mean number of new leaf pairs was higher with 2.5 µM IBA (4.20 ± 0.36) **(Figure 3.10)**. IBA (5.0 µM) also resulted in a significantly higher MNR compared to the untreated and treated controls **(Figure 3.10b)**, while its MRL was significantly different from the treated control **(Figure 3.10a)**.

For IAA alone, there was no single concentration which was better than the rest. At 2.5 µM IAA, the highest MRL was achieved (42.01 ± 5.24 mm), at 5.0 µM, MNR was the highest, and 10.0 µM had the highest MNLP (3.50 ± 0.54) **(Figure 3.10)**.

The lowest rooting parameters were found in the treated control (13.18 ± 1.78 and 5.20 ± 0.36 mm for MRL and MNR respectively), whereas these parameters were about average in the untreated control (39.48 ± 9.69 mm and 7.30 ± 1.17 for MRL and MNR respectively) **(Figure 3.10)**.

Although there were no significant differences among MNLPs, 4 of 6 auxin treatments resulted in values higher than both control treatments. Also, 5 of 6 treatments had a higher MNLP than the untreated control. Only 5.0 µM IAA had the same value as this control (2.70 ± 0.56) **(Figure 3.10b)**.

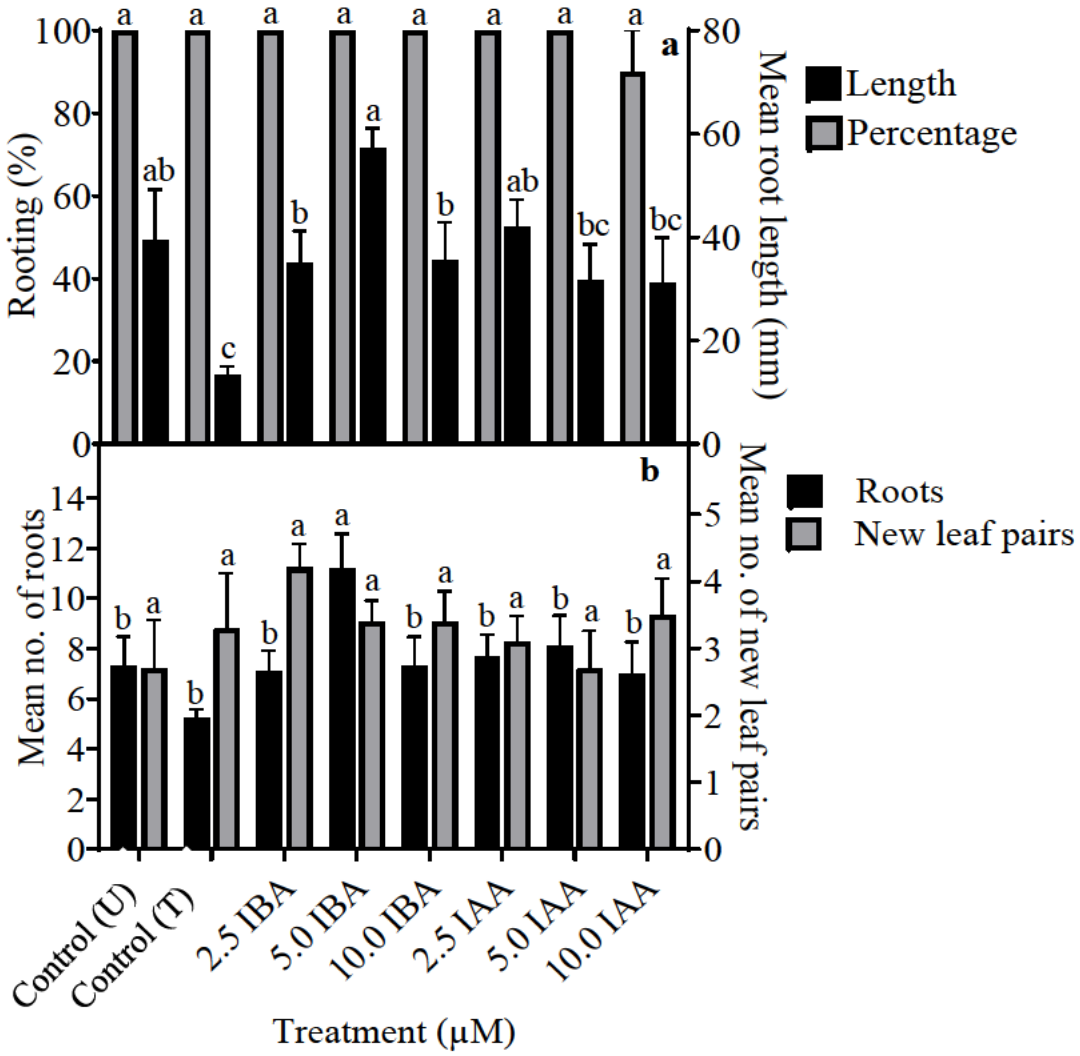


Figure 3.10: Rooting percentage and mean root length **(a)** and mean number of roots and new leaf pairs **(b)** of *Sceletium tortuosum* cuttings from different auxin and control treatments after 21 days *ex vivo*. Letters for controls are according to: U = untreated; T = treated. Error bars show SE of the mean. Bars with different letters indicate significant differences between treatments ($P \leq 0.05$).

Findings from control treatments indicated that a short pulse in water gave a negative response compared to no pulse at all **(Figure 3.10)**. A similar finding was reported for *Vitellaria paradoxa*, where cuttings that were not dipped in water had a significantly high MNR and MRL, as compared to cuttings that were dipped **(YEBOAH *et al.*, 2009)**. These researchers suggested that submerging cut-ends in water may have caused auxins and nutrients to leach out of cuttings.

The high value achieved for leaf pairs from 2.5 µM IBA and 10.0 µM IAA may be explained by the GMPs' stage in the life cycle. Plants were flowering at the time of the study. Flowers are formed at the ends of shoots or the ends of lateral shoot/nodal segments. One to four new nodal segments may emerge from a single node, particularly during flowering (results not shown). Therefore, GMPs may have had high cytokinin levels in shoots **(BERNIER *et al.*, 1990; CORBESIER *et al.