# ESTABLISHMENT OF MICROPROPAGATION AND CRYOPRESERVATION PROTOCOLS FOR THE CRITICALLY ENDANGERED MEDICINAL PLANT, *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt.

**VILOSHANIE REDDY**

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# Establishment of micropropagation and cryopreservation protocols for the critically endangered medicinal plant, *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt.

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A thesis submitted to the School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, for the degree of Doctor of Philosophy in Science.

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As the candidate's supervisor and co-supervisor, we have approved this thesis for submission.

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# ABSTRACT

In South Africa, more than 80% of rural communities rely on the trade of medicinally important plant species. Overexploitation coupled with biotic, abiotic, and climate change-induced stresses have reduced these wild populations. Since the Sustainable Development Goals adopted by the United Nations recognise the importance of protecting the planet's natural resources to alleviate poverty and improve healthcare worldwide, increasing production and implementing ex situ long-term conservation strategies for these plant genetic resources is essential.

The Zingiberaceae species *Siphonochilus aethiopicus*, a perennial geophyte, is amongst the top ten sought-after traditional medicinal plants in South Africa, which has led to its over-exploitation and consequent critically endangered status. The anti-inflammatory activity of the plant's major phytocompound, a furanoterpernoid called siphonochilone, from the rhizomes and roots has prompted further interest in this species by the pharmaceutical industry. Vegetative propagation via separated rhizome pieces containing buds is the only feasible method to grow this species due to poor seeding, which further complicates long-term storage through seed banking. Hence, an alternate long-term ex situ conservation strategy is needed for this species. Furthermore, established micropropagation protocols result in low yields and there are no reports on the siphonochilone content in the rhizomes and roots of regenerated plants. This study, therefore, aimed to develop conservation strategies for *Siphonochilus aethiopicus* through micropropagation and cryopreservation. A further aim was to validate and quantify siphonochilone in micropropagated clones and to compare this with conventionally propagated plants.

During the establishment of in vitro cultures of *S. aethiopicus,* decontamination of rhizome-derived bud explants proved challenging. A multi-step procedure incorporating a fungicide and a hypochlorite-based surface disinfectant yielded a small number of sterile buds. Thereafter, buds were cultured on full-strength Murashige and Skoog (MS) basal salts and vitamins, 0.09 M sucrose, 5 mg $l^{-1}$ 6-benzylaminopurine (BAP) and 4 g $l^{-1}$ Gelrite® to promote development into shoots. Shoot apices were then excised and transferred to multiplication medium for 42 days. Multiplied shoots were elongated with a reduced BAP concentration (0.1 mg $l^{-1}$) for 28 days. Elongated shoots were then rooted on medium containing 1 mg $l^{-1}$ indole-3-acetic acid

(IAA) for 28 days. Acclimatisation (42 days) was successful, with 100% survival. The plant yield in this study was much higher (13 plants per initial rhizome bud) than conventional vegetative propagation (a single plant per bud) or previously established micropropagation protocols (3 – 4 plants per bud). The mature two-year-old post-acclimatised micropropagated plants produced rhizomes (35.80 ± 6.11 g) and roots (15.70 ± 2.00 g) with higher biomasses compared to conventionally propagated plants (3.73 ± 2.48 g and 1.14 ± 0,22 g, respectively). Gas Chromatography-Mass Spectrometry (GC-MS) analyses identified siphonochilone in micropropagated and soil-grown plants. There were no significant differences in siphonochilone yield in rhizomes from the two-year-old micropropagated plants compared to that from field-cultivated plants. However, the root siphonochilone yield in the mature two-year-old post-acclimatised micropropagated plants was higher (0.03120 ± 0.1109 to 0.4498 ± 0.1081 g $g^{-1}$extract) than in roots of conventionally propagated plants (0.0435 ± 0.0136 g $g^{-1}$extract).

To establish a cryopreservation protocol using in vitro-derived shoot apices, various cryopreparative and sequential dehydration procedures prior to cryostorage were evaluated. The shoot apices were precultured on sucrose-enriched medium and cryoprotected prior to vitrification and cryostorage. Shoot apices that were precultured on MS basal salt and vitamin medium with 0.09 M sucrose and 3 g $l^{-1}$ Gelrite® for 48 h and then with 0.3 M sucrose in the dark for 24 h, followed by sequential dehydration, showed the highest viability and survival (58.33%) and a water content reduced to 0.92 ± 0.36 g $g^{-1}$ dry weight of explants prior to cooling. The droplet-vitrification of the apices after this sequential dehydration process followed by cooling and storage in liquid nitrogen for 24 h resulted in 66.67% regrowth of the cryostored shoot apices following thawing, rehydration and recovery. Development of the shoot apex into a single shoot took place 90 days after retrieval from liquid nitrogen followed by elongation (28 days) and in vitro rooting (28 days). The plantlets generated from cryostored explants produced a single shoot and root, in contrast to untreated shoot apices (control) which produced robust shoots and roots in vitro. The successful recovery of cryostored explants represents the initial step towards long-term conservation of *S. aethiopicus*. Further research is recommended to improve explant recovery rates. Additionally, research is necessary to determine the presence and levels of siphonochilone in plants regenerated from cryostored explants. The improved micropropagation protocol for *S. aethiopicus* established in this study using in vitro-derived shoot apices, provide a viable recovery method for successful plant production following cryopreservation. Nonetheless, plant yields can be improved by

optimising multiplication steps in temporary immersion culture systems, siphonochilone yield can be enhanced by investigating cell suspension cultures for phytocompound extraction, and optimisation of certain steps of the developed cryopreservation protocol can promote higher explant recovery.

# ABBREVIATIONS and ACRONYMS

| | |
|---|---|
| Ac | acetate |
| ANOVA | Analysis of variance |
| aq | aqueous |
| BAP | 6-benzylaminopurine |
| $Ca(OCl)_2$ | calcium hypochlorite |
| $CaCl_2.2H_2O$ | calcium chloride dihydrate |
| CBD | Convention on Biological Diversity |
| c | concentration |
| cc | column chromatography |
| $CDCl_3$ | deuterated chloroform |
| ch | chloroplast |
| cm | cell membrane |
| cw | cell wall |
| cx | cytoplasmic matrix |
| $^{13}$C NMR | C-13 nuclear magnetic resonance spectroscopy |
| D | direct shoot regeneration |
| dmb | dry mass basis |
| DW | dry weight |
| ea | ethyl acetate |
| EMTC | eThekwini Municipal Tissue Culture |
| EtOH | ethanol |
| DMSO | dimethylsulphoxide |
| $g\ g^{-1}$ | gram per gram |
| GC-MS | gas chromatography-mass spectroscopy |
| GDPs | Gross Domestic Products |
| GSPC | Global Strategy for Plant Conservation |
| HCl | hydrochloric acid |
| $^{1}$H NMR | H-1 nuclear magnetic resonance spectroscopy |
| hx | hexane |
| IAA | indole-3-acetic acid |
| IBA | indole-3-butyric acid |

