Elicitation effects on *Sceletium tortuosum* growth, physiology, and mesembrine-type alkaloid production *in vitro*

By

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Submitted in fulfilment of the requirements of the
degree of Doctor of Philosophy (Biological Sciences)

Research Centre for Plant Growth and Development

School of Life Sciences

University of KwaZulu-Natal

Pietermaritzburg Campus

February 2025

DECLARATION BY STUDENT

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

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Publication 1: A. Sreekissoon, W. Chen, A. M. Viljoen, J. F. Finnie, J. Van Staden (2025). The effects of light, salt, and osmotic stress on *Mesembryanthemum tortuosum* growth and mesembrine-type alkaloid production *in vitro* **(Accepted by *Physiologia Plantarum*)**

Contributions: A. Sreekissoon performed all experiments, sample preparation, data and statistical analyses, and was responsible for writing of the manuscript, under the supervision of J. F. Finnie and J. Van Staden. W. Chen and A. M. Viljoen procured the necessary materials and performed UPLC-MS analyses. All authors reviewed and edited the manuscript.

Publication 2: A. Sreekissoon, W. Chen, A. M. Viljoen, J. F. Finnie, J. Van Staden (2025). Biostimulant effects on growth and mesembrine-type alkaloid production of *Mesembryanthemum tortuosum in vitro* **(In Preparation)**

Contributions: A. Sreekissoon performed all experiments, sample preparation, data and statistical analyses, and is responsible for writing of the manuscript, under the supervision of J. F. Finnie and J. Van Staden. W. Chen and A. M. Viljoen procured the necessary materials and performed UPLC-MS analyses.

Publication 3: A. Sreekissoon, W. Chen, A. M. Viljoen, J. F. Finnie, J. Van Staden (2025). Popular plant growth regulators influence growth, morphology, and alkaloid production of *Mesembryanthemum tortuosum* **(In Preparation)**

Contributions: A. Sreekissoon performed all experiments, sample preparation, data and statistical analyses, and is responsible for writing of the manuscript, under the supervision of J. F. Finnie and J. Van Staden. W. Chen and A. M. Viljoen procured the necessary materials and performed UPLC-MS analyses.

Signed……… [redacted] ……..

ACKNOWLEDGEMENTS

I would like to thank:

- First, Almighty God. If not for Your guidance and grace, I would not have been able to walk this long and winding road. It’s been a crazy journey, but I am grateful for it all.
- Prof. J. Van Staden. For your guidance, support, patience, and positive reinforcement. I am honoured to have studied under you for all these years. Your perseverance and determination are characters I will always aspire to, in science and in life.
- Prof J. F. Finnie. For your guidance, understanding, and support. You always had the right words to say on a bad day. Thank you for all the times you’ve been “pedantic” – you’ve made me a better researcher.
- Prof. A. M. Viljoen and Dr. W. Chen. For performing crucial UPLC-MS analyses for this project. Thank you for allowing me to be involved in the process.
- Dr. M. G. Kulkarni. I appreciate your constant willingness to assist with any technical or statistical issue. Your open-door policy has helped me through several challenging circumstances.
- Dr. C. Van Rensburg. For your assistance with UPLC-MS data analysis. Thank you for the time you contributed towards this project.
- Ms. L. Warren. For your assistance with all administrative tasks. I appreciate your constant care, kindness, and meticulousness.
- Mr. R. Chetty. For being there to assist me with anything and everything, whenever I needed you. Thank you for your care, support, and for always lifting my spirits.

- My parents. For their unconditional love, patience, support, and understanding. Thanks to you, I have reached this glorious milestone.

- I am grateful to the National Research Foundation (NRF) and the Oppenheimer Memorial Trust (OMT) for their financial support for the duration of this project.

Abstract

Sceletium tortuosum is an immensely valuable South African succulent. The plant is well-known for its versatility in traditional medicine. Its' mesembrine-type alkaloids (mesembranol, mesembrenol, mesembrenone, and mesembrine) have shown remarkable potential as therapeutic agents for numerous disorders, including depression, anxiety, diabetes, epilepsy, and Alzheimer's disease. Despite the tremendous value of these phytochemicals, investigations into improving alkaloid production in this species are virtually non-existent, and commercial alkaloid sources are not readily available. Hence, this research aimed to determine the effects of elicitation techniques on growth and mesembrine-type alkaloid production of *S. tortuosum in vitro*.

Elicitors were applied to nodal explant cultures for 28 days. Growth was assessed by determining the mean number of new leaf pairs, mean number of roots, and the mean length of the longest root. Mesembrine-type alkaloids were extracted from plant material and quantified with UPLC-MS.

Light (0, 8, 16, and 24 H), salt (0, 50, 100, and 200 μM NaCl), and osmotic stress (0, 25, 50, and 100 g/L polyethylene glycol (PEG)) were applied. Growth was optimized in 24 H light as plantlets produced the highest mean number of new leaf pairs (2.79 ± 1.89) and roots (2.60 ± 1.60). Total alkaloid contents were significantly increased by 0 H light (9.16 ± 0.12 μg/mg DW) and 100 g/L PEG (6.74 ± 0.30 μg/mg DW). Growth effects and conservation of secondary metabolite pools in the presence of salt confirmed a halophytic nature for *S. tortuosum*.

Colorimetric methods were used to quantify primary metabolites (total carbohydrates, starch, proteins, and proline) in stress-treated plantlets. Treatment with 50 g/L PEG resulted in peak quantities of starch (130.10 ± 0.16 μg/mg FW) and proline (24.73 ± 0.06 μg/mg FW), while 100 g/L PEG produced the highest protein concentration (71.60 ± 0.30 μg/mg FW). Protein levels were sensitive to osmotic stress, suggesting enhanced synthesis of stress-response proteins. Salt treatments (100 and 200 µM NaCl) yielded the highest carbohydrate concentrations (383.3 ± 1.40 and 323.1 ± 0.72 µg/mg FW, respectively), but only slight increases in proline concentrations (17.07 and 16.10 µg/mg FW, respectively) relative to the control (15.40 µg/mg FW). This provided further evidence of a halophytic profile for the species.

Plant growth regulators (indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA), kinetin (KIN) and benzylaminopurine (BAP) were applied to tissue cultures at concentrations of 0, 2.5, 5, 10, 15, and 20 µM. Plantlets treated with IBA were the healthiest, with 20 µM IBA resulting in the highest mean number of roots (14.18 ± 4.66) and mean root length (6.28 ± 2.59 mm). Treatments with NAA and cytokinins caused unhealthy shoot and root proliferation, respectively. However, KIN treatments resulted in the highest total alkaloid contents (5.51–7.77 µg/mg DW), with the maximum amount achieved by 5 µM KIN.

Various concentrations of biostimulants (smoke water (SW), Kelpak® (KEL), vermicompost leachate (VCL), sodium humate (SH)) were tested. Smoke water and SH showed the most potential for growth improvements. Treatment with 5 mg/L SH resulted in the highest mean number of new leaf pairs (1.67 ± 1.20), while 1:2000 SW resulted in the highest mean number of roots and mean root length (2.57 ± 2.95 and 4.14 ± 6.69 mm, respectively). Significant increases in total alkaloid contents were recorded for 1:1000 and 1:2000 SW treatments (4.95 ± 0.23 and 4.72 ± 0.06 µg/mg DW, respectively). Positive responses were inversely proportional to SW concentration and directly proportional to SH concentration.

Callus cultures were successfully initiated with 2.5 µM 2,4-D. Cell suspension cultures were supplemented with PGRs (KIN, BAP, NAA, 2,4-dichlorophenoxyacetic acid (2,4-D) (NAA, 2,4-D, KIN, BAP) and amino acids (methionine, tyrosine, phenylalanine) at concentrations of 0, 2.5, 5, and 10 µM. After a 37-day incubation, culture growth and alkaloid contents were assessed.

Treatments with 5 and 10 µM 2,4-D µM resulted in the highest mean total cell number and fresh weight ($1.59 \times 10^{11} \pm 1.19 \times 10^{11}$ cells/mL and 2.82 ± 0.95 g, respectively). Alkaloids were present in cells and culture media. Total alkaloid contents were optimized by BAP treatments (3.36–4.51 μg/mg DW) and 10 μM 2,4-D (3.70 ± 0.40 μg/mg DW). Among the amino acids tested, phenylalanine (2.5 μM) produced the highest mean fresh weight (2.36 ± 0.28 g), and total alkaloid content (3.32 ± 0.24 μg/mg DW). However, the efficacy of 2,4-D over phenylalanine was proven by the alkaloid contents obtained in a later experiment (2.73 ± 0.94 and 1.29 ± 0.35 μg/mg DW, respectively).

Several treatments successfully improved growth and alkaloid production of *S. tortuosum*. These methods could prove valuable to small- and large-scale *Sceletium* growers, along with biotechnologists and pharmaceutical companies. The growing need for commercial availability of these alkaloids could be satiated with appropriate use of the methodologies described.

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2,4-D	2,4-dichlorophenoxyacetic acid
5-HT	Serotonin
ANOVA	Analysis of variance
BAP	Benzylaminopurine
BSA	Bovine serum albumin
DMRTs	Duncan's Multiple Range Tests
DW	dry weight
FW	fresh weight
H	hour/s
IAA	Indole acetic acid
IBA	Indole-3-butyric acid
KAR1	Karrikin1
KAR1-6	Karrikins
KEL	Kelpak®
KIN	Kinetin
MS	Murashige and Skoog (1962)
NAA	Naphthaleneacetic acid
NaCl	Sodium chloride (salt)
PAR	Photosynthetic active radiation
PBS	phosphate-buffered saline

PEG	Polyethylene glycol
PGR	Plant growth regulator
ROS	reactive oxygen species
SH	sodium humate
SW	Smoke water
TMB	trimethylbutenolide
UPLC-MS	Ultra Performance Liquid Chromatography-Mass Spectrometry
VCL	Vermicompost leachate

Chapter 1: Literature review

1.1 Classification, description, and distribution of *Sceletium*

Sceletium tortuosum (L.) N.E. Br. belongs to the Caryophyllales order and is part of the Aizoaceae family (Klak *et al.*, 2003). There has been much controversy over the classification of this species. It was initially classified as *Sceletium*, a genus which contained seven other species (*S. emarcidum, S. exalatum, S. rigidum, S. crassicaule, S. expansum, S. strictum, S. varians*) (Gerbaulet, 1996; Klak *et al.*, 2003). Over the years, however, several authors have motivated for the species to be classified into the *Phyllobolus* and *Mesembryanthemum* genera (Bittrich, 1986; Klak *et al.*, 2007). As of late, *S. tortuosum* has been reduced to synonymy, making *Mesembryanthemum tortuosum* the accepted scientific name (Plants of the World Online, 2023a). Despite this taxonomic adjustment, the majority of publications from recent years continue to make reference to the synonym, *S. tortuosum*. Thus, for ease of reference, the synonym will be carried through in this work.

Plants belonging to the original *Sceletium* genus acquired their name from the skeletonized appearance of withering leaves **(Figure 1.1A)**. The plants' dried leaves cover and protect new leaves from adverse environmental conditions (Chesselet, 2005). *Sceletium tortuosum* is a succulent which contains idioblasts in the leaves for the storage of water or other metabolites (Gericke and Viljoen, 2008; Tulyananda and Nilsen, 2017). Flowers are yellow, white, or pink, with multiple thread-like petals **(Figure 1.1B)**. Its reproduction relies on insect pollination, and seed dispersal occurs from hygrochastic fruit capsules (Chesselet, 2005; Gericke and Viljoen, 2008).

Plants are scrambling or decumbent shrubs found in arid to semi-arid areas (Gericke and Viljoen, 2008). They thrive in partial shade environments, particularly in the shade provided by other plants. Their life span is approximately 3–5 years (Chesselet, 2005; Smith *et al.*, 1996). The species is endemic to south-western regions of South Africa **(Figure 1.2)**.

Sceletium tortuosum is classified as a threatened species due to its shrinking natural populations (Gericke and Viljoen, 2008; Plants of the World Online, 2023b).

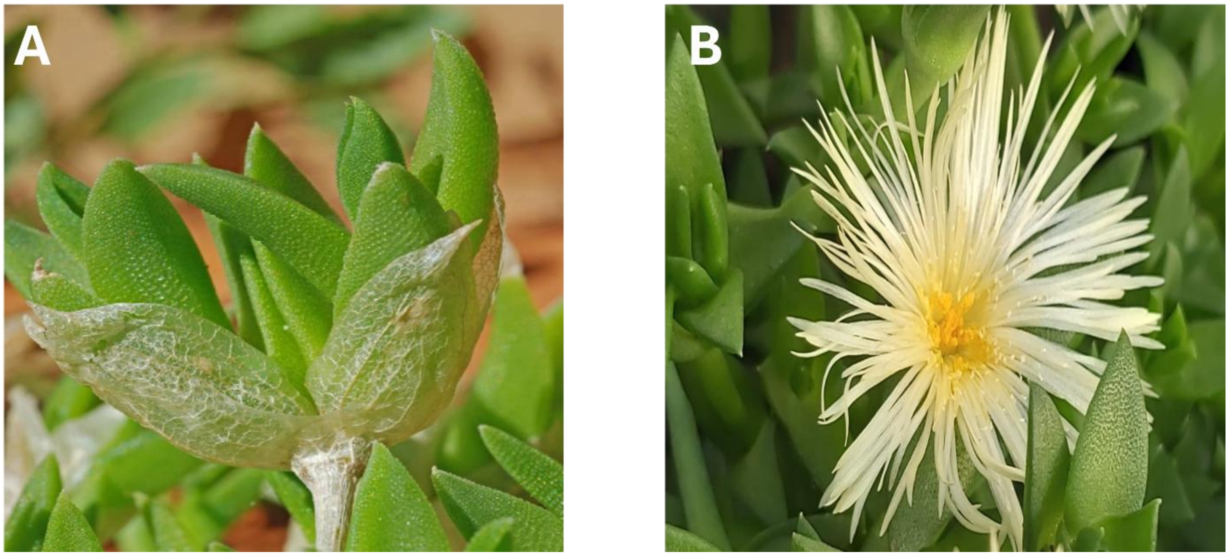


Figure 1.1: *Sceletium tortuosum* with skeletonized leaves (adapted from Gericke and Viljoen (2008)) **(A)**, and in flower **(B)**

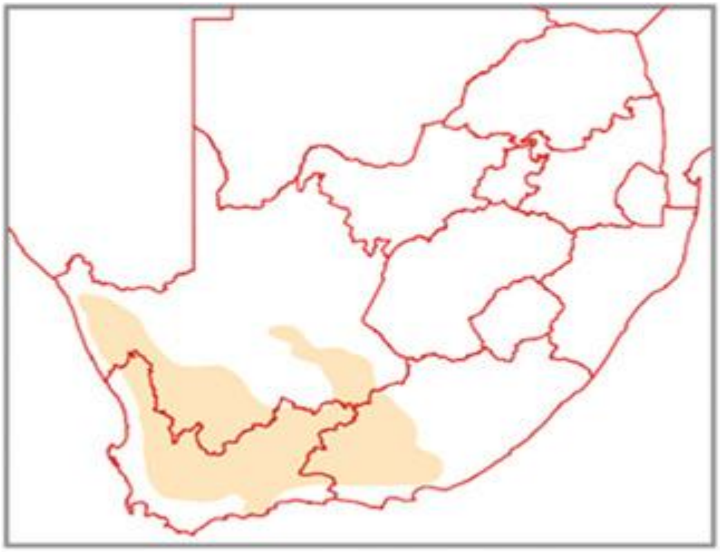


Figure 1.2: Distribution of *Sceletium tortuosum* in South Africa (adapted from Viljoen *et al.* (2023))

1.2 Traditional uses and preparations of *Sceletium*

Sceletium tortuosum was discovered by the KhoiSan people of the Cape at least three centuries ago (Waterhouse *et al.*, 1979). Its common names include kanna, channa, and kougoed (Gericke and Viljoen, 2008). Historically, channa referred to various members of the *Sceletium* genus (e.g. *S. tortuosum*, *S. expansum*) (Lewin, 1998). Thus, the uses described may also apply to other members of the genus.

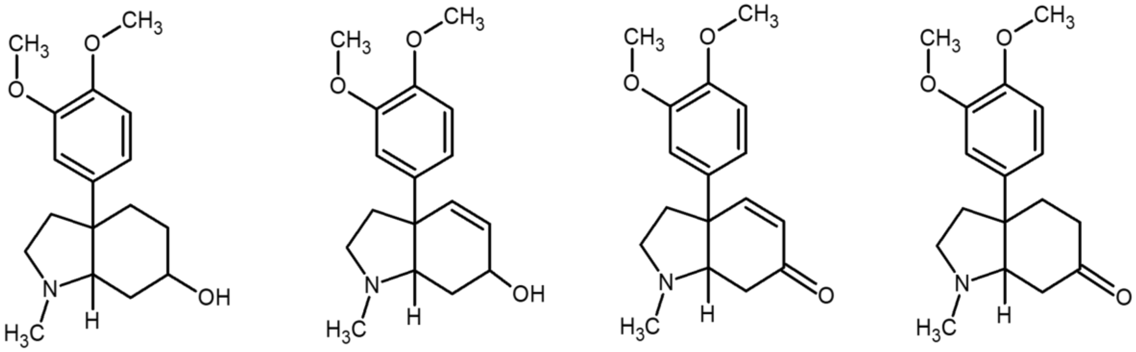
Sceletium was utilized in a variety of ways and the method of preparation often dictated its effects. The fresh plant reportedly had the mildest effects and was suitable for infants and young children (Gericke and Viljoen, 2008; Smith *et al.*, 1996). Mothers would mix some of the plants' juice with milk or water to treat colic in babies (Smith *et al.*, 1996). The raw plant was used to relieve pain and gastrointestinal disturbances. It would also elicit calmness and remedy sleeplessness (Digby, 2005; Rood, 1994; Smith *et al.*, 1996). *Sceletium* was also used in teas and tinctures to achieve these effects (Pappe, 1868; Watt and Breyer-Brandwijk, 1962).

The common name, kougoed, means "something to chew". This name often refers to *S. tortuosum* in its fermented state (Watt and Breyer-Brandwijk, 1962). Two preparation methods have been reported (Smith *et al.*, 1996). Fermentation involved crushing the plant with stones and placing it in an animal skin or plastic bag. The bag would be left in the sun for several days, then the plant contents would be laid out to dry in the sun. A shorter, baking method, involved digging a hole underneath a fire, placing the plant inside, and covering with hot sand for an hour. The plants' effects were said to be more potent after fermentation (Smith *et al.*, 1996).

The Khoi and Nama people would chew on kougoed to relieve their thirst and hunger (Forbes, 1986; Watt and Breyer-Brandwijk, 1962). Otherwise, it was used recreationally for its euphoric and intoxicating effects (Forbes, 1986; Smith *et al.*, 1996). It was stated that if the plant was chewed immediately after fermentation, its intoxicating effects would be experienced. Apart from chewing, kougoed was also smoked and used as snuff (Forbes, 1986; Gericke and Viljoen, 2008).

1.3 Chemistry of *Sceletium* alkaloids

Sceletium tortuosum possesses a variety of alkaloids belonging to the crinane class (Gericke and Viljoen, 2008). There are 15 compounds within this class, all with a 3a-aryl-*cis*-octahydroindole skeleton (Patnala and Kanfer, 2017). The most well-studied alkaloids within this group are the principal mesembrine-type alkaloids, namely, mesembranol, mesembrenol, mesembrenone, and mesembrine **(Figure 1.3)**. These alkaloids were thought to be responsible for the psychoactive effects of the plant, thus leading to further investigations.



Mesembranol Mesembrenol Mesembrenone Mesembrine

Figure 1.3: Principal mesembrine-type alkaloids (adapted from Terburg *et al.* (2013))

Early speculations into the biosynthetic pathways of mesembrine-type alkaloids began with Bodendorf and Kloss (1961). However, it was Jeffs and colleagues that successfully elucidated the pathway for mesembrine biosynthesis. Through a series of experiments, they determined that phenylalanine, tyrosine, and methionine were involved (Jeffs *et al.*, 1971). They showed that 4’-hydroxycinnamic acid and *N*-functionalized tyramine (derived from phenylalanine and tyrosine, respectively), form *bis*-spirodienone. This intermediate is utilized to form sceletenone, which undergoes oxidation to generate the octahydroindole skeleton of mesembrine-type alkaloids (Jeffs *et al.*, 1974a; Jeffs *et al.*, 1974b; Jeffs *et al.*, 1976; Jeffs *et al.*, 1978). These researchers also established that this pathway leads to the formation of mesembrenone first. Thereafter, mesembrenone is converted to mesembranol, mesembrenol, and mesembrine (Jeffs *et al.*, 1978).

Several chemical syntheses of mesembrine have been reported (Czekelius, 2018), the first of which was achieved by Shamma and Rodriguez (1965). A fairly recent protocol allows for the generation of other mesembrine-type alkaloids from mesembrine (Verma *et al.*, 2018). However, it is worth noting that the majority of these syntheses involve multi-step reactions, and the action of a metal catalyst, such as palladium, gold, or platinum (Czekelius, 2018; Shamma and Rodriguez, 1965; Smith and Brown, 2017). Thus, the usefulness of these syntheses is likely limited by the cost of purchasing these precious metals.

1.4 *Sceletium* materials and their use in pharmacological studies

Initial studies on the effects of *S. tortuosum* began with the use of dried plant material (Hirabayashi *et al.*, 2002; Hirabayashi *et al.*, 2005). Thereafter, extracts of the plant were prepared via different methods (e.g. methanol-chloroform, aqueous-ethanol, acid-base extractions) (Loria *et al.*, 2014; Shikanga *et al.*, 2012; Smith, 2011). Over time, standardized extracts of *S. tortuosum* alkaloids were developed, namely, Zembrin® and Trimesemine™ (Coetzee *et al.*, 2016; Zembrin®, 2025). Zembrin® is reportedly prepared as an aqueous-ethanolic extract (purified water 30%, ethanol 70% (v/v)), and spray-dried onto a maltodextrin carrier (Terburg *et al.*, 2013). Mesembrine-type alkaloids make up 0.42% (w/w) of Zembrin®. Its alkaloid composition is mesembrenone + mesembrenol > 70% and mesembrine < 20% (Harvey *et al.*, 2011). The total alkaloid content of Trimesemine™ was found to be 3% (w/w), with mesembrine constituting > 80% of the alkaloid fraction (Coetzee *et al.*, 2016). Studies that tested *S. tortuosum* materials are described in **Table 1.1**.

Table 1.1: Effects and therapeutic potential of *Sceletium tortuosum* materials as determined by *in vivo* and *in vitro* studies

Test subjects / bioassays	**Type of extract**	**Effects and therapeutic potential**	**Reference**
Dogs and cats	Dried plant material *Sceletium tortuosum*	Dementia	Hirabayashi *et al*. (2002); Hirabayashi *et al*. (2005)
Radioligand binding assay Phosphodiesterase activity assay	Zembrin®, mesembrenone, mesembrine, and mesembrenol	Mesembrine and mesembrenol inhibited serotonin (5-HT) transport Mesembrenone inhibited 5-HT transport and phosphodiesterase-4 (PDE4) Depression, anxiety, schizophrenia, improving cognitive function	Harvey *et al*. (2011)
Anti-HIV assays	*Sceletium* extracts	Inhibition of Human Immunodeficiency Virus-1 (HIV-1) reverse transcriptase and protease Free radical scavenging activity HIV, depression, anxiety	Kapewangolo *et al*. (2016)
Human astrocytes and mouse hippocampal neurons	Trimesemine™	Down-regulation of 5-HT transporter Monoamine releasing agent Mild inhibition of acetylcholinesterase (AChE) and monoamine oxidase-A (MAO-A) Depression, Alzheimer's and Parkinson's diseases, attention deficit disorders	Coetzee *et al*. (2016)

Human astrocytes	*Sceletium* extracts	Neuroprotective effects Limiting inflammation Chronic diseases and associated neurodegeneration, obesity, diabetes	Bennett *et al*. (2018)
Human monocytes	Trimesemine™	Cytoprotective effects Mild anti-inflammatory properties Depression, psychologically-induced systemic low-grade inflammation	Bennett and Smith (2018)
Human adrenocortical carcinoma cells	Trimesemine™	Reduced cortisol, androstenedione, and aldosterone levels Stress, hypertension	Johannes (2018)
Male Wistar rats	*Sceletium* extract (containing mesembrine)	Decreased corticosterone and interleukin-2 (IL-2) levels Stress, anxiety	Smith (2011)
Sprague-Dawley rats	*Sceletium* extracts containing all four mesembrine-type alkaloids, mesembrine	Depression, pain	Loria *et al*. (2014)
Male BALB/c mice	*Sceletium* extract containing all four mesembrine-type alkaloids	Depression	Schell (2014)
Male Silver Laced Wyandotte chicks	*Sceletium* leaf extract (containing mesembrine)	Anxiety	Carpenter *et al*. (2016)
Fischer rats	Zembrin®	Depression, pain, improving cognitive function	Dimpfel *et al*. (2016c)

Live rats and slices of the hippocampal regions	Zembrin®, mesembrenone, mesembrine, mesembranol, mesembrenol	Mesembranol, mesembrenol, and Zembrin® attenuated α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) mediated transmission Epilepsy	Dimpfel *et al.* (2018)
Sprague Dawley rats	Zembrin® (in addition to methamphetamine)	Increased striatal and hippocampal monoamine levels Drug addiction	Postma (2021)
Flinders Sensitive Line rats and Flinders Resistant Line rats	Zembrin®	Depression	Gericke *et al.* (2022)
Zebrafish larvae	Zembrin®, mesembranol, mesembrenol, mesembrenone, mesembrine	Depression, anxiety	Gericke *et al.* (2024)
Humans	Zembrin®	Reduced amygdala reactivity and amygdala-hypothalamus coupling Anxiety	Terburg *et al.* (2013)
Humans	Zembrin®	Alzheimer's disease, improving cognitive function, mood, and sleep	Chiu *et al.* (2014)
Humans (ages 40–75)	Zembrin®	Depression, anxiety, improving cognitive function	Dimpfel *et al.* (2016a)
Humans (ages 50–80)	Zembrin®	Anxiety, improving cognitive function and mood	Dimpfel *et al.* (2016b)

Humans	Zembrin®	Anxiety	Reay *et al.* (2020)
Humans	Zembrin®	Pain, mood stabilizer	Berry *et al.* (2021)

1.5 Safety studies and adverse effects

Several of the abovementioned studies **(Table 1.1)** monitored adverse effects. Animal studies involving rats produced some undesirable effects, such as ataxia, immune suppression, and inflammatory responses (e.g. increased gut secretion) (Loria *et al.*, 2014; Smith, 2011). However, Murbach *et al.* (2014) conducted a comprehensive safety assessment on rats using a wide dosage range of Zembrin® (0–5000 mg/kg body weight), and reported no adverse effects.

Trimesemine™, however, proved to have some negative effects. Testing with human adrenocortical carcinoma cells resulted in decreased testosterone levels. Additionally, high doses of the extract (0.5 mg/mL) adversely affected human embryonic kidney cells and rat testis cultures (Johannes, 2018).

In terms of studies performed on humans, Zembrin® appeared to have some adverse effects. Chiu *et al.* (2014) reported gastrointestinal disturbance, skin irritation, decreased appetite, weight loss, and interrupted sleep in their cognitive function study. The safety and tolerability of Zembrin® was thoroughly evaluated by Nell *et al.* (2013). Participants reported head and abdominal pain, respiratory tract infections, and influenza. In both cases though, adverse effects were more prevalent in the control group (Chiu *et al.*, 2014; Nell *et al.*, 2013).

1.6 *Sceletium tortuosum* in practice

Sceletium tortuosum has been incorporated into clinical practice. Psychiatrists and psychopharmacologists have prescribed dried *Sceletium* material, as well as Zembrin®, to patients suffering with depression and anxiety disorders (Gericke, 2001; Gericke *et al.*, 2017). The patients reported elevation in mood, improvements in sleeping patterns, greater capacity to deal with stress, and decreased anxiety (Gericke, 2001). *Sceletium tortuosum* also proved to be an effective substitute for the anti-depressant, Citalopram (Gericke *et al.*, 2017). Prescribers noted that withdrawal symptoms from *S. tortuosum* were dose-dependent, with doses lower than 100 mg/day precluding these effects (Gericke, 2001; Gericke *et al.*, 2017).

Sceletium tortuosum is incorporated into several dietary supplements, teas, tablets, and capsules, all of which are easily available online (Manganyi *et al.*, 2021). Many of these products are unregulated though (Patnala and Kanfer, 2021), and the alkaloid profile of these nutraceuticals is uncertain (for example, see Green square (2025)). The only commercially available standardized extract is Zembrin®. It is primarily used in the manufacture of elev8™, which is marketed as a mood booster, and an aid for coping with stress (elev8™, 2020). Commercial *Sceletium* farms supply the plant material necessary to produce Zembrin® (Zembrin®, 2025).

1.7 Investigations on *Sceletium tortuosum* and associated alkaloids

Despite these commercial farming endeavours, there remains to be a commercially available source of pure mesembrine-type alkaloids (Krstenansky, 2017; Shikanga *et al.*, 2012). In fact, only a handful of studies have focused on the alkaloid contents of *S. tortuosum*. Zhao *et al.* (2018) evaluated the chemotypic variation among *S. tortuosum* plants found in different regions of South Africa. These authors were able to conclude that alkaloid profiles varied based on population location. Reddy *et al.* (2022) later confirmed this finding, and showed that chemotypic variation also exists within species populations.

Several researchers took special interest in the effects of fermentation on alkaloid quantities of *S. tortuosum*. Smith *et al.* (1998) fermented plant material in a manner similar to the traditional method **(Section 1.2)**. They established that total alkaloid content increased after fermentation. Additionally, they reported a considerable increase in mesembrenone, coupled with decreases in mesembrine and mesembrenol quantities. Patnala and Kanfer (2009) reported similar effects on mesembrenone and mesembrine concentrations following fermentation. These studies utilized several plants for replication (Patnala and Kanfer, 2009; Smith *et al.*, 1998).

Chen and Viljoen (2019) used sections of a single plant in their investigation. Fermentation proved to increase total alkaloid contents; however, they recorded a decrease in mesembrenone and an increase in mesembrine. Recently, Koroleva *et al.* (2024) reported that fungal activity, brought about by the fermentation process, was responsible for alterations to the alkaloid profile.

Precious few studies have been concerned with plant growth and the resulting alkaloid contents. In 2020, Faber and colleagues grew *S. tortuosum* hydroponically and determined that growth substrate and fertigation frequency influenced alkaloid production. In terms of *in vitro* works, propagation strategies were reported for *S. tortuosum*, i.e. plant tissue culture and germination studies (Sreekissoon *et al.*, 2021a; Sreekissoon *et al.*, 2021b). However, treatment effects on alkaloid composition were not evaluated (Sreekissoon *et al.*, 2021b). Makunga *et al.* (2022) initiated various cultures and assayed alkaloid contents. These researchers were able to obtain alkaloid quantities similar to those of wild-type plants. However, they did not attempt to optimize alkaloid production.

1.8 Plant biotechnology for medicinal plants

Plant tissue culture methods are advantageous for medicinal plant species. The strict control of factors such as temperature, light, water, and nutrient availability, allows for propagation of pathogen-free plantlets, without interference from fluctuating environmental factors (Jain *et al.*, 2023). *In vitro* strategies can effectively generate multiple plantlets in a fraction of the time it would require *in situ* (Hasnain *et al.*, 2022).

Tissue culture practices are extremely useful for inducing and improving secondary metabolite production. However, the application of elicitors is paramount for the manipulation of secondary compound biosynthesis (Narayani and Srivastava, 2017; Ozyigit *et al.*, 2023). Elicitors include stress, chemical substances (e.g. plant growth regulators (PGRs), inorganic salts), and bacteria- and fungi-derived compounds (Jamwal *et al.*, 2018; Narayani and Srivastava, 2017). Elicitors may be applied at the cell, tissue, organ, and whole plant level (Ozyigit *et al.*, 2023).

In vitro elicitation for the enhancement of secondary metabolite production is well-documented. Prime examples include the over-production of the pharmaceutically valuable alkaloids, taxol, vincristine, vinblastine, and colchicine (Alam *et al.*, 2017; Expósito *et al.*, 2009; Mahendran *et al.*, 2018). Elicitation techniques are valuable tools for boosting secondary metabolite production, without involving more complex processes like those of genetic engineering (Kowalczyk *et al.*, 2020).

1.9 Purpose of this study

Mesembrine-type alkaloids have tremendous pharmacological significance, and the list of potential therapeutic applications continues to grow **(Table 1.1)**. However, there is a distinct gap in the literature between the potential uses of mesembrine-type alkaloids, and avenues to enhance alkaloid production to increase their availability. The demand for a commercial source of mesembrine-type alkaloids has been present for years (Krstenansky, 2017). Such a source would undoubtedly hasten research and development, as well as pharmaceutical manufacture. *In vitro* methods could be the key to the development of a commercial source of these valuable phytochemicals. Thus, this study aims to utilize elicitation techniques to manipulate and monitor *S. tortuosum* growth *in vitro*, with the goal of enhancing alkaloid production.

1.10 References

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Chapter 2: Effects of abiotic stress on growth and alkaloid production in tissue culture

2.1 Introduction

Various types of abiotic stress are experienced by plants in nature. These include water, salt, light, temperature, nutrient, and heavy metal stress. All plants must encounter most, if not all of these stresses in some capacity during their life cycle (Zhang *et al.*, 2023). The abiotic stresses relevant to this investigation will be discussed further.

2.1.1 Stress effects on plant growth and physiology

2.1.1.1 Water stress

Water stress can occur due to flooding or drought. Both phenomena have consequences for photosynthesis, respiration, plant growth, and secondary metabolism (Farooq *et al.*, 2012; Salehi-Lisar and Bakhshayeshan-Agdam, 2016). Flooding refers to waterlogged soil and plant submergence (Jackson and Colmer, 2005). This decreases the availability of oxygen, carbon dioxide, and light (Armstrong and Drew, 2002; Jackson and Ram, 2003). Drought, however, is the more common cause of water stress in plants. Drought stress decreases chlorophyll contents and lowers photosynthesis and respiration (Atkin and Macherel, 2009; Farooq *et al.*, 2012; Flexas *et al.*, 2006; Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

There are three mechanisms utilized by plants to cope with drought stress, i.e. drought escape, drought avoidance, and drought tolerance (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). The escape strategy usually applies to plants with a short life cycle, with the intention of completing said life cycle prior to encountering drought stress (Meyre *et al.*, 2001). Drought avoidance strategies can include reductions in shoot and root growth (Farooq *et al.*, 2012). Leaf number, size, area, and stomata number and size may be reduced to mitigate water loss due to transpiration (Lei *et al.*, 2006; Sinclair and Muchow, 2001). Contrastingly, root growth may be increased (proliferation, length and depth) to aid in the acquisition of water from surrounding regions (Matsui and Singh, 2003; Wang and Yamauchi, 2006).

Drought tolerance mechanisms involve changes at a physiological level. This can take the form of osmotic adjustment (increased accumulation of compatible solutes to reduce water potential) which improves water uptake (Chimenti *et al.*, 2002; Serraj and Sinclair, 2002). Through this adjustment, leaf turgor is also maintained, aiding in carbon dioxide intake (Kiani *et al.*, 2007). The phytohormone balance may be altered, resulting in greater quantities of growth-inhibiting hormones (e.g. abscisic acid). These substances limit growth, as well as induce stomatal closure to restrict water loss (Davies and Zhang, 1991; Farooq *et al.*, 2009). Furthermore, production of secondary metabolites is enhanced to reduce damage caused by reactive oxygen species (ROS) generated by the stress (Farooq *et al.*, 2012; Li, 2008; Rout and Shaw, 2001).

2.1.1.2 Salt stress

Salt stress can occur as a result of drought stress. Essentially, dehydration of the soil results in higher salt concentrations (Ma *et al.*, 2020). Excess salt in the soil lowers its osmotic potential making water uptake difficult (Parihar *et al.*, 2015; Rasool *et al.*, 2013). Thus, salt stress elicits many of the same physiological responses as drought stress, including osmotic adjustment, and reduced photosynthesis and respiration. Increased production of ROS and secondary metabolites occurs, and the phytohormone balance may be affected (Chandna *et al.*, 2013; Rasool *et al.*, 2013).

Increased salt (NaCl) concentrations result in plants having increased levels of Na^+ and Cl^- ions. This can affect the assimilation of other nutrients and ions (e.g. K^+ and Ca^{2+}), thereby disrupting ion and nutrient balances (Rasool *et al.*, 2013). Various plant species employ an avoidance mechanism to cope with high salinity levels, namely salt exclusion. This occurs by reducing the permeability of roots to salt ions, thus lowering salt uptake (Chandna *et al.*, 2013; Flowers and Hajibagheri, 2001).

Species known as halophytes are capable of surviving and reproducing in conditions with high salinity levels (Mohammed *et al.*, 2023). In fact, these plants can respond better to saline conditions than to saline-free conditions (Lee *et al.*, 2016; Lokhande *et al.*, 2011). Halophytes have evolved an array of adaptations and mechanisms to allow them to tolerate high salinity levels. Morphologically, leaves may be succulent with a thickened epidermis, allowing for water storage and a reduction of salt concentration (Flowers and Colmer, 2015; Grigore and Toma, 2021; Van Zelm *et al.*, 2020). Salt glands and salt hairs are often present on shoots and leaves to allow for salt excretion (Chandna *et al.*, 2013; Popp, 1995). Ion compartmentalization (concentrating Na^+ and Cl^- to vacuoles) and ion retrieval (removal of Na^+ from xylem sap) mechanisms are employed to reduce salt accumulation and limit disruptions to normal metabolism (Flowers *et al.*, 2015; Munns and Tester, 2008).