*, 2003)**, and signalling for further shoot proliferation had already commenced. So, even after cuttings were taken, shoot proliferation had to be completed before signalling for increased root proliferation and elongation could occurr. This hypothesis would explain the high MNLPs together with the low MNRs and MRLs for 2.5 µM IBA and 10.0 µM IAA **(Figure 3.10)**. Additionally, the disruption of auxin flow by severing shoots may have caused new leaf pairs to emerge on the previous nodal segments **(ALONI *et al.*, 2006)**.

Auxin levels and roles would have been significantly altered in GMPs due to flowering **(D'ALOIA *et al.*, 2011)**. The primary role of endogenous auxins would have been more inclined towards reproduction and less inclined towards root proliferation **(CHENG and ZHAO, 2007; FRICK and STRADER, 2017)**. The HSPs or hormone balance was likely disrupted by first severing shoots from mother plants and then removing flowers from shoots. Signalling would have had to occur to either re-direct auxins away from shoots (for flower production **(ALONI *et al.*, 2006)**) to cut-ends for root induction; or signal root proliferation with the uptake of exogenous auxins **(D'ALOIA *et al.*, 2011; OVERVOORDE *et al.*, 2010; ROBERT and FRIML, 2009)**.

Auxins were not essential to induce rooting in *S. tortuosum* cuttings as 100% rooting was observed with both control treatments **(Figure 3.10)**. This can be attributed to the state of GMPs. It has been reported that cuttings which have high auxin levels (when excised from the mother plant) produce more roots **(WEIGEL *et al.*, 1984)**. Despite the fact that root proliferation was induced across all treatments and controls, auxins were effective in improving rooting parameters **(Figure 3.10)**. This finding is supported by **HAISSIG (1974)**, who stated that auxins are able to enhance root proliferation even in cuttings that root easily.

Discrepancies between *in vitro* and *ex vivo* rooting may be explained by the specific auxins used and their concentrations, in conjunction with endogenous PGR levels and hormone signalling and transport which was ongoing when shoots were severed from GMPs **(D'ALOIA *et al.*, 2011; JARVIS, 1986; LI *et al.*, 2009; OVERVOORDE *et al.*, 2010; WEIGEL *et al.*, 1984)**. It should also be noted that GMPs were subjected to different conditions compared to

in vitro multiplication plantlets, which were especially influenced by seasonal changes and weather conditions. These plants were not acclimated to an exogenous supply of auxin. This could explain the better responses achieved by an intermediate concentration of IBA (5.0 µM), as opposed to the highest concentration **(Figure 3.10)**.