| | |
|---|---|
| MeOH | methanol |
| MS | Murashige and Skoog basal salts and vitamins |
| m | mitochondrion |
| n | nucleus |
| PGRs | plant growth regulators |
| NAA | naphthalene acetic acid |
| NaDCC | sodium dichloroisocyanurate |
| NaOCl | sodium hypochlorite |
| NaOH | sodium hydroxide |
| SDGs | Sustainable Development Goals |
| TIS | temporary immersion system |
| TLC | thin layer chromatography |
| $HgCl_2$ | mercuric chloride |
| kPa | kilopascal |
| p value | probability value |
| PGRs | plant growth regulators |
| PPFD | photosynthetic photon flux density |
| PVS | Plant Vitrification Solution |
| spp. | species |
| Tg | glass transition temperature |
| $\mu mol\ m^{-2}\ s^{-1}$ | micromole per metre squared per second |
| U | units |
| UN | United Nations |
| UNEP | United Nations Environmental Programme |
| UKZN | University of KwaZulu-Natal |
| UV | ultraviolet |
| v | volume |
| v/v | volume per volume |
| WC | gravimetric water content |
| WHO | World Health Organisation |
| w/v | weight per volume |

# DECLARATIONS

## DECLARATION 1 – PLAGIARISM

I, **Viloshanie Reddy** declare that

1. The research reported in this thesis is my original research, except where otherwise indicated.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. their words have been re-written but the general information attributed to them have been referenced, and
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Signed: [redacted]................. Date..............................

Viloshanie Reddy (Student Number: 983172719)

As the supervisors, we ....

Superv [redacted] Date
Sign

Co-Supervisor: Dalia Varghese Date 7 April 2025
Sign [redacted]

# DECLARATION 2 – PUBLICATIONS AND CONFERENCE PARTICIPATION

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis, including publications in preparation, submitted, *in press* and published.

**Details of publications:**

Publication 1: Reddy, V., Varghese, D., and Shaik, S. Towards long-term conservation of *Siphonochilus aethiopicus*, a critically endangered South African medicinal species: An improvement on micropropagation using in vitro shoot apices. *South African Journal of Botany*. (published: https://doi.org/10.1016/j.sajb.2025.03.017)

Publication 2: Reddy, V., Varghese, D., and Shaik, S. Evaluation of biomass accumulation and quantification of siphonochilone in rhizomes and roots of micropropagated and conventionally propagated *Siphonochilus aethiopicus*. *Journal of Ethnopharmacology*. (In preparation)

Publication 3: Reddy, V., Varghese, D., and Shaik, S. Vitrification-based cryopreservation of *in vitro* shoot apices of *Siphonochilus aethiopicus*. CryoLetters. (In Preparation)

**Details of symposia and conference participation:**

Presentation 1: 8TH International Workshop on Desiccation Sensitivity and Tolerance across Life Forms. 15 – 19 January 2024, Limpopo, South Africa. Towards vitrification-based cryopreservation of *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt. using in vitro shoot apices. V. Reddy, D. Varghese and S. Shaik (oral presentation)

Presentation 2: University of KwaZulu-Natal, College of Agriculture, Engineering and Science Postgraduate Research & Innovation Symposium 2024. 29 – 30 October 2024, KwaZulu-Natal, South Africa. Establishment of micropropagation and cryopreservation protocols for the critically endangered medicinal plant, *Siphonochilus aethiopicus*. V. Reddy, D. Varghese and S. Shaik (oral presentation)

Presentation 3: Conservation of Exceptional Plants Symposium 2025. 8 - 9 April 2025, Virtual Symposium. Establishment of micropropagation and cryopreservation protocols for the critically endangered medicinal plant, *Siphonochilus aethiopicus*. V. Reddy, D. Varghese and S. Shaik (Abstract accepted for a poster presentation)

For all the above publications and presentations, my role included carrying out all the experimental work and writing of the publications, designing the presentation slides and poster along with guidance from my supervisors. Where other authors carried out aspects of the experimental, I either interpreted or consulted with them to understand the data. Co-authors contribution was also that of an editorial nature, checking on the scientific content in their field, and my correct interpretation of the data in their field. Based on their expertise, they may have added minor parts to the manuscripts and presentations.

Signed: [redacted] ………..
Viloshanie Reddy

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# DEDICATION

This study is dedicated to Professor Norman W. Pammenter and the late Professor Patricia Berjak.

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# Chapter One: Introduction and Literature Review

## 1.1 Plant biodiversity

The term ‘biodiversity’ defines the variations in all life forms on Earth. Plants are recognised as an essential part of the world’s biological diversity (Sharma and Sharma, 2013). An estimated 500,000 terrestrial plant species have been identified globally (Sharrock et al., 2014) with the majority being angiosperms (Pimm, 2021). Angiosperms comprise flowering plants that produce seeds within enclosed ovaries and are further categorised into monocotyledon and dicotyledon subclasses (Dahlgren, 1980). The interplay of biotic and abiotic factors determines species richness in a specific environment. Geographic, regional, and climatic conditions determine global plant species distribution. The global diversity of flowering plants represents the range of adaptive strategies plants express to survive in different terrestrial ecosystems. The relationship that exists between plants, animals and microbial organisms is fundamental to all ecosystem services.

Global plant biodiversity underpins all ecosystems, since plants are the primary producers in the food chain. They also play a vital role in maintaining the planet’s primary environmental balance and stability by providing valuable ecosystem services such as oxygen. Plants are also essential for human well-being since we rely on them for food, fibre, medicines, and so forth. Historical evidence from many parts of the world highlights the important role of plants in the healthcare practices of some of the greatest ancient civilisations (Antonelli et al., 2020). Currently, an estimated 70,000 species are still used for traditional medicinal purposes worldwide; this accounts for more than one-tenth of the global plant species diversity (Kundu *et al.*, 2018). Since plants are vital for sustaining life, widely used as a primary source of healthcare, contribute to over half the global gross domestic products (GDPs) and support diverse cultural and spiritual activities that also have economic value, it is critical that they are safeguarded for future generations. Therefore, amongst the 17 interconnected United Nations Sustainable Development Goals (SDGs) (the blueprint to achieve a better and more sustainable future for all), Goal 15 (Life on Land) is targeted to protect and restore terrestrial ecosystems, sustain and manage forests to combat desertification, halt and reduce land degradation and stop biodiversity loss (Purushothaman and Mol, 2023).