Salt stress affects cell enlargement and elongation, thus impacting root and shoot growth (Van Zelm *et al.*, 2020). If not adequately mitigated by avoidance or tolerance strategies, it will result in stunted plant growth, which can eventually lead to death (Flowers and Colmer, 2015; Rasool *et al.*, 2013).

2.1.1.3 Light stress

Light stress can be experienced in terms of high or low light (irradiance, intensity, or photoperiod) (Yang *et al.*, 2019). Naturally, light fluctuations are experienced on a regular basis, on account of diurnal, seasonal, and climatic changes (Yang *et al.*, 2019). Plants are able to acclimate to these variations with morphological changes (leaf diameter, number and density of stomata), and/or physiological changes (chloroplast avoidance movement, alterations in photosynthetic rate, and investments in secondary metabolism) (Kasahara *et al.*, 2002; Yang *et al.*, 2019).

High light can induce photoinhibition if the amount of light absorbed is more than what can be used for photosynthesis (Adams *et al.*, 2013). It can also cause chlorophyll degradation and damage to the photosynthetic apparatus (photosystems I and II), thereby reducing photosynthetic rate (Keren and Krieger-Liszkay, 2011; Wimalasekera, 2019). Excessive exposure could cause genetic abnormalities and damage to various components of primary metabolism (e.g. proteins, lipids) (Hollósy, 2002).

On the other hand, high light may increase photosynthetic rate, photosynthetic dry mass, and carbohydrate accumulation (Liu and Su, 2016; Wimalasekera, 2019; Yang *et al.*, 2019). Longer photoperiods can result in increased shoot and root growth (Adams and Langton, 2005). In fact, continuous light increased leaf number and growth rate for a number of crop species (Sysoeva *et al.*, 2010).

In comparison, low light has been known to decrease photosynthesis and respiration (Sims and Pearcy, 1991). Low light can negatively impact plant growth, in terms of roots and shoots (Fløistad and Eldhuset, 2017; Kasperbauer and Hunt, 1992; Reich *et al.*, 1998). Size of plants and their reproductive structures can be reduced by low light conditions (Kilkenny and Galloway, 2008). Juvenile vegetative growth may be prolonged with low light conditions, thus delaying the transition to the adult vegetative growth phase (Xu *et al.*, 2021).

Plant species have a variety of mechanisms for dealing with periods of low light, particularly if these species dwell under plant canopies or the shade provided by neighbouring plants (Lichtenthaler, 1996; Valladares and Niinemets, 2008). In some cases, shade avoidance strategies are employed. Typically, shoots elongate towards the light, and away from the shade (De Wit *et al.*, 2016; Franklin, 2008).

Alternatively, shade-tolerance behaviours are exhibited (Gommers *et al.*, 2013). These can include increases in leaf size, but decreases in leaf lamina thickness and stomatal density (Valladares and Niinemets, 2008). Chlorophyll content increases, along with carbohydrate storage (Valladares and Niinemets, 2008; Walters and Reich, 2000). The root:shoot ratio may be lowered, but the relative growth rate is high (Valladares and Niinemets, 2008).

Various researchers reported that plants adapted to low light are unable to grow in high light (Davis *et al.*, 2011; Lichtenthaler, 1996). However, the native habitat of a species may not provide an accurate indication of the appropriate light environment. For instance, *Acer saccharum* and *Ostrya virginiana* are shade-tolerant species, yet they exhibited high photosynthetic and relative growth rates in the presence of high light (Walters *et al.*, 1993). Semchenko *et al.* (2012) also reported that shade improved growth of various grassland species, including those originating from high light areas. Ultimately, plant perception of light stress is species-dependent and difficult to predict without experimentation.

2.1.2 Stress effects on secondary metabolism

Stress events trigger the production of ROS. These free radicals can cause damage to cellular components, thus impeding normal plant metabolism and growth. In the event of stress, growth may be sidelined, in order for the plant to make necessary investments in secondary metabolism (Akula and Ravishankar, 2011; Gommers *et al.*, 2013; Punetha *et al.*, 2022).

Secondary metabolites (flavonoids, phenols, terpenes, alkaloids, carotenoids, anthocyanins, etc.) form part of the plant defense system (Akula and Ravishankar, 2011; Punetha *et al.*, 2022). Flavonoids, phenols, and terpenoids act as signalling compounds and ROS scavengers. Flavonoids, carotenoids, and anthocyanins protect against high light damage and ultraviolet radiation (Cheynier *et al.*, 2013; Punetha *et al.*, 2022; Zhao *et al.*, 2022). Alkaloids are better known for acting in defense against biotic stress (Yeshi *et al.*, 2022). However, abiotic stresses cause marked differences in alkaloid accumulation (Amirifar *et al.*, 2022; Yeshi *et al.*, 2022). Thus, it is thought that alkaloids also have protective roles in situations of oxidative stress. They may be responsible for preventing ROS production and damage (Lee *et al.*, 2016; Radácsi *et al.*, 2010). They may also function in repairing the damage caused (Matsuura and Fett-Neto, 2015).

Medicinal plants, in particular, are capable of up-regulating secondary metabolite production in events of stress (Punetha *et al.*, 2022). For instance, alkaloids, phenols, and flavonoids were increased under salt stress conditions in *Plantago ovata* (Punetha *et al.*, 2022). Production of anti-cancer alkaloids was increased in *Camptotheca acuminata* and *Catharanthus roseus* exposed to drought stress (Liu, 2000; Yahyazadeh *et al.*, 2021). Under light stress, high light increased secondary metabolite production in *Camellia sinensis*, *Coffea arabica*, and *Erigeron breviscapus* (Koshiishi *et al.*, 2000; Kurata *et al.*, 1997; Zhou *et al.*, 2016). Other species showed higher accumulations under low light, or in the absence of light (Aly *et al.*, 2010; Hou *et al.*, 2010; Sauerwein *et al.*, 1992).

Due to such enhancements in secondary metabolite production, stress application is thought to be a viable method for increasing quantities of phytochemicals with high value for the pharmaceutical industry (Punetha *et al.*, 2022; Yeshi *et al.*, 2022). However, stress conditions require rigorous testing in order to minimize adverse effects on growth. Nevertheless, the prospect of striking a balance between sustainable growth and heightened secondary metabolite production is worthy of investigation.

2.1.3 Aims and objectives

This research aimed to determine the effects of different types of abiotic stress (light, salt, and osmotic stress) on (1) *in vitro* plantlet growth and (2) mesembrine-type alkaloid production.

To satisfy the aims, the following objectives were set:

1) Prepare standard Murashige and Skoog (1962) (MS) medium, and MS media containing various concentrations of NaCl and polyethylene glycol (PEG),
2) Utilize nodal segments from PGR-free multiplication cultures to inoculate experimental cultures,
3) Incubate light stress cultures with different photoperiods, and salt and osmotic stress cultures with constant light for 28 days,
4) Upon harvest, record growth parameters of plantlets (number of new leaf pairs, number of roots, and length of the longest root),

5) Oven-dry plant material and subject to alkaloid extraction protocol and filtration,
6) Quantify mesembrine-type alkaloids using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS), and
7) Conduct a comparative analysis of growth responses and alkaloid production.

2.2 Materials and methods

2.2.1 Plant material

Sceletium tortuosum plants were identified and collected by Dr J. H. de Lange from 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) in Pietermaritzburg. Plants were potted in a 1:1 mixture of soil and vermiculite. Various cuttings were set to maintain a healthy stock of mother plant material. Plants were maintained outdoors in a partially shaded area, with manual watering as necessary.

2.2.2 Media preparation and *in vitro* multiplication procedures

Murashige and Skoog (1962) medium was prepared for *in vitro* multiplication. Two types of multiplication media were prepared, i.e. containing 2.5 µM indole-3-butyric acid (IBA) and PGR-free media. Media contained 3% sucrose, 0.01% myo-inositol, and 0.8% agar (OXOID, Bacteriological Agar No. 1). Media were adjusted to a pH of 5.8 ± 0.02 before dispensing into tissue culture vessels (20 mL each). Media, along with glassware and instruments, were autoclaved (121°C, 1 bar) for 15 min.

Nodal segments were obtained from mother plants and de-contaminated according to Sreekissoon *et al.* (2021). Briefly, plant material was washed under running tap water and placed in a sterile glass jar. The following immersion steps took place with constant shaking (120 rpm) on an orbital shaker: (1) distilled water containing 2–3 drops of Tween 20 for 20 min, (2) Benlate (1 g/L) for 40 min, (3) streptomycin sulphate (0.05%) for 20 min. Plant material was transferred to a sterile laminar flow bench and immersed in 20% hydrogen peroxide for 20 min, then submerged in 70% ethanol for 30 seconds. Finally, plant material was rinsed three times with sterile distilled water.

Nodal explants were then inoculated onto multiplication media. Plantlets were grown and maintained on this medium throughout the course of experimentation. Cultures were incubated at 25 ± 3 °C under constant light (Photosynthetic active radiation (PAR) = 75 $\mu mol.m^{-2}.s^{-1}$).

2.2.3 Experimental conditions

Light stress experiments made use of the PGR-free MS medium as previously prepared. Salt stress was mimicked by preparing MS medium containing NaCl concentrations of 0, 50, 100 and 200 µM. For water stress, MS medium contained 0.3% phytagel instead of agar. Polyethylene glycol 6000 was used to supplement media at concentrations of 25, 50 and 100 g/L. The control treatment was prepared without PEG.

Nodal explants were excised from PGR-free multiplication cultures and used to inoculate the experimental media. Each treatment comprised of 5–6 replicates comprised of five explants each. All cultures were incubated at 25 ± 3°C. Salt and osmotic stress cultures were incubated with constant light (PAR = 72 $\mu mol.m^{-2}.s^{-1}$). Photoperiods of 0 H, 8 H (PAR = 36 $\mu mol.m^{-2}.s^{-1}$), 16 H (PAR = 68 $\mu mol.m^{-2}.s^{-1}$), and 24 H light (PAR = 62 $\mu mol.m^{-2}.s^{-1}$) were utilized in the light stress experiment. Twenty-four-hour light served as the control in the light stress experiment. Cultures were maintained under these conditions for 28 days. Upon harvest, the number of roots, newly-emerged leaf pairs, and length of the longest root was recorded.

2.2.4 Alkaloid extraction and analysis

Intact plantlets were oven-dried at 40°C. Dried plant material was then powdered using liquid nitrogen and a mortar and pestle. The resulting powders were prepared to a concentration of 20 mg/mL with HPLC grade methanol. Samples were sonicated for 20 min in an ultrasound bath (BRANSON 2210). Following this, samples were syringe-filtered (0.20 µm) (MACHEREY-NAGEL CHROMAFIL® Xtra PTFE-20/25) into autosampler vials for UPLC-MS analysis.

Mesembrine-type alkaloids were extracted and purified according to the method described by Shikanga *et al.* (2011). These served as chemical standards for this investigation. Ultra Performance Liquid Chromatography (UPLC) analyses were performed on a Waters Acquity I Class Ultra Performance Liquid Chromatographic system with photo-diode array (PDA) detector (Waters, Milford, MA, USA). Separation was achieved on an Acquity UPLC BEH C_{18} column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 35°C. The mobile phase consisted of 0.1% ammonium hydroxide in water (solvent A) and 90% acetonitrile (solvent B) at a flow rate of 0.3 mL/min; a gradient elution was as follows: 80% A: 20% B to 60% A: 40% B in 2 min, changed to 50% A: 50% B in 4.5 min, back to initial ratio in 0.2 min and equilibrating the system for 1.8 min. The total running time was 8.5 min. The samples were injected in the mobile phase with an injection volume of 1.0 µL (full-loop injection). All samples were injected twice. Data were collected and processed by chromatographic software Masslynx 4.2. The quantitative aspect of the method was validated by determining the linearity, limit of detection (LOD), and limit of quantification (LOQ) **(Table 2.1)**.

The same column, elution gradient, and flow rate were used for the UPLC-MS analysis. Mass spectrometry (G_3QTof, Waters) was carried out in the positive ion electrospray mode. Nitrogen was used as the desolvation gas at a flow rate of 500 L/H, while maintaining a desolvation temperature of 350°C. The source temperature was 100°C and the capillary and cone voltages were set to 3000 and 38 V, respectively. Data, collected over the range *m/z* 100 to 1000, were centroided during acquisition using independent reference lock-mass ions via the LockSpray™ interface to ensure mass accuracy and reproducibility.

Table 2.1: Linearity, limit of detection (LOD), and limit of quantification (LOQ) of UPLC-PDA method

Compound	Linear range (µg/mL)	Regression equation	r^2	LOD (µg/mL)	LOQ (µg/mL)
Mesembranol	0.5-100	Y=3165X-280	0.9999	0.256	0.777
Mesembrenol	0.5-100	Y=3946X-35	0.9999	0.222	0.672
Mesembrenone	0.5-100	Y=6568X-604	0.9998	0.218	0.659
Mesembrine	0.5-100	Y=3795X-55	0.9998	0.332	1.007

All experimental data were analyzed with SPSS V29 using one-way analysis of variance (ANOVA), and Duncan's Multiple Range Tests (DMRTs). Mean and standard deviation values calculated by the software were then plotted using Graphpad Prism V5.

2.3 Results

2.3.1 Plantlet growth

The effects of abiotic stress treatments on plantlet growth are illustrated in **Figure 2.1.** Notable differences were observed between treatments for each growth parameter. The mean number of new leaf pairs increased in direct proportion to increasing photoperiod **(Figure 2.1A)**. Overall, 24 H light (2.79 ± 1.89) resulted in the highest mean number of new leaf pairs, followed by 16 H light (2.66 ± 1.23). Zero H light and 100 g/L PEG resulted in the lowest values for this parameter (0.24 ± 0.38 and 0.18 ± 0.17, respectively). The mean number of new leaf pairs resulting from 0 H light was significantly lower than those obtained from 16 H and 24 H light. Additionally, all PEG treatments (25–100 g/L PEG) resulted in mean numbers that were significantly lower than the PEG control (0.18 ± 0.17). Within the salt treatment group, 100 and 200 µM NaCl were responsible for the highest mean numbers of new leaf pairs (2.14 ± 1.60 and 2.11 ± 1.53, respectively) **(Figure 2.1A)**.

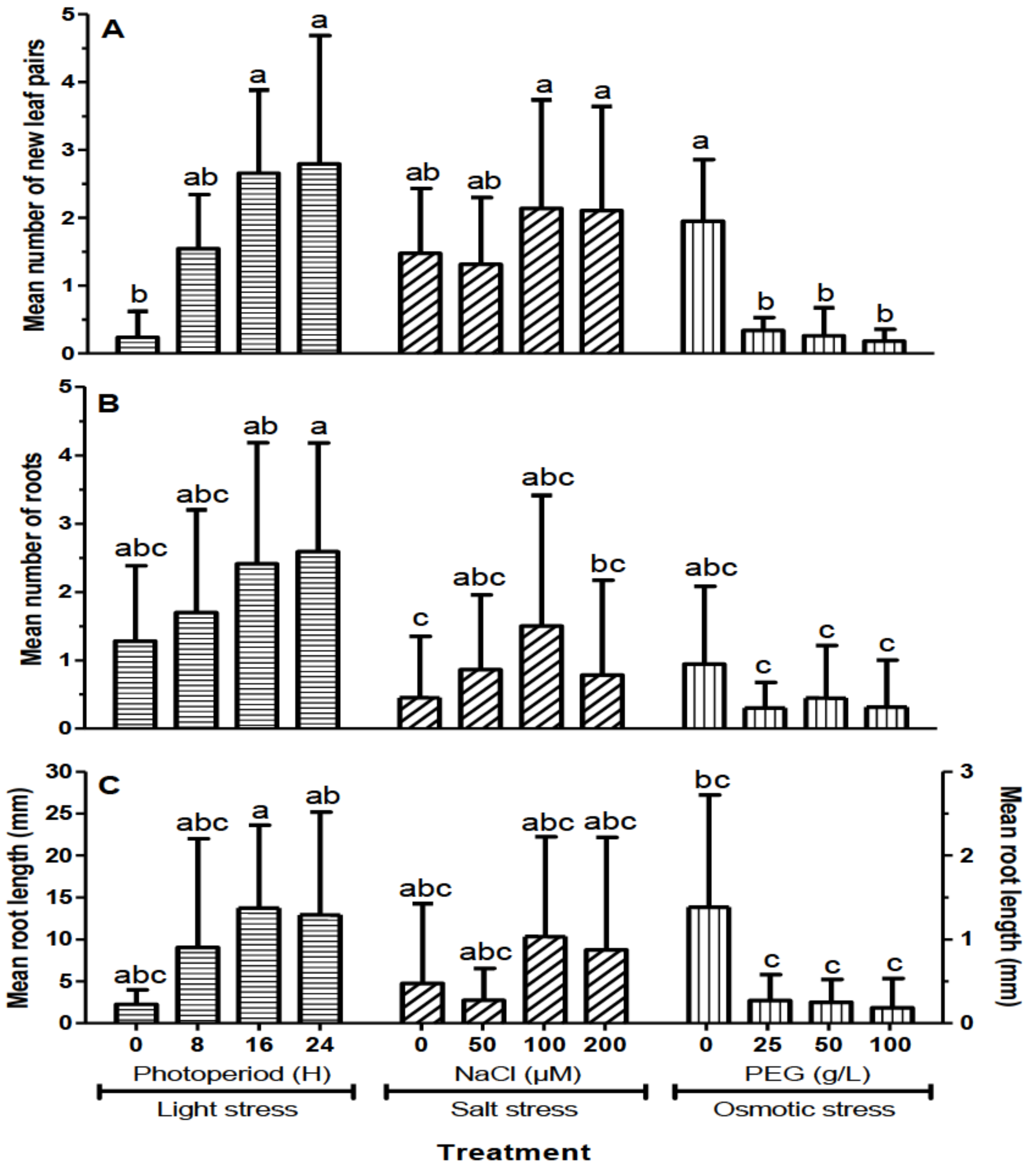

Figure 2.1: Mean number of newly emerged leaf pairs **(A)**, mean number of roots **(B)**, and mean length of the longest root **(C)** of *Sceletium tortuosum* plantlets following exposure to varying levels of light, salt, and osmotic stress for 28 days (PEG = polyethylene glycol). Error bars show standard deviation (n = 5–6). Different letters indicate statistically significant differences (p < 0.05) between treatments. NB. Mean root length for osmotic stress treatments to be read from right y-axis.

The mean number of roots increased in direct proportion to increasing photoperiod, peaking at 24 H light (2.60 ± 1.60) **(Figure 2.1B)**. A similar trend can be seen for salt stress; however, the highest mean number of roots was achieved at 100 µM NaCl (1.50 ± 1.91), with a decline observed at 200 µM NaCl (0.79 ± 1.39). In terms of osmotic stress, PEG treatments (25–100 g/L PEG) produced lower mean root numbers than the PEG control (0.94 ± 1.14), although these reductions were not statistically significant **(Figure 2.1B)**.

With respect to **Figure 2.1C**, mean root lengths were highest in the 16 H and 24 H light treatments (13.75 ± 9.87 mm and 12.91 ± 12.27 mm, respectively). Darkness (0 H light) was responsible for the lowest mean root length in this treatment group (2.26 ± 1.72 mm). Mean root lengths were ultimately lowest in the osmotic stress group as declines were observed as the level of stress increased. The shortest mean root length was obtained from the 100 g/L PEG treatment (0.19 ± 0.35 mm). Mean root lengths obtained from 100 and 200 µM NaCl (10.30 ± 11.94 mm and 8.76 ± 13.41 mm, respectively) were much greater than that of the NaCl control (4.75 ± 9.51 mm) and 50 µM NaCl (2.73 ± 3.83 mm) **(Figure 2.1C)**. Significant differences were not apparent within the salt stress group for any of the parameters evaluated **(Figure 2.1)**.

Overall, the most positive growth effects were elicited by 24 H and 16 H light, while the most negative effects were observed in the osmotic stress treatments. Salt proved to have variable effects on growth, however, raised salinity (particularly 100 µM NaCl) influenced growth more positively than the saline-free control **(Figure 2.1)**.

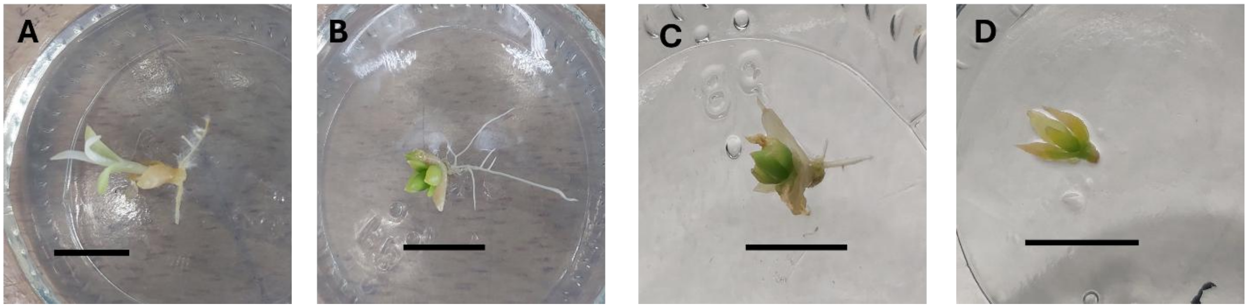

Figure 2.2: *Sceletium tortuosum* cultures following exposure to 0-hour light (A), 24-hour light (B), 0 g L-1 PEG (C), and 100 g L-1 PEG (D) for 28 days (PEG = polyethylene glycol)

The healthiest plantlets were obtained from the 24 h light treatment (Figure 2B). SImilaritites in growth were observed between the 24 h light and 0 g L^{-1} PEG treatments (Figure 2B and 2C). Zero h light caused a visible reduction in chlorophyll in comparison to plantlets from other treatments (Figure 2). However, 100 g L^{-1} PEG produced plantlets with severely stunted growth, in terms of roots and shoots (Figure 2D).

2.3.2 Alkaloid production

All four principal mesembrine-type alkaloids were identified and quantified in *S. tortuosum* samples. A chromatogram generated from an experimental sample is shown below **(Figure 2.3)**.

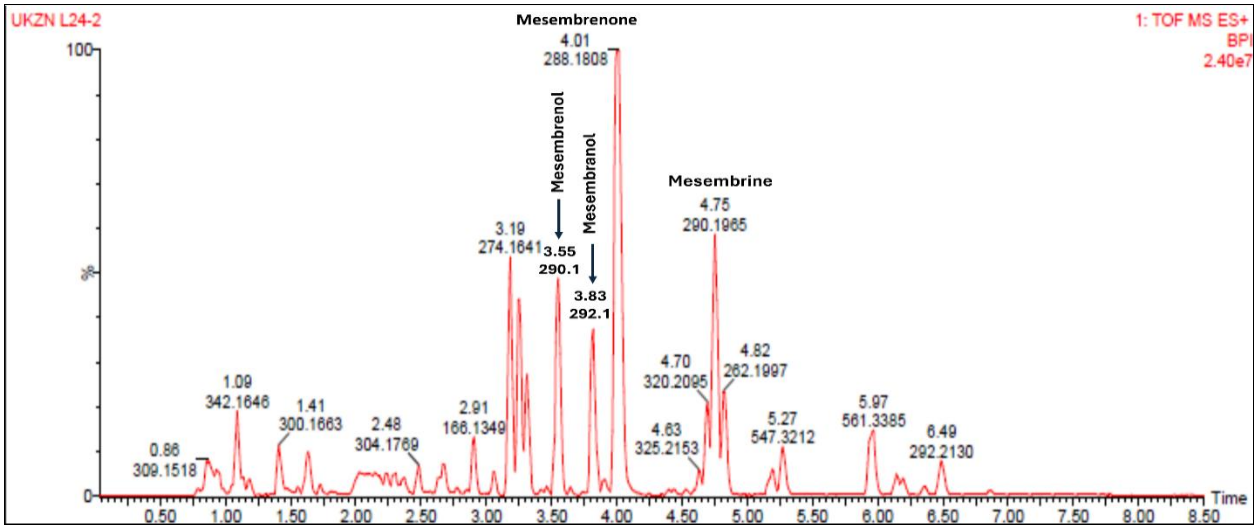

Figure 2.3: Representative chromatogram obtained from UPLC-MS analysis of a *Sceletium tortuosum* sample (24-hour light treatment). Retention times and *m/z* (mass) of mesembrine-type alkaloids are shown.

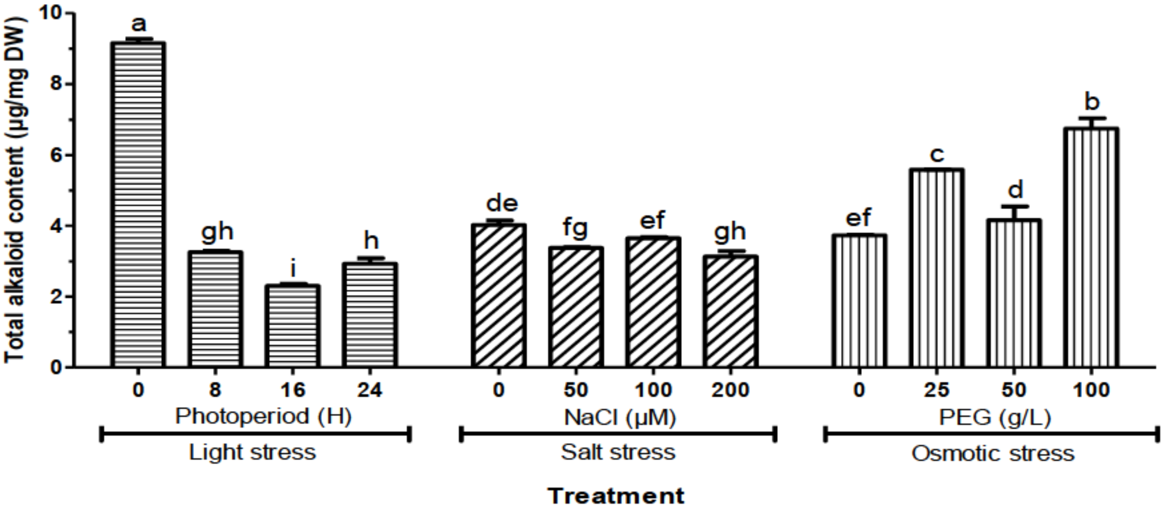

Figure 2.4: Total mesembrine-type alkaloid contents of *Sceletium tortuosum* samples generated from light, salt, and osmotic stress treatments (PEG = polyethylene glycol). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

The highest alkaloid content was obtained from the 0 H light treatment (9.16 ± 0.12 µg/mg DW). Following this, 100 g/L PEG resulted in a total alkaloid content of 6.74 ± 0.30 µg/mg DW **(Figure 2.4)**.

Within the osmotic and light stress groups, the PEG control (3.73 ± 0.014 µg/mg DW) and 16 H light (2.30 ± 0.057 µg/mg DW) resulted in the lowest alkaloid content, respectively. The latter treatment was responsible for the lowest total alkaloid content overall. Total alkaloid contents resulting from salt stress treatments were similar to each other (3.13–3.65 µg/mg DW) and the NaCl control (4.02 ± 0.13 µg/mg DW) **(Figure 2.4)**.

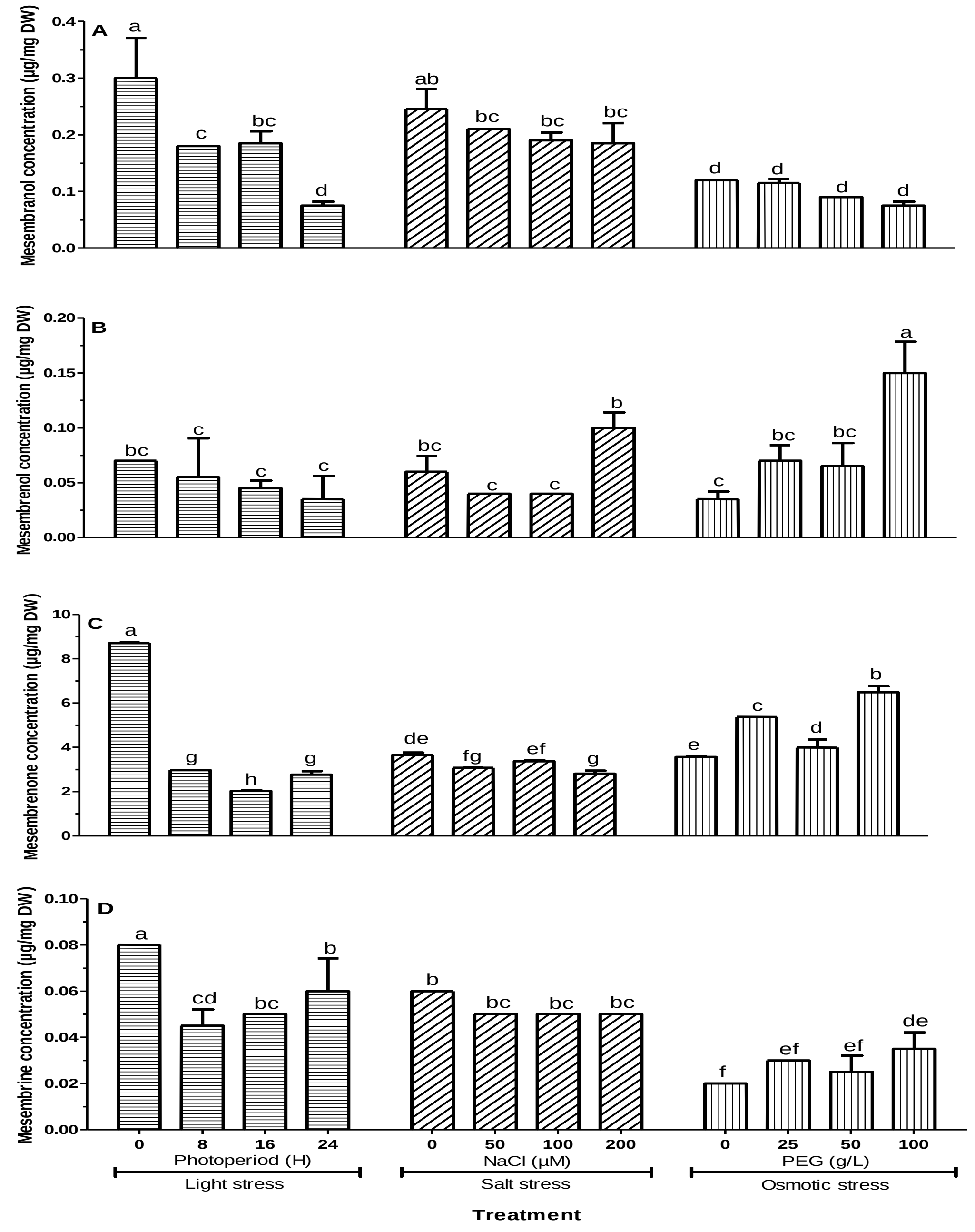

Figure 2.5: Concentrations of mesembranol **(A)**, mesembrenol **(B)**, mesembrenone **(C)**, and mesembrine **(D)** contained in *Sceletium tortuosum* samples obtained from light, salt, and osmotic treatments (PEG = polyethylene glycol). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Significant differences were apparent among treatments for each of the alkaloids evaluated **(Figure 2.5)**. Mesembranol concentration peaked in the 0 H light treatment (0.30 ± 0.071 µg/mg DW), whereas 24 H light was responsible for the lowest concentration in this group (0.075 ± 0.0071 µg/mg DW). Osmotic stress treatments resulted in concentrations that were markedly similar to the 24 H light treatment, with 100 g/L PEG also having a concentration of 0.075 ± 0.0071 µg/mg DW. The NaCl and PEG controls contained high concentrations of mesembranol (0.25 ± 0.035 and 0.12 µg/mg DW, respectively), with decreases observed with increasing degrees of stress in both treatment groups. However, the differences observed within these groups were not statistically significant **(Figure 2.5A)**.

Within the light stress group, mesembrenol concentration was highest for 0 H light (0.070 µg/mg DW). Mesembrenol concentration declined as photoperiod increased, however these treatments were not significantly different from 0 H light **(Figure 2.5B)**. For salt and osmotic stress groups, 200 µM NaCl (0.10 ± 0.014 µg/mg DW) and 100 g/L PEG (0.15 ± 0.028 µg/mg DW) resulted in peak concentrations of mesembrenol. The 24 H light treatment, along with the PEG control resulted in the lowest mesembrenol concentrations (0.035 µg/mg DW). Interestingly, 50 and 100 µM NaCl were responsible for the lowest mesembrenol concentrations (0.040 µg/mg DW) in the salt treatment group. In fact, these treatments produced mesembrenol concentrations that were significantly lower than 200 µM NaCl (0.10 ± 0.014 µg/mg DW) **(Figure 2.5B)**.

Mesembrenone was the most abundant alkaloid across all samples **(Figure 2.5)**. Zero H light was responsible for the highest mesembrenone concentration (8.71 ± 0.042 µg/mg DW), followed by 100 g/L PEG (6.49 ± 0.28 µg/mg DW). Within light and osmotic stress groups, 16 H light (2.03 ± 0.028 µg/mg DW) and the PEG control (3.57 ± 0.0071 µg/mg DW) resulted in the lowest concentrations of mesembrenone. With regards to salt, concentrations ranged from 2.81–3.37 µg/mg DW, with the maximum found in the salt control (3.66 ± 0.099 µg/mg DW) **(Figure 2.5C)**.

Mesembrine concentration peaked in the 0 H light treatment (0.080 µg/mg DW) **(Figure 2.5D)**. Concentration was significantly lowered by 8 H light (0.045 ± 0.0071 µg/mg DW), but increases were observed from 8 H to 24 H (max. 0.060 ± 0.014 µg/mg DW). The lowest levels of mesembrine stemmed from the osmotic stress group. The PEG control resulted in the overall lowest concentration of this alkaloid (0.020 µg/mg DW), followed by 50, 25, and 100 g/L PEG (0.025 ± 0.0071; 0.030; and 0.035 ± 0.0071 µg/mg DW, respectively). Mesembrine concentrations achieved by the salt treatments were significantly higher than the osmotic stress treatments. In fact, all three levels of salt stress resulted in the same amount of mesembrine (0.050 µg/mg DW) **(Figure 2.5D)**. Interestingly, concentrations of mesembranol, mesembrenol, and mesembrine were similar for all degrees of salt stress **(Figure 2.5)**.

Table 2.2: Mesembrine-type alkaloid concentrations from the most stressful treatments (this study) and similar plant material assayed by other research groups

	Mesembranol (mg/kg DW)	**Mesembrenol (mg/kg DW)**	**Mesembrenone (mg/kg DW)**	**Mesembrine (mg/kg DW)**
Zhao *et al.* (2018) **Field-grown**	364.4	182.2	377.9	172.5
Makunga *et al.* (2022) ***In vitro*-derived (control)**	1034.3	190.1	34	574.3
This study **0 H light**	300	70	8710	80
100 g/L PEG	75	150	6490	35
200 µM NaCl	190	100	2810	50

*Concentrations from this study were converted from µg/mg to mg/kg using: $\mathcal{X}$ µg/mg x 1000

2.4 Discussion

2.4.1 Plantlet growth

This study was performed in the absence of PGRs in the tissue culture media. This makes it abundantly clear that *S. tortuosum* growth, in terms of root and shoot development, can occur without supplementation. It has been suggested that the plant contains sufficient levels of endogenous auxins to support root development (Makunga *et al.*, 2022). Additionally, a study conducted by our group (Sreekissoon *et al.*, 2021), proved that *S. tortuosum* contains substantial levels of endogenous cytokinins, thus accounting for its successful shoot development.

The effects of different types of abiotic stress on plant growth has shown variable results. Light stress exhibited extraordinary effects on plantlet growth, development, and alkaloid production. Plantlets grew optimally under the 24 H photoperiod **(Figure 2.1)**. Although this can be perceived as an extreme stress, plantlets tissues may have acclimated to this light regime because it was initially used to grow plant material for the study (Vialet-Chabrand *et al.*, 2017). Proof for this acclimation hypothesis could be the reduction in growth observed with shorter photoperiods, with growth optimization in 24 H light **(Figure 2.1)**.

Interestingly, plantlets were able to grow in darkness **(Figures 2.1** and **2.2A)**. In nature, *S. tortuosum* typically grows under shrubbery or in partial shade (Chesselet, 2005). This is indicative of the species' adaptations to low light. Hence, growth in darkness could be a result of shade-tolerance behaviours at work (Valladares and Niinemets, 2008). Lowering dark respiration, investing in storage, and shifting from growth maximization to utilizing resources sparingly, are some of the mechanisms employed (Canham *et al.*, 1999; Craine and Reich, 2005; Gaucher *et al.*, 2005; Gommers *et al.*, 2013; Grubb, 1998; Walters and Reich, 2000). Additionally, the 24 H photoperiod utilized for initially growing study material likely enhanced photosynthetic rate. This would have resulted in a significant accumulation of carbohydrates (Dorais *et al.*, 1996; Proietti *et al.*, 2023). Such reserves may have aided in growth during the dark period.

Osmotic stress had detrimental effects on the three parameters evaluated **(Figure 2.1)**. In general, plants are highly sensitive to changes in water availability, as it impacts growth and can ultimately lead to death (Osakabe *et al.*, 2014). *Sceletium tortuosum* is commonly found in arid regions, however, it is not known to be a drought-tolerant species. The effects of osmotic stress on the species have not been reported. However, the closest analogy was found in Faber *et al.*'s (2020) greenhouse study, which involved fertigating plants with a nutrient solution 1–5 times in a 5-week period. Plant growth was optimal with fertigation taking place at 3-week intervals, as compared to less frequent fertigation regimes. From this, along with findings from the present study **(Figure 2.1)**, it can be inferred that *S. tortuosum* is drought-sensitive.

Drought-sensitive species have been known to exhibit reduced growth potential in response to the stress. For instance, shoot growth and plant weights of *Myrtus communis* and *Carrizo citrange* were significantly reduced by increasing PEG concentration in culture media (Simsek, 2018; Tafreshi *et al.*, 2021). In the same manner, PEG decreased rooting percentages in cultures of *Trachyspermum ammi* (Niazian *et al.*, 2021).