Nevertheless, IBA was the best auxin for root induction. This finding is substantiated by other researchers who also determined that IBA was more effective than other auxins for the rooting of cuttings. **TOPACOGLU *et al.* (2016)** found that IBA was more successful than IAA and NAA for rooting *Ficus benjamina* cuttings. **PALANISAMY *et al.* (1998)** determined that IBA resulted in a higher MNR for *Azadirachta indica* and *Pongamia pinnata* cuttings compared to control treatments. The efficacy of IBA was shown in *P. pinnata* by **KESARI *et al.* (2009)**. This very same effect of IBA was exhibited by the optimal concentration (5.0 µM) used in this study **(Figure 3.10)**.

Findings regarding the rooting capabilities of IBA were congruent with results from the *in vitro* study. As evidenced by these findings, IBA does indeed appear to control root proliferation **(FATTORINI *et al.*, 2017)** in *S. tortuosum* as it does in *Arabidopsis thaliana*.

3.4. CONCLUSIONS

Seasonal changes affected contamination in tissue culture. In light of preliminary investigations and variations in contamination observed throughout the year, a stepwise sterilization protocol was developed (pre-sterilization + 20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min) + 0.05% streptomycin sulphate (20 min) + post-sterilization). In Spring, it was determined that 2.5% NaOCl for 10 min gave the lowest percentage of contamination followed by the step-wise protocol. It is recommended that NaOCl be the first sterilant tested for future *in vitro* studies. In the event that this treatment is ineffective, the step-wise protocol should be employed. During this investigation, bacterial contamination was rife. Thus, endophytic bacteria within *S. tortuosum* should be investigated at different times of the year.

Shoot nodal explants proliferated healthy shoots which elongated when placed on MS medium supplemented with 2.5 µM IBA only. Treatments with 2.5 µM IBA induced flowering *in vitro*, although this auxin alone resulted in overall healthier plantlets and flowers than when it was used in combination with a cytokinin (2.5 µM mT).

Standard MS medium was able to induce root proliferation in shoot nodal explants of *S. tortuosum* during the rooting investigation. Murashige and Skoog media supplemented with IBA or IAA effectively enhanced rooting parameters at certain concentrations. A concentration of 10.0 µM IBA had the largest impact on rooting percentage and mean number of roots, while 5.0 µM IAA also effectively induced root proliferation and the highest mean root length. In addition to rooting capabilities, auxins impacted on shoot proliferation of *S. tortuosum*. The optimal treatment (10.0 µM IBA) resulted in the highest mean number of new leaf pairs after the 21-day trial, however shoot proliferation also occurred on hormone-free MS medium.

Ex vivo cuttings rooted well regardless of the treatments tested. However, 5.0 µM IBA was the best treatment in terms of mean root number and length, and 2.5 µM IBA resulted in the highest mean number of new leaf pairs.

IBA was consistently better than IAA for the improvement of growth parameters *in vitro* and *ex vivo*. These findings indicate that auxins play significant roles in root and shoot proliferation, as well as flower development in *S. tortuosum*. Although, endogenous cytokinin levels may have contributed to shoot proliferation and development. Thus, endogenous hormone levels should be investigated in greenhouse mother plants and *in vitro* plantlets at different stages of development. Due to the value of this species in pharmacology, these methods and additional *in vitro* methods should be investigated to determine their effects on mesembrine-type alkaloid content.

CHAPTER 4: GENERAL CONCLUSIONS

Sceletium tortuosum was successfully propagated by several *in vitro* and *ex vivo* methods. *In vitro* seed germination trials showed that various chemicals and treatments can be useful for improving germination percentages, although smoke-water proved to be the most effective stimulant. The optimum temperature for germination was 20°C and constant light was an appropriate condition. Pulse treatments with SW improved germination parameters with increasing pulse time. Twenty-four-hour treatments had the most positive results. The best treatments in terms of mean germination percentage and germination rate were 1:1000 SW (24 h) and 1:2000 SW (24 h). These treatments yielded 83 ± 1.9% germination and 6.35 seeds germinated/day, respectively.

The treatment which gave the highest mean germination percentage (1:1000 SW 24 h), also yielded the highest vigour index (191.69 ± 7.82). However, this treatment and 1:2000 SW (24 h) resulted in seedlings that were smaller than those obtained from shorter pulse treatments. Therefore, it is recommended that seeds are germinated on nutrient media or transferred to a nutrient medium soon after germination.