### 1.1.1 Plants used in traditional medicinal practices worldwide

Numerous wild plant species indigenous to a localised region with cultural and traditional medicinal importance continue to provide the primary healthcare needs for many rural communities worldwide. The World Health Organisation (WHO) also acknowledges the pivotal role of herbal medicines as the initial source of healthcare in regions with limited or no access to allopathic healthcare practices (WHO, 2019). India, China, Colombia, and South Africa are amongst the top four countries that rely significantly on plants in traditional medicinal and cultural practices (Antonelli et al., 2020). Traditional Chinese medicine uses approximately 11,146 plant species; traditional Indian medicine relies on over 7,500 different plants (Global Strategy for Plant Conservation, 2011), and South African traditional medicinal practices rely on more than 3,000 plant species (Xego et al., 2016) from its estimated 30,000 indigenous species.

The application of advanced chemical analyses to elucidate phytochemical compounds from medicinal plants has prompted further traditional medicinal use. The use of these plant-based products for healthcare has increased from 8 to 15% per year in Europe, North America, and Asia ( Chen et al., 2016a; Applequist et al., 2020). Phytochemicals produced by South African indigenous species in the global pharmaceutical industry have also increased significantly in recent decades and continues to increase at a rate of 6% per year (Howes *et al.*, 2020).

### 1.1.2 Indigenous South African plants used in traditional medicinal practices

South Africa is globally recognised as a megadiverse hotspot (Antonelli et al., 2020), with its plant diversity contributing to 10% of the global variety of higher plants. Owing to various cultural beliefs and practices, more than 80% of the South African rural population, rely on many of these plants as traditional medicines ('*muthi'*) instead of allopathic drugs for their primary healthcare needs (Ndhlala et al., 2011; Khan and Ahmad, 2019). The rural economy also depends on the trade of medicinal plants harvested from the wild. Two of South Africa's largest '*muthi'* markets are located in Johannesburg (Gauteng Province) and Durban (KwaZulu-Natal Province). Furthermore, two of the most sought-after species in South Africa are *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt. from the Zingiberaceae family, commonly referred to as African ginger or wild ginger, and *Warburgia salutaris*, known as the pepper bark tree (Ndhlala et al., 2011). This study focuses on *S. aethiopicus.*

### *1.1.2.1 Siphonochilus aethiopicus*

#### 1.1.2.1.1 Distribution in Africa

*Siphonochilus aethiopicus*, a relative of the commercially traded ginger (*Zingiber officinale* Rosc.) is the only Zingiberaceae species native to Africa (Gordon-Gray et al., 1989). Although *S. aethiopicus* is naturally distributed from tropical Africa to southern Africa, over-harvesting for the traditional medicinal plant trade has led to this species being endangered in Swaziland (EN A1d) (Braun et al., 2004) and critically endangered in South Africa (CR A4acd) (Lötter et al., 2022). Currently, only small, wild populations are found sporadically distributed from the Letaba catchment in the Limpopo Lowveld region to Swaziland (Fig. 1.1). The limited wild populations in protected areas are further challenged due to illegal harvesting for commercial gain and has resulted in an extinct status in the KwaZulu-Natal Province in South Africa (Dlamini and Dlamini, 2002). The status of the natural distribution of *S. aethiopicus* in other parts of Africa remains unknown, as last reported in the 17th meeting of the Conference of the Parties (CoP17 2016); since then, there have been no further reports on assessments having been conducted in those regions. The current extinct status of *S. aethiopicus* in KwaZulu-Natal has led to amendments to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (United Nations, 2011) from its Appendix I status to an Appendix II status (UNEP, 2024). This amendment was implemented to further regulate the illegal cross-border trade of *S. aethiopicus* in the southern African region addressed at CoP17 (2016).

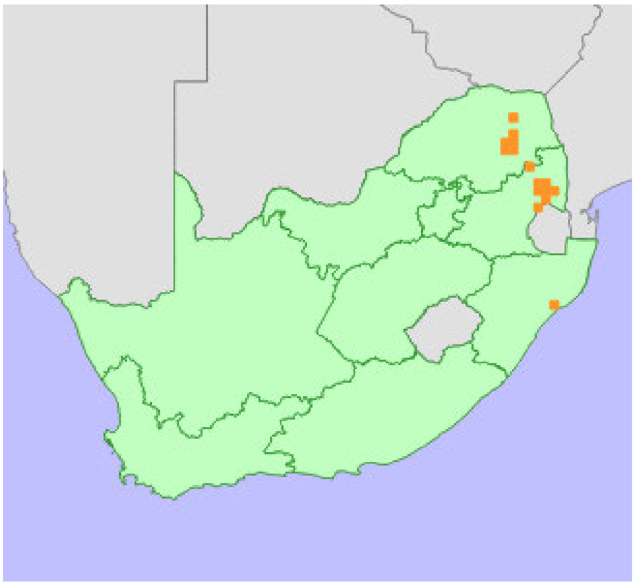


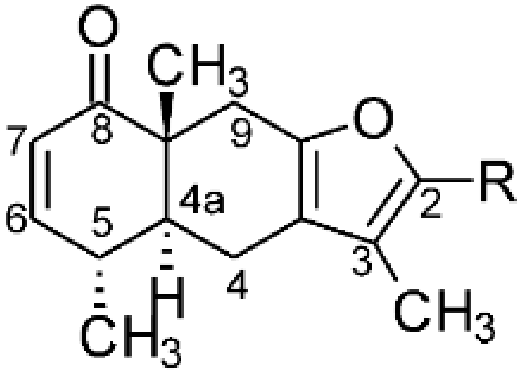
**Figure 1.1:** The original natural distribution pattern of *Siphonochilus aethiopicus* in southern Africa (represented by orange squares on the map) (Lötter et al., 2022).