Remarkably, growth was more favourable in darkness than under osmotic stress **(Figures 2.1** and **2.2)**. Lowered osmotic potential could have hindered photosynthesis, impeded adequate break-down of stored sugars, and slowed transport of resources to necessary regions (Akıncı and Lösel, 2012; Cramer *et al.*, 2011). However, effective shade-tolerance strategies, coupled with sufficient water availability, improved growth capabilities (Craine and Reich, 2005; Gommers *et al.*, 2013).

Raised salinity (100 and 200 µM NaCl) improved plantlet growth more so than the control treatment **(Figure 2.1)**. This points to the species having a halophytic nature. Halophytes are able to tolerate raised salinity and can thrive in such environments (Chen and Wang, 2024). For halophytic species, *Salicornia herbacea* and *Sesuvium portulacastrum*, growth was optimized with heightened salinity as compared to saline-free controls (Lee *et al.*, 2016; Lokhande *et al.*, 2011).

Many members of the *Mesembryanthemum* genus are well-known halophytes, including *M. crystallinum* and *M. edule* (Falleh *et al.*, 2013; Yang and Yen, 2002). Interestingly, Von Willert *et al.* (1977) noted that *Sceletium joubertii* (now reduced to synonymy as *S. tortuosum*) showed high accumulations of Na^+ and Cl^-. Based on this, the researchers suggested that the species was halophilic. The present findings lend support for this early notion.

2.4.2 Alkaloid production

Impressively, 0 H light yielded the highest total alkaloid content and maximum concentrations of three of the four alkaloids **(Figures 2.4 and 2.5)**. Alkaloids are typically produced at higher levels during periods of stress (Akula and Ravishankar, 2011; Muthusamy and Lee, 2024). Thus, it is likely that heightened alkaloid contents were a consequence of the shock and stress experienced with the transfer from high light to darkness.

Our results contradict previous studies reporting that light more effectively enhanced alkaloid production (Koshiishi *et al.*, 2000; Kurata *et al.*, 1997). However, Waller and Nowacki (1978) reported on early studies involving *Nicotiana tabacum* seedlings that produced higher alkaloid quantities in darkness than in light. Sauerwein *et al.* (1992) found that alkaloid quantities of transformed root cultures of *Hyoscyamus albus* were enhanced with darkness incubation. Similar to these results, *Hyoscyamus muticus* cultures in the absence of light also responded with decreased growth, but increased alkaloid production (Aly *et al.*, 2010).

Medicinal plants tend to respond to water deficits by increasing their secondary metabolite contents (Faber *et al.*, 2021; Punetha *et al.*, 2022). In the case of *S. tortuosum*, this was evidenced by the large alkaloid fractions obtained from the osmotic stress treatments **(Figure 2.4)**. Faber *et al.* (2020) showed that the highest total alkaloid contents were found in *S. tortuosum* plants fertigated once in 3–4 weeks, as opposed to more frequently. Additionally, Makunga *et al.* (2022) dehydrated *S. tortuosum* plantlets by exposing cultures to laminar air flow. The resulting plant material contained higher concentrations of mesembrine-type alkaloids as compared to the control treatment.

Other medicinal plant species have also benefitted from induced drought stress. For instance, alkaloid production was improved in *Trigonella foenum* and *Catharanthus roseus* cultures with the addition of PEG to culture media (Abdulwahid *et al.*, 2024; Alalwani and Alrubaie, 2016). The findings of Razavizadeh and colleagues (2019) are remarkably similar to the present study. They showed that PEG application (0–80 g/L) reduced growth of *Thymus vulgaris* whilst simultaneously increasing secondary metabolite content.

Notably, total alkaloid contents were relatively constant in the salt stress group, as compared to the light and osmotic stress groups **(Figure 2.4)**. Additionally, the concentrations of all alkaloids were in a similar range for all salt treatments **(Figure 2.5)**. The inherent ability of a species to maintain a moderate production of phytochemicals in the presence of raised salinity is further evidence of a halophytic profile (Buhmann and Papenbrock, 2013; Nikalje *et al.*, 2018).

Mesembrythemum crystallinum responded to salt stress (400 mM NaCl) with an increased accumulation of polyols (compounds necessary for secondary metabolite production) (Agarie *et al.*, 2009). This could in turn result in an increase in secondary metabolite production. This notion could explain the elevated mesembrenol concentration caused by the highest degree of salt stress in this investigation **(Figure 2.5B)**.

Lee *et al*. (2016) reported that indole alkaloid concentrations increased with increasing salinity in *Salicornia herbacea* plants. Indole alkaloids (which includes mesembrine-type alkaloids) perform a protective function by reducing oxidative damage caused by stress (Cordell, 2013; Lee *et al.*, 2016). Thus, it can be postulated that mesembrenol has a more pivotal protective role than mesembrenone. This could account for the increase in mesembrenol coinciding with a reduction of mesembrenone **(Figure 2.5B and C)**. Fundamentally, these works suggest that much higher salt concentrations may be appropriate for increasing the concentrations of the remaining mesembrine-type alkaloids.

Mesembrenol concentrations were highest for the 100 g/L PEG, 200 µM NaCl, and 0 H light treatments **(Figure 2.5B)**. This could be an indication of a cellular protective function of this alkaloid during high levels of stress (Lee *et al.*, 2016). Thus, these treatments may be regarded as the most stressful.

To date, only a single other study has evaluated mesembrine-type alkaloid production *in vitro* (Makunga *et al.*, 2022). The authors investigated morphotypic variation in *S. tortuosum* cultures and analyzed alkaloid contents of the resulting microshoots. However, they also analyzed whole plantlets as part of their control. Essentially, plantlets from the present study contained much lower levels of mesembrine-type alkaloids, with the exception of mesembrenone **(Table 2.2)**. Different experimental conditions (6-month incubation, 16 H light, BAP-containing medium, etc.) may be responsible for this discrepancy. More likely though, is the possibility that different chemotypes were used in these investigations.

Zhao *et al.* (2018) reported that *S. tortuosum* has various chemotypes. They collected plants from several regions of the Cape and analyzed alkaloid contents. Most chemotypes contained mesembrine as the major alkaloid, however specimens collected from Oudtshoorn contained mesembrenone in the highest quantity **(Table 2.2)**. This is consistent with the results from all samples in this study **(Figures 2.3 and 2.5)**. Furthermore, our alkaloid quantities are comparable to the wild-types reported in the Zhao *et al.*'s (2018) study **(Table 2.2)**. In fact, the mesembrenone quantities achieved by our most stressful treatments are 7–23-fold higher than the wild-type. Collectively, this shows a conservation of genetic and chemotypic diversity, even years after collection and re-establishment under various growth conditions. Additionally, it shows that mesembrenone concentration can be enhanced far beyond what is found in nature using stress conditions.

2.5 Conclusions

Plantlets grew optimally under 24 H and 16 H photoperiods, while osmotic stress proved detrimental to plantlet health. Growth was observed with incubation in darkness. Moreover, growth was more optimal in darkness than for osmotic stress treatments. These results were indicative of shade tolerance and drought-sensitivity behaviours.

Saline conditions (100 and 200 µM NaCl) improved growth in comparison to the control. Additionally, the resulting alkaloid contents and quantities from salt treatments were remarkably similar and conserved. These findings confirm that *S. tortuosum* is indeed a halophyte.

Zero H light and 100 g/L PEG achieved the highest total alkaloid contents. Mesembranol, mesembrenone, and mesembrine concentrations were optimal in plantlets incubated in darkness, while mesembrenol concentration was highest in osmotic stress treatment.

Notably, 0 H light, 100 g/L PEG, and 200 µM NaCl were responsible for the highest mesembrenol concentrations. It was postulated that mesembrenol plays a crucial protective role against potential oxidative damage arising from these stress conditions. These treatments were considered the most stressful to *S. tortuosum* plantlets.

Ultimately, 0 H light permitted growth whilst simultaneously increasing alkaloid contents. However, given that growth was suboptimal in darkness, it should be further investigated how long plantlets can survive under this condition, and whether alkaloid production can be further enhanced in this way. Light quality and shade are factors that warrant further examination for the species.

2.6 References

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Chapter 3: Effects of abiotic stress on primary metabolite accumulation in tissue culture

3.1 Introduction

3.1.1 Carbohydrates and starch

Carbohydrates and starch are the primary sources of carbon in plants. Effective utilization, transport, and storage of carbon are essential for healthy growth and development (Apriyanto *et al.*, 2022; MacNeill *et al.*, 2017). Chief among the carbohydrates is glucose, as it is crucial for respiration. Furthermore, glucose is necessary for cell wall formation, chlorophyll production, protein synthesis, and a myriad of other necessities in plants (Siddiqui *et al.*, 2020).

Since glucose is highly reactive, it is not translocated from one region to another. Instead, it is converted to sucrose, which serves as a transport carbohydrate (Geiger, 2020). Once translocated to the necessary organs, sucrose may be utilized or stored (Lemoine, 2000). Alternatively, glucose is polymerized to form starch granules for storage (Dong and Beckles, 2019).

A source-sink relationship exists for carbohydrates and starch. Essentially, leaves are the primary source of free glucose as it is generated from photosynthesis. However, glucose may be needed to support respiration and encourage growth in other organs, e.g. roots. In this instance, leaves are the carbohydrate source, while roots are the carbohydrate sink. In the absence of free glucose, starch is metabolized to supply carbohydrates to the necessary regions, and the source-sink relationship remains (Dong and Beckles, 2019; MacNeill *et al.*, 2017).

Starch supports respiration and growth in periods of darkness, seasonal regrowth, and stress conditions (MacNeill *et al.*, 2017; Zeeman and Solhaug, 2022). In stressful situations, starch may accumulate or degrade (Thalmann *et al.*, 2016; Wang *et al.*, 2013). Its degradation increases carbohydrates levels (Boyer, 2010). The resulting carbohydrates may be used to support primary metabolic functions, and they may act as osmoprotectants (Dong and Beckles, 2019; Zeeman and Solhaug, 2022).

Starch, unsurprisingly, affects secondary metabolism through its release of usable carbohydrates (Ibrahim *et al.*, 2012). Carbohydrates provide the necessary energy and carbon skeletons for amino acid synthesis (Trovato *et al.*, 2021). Amino acids are in turn used to generate secondary metabolites. Thus, increased carbohydrate contents can positively influence phytochemical production (Ibrahim and Jaafar, 2012; Khan *et al.*, 2018).

3.1.2 Proteins

Proteins are formed from amino acids (Day, 1996). They are used in the structural formation of various cell components and machinery (Guerra-Guimarães *et al.*, 2016; Jain *et al.*, 2022). For instance, enzymes, which are essential for the catalysis of countless chemical reactions, are proteins (Day, 1996). Proteins also have regulatory roles and are involved in signalling and transport (Day, 1996; Zhou *et al.*, 2022). Additionally, they play protective and defensive roles against abiotic and biotic stresses (Jain *et al.*, 2022; Zhou *et al.*, 2023). Certain proteins (e.g. heat shock proteins, dehydrins, ROS scavenging enzymes) are generated exclusively for stress tolerance purposes (Hanin *et al.*, 2011; Sun *et al.*, 2002; Wang *et al.*, 2019).

Transcription factors are also proteins, and are involved in stress detection, and the induction of genes responsible for secondary metabolite production (Jan *et al.*, 2021). Furthermore, proteins contribute to secondary metabolism through the action of biosynthetic enzymes (Rischer *et al.*, 2013). They are also involved in phytochemical transport (Shitan, 2016).

3.1.3 Proline

The amino acid proline is characterized as a stress marker (Boscaiu *et al.*, 2013). This is due to its accumulation in stress conditions (e.g. salinity, water, temperature stress) (Salam *et al.*, 2023). As such, proline contributes to stress amelioration or recovery (Hayat *et al.*, 2012). It up-regulates ROS scavenging, protects the photosynthetic apparatus, and improves stress tolerance (Brini and Saibi, 2023; El Moukhtari *et al.*, 2020). Proline acts as an osmoprotectant under conditions of water and salt stress (Salam *et al.*, 2023). Increased tolerance to heat, drought, and salt, have been triggered by enhanced proline levels (El Moukhtari *et al.*, 2020; Hanif *et al.*, 2021).

Proline has been implicated in signalling, protein synthesis, and enzyme activity (Nanjo *et al.*, 1999; Rady *et al.*, 2016). Improvements in starch and sucrose metabolism have been reported in its presence (Zuo *et al.*, 2022). Furthermore, proline impacts secondary metabolism (Zuo *et al.*, 2022). Although rarely a direct precursor for phytochemical biosynthesis, proline has been found to increase secondary metabolite production, likely due to coupling of the proline cycle to the pentose phosphate pathway. The products of this pathway have implications for the shikimate pathway, which generates precursors essential for the biosynthesis of various secondary metabolites (Funayama and Cordell, 2014; Perassolo *et al.*, 2013; Zuo *et al.*, 2022).

Stress conditions have significant effects on primary metabolites. Moreover, these metabolites have consequences for secondary metabolism. Thus, four primary metabolites (carbohydrates, starch, proteins, and proline) were evaluated in *S. tortuosum* under stress conditions.

3.1.4 Aims and objectives

This experiment aimed to (1) determine the effects of stress on primary metabolite concentrations, and (2) evaluate possible correlations between primary and secondary metabolites, and growth of *S. tortuosum*.

The following objectives were set:

1) Replicate the stress experiment utilizing the original experimental conditions **(Section 2.2.3)**,
2) Upon culture harvest, perform carbohydrate, starch, protein, and proline extraction procedures,
3) Quantify primary metabolites using a spectrophotometer,
4) Conduct a comparative analysis of primary metabolite concentrations, and
5) Discuss primary metabolites in terms of secondary metabolites, and *S. tortuosum* growth.

3.2 Materials and methods

Stress treatments, explant source, and experimental conditions were as described in **Section 2.2.3**.

Each of the following bioassays made use of 200 mg fresh plant material (intact plantlets) obtained from stress cultures incubated for 28 days. Plant material was homogenized using a tissue grinder (IKA® ULTRA-TURRAX®).

3.2.1 Carbohydrates and starch

Quantification methods were as described by Sadasivam and Manickam (1996), with some modifications. A glucose stock solution (1 mg/mL) was used to prepare standard concentrations of 0, 20, 40, 60, 80, and 100 µg/mL with distilled water. The reagent was prepared using 95% ice-cold sulphuric acid and anthrone (final conc. 2 mg/mL anthrone).

For carbohydrate quantification, plant material was homogenized with 4 mL 2.5 M hydrochloric acid and heated in a boiling water bath for three hours. Test tubes were cooled to room temperature before neutralizing the solutions with sodium carbonate (i.e. until effervescence ceased). The volume of all samples was adjusted to 10 mL with distilled water. The tubes were then centrifuged at 3 000 *g* for 15 min at 25°C.

For starch quantification, plant material was homogenized with 6 mL hot ethanol (80%) and centrifuged at 3 000 *g* for 15 min at 25°C. Samples were then rinsed three times with hot ethanol (80%), and the residues were dried over a boiling water bath. Following this, 2 mL distilled water and 3 mL perchloric acid (52%) were added to the residues and the tubes were placed on ice for 20 min.

Aliquots (500 µL) from both sets of samples were placed in separate test tubes and the volume was made up to 1 mL with distilled water. Anthrone reagent (2 mL) was added to all sample and standard tubes. The tubes were then heated in a boiling water bath for 8 min, then cooled on ice. The tubes were vortexed briefly and the absorbance of samples was measured at 630 nm using a spectrophotometer (JENWAY Genova Plus). The zero standard concentration was used as the blank for the spectrophotometer.

3.2.2 Proteins

Protein was quantified using the Bradford (1976) method with some modifications. Bovine serum albumin (BSA) was used as a standard and prepared to a concentration of 100 µg/mL with phosphate-buffered saline (PBS). Standard concentrations of 0, 20, 40, 60, 80, and 100 µg/mL BSA were prepared using PBS.

Plant material was homogenized with 6 mL PBS and centrifuged at 15 000 *g* for 15 min at 4°C. Aliquots (1 mL) of supernatant were placed in new test tubes. Bradford reagent (1 mL) was added to all sample and standard tubes, which were then briefly vortexed and allowed to stand for 5–10 min. Absorbance was read at 595 nm using a spectrophotometer. The zero standard concentration was used as the blank for the spectrophotometer.

3.2.3 Proline

The methods of Sadasivam and Manickam (1996) and Shabnam *et al.* (2016) were adapted for proline quantification. A stock solution of proline (1 mg/mL) was used to prepare standard concentrations of 0, 20, 40, 60, 80, and 100 µg/mL with distilled water. Using glacial acetic acid and ninhydrin, the reagent was prepared (final conc. 1.25% ninhydrin).

Plant material was homogenized with 4 mL sulphosalicylic acid (3%) and centrifuged at 15 000 *g* for 15 min at 4°C. Aliquots (1 mL) of supernatant were transferred to new test tubes. Ninhydrin reagent (2 mL) was added to all sample and standard tubes, prior to heating in a boiling water bath for 30 min. Thereafter, the tubes were cooled on ice, vortexed briefly, and absorbance was measured at 520 nm using a spectrophotometer. The zero standard concentration served as the blank.

3.2.4 Data and statistical analysis

Calibration curves were plotted using the absorbance data from the prepared standards. Sample concentrations were extrapolated from the calibration curve. Dilution factors were taken into account and the final metabolite concentrations were calculated.

Primary metabolite data were analysed by either one-way ANOVA with DMRTs, or Kruskal-Wallis test with pairwise comparisons. Mean and standard deviation values calculated by the software were plotted using Graphpad Prism V5. Linear regression analyses were conducted using primary metabolite and secondary metabolite data (total alkaloid contents obtained from **Chapter 2**). All statistical analyses were performed using SPSS V29.

3.3 Results

Abiotic stress treatments affected carbohydrate, starch, protein, and proline concentrations in *S. tortuosum* plantlets **(Figure 3.1)**. Carbohydrate concentrations were highest in the salt stress treatment group, as a result of 100 and 200 µM NaCl (383.3 ± 1.40 and 323.1 ± 0.72 µg/mg FW, respectively) **(Figure 3.1A)**. Within the osmotic stress group, some fluctuations were apparent; however, a general downward trend was observed as carbohydrate concentrations declined with increasing stress level. As a result, 100 g/L PEG yielded the lowest carbohydrate concentration (85.37 ± 0.15 µg/mg FW). Contrastingly, carbohydrate concentrations increased with longer photoperiods (8–24 H; 81.47–178.87 µg/mg FW). The overall lowest carbohydrate concentrations were as a result of 0 and 8 H light treatments (82.07 ± 0.29 and 81.47 µg/mg FW, respectively). These two treatments were statistically similar **(Figure 3.1A)**.

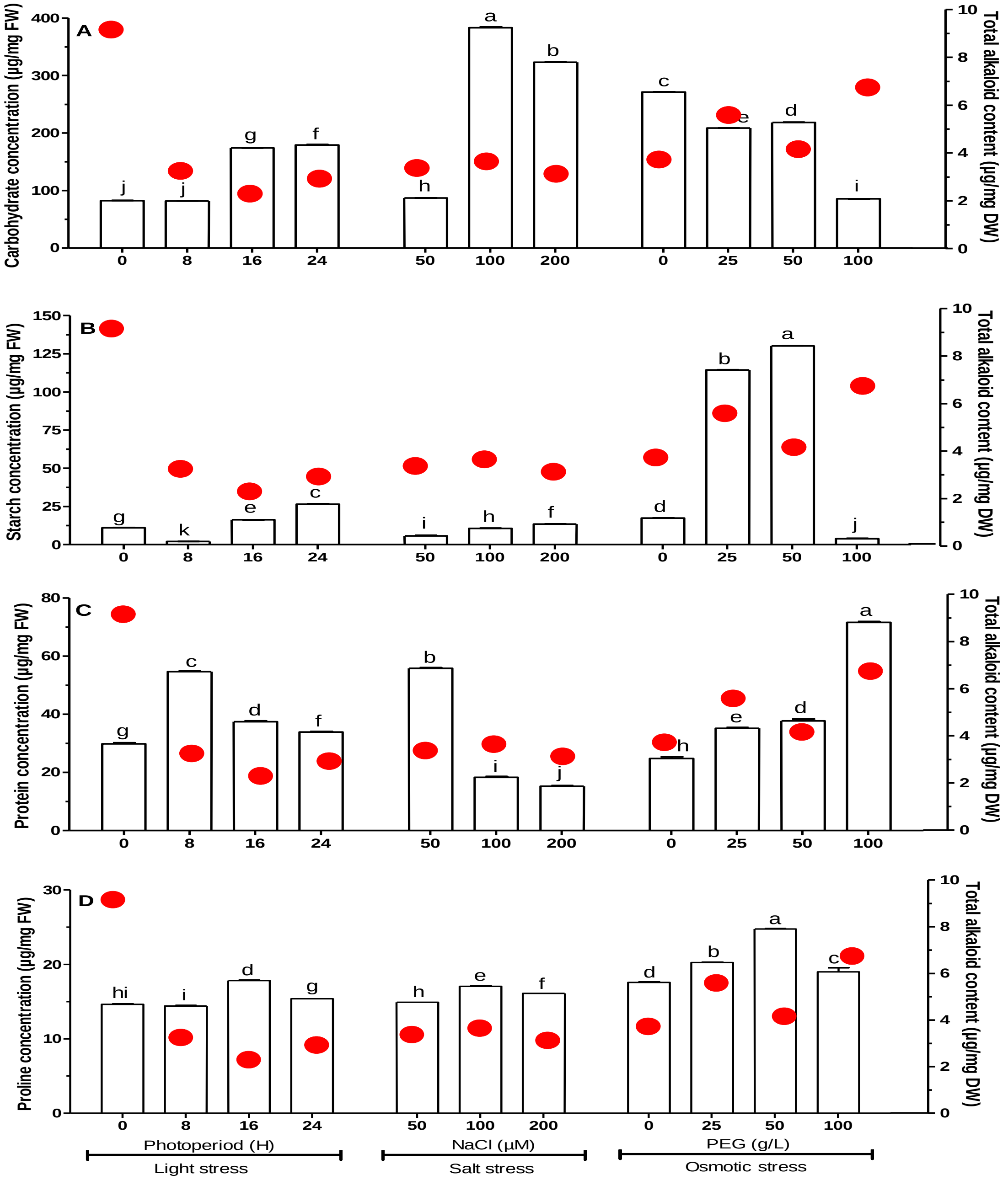

Figure 3.1: Concentrations of carbohydrates **(A)**, starch **(B)**, proteins **(C)**, and proline **(D)** in *Sceletium tortuosum* plantlets exposed to various abiotic stress treatments for 28 days. The 24 H light treatment serves as the control for light and salt stress. Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments. Scatterplots (red) show total alkaloid contents obtained from **Chapter 2**.

Concentrations of the remaining metabolites were highest in the osmotic stress group **(Figure 3.1B-D)**. Within this group, starch concentration increased in accordance with the level of osmotic stress (17.33–130.10 μg/mg FW), with the exception of 100 g/L PEG (3.82 ± 0.23 μg/mg FW) **(Figure 3.1B)**. Similarly, starch concentration increased in accordance with salt concentration (5.79–13.39 μg/mg FW), and increasing photoperiod (8–24 H; 2.02–26.48 μg/mg FW). However, 0 H light resulted in a higher starch concentration than 8 H light (11.02 ± 0.23 and 2.02 ± 0.18 μg/mg FW, respectively) **(Figure 3.1B)**.

Protein concentrations increased as the stress level was elevated in the osmotic stress group. Hence, 100 g/L PEG resulted in the highest protein concentration (71.60 ± 0.30 μg/mg FW) **(Figure 3.1C)**. Among salt stress treatments, 50 μM NaCl achieved the highest protein concentration (55.82 ± 0.17 μg/mg FW). Further increases in salt concentration resulted in decreases in protein concentration (18.29 ± 0.32 and 15.19 ± 0.27 μg/mg FW, for 100 and 200 μM NaCl, respectively). A similar trend was observed for the light stress group, where protein concentration decreased from 8–24 H (54.67–33.92 μg/mg FW), although 0 H resulted in the lowest protein concentration (29.84 ± 0.34 μg/mg FW) **(Figure 3.1C)**.

Proline concentrations were enhanced by increasing levels of osmotic stress, up until 50 g/L PEG (17.57–24.73 μg/mg FW) **(Figure 3.1D)**. However, 100 g/L PEG lowered proline concentration (19.00 ± 0.56 μg/mg FW). Proline concentrations were in a similar range for salt stress treatments (14.90–17.07 μg/mg FW). Within the light stress group, 16 H was responsible for the highest proline concentration (17.83 ± 0.058 μg/mg FW). Zero and 8 H light treatments resulted in the lowest proline concentrations overall (14.63 ± 0.058 and 14.40 ± 0.10 μg/mg FW). These treatments were found to be statistically similar **(Figure 3.1D)**.

Table 3.1: Linear regression analyses on the correlation between primary metabolite concentrations obtained from *Sceletium tortuosum* plantlets exposed to abiotic stress treatments

Variables tested	**R^2**	**F**	**t**	**Sig.**
Carbohydrate concentration **x** Starch concentration	0.029	0.913	0.956	0.347
Carbohydrate concentration **x** Proline concentration	0.058	1.911	1.382	0.177
Carbohydrate concentration **x** Protein concentration	0.643	55.925	-7.478	**<0.001**
Starch concentration **x** Proline concentration	0.694	70.410	8.391	**<0.001**
Starch concentration **x** Protein concentration	0.015	0.458	-0.677	0.504
Proline concentration **x** Protein concentration	0.001	0.022	0.148	0.883

R^2 indicates the correlation coefficient; Sig. = significance of the correlation ($p < 0.05$). F and t statistics are provided. Values in bold are statistically significant.

There appeared to be a correlation between carbohydrate and protein concentrations. For salt stress treatments, a decline in carbohydrates coincided with an increase in protein concentration, and vice versa **(Figure 3.1A and C)**. Light stress treatments increased carbohydrate and starch concentrations whilst decreasing protein concentration. However, the 0 H treatment did not conform to this pattern **(Figure 3.1A-C)**. With regards to osmotic stress, decreases in carbohydrate concentration were consistent with increases in starch, protein, and proline concentrations **(Figure 3.1)**. Additionally, 8 H light, 50 µM NaCl, and 100 g/L PEG resulted in extremely low carbohydrate and starch concentrations, but protein concentrations were enhanced. Protein concentrations from these treatments were significantly higher than the respective controls **(Figure 3.1A-C)**. A statistically significant correlation was apparent between carbohydrate and protein concentrations, however not between starch and carbohydrates, or starch and protein **(Table 3.1)**.

Based on **Figure 3.1**, a relationship was also thought to exist between starch and proline concentrations. For instance, high starch concentrations were observed for 25 and 50 g/L PEG, and this coincided with the highest concentrations of proline. Also, the 100 g/L PEG treatment resulted in minimal starch and a lowered proline concentration **(Figure 3.1A and D)**. Furthermore, starch concentrations from light and salt stress treatments were relatively low, as was the case for proline concentrations **(Figure 3.1C and D)**. A significant correlation between these two metabolites was supported statistically **(Table 3.1).**

Table 3.2: Linear regression analyses on the correlation between total mesembrine-type alkaloid content and primary metabolite concentrations obtained from *Sceletium tortuosum* plantlets exposed to abiotic stress treatments

Variables tested	**R^2**	**F**	**t**	**Sig.**
Total alkaloid content **x** Carbohydrate concentration	0.154	3.652	-1.911	0.070
Total alkaloid content **x** Starch concentration	0.004	0.086	0.293	0.773
Total alkaloid content **x** Proline concentration	0.002	0.033	0.182	0.857
Total alkaloid content **x** Protein concentration	0.029	0.589	0.767	0.452

R^2 indicates the correlation coefficient; Sig. = significance of the correlation ($p < 0.05$). F and t statistics are provided.

There were no observable relationships between primary and secondary metabolite contents **(Figure 3.1)**. For instance, the lowest carbohydrate concentrations were achieved by the 0 and 8 H light treatments (82.07 ± 0.29 and 81.47 µg/mg FW), however, total alkaloid content was highest in the 0 H treatment, and significantly lower in the 8 H treatment (9.16 and 3.25 µg/mg DW, respectively) **(Figure 3.1A)**. Additionally, the highest protein concentration was achieved by 100 g/L PEG (71.60 ± 0.30 µg/mg FW), and a significantly lower protein concentration resulted from 0 H light (29.84 ± 0.34 µg/mg FW). Yet, these treatments were responsible for the highest alkaloid yields (6.74 and 9.16 µg/mg DW, for 100 g/L PEG and 0 H light, respectively) **(Figure 3.1C)**. Upon statistical analysis, significant correlations between primary metabolites and total alkaloid content were not identified **(Table 3.2)**.

3.4 Discussion

3.4.1 Osmotic stress

Carbohydrate concentrations were reduced as osmotic stress increased **(Figure 3.1A)**. This was unsurprising, as drought stress reduces photosynthesis, thus lowering carbohydrate concentration (Reddy *et al.*, 2004). Together with reductions in total carbohydrates, starch concentrations were elevated **(Figure 3.1A and B)**. It has been documented that water deficits increase the conversion of sugars to starch (Dong and Beckles, 2019; Liu *et al.*, 2019). In contrast, starch may be metabolized under osmotic stress to serve as an alternate carbon source (Boyer, 2010; Thalmann *et al.*, 2016). Therefore, it is possible that the 100 g/L PEG treatment severely reduced carbohydrate concentration, leading to enhanced starch degradation to increase carbohydrate availability **(Figure 3.1A and B)**.

Protein concentrations increased as the level of osmotic stress was raised **(Figure 3.1C)**. This was contrary to the findings of Lai *et al.* (2007), who reported lowered protein contents with increasing osmotic stress. However, Pakzad *et al.* (2023) found that a number of proteins were over-expressed by *Pistacia vera* under osmotic stress. Riccardi *et al.* (1998) reported that osmotic stress can trigger the expression of stress-response proteins, e.g. dehydrins and response to abscisic acid (RAB) proteins. Additionally, proteins that may not be directly related to osmotic stress can also be induced, e.g. heat shock proteins (Riccardi *et al.*, 1998). The increased synthesis of proteins may explain the decrease in carbohydrate and starch concentrations observed for the most severe osmotic treatment **(Figure 3.1A-C)**.

Proline levels were most elevated in the osmotic stress group **(Figure 3.1D)**. This is likely due to the amino acids' multifunctional roles in osmotic stress response, including osmotic adjustment, protection of cell structures, and redox scavenging (Verslues and Sharma, 2010). Notably though, a decrease in proline concentration was noted for 100 g/L PEG. This coincided with a substantially large protein concentration **(Figure 3.1C and D)**. It has been established that proline is utilized in the synthesis of proteins. Such proteins are essential for cell wall modification, signal transduction, and stress tolerance (Kavi Kishor *et al.*, 2015). Proline-rich proteins were found to accumulate in response to drought stress (Battaglia *et al.*, 2007). Thus, under extreme osmotic stress (100 g/L PEG), proline level likely decreased to allow for the synthesis of other essential metabolites.

3.4.2 Salt stress

Salt stress affects enzymes associated with photosynthesis and sugar synthesis (Fan *et al.*, 2011; Zhao *et al.*, 2019). It was reported that low levels of salt stress up-regulated these enzymes in *Salicornia europaea* (Fan *et al.*, 2011). This could account for the raised protein concentration caused by the 50 µM NaCl treatment **(Figure 3.1C)**. Furthermore, salinity stress enhanced protein accumulation in the closely related halophyte, *Mesembryanthemum crystallinum* (Thomas and Bohnert, 1993).

Fan *et al.* (2011) found that high salt concentrations resulted in reduced enzyme levels. This phenomenon was also observed in *Oryza sativa*, and elevated sucrose concentration was the outcome (Udomchalothorn *et al.*, 2009). Similarly, reduced protein concentration and increased carbohydrate concentration were observed for 100 and 200 µM NaCl **(Figure 3.1A and C)**. These earlier reports lend support for the correlation observed between carbohydrate and protein concentrations **(Figure 3.1; Table 3.1)**.

Starch concentrations were quite low in comparison to carbohydrate levels. Additionally, starch concentrations were significantly lower for salt treatments as compared to the control **(Figure 3.1A and B)**. It is well-established that starch concentrations decrease under salinity stress (Parida and Das, 2005). However, it was also discovered that lowered starch content correlated with increased sugar content in *Setaria sphacelate* and *Bruguiera parviflora* (da Silva and Arrabaça, 2004; Parida *et al.*, 2002). This is strikingly similar to the behaviour of these metabolites in the current study **(Figure 3.1A and B)**. It was suggested that starch metabolism is more disturbed by salinity stress than sucrose synthesis (da Silva and Arrabaça, 2004).

Regardless, slight increases in starch concentration were observed with increasing salinity levels. This may simply be attributed to the substantial amounts of carbohydrates present **(Figure 3.1A and B)**. An abundance of carbohydrates has been known to encourage the conversion of sugars to starch to prevent over-accumulation of sugars (Dong and Beckles, 2019). Such an accumulation could negatively affect the expression of genes involved in photosynthesis, osmolyte synthesis, as well as sucrose metabolism (Rosa *et al.*, 2009).

Several researchers showed that proline accumulation increases in accordance with the level of salt stress (Pingle *et al.*, 2022; Poustini *et al.*, 2007; Wang *et al.*, 2015). Yet, the proline concentrations recorded in this study did not increase in such a manner **(Figure 3.1D)**. Poustini *et al.* (2007) reported that salt-tolerant wheat cultivars had much lower proline accumulations than salt-sensitive cultivars (5.2- vs 27.4-fold increases compared to the control). Salt treatments that enhanced proline accumulation also resulted in concentrations that were not much higher than the control treatment **(Figure 3.1D)**. These findings provide further evidence for *S. tortuosum* as a halophyte.

Plants can accumulate a diverse array of osmolytes under salt stress conditions, e.g. glycine betaine, polyamines, and sugars (Mansour and Ali, 2017; Sharma *et al.*, 2019). Halophytes, in particular, often accumulate ions (e.g. Na^+ and Cl^-) to support osmotic adjustment and reduce the need to synthesize osmolytes such as proline (Song *et al.*, 2009). Furthermore, carbohydrates sometimes serve as osmolytes in halophytes (Maimaiti *et al.*, 2016). This could account for the increased carbohydrate concentrations resulting from 100 and 200 µM NaCl treatments.

3.4.3 Light stress

Carbohydrate and starch concentrations increased together with increasing light exposure for 8–24 H treatments **(Figure 3.1A and B)**. Longer light exposures increase photosynthetic rate, hence the production of carbohydrates increases. This can cause a carbohydrate surplus, leading to increased sugar conversion to starch (Wu *et al.*, 2024). Supporting this, Luo *et al.* (2021) and Gibon *et al.* (2009) showed that shorter photoperiods decreased carbohydrate concentrations in *Pinus tabuliformis* and *Arabidopsis thaliana*. Additionally, starch is typically metabolized at night (Graf *et al.*, 2010). Thus, the increases in starch concentration in 8–24 H treatments can be explained by the reducing dark period.

Protein concentration was lowest in the 0 H light treatment **(Figure 3.1C)**. Darkness and extremely short photoperiods (e.g. 2 H) have caused significant decreases in protein contents in *A. thaliana* (Gibon *et al.*, 2009; Hannemann *et al.*, 2009). There appeared to be a relationship between protein and starch concentrations, though this was not supported statistically **(Figure 3.1B and C; Table 3.1)**. Regardless, a similar relationship was previously reported, i.e. decreasing protein concentration resulted in increased starch quantities (Gibon *et al.*, 2009; Hannemann *et al.*, 2009). It was suggested that: (1) reduced protein content lowers respiration such that less starch degradation is required to sustain respiration, and (2) protein synthesis may remain low to conserve energy (Gibon *et al.*, 2009).

Ultimately, protein concentration was higher in light than in darkness **(Figure 3.1C)**. This positive effect of light has been documented in several species, including *Glicine max* and *Raphanus sativus* (Mastropasqua *et al.*, 2020). However, it is worth noting that protein concentrations obtained from light (24 H) and dark treatments were somewhat similar, relative to the remaining light stress treatments **(Figure 3.1C)**. Watanabe *et al.* (2022) also noticed similarities in protein levels in *Oryza sativa* seedlings incubated under light and dark conditions. These researchers postulated that increased nutrient availability (from MS medium) significantly enhanced protein synthesis. Furthermore, light conditions were not found to affect the expression of protein-encoding genes (Granlund *et al.*, 2009).

3.4.4 Correlations between primary metabolites

It was consistent amongst the stresses tested that reductions in carbohydrate concentration coincided with increases in protein concentration. If not, the inverse was apparent **(Figure 3.1A-C)**. This correlation between carbohydrate and protein concentrations was statistically significant **(Table 3.1)**. This suggests that carbohydrates (and reserves) were utilized to cope with stressful conditions (Krasavina *et al.*, 2014). In this way, carbohydrates provided the essential building blocks for amino acids, and thus, protein synthesis (Trovato *et al.*, 2021). This ties in with the elevated protein concentrations observed **(Figure 3.1C)**. As previously mentioned **(Section 3.4.1),** various stress-response proteins (e.g., osmotin, germin, heat-shock proteins, dehydrins) and enzymes (e.g. ROS scavenging enzymes) are synthesized to cope with stress (Athar *et al.*, 2022; Zhou *et al.*, 2023).

Additionally, there was a significant correlation between starch and proline concentrations **(Table 3.1)**. Other authors have reported similar correlations between these two metabolites (Dien *et al.*, 2019; Đukić *et al.*, 2021). As starch is simply a carbohydrate reserve, increased quantities of starch lead to increased starch degradation, particularly under stress conditions. This provides energy, as well as components that are essential for proline synthesis. Thus, high levels of starch can induce high levels of proline (Zanella *et al.*, 2016).