The *ex vivo* germination trial further supported 1:1000 SW (24 h) as the best pulse treatment. This treatment resulted in the highest percentage of seedling emergence (10%) and the highest seedling emergence rate (0.352 seedlings emerged/day). These parameters are significantly lower than the germination parameters achieved *in vitro.* It is postulated that dormancy may have been acquired (secondary or seed-coat dormancy), due to storage and/or environmental and experimental conditions. In order to investigate dormancy in these seeds, further scanning electron microscopy imaging is required to determine seed characteristics, along with the testing of dormancy-breaking treatments.

A step-wise sterilization protocol was developed for the initiation of plant tissue cultures (pre-sterilization + 20% H_2O_2 (20 min) + 1 g/L Benomyl (40 min) + 0.05% streptomycin sulphate (20 min) + post-sterilization). This protocol was developed and used throughout the year (± 6 months) and proved to be reliable in the Spring sterilization experiment (20 ± 9.2% contamination). During this experiment, it was discovered that 2.5% NaOCl was also an effective sterilant, yielding 10 ± 6.9% contamination. It was noticed that bacteria were the only

source of contamination, suggesting that these contaminants may be endophytic and may be more prevalent during certain seasons. These findings highlight the need for investigating endophytic bacteria in this species at different times of the year.

The step-wise protocol allowed for sterile shoot multiplication cultures to be initiated. Shoot nodal explants inoculated on 2.5 µM IBA exhibited root and shoot proliferation and elongation as well as flowering. The fact that these morphogenetic responses were possible on an auxin-only medium suggested that auxins play a key role in shoot and root development of this species. The addition of a cytokinin (2.5 mT) was detrimental to plant growth and morphology. High endogenous cytokinin levels at culture initiation or increased production of cytokinins *in vitro* may have been contributing factors to these results.

During the *in vitro* rooting study, all treatments (including the control) successfully produced rooted plantlets (25–55%). However, 10.0 µM IBA resulted in the highest rooting percentage (55 ± 11.4%), mean number of roots (3.80 ± 0.83) and mean number of new leaf pairs (4.65 ± 0.67). However, the longest mean root length originated from plantlets in the 5.0 µM IAA treatment. The highest concentration of IBA (10.0 µM) having the most positive effect on growth parameters could be due to the acclimation of *in vitro* plantlets to 2.5 µM IBA. Nevertheless, this finding supports the previous conclusion that auxins have a number of crucial, active roles within this plant.

Rooting percentages of *ex vivo* cuttings were much higher than those obtained from the *in vitro* study (90–100%). This was likely influenced by the endogenous hormone levels of greenhouse-maintained mother plants which were flowering at the time of the experiment. IBA (5.0 µM) had the most significant effects on rooting parameters, with the highest mean number of roots (11.20 ± 1.37) and the longest mean root length (57.18 ± 3.85 mm). Although the highest mean number of new leaf pairs was not significantly affected by treatment, 2.5 µM IBA was responsible for the highest value of this growth parameter (4.20 ± 0.36).

All vegetative propagation investigations have shown a stronger effect of IBA as opposed to IAA thus, it is concluded that this hormone is responsible for controlling root proliferation in *S. tortuosum*. Further, it is postulated that IBA controls other morphogenetic responses (shoot proliferation and development).

In vitro-generated plantlets from all treatments were able to acclimatize to greenhouse conditions (45–90%). The control treatment was the most successfully acclimatized (90 ± 6.9%), despite some plantlets having non-functional or no roots at all when transplanted *ex*

vitro. This suggests a heightened ability of control plantlets to continue root proliferation in the *ex vitro* environment.

The endogenous hormone levels of *S. tortuosum* requires extensive investigation, along with the functioning of hormone signalling pathways. Such investigations will shed light on the effects of specific plant growth regulators within these plants, and the seasons and conditions which enhance these effects.

This investigation provides various methods for the propagation of *S. tortuosum*, which can aid in conservation efforts and replenishment of natural populations. This information will also be valuable to researchers and pharmaceutical companies to aid them in obtaining and multiplying plant material for research and alkaloid extraction.

Due to the potential of mesembrine-type alkaloids in pharmacology, it is crucial to determine the effects of these studied methods, and additional *in vitro* methods (e.g. cell suspension culture) on alkaloid content. In this way, it will be possible to ascertain the optimum method for enhancing alkaloid production, which will be the key to creating a commercially available source of these valuable mesembrine-type alkaloids.

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