### 1.1.2.1.2 Biology, cultivation and uses

*Siphonochilus aethiopicus* is a perennial geophytic angiospermous monocot (Fig. 1.2 a) (Gordon-Gray et al., 1989). Its leaves are long and tapered with false stems of approximately 600 mm in length with thickened, aromatic, cone-shaped rhizomes producing tuberous roots which dry down during the winter months (Fig. 1.2 b). *Siphonochilus aethiopicus* produces bisexual, white, yellow and mauve-coloured flowers that are short-lived (2 - 3 days). The flowers arise directly from the rhizome at ground level. When pollination and fertilisation take place, the seeds develop in fruits close to the ground and may take up to a year to germinate (Gordon-Gray et al., 1989). The seeds are difficult to locate because the fruit is produced at ground level, and seeding is poor (Crouch et al., 2008), which complicates natural propagation. Therefore, *S. aethiopicus* is primarily cultivated via vegetative propagation by separating rhizomes with emerging buds and planting them in open fields (Williams et al., 2013; Salmina, 2017). In vitro methods have also been developed to propagate this species (Hannweg et al., 2016; Kunene et al., 2018), however, these protocols are limited by the difficulty in eliminating microbial organisms in explants, and low rates of multiplication. Micropropagation of *S. aethiopicus* is currently applied at the Tissue Culture Section, Production and Display division of the Parks Department, eThekwini Municipality (Durban, South Africa) to mass-produce plants that are largely channelled to the municipal indigenous medicinal plant nursery where the distribution is limited to traditional herbal traders and healers who use the rhizomes and roots (Berjak et al., 2011). In so doing, the impact of harvesting from the wild is decreased to some degree.

**Figure 1.2:** *Siphonochilus aethiopicus*, a perennial geophytic angiospermous monocot with mauve-coloured flowers; above-ground parts arising directly from the rhizome (a) that dries down during the dry winter months (b).

Traditional healers harvest rhizomes with their attached roots, dry and grind them down to a fine powder before adding to water. The mixtures are then used as herbal concoctions to treat ailments such as coughs, colds, influenza-like symptoms, malaria, menstrual disorders and stomach infections ( Lindsey, 1999; Street and Prinsloo, 2013; Adebayo et al., 2021); they also find use as a protective charm in cultural practices (Cunningham, 1993). In East Africa, the rhizomes are used as a spice (Williams et al., 2013; Ndhlovu et al., 2021). The major active phytocompound, first isolated from *S. aethiopicus* rhizomes, is the furanoterpernoid (4aα,5β,8α)-3,5,8a-trimethyl4,4a,9-tetrahydo-naphtho[2,3-b]-furan-8(5H) (Fig. 1.3) (Holzapfel et al., 2002). It has also been reported in roots and given the common name, siphonochilone (Viljoen et al., 2002). This compound was shown to have anti-plasmodial activities (Light, 2002) and anti-inflammatory properties (Lategan et al., 2009; Adebayo et al., 2021) encouraging its application in the pharmaceutical industry (Blumenthal, 2009; Adebayo et al., 2021). The root and rhizome extracts are also added to commercial health beverages (Mudau et al., 2022).



**1** **R = H**

**2** **R = OH**

**3** **R = OCOCH$_3$**

**Figure 1.3:** Structure of siphonochilone isolated from *S. aethiopicus* rhizomes and roots (Holzapfel et al., 2002)

### 1.1.3 Threats to plant biodiversity

Research has shown that 60,000 to 100,000 plant species are threatened worldwide (Antonelli et al., 2020) and their populations are decreasing. The key drivers of this biodiversity loss include the spread of invasive alien species, natural disturbances, pollution and climate change (Xego et al., 2016; Khan and Ahmad, 2019). Other contributing factors are human-imposed stressors such as overexploitation through unsustainable harvesting, and urbanisation ( World Health Organization and Convention on Biological Diversity, 2015; Chen et al., 2016a). Destruction of natural ecosystems has been further exacerbated due to unsustainable food consumption in developed regions and persistent poverty in developing regions (Larsen et al., 2011; Pence et al., 2017). Ecosystems worldwide have also been negatively affected by agricultural expansion (Potter et al., 2017). Clearing natural vegetation for agricultural production already accounts for 39% of the total 54% of the land cleared for human use (Purushothaman and Mol, 2023). The continued global population expansion and the demand for more food will require the clearing of even more natural ecosystems for agricultural production (Chen et al., 2016 b). This will further destroy habitats and natural vegetation structures, leading to an increased loss of plant biodiversity in these habitats.

Medicinal plants in particular, are increasingly vulnerable based on the rising demands of plant-based drugs worldwide (Kundu et al., 2018; Antonelli et al., 2020). Medicinal plant populations are declining; consequently, some of the most highly utilised medicinal plant families now have a great proportion of threatened species (Antonelli, et al. 2020). As species vanish, the opportunity for future resource use is also lost. With the loss of the ultimate source of phytochemicals, even those yet undiscovered, to treat illnesses increases, and the chances of providing remedies for some of the deadliest diseases, are drastically reduced (Khan and Ahmad, 2019). Although natural evolutionary processes have influenced the successful re-establishment of some species, in other instances they have completely ceased to exist in certain regions (extinction), as is the case for *S. aethiopicus*. Extinction is a natural, irreversible process; unfortunately, with increased human activities, the rate of species extinction has increased several times more than the predicted natural rates (Mittermeier et al., 1998; Larsen et al., 2011). Since plant biodiversity conservation is considered critical for human well-being worldwide (Cruz-Cruz et al., 2013), plant biodiversity loss has become a global dilemma that requires immediate and long-term solutions guided by international, national, and local interventions (Sharrock, 2012), some of which are explored in this work.

## 1.2 Strategies for germplasm conservation

### 1.2.1 Determining priorities

Determining priorities for biodiversity conservation is a complex task in which several factors need consideration. Global conservation planning is guided by the framework of vulnerability and irreplaceability measures (Turner et al., 2007). Vulnerability measures the risk of extinction of a species within a specific region, while irreplaceability measures the frequency of species distribution across a range of geographic regions, and the extent to which a spatial substitute exists for securing biodiversity (Myers et al., 2000). Hence, using this framework, conservation priorities are centralised around regions of high species diversity and high levels of endemism (Mittermeier et al., 1998).

Myers et al. (2000) applied the principles of irreplaceability and vulnerability to guide global scale conservation planning when biodiverse ′hotspots′ were first described based on high levels of plant endemism coupled with accelerated rates of habitat loss. Although biodiversity hotspots represent 2.3% of the earth, together they contain around 50% of the world's endemic plant species and 42% of all terrestrial vertebrates (Antonelli et al., 2020). Hotspots have already lost 86% of their original habitat and are considered threatened by extinction (Mittermeier et al., 1998; Purushothaman and Mol, 2023). After the first list of ten biodiverse hotspots were identified (Myers et al., 2000), subsequent global assessments (including various quantitative thresholds for identifying biodiverse hotspots) have included 35 regions. Most of these hotspots are in tropical forests. Three of the 35 biodiverse regions in the world are in South Africa; these include, the Cape Floristic region, the Succulent Karoo and the Maputuland-Pondoland-Albany region (Reyers and McGeoch, 2007). Although hotspots are not formally recognised or governed areas, identification of a region as a biodiversity hotspot increases the potential for conservation investment. In April 2002, the Conference of the Parties to the Convention on Biological Diversity (CBD) adopted the Global Strategy for Plant Conservation (GSPC) at the Hague, Netherlands, to address the continuous loss of biodiversity ( United Nations, 1992; Larsen et al., 2011).