3.4.5 Primary metabolites in relation to growth and secondary metabolites

Osmotic stress treatments resulted in the highest proline concentrations; however, negative growth effects were apparent within this treatment group **(Figure 3.1D; Chapter 2)**. This may be attributed to the over-accumulation of proline, which can cause toxicity and adversely impact plant growth (Hayat *et al.*, 2012). This would explain the low growth performance of osmotic stress treatments in comparison to other treatments **(Chapter 2)**. Based on this postulation, the significantly reduced proline accumulation in the 0 H light treatment could account for the more positive plant growth **(Figure 3.1D; Chapter 2)**.

Proline, however, should not be solely relied upon as an indicator of plant stress. This notion is largely based on the 0 H light treatment, which was regarded as one of the most stressful treatments **(Chapter 2)**. Yet, proline concentration proved to be extremely low **(Figure 3.1D)**. Moreover, healthy plantlet growth was documented for 16 H and 24 H light treatments, yet there were marked differences in proline concentrations between these treatments **(Chapter 2; Figure 3.1D)**. As previously mentioned **(Section 3.4.2)**, proline is not the only osmolyte that accumulates under stress conditions (Hayat *et al.*, 2012; Shim *et al.*, 2023). Furthermore, synthesis of other osmolytes, enzymes, and proteins could have taken precedence over proline, perhaps due to their more specialized roles in combating stress (Maimaiti *et al.*, 2016; Mansour and Ali, 2017).

There were no clear relationships between the four primary metabolites and total alkaloid contents of *S. tortuosum* **(Figure 3.1; Table 3.2)**. However, it is worth noting that a plethora of compounds form part of the carbohydrate and protein groups, and individual compounds were not specifically quantified. Additionally, lipids and other amino acids were not taken into account in this experiment. Therefore, other metabolites could be significantly contributing to alkaloid production (Maeda, 2019).

3.5 Conclusions

Osmotic stress treatments resulted in the most prominent shifts in primary metabolism. Increasing levels of stress resulted in increases in starch, protein and proline concentrations, together with decreases in carbohydrate concentrations. It was suggested that carbohydrate concentrations decreased to allow for increased protein synthesis and starch accumulation.

Growth reductions were evident for osmotic stress treatments **(Chapter 2)** which could be due to the over-accumulation of proline. The reduced proline concentration exhibited by the most stressful osmotic treatment (100 g/L PEG) was indicative of alternate uses for proline, such as for protein synthesis to improve stress tolerance.

A trend was observed between carbohydrates and proteins obtained from salt stress-treated plantlets. It was postulated that low levels of salt stress affect carbon metabolism, while higher levels impact protein (enzyme) synthesis. Alterations to proline concentration were similar to the control, and not nearly as severe as for osmotic stress. The lack of prominent changes in proline metabolism was further confirmation of a halophytic profile for *S. tortuosum*.

Increases in light exposure resulted in increases in carbohydrate and starch concentrations. This was likely due to increased photosynthesis and sugar storage as a result of longer light exposures. Additionally, starch degradation was possibly reduced by shortening the dark period.

Proline concentrations did not provide an accurate measure of stress in *S. tortuosum*, thus the quantification of additional osmolytes is imperative. It is certainly worth investigating ion accumulation in *S. tortuosum* under stress conditions. Additionally, examining stricter groups of primary metabolites could provide clarity regarding relationships with secondary metabolites.

3.6 References

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Chapter 4: Effects of plant growth regulators in tissue culture

4.1 Introduction

Plant growth regulators are substances that occur naturally in plants. They, together with synthetic forms that have been created, are used for inducing and manipulating plant growth. There are five well-recognized groups of PGRs, i.e. auxins, cytokinins, gibberellins, abscisic acid, and ethylene. For the purpose of this study, only auxins and cytokinins are relevant and will be discussed further.

4.1.1 Auxins

Auxins are biosynthesized in the roots and shoots of plants (Ljung *et al.*, 2005). They play significant roles in cell division, differentiation, and elongation (Di Mambro *et al.*, 2017; Ding and Friml, 2010; Perrot-Rechenmann, 2010; Teale *et al.*, 2006). Auxins are responsible for the induction and formation of roots (Overvoorde *et al.*, 2010; Saini *et al.*, 2013). They control apical dominance, and influence leaf formation and flowering (Aloni *et al.*, 2006; Scarpella *et al.*, 2010; Tanaka *et al.*, 2006). Plant photo- and gravi-tropism responses are due to the presence of auxins (Teale *et al.*, 2006).

Indole acetic acid (IAA) is the most common naturally occurring auxin, though natural indole-3-butyric acid (IBA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) have also been reported (George *et al.*, 2008; Magnus *et al.*, 1997). Several synthetic auxins also exist, e.g. naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). These auxins, along with IAA and IBA are routinely employed in plant tissue culture (George *et al.*, 2008). It was stated that NAA and IBA were the most used auxins, followed by 2,4-D, and IAA (Phillips and Garda, 2019).

Auxins are typically employed to induce rooting. However, they may be used in combination with other PGRs for shoot multiplication (George *et al.*, 2008; Phillips and Garda, 2019). Most often, 2,4-D is utilized for inducing somatic embryogenesis and callus cultures (Keshvari *et al.*, 2018; Naz *et al.*, 2017; Raghavan, 2004).

With respect to IBA, NAA, and IAA, the appropriate concentration and/or combination of PGRs for root and shoot proliferation are explant- and species-dependent (Mehalaine and Chenchouni, 2021). For instance, optimal rooting of *Rotula aquatica* was observed with NAA, rather than IBA and IAA (Martin, 2003). Conversely, *in vitro* rooting of *Plectranthus amboinicus* was improved by IBA, rather than IAA and NAA (Rahman *et al.*, 2015). Superior rooting was also reported with the use of IAA in *Hemidesmus indicus* cultures (Purohit *et al.*, 2014). In some cases, combination treatments are more appropriate. Kumlay (2014) used a combination of auxin (IBA, NAA, or IAA) and gibberellic acid, stating that this was ideal for shoot and root induction of *Solanum tuberosum*.

Auxins influence secondary metabolite production (Jamwal *et al.*, 2018). They play a role in inducing and regulating the biosynthesis of flavonols and terpenoids (Ke *et al.*, 2021; Lewis *et al.*, 2011). Auxin, together with light, increases the expression of alkaloid biosynthesis genes (Koirala *et al.*, 2023).

Alkaloid production can be enhanced with auxin application. However, certain auxins may be more suitable than others. *Stemona curtisii* had the highest alkaloid yield with IBA, rather than IAA and NAA (Montri *et al.*, 2023). However, *Catharanthus roseus* cultures showed enhanced alkaloid biosynthesis in the presence of NAA and IAA. For this species, 2,4-D inhibited alkaloid production (Merillon *et al.*, 1989; Whitmer *et al.*, 1998).

4.1.2 Cytokinins

Cytokinins are often synthesized in roots, but production in shoots has also been documented (Sosnowski *et al.*, 2023). Cytokinins are key role players in cell division, expansion, and differentiation (Di Mambro *et al.*, 2017; Wu *et al.*, 2021). They are crucial for the formation and maintenance of shoot apical meristems. They are involved in the generation of leaf primordia, determination of leaf morphology, and flowering (Wu *et al.*, 2021; Wybouw and De Rybel, 2019). Cytokinins' roles also extend to below-ground organs. Cytokinins regulate root characteristics, including root meristem size, root elongation, and lateral root proliferation (Ioio *et al.*, 2007; Osugi and Sakakibara, 2015).

Zeatin and isopentenyladenine are the most abundant naturally occurring cytokinins (Svolacchia and Sabatini, 2023). However, various other cytokinins exist, including 6-benzylaminopurine (BAP), kinetin (KIN), and topolins (Aremu *et al.*, 2012). These substances are commonly used in plant tissue culture to induce shoot organogenesis (George *et al.*, 2008).

Similar to auxins, the appropriate cytokinin and concentration for inducing specific effects varies. For example, optimal shoot proliferation of *Trichosanthes dioica* occurred with BAP, rather than KIN (Tiwari *et al.*, 2010). However, the inverse was apparent for *Cucumis sativus* and *Stevia rebaudiana* (Abu-Romman *et al.*, 2015; Pradhan and Dwivedi, 2016). For some species, tissue culture media supplemented with more than one cytokinin gave the best results (Ashraf *et al.*, 2014; Mehta *et al.*, 2012). Cytokinin and auxin combinations can result in optimal shoot multiplication and growth (Islam and Bari, 2013; Ramak *et al.*, 2011).

Cytokinin type and concentration can have substantial impacts on secondary metabolite production (Grzegorczyk-Karolak *et al.*, 2015). Phenolic and flavonoid contents of *Huernia hystrix* were enhanced by supplementing media with BAP and *meta*-topolin (*m*T) (Amoo and Van Staden, 2013). Kinetin and BAP enhanced alkaloid production in *Solanum erianthum* and *Catharanthus roseus* (Sarkar and Banerjee, 2021; Sidkey, 2020). Cytokinins are likely involved in the up-regulation of genes for alkaloid biosynthetic pathways (Papon *et al.*, 2005).

Plant growth regulator combination treatments may be beneficial for phytochemical production. Several authors reported increased phenolic and flavonoid contents with cytokinin and auxin treatments. In *Origanum vulgare*, BAP + IBA was ideal, however BAP/*m*T + NAA worked well for *Huernia hystrix* (Amoo and Van Staden, 2013; Karalija *et al.*, 2016).

Even though plant responses to PGRs are unpredictable, these regulatory substances provide a promising avenue for the induction and enhancement of plant growth and phytochemical production (George *et al.*, 2008).

4.1.3 Aims and objectives

This research aimed to determine the effects of various PGRs on (1) *in vitro* plantlet growth, and (2) mesembrine-type alkaloid production.

To satisfy the aims, the following objectives were set:

1) Prepare MS medium supplemented with IBA, NAA, BAP, and KIN at various concentrations,
2) Utilize nodal segments from PGR-free multiplication cultures to inoculate experimental cultures,
3) Incubate all cultures for 28 days with constant light and temperature,
4) Record growth parameters (number of new leaf pairs, number of roots, and length of the longest root) upon harvest,
5) Oven-dry plant material and perform alkaloid extraction procedures,
6) Quantify mesembrine-type alkaloids using UPLC-MS, and
7) Perform a comparative analysis of the treatments based on growth and alkaloid production.

4.2 Materials and methods

Experimental tissue culture media were prepared by fortifying Murashige and Skoog (1962) (MS) media with IBA, NAA, BAP, and KIN at concentrations of 0, 2.5, 5, 10, 15 and 20 µM. All media contained 3% sucrose, 0.01% myo-inositol, and 0.8% agar (OXOID, Bacteriological Agar No. 1). Media were adjusted to a pH of 5.8 ± 0.02 and dispensed into tissue culture vessels (approx. 20 mL per vessel). All media, glassware, and instruments were autoclaved at 121°C, 1 bar, for 15 min.

Nodal explants were excised from PGR-free multiplication cultures **(Section 2.2.2)** and used to inoculate the prepared media. Each treatment consisted of 4–6 replicates, each with 4–6 nodal segments. Cultures were incubated at 25 ± 3°C under constant light (PAR = 72 $\mu mol.m^{-2}.s^{-1}$) for 28 days. On the final day of the experiment, growth parameters (number of roots, length of the longest root, and number of newly-emerged leaf pairs) were recorded. Alkaloid extraction and analyses were performed as per **Section 2.2.4**.

4.3 Results

4.3.1 Plantlet growth

Plant growth regulator treatments substantially influenced *S. tortuosum* growth. Significant differences were observed amongst treatments for all growth parameters **(Figure 4.1)**. The highest mean numbers of new leaf pairs were found in the BAP and KIN treatment groups. Highest among these were achieved by 10 µM BAP (9.54 ± 5.84) and 15 µM KIN (6.54 ± 4.81). All concentrations of BAP and KIN resulted in higher mean numbers than the control treatment (1.94 ± 0.73). Indole-3-butyric acid treatments achieved mean numbers (1.72–2.29) that were statistically similar to that of the control **(Figure 4.1A)**. Only 5 and 15 µM IBA (2.23 ± 0.68 and 2.29 ± 1.13) resulted in higher mean numbers than the control treatment (1.94 ± 0.73), though these differences were not statistically significant. Naphthaleneacetic acid resulted in the lowest mean numbers of new leaf pairs for all concentrations tested (0–0.39), with 15 µM NAA having produced no new leaf pairs **(Figure 4.1A)**.

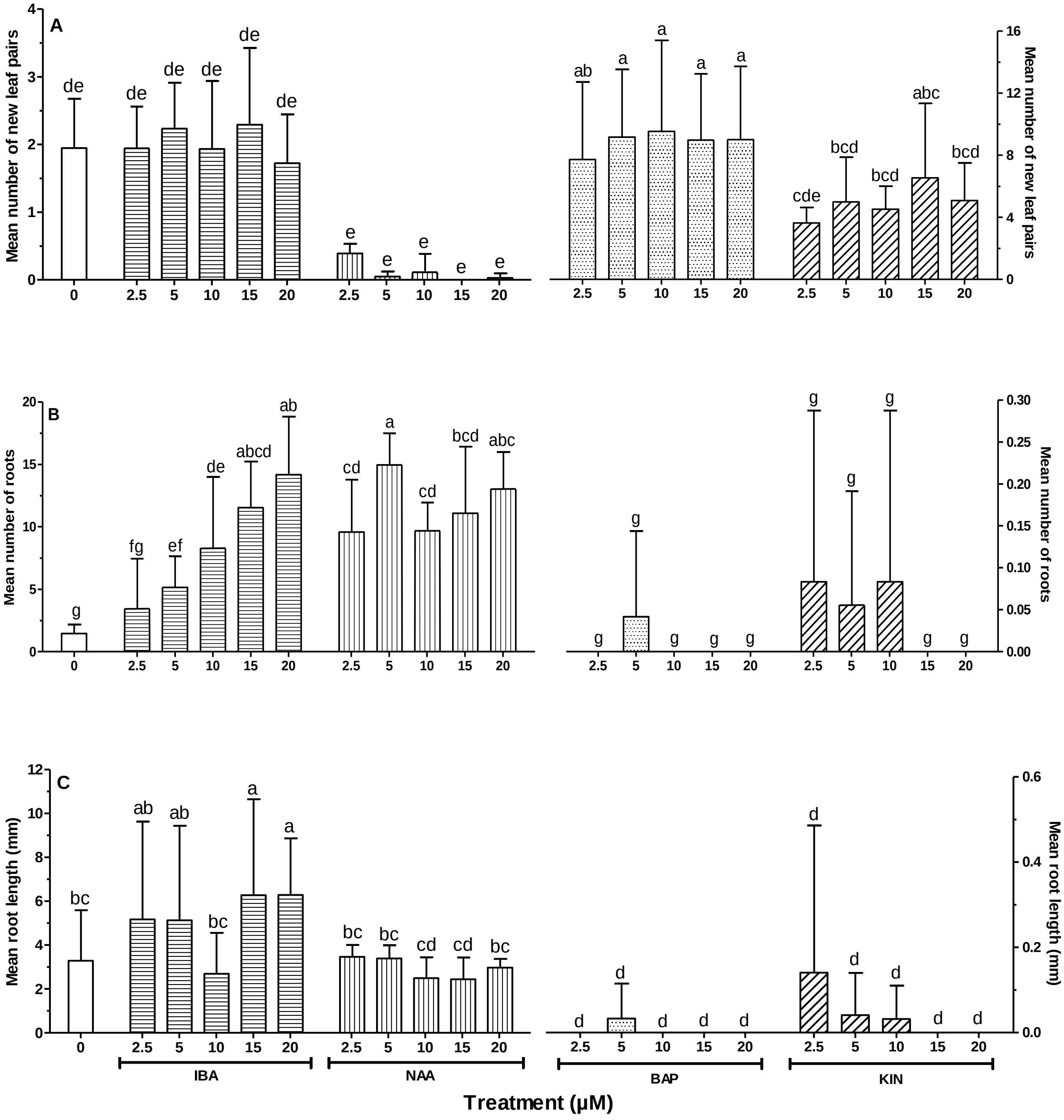

Figure 4.1: Mean number of newly emerged leaf pairs **(A)**, mean number of roots **(B)**, and mean length of the longest root **(C)** of *Sceletium tortuosum* plantlets following exposure to various plant growth regulators for 28 days (IBA = indole-3-butyric acid; NAA = naphthaleneacetic acid; BAP = benzylaminopurine; KIN = kinetin). Error bars show standard deviation (n = 4–6). Different letters indicate statistically significant differences ($p < 0.05$) between treatments. NB. BAP and KIN treatments to be read from the right y-axis.

Mean number of roots were highest within IBA and NAA treatment groups. With the exception of 2.5 µM IBA, mean root numbers from all auxin treatments were significantly higher than the control treatment (1.46 ± 0.73) **(Figure 4.1B)**. Concentrations of 5 µM NAA (14.96 ± 2.53) and 20 µM IBA (14.18 ± 4.66) resulted in the highest mean numbers of roots among all treatments. Increasing concentrations of IBA resulted in increases in the mean number of roots. 6-Benzylaminopurine and kinetin were responsible for the lowest mean numbers of roots, where 4 and 2 of the concentrations tested produced no roots **(Figure 4.1B)**. Mean number of roots peaked for these PGRs with 2.5 and 10 µM KIN (0.083 ± 0.20) and 5 µM BAP (0.042 ± 0.10).

The longest mean root lengths were found in the IBA treatment group **(Figure 4.1C)**. Within this group, 15 and 20 µM IBA resulted in the highest mean root lengths (6.27 ± 4.37 and 6.28 ± 2.59 mm, respectively). Concentrations of 2.5 and 5 µM were slightly lower (5.13–5.17 mm), with the lowest mean root length stemming from 10 µM IBA (2.69 ± 1.87 mm). The NAA treatments achieved root lengths (2.44–3.46 mm) that were statistically similar to the control treatment (3.28 ± 2.30 mm). The lack of roots for many BAP and KIN treatments led to no measurable root lengths for those treatments **(Figure 4.1B** and **C)**. Among the cytokinin treatments with roots, 2.5 µM KIN resulted in the longest mean root length (0.14 ± 0.34 mm). This was not significantly different from the rest of the cytokinin treatments **(Figure 4.1C)**.

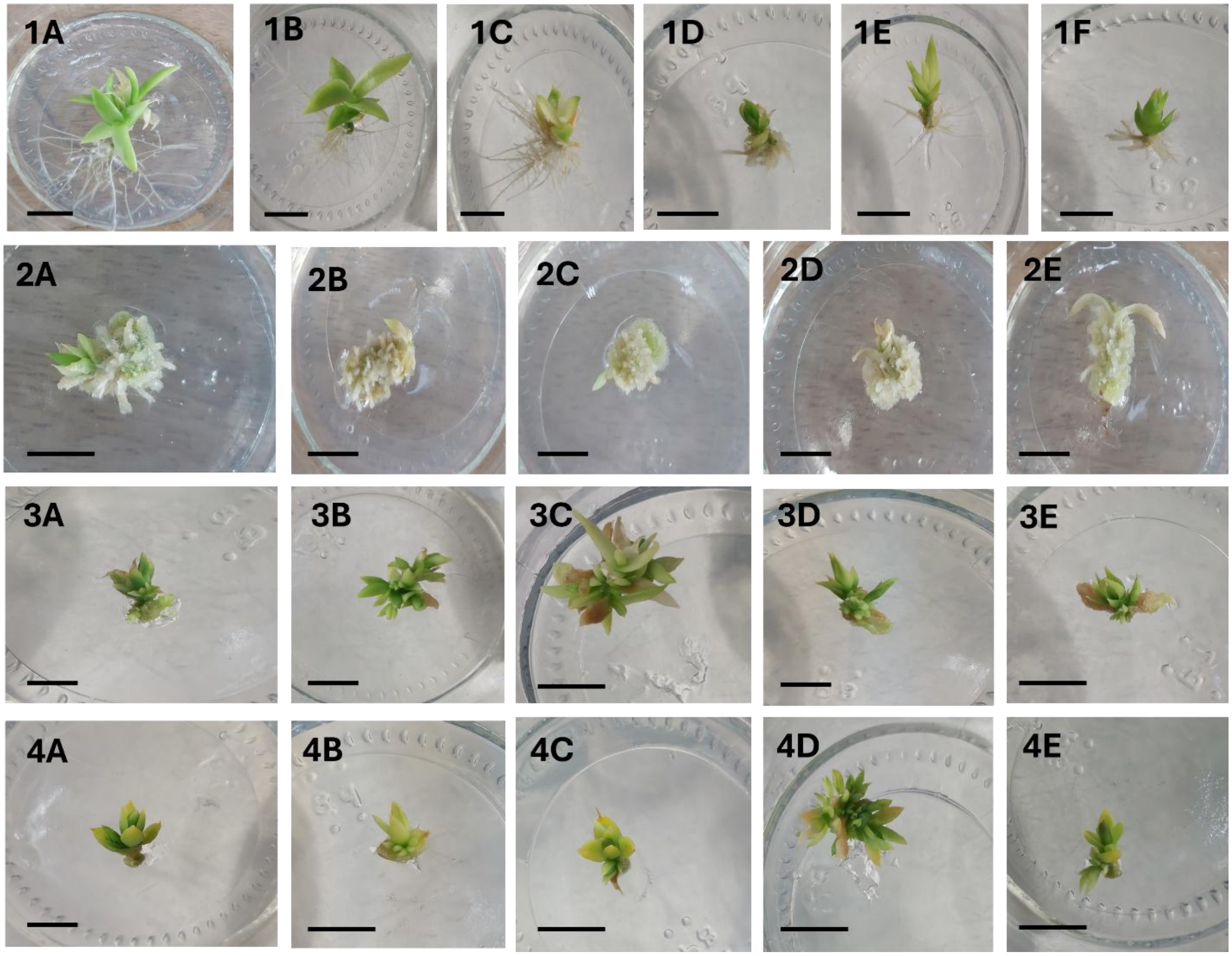

Figure 4.2: *Sceletium tortuosum* plantlets obtained from the control **(1A)**, IBA **(1B-F** = 2.5, 5, 10, 15, 20 µM IBA**)**, NAA **(2A-E** = 2.5, 5, 10, 15, 20 µM NAA**)**, BAP **(3A-E** = 2.5, 5, 10, 15, 20 µM BAP**)**, and KIN **(4A-E** = 2.5, 5, 10, 15, 20 µM KIN**)** treatments after 28 days. Scale bars indicate 1 cm.

Control plantlets showed healthy root and shoot development **(Figure 4.2 (1A))**. Plantlets obtained from IBA treatments exhibited similar balanced root and shoot growth **(Figure 4.2 (1B-F))**. However, 10 µM IBA resulted in a slight decline in plantlet growth **(Figure 4.2 (1D))**. Indole-3-butyric acid produced plantlets with elongated primary roots, and adventitious secondary roots were evident in some treatments **(Figure 4.2 (1B-F))**.

Plantlets generated using NAA showed substantial root proliferation **(Figure 4.2 (2A-E))**. However, these plantlets formed only thick primary roots **(Figure 4.2 (2A-E))**. Only the lowest concentration (2.5 µM NAA) showed signs of producing new leaf pairs **(Figure 4.2 (2A))**.

Over-proliferation of shoots (leaves) was observed for all BAP treatments **(Figure 4.2 (3A-E))**. The high proliferation of shoots resulted in the new leaf pairs being small, often crowded together, and lacking visible internodes. With regards to BAP, numbers of new leaf pairs increased from 2.5 µM to10 µM BAP **(Figure 4.2 (3A-C))** and decreased with 15 and 20 µM BAP **(Figure 3.2 (3D-E))**. Contrastingly, leaf proliferation was highest for 15 µM KIN **(Figure 4.2 (4D))**. Shoot growth was more limited for the remaining KIN treatments **(Figure 4.2 (4A-C; 4E))**.

4.3.2 Alkaloid production

The principal mesembrine-type alkaloids were successfully identified and quantified in *S. tortuosum* samples. A chromatogram generated from an experimental sample is shown below **(Figure 4.3)**.

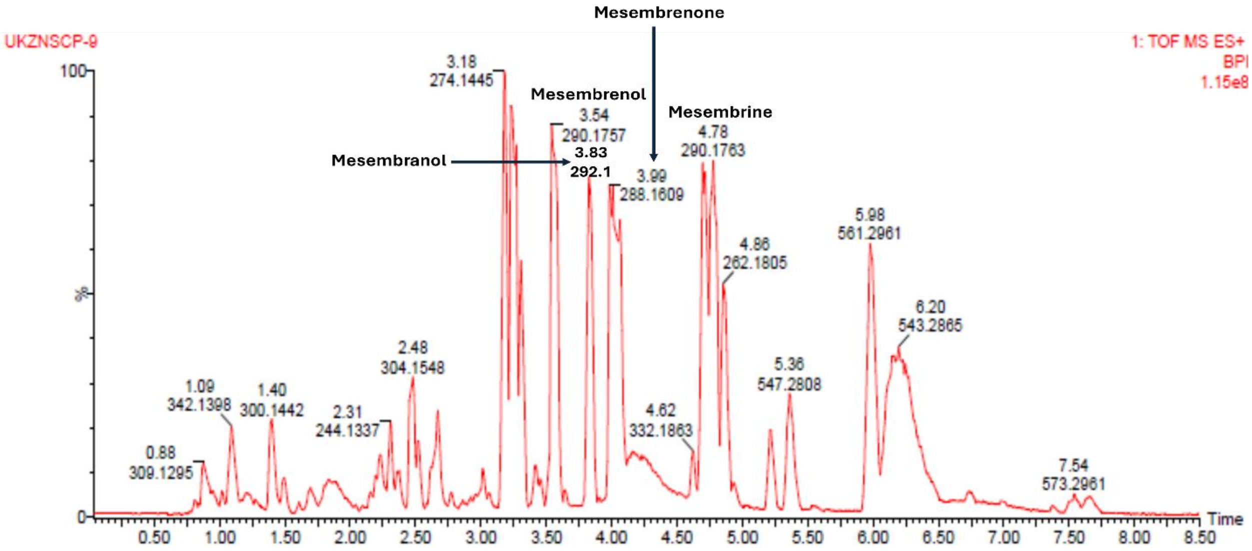

Figure 4.3: Representative chromatogram obtained from UPLC-MS analysis of a *Sceletium tortuosum* sample (control treatment). Retention times and *m/z* (mass) of mesembrine-type alkaloids are shown.

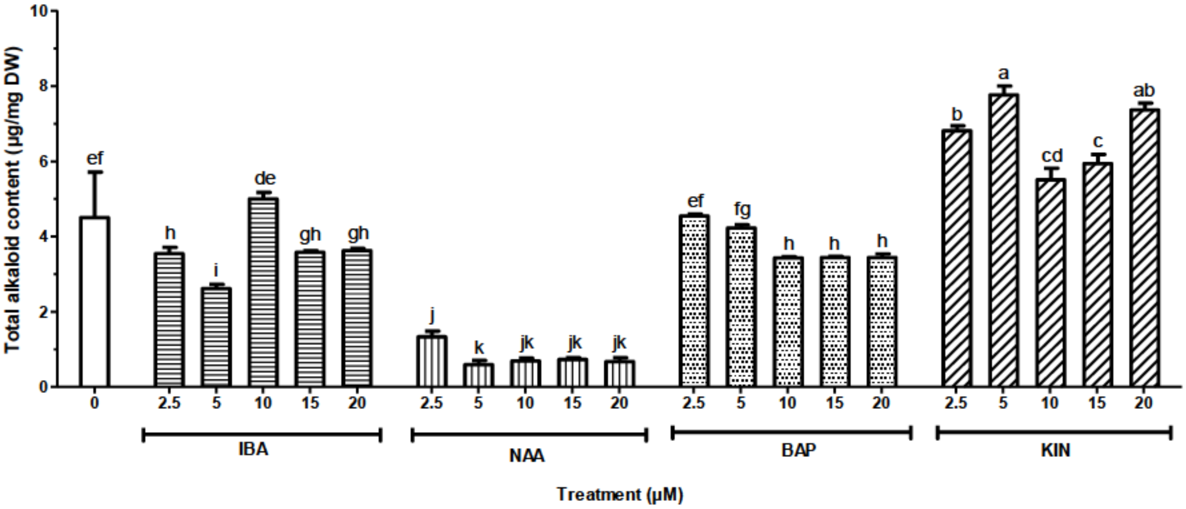

Figure 4.4: Total mesembrine-type alkaloid contents of *Sceletium tortuosum* samples generated from plant growth regulator treatments (IBA = indole-3-butyric acid; NAA = naphthaleneacetic acid; BAP = benzylaminopurine; KIN = kinetin). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences (p < 0.05) between treatments.

Noteworthy differences were observed among the treatments tested **(Figure 4.4)**. Kinetin treatments resulted in the highest total alkaloid contents amongst the PGRs tested. The highest total alkaloid contents were found in the 5 µM KIN treatment, followed by 20 µM KIN (7.77 ± 0.23 and 7.36 ± 0.18 µg/mg DW, respectively). The lowest alkaloid contents resulted from the NAA treatment group (0.59–1.33 µg/mg DW). All NAA treatments were outperformed by the control treatment **(Figure 4.4)**. Within the IBA treatment group, 10 µM IBA achieved in the highest total alkaloid content (5.00 ± 0.18 µg/mg DW), though this was not significantly different from the control treatment (4.50 ± 1.21 µg/mg DW). Total alkaloid contents from BAP treatments (3.43–4.55 µg/mg DW) were similar to the control and IBA treatments **(Figure 4.4)**.

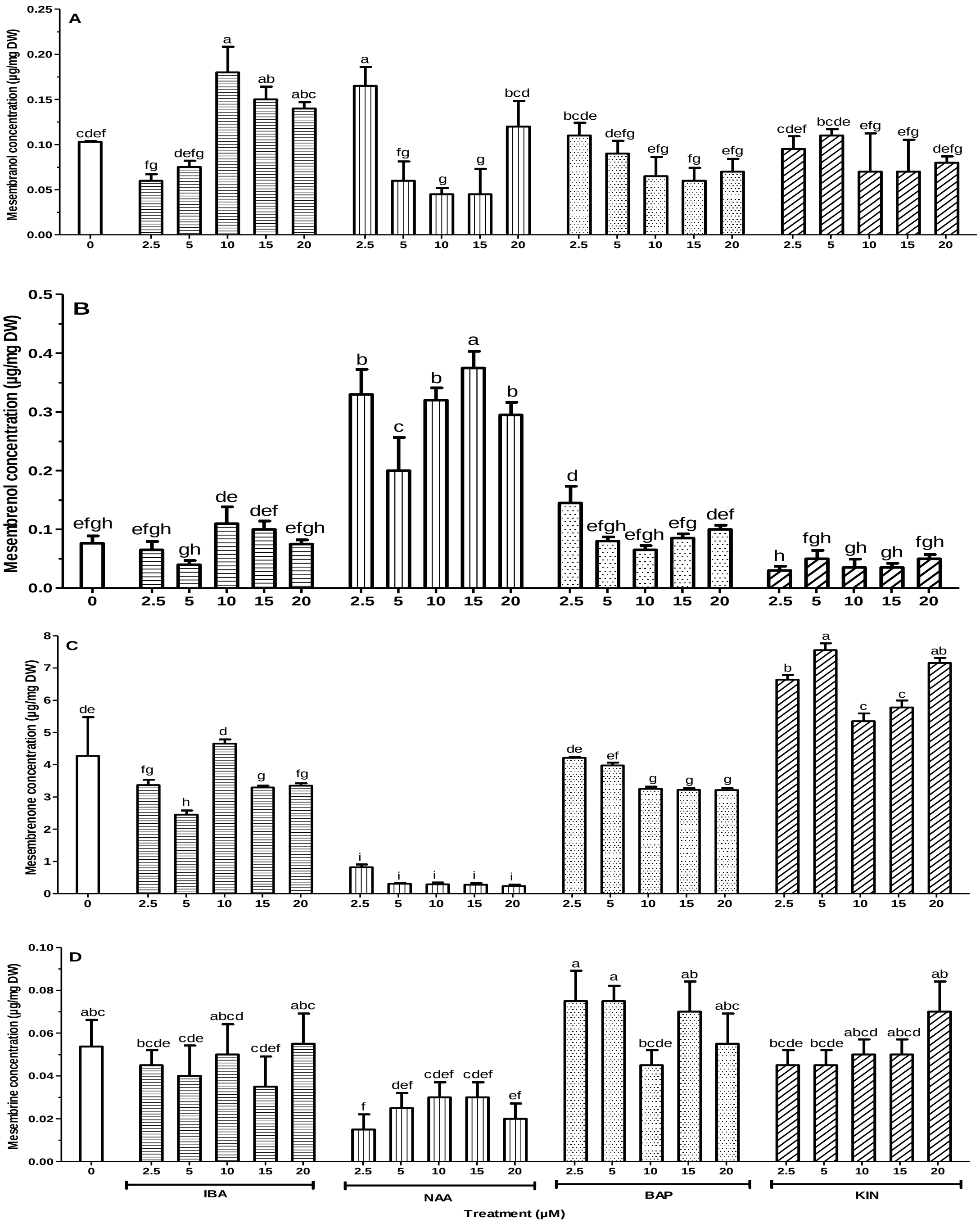

Figure 4.5: Concentrations of mesembranol **(A)**, mesembrenol **(B)**, mesembrenone **(C)**, and mesembrine **(D)** contained in *Sceletium tortuosum* samples obtained from plant growth regulator treatments (IBA = indole-3-butyric acid; NAA = naphthaleneacetic acid; BAP = benzylaminopurine; KIN = kinetin). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Significant differences were observed between treatments for mesembrine-type alkaloid concentrations **(Figure 4.5)**. Mesembranol concentrations were highest within IBA and NAA treatment groups **(Figure 4.5A)**. Peak amounts were reached by 10 µM IBA and 2.5 µM NAA (0.18 ± 0.028 and 0.17 ± 0.021 µg/mg DW, respectively). Interestingly, the NAA treatment group also resulted in the lowest mesembranol concentrations overall. This was achieved by 10 and 15 µM NAA (0.045 µg/mg DW). These concentrations were significantly lower than that of the control treatment (0.10 ± 0.00088 µg/mg DW). Within BAP and KIN treatment groups, medium–high concentrations (10 and 15 µM) of both PGRs resulted in the lowest mesembranol concentrations (0.060–0.070 µg/mg DW). None of the BAP and KIN concentrations resulted in mesembranol quantities significantly higher than the control **(Figure 4.5A)**.

The highest mesembrenol concentrations were achieved by the NAA treatment group **(Figure 4.5B)**. In this group, 15 µM resulted in the highest concentration of mesembrenol (0.38 ± 0.028 µg/mg DW), while 5 µM was responsible for the lowest concentration (0.20 ± 0.057 µg/mg DW) **(Figure 4.5B)**. The KIN treatment group resulted in the lowest mesembrenol quantities. The lowest concentration was achieved by 2.5 µM KIN (0.030 ± 0.042 µg/mg DW). Within the BAP treatment group, 2.5 µM was responsible for the highest mesembrenol concentration (0.15 ± 0.028 µg/mg DW). The majority of the remaining BAP treatments, along with all IBA treatments, yielded concentrations that were similar to the control treatment (0.076 ± 0.012 µg/mg DW) **(Figure 4.5B)**.

Mesembrenone concentrations peaked in the KIN treatment group **(Figure 4.5C)**. The highest concentrations were achieved by 5 and 20 µM KIN (7.56 ± 0.21 and 7.16 ± 0.16 µg/mg DW, respectively). Within the KIN group, 10 µM resulted in the lowest mesembrenone quantity (5.36 ± 0.24 µg/mg DW). The NAA treatment group yielded mesembrenone quantities that were extremely low (0.24–0.82 µg/mg DW) in comparison to all other treatments. The maximum concentration within this group was as a result of 2.5 µM NAA. Treatments of 10–20 µM BAP resulted in mesembrenone concentrations that were remarkably similar (3.22–3.26 µg/mg DW). All IBA and BAP treatments were unable to yield mesembrenone concentrations that were significantly higher than the control (4.27 ± 1.21 µg/mg DW) **(Figure 4.5C)**.

Mesembrine concentrations were highest in the BAP treatment group, and lowest in the NAA group **(Figure 4.5D)**. Within the BAP treatment group, 2.5 and 5 µM resulted in the highest mesembrine concentrations (0.075 µg/mg DW). With regards to the NAA treatment group, 2.5 µM was responsible for the lowest mesembrine concentration (0.015 ± 0.0071 µg/mg DW). The remaining NAA treatments achieved concentrations only marginally higher than this value (0.020–0.030 µg/mg DW). The control treatment (0.054 ± 0.012 µg/mg DW) outperformed all IBA and NAA treatments, with the exception of 20 µM IBA (0.055 ± 0.014 µg/mg DW). Kinetin (20 µM) resulted in the highest mesembrine concentration within the treatment group (0.070 ± 0.014 µg/mg DW). Concentrations of 2.5–15 µM KIN resulted in quantities of mesembrine that were similar to each other (0.045–0.050 µg/mg DW) **(Figure 4.5D)**.

4.4 Discussion

4.4.1 Plantlet growth

Cytokinin treatments induced exceptionally high mean numbers of new leaf pairs **(Figure 4.1)**. Cytokinins encourage axillary bud proliferation, thereby interfering with the role of auxins in apical dominance (George *et al.*, 2008; Ibrahim *et al.*, 2008). This would explain the over-proliferation of shoots and leaves. Despite enhanced shoot growth, plantlets could not be deemed healthy **(Figure 4.2 (3A-E; 4A-E))**. Due to the growth form exhibited, long-term survival of cytokinin-treated plantlets is unlikely. Additionally, the lack of visible internodes renders these plantlets unsuitable for use in multiplication procedures. Similar effects of cytokinin treatments were observed with *Stevia rebaudiana* (Ibrahim *et al.*, 2008).