### 1.2.2 The global strategy

The GSPC provides an overall framework to address plant conservation through various measures at both global and national levels (Lefebvre, 2022; Royal Botanic Gardens, 2022). This framework has significant relevance to developing and prioritising conservation strategies for biodiversity hotspots. Although an ecosystem approach (in situ) to biodiversity, conservation is the primary objective of the CBD; improved long term conservation of plant biological diversity requires that in some circumstances, when necessary, ex situ measures must be employed to complement in situ measures (Thormann et al., 2006; Griffith et al., 2015). Therefore, to ensure the effective, long-term conservation of threatened plant species, Target 8 of the GSPC emphasises that: ‘At least 75% of threatened plant species in ex situ collections, preferably in the country of origin, and at least 20% available for recovery and restoration programmes’ (UNEP, 2011; Sharrock et al., 2014) must be considered as biodiversity hotspots.

### 1.2.3 Ex situ methods

Ex situ conservation refers to the protection of species outside their natural habitat, such as botanic gardens, cultivated protected areas, nurseries, seed banks and so forth (Thormann et al., 2006; Royal Botanic Gardens, 2022). While in situ conservation techniques are the preferred method of preserving biodiversity, habitat degradation has made in situ conservation difficult for many species (Thormann et al., 2006). Therefore, ex situ conservation has become an alternative method of protecting many species facing extinction.

While ex situ conservation techniques can be useful, the space and the labour required to maintain field collections need consideration. Furthermore, large expanses of land are in demand for urbanisation and agricultural purposes, limiting land availability for both in situ (Thormann et al., 2006) and ex situ conservation (Sharma and Sharma, 2013; Purushothaman and Mol, 2023). Long-term plant germplasm storage in seed banks is another useful ex situ conservation tool to conserve threatened plant species from extinction (Engelmann, 2012). However, long-term plant germplasm storage in seed banks is impossible for all threatened species, especially in cases where seeds cannot be stored in the long term without viability loss ( Sharma and Sharma, 2013; Reed, 2017).

Ex situ conservation through seed banking also becomes impossible for species (particularly threatened species) that are poor-seeding, and in rare instances when seeds are produced, they are nonviable (Kalaiselvi et al., 2017). The diversity of these species can only be maintained in field collections (Pence et al., 2020). However, maintaining field collections in the long term has serious limitations, including the need for adequate land space, and intensive labour costs associated with maintaining the field collections, and their maintenance and for securing the site from illegal harvesting (Griffith et al., 2015; Purushothaman and Mol, 2023). Species that are difficult to conserve through ‘conventional’ long-term storage through seed banking have been categorised as ‘exceptional’ plant species (Pence, 2014).

While in situ (nature reserves) and ex situ (botanic gardens, seed banks) conservation were initially considered as common approaches to plant biodiversity conservation, in vitro conservation has proven to be a favourable alternative (Berjak et al., 2011; Engelmann, 2011; Engelmann, 2014; Kundu et al., 2018). Various studies have expounded on the impact of biotechnology in conserving plant germplasm in tropical and subtropical regions (Berjak et al., 2011; Moyo et al., 2011; Sharma and Sharma, 2013; Walters et al., 2013; Pence, 2014; Pence et al., 2020) with biotechnology-based techniques being developed in several laboratories worldwide (Cruz-Cruz et al., 2013). These tools are not intended to replace conventional ex situ conservation methods, instead, they are considered complementary, offering greater opportunities for conservation (Hartzell, 2011; Moyo et al., 2011; Pence, 2014; Pritchard et al., 2014; Reed, 2017; Pence et al., 2020). As the danger of extinction is constantly increasing, high-priority species requiring biotechnological tools to be conserved ex situ should be identified (Reyers and McGeoch, 2007), these include the ‘exceptional’ plant species (Berjak et al., 2011; Pence et al., 2020), such as *S. aethiopicus*.

## 1.3 In vitro culture

Over the past four decades, tissue culture technology has become an increasingly important tool in research, particularly in the commercial, agricultural and horticultural industries, and in plant biodiversity conservation (Chaturvedi et al., 2007; Hartzell, 2011; Moyo et al., 2011; Sharma and Sharma, 2013; Gaidamashvili and Benelli, 2021). Plant tissue culture is a technique whereby plant organs such as the growing points of shoots, roots and leaf initials with preserved genetic integrity and cellular differentiation potential, continue to grow when transferred to suitable culture conditions in a controlled in vitro growing environment (George and Manuel, 2013). Organised growth takes place when the organ or piece of tissue (an explant) is placed in vitro on a suitable culture medium or when the culture of previously unorganised tissue (callus) is transferred to appropriate culture conditions. Although the natural growth of higher plants relies on differentiated cellular structure and organised growth, unorganised growth is frequently encountered when pieces of whole plants are grown in vitro (George et al., 2008). Nevertheless, in vitro culture of plant tissue has the potential to generate whole plants under appropriate controlled environmental growing conditions.

The scientific process whereby plant cells, tissues, or organs are used to regenerate whole plants in an aseptic, controlled growing environment is termed micropropagation (George et al., 2008). Plant organs can be directly generated from an explant (plant tissues used for propagation) or from undifferentiated cell clumps (callus) that are generated on an explant. Shoots, buds, roots, and even embryos (somatic embryos) generated in vitro, can progressively develop into plantlets and eventually survive under natural environmental conditions (George and Manuel, 2013). Micropropagation protocols are established on a case-by-case basis, by determining the medium components and environmental conditions for each morphogenic stage (Rout et al., 2001). Basic media formulations include the addition of macronutrients and micronutrients. Established micropropagation protocols include morphogenic stages that culminate in the formation of plants through direct or indirect organogenesis and somatic embryogenesis (George et al., 2008).

Micropropagation involves several key stages; explant selection and decontamination, are the initial stages where in a healthy piece of plant tissue (explant) is chosen from a selected mother plant. This explant is then decontaminated to eliminate any microorganisms that could impede growth. This is followed by shoot initiation and multiplication, and thereafter, the shoots are induced to develop roots to become complete plantlets. The rooted plantlets are gradually introduced (acclimatised) to greenhouse conditions to prepare them for independent growth outside the controlled laboratory environment. Acclimatisation is the final stage of micropropagation when the survival of in vitro plantlets ex vitro determines the success of the established micropropagation protocol.