Due to the natural occurrence of PGRs in plants, exogenous applications may disrupt the internal phytohormone balance (Bhojwani and Razdan, 1986; George *et al.*, 2008). *Sceletium tortuosum* contains high levels of endogenous cytokinins (e.g. *para*-topolin, N^6-

isopentenyladenosine riboside, and other cytokinin glucosides) (Sreekissoon *et al.*, 2021). Thus, supplying plantlets with additional cytokinin may have caused a hormonal imbalance, resulting in the negative growth effects observed **(Figure 4.2 (3A-E; 4A-E))**.

Notably, BAP and KIN treatments diminished root growth, even at low concentrations of BAP (2.5 µM) **(Figure 4.1B)**. This was not entirely unusual, as cytokinins in tissue culture typically induce shoot proliferation (Hill and Schaller, 2013). Root proliferation might require exogenous auxins in the tissue culture medium (Faisal *et al.*, 2018; Naz *et al.*, 2015; Siddique and Anis, 2007). In many cases, high auxin:cytokinin ratios are most conducive to root induction (Skoog and Miller, 1957; Yu *et al.*, 2017).

In terms of shoot proliferation, IBA treatments were more successful than NAA treatments **(Figures 4.1** and **4.2 (1B-F; 2A-E))**. It was previously reported that NAA treatments reduced shoot growth in various fruit species (Boswell *et al.*, 1976; Morris and Cawthon, 1981; Nauer and Boswell, 1978). Other researchers reported that increasing concentrations of NAA reduced the number and length of shoots of *Stevia rebaudiana* and *Valeriana jatamansi* plantlets (Ibrahim *et al.*, 2008; Nazir *et al.*, 2022).

Naphthaleneacetic acid treatments enhanced root proliferation, however, root growth was not significantly improved in comparison to the control **(Figure 4.1B** and **C)**. Additionally, thick roots were observed **(Figure 4.2 (2A-E))**. Faustinelli *et al.* (2009) reported that NAA promoted root thickening more so than elongation. Various researchers who have utilized NAA in combination with cytokinins have not reported this issue (Asadi *et al.*, 2009; Rehman *et al.*, 2023). This suggests that perhaps the effects of NAA need to be balanced out with a suitable cytokinin (George *et al.*, 2008; Yu *et al.*, 2017).

Root growth was optimized in the presence of IBA as compared to NAA **(Figure 4.1B** and **C; Figure 4.2 (1B-F; 2A-E))**. Synthetic indole auxins (such as IBA) may play a role in the protection of endogenous auxins (typically IAA) (George *et al.*, 2008; Maeda and Thorpe, 1979). On the other hand, NAA is thought to inhibit IAA synthesis, and/or encourage its degradation or conjugation (George *et al.*, 2008; Zažimalová *et al.*, 1995). This could account for the differential growth effects observed from these two auxins. For many species, including *Panax ginseng*, *Stevia rebaudiana*, and *Rhododendron* cultivars, IBA has been the preferential auxin for plant growth (Elmongy *et al.*, 2018; Ibrahim *et al.*, 2008; Kim *et al.*, 2003). Similarly, IBA-treated plantlets were the healthiest, with adequate root and shoot systems **(Figures 4.1** and **4.2)**.

Additionally, the control treatment produced healthy plantlets **(Figures 4.1** and **4.2 (1A))**. Other species have also exhibited shoot and root proliferation in the absence of PGRs (Ibrahim *et al.*, 2008; Wijaya *et al.*, 2022). This finding is also supported by earlier research documenting healthy *S. tortuosum* growth on hormone-free medium (Sreekissoon *et al.*, 2021). In fact, that was the basis of utilizing PGR-free MS medium for *in vitro* multiplication.

4.4.2 Alkaloid production

Various BAP and KIN treatments resulted in little to no root growth, yet total alkaloid contents were higher than for rooted plantlets (control, IBA, and NAA treatments) **(Figures 4.1B** and **4.4)**. This is likely due to the fact that the highest levels of mesembrine-type alkaloids are ordinarily found in *S. tortuosum* shoots (Faber *et al.*, 2020).

Despite greater mean numbers of new leaf pairs from the BAP group, compared to the KIN group, total alkaloid contents were higher for KIN treatments **(Figures 4.1A** and **4.4)**. Bozsó and Barna (2021) determined that KIN activated flavonoid biosynthetic genes, while BAP suppressed the expression of these genes. Our results point towards similar activation and suppression effects caused by these PGRs, but with respect to the alkaloid biosynthetic pathway instead. Additionally, Steinhart *et al.* (1964) reported that KIN increased levels of a key enzyme involved in the synthesis of the alkaloids, hordenine and *N*-methyltyramine. This cytokinin may also up-regulate transcription factors involved in alkaloid biosynthesis (Barciszewski *et al.*, 1999). Alkaloid production was reportedly improved with KIN in *Coffea arabica*, *Trigonella foenum-graecum*, and *Nicotiana rustica* (Acidri *et al.*, 2020; Creus and Barcelo, 1988; Dar and Uddin, 2018).

BAP treatments resulted in moderate total alkaloid contents. Within the BAP treatment group, the highest total alkaloid contents and alkaloid concentrations were achieved by the lowest two concentrations tested (2.5 and 5 µM BAP) **(Figures 4.4** and **4.5)**. Sparzak-Stefanowska *et al.* (2019) reported that alkaloid contents were optimal in the presence of low levels of BAP (2.22 µM and 4.44 µM). They showed that higher BAP concentrations caused reductions in alkaloid contents. Such a reduction was also observed for mesembrine-type alkaloids **(Figure 4.4)**.

Indole-3-butyric acid treatments did not significantly increase total alkaloid contents beyond that of the control treatment **(Figure 4.4)**. Furthermore, concentrations of the individual alkaloids were not significantly improved by IBA, with the exception of 10 µM **(Figure 4.5)**. A similar consequence of IBA application was reported for *Nicotiana rustica* (Creus and Barcelo, 1988). Moreover, this reduction in secondary metabolism may be due to the healthy, balanced root and shoot growth observed with IBA treatments **(Figures 4.1** and **4.2 (1B-F))**. Without external stressors, secondary metabolite production remained comfortably low (Ncube and Van Staden, 2015). However, when plantlet growth was slightly less favourable (10 µM IBA), total alkaloid content and mesembranol concentration increased **(Figures 4.2 (1B-F)**, **4.4,** and **4.5)**. This suggests that *S. tortuosum* is highly sensitive, and begins investing in secondary metabolism as soon as growth is less than optimal (Li *et al.*, 2020).

Total alkaloid content was largely dependent on the mesembrenone concentration of samples **(Figures 4.4** and **4.5)**. This makes sense as mesembrenone is the major alkaloid of the chemotype utilized **(Figure 4.3)**. Interestingly, mesembrenone concentration declined drastically in the NAA treatment group **(Figure 4.5C)**. This coincided with remarkable increases in mesembrenol concentrations **(Figure 4.5B)**. These findings are indicative of the mesembrenone pool diminishing to allow for mesembrenol concentration to increase. This is consistent with the literature as Jeffs *et al.* (1978) reported that mesembrenone converts to mesembranol, mesembrenol, and mesembrine.

Despite evidence of such a conversion, NAA treatments were responsible for the lowest total alkaloid contents in comparison to all other treatments **(Figure 4.4)**. This was probably due to the fact that mesembrine-type alkaloids are primarily located in *S. tortuosum* shoots (Faber *et al.*, 2020). Treatments with NAA almost completely eliminated the production of new leaf pairs **(Figures 4.1** and **4.2 (2A-E))**.

Strictly speaking, abiotic stress was not applied to NAA-treated plantlets. However, the growth form of these plantlets was far from ideal **(Figure 4.2 (2A-E))**. In fact, many of the roots produced were quite short, and did not make contact with the medium. This could have led to reduced water uptake, mimicking drought stress. Furthermore, the lack of new leaves undoubtedly reduced photosynthesis (Valladares *et al.*, 2003). These inadvertently created stresses would have increased ROS levels. It was previously stated that alkaloids reduce the harmful effects of ROS (Lee *et al.*, 2016). Mesembrenol, in particular, was suggested to have a pivotal protective role in stressful situations **(Chapter 2)**. Thus, the marked increases in the levels of mesembrenol lend support for this hypothesis.

4.5 Conclusions

Plantlets treated with IBA were the healthiest, overall. In particular, 20 and 15 µM IBA resulted in the highest mean number of roots and mean root length. All IBA treatments produced mean numbers of new leaf pairs that were similar to the control-treated plantlets The remaining treatment groups (NAA, BAP, and KIN) generated plantlets with severely unbalanced growth. Regardless of the positive growth responses observed in IBA treatment group, these plantlets did not have the highest total alkaloid content.

Kinetin-treated plantlets had moderate mean numbers of new leaf pairs, but exhibited diminished root growth. The growth form of these plantlets was likely due to a hormonal imbalance caused by exogenous cytokinin application. Kinetin treatments were responsible for optimum total alkaloid contents, and the highest concentrations of mesembrenone. This heightened alkaloid production may be attributed to KIN triggering the up-regulation of enzymes and pathways associated with alkaloid biosynthesis.

Concentrations of the remaining mesembrine-type alkaloids were optimized by different PGRs. Mesembranol concentration was highest in the IBA group (10 µM IBA), mesembrine concentrations were highest in the BAP group (2.5 and 5 µM BAP), and mesembrenol concentrations were optimized in the NAA treatment group. The potential of mesembrenol to behave in a cellular protective capacity is once again suggested.

In terms of multiplication, IBA treatments proved the most suitable. However, alkaloid production was most enhanced by KIN treatments, especially the size of alkaloid pools. Selection of the ideal treatment would depend on the desired mesembrine-type alkaloid. It is recommended that KIN be investigated to ascertain its role in alkaloid biosynthesis. Furthermore, it may be beneficial to examine the effects of combination treatments (e.g. KIN + auxin) on growth and mesembrine-type alkaloid production of *S. tortuosum*.

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Chapter 5: Effects of plant biostimulants in tissue culture

5.1 Introduction

Biostimulants include any substance that is beneficial to plants, without being a nutrient, pesticide, or soil improver (Du Jardin, 2015). These substances are usually applied at low concentrations and can have considerable influences on plant growth and metabolism (Bulgari *et al.*, 2015). Biostimulants include plant and seaweed extracts, micro-organisms (bacteria and fungi), and humic substances, among others (Du Jardin, 2015). The four biostimulants utilized in this study will be elaborated upon.

5.1.1 Smoke water

Smoke water (SW) is generated by burning vegetation and channelling the smoke through water (Gupta *et al.*, 2020). The resulting SW contains numerous chemical compounds and growth regulatory substances that can influence plant growth (Baldwin *et al.*, 1994; Gupta *et al.*, 2020). The most well-known compounds are germination- and growth-stimulating compounds known as karrikins (KAR_{1-6}). Karrikin1 (KAR_1) is apparently the most potent of the group (Kępczyński and Kępczyńska, 2023; Singh *et al.*, 2023). Smoke water also contains the growth inhibitory compound, trimethylbutenolide (TMB) (Light *et al.*, 2010). To reduce the inhibitory effects of TMB, SW is diluted prior to administration to plants (Gupta *et al.*, 2020; Light *et al.*, 2010). The appropriate dilution and its effects are species-specific. For instance, 1:1000 SW improved growth of *Isatis indigotica* (Zhou *et al.*, 2011). Yet, 1:500 was more appropriate for *Solanum lycopersicum* (Kulkarni *et al.*, 2008).

Smoke water has elicited positive effects on the germination and seedling vigour of many species (Khatoon *et al.*, 2020). Growth improvements are also apparent at later stages of the plant life cycle. Smoke water has effectively enhanced shoot and root growth of bananas, onions, and tomatoes (Aremu *et al.*, 2012b; Kulkarni *et al.*, 2008; Kulkarni *et al.*, 2010). Its effects also extend to bulb growth and fruit development (Kulkarni *et al.*, 2008; Kulkarni *et al.*, 2010).

Smoke water plays a role in plant secondary metabolism (Soós *et al.*, 2009). Up-regulation of flavonoid and terpenoid biosynthesis have been documented (Sun *et al.*, 2020). Improved phenolic, flavonoid, and tannin contents were observed in some species, including *Tulbaghia ludwigiana* and *Aloe arborescens* (Aremu *et al.*, 2012a; Aremu *et al.*, 2014b; Kulkarni *et al.*, 2014). Essential oil and photochemical (e.g. indigo) production were also enhanced with SW application (Chhaya, 2019; Uddin *et al.*, 2023; Zhou *et al.*, 2011).

5.1.2 Kelpak®

Kelpak® (KEL) is a seaweed extract prepared from *Ecklonia maxima*. Cold cellular-burst technology is used to release the cellular components of kelp cells (Kelpak®, 2023). The substances released are collected as a liquid, which is then diluted for the final product (Stirk *et al.*, 2020). Kelpak® contains nutrients and PGRs, such as auxins, cytokinins, gibberellins, abscisic acid, 1-aminocyclopropane-1-carboxylic acid (ACC; an ethylene precursor), brassinosteroids, and phlorotannins (Khan *et al.*, 2009; Nelson and Van Staden, 1985; Rengasamy *et al.*, 2015a; Stirk *et al.*, 2004; Stirk *et al.*, 2014). Thus, the constituents of KEL elicit numerous effects on plant growth and development (Stirk *et al.*, 2020). They may also activate the biosynthetic pathways of phytohormones (Stirk *et al.*, 2020).

Germination and growth have been enhanced due to KEL application. Kelpak® increased root and shoot growth in *Pelargonium peltatum* (Krajnc *et al.*, 2012). Yordanova and Filipov (2023) reported stem elongation and increased numbers of leaves and fruit in cucumbers. Plants under stress have also benefitted from KEL application. Nutrient deficient plants showed increased root and shoot growth in the presence of KEL (Masondo *et al.*, 2019; Papenfus *et al.*, 2013). Heat and salt stress effects were reduced with KEL in the crop species, *Vigna unguiculata* and *Cucurbita pepo* (Rouphael *et al.*, 2017; Voko *et al.*, 2022).

The bioactive compounds contained in seaweed extracts can trigger plant stress responses (Rengasamy *et al.*, 2015b; Stirk *et al.*, 2020). Up-regulation of enzymes involved in antioxidant systems and flavone precursor synthesis have been reported (Fan *et al.*, 2011). Kelpak® increased phenolic and flavonoid contents of *Eucomis autumnalis* (Aremu *et al.*, 2016). Additionally, phenolic and carotenoid contents were increased in *Pelargonium peltatum* (Krajnc *et al.*, 2012). Other commercial seaweed extracts have improved the production of terpenoids and other phytochemicals (Fan *et al.*, 2011; Sharma *et al.*, 2015).

5.1.3 Humic substances

Humic substances consist of humic acid, fulvic acid, and humin. These substances are formed in soil by the degradation of organic matter (Huang and Hardie, 2009). Humic acid is also available in salt forms, e.g. potassium and sodium humate (SH). These are also considered biostimulatory compounds (Nazarov *et al.*, 2020). Humic substances are known for enhancing nutrient uptake (El-Naqma, 2020). Furthermore, they have positive effects on nitrogen assimilation, glycolysis, and the Krebs cycle. This is due to their ability to up-regulate levels and activities of crucial enzymes (e.g. glutamine synthetase and glyceraldehyde-3-phosphate dehydrogenase (GADPH)) (Nunes *et al.*, 2019). Such alterations lead to increased investments in growth. Growth improvements have been documented at the whole-plant level in wheat and sugar beet (Rathor *et al.*, 2024; Sanchez Conde *et al.*, 1972). Positive effects on fruit yield have also been reported (Varga and Ducsay, 2003)

The improved nutrient status of plants is thought to contribute to secondary metabolism (Nardi *et al.*, 2021). However, it has been established that humic substances influence stress response by up-regulating enzymatic and non-enzymatic antioxidant systems (García *et al.*, 2016). Humic substances influence key pathways associated with secondary metabolite production, including phenylalanine and phenylpropanoid pathways (Xu *et al.*, 2024; Zhang *et al.*, 2023). These actions lead to enhanced flavonoid biosynthesis (Xu *et al.*, 2024; Zhang *et al.*, 2023). Increases in phenols, terpenes, anthocyanins, and carotenoids have also been reported (Aminifard *et al.*, 2012; Gomes *et al.*, 2019).

5.1.4 Vermicompost leachate

Vermicompost is generated by worms (most commonly, *Eisenia* spp.). The worms feed on organic material (e.g. vegetable waste, cattle manure, etc.), and excrete their castings (waste) (Dominguez and Edwards, 2011). Vermicompost leachate (VCL) is created and collected by passing water through the vermicompost (Aremu *et al.*, 2015; Canellas *et al.*, 2002). It contains PGRs, including cytokinins, auxins, gibberellins, abscisic acid, and brassinosteroids (Aremu *et al.*, 2015). Various nutrients (e.g. nitrogen, phosphorus, potassium, and calcium) are also present, along with humic substances (Aremu *et al.*, 2015; Atiyeh *et al.*, 2002; Benazzouk *et al.*, 2020).

Vermicompost leachates have been used in numerous plant studies, and proved to enhance various aspects of plant growth (Blouin *et al.*, 2019). Leaf and bulb size of *Drimiopsis maculata* increased with VCL application (Dube *et al.*, 2018). Improved shoot growth and plant weight of *Amaranthus hybridus* was also documented (Ngoroyemoto *et al.*, 2019). Furthermore, the humic acids in VCL affect not only nutrient uptake, but root emergence, elongation, and fruit yield (Canellas *et al.*, 2002; El-Hameid and Adel, 2018; Gomes *et al.*, 2019).

Vermicompost and associated leachates have been beneficial in terms of secondary metabolism of medicinal plants. Increases in terpenoids, flavonoids, phenols, and alkaloids have been described (Aremu *et al.*, 2014a; Dube *et al.*, 2018; Kaur *et al.*, 2022; Manzano-Gómez *et al.*, 2021). It was proposed that this biostimulant influences the regulation of enzymes within the mevalonic acid pathway (crucial for terpenoid biosynthesis) (Souffront *et al.*, 2022). Various research groups showed that VCL application further improved secondary metabolite contents under stress conditions (Benazzouk *et al.*, 2020; Kosem *et al.*, 2022; Masondo *et al.*, 2016).

5.1.5 Aims and objectives

This research aimed to determine the effects of various biostimulants on (1) *in vitro* plantlet growth, and (2) mesembrine-type alkaloid production.

To satisfy the aims, the following objectives were set:

1) Prepare MS medium supplemented with various concentrations of SW, KEL, VCL, and SH,
2) Utilize nodal segments from PGR-free multiplication cultures to inoculate experimental cultures,
3) Incubate all cultures for 28 days with constant light and temperature,
4) Record growth parameters (number of new leaf pairs, number of roots, and length of the longest root) upon harvest,
5) Oven-dry plant material and perform alkaloid extraction procedures,
6) Quantify mesembrine-type alkaloids using UPLC-MS, and
7) Perform a comparative analysis of the treatments based on growth and alkaloid production.

5.2 Materials and methods

Murashige and Skoog (1962) (MS) media were supplemented with various concentrations of SW (1:2000, 1:1000, 1:500 (v/v)), KEL (1:40, 1:20, 1:10 (v/v)), VCL (1:20, 1:10, 1:5 (v/v)), and SH (1.5, 2.5, 5 mg/L). Smoke water was produced by the standardized protocol published by Gupta *et al.* (2020). Kelpak® was purchased from Starke Ayres. Vermicompost leachate was purchased from Wizzard Worms. Sodium humate was purchased from Sigma Aldrich. All media contained 3% sucrose, 0.01% myo-inositol, and 0.8% agar (OXOID, Bacteriological Agar No. 1). Media were adjusted to a pH of 5.8 ± 0.02 and dispensed into tissue culture vessels (approx. 20 mL per vessel). All media, glassware, and instruments were autoclaved at 121°C, 1 bar, for 15 min.

Nodal explants were excised from PGR-free multiplication cultures **(Section 2.2.2)** and used to inoculate the experimental media. Each treatment consisted of 4–6 replicates, each with 4–6 nodal segments. Cultures were incubated at 25 ± 3 °C under constant light (PAR = 7.18 $\mu mol.m^{-2}.s^{-1}$) for 28 days. On the final day of the experiment, growth parameters (number of roots, length of the longest root, number of newly-emerged leaf pairs) were recorded. Alkaloid extraction and analyses were performed as outlined in **Section 2.2.4**.

5.3 Results

5.3.1 Plantlet growth

Plant biostimulants influenced *S. tortuosum* growth. However, treatment effects were only significant for shoot growth **(Figure 5.1)**. Specifically, the mean number of new leaf pairs obtained from 5 mg/L SH and 1:500 SW treatments were significantly different **(Figure 5.1A)**. The highest mean number of new leaf pairs was observed with 5 mg/L SH (1.67 ± 1.20). Smoke water (1:500) was responsible for the lowest mean number of new leaf pairs (0.44 ± 0.38). Mean numbers of new leaf pairs increased with increasing concentrations of SH. In contrast, SW and VCL treatments resulted in decreasing mean numbers as concentration increased **(Figure 5.1A)**.

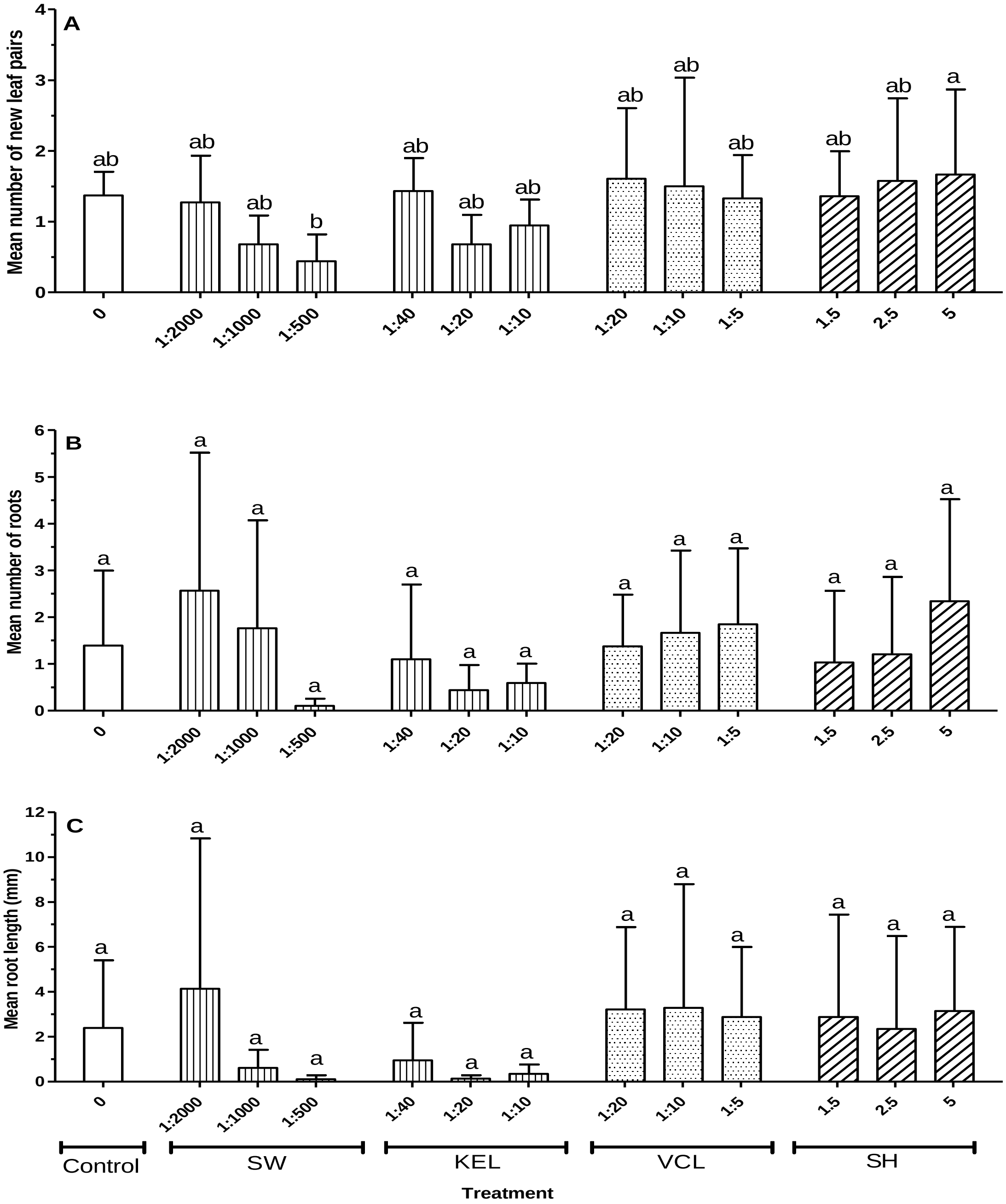

Figure 5.1: Mean number of newly emerged leaf pairs **(A)**, mean number of roots **(B)**, and length of the longest root **(C)** of *Sceletium tortuosum* plantlets following exposure to various biostimulants for 28 days (SW = smoke water; KEL = Kelpak®; VCL = vermicompost leachate; SH = sodium humate). Error bars show standard deviation (n = 4–6). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Statistically significant differences were not evident for mean number of roots and mean root length **(Figure 5.1B** and **C)**. In terms of mean number of roots, 1:2000 SW resulted in the highest mean number (2.57 ± 2.95), whereas the 1:500 SW treatment achieved the lowest mean number of roots (0.11 ± 0.15). Kelpak® treatments resulted in mean root numbers that were lower than the control treatment (1.39 ± 1.61). Increases in mean number of roots were observed with increasing concentrations of VCL and SH. The inverse was apparent with regards to SW, i.e. mean root numbers decreased with increasing concentration **(Figure 5.1B)**.

The same trend was observed for mean root lengths with increasing concentrations of SW **(Figure 5.1C)**. The longest mean root length amongst all biostimulant treatments was achieved by 1:2000 SW (4.14 ± 6.69 mm). However, 1:1000 and 1:500 SW (0.62 ± 0.80 and 0.11 ± 0.17 mm, respectively) resulted in mean root lengths that were shorter than the control treatment (2.39 ± 3.01 mm). Kelpak® treatments (0.14–0.95 mm) also underperformed relative to the control treatment. Vermicompost leachate and SH treatments resulted in mean root lengths that were longer than the control, with the exception of 2.5 mg/L SH (2.34 ± 4.14 mm) **(Figure 5.1C)**.

5.3.2 Alkaloid production

Mesembranol, mesembrenol, mesembrenone, and mesembrine were identified and quantified in *S. tortuosum* samples. A chromatogram generated from an experimental sample is shown in **Figure 5.2**.

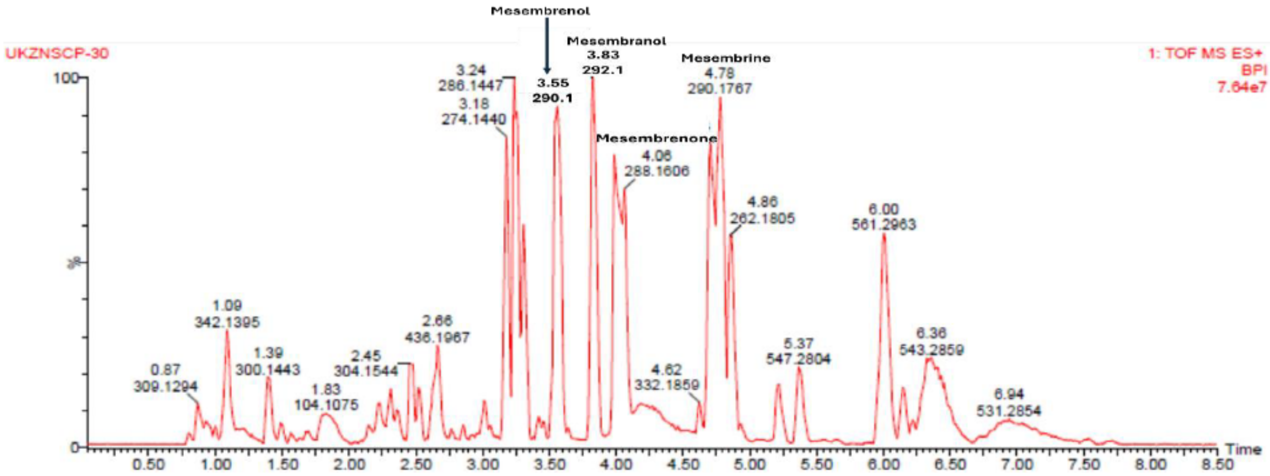

Figure 5.2: Representative chromatogram obtained from UPLC-MS analysis of a *Sceletium tortuosum* sample (control treatment). Retention times and *m/z* (mass) of mesembrine-type alkaloids are shown.

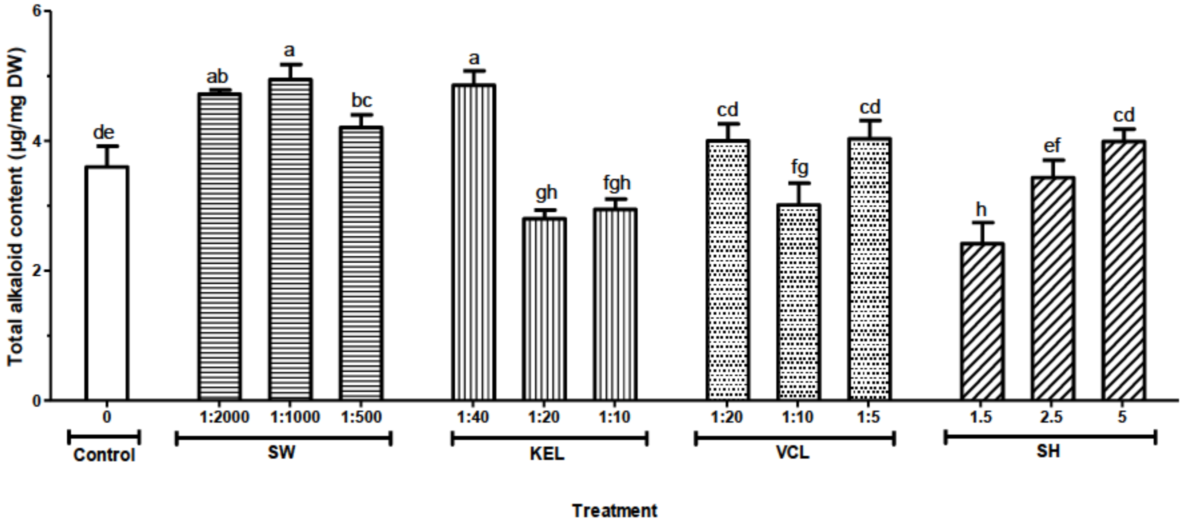

Figure 5.3: Total mesembrine-type alkaloid contents of *Sceletium tortuosum* samples generated from biostimulant treatments (SW = smoke water; KEL = Kelpak®; VCL = vermicompost leachate; SH = sodium humate). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Statistically significant differences were observed amongst the biostimulant treatments **(Figure 5.3)**. Overall, 1:1000 SW and 1:40 KEL resulted in the highest total alkaloid contents (4.95 ± 0.23 and 4.86 ± 0.22 µg/mg DW, respectively). All smoke water treatments yielded high total alkaloid contents (4.21–4.95 µg/mg DW). In fact, alkaloid contents obtained from the SW group, along with 1:40 KEL, were significantly higher than that of the control treatment (3.60 ± 0.32 µg/mg DW). The lowest total alkaloid contents resulted from 1.5 mg/L SH (2.42 ± 0.33 µg/mg DW). Interestingly, as SH concentration increased, total alkaloid content increased **(Figure 5.3)**.

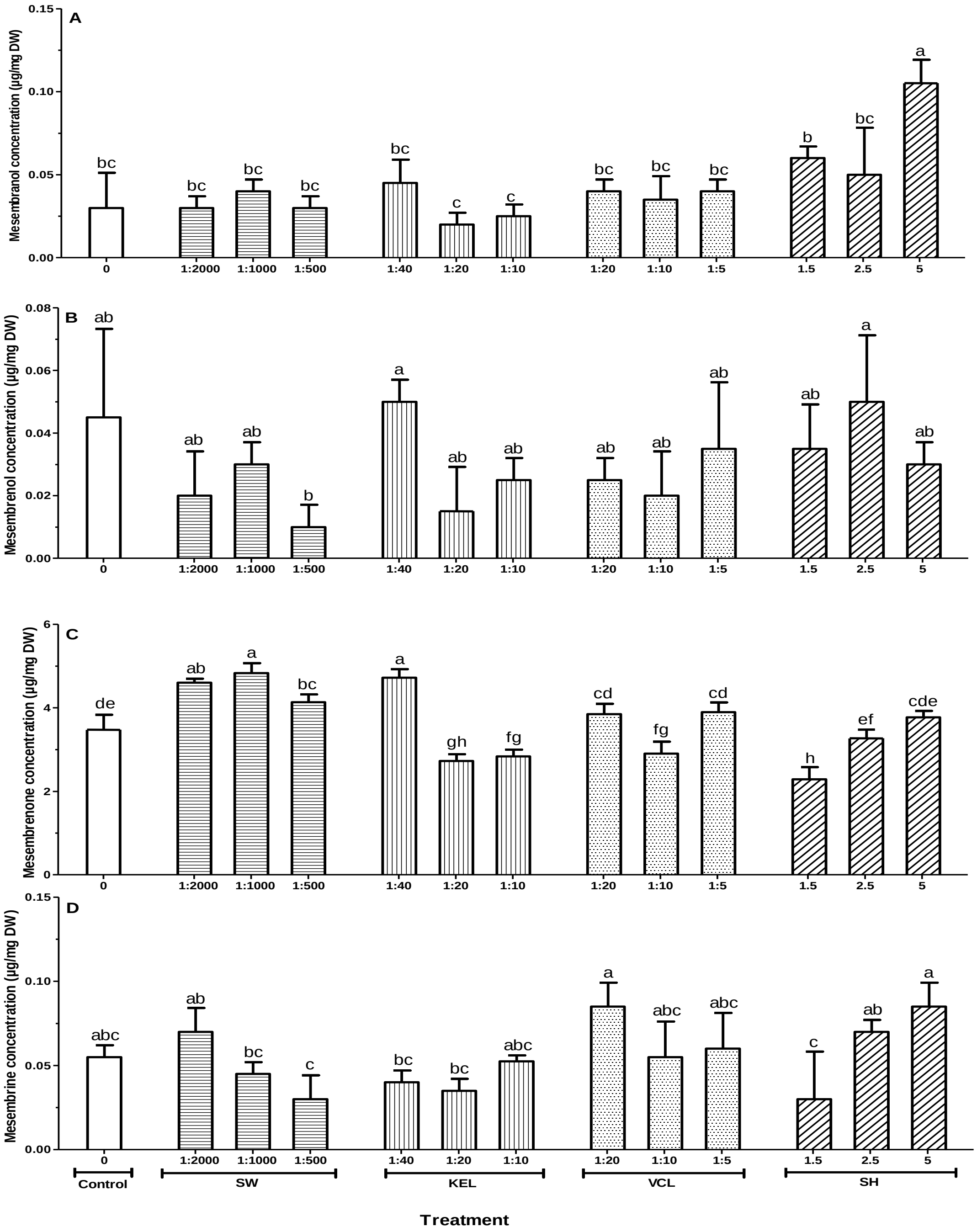

Figure 5.4: Concentrations of mesembranol **(A)**, mesembrenol **(B)**, mesembrenone **(C)**, and mesembrine **(D)** contained in *Sceletium tortuosum* samples obtained from biostimulant treatments (SW = smoke water; KEL = Kelpak®; VCL = vermicompost leachate; SH = sodium humate). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Notable differences were observed between treatments for each of the alkaloids quantified **(Figure 5.4)**. Among all treatments, 5 mg/L SH achieved the highest concentration of mesembranol (0.11 ± 0.014 µg/mg DW) **(Figure 5.4A)**. This was followed by 1.5 and 2.5 mg/L SH (0.060 ± 0.0071 and 0.050 ± 0.028 µg/mg DW, respectively). Notably, only 5 mg/L SH resulted in a mesembranol concentration that was significantly higher than the control treatment (0.030 ± 0.021 µg/mg DW). Kelpak® treatments (1:20 and 1:10) resulted in the lowest concentrations of mesembranol overall (0.020 and 0.025 ± 0.0071 µg/mg DW) **(Figure 5.4A)**.

Mesembrenol concentrations peaked with 1:40 KEL and 2.5 mg/L SH (0.050 µg/mg DW) **(Figure 5.4B)**. Although the concentrations achieved were high, they were not significantly different from that of the control treatment (0.045 ± 0.028 µg/mg DW). They were, however, significantly higher than 1:500 SW (0.010 ± 0.0071 µg/mg DW), which was responsible for the lowest mesembrenol concentration among all biostimulants.

Mesembrenone concentrations were highest in the 1:1000 SW and 1:40 KEL treatments (4.83 ± 0.24 and 4.73 ± 0.21 µg/mg DW, respectively) **(Figure 5.4C)**. This KEL treatment, along with all SW treatments, resulted in mesembrenone concentrations that were significantly higher than the control treatment (3.47 ± 0.36 µg/mg DW). Contrastingly, the remaining KEL treatments lowered the concentrations of mesembrenone more so than the control (2.73 ± 0.16 and 2.84 ± 0.16 µg/mg DW, for 1:20 and 1:10 KEL, respectively). Mesembrenone concentrations increased in direct proportion to SH concentration. Within this biostimulant group, the highest quantity of mesembrenone was achieved by 5 mg/L SH (3.77 ± 0.16 µg/mg DW). Vermicompost leachate treatments (1:20 and 1:5) yielded mesembrenone concentrations above that of the control (3.85 ± 0.25 and 3.90 ± 0.23 µg/mg DW, respectively), however there were no significant differences between these treatments **(Figure 5.4C)**.

Sodium humate (5 mg/L) and 1:20 VCL treatments resulted in the highest mesembrine concentration of all biostimulant treatments tested (0.085 ± 0.014 µg/mg DW) **(Figure 5.4D)**. Sodium humate (1.5 mg/L) also resulted in one of the lowest mesembrine concentrations

(0.030 ± 0.028 µg/mg DW). Interestingly, increased concentrations of SH resulted in elevated quantities of mesembrine **(Figure 5.4D)**. Smoke water treatments showed the inverse, i.e. raised concentration of SW decreased mesembrine concentrations. Hence, 1:500 SW resulted in 0.030 ± 0.014 µg/mg DW mesembrine. Kelpak® treatments underperformed relative to the control (0.055 ± 0.0071 µg/mg DW), however the differences between these treatments were not statistically significant **(Figure 5.4D)**.

5.4 Discussion

5.4.1 Plantlet growth

A previous study (Sreekissoon *et al.*, 2021a) investigating the effects of SW on *S. tortuosum* germination showed increases in seedling shoot length for the lowest concentrations of SW tested (1:2000 and 1:2500 SW). The present findings are consistent with this, as shoot number was maximized with the lowest SW concentration **(Figure 5.1A)**. In fact, root and shoot growth improved as SW concentration decreased **(Figure 5.1)**. Ghazanfari *et al.* (2012) reported similar growth improvements in *Brassica napus* with low, rather than high, concentrations of SW. It has been suggested that plant responses to SW are hormetic (i.e. low concentrations are stimulatory, while high concentrations are inhibitory) (Calabrese and Agathokleous, 2021; Garrido *et al.*, 2023).

Decreases in *S. tortuosum* plantlet growth were observed with medium–high concentrations of SW **(Figure 5.1)**. It is well-established that SW contains KAR_1 and TMB (Garrido *et al.*, 2023; Singh *et al.*, 2023). Highly concentrated SW solutions contain high levels of TMB, which can suppress the stimulatory effect of KAR_1 (Gupta *et al.*, 2020; Light *et al.*, 2010). This could explain the retardation of growth observed for the 1:500 and 1:1000 SW treatments **(Figure 5.1)**. Other researchers have also reported reductions in growth with high SW concentrations (2% and higher) (Abou El-Nour, 2021; Garrido *et al.*, 2023).

Within the KEL treatment group, 1:40 KEL resulted in optimal root and shoot growth. However, plantlet growth was not significantly improved in comparison to the control and other biostimulants **(Figure 5.1)**. Aremu *et al.* (2016) determined that KEL application increased cytokinin contents. This could have potentially affected *S. tortuosum* plantlet growth, due to the already high endogenous cytokinin contents (Sreekissoon *et al.*, 2021b). Exogenous cytokinins can interfere with auxin effects (George *et al.*, 2008). This could explain the negative effects on root growth **(Figure 5.1B** and **C)**.

Robertson-Andersson *et al.* (2006) reported that 1:500 KEL had detrimental effects on the growth of *Ulva lactuca* maricultures. Notably, this concentration is several-fold lower than the concentrations used in the present study. Thus, it is possible that the concentrations utilized exceeded what was necessary and appropriate for healthy and improved plant growth.

Interestingly, VCL treatments improved growth more so than KEL treatments. Additionally, growth parameters were similar among all VCL treatments tested **(Figure 5.1)**. This may be attributed to the composition of VCL, i.e. it contains humic acids in addition to PGRs (Aremu *et al.*, 2015; Atiyeh *et al.*, 2002). Regardless, VCL-treated plantlets did not exhibit growth that was significantly better than the control treatment. Aremu *et al.* (2014a) reported that VCL failed to enhance growth of *Eucomis autumnalis*, *Tulbaghia ludwigiana*, and *Tulbaghia violacea*. They did, however, notice growth improvements when VCL was applied to nutrient-stressed plants. The present study supplied adequate nutrients to plantlets in the culture media, which could explain the non-significant growth effects **(Figure 5.1)**.

Sodium humate produced healthier plantlets than KEL treatments **(Figure 5.1)**. Humic acids exhibit auxin-like effects, in addition to their ability to up-regulate cytokinin biosynthesis (Nardi *et al.*, 2017; Zhang *et al.*, 2023). These metabolic effects of SH may have been more conducive to growth, as compared to KEL, which contains PGRs. These PGRs may have interfered with endogenous PGRs, thereby adversely affecting growth (Aremu *et al.*, 2016). In comparison to VCL treatments, SH resulted in similar consistencies in growth **(Figure 5.1)**. These results suggest that humic substances play a key role in regulating growth of *S. tortuosum*.

Growth parameters improved as the concentration of SH was increased **(Figure 5.1)**. Sodium humate increases nutrient uptake (El-Naqma, 2020; Varga and Ducsay, 2003). As ample nutrients were supplied in the culture media, these findings imply that nutrient uptake and utilization was facilitated according to the concentration of SH. In the past, SH application together with nutrients yielded promising results. For instance, shoot growth (leaf length, area, and shoot weight) of wheat and cucumber was improved (Mora *et al.*, 2010; Nazarov *et al.*, 2020). However, Nunes *et al.* (2019) showed that humic acids improved root development (root number, diameter, and length), even in the absence of nutrients.

The highest concentration of SH (5 mg/L) resulted in all three growth parameters surpassing the control treatment **(Figure 5.1)**. Van de Venter *et al.* (1991) documented optimal growth of lettuce, onion, and cantaloupe with concentrations of 500 and 1000 mg/L SH. Based on this, perhaps elevated SH concentrations could further enhance growth of *S. tortuosum*.

5.4.2 Alkaloid production

Increased secondary metabolite production is characteristic of plant stress response (Aguirre-Becerra *et al.*, 2021). Soós *et al.* (2009) reported that SW application resulted in the over-expression of stress-responsive genes. This could explain the high total alkaloid contents observed for all SW treatments **(Figure 5.3)**.

Within the SW treatment group, lower concentrations of SW (1:1000 and 1:2000) resulted in the highest concentrations of each of the mesembrine-type alkaloids **(Figure 5.4)**. This is contrary to various studies showing that 1:500 SW was the most suitable concentration for increasing secondary metabolite production (Aremu *et al.*, 2014b; Uddin *et al.*, 2023; Zhou *et al.*, 2011).

Hormetic responses are not only applicable to growth, but affect other aspects of plants, including secondary metabolism (Vargas-Hernandez *et al.*, 2017). This could explain the raised alkaloid concentrations and total alkaloid contents with lower SW concentrations **(Figures 5.3** and **5.4)**. Furthermore, these enhancements at low SW concentrations correlate with the growth improvements observed **(Figure 5.1)**. This study could be an example of hormetic SW responses exhibited at a whole-plant level.

A concentration of 1:40 KEL resulted in one of the largest alkaloid fractions overall **(Figure 5.3)**. Additionally, this treatment resulted in mesembranol, mesembrenol, and mesembrenone concentrations which exceeded the control treatment **(Figure 5.4A-C)**. Aremu *et al.* (2016) found that 1:40 KEL increased phytochemical levels in *Eucomis autumnalis*. More recently, this concentration (1:40 KEL) also increased terpenoid and flavonoid contents of *Abelmoschus esculentus* (Aremu *et al.*, 2022). However, even though this concentration increased the levels of some phytochemicals, others benefitted from higher KEL concentrations (e.g. 1:20) (Aremu *et al.*, 2022; Aremu *et al.*, 2016). Similarly, mesembrine concentration was most enhanced by 1:10 KEL, rather than 1:40 KEL **(Figure 5.4D)**. Thus, the ideal KEL concentration is dependent on the desired secondary metabolite/s.

Aremu *et al.* (2014a) and Masondo *et al.* (2016) reported that VCL application increased phytochemical contents, rather than growth, of nutrient-deficient *Ceratotheca triloba, Eucomis autumnalis*, and *Tulbaghia* spp. Unfortunately, significant increases in neither growth nor alkaloid production were found in the current investigation **(Figures 5.1, 5.3,** and **5.4)**. It is possible that VCL enhances secondary metabolite production more effectively under conditions of abiotic stress. Benazzouk *et al.* (2020) stated that VCL improved phytochemical production of salt-stressed *Solanum lycopersicum*. Similarly, water-stressed *Ocimum basilicum* exhibited increased levels of phenols (caffeic and rosmarinic acids) and the terpenoid, estragole (Kosem *et al.*, 2022).

Sodium humate (5 mg/L) resulted in the highest total alkaloid contents among SH treatments **(Figure 5.3)**. Additionally, concentrations of mesembrenone and mesembrine increased proportionally to SH concentration **(Figure 5.4C** and **D)**. These findings build upon our earlier postulation (↑SH concentration = ↑nutrient uptake; see **5.4.1**), suggesting that heightened nutrient uptake also correlates with increased secondary metabolite production. Alkaloids are nitrogen-containing compounds, thus increased nitrogen levels impact on alkaloid contents (Demeyer and Dejaegere, 1998; Nunes *et al.*, 2019; Sanders *et al.*, 1990). Lovkova *et al.* (2006) reported that molybdenum, cobalt, and copper increased alkaloid production in *Papaver somniferum*. These elements were present in MS media (Murashige and Skoog, 1962). Their increased uptake could have contributed to the increases in alkaloid production.

Within the SH treatment group, 5 mg/L SH resulted in the highest concentrations of mesembranol, mesembrenone, and mesembrine **(Figure 5.4)**. Sodium humate has been implicated in secondary metabolite biosynthesis. Xu *et al.* (2024) recently reported that SH influenced the phenylalanine pathway, which led to increased flavonoid biosynthesis in *Citrus limon*. Phenylalanine and tyrosine (products of the phenylalanine pathway) are crucial for the biosynthesis of mesembrine-type alkaloids (Jeffs *et al.*, 1971). Thus, SH may have some influence on alkaloid production in *S. tortuosum*.

5.5 Conclusions

Biostimulants were unable to significantly enhance growth. However, growth improvements were noted with decreasing concentrations of SW. Kelpak® (1:40) resulted in optimal growth within the KEL treatment group, but root growth parameters were low for all KEL concentrations. Sodium humate resulted in increased growth parameters with increasing concentrations. Vermicompost leachate treatments showed the least variable growth amongst the biostimulant groups.

Smoke water treatments (1:1000 and 1:2000) yielded very large total alkaloid fractions. Within the SW group, 1:1000 SW resulted in the highest concentrations of mesembrenone, mesembranol, mesembrenol. The effects of SW on growth and alkaloid production were attributed to a hormetic response of *S. tortuosum*. Low concentrations of SW (1:1000 and 1:2000) are recommended for future use in *S. tortuosum*. Increased dilutions of SW may have the potential to further improve growth and mesembrine-type alkaloid production.

Kelpak® (1:40) also yielded one of the highest total alkaloid contents. This treatment, along with 2.5 mg/L SH, optimized mesembrenol concentration. Mesembrine and mesembranol levels were highest with 5 mg/L SH. Notably, SH increased total alkaloid contents, and mesembrenone and mesembrine concentrations in a dose-dependent manner. Improvements in growth and alkaloid production were likely due to increased nutrient uptake caused by SH. Greater concentrations of SH are worthy of testing in *S. tortuosum*.

Overall, no single biostimulant treatment can be deemed ideal for enhancing growth and alkaloid production simultaneously. However, low SW concentrations (≤ 1:1000), and high SH concentrations (≥ 5 mg/L) appear to be the most promising. Treatment selection will be dependent on the target alkaloids.

5.6 References

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Chapter 6: Effects of plant growth regulators and amino acid precursors in cell culture

6.1 Introduction

Cell suspension cultures are considered a viable method for enhancing secondary metabolite production of medicinal plants (Murthy *et al.*, 2014). Evaluation of the growth potential and phytochemical productivity of such cultures is essential for determining whether this technique is appropriate for a particular plant species. Based on culture performance and yield, cell cultures can be scaled-up in the form of continuous culture using large bioreactors (Bhatia, 2015; Yue *et al.*, 2016). In this way, high yields of valuable secondary metabolites can be obtained (Murthy *et al.*, 2014).

Manipulation of cell secondary metabolism can take place with the use of elicitors, such as PGRs and precursor compounds (Twaij and Hasan, 2022). Precursors are selected based on their involvement in the pathways responsible for synthesis of the desired compounds (Murthy *et al.*, 2014). The elicitors utilized in the current investigation will be expanded upon below.

6.1.1 PGRs in cell culture

Auxins and cytokinins play key roles in cell division, elongation, and expansion (George *et al.*, 2008; Perrot-Rechenmann, 2010). As explained in **Chapter 4**, the effects of PGRs on growth and phytochemical production depend upon the species and its metabolites (Aly *et al.*, 2010; Panda *et al.*, 1992).

The auxin, 2,4-D, has been utilized extensively in callus and cell suspension cultures (Karuppusamy, 2009). Often, this PGR alone is sufficient for callus formation, which is essential for the induction of cell cultures (Nikolaeva *et al.*, 2009; Tahir *et al.*, 2011).

However, the effect of this auxin on the promotion of secondary metabolite production is debatable. For instance, berberine alkaloid production was improved with 2,4-D addition to cell cultures of *Thalictrum minus* (Nakagawa *et al.*, 1986). Additionally, callus cultures of *Polyalthia bullata* and *Pinellia ternata* exhibited enhanced alkaloid production in the presence of 2,4-D (Liu *et al.*, 2010; Zaman *et al.*, 2021). Contrarily, 2,4-D reportedly inhibited alkaloid synthesis in cell cultures of *Catharanthus roseus* and *Lycopodiella inundata* (Bienaimé *et al.*, 2015; Gantet *et al.*, 1997).

Other auxins (e.g. NAA, IAA, and IBA) also proved to have stimulatory effects on culture growth and phytochemical yield. Naphthaleneacetic acid improved biomass and alkaloid production in *Vernonia cinerea* cultures (Maheshwari *et al.*, 2007), while phytochemical contents of *Sericostoma pauciflorum* and *Morus alba* cultures were most enhanced by IBA and IAA, respectively (Jain *et al.*, 2012; Lee *et al.*, 2011).

Cytokinins also elicit powerful effects in cell and callus cultures. Kinetin and BAP improved growth and secondary metabolite production in *Melia azedarach*, *Artemisia absinthium*, and *C. roseus* cultures (Ahmadpoor *et al.*, 2023; Mekky *et al.*, 2018; Zia *et al.*, 2007).

Callus cultures may be initiated using a combination of auxin and cytokinin (Normasari *et al.*, 2023). Equal ratios of these PGRs usually produces callus (Skoog and Miller, 1957). Under these circumstances, researchers may carry forward the PGR combination into cell suspension culture. In fact, the vast majority of suspension culture studies make use of PGR combination treatments (Karuppusamy, 2009; Mekky *et al.*, 2018). Kaňuková *et al.* (2024) reported that growth of stem cell cultures was achieved using 2,4-D and BAP. Similarly, cell culture growth and phytochemical production of *Melia azedarach* and *Vaccinium corymbosum* were optimal in the presence of 2,4-D and BAP/KIN (Ahmadpoor *et al.*, 2023; Rybin *et al.*, 2024). Numerous other combinations have also influenced culture productivity (Karuppusamy, 2009; Mekky *et al.*, 2018).

6.1.2 Precursors in cell culture

Secondary metabolite precursors include amino acids and fatty acids, along with various other compounds derived from primary metabolites (Namdeo *et al.*, 2007; Ramadani and Jadid, 2024). Precursors are selected based on their involvement in the synthesis of the desired secondary metabolite(s) (Ramadani and Jadid, 2024). The basic premise of precursor-feeding is that supplementing cultures with compounds essential for phytochemical biosynthesis will increase the production of said phytochemicals (Ramadani and Jadid, 2024). Much research has shown the efficacy of precursors for the enhancement of alkaloid, phenolic, flavonoid, and terpenoid contents in culture systems (Dasari *et al.*, 2020; Kundu *et al.*, 2018; Moreno *et al.*, 1993). For instance, concentrations of strictosidine and ajmalicine (alkaloids) increased 5–11-fold with precursor application (Moreno *et al.*, 1993).

Most alkaloids are derived from amino acids (Wansi *et al.*, 2013). Specifically, phenylalanine and tyrosine are involved in the biosynthesis of numerous alkaloids originating from several different plant families (Seigler, 1998). Both amino acids are involved in biosynthetic pathways of norbelladine, which leads to the production of galanthamine, lycorine, and crinine alkaloids (Amaryllidaceae) (Desgagné-Penix, 2021). These precursors have also been implicated in the synthesis of tropane alkaloids, e.g. scopolamine and hyoscyamine (Solanaceae), and mesembrine-type alkaloids (Mesembryanthemaceae) (Bedewitz *et al.*, 2014; Jeffs *et al.*, 1971).

Methionine is a precursor to *S*-adenosylmethionine, which is part of the biosynthesis of various phytochemicals, including hordenine and isoquinoline alkaloids (Matchett *et al.*, 1953; Morishige *et al.*, 2000; Ravanel *et al.*, 1998). Purine alkaloids, such as caffeine, theobromine, and theacrine are also formed via methionine-dependent pathways (Ashihara *et al.*, 2013; Jia *et al.*, 2024; Zhou *et al.*, 2022).

Precursors may be supplied to cultures for the duration of incubation or for a short period of the culture (Rakesh and Praveen, 2022; Suryawanshi *et al.*, 2022). The appropriate time to supplement cultures can be selected randomly, however determination of the cell cycle (i.e.

lag, exponential, linear, deceleration, and stationary phases) is useful for deciding the optimal timeframe to supplement, subculture, or harvest cultures (Naill and Roberts, 2005; Volpe and Eremenko, 1973).

In terms of *S. tortuosum*, Jeffs and colleagues (1971) determined that methionine, tyrosine, and phenylalanine are involved in the biosynthesis of mesembrine-type alkaloids. Based on this knowledge, these amino acids were selected as precursors for the current investigation. Additionally, prior work (see **Chapter 4**) has shown the efficacy of PGRs for enhancing mesembrine-type alkaloid production. Thus, the effects of these elicitors on *S. tortuosum* cell cultures was investigated.

6.1.3 Aims and objectives

These investigations aimed to determine the effects of PGRs and amino acid precursors on the growth and alkaloid production of *S. tortuosum* in cell suspension cultures. The following objectives were set:

1) Initiate callus cultures using *in vitro*-derived leaf material and 2,4-D MS (agar) medium,
2) Initiate cell suspension cultures using callus from (1) and 2,4-D MS (liquid) medium,
3) Monitor growth of cell suspensions by performing total cell counts and recording cell fresh weights for 50 days,
4) Based on the findings of (3), determine the appropriate time(s) to terminate subsequent experiments,
5) Initiate PGR and precursor-feeding experiments using various PGRs and amino acids supplied at different concentrations,
6) Upon termination of experiments, record total cell number, cell fresh weight, and cell viability,
7) Freeze-dry cells and liquid culture media and perform alkaloid extractions,
8) Analyze culture components using UPLC-MS, and

9) Determine the optimal PGR(s) and precursor(s) for culture growth and alkaloid accumulation in cell suspensions of *S. tortuosum*.

6.2 Materials and Methods

6.2.1 Callus induction

Callus induction medium was prepared by supplementing Murashige and Skoog (1962) (MS) medium with 2.5 µM 2.4-D. Specifications of medium preparation were as described in **Section 2.2.2**. Plant material was obtained from IBA-containing multiplication cultures (2.5 µM IBA). Leaves were cut into pieces (approx. 0.5 cm^2) and inoculated onto the callus induction medium. Cultures were incubated with constant temperature (25 ± 3°C) and light (PAR = 73 $\mu mol.m^{-2}.s^{-1}$) for 17 days.

6.2.2 Cell cycle determination

Liquid MS medium supplemented with 2.5 µM 2.4-D was prepared. The medium was dispensed into 100 mL Erlenmeyer flasks (20 mL per flask). The flasks were stoppered with cotton wool bungs and covered with aluminium foil prior to autoclaving at 121°C, 1 bar, for 15 min.

Calli were removed from callus induction medium at the laminar flow bench and cut into small pieces (1–3 mm). For each flask, 4–6 intact pieces of callus were used (approx. 0.05 g in total). Cell suspension flasks were incubated under constant light (PAR = 10 $\mu mol.m^{-2}.s^{-1}$) and temperature (25 ± 3°C) on an orbital shaker set to 100 rpm for 50 days.

Every alternate day, three flasks were removed from the shaker and a small volume (< 1 mL) of the medium / suspension was taken to perform a total cell count. This was done using a Fuchs-Rosenthal haemocytometer, according to the method described by Warner and Edwards (1989). Briefly, a single drop of the medium was applied to the haemocytometer and all intact cells in the numbered squares **(Figure 6.1)** were counted. When cells were clustered, making counting difficult, the suspension was diluted with distilled water. Cell counting was performed using both sides of the haemocytometer to obtain an average for each replicate.

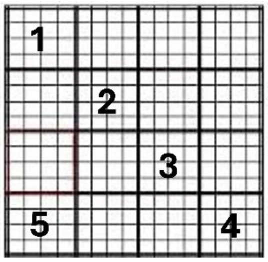

Figure 6.1: Counting grid of Fuchs-Rosenthal haemocytometer

Total cell number/mL was calculated using: $\frac{Cell\ number\ from\ 5\ squares}{5}$ x dilution factor x 10 000 = total cell number/mL

The contents of the flask were then poured out under vacuum filtration and fresh weight of the cells was recorded.

6.2.3 Cell suspension culture experiments

Liquid MS medium was fortified with NAA, 2,4-D, BAP, or KIN. Amino acid precursors, methionine, tyrosine, and phenylalanine were also tested. For each PGR / precursor, concentrations of 2.5, 5, and 10 µM were prepared. Twenty mL of medium was dispensed into 100 mL Erlenmeyer flasks. Each treatment comprised of three replications. Callus obtained from the induction medium was cut into small pieces and used to inoculate the treatment flasks (4–6 calli = ~ 0.05 g per flask). All flasks were incubated for 37 days under constant temperature (25 ± 3°C) and light (PAR = 10 µmol.m^{-2}.s^{-1}. On the 37th day, cultures were harvested. Sample photos of cultures are shown in **Figure 6.2**.

Figure 6.2: Cell suspension cultures on an orbital shaker during the experimental period **(A)**, and prior to harvest at the end of the experiment **(B)**

6.2.4 Cell culture harvest procedures

6.2.4.1 Cell counting and fresh weight determination

One mL of each replicate was removed, and a total cell count was performed as described in **Section 6.2.2**. A viable cell count was attempted by first staining the cells. For this, an additional 1 mL of the suspension was aliquoted into an Eppendorf tube and 25 µL of Evan's Blue (10 mg/mL) was introduced (Castro-Concha *et al.*, 2006). The mixture was incubated for 1 H at room temperature in darkness. Sample tubes were then centrifuged at 14 000 rpm for 1 min with a HERMLE Z 160 M desktop centrifuge. Samples were then rinsed three times with distilled water. Lastly, 1 mL of distilled water was added to rinsed cells. A drop of this cell solution was placed on the haemocytometer for cell counting.

It was clear from viewing undiluted samples that viable cells were present, but very few, if any could be counted using this method. Thus, cells were viewed using an Olympus AX70 compound light microscope coupled to a Nikon Digital Sight DS-U3. The NIS Elements Program was used to capture images and insert scale bars.

Contents of the experimental flasks were emptied out under vacuum filtration and weights of the cells were recorded. The cells and remaining liquid media were retained (separately) and stored at -80°C.

6.2.4.2 Sample preparation, alkaloid extraction, and analysis

The retained cells and liquid media were freeze-dried. Cell samples were powdered with a mortar and pestle, however this was not possible for the media samples as freeze-drying resulted in a sticky resin. To obtain a dried medium component, the samples were heated to ~ 60°C using a hot plate. HPLC grade methanol (10–15 mL) was added to loosen and dissolve the resin. Thereafter, samples were dried in an oven at 100°C. This yielded a product which could be scraped and weighed accurately for extraction procedures.

Dried cell and media samples were sonicated with HPLC grade methanol (10–20 mg/mL) for 20 min. The samples were then filtered through a 0.20 µm syringe-filter (MACHEREY-NAGEL CHROMAFIL® Xtra PTFE-20/25) and collected in autosampler vials. Sample and data analyses were as stated in **Section 2.2.4**.

6.2.5 Experimental conditions and challenges

Experiment one: Flasks were incubated with constant light (PAR = 10 $\mu mol.m^{-2}.s^{-1}$) and shaking (100 rpm) for 37 days. However, due to electrical issues, one of two shakers malfunctioned, overheated, and shaking ceased. Flasks from this shaker were transferred to alternative shakers for the remainder of the experiment. Additionally, after harvest and storage of the culture materials, an electrical outage resulted in no power to the building for almost three weeks. This led to samples defrosting, and the introduction of microbes into samples. As mesembrine-type alkaloids are stable at a wide range of temperatures, samples were processed regardless of the adversities experienced. Microbial growth was removed by slightly defrosting samples and dissecting away microbial growth using a scalpel. Samples were then subjected to drying, extraction, and analysis as stated above.

The experiment was repeated (experiment two), but similar difficulties were experienced, i.e. equipment malfunctions leading to more severe heating of culture flasks. Shaking became uneven, with more pronounced movement on one side of the shaker as compared to the other. This led to inconsistencies in growth within treatments. Harvest, drying and extraction procedures took place as previously stated, however due to large discrepancies in alkaloid quantities, these results are not reported.

Experiment three was conducted with only the optimal treatments (10 µM 2,4-D and 2.5 µM phenylalanine) and a control treatment. These treatments were selected based on the fresh weights obtained from the first experiment. Flasks were incubated with constant temperature (25 ± 3°C), light (PAR = 12 µmol.m^{-2}.s^{-1}) and shaking (110 rpm) for 37 days.

6.3 Results

6.3.1 Cell cycle

The cell cycle of *S. tortuosum* cultures is depicted in **Figure 6.3**. Mean total cell number remained relatively low during the lag phase, i.e. from Day 6–12 (4.8 – 5.5 x 10^4 cells/mL). The total cell number then increased gradually from Day 14 (2.2 x 10^4 cells/mL) until Day 32 (3.3 x 10^6 cells/mL). A steep increase was then observed between Day 32 and Day 34 (4.8 x 10^7 cells/mL). From Day 34 onwards, slight fluctuations were observed during the stationary phase, however cell number remained relatively constant until Day 50 (1.3 x 10^8 cells/mL) **(Figure 6.3)**.

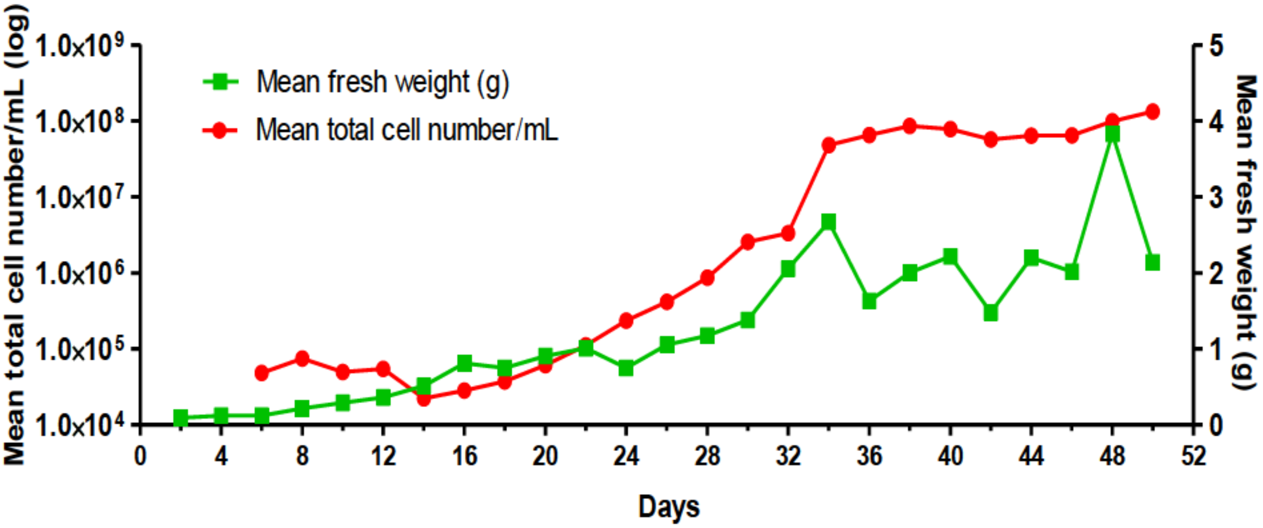

Figure 6.3: Mean total cell number/mL and mean fresh weight of *Sceletium tortuosum* cells grown in liquid MS medium containing 2.5 μM 2,4-dichlorophenoxyacetic acid during the 50-day incubation period

Mean fresh cell weights increased gradually from Day 2 (0.091 g) until Day 16 (0.81 g) **(Figure 6.3)**. Some fluctuations were observed from Day 16–24 (0.81–0.75), followed by continuous increases until Day 34 (2.67 g). Further fluctuations were recorded, with increases from Day 36–40 (1.63–2.22 g), and Day 42–44 (1.48–2.20 g). A final sharp increase was observed between Day 46–48 (2.02–3.83 g), which was responsible for the peak fresh weight throughout the duration. Thereafter, fresh weight decreased from Day 48–50 (3.83–2.14 g) **(Figure 6.3)**.

6.3.2 PGRs and culture growth

Plant growth regulators exerted considerable influence on the growth of *S. tortuosum* cell cultures. Significant differences were observed between treatments **(Figure 6.4)**. The highest mean total cell numbers were achieved by 5 μM 2,4-D and 10 μM NAA (1.59 x 10^{11} ± 1.19 x 10^{11} and 1.44 x 10^{11} ± 9 x 10^{10} cells/mL, respectively) **(Figure 6.4A)**. These treatments resulted in cell numbers that were significantly higher than the control treatment (3.23×10^{10} ± 7.42×10^{9} cells/mL). The overall lowest mean total cell number originated from the 10 µM KIN treatment (2.01×10^{10} ± 7.25×10^{9} cells/mL) **(Figure 6.4A)**.

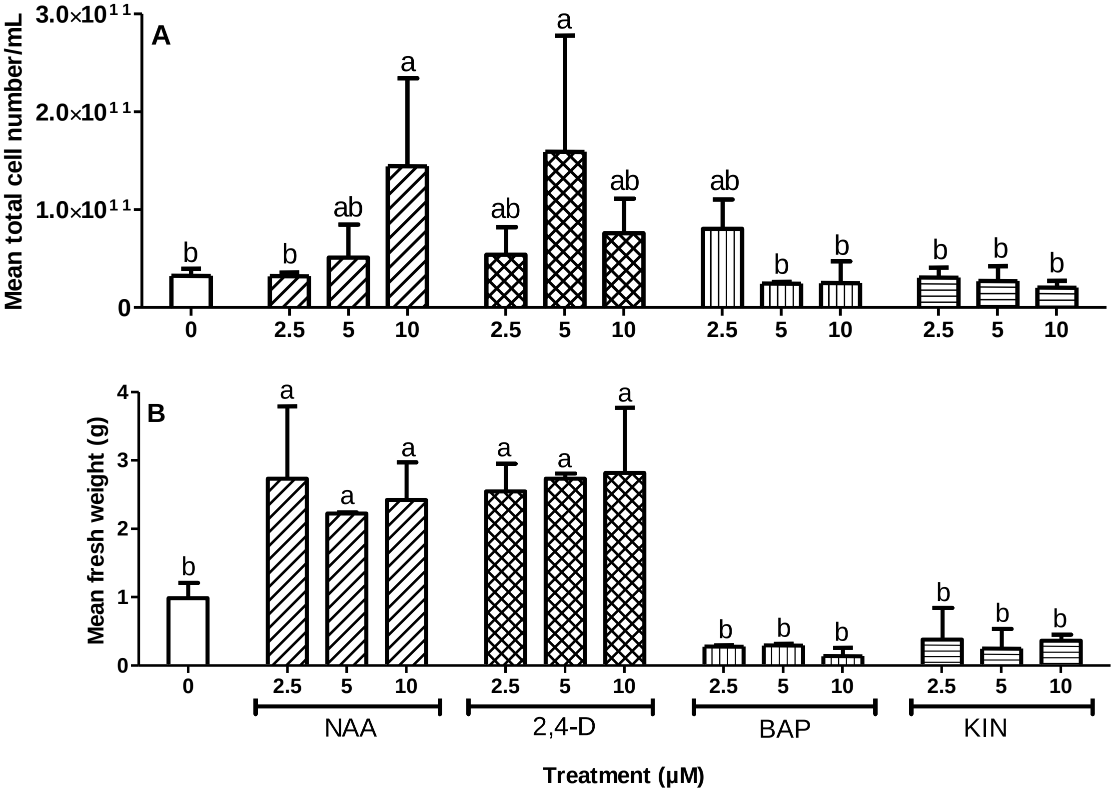

Figure 6.4: Mean total cell number/mL **(A)** and fresh weight of *Sceletium tortuosum* cells **(B)** after exposure to different plant growth regulator treatments for 37 days (NAA = naphthaleneacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = benzylaminopurine; KIN = kinetin). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Regarding mean fresh weight of cells, the 2,4-D (2.55–2.82 g) and NAA (2.22–2.73 g) treatment groups resulted in consistently high weights, which were significantly higher than the control and all cytokinin treatments **(Figure 6.4B)**. Within the 2,4-D group, 10 µM was responsible for the highest mean fresh weight (2.82 ± 0.95 g). Among the NAA treatments, 2.5 µM achieved the highest fresh weight (2.73 ± 1.06 g). Kinetin (0.24–0.38 g) and BAP (0.14–0.29 g) treatment groups obtained low fresh weights, all of which were exceeded by the control treatment (0.98 ± 0.23 g) **(Figure 6.4B)**.

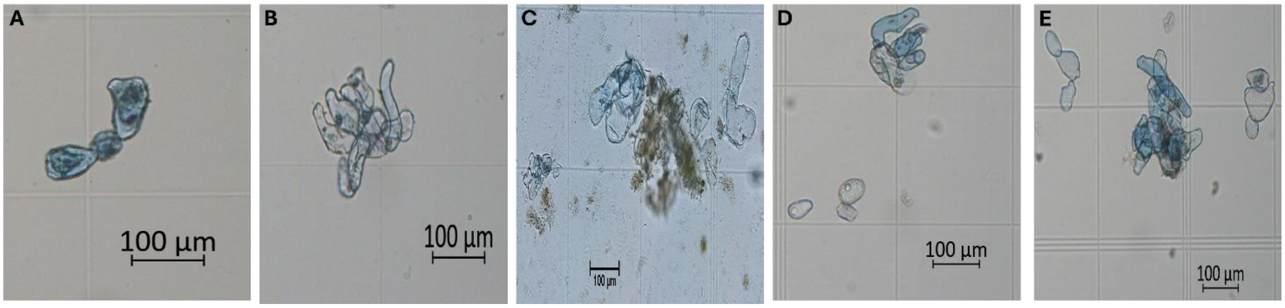

Figure 6.5: Microscopic images of viable *Sceletium tortuosum* cells obtained from the control **(A)**, 5 µM 2,4-D **(B)**, 10 µM 2,4-D **(C)**, 2.5 µM NAA **(D)**, and 10 µM NAA **(E)** treatments. Scale bars indicate 100 µm.

Cells from the control treatment conformed to a spherical or oval shape **(Figure 6.5A)**. Treatments with 2,4-D resulted in cell elongation and enlargement **(Figure 6.5B** and **C)**. Naphthaleneacetic acid-treated cultures also exhibited cell elongation, however this was more pronounced with 10 uM NAA as compared to 2.5 uM NAA **(Figure 6.5D** and **E)**.

6.3.3 PGRs and alkaloid production

6.3.3.1 Cells

Notable differences were observed among treatments for cellular alkaloid contents and all quantified alkaloids **(Figure 6.6)**. The highest alkaloid contents were found in the BAP and 2,4-D treatment groups **(Figure 6.6A)**. Within each of these groups, 2.5 µM BAP (4.35 ± 0.74 µg/mg DW) and 10 µM 2,4-D (2.03 ± 0.28 µg/mg DW) were responsible for the maximum alkaloid contents. Alkaloid contents increased with increasing concentrations of 2,4-D. Treatments with NAA (0.48–1.03 μg/mg DW) and KIN (0.56–0.94 μg/mg DW) resulted in alkaloid contents that were similar the control treatment (1.03 ± 0.59 μg/mg DW) **(Figure 6.6A)**.