Micropropagation offers numerous advantages over conventional propagation; most established micropropagation protocols have benefitted research laboratories, commercial horticultural, agriculture, forestry production systems and in vitro conservation laboratories (Caton, 2008; Watt, 2012; Hlatshwayo et al., 2020). Micropropagation facilitates continuous, rapid, and high-quality plant propagation year-round, including rapid propagation of large numbers of plants, production of disease-free plants and multiplication of rare or endangered species. It has been shown to overcome certain problems associated with field collections and seed storage of certain threatened species, albeit possible challenges during the various propagation steps. One of the critical steps is eliminating exogenous (surface) microorganisms from the explant, as they can compete with the plant tissue for nutrients and cause stunted growth or death of the cultures (Neidz and Bausher. 2002; Miyazaki et al., 2010; Moreno-Vázquez et al., 2014). To prevent this, explants are surface decontaminated with disinfectants, one of the most common being sodium hypochlorite (Khatun et al., 2016). However, some endogenous (internal) contaminants are difficult to eliminate despite those treatments (Miyazaki et al., 2010). Therefore, researchers are continuously exploring more improved methods to detect and eliminate endogenous microbes using antibiotics or antimicrobial agents (Falkiner, 1969; Neidz et al., 2002; Webster and Mitchell, 2003; Thomas, 2004). Eliminating surface contaminants on explants derived from field-grown plants can be particularly challenging, and in such explants rigorous decontamination procedures must be applied. The presence of microbial contaminants can, however, lead to low recovery and survival of explants, and subsequent shoot initiation in vitro, as reported for the species of choice in the present study (two to three shoots per explant initiated in vitro), by Hannweg et al. (2016) and Kunene et al. (2018).

Typically, plant cultures are supported by a semi-solid substrate comprising essential nutrients and a gelling agent. The use of semi-solid substrate in micropropagation protocols has limitations as there is the need for regular sub-culturing (every 28 to 42 days) since nutrients are depleted from the small media volumes (10 – 40 ml) depending on the size of the culture vessel, and this is also accompanied by associated sub-culturing labour costs (Watt, 2012; Georgiev et al., 2014; Welander et al., 2014). However, scaled-up automated production systems can be used to overcome these limitations, increase multiplication rates and reduce the handling frequency during the micropropagation steps (Watt, 2012; Georgiev et al., 2014; Gómez et al., 2017; De Carlo et al., 2021). However, semi-solid media containing gelling agents complicates automation (Watt, 2012). Liquid-based media devoid of inert gelling agents, on the other hand, allow for closer contact and uniform access of nutrients to the plant cultures, and the nutrients can be replenished without changing the culture vessel. Additionally, it is possible to sterilise cultures through ultrafiltration, as liquid. Liquid-based culture systems also use larger containers than those used for conventional semi-solid-based cultures (Watt, 2012; Welander et al., 2014; Carvalho et al., 2019;). Although there are advantages to liquid-based systems, cultures may undergo asphyxiation and are more prone to physiological disorders such as hyperhydricity (Polivanova and Bedarev, 2022). Hyperhydricity (glassiness) is the most common physiological disorder associated with in vitro cultivation of plants (Kevers et al., 2004). Hyperhydric (glassy) shoots are characterised by thickened stems, short internodes and translucent, fragile, elongated and twisted leaves (George et al., 2008). However, Ziv (2005) identified the liquid-based bioreactor culture systems with temporary immersion and semi-automated control to overcome the problems associated with liquid-based culture systems (Georgiev et al., 2014; Gómez et al., 2017; Carvalho et al., 2019; De Carlo et al., 2021).

In addition to micropropagation, in vitro culture techniques can also be used for plant germplasm conservation. The application of in vitro culture techniques to preserve plant genetic resources has evolved significantly over the years (Pence, 2014; Pence et al., 2020). The central concern is to conserve the germplasm in the short- or medium-term under controlled, slow-growth conditions. However, preserving plant material through in vitro culture requires continuous labour-intensive subcultures to maintain the in vitro culture which also increases the possibility of somaclonal variation (induced genetic irregularities in clones due to continuous subculture of the plant cultures over time) (Panis and Lambardi, 2006; George et al., 2008; Panis et al., 2020). To circumvent these challenges, cryopreservation is considered an alternative for the long-term conservation of plant germplasm (Li et al., 2017; Vollmer et al., 2017; Kundu et al., 2018).