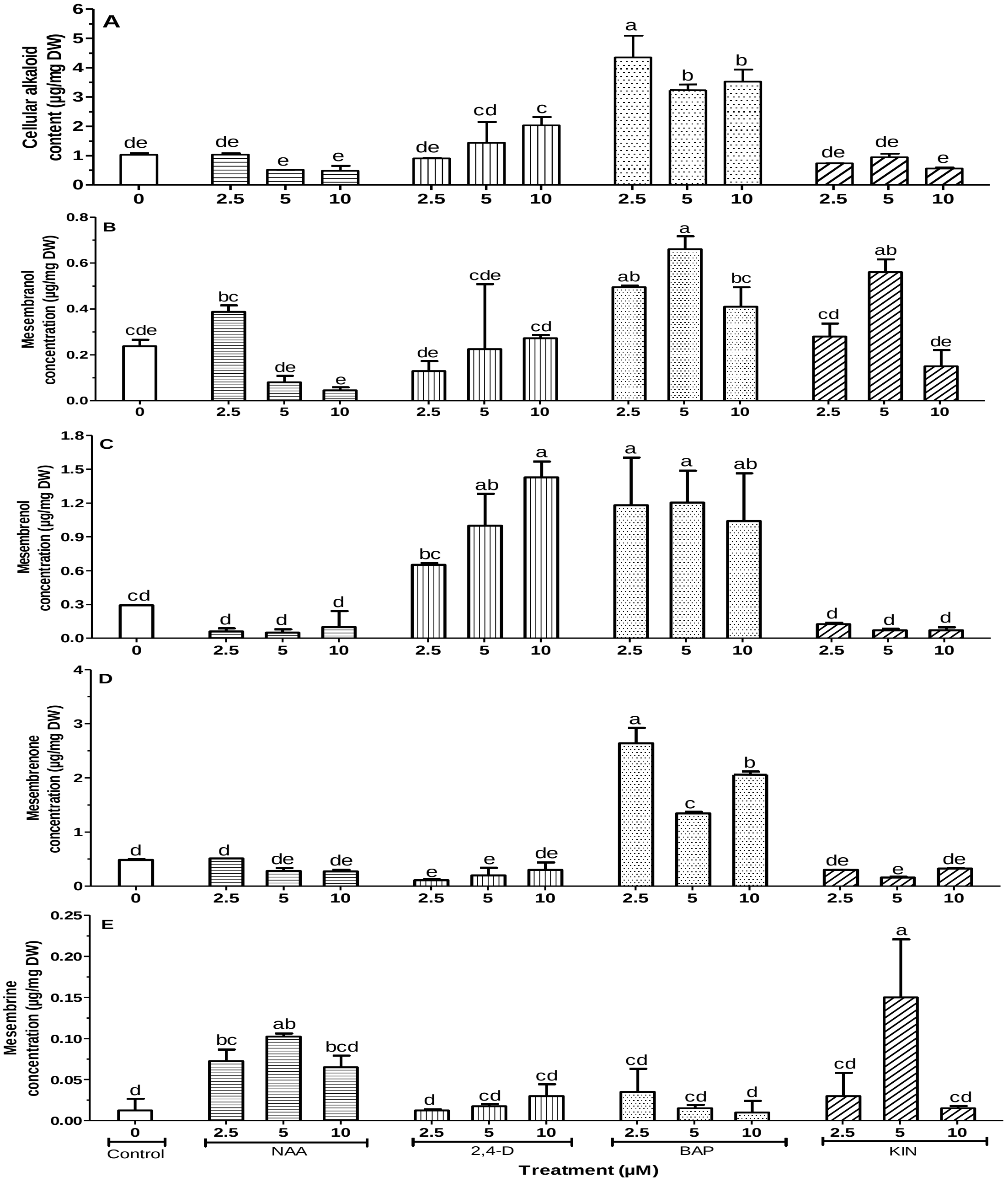

Figure 6.6: Alkaloid content of *Sceletium tortuosum* cells **(A)**, and individual concentrations of mesembranol **(B)**, mesembrenol **(C)**, mesembrenone **(D)**, and mesembrine **(E)** after exposure to different plant growth regulator treatments for 37 days (NAA = naphthaleneacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = benzylaminopurine; KIN = kinetin). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

The highest mesembranol concentration was achieved by 5 µM BAP (0.66 ± 0.057 µg/mg DW) **(Figure 6.6B)**. Kinetin (5 µM) also resulted in a mesembranol concentration that was significantly higher than the control treatment (0.56 ± 0.057 and 0.24 ± 0.028 µg/mg DW, respectively). In contrast, the lowest mesembranol concentration resulted from 10 µM NAA (0.45 ± 0.014 µg/mg DW). Interestingly, a step-wise decrease in mesembranol concentration was observed with increasing NAA concentrations. The opposite was observed for 2,4-D, i.e. increasing 2,4-D concentration resulted in increased mesembranol concentration **(Figure 6.6B)**.

Mesembrenol levels were highest for 2,4-D and BAP treatments **(Figure 6.6C)**. Once again, proportional increases were observed with increasing concentrations of 2,4-D, with 10 µM resulting in the highest quantity of mesembrenol (1.43 ± 0.14 µg/mg DW). Treatments with BAP (2.5 and 5 µM) also resulted in high mesembrenol quantities (1.18 ± 0.42 and 1.21 ± 0.28 µg/mg DW, respectively). Mesembrenol concentrations were quite low within NAA (0.05–0.1 µg/mg DW) and KIN (0.07–0.13 µg/mg DW) treatment groups. Concentrations from these groups were surpassed by that of the control treatment (0.29 ± 0.0028 µg/mg DW) **(Figure 6.6C)**.

Mesembrenone concentrations peaked in the BAP treatment group (1.35–2.64 µg/mg DW), where 2.5 µM was responsible for the maximum amount **(Figure 6.6D)**. The lowest mesembrenone concentration was obtained from 2.5 µM 2,4-D (0.11 ± 0.014 µg/mg DW). The NAA, 2,4-D, and KIN treatment groups yielded mesembrenone concentrations that were similar to, or lower than that of the control treatment (0.49 ± 0.014 µg/mg DW) **(Figure 6.6D)**.

Mesembrine concentration was highest for 5 µM KIN (0.15 ± 0.071 µg/mg DW) **(Figure 6.6E)**. Only 5 µM KIN, and 2.5 and 5 µM NAA (0.073 ± 0.014 and 0.10 ± 0.0035 µg/mg DW, respectively) yielded mesembrine quantities that were significantly higher than the control treatment (0.013 ± 0.014 µg/mg DW). All remaining treatments yielded mesembrine concentrations that were similar to the control **(Figure 6.6E)**.

6.3.3.2 PGRs and culture media

Alkaloids were present in liquid culture media. Plant growth regulator treatments significantly affected the amount of alkaloids that exuded into media **(Figure 6.7)**. The highest alkaloid content was obtained from 10 µM 2,4-D (1.67 ± 0.11 µg/mg DW) **(Figure 6.7A)**. Treatment with 2.5 µM NAA, also resulted in a significantly large fraction (1.41 ± 0.11 µg/mg DW). However, 5 µM NAA resulted in the absence of an alkaloid fraction altogether. The control treatment (0.34 ± 0.042 µg/mg DW) yielded a larger alkaloid fraction than those obtained from the BAP and KIN treatment groups **(Figure 6.7A)**.

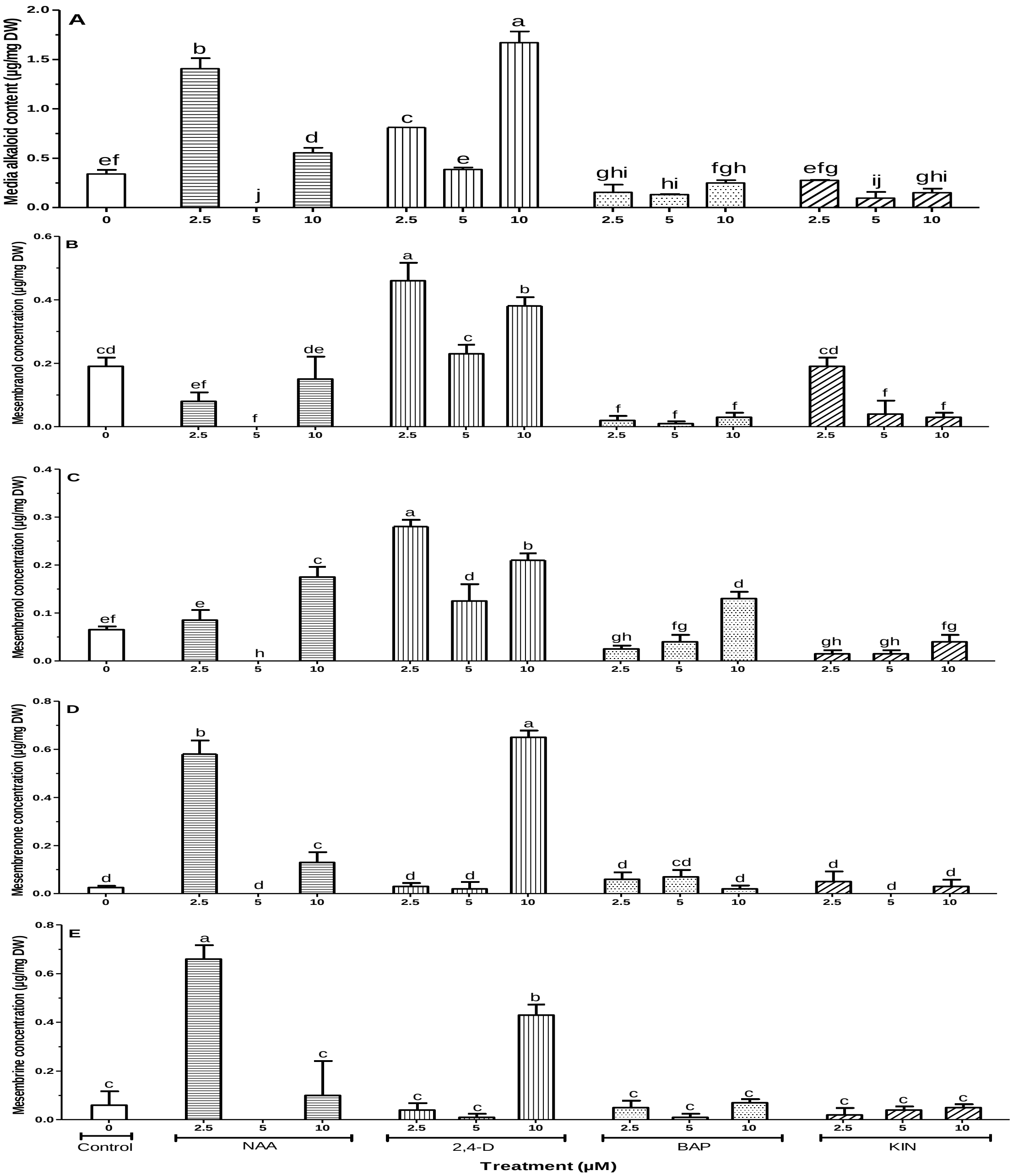

Figure 6.7: Alkaloid content in culture media **(A)**, and individual concentrations of mesembranol **(B)**, mesembrenol **(C)**, mesembrenone **(D)**, and mesembrine **(E)** after exposure to different plant growth regulator treatments for 37 days (NAA = naphthaleneacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = benzylaminopurine; KIN = kinetin). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Mesembranol concentration was highest in the 2,4-D treatment group, with 2.5 and 10 μM resulting in optimal concentrations (0.46 ± 0.057 and 0.38 ± 0.028 μg/mg DW, respectively) **(Figure 6.7B)**. 6-Benzylaminopurine treatments yielded mesembranol concentrations (0.01–0.03 μg/mg DW) that were significantly lower than that of the control treatment (0.19 ± 0.028 μg/mg DW) **(Figure 6.7B)**.

Mesembrenol concentrations were also optimized by 2,4-D treatments **(Figure 6.7C)**. The highest mesembrenol concentrations were achieved by 2.5 μM and 10 μM 2,4-D (0.28 ± 0.014 and 0.21 ± 0.014 μg/mg DW, respectively). Most BAP and KIN treatments yielded mesembrenol quantities that were lower than the control treatment (0.065 ± 0.0071 μg/mg DW). Although, 5 μM NAA produced no mesembrenol, the other NAA treatments (0.085 ± 0.021 and 0.18 ± 0.021 μg/mg DW, for 2.5 and 10 μM, respectively) resulted in higher mesembrenol levels than the control **(Figure 6.7C)**.

The highest mesembrenone concentrations stemmed from 10 μM 2,4-D and 2.5 μM NAA (0.65 ± 0.028 and 0.58 ± 0.057 μg/mg DW) **(Figure 6.7D)**. These treatments, along with 10 μM NAA (0.13 ± 0.42 μg/mg DW) were significantly higher than that of the control treatment (0.025 ± 0.0071 μg/mg DW). Consistently low mesembrenone concentrations were observed from the BAP (0.02–0.07 μg/mg DW) and KIN (0–0.05 μg/mg DW) treatment groups. Mesembrenone was completely absent from 5 μM KIN and 5 μM NAA treatments **(Figure 6.7D)**.

Mesembrine concentrations peaked in the NAA and 2,4-D treatment groups **(Figure 6.7E)**. Naphthaleneacetic acid (2,5 μM) and 10 μM 2,4-D resulted in the highest concentrations (0.66 ± 0.057 and 0.43 ± 0.042 μg/mg DW). These concentrations exceeded the control treatment (0.06 ± 0.057 μg/mg DW). All other treatments yielded mesembrine quantities similar to that of the control. Only 5 μM NAA produced no mesembrine **(Figure 6.7E)**.

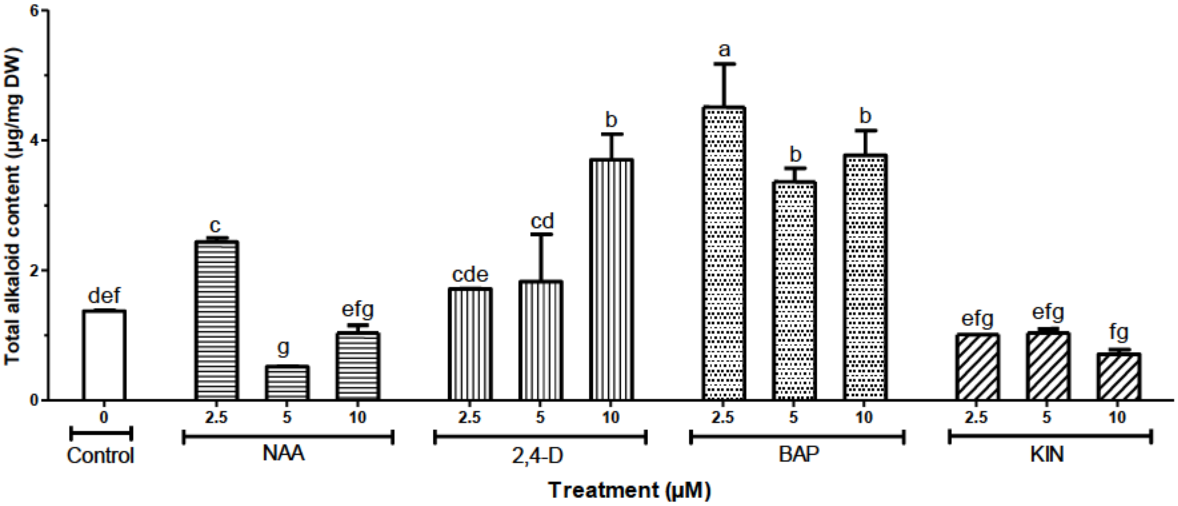

Figure 6.8: Total alkaloid contents (cells + media) of plant growth regulator-treated suspension cultures of *Sceletium tortuosum* (NAA = naphthaleneacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = benzylaminopurine; KIN = kinetin). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p <$ 0.05) between treatments.

Total alkaloid content was highest for 2.5 and 10 µM BAP (4.51 ± 0.66 and 3.77 ± 0.38 µg/mg DW, respectively), followed by 10 µM 2,4-D (3.70 ± 0.40 µg/mg DW). Increasing concentrations of 2,4-D improved the total alkaloids contents accordingly. The lowest total alkaloid content was achieved by 5 µM NAA (0.52 ± 0.0071 µg/mg DW) **(Figure 6.8)**.

Table 6.1: Mesembrine-type alkaloid concentrations of cells/calli originating from BAP treatments from this experiment and Makunga *et al.*'s (2022) study

Study and treatment	Mesembrenol (mg/kg DW)	Mesembranol (mg/kg DW)	Mesembrenone (mg/kg DW)	Mesembrine (mg/kg DW)
Makunga *et al.* (2022) **New callus growth (4.44 µM BAP)**	4.0	37.0	0	3.7
Original inoculum (4.44 µM BAP)	10.9	64.6	26.9	21.4
This study **2.5 µM BAP**	1180	500	2640	35
5 µM BAP	1210	660	1350	15
10 µM BAP	1040	410	2060	10

*Concentrations from this study were converted from µg/mg to mg/kg using: $\mathcal{X}$ µg/mg x 1000

Table 6.1 shows a comparison between the cells generated from BAP treatments in the current experiment, and the callus produced from a BAP treatment (4.44 µM) tested by Makunga *et al.* (2022). Cell lines with the highest alkaloid concentrations are presented in the table. The BAP treatments used in the present study resulted in mesembranol, mesembrenol, and mesembrenone concentrations that were between 11- and 303-fold higher than those expressed by Makunga *et al.* (2022). In terms of mesembrine, the 2.5 µM BAP treatment exceeded both of the concentrations expressed in the publication.

6.3.4 Amino acid precursors and culture growth

Amino acid precursors altered *S. tortuosum* culture growth. Considerable differences between treatments were noted **(Figure 6.9)**. The highest mean total cell number was achieved by the control treatment (2.12 x 10^{11} ± 4.53 x 10^{10} cells/mL), followed by 10 µM tyrosine ($1.74 \times 10^{11} \pm 1.08 \times 10^{11}$ cells/mL). The lowest cell count was observed in the 5 µM tyrosine treatment ($1.70 \times 10^{10} \pm 8.49 \times 10^{9}$ cells/mL) **(Figure 6.9A)**.

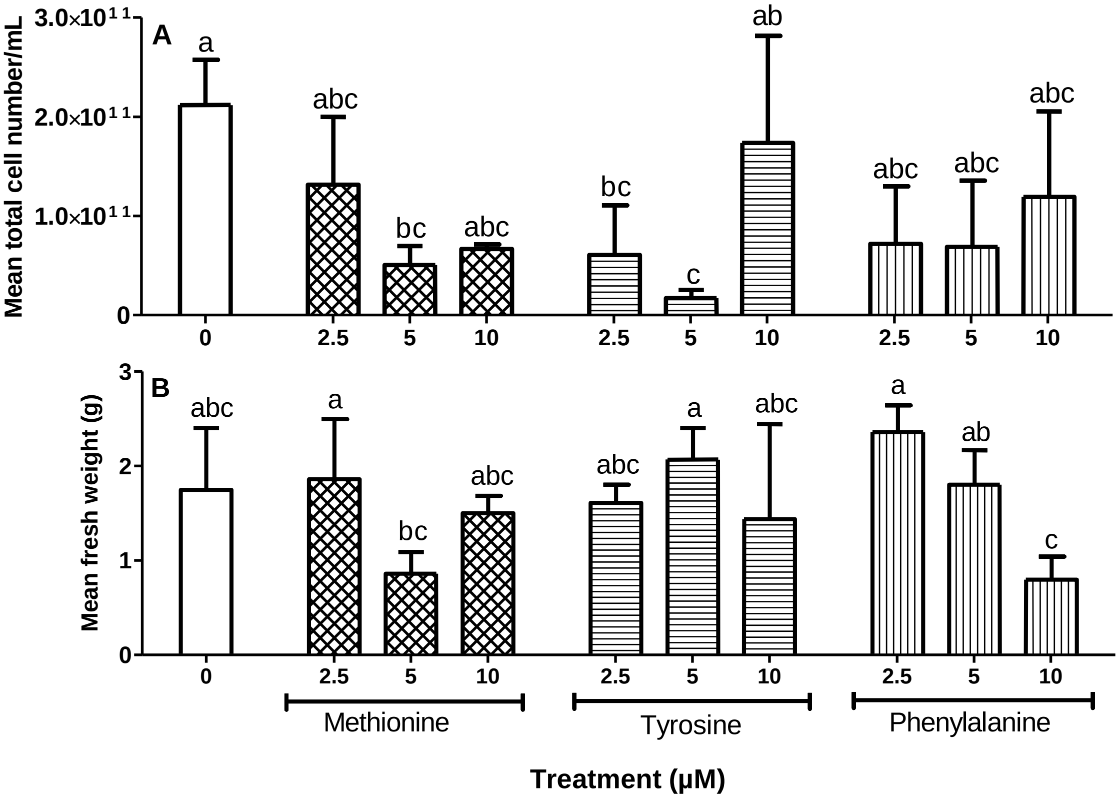

Figure 6.9: Mean total cell number/mL **(A)** and mean fresh weight **(B)** of *Sceletium tortuosum* cells after exposure to different amino acid precursor treatments for 37 days. Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

The highest mean fresh weights were achieved by 2.5 µM phenylalanine (2.36 ± 0.28 g), 5 µM tyrosine (2.07 ± 0.34 g), and 2.5 µM methionine (1.86 ± 0.63 g) **(Figure 6.9B)**. Although these treatments resulted in weights that exceeded the control treatment (1.75 ± 0.65 g), these increases were not statistically significant. The lowest mean fresh weight was as a result of 10 µM phenylalanine (0.80 ± 0.25 g) **(Figure 6.9B)**.

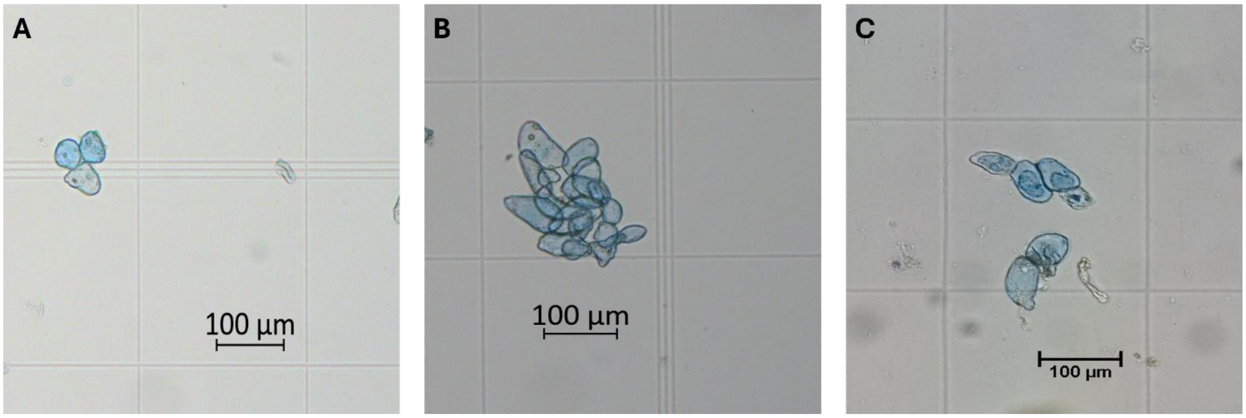

Figure 6.10: Microscopic images of viable *Sceletium tortuosum* cells obtained from control **(A)**, 10 µM tyrosine **(B)**, and 2.5 µM phenylalanine **(C)** treatments. Scale bars indicate 100 µm.

Cells obtained from the control treatment were spherical in shape and the smallest in size **(Figure 6.10A)**. Cell enlargement was visible among precursor-treated cells. Most cells were oval or elliptical in shape **(Figure 6.10B** and **C)**.

6.3.5 Amino acid precursors and alkaloid production

6.3.5.1 Cells

Amino acid precursors had some impact on the alkaloid contents and concentrations of *S. tortuosum* cells. However, significant differences were only apparent between treatments for mesembrenol concentration **(Figure 6.11)**. With regards to alkaloid contents, 2.5 µM methionine and 2.5 µM tyrosine yielded the highest cellular alkaloid contents (0.49 ± 0.13 and 0.40 ± 0.21 µg/mg DW, respectively) **(Figure 6.11A)**. For both these precursors, alkaloid contents decreased as precursor concentration increased. Contrastingly, the lowest alkaloid content was obtained from 10 µM methionine (0.27 ± 0.042 µg/mg DW) **(Figure 6.11A)**.

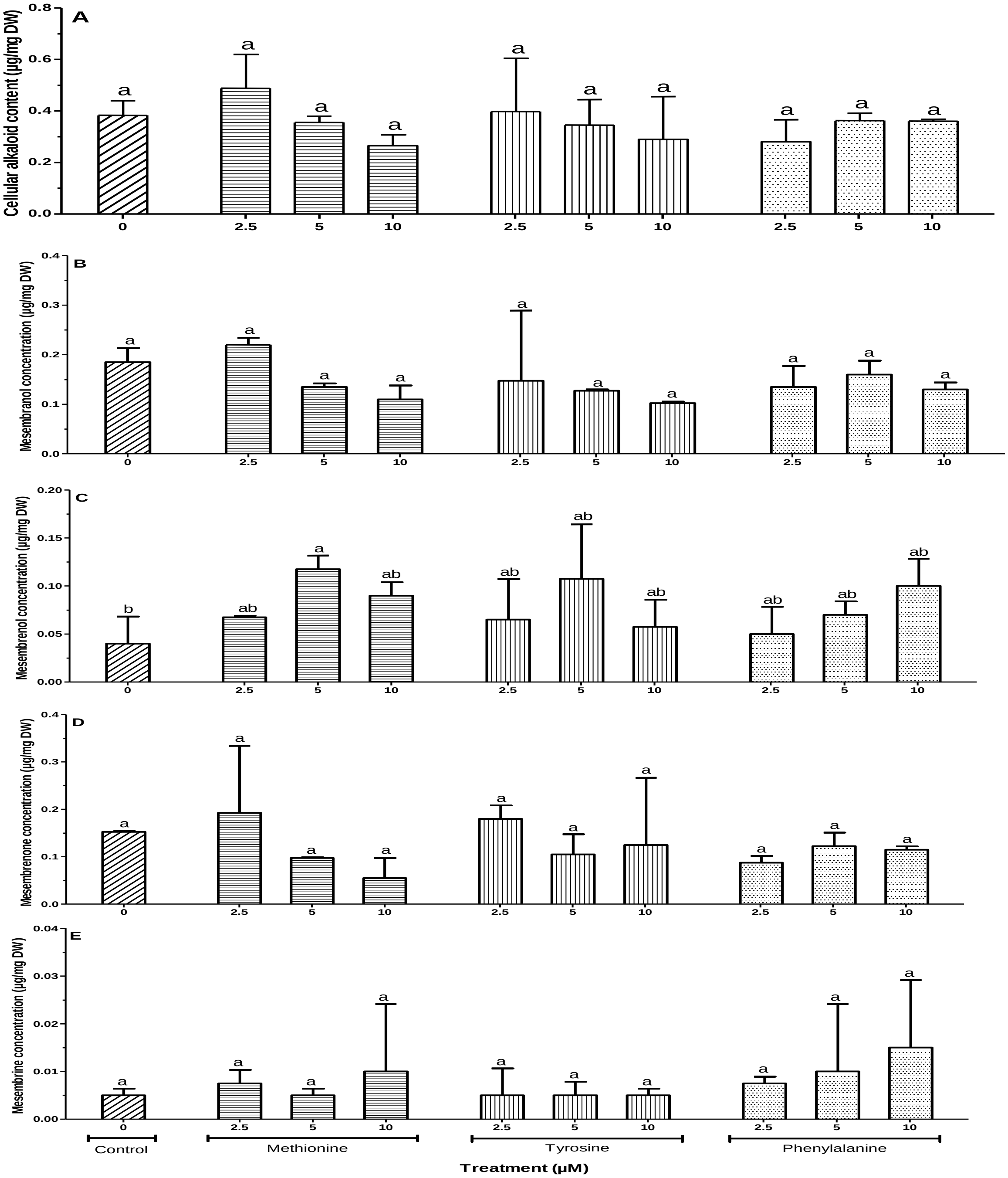

Figure 6.11: Alkaloid contents of *Sceletium tortuosum* cells **(A)**, and individual concentrations of mesembranol **(B)**, mesembrenol **(C)**, mesembrenone **(D)**, and mesembrine **(E)** after exposure to different amino acid precursor treatments for 37 days. Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

The highest mesembranol concentration was achieved by the 2.5 µM methionine treatment (0.22 ± 0.014 µg/mg DW) **(Figure 6.11B)**. This was the only treatment which had a higher mesembranol concentration than the control (0.19 ± 0.028 µg/mg DW), however this did not result in a statistically significant difference. Tyrosine (10 µM) was responsible for the lowest mesembranol concentration (0.10 ± 0.0028 µg/mg DW) **(Figure 6.11B)**.

All treatments resulted in mesembrenol quantities that were higher than the control treatment (0.04 ± 0.028 µg/mg DW). Mesembrenol concentration was optimized by 5 µM methionine (0.12 ± 0.014 µg/mg DW), which had a significantly higher concentration than the control **(Figure 6.11C)**.

Mesembrenone concentration was highest for 2.5 µM methionine (0.19 ± 0.14 µg/mg DW), and lowest for 10 µM methionine (0.055 ± 0.042 µg/mg DW) **(Figure 6.11D)**. The control treatment (0.15 ± 0.0014 µg/mg DW) yielded mesembrenone quantities that surpassed all treatments, with the exception of 2.5 µM tyrosine (0.18 ± 0.028 µg/mg DW) and 2.5 µM methionine (0.19 ± 0.14 µg/mg DW) **(Figure 6.11D)**.

Mesembrine concentration increased in accordance with phenylalanine concentration, with the highest quantity achieved by 10 µM phenylalanine (0.015 ± 0.014 µg/mg DW) **(Figure 6.11E)**. Methionine (5 µM) was responsible for the lowest level of mesembrine (0.0050 ± 0.0014 µg/mg DW). All tyrosine treatments resulted in the same mesembrine concentration as the control treatment (0.0050 µg/mg DW) **(Figure 6.11E)**.

6.3.5.2 Culture media

Amino acid precursor treatments had significant effects on the alkaloid contents of media and individual mesembrine-type alkaloids **(Figure 6.12)**. The control (0.030 ± 0.028 µg/mg DW) and 10 µM methionine (0.020 ± 0.021 µg/mg DW) resulted in the smallest alkaloid fractions overall **(Figure 6.12A)**. However, 2.5 µM phenylalanine resulted in the largest alkaloid fraction (3.04 ± 0.15 µg/mg DW). The phenylalanine treatment group showed decreases in alkaloid contents as the precursor concentration increased **(Figure 6.12A)**.

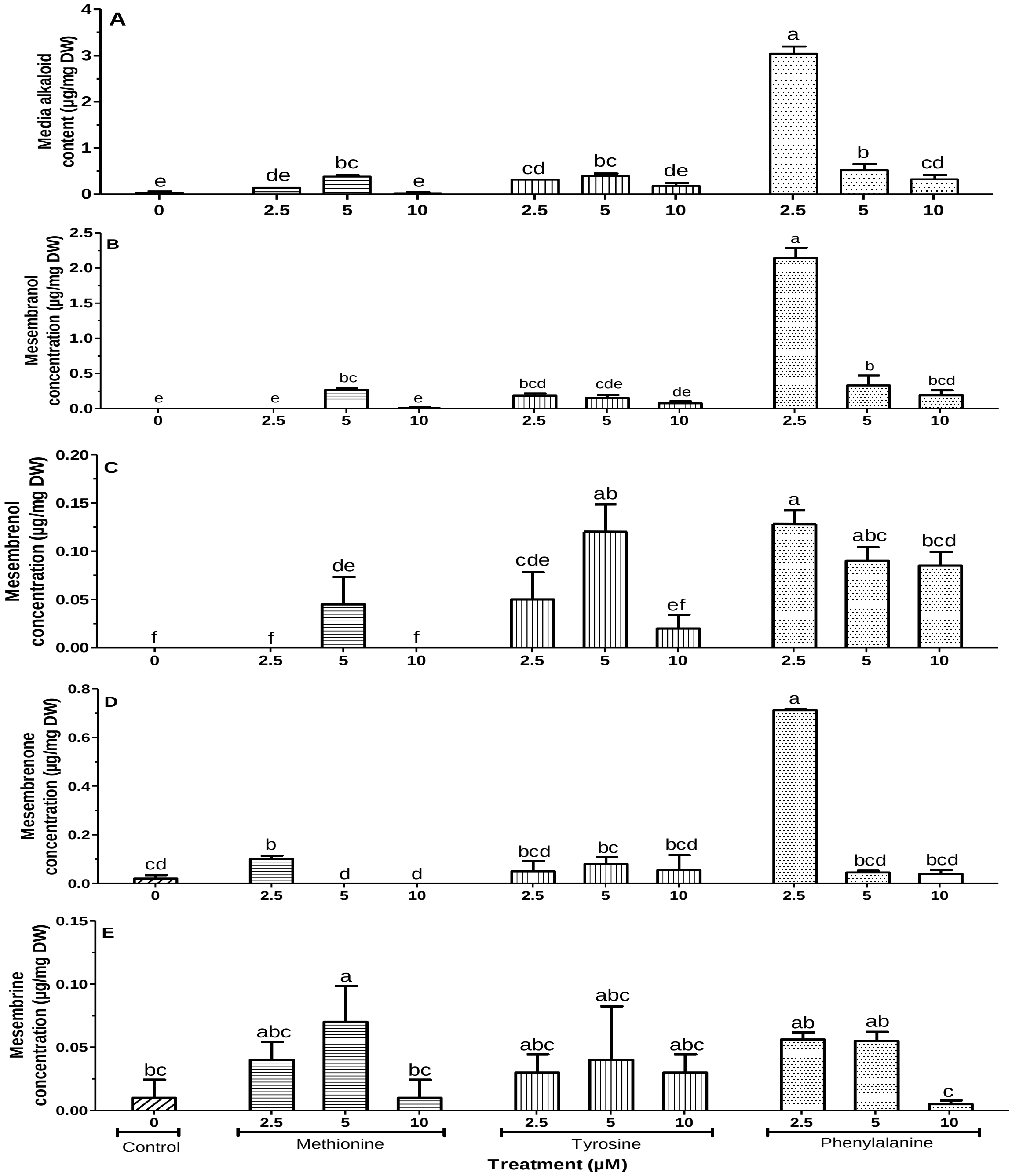

Figure 6.12: Alkaloid contents in *Sceletium tortuosum* culture media **(A)**, and individual concentrations of mesembranol **(B)**, mesembrenol **(C)**, mesembrenone **(D)**, and mesembrine **(E)** after exposure to different amino acid precursor treatments for 37 days. Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Mesembranol, mesembrenol and mesembrenone concentrations were optimized by the 2.5 µM phenylalanine treatment (2.14 ± 0.14, 0.13 ± 0.014, and 0.71 ± 0.0042 µg/mg DW, respectively). Quantities of all four alkaloids were reduced as phenylalanine concentration increased **(Figure 6.12)**.

With regards to mesembranol, the same inversely proportional trend was observed for tyrosine treatments, which resulted in 2.5 µM achieving the highest mesembranol quantity within the treatment group (0.19 ± 0.028 µg/mg DW) **(Figure 6.12B)**. In terms of mesembrenol, 5 µM tyrosine yielded a high concentration of mesembrenol (0.12 ± 0.028 µg/mg DW), similar to the peak concentration obtained from 2.5 µM phenylalanine (0.13 ± 0.014 µg/mg DW) **(Figure 6.12C)**. The control treatment, and 2.5 and 10 µM methionine did not result in detectable levels of both these alkaloids **(Figure 6.12C)**.

The control (0.020 ± 0.014 µg/mg DW) treatment yielded a quantity of mesembrenone that was similar to all treatments, except 2.5 µM phenylalanine (0.71 ± 0.0042 µg/mg DW). Methionine (2.5 µM) resulted in a mesembrenone concentration of 0.10 ± 0.014 µg/mg DW, however higher methionine concentrations did not result in any quantifiable mesembrenone **(Figure 6.12D)**.

Methionine (5 µM) was responsible for the highest mesembrine quantity overall (0.070 ± 0.028 µg/mg DW) **(Figure 6.12E)**. Following this, 2.5 and 5 µM phenylalanine resulted in the highest mesembrine concentrations (0.056 ± 0.0057 and 0.055 ± 0.0071 µg/mg DW). The lowest yield of mesembrine was obtained by 10 µM phenylalanine (0.0050 ± 0.0028 µg/mg DW) **(Figure 6.12E)**.

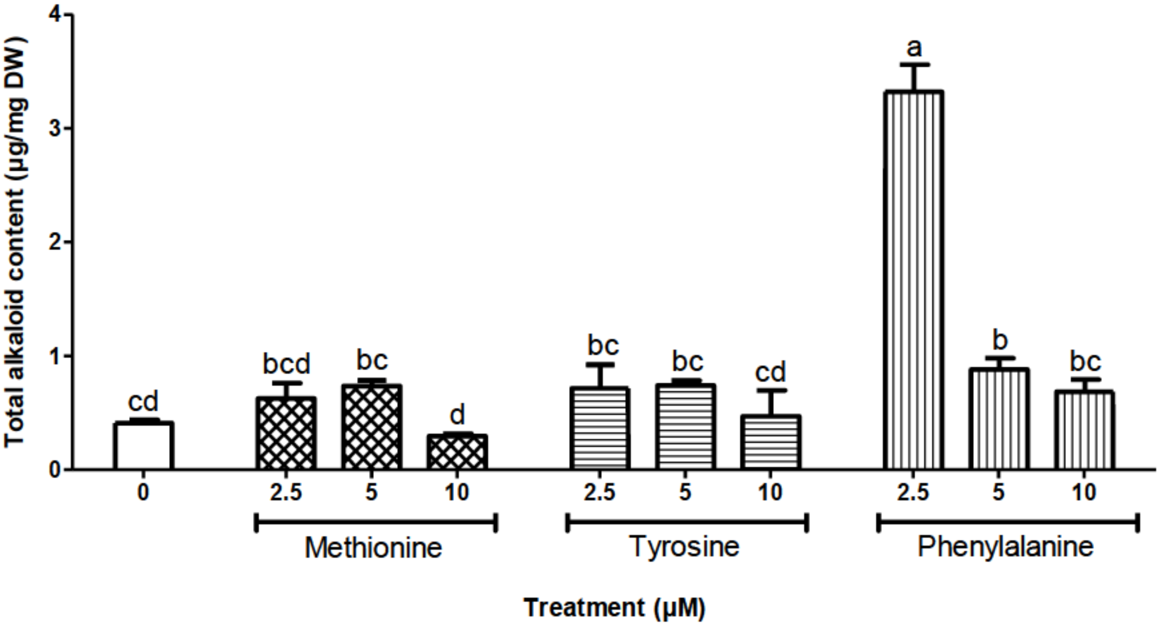

Figure 6.13: Total alkaloid contents (cells + media) of precursor-treated suspension cultures of *Sceletium tortuosum*. Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Phenylalanine (2.5 µM) yielded the highest total alkaloid content (3.32 ± 0.24 µg/mg DW). Methionine (10 µM) was responsible for the lowest total alkaloid content (0.30 ± 0.021 µg/mg DW). The control treatment (0.41 ± 0.028 µg/mg DW) exceeded the yield of only this methionine treatment, however these treatments were not significantly different from each other. Total alkaloid contents of all remaining treatments (0.41–3.32 µg/mg DW) were higher than the control **(Figure 6.13)**.