## 1.4 Cryopreservation

Cryopreservation allows plant germplasm to be stored at ultralow temperatures (such as -196°C in liquid nitrogen [LN] or in its vapour phase [-160 to -190°C]), at which virtually all cellular activities remain suspended, thereby allowing long-term conservation. The commonly used plant parts for cryopreservation are seeds or zygotic embryos, however, for species that are known as ‘poor seeders’ for which vegetative propagation is the only method of propagation, the choice of explants are vegetative tissue derived in vitro (Engelmann, 2014; Hubel and Skubitz, 2017; Pence et al., 2020). The storage of plant germplasm other than seed in LN has routinely been used for over two decades for a wide range of plant species (Yamuna et al., 2007; Varghese et al., 2009; Hajari et al., 2011; Yap et al., 2011; Kundu et al., 2018). However, when living plant tissue is stored at sub-zero temperatures, ice crystals form (Sershen et al., 2010; Teixeira et al., 2013). These crystals cause irreversible damage to the internal cell ultrastructure and organelles, including the cell membranes, reducing their selective permeability; tissue and cell death, and loss of viability are inevitable outcomes upon recovery from sub-zero temperature (Engelmann, 2012; Kami, 2012). Avoidance of intracellular freezing during rapid cooling in LN is critical to ensure successful cryopreservation (Sershen et al., 2010; Hajari et al., 2011; Quain and Berjak, 2012). Although scientists have tested several techniques to preserve plant tissues in LN without causing irreversible cell damage upon cooling, the greatest progress towards developing plant cryopreservation protocols was possible after Sakai *et al.* (1960) described a vitrification-based approach. Vitrification-based cryopreservation is a process whereby cells and meristematic tissue are osmotically dehydrated stepwise in a highly concentrated vitrification solution, that dehydrates the cytosol without causing injury and turns it into a stable glassy state when plunged into LN (Engelmann, 2012; Engelman, 2014, 2012; Pence, 2014; Hubel and Skubitz, 2017; Reed, 2017). Vitrification is the only freeze-avoidance mechanism that allows hydrated cells, tissues and organs to survive at ultra-low temperature (Sakai et al., 2008). Sakai et al. (1990, 1991) developed a glycerol-based plant vitrification solution with low viscosity (PVS2). This solution remained outside the cell's cytosol during dehydration, achieved a supercooled state below -100°C, and ultimately solidified at -115°C. Sakai (1997) then developed valuable cryogenic procedures for cryopreservation of cultured plant meristems. Vitrification solutions can supercool down to a temperature below -100°C and finally transform into a metastable glass at the glass transition temperature (Tg) (-110°C) at a practical cooling rate. By promoting vitrification of the tissue, water is prevented from forming ice crystals during the cooling process; this can be accomplished by replacing the water present in the cells with cryoprotecting substances and rapidly freezing the explant (Engelmann, 2011; Enegelman, 2014; Martinez-Montero et al., 2012). During vitrification, the solution in the cells of the explant transforms into an amorphous glass state when cooled to the ultralow temperature of LN (Teixeira et al., 2013). The glass fills the spaces in the tissue, minimises tissue collapse or solute loss from within the cells, and maintains pH stability during the dehydration process (Nadarajan and Pritchard, 2014). The glassy state exhibits lower water vapour pressure during cryopreservation than the corresponding ice crystal state, thereby preventing further dehydration (Nadarajan et al., 2008). Vitrification-based cryopreservation procedures reduce the damaging effects of ice crystal formation during freezing. Subsequent to the development of PVS2, another plant vitrification solution (PVS3), was reported to provide improved cryoprotection for some species during cooling in LN (Zamecnik et al., 2021). As such, vitrification-based cryopreservation protocols are widely used and their popularity has increased steadily since the successful use of PVS2 decades ago (Varghese et al., 2009;Chen et al., 2011; Hajari et al., 2011; Yap et al., 2011; Sen-Rong and Ming-Hua, 2013; Pawlowska and Szewczyk-Taranek, 2014; Sharma et al., 2017; Kundu et al., 2018). At present, cryopreservation is routinely applied to in vitro-derived shoot apices or somatic embryos using vitrification (Sakai and Engelmann, 2007), encapsulated vitrification (Sakai et al., 2008) or droplet-vitrification for osmotic dehydration ( Pence, 2014; Martinez-Montero et al., 2014; Popova et al., 2016; Vollmer et al., 2017; Kundu et al., 2018). Vitrification-based techniques are appropriate to freeze complex organs such as apical meristems and somatic embryos that comprise a variety of cell types, each with unique requirements during freeze-induced dehydration (Engelmann, 2014). However, optimisation of these techniques is essential. One of the main criteria is for the whole or most of an apical meristem to remain alive to allow direct and organised regrowth upon recovery from cryostorage (Varghese et al., 2009; Sen-Rong and Ming-Hua, 2013; Pawlowska and Szewczyk-Taranek, 2014; Wilms et al., 2020; Koroleva et al., 2023).

For a cryopreservation protocol to be successful, experimental conditions for each of its successive steps should be optimised and several parameters must be considered during the establishment of the protocol (Engelmann, 2014). These include, but are not limited to, the type and size of explants, the type of cryoprotectants used, and the cooling rates. Small in vitro explants such as apical and axillary shoot apices are the explants of choice, since they are exceedingly small (0.5 – 1 mm) and have much lower water content than larger explants (Engelmann, 2014; Wilms et al., 2020). Recovery and regrowth conditions of explants retrieved from cryostorage also require careful consideration (Popova et al., 2023) and are dependent on a suitable micropropagation protocol (Quain and Berjak, 2012; Engelmann, 2014; Kalaiselvi et al., 2017; Koroleva et al., 2023). In addition, the preculture of the explant on a medium with high sucrose concentrations is often found to be effective for successful cryopreservation (Feng et al., 2013; Zamecnik et al., 2021) due to its cryoprotective effects on cell membranes (Uemura and Steponkus, 2003). As such, long-term conservation of germplasm of clonally propagated plants is now possible, largely due to the availability of highly efficient in vitro culture methods and cryopreservation techniques (Gantait et al., 2014; Pence, 2014; Popova et al., 2016; Li et al., 2017).

Currently, there is a pressing need to develop strategies for the long-term conservation of *S. aethiopicus* due to its critically endangered status. To date, there have been no published reports on the development of a cryopreservation protocol for this species, although there are reports on successful cryopreservation of shoot tips of *Zingiber officinale* (Yamuna et al., 2007) using vitrification techniques and *Kaempferia galangal* L. (Preetha et al., 2021) using encapsulation-dehydration; both these species belong to the same family as *S. aethiopicus.* Development of a vitrification-based cryopreservation protocol for *S. aethiopicus* first requires the establishment of a micropropagation protocol from in vitro-derived shoot apices. This would ensure the regeneration of plantlets once the explants are retrieved from cryostorage. In the case of medicinal plant species such as *S. aethiopicus*, once micropropagation and cryopreservation protocols have been developed, it is also important to compare the major phytocompound content of the clones or recovered plantlets, respectively, with the mother plants (Kundu et al., 2018) to confirm metabolic stability. Such outcomes hold important implications for the continued medicinal use of this species.

## 1.5 Aims and objectives

This study aimed to develop conservation strategies for the critically endangered, *Siphonochilus aethiopicus* through micropropagation and cryopreservation. A further aim was to validate and quantify siphonochilone in micropropagated clones.

To achieve these aims, the following objectives were set out:

- To evaluate the use of in vitro-derived shoot apices, generated from buds of rhizomes, to improve the micropropagation of *S. aethiopicus* (compared to that in the literature).
- To establish a cryopreservation protocol for *S. aethiopicus* using in vitro-derived shoot apices.
- To isolate, characterise and quantify siphonochilone in rhizomes and roots of mother plants and micropropagated clones of *S. aethiopicus*, using nuclear magnetic resonance (NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS), respectively.

## 1.6 Structure of the thesis

This thesis is structured in a publication format with the initial chapter being a conflated general introduction and literature review, and the final chapter, the conclusions and recommendations. The remaining three chapters focus on each objective of the study. The five chapters are as follows:

- Chapter One presents the general introduction and the importance of long-term ex situ conservation of plants used in traditional medicinal practices. Focus is placed on the importance of plant biodiversity in general and of medicinal plants in particular and further outlines the challenges associated with long term conservation of these species. The literature review describes the different biotechnological tools that can assist in overcoming these challenges. The aim and objectives of this study are also included in this chapter.
- Chapter Two outlines the effectiveness of using in vitro-derived shoot apices, sourced from in vitro inoculated mother plant rhizome buds, in improving published micropropagation protocols of *S. aethiopicus*.
- Chapter Three describes the procedures towards establishing and optimising a cryopreservation protocol for in vitro-derived *S. aethiopicus* shoot apices.
- Chapter Four details the isolation, characterisation (NMR spectroscopy) and quantification (GC-MS) of the active phytocompound, siphonochilone, from rhizomes and roots of mother plants of *S. aethiopicus*, and its micropropagated clones.
- Chapter Five discusses on the interconnectivity of the results and provides a general conclusion and recommendations for future research.