6.3.6 Third (optimized) experiment

6.3.6.1 Culture growth

Aspects of culture growth from the optimized experiment are shown in **Figure 6.14**. Plant growth regulator treatment (2,4-D) was responsible for the highest mean total cell number ($5.22 \times 10^9 \pm 3.03 \times 10^9$ cells/mL), and fresh weight of cells (3.26 ± 0.41 g). The lowest mean total cell number ($3.76 \times 10^9 \pm 8.49 \times 10^8$ cells/mL) and mean fresh weight (1.01 ± 0.84 g) resulted from the 2.5 µM phenylalanine treatment **(Figure 6.14)**. For both growth parameters, 2,4-D resulted in a significantly higher mean total cell number and fresh weight than the remaining two treatments.

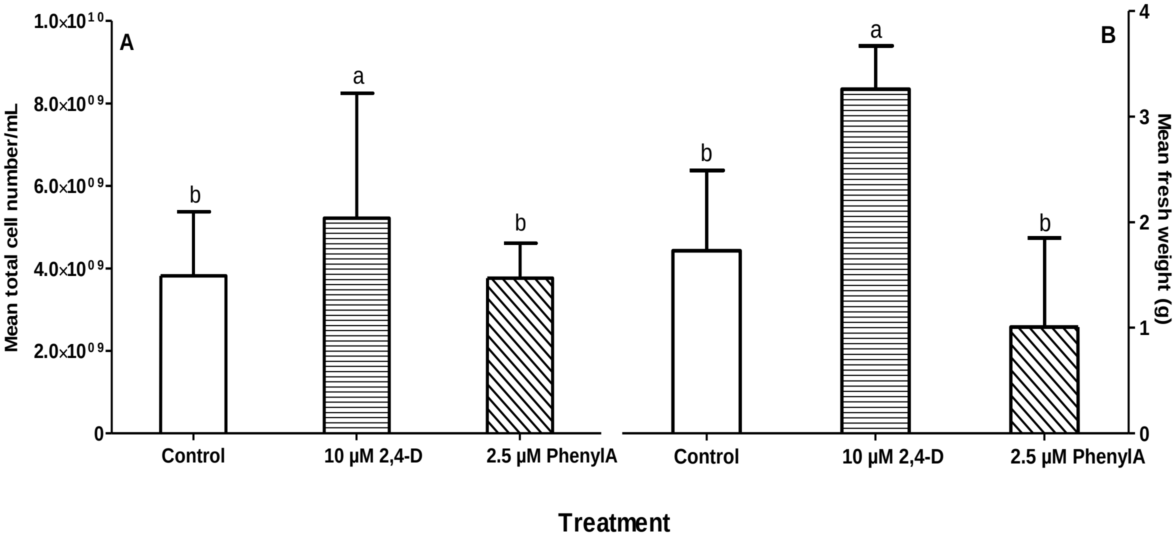

Figure 6.14: Mean total cell number/mL **(A)** and mean fresh weight **(B)** of *Sceletium tortuosum* cells from the third experiment (PhenylA = phenylalanine). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

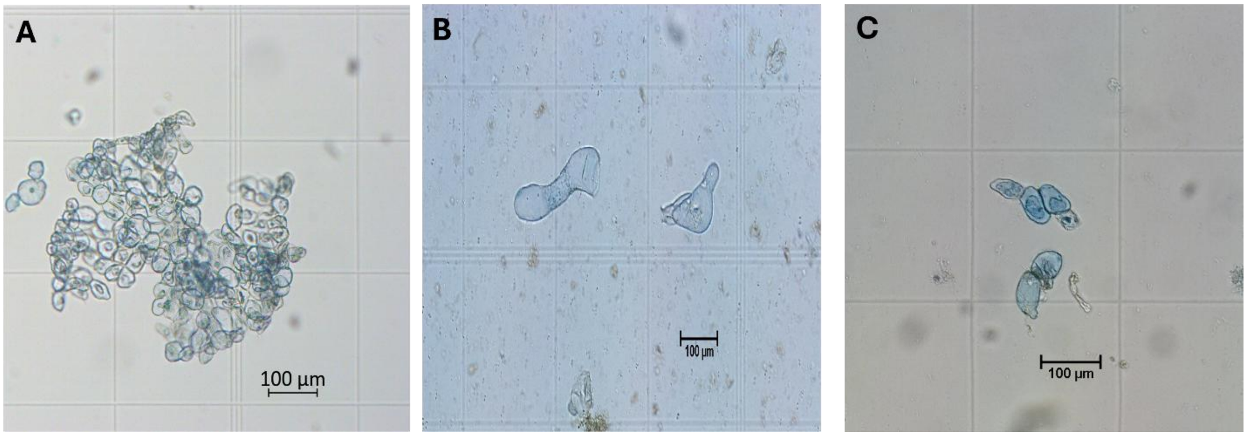

Figure 6.15: Microscopic images of viable *Sceletium tortuosum* cells obtained from control **(A)**, 10 µM 2,4-D **(B)**, and 2.5 µM phenylalanine **(C)** treatments. Scale bars indicate 100 µm.

Cells resulting from the 2,4-D treatment showed considerable elongation and enlargement compared to phenylalanine- and control-treated cells. Cells obtained from the latter two treatments were more rigid, and oval or elliptical in shape **(Figure 6.15)**.

6.3.6.2 Alkaloid production

The resulting alkaloid contents of cells, media, and combined alkaloid contents from the optimized experiment are illustrated in **Figure 6.16.** The control treatment was responsible for the lowest alkaloid contents in cells and media (0.73 ± 0.14 and 0.30 µg/mg DW, respectively) **(Figure 6.16)**. Alkaloid content of cells was optimized by 2.5 µM phenylalanine (0.84 ± 0.25 µg/mg DW), while 10 µM 2,4-D yielded the highest alkaloid content in the culture medium (1.91 ± 0.53 µg/mg DW). Notably, significant differences were only apparent for total alkaloid content of the medium. In this instance, 10 µM 2,4-D produced a significantly higher alkaloid content than the control (0.30 µg/mg DW) and 2.5 µM phenylalanine (0.45 ± 0.091 µg/mg DW). Overall, the 2,4-D treatment resulted in the highest combined alkaloid content (2.73 ± 0.94 µg/mg DW) **(Figure 6.16)**.

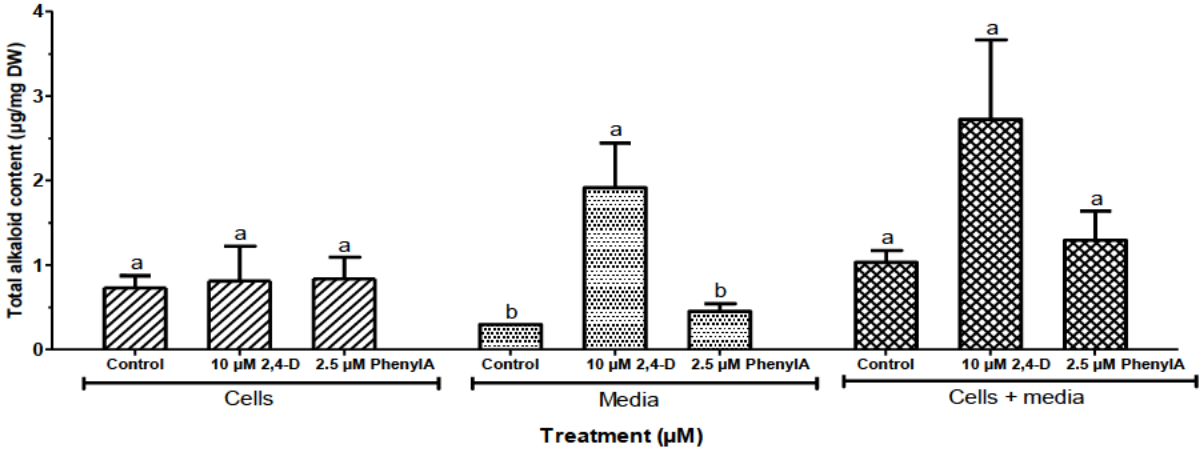

Figure 6.16: Total alkaloid contents of *Sceletium tortuosum* cells, media, and whole cultures obtained from experiment three (2,4-D = 2,4-dichlorophenoxyacetic acid; PhenylA = phenylalanine). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

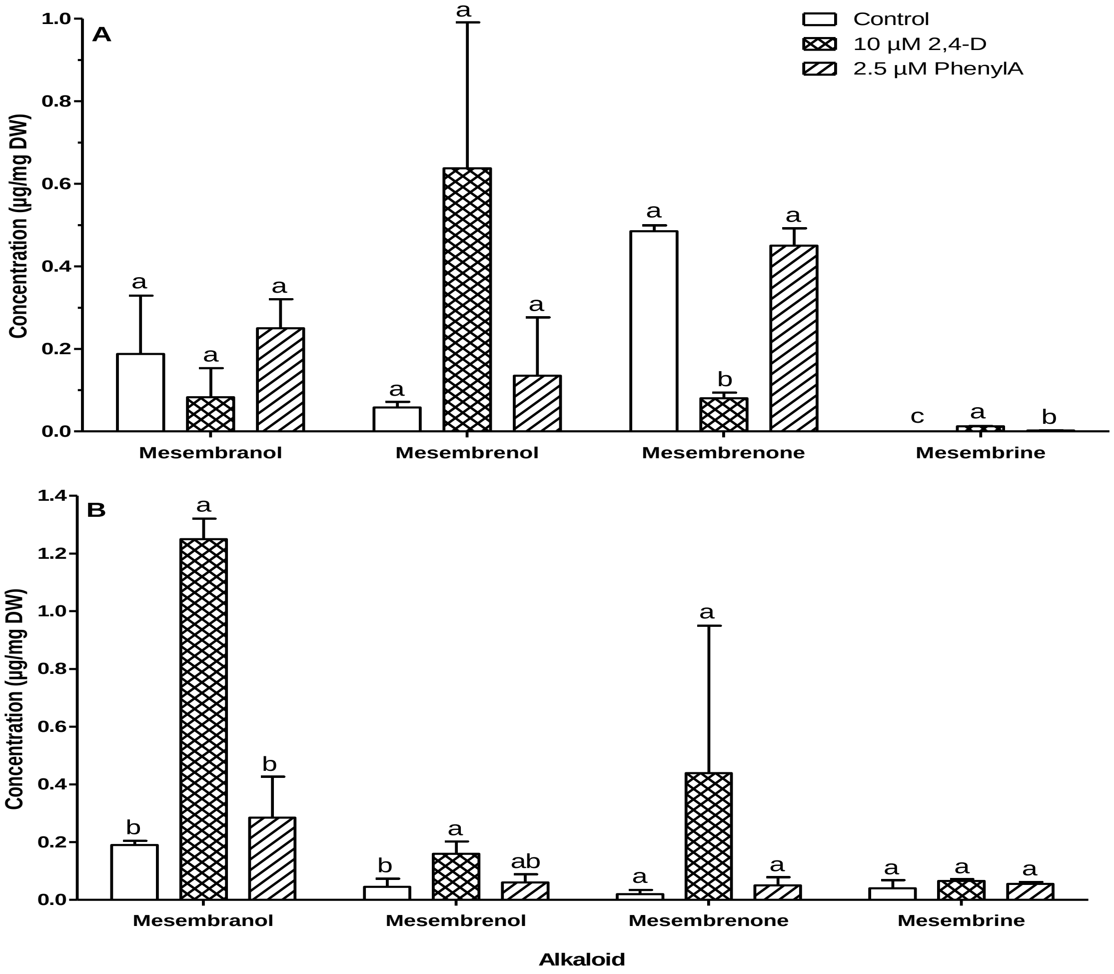

Figure 6.17: Concentrations of mesembrine-type alkaloids obtained from *Sceletium tortuosum* cells **(A)**, and culture media **(B)** from experiment three (2,4-D = 2,4-dichlorophenoxyacetic acid; PhenylA = phenylalanine). Error bars show standard deviation (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

With regards to alkaloids present in cells, 10 µM 2,4-D was responsible for the highest concentrations of mesembrenol (0.64 ± 0.35 µg/mg DW) and mesembrine (0.013 µg/mg DW) **(Figure 6.17A)**. However, concentrations of mesembranol (1.25 ± 0.071 µg/mg DW), mesembrenol (0.16 ± 0.042 µg/mg DW), mesembrenone (0.44 ± 0.51 µg/mg DW), and mesembrine (0.065 ± 0.0071 µg/mg DW) were optimized by the 2,4-D treatment in the culture medium **(Figure 6.17B)**. A trend was observed for the 2,4-D treatment, i.e. high alkaloid contents (and concentrations) in cells resulted in low alkaloid contents in the media, and vice versa **(Figures 6.16** and **6.17)**.

6.3.7 Comparative analysis

With regards to the 2,4-D treatment, the initial experiment showed a higher total cell number (7.6 x 10^{10} ± 3.5 x 10^{10} cells/mL), and total alkaloid content (3.70 ± 0.40 µg/mg DW) **(Figures 6.4A, 6.8** vs **6.14, 6.16)**. However, the final experiment resulted in a higher mean fresh weight for 10 µM 2,4-D (3.26 ± 0.41 g) **(Figure 6.4B** vs **6.14B)**. For the first experiment, alkaloid content was higher in cells than media, however, inverse was apparent in the third experiment **(Figures 6.6A, 6.7A** vs **6.16)**.

The phenylalanine treatment resulted in a higher total cell number (7.2 x 10^{10} ± 5.8 x 10^{10} cells/mL) and fresh weight (2.36 ± 0.28 g) in the first experiment **(Figures 6.9** vs **6.14)**. The total alkaloid content was higher in the initial experiment (3.32 ± 0.24 µg/mg DW), with most of the alkaloids found in the medium. Contrastingly, alkaloid content was significantly lowered in the final experiment (1.30 ± 0.35 µg/mg DW) and cells had the highest alkaloid accumulation **(Figures 6.11A, 6.12A, 6.13** vs **6.16)**.

6.4 Discussion

6.4.1 PGRs and culture growth

The cell cycle of *S. tortuosum* conformed to the sigmoidal growth curve. Lag, exponential, and stationary phases were easily observed **(Figure 6.3)**. This type of growth is characteristic of suspension cultures that are incubated with constant light, temperature, and aeration (Bhatia, 2015).

Total cell number was not directly correlated with fresh weight. All NAA and 2,4-D treatments had high fresh weights, but only two of these six treatments also had high total cell numbers **(Figure 6.4)**. Culture growth via cell elongation, rather than increases in cell number, are associated with suspension cultures (Santos *et al.*, 2016). Furthermore, auxins are involved in cell elongation and expansion (George *et al.*, 2008; Lin *et al.*, 2021). These growth characteristics were observed in the NAA and 2,4-D treatments in the microscopic analysis **(Figure 6.4)**.

Total cell numbers were often higher for auxin treatments as compared to cytokinin treatments **(Figure 6.4A)**. Auxins are known to trigger cell division in suspension cultures. In fact, the absence of auxin can cause cell division to slow down or cease (Doerner *et al.*, 2000; Perrot-Rechenmann, 2010). Thus, the absence of auxin in cytokinin-treated cultures could explain the significantly lowered total cell numbers **(Figure 6.4A)**.

6.4.2 PGRs and alkaloid production

6.4.2.1 Cells

Despite the more favourable growth parameters observed for auxin treatments, cytokinin application (BAP) yielded the largest alkaloid fractions **(Figures 6.4** and **6.6A)**. Treatments with BAP improved total alkaloid contents beyond that of the control and various other PGR treatments **(Figure 6.6A)**. Other researchers have also reported the efficacy of BAP, rather than KIN and NAA, for enhancing phytochemical production *in vitro* (Decendit *et al.*, 1992; Yahia *et al.*, 1998; Zia *et al.*, 2007).

It was previously shown that KIN was the optimal cytokinin for alkaloid production in *S. tortuosum* plantlets (see **Chapter 4**), yet alkaloid production was favoured by BAP in cell cultures **(Figures 6.6** and **6.8)**. Notably, Decendit *et al.* (1992) reported that BAP was the optimal cytokinin for alkaloid production in *C. roseus* cell cultures, however Mekky *et al.* (2018) showed KIN to be more appropriate for callus cultures. These reports, together with current findings, suggest that alkaloid biosynthesis differs at the cell versus organ / whole plant level. Thus, culture type is a factor in determining the most suitable PGR (Shukor *et al.*, 2013). Nevertheless, cytokinins are involved in alkaloid biosynthetic pathways, likely inducing the up-regulation of crucial genes (Papon *et al.*, 2005; Yahia *et al.*, 1998). Such an effect may have programmed cells for secondary metabolite biosynthesis, rather than active cell division and growth. This could explain the increased alkaloid contents coupled with low cell counts and mean fresh weights in the BAP treatment group **(Figures 6.4** and **6.6A)**.

Interestingly, BAP significantly influenced mesembrenone, mesembranol, and mesembrenol levels **(Figure 6.6)**. Mesembrenone converts to the other three mesembrine-type alkaloids (Jeffs *et al.*, 1978), and accounts for the majority of the alkaloid pool (see **Chapters 2**, **4**, and **5**). These results suggest that there must have been a substantial increase in the mesembrenone pool, leading to increases in mesembranol and mesembrenol concentrations, whilst maintaining a large mesembrenone pool **(Figure 6.6B-D)**.

Makunga *et al.* (2022) conducted a cell culture experiment utilizing 1 mg/L BAP (= 4.44 µM BAP). These authors analyzed the resulting calli after 4-week incubations and multiple subculture cycles. Interestingly, the three of the four principle mesembrine-type alkaloids were found at much lower concentrations in comparison to this study **(Table 6.1)**. The primary cause of the differences between alkaloid concentrations was likely due to determination of the cell cycle. This improved our understanding of the appropriate time to terminate cell cultures. The present study harvested cultures 9 days later than Makunga *et al.* (2022). Nonetheless, the aforementioned study lends support for our findings that BAP-supplementation is conducive to mesembrine-type alkaloid production.

Several researchers have reported that 2,4-D suppressed alkaloid production in cell cultures (Bienaimé *et al.*, 2015; Whitmer *et al.*, 1998). However, significant enhancements in alkaloid accumulation were observed in the presence of 2,4-D, particularly at 10 µM **(Figure 6.6A)**. Our results are more in line with those of Zaman *et al.* (2021). They reported increased alkaloid production in *Polyalthia bullata* cultures with the use of 2,4-D. The present findings indicate that 2,4-D should not be regarded as a suppressor of alkaloid biosynthesis. Rather, its efficacy in enhancing biosynthesis may be species-specific, as is the case with PGRs in general (Assaf *et al.*, 2022).

Mesembrenone levels were low in the presence of 2,4-D, but mesembranol and mesembrenol concentrations were high. This is suggestive of a sizeable increase in the mesembrenone pool, which allowed for alkaloid conversions, but resulted in a significantly reduced concentration of this primary alkaloid **(Figure 6.6B-D)**.

Some KIN and NAA treatments increased mesembranol and mesembrine concentrations **(Figure 6.6B** and **E)**. Ahmadpoor *et al.* (2023) showed that different PGR combinations improved the production of different secondary metabolites. Thus, the production of individual alkaloids may be favoured by certain PGRs more so than others.

6.4.2.2 Media

It was previously expressed that 2,4-D treatments caused the size of the mesembrenone pool to diminish allowing for full conversion to mesembranol and mesembrenol **(Section 6.4.2.1)**. However, in the case of 10 µM 2,4-D, a remarkably high mesembrenone concentration was recorded in the medium **(Figure 6.7A)**. Thus, the absence of mesembrenone in cells was due its leaching / exudation into the culture medium **(Figures 6.6** and **6.7)**. Interestingly, despite substantial alkaloid quantities found within cells, all alkaloids were also present in the culture medium **(Figure 6.7)**. Plant growth regulators can be classified as elicitors, and elicitation is known to enhance the secondary metabolite exudation (Cai *et al.*, 2012; Jamwal *et al.*, 2018). Thus, 2,4-D may not be suppressing alkaloid production, as expressed by other researchers (Bienaimé *et al.*, 2015; Whitmer *et al.*, 1998); instead, production may be stimulated, followed by the exudation of phytochemicals into the medium.

Several auxin treatments resulted in a higher proportion of individual alkaloids in culture media as opposed to cells. In comparison, alkaloids were not present in considerable quantities in culture media for BAP and KIN treatments **(Figures 6.6** and **6.7)**. These findings suggest that auxins enhance secondary metabolite exudation in cell cultures of *S. tortuosum*.

6.4.3 Amino acid precursors and culture growth

Most treatments resulted in total cell numbers similar to the control treatment **(Figure 6.9A)**. The ability of the control treatment (MS medium devoid of elicitors) to sustain cell growth and differentiation is less common. However, Hoffmann and Hoffmann-Tsay (1994) reported active growth of carrot protoplast cultures in basal MS medium. They suggested that the habituation of cells, caused by initial growth in auxin-containing medium, provided the auxin necessary to induce cell division and growth. This reasoning could account for the success of the control treatment **(Figure 6.9A)**.

Fresh weight of cells was highest for 2.5 µM phenylalanine and 5 µM tyrosine treatments, however weights were not significantly higher than the control **(Figure 6.9B)**. This is supported by images showing that precursor treatments did not result in significant cell elongation or enlargement, in comparison to the control **(Figure 6.10)**. Amino acid precursors can influence cell growth, but the effects are concentration- and time-dependent (Rakesh and Praveen, 2022). For instance, a low phenylalanine concentration (500 mg/L) present in the medium for a long time (9 days) increased cell biomass more so than a higher concentration (1000 mg/L) present for the same amount of time (Rakesh and Praveen, 2022). This report suggests that (1) higher precursor concentrations and (2) short exposure periods could be more beneficial for cell growth of *S. tortuosum*.

6.4.4 Amino acid precursors and alkaloid production

The lowest concentrations of methionine and tyrosine yielded cellular alkaloid contents that were higher than the control treatment **(Figure 6.11A)**. Jeffs *et al.* (1971) reported that all three amino acids showed some level of incorporation into the mesembrine-type alkaloids. Interestingly, incorporation in order of percentage was methionine > tyrosine >

phenylalanine. This may explain the preferential effects observed for the former two amino acids on alkaloid contents in cells **(Figure 6.11A)**. However, Jeffs *et al.* (1971) also showed that the percentage of amino acid incorporation differed at different stages of growth. This reinforces the notion that the time point and period of exposure to precursors is of prime importance for the biosynthesis of secondary metabolites (Rakesh and Praveen, 2022). Further research has also shown that precursor feeding at selected times, rather than the entire incubation period, enhanced phytochemical production (Arano-Varela *et al.*, 2020; Raghavendra *et al.*, 2012; Rakesh and Praveen, 2022).

Phenylalanine (2.5 µM) resulted in substantial accumulations of alkaloids in the medium, as opposed to within cells **(Figure 6.12A)**. Jeffs *et al.* (1971) elucidated that the aromatic ring of mesembrine-type alkaloids is derived from phenylalanine. Thus, the role of phenylalanine in alkaloid production is paramount. This could account for the marked increases in alkaloid accumulation observed for the 2.5 µM phenylalanine treatment **(Figure 6.12A)**. Additionally, several authors have reported increased alkaloid yields in cultures supplied with phenylalanine (Mahood and Alwash, 2018; Urmantseva *et al.*, 2005).

Elevated concentrations of phenylalanine reduced the alkaloid content of *Moringa oleifera* cultures (Mahood and Alwash, 2018). This lends support for our findings, i.e. alkaloid contents in the media were most enhanced by the lowest precursor concentrations (2.5 µM) **(Figure 6.12A)**. Several other species also responded best to low precursor concentrations (Rakesh and Praveen, 2022; Suryawanshi *et al.*, 2022).

6.4.5 Comparisons and discrepancies

There were some inconsistencies observed within the first experiment. With regards to culture media, 5 µM NAA resulted in a lack of mesembrine-type alkaloids, despite higher (2.5 µM) and lower (10 µM) NAA concentrations causing alkaloid accumulation. Similarly, low (2.5 µM) and high (10 µM) 2,4-D concentrations resulted in high alkaloid accumulations, while 5 µM significantly lowered alkaloid quantities **(Figure 6.7)**. Such results may be best explained by the challenges encountered during the experiment, i.e. heating of some of the culture flasks. Incubation temperature can influence culture growth and phytochemical yield, with high temperatures possibly causing cell damage and death (Hasanuzzaman *et al.*, 2013; Yue *et al.*, 2016).

Additionally, there were marked differences in culture growth and alkaloid contents between the first and final experiments. Treatments from the first experiment performed better, in terms of cell number **(Figures 6.4A** and **6.9A** vs **6.14A)** and alkaloid yield **(Figures 6.6A, 6.7A** vs **6.16)**. This could also be due to the unintended temperature increase experienced by some of the cultures. Elevated temperatures can enhance alkaloid production. This was reported for *C. roseus* cultures (Courtois and Guern, 1980). Temperature has not yet been evaluated for cell cultures of *S. tortuosum*, however the discrepancies in these experiments strongly suggest that it affects culture productivity.

6.5 Conclusions

Auxin application significantly enhanced cell division and growth. The bulk of alkaloid contents was found in cells, rather than the culture media in the first PGR experiment. Peak alkaloid quantities were achieved by BAP treatments (2.5 and 10 μM) and 10 μM 2,4-D. However, cell growth was optimized by the 2,4-D treatment. The efficacy of 2,4-D for improving alkaloid production was confirmed in the third experiment, although total cell number and alkaloid contents were optimal in experiment one (elevated temperatures). Auxins seemed to increase alkaloid exudation.

Amino acid precursor treatments, particularly 2.5 μM phenylalanine, improved alkaloid contents to an extent, but not more so than the PGR treatments mentioned. Interestingly, the first experiment (elevated temperatures) resulted in higher cell number, fresh weight, and total alkaloid contents than the third experiment (25°C). These findings indicate that the optimal temperature for suspension cultures of *S. tortuosum* is higher than 25°C.

Collectively, these findings indicate that culture media are an important source of mesembrine-type alkaloids, irrespective of whether PGRs or amino acid are utilized for elicitation. Treatment with 2,4-D (10 µM) appears to be the most appropriate for *S. tortuosum* cultures – in terms of growth and alkaloid production. Investigating higher concentrations of 2,4-D in suspension cultures may be beneficial. Additionally, treatments with BAP significantly enhanced alkaloid production and is worthy of further testing. Combination treatments of PGRs, for example 2,4-D + BAP, may also promote culture growth and alkaloid yields.

Precursor-feeding with phenylalanine presents a viable option for enhancing alkaloid production in suspension cultures. However, the evaluation of several different concentrations is necessary, along with selected (short) exposures to the precursor. Temperature proved to play an important role in the productivity of suspension cultures of *S. tortuosum*. Examination of this factor could be fundamental for improving culture yield.

Overall, significantly high yields of mesembrine-type alkaloids were produced by some PGR and precursor treatments. Cell suspension culture methodologies could indeed be scaled-up for use in continuous culture systems.

6.6 References

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Chapter 7: General conclusions and recommendations

The overarching aim of investigation was achieved, i.e. elicitation strategies successfully manipulated growth and improved mesembrine-type alkaloid production of *S. tortuosum*. However, alkaloid enhancements and growth improvements very rarely occurred concurrently. In terms of stress elicitation, healthy plantlet growth was observed in the 24 H treatment (2.79 ± 1.89 new leaf pairs and 2.60 ± 1.60 roots), but total alkaloid content was quite low (2.93 ± 0.16 µg/mg DW). Plantlets arising from 0 H light and 100 g/L PEG treatments yielded the highest total alkaloid contents (9.16 ± 0.12 and 6.74 ± 0.30 µg/mg DW, respectively), but growth was less than optimal.

Plantlet incubation in darkness is recommended for alkaloid optimization for this species. An added benefit of this treatment is its simplistic execution, as it does not require PGRs and chemicals (e.g. PEG) However, the incubation period should be extended to determine the effects on plantlet growth, survival, and alkaloid contents. Otherwise, short photoperiods (1–6 H) may be beneficial for improving these parameters. Light quality should also be investigated for this species. Despite the negative effects of osmotic stress on plantlet growth, PEG treatments could be worth evaluating in more established plantlets. This may reduce the negative growth effects, whilst improving alkaloid production.

The osmotic stress group produced the highest concentrations of starch (130.10 ± 0.16 µg/mg FW; 50 g/L PEG), protein (71.60 ± 0.30 µg/mg FW;100 g/L PEG), and proline (24.73 ± 0.06 µg/mg FW; 50 g/L PEG). Increasing osmotic stress level reduced carbohydrate concentrations whilst simultaneously increasing protein concentrations. Carbohydrates were thought to be utilized for the production of specialized stress-response proteins. Additional studies on the identification and quantification of such proteins could provide some insights into the stress responses of this species.

Salt treatments (100 and 200 µM NaCl) improved plantlet growth in comparison to the control. In particular, 100 µM NaCl resulted in the optimal mean number of new leaf pairs (2.14 ± 1.60), roots (1.50 ± 1.91), and mean root length (10.30 ± 11.94 mm). Salt treatments also produced lower alkaloid yields (3.13–3.65 µg/mg DW) than the control treatment (4.02 ± 0.13 µg/mg DW), and little variation was observed among mesembranol (0.19–0.25 µg/mg DW), mesembrenone (2.81–3.66 µg/mg DW), and mesembrine (0.05–0.06 µg/mg DW) quantities.

The highest carbohydrate concentrations were found in plantlets treated with 100 and 200 µM NaCl (383.3 ± 1.40 and 323.1 ± 0.72 µg/mg FW, respectively). Interestingly, proline concentrations obtained from these treatments (17.07 ± 0.06 and 16.10 µg/mg FW, respectively) were not much higher than the control (15.40 µg/mg FW). The effects of salinity on *S. tortuosum* thus provided confirmation of a halophytic nature.

The most stressful treatments were expected to be reflected in proline concentrations, though, this was not apparent in every case. This may have been due to species' halophytic nature and its ability to accumulate and synthesize other osmolytes (e.g. Na^+ and Cl^- ions, glycine betaine, and trehalose). Quantification of these components is necessary to gain a better understanding of stress response in *S. tortuosum*, as well to detect any relationships with secondary metabolites.

These stress experiments provided abundant evidence of a halophytic profile for this species. Given that the plant has received excessive attention in recent decades, it is surprising that this notion was not previously confirmed. This knowledge provides a new avenue of investigation for *S. tortuosum*. Further research will be required to ascertain the effects of higher salt concentrations on growth and alkaloid production of *S. tortuosum*.

The apparent trade-off between healthy plantlet growth and enhanced alkaloid production was also evident in the PGR experiment. Plant growth regulators induced several growth forms. By far, the healthiest were IBA-treated plantlets. In particular, 20 µM IBA produced the highest mean number of roots (14.18 ± 4.66), and mean root length (6.28 ± 2.59 mm), and 15 µM IBA resulted in the highest mean number of new leaf pairs (2.29 ± 1.13). However, total alkaloid contents from these treatments were not exceptionally high (2.61–5.00 µg/mg DW). On the other hand, cytokinin treatments elicited unbalanced growth, inclined towards shoot development (3.64–6.54 and 7.73–9.54 new leaf pairs, for KIN and BAP, respectively). Yet, 5 µM KIN resulted in the largest alkaloid fraction (7.77 ± 0.23 µg/mg DW). Thus, evaluating combination treatments of PGRs (e.g. IBA + KIN) may be worthwhile as they may be able to improve both growth and alkaloid production.

Optimistically, biostimulants showed the most potential for improving these responses in *S. tortuosum*. Smoke water (1:2000) resulted in the highest number of roots (2.57 ± 2.95) and mean root length (4.14 ± 6.69 mm). Sodium humate (5 mg/L) optimized the mean number of new leaf pairs (1.67 ± 1.20). Growth improved as SW concentration decreased and as SH concentration increased. Significantly large alkaloid fractions were obtained from 1:1000 and 1:2000 SW treatments (4.95 ± 0.23 and 4.72 ± 0.06 µg/mg DW, respectively). Additionally, increases in SH concentration resulted in proportional increases in mesembrenone and mesembrine concentrations, along with total alkaloid content (3.99 ± 0.19 µg/mg DW; 5 mg/L SH).

Based on these positive responses, these biostimulants are certainly worthy of further investigation. Greater dilutions of SW and greater concentrations of SH could boost growth and alkaloid production. These findings indicate that, perhaps, a balance could be achieved between healthy growth and increased alkaloid production.

Callus and cell suspension cultures were successfully initiated using 2.5 µM 2,4-D. Auxin application significantly enhanced total cell numbers and cell fresh weights, with 5 and 10 µM 2,4-D responsible for optimization of these parameters ($1.59 \times 10^{11} \pm 1.19 \times 10^{11}$ and 2.82 ± 0.95 g, respectively). Alkaloids were present in cells and culture media for most of the PGR and amino acid treatments tested. Treatments with 2.5 µM BAP and 10 µM 2,4-D resulted in substantially high total alkaloid contents (4.51 ± 0.66 and 3.70 ± 0.40 µg/mg DW µg/mg DW). Interestingly, auxin treatments seemed to enhance alkaloid exudation into the medium.

Amongst the amino acid precursors, only 2.5 µM phenylalanine improved mean fresh weight of cells (2.36 ± 0.28 g) and resulted in a significant increase in total alkaloid content (3.32 ±

0.24 µg/mg DW). In the final experiment, 10 µM 2,4-D enhanced total alkaloid content more so than 2.5 µM phenylalanine (2.73 ± 0.94 and 1.29 ± 0.35 µg/mg DW, respectively).

Cell suspension culture experiments revealed that elicitation with PGRs was more efficient than elicitation with amino acids. This was unexpected, as the amino acids utilized are known precursors of the mesembrine-type alkaloids. By providing these essential components, it was assumed that alkaloid production would be significantly enhanced, however, this was not the case. Future studies incorporating precursors into suspension cultures of *S. tortuosum* should focus on higher concentrations of these compounds. Additionally, the period of exposure to precursors should be investigated.

Auxin (2,4-D) had the most profound effects culture growth and alkaloid production. However, due to the positive effects of BAP treatments on alkaloid production, combination PGR treatments (e.g. BAP + 2,4-D) should also be tested for their efficacy in cell suspensions of *S. tortuosum*. Temperature exerted some influence on alkaloid production, thus signifying another factor that warrants investigation in cell cultures of this species. Optimizing cell suspension protocols for this species should be a priority.

To provide further perspective; an extremely small amount of plant material (~0.05 g callus tissue) was utilized, and cultures produced approximately half the total alkaloid content of the highest-yielding plantlets (4.51 ± 0.66 µg/mg DW; 2.5 µM BAP and 9.16 ± 0.12 µg/mg DW; 0 H light). This indicates that cell suspension cultures are certainly worth scaling-up for continuous culture systems. In this way, the maintenance and multiplication of cells would allow for continuous alkaloid production in cells, as well as continuous exudation of alkaloids into media. The periodic replacement of the culture medium would easily allow for alkaloid extraction. This would be a novel method for producing mesembrine-type alkaloids and could indeed lead to the creation of a sustainable commercial source of these phytochemicals.

Overall, these elicitation strategies provided useful information that can extend beyond the *in vitro* environment. For instance, salt and osmotic (drought) stress can be mimicked under greenhouse conditions. Due to the influence of light (photoperiod) *in vitro*, shade should be investigated *in situ*.

Biostimulants (SW and SH) can also be applied to *in situ* plants. The positive effects elicited by SH and VCL treatments were thought be due to humates. Since humates are a product of organic waste decomposition, the addition of compost to growth substrates could be beneficial for *S. tortuosum*. Additionally, the halophytic characteristics described, indicate that plants can grow under low levels of salinity. Collectively, the present findings carry substantial implications for *Sceletium* growers and provide some direction for *in situ* studies.

Mesembrenone was the primary alkaloid in the chemotype used in these studies. However, mesembrine-rich chemotypes appear most frequently in the literature. Thus, further investigation is necessary to determine whether the treatments described elicit similar effects on the mesembrine-rich chemotype. An alternative starting point for *in vitro* elicitation studies would be the selection of a chemotype rich in the alkaloid of interest. In this way, elicitation can be aimed at improving the production of the target alkaloid.

Transcriptomics and metabolomics studies are essential for this species. Studies of these types, used in the context of elicitation, would provide key information on elicitor-specific effects at the genetic (transcriptomic) level, and the knock-on effects for alkaloid biosynthetic pathways. This approach would be especially useful for clarifying cytokinin effects, as BAP and KIN elicited different responses when applied in different types of cultures. Once these effects are known, elicitation strategies could be further exploited for the over-production of mesembrine-type alkaloids.

The aforementioned *omics* studies could also provide key information regarding the chemotypes of this species. They could reveal differences in gene expression, and how this translates to different primary alkaloids. This could provide researchers with novel ideas to induce the expression of crucial genes, possibly with the use of elicitation. This may make it possible to alter chemotype based on the target alkaloids.

Elicitation techniques may, in this day in age, be regarded as quite simplistic, in comparison to genetic techniques. However, based on the current evidence, the reason these techniques persist and continue to be utilized in research, is clear. These techniques proved their efficacy in eliciting growth and enhancing secondary metabolite production far beyond that of control treatments. In every experiment performed, several treatments successfully improved alkaloid production.

Genetic engineering studies present an alternate avenue of investigation, especially with novel techniques and technologies emerging. However, simpler methodologies (elicitation) should be fully exploited prior to pursuing the genetic modification route. There is much to be explored in terms of elicitor types, and their effects on this species and its' mesembrine-type alkaloids.

The only caveat to elicitation is that careful consideration should be undertaken when establishing protocols for continuous use. Although the goal of elicitation is principally to enhance secondary metabolite production, the health of plant material during this process must also be taken into account. This will aid in the development of sustainable elicitation methods, rather than wasteful ones.