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# Chapter Two: Towards long-term conservation of *Siphonochilus aethiopicus*, a critically endangered South African medicinal species: An improvement on micropropagation using in vitro shoot apices

(Published manuscript)

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## Abstract

*Siphonochilus aethiopicus*, a critically endangered medicinal plant, faces threats from overharvesting, habitat destruction and climate change. While vegetative propagation is practiced, its limited success cannot meet demand and necessitates efficient micropropagation alternatives. However, current protocols rely on multiplying rhizome-derived bud explants which have high microbial loads in addition to being too large for cryopreservation. Furthermore, plant yield is low. The current study, therefore, presents an improved micropropagation protocol that uses minute (0.5 – 1 mm) in vitro-derived shoot apices following rhizome bud culture, which results in higher yields than that reported in the literature. Emerging buds from post-dormant rhizomes were subjected to a multi-step decontamination procedure which included 30- and 20-minute soaks in fungicides, 0.2% Benlate® and 0.5% sodium dichloroisocyanurate, respectively, with three sterile distilled water rinses in between. Clean and healthy-looking buds were then streaked on Petri dishes containing a bacterial screening medium, which did not show any bacterial growth after 48 h of incubation. Buds were then cultured on full-strength Murashige and Skoog (MS) basal salts and vitamins, 0.09 sucrose, 5 mg $l^{-1}$ 6-benzylaminopurine (BAP) and 4 g $l^{-1}$ Gelrite® to promote shoot development following which axillary shoot apices (0.5 – 1 mm) were excised and multiplied on the same medium. Multiplied shoots (4 per shoot apex) were elongated with reduced BAP (0.1 mg $l^{-1}$), followed by in vitro rooting on MS medium containing 1 mg $l^{-1}$ indole-3-acetic acid (IAA), which resulted in 88 ± 12% rooted shoots. Thereafter, 100% of plantlets first survived acclimatisation on a peat and vermiculite substrate and then transplantation to a coarse river sand and potting medium mixture, surpassing a previous method with 87% acclimatisation success. The plant yield in this study was higher (13 plants per bud) than conventional vegetative propagation (a single plant per bud) or previously established micropropagation protocols (3 – 4 plants per bud). The improved protocol here presented will make significant contributions to plant demand, and also offers suitably sized explants for cryopreservation of *S. aethiopicus*.

**Keywords:** climate change; explants; geophyte; rhizomes; sodium dichloroisocyanurate; wild ginger

## 2.1 Introduction

South Africa is recognised as a rich floral diversity hotspot (Mittermeier et al., 1998; Conservation International, 2011), with an estimated 30,000 indigenous plant species that contribute 10% of the global variety of higher plants. Across the global diversity of species, many are utilised as herbal and traditional medicines for the primary healthcare needs of approximately 80% of rural communities (WHO, 2019). South African rural communities are no exception, with the vast majority relying on *muthi* (traditional medicine) instead of allopathic drugs (Adebayo et al., 2021; Mudau et al., 2022). Traditional medicines are associated with cultural beliefs and values that have been passed on through generations (Ozioma and Okaka, 2019). The trade of medicinal plants harvested from the wild has also had a positive financial impact on the rural South African economy (Khan and Ahmad, 2019). However, due to their increased demand, many of these plants have been indiscriminately over-harvested; this, together with habitat destruction (Ndhlovu et al., 2021) and climate change-imposed stresses (Applequist et al., 2020), has severely reduced natural populations. *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt, a perennial geophyte from the Zingiberaceae family, commonly known as African ginger or wild ginger, is a primary example of such a species. Since *S. aethiopicus* is a perennial geophyte, it depends on seasonal temperature increases to initiate dormancy breaking. However, climate change-related fluctuations in weather patterns, particularly prolonged low winter temperatures (15-20°C) instead of the typical warmer temperatures (22-25°C) in the plant’s environment, pose challenges to dormancy breaking, vegetative propagation, and the necessary scaled-up production. Traditionally, the aromatic cone-shaped rhizomes of *S. aethiopicus* are harvested to treat coughs, colds, influenza-type illnesses and symptoms, malaria and menstrual disorders; it is also added to beverages (mostly ginger beer) consumed during cultural ceremonies

(Cunningham, 1993; Gericke, 2011; Adebayo et al., 2021). The major active phytocompound isolated from the essential oil of both the roots and rhizomes is a furanoterpenoid (4aα,5β,8α)-3,5,8a-trimethyl4,4a,9-tetrahydo-naphtho[2,3-b]-furan-8(5H) (Holzapfel et al., 2002), commonly known as siphonochilone (Viljoen et al., 2002). The isolation of this compound and its documented anti-inflammatory (Lategan et al., 2009), antimalarial (Blumenthal, 2009) and antiplasmodial properties (Light, 2002) have further encouraged its use in the pharmaceutical industry (Blumenthal, 2009; Adebayo et al., 2021).

Although *S. aethiopicus* is documented to be naturally distributed from tropical Africa to Southern Africa, it is listed amongst the top ten most sought-after and over-harvested species by traditional healers, making it endangered in Swaziland (EN A1d) (Braun et al., 2004) and critically endangered in South Africa (CR A4acd) (Lötter, et al., 2022). Currently, small wild populations are sporadically distributed from the Letaba catchment area in the Limpopo Lowveld region in South Africa, to Swaziland (Lötter et al., 2022). The in situ conservation of wild populations of this species in protected areas is hampered by illegal harvesting and trade. At the 17th meeting of the Conference of the Parties (CoP17, 2016), its critically endangered status in South Africa led to the amendment of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (UNEP, 2011) from its Appendix I status to an Appendix II status (at which it currently stands) to further regulate illegal cross-border trade (CoP17, 2016).

In efforts to alleviate further harvesting stress on the remaining wild populations and to restore natural populations in protected areas in South Africa, *S. aethiopicus* is cultivated to a limited extent in some parts of the country. However, these cultivated populations have been, and are still, propagated from mother plant stocks originating from a few individual rhizomes collected from a single population in the Twinstreams region in Mtunzini, KwaZulu-Natal (Ndhlala et al., 2011). A major limitation of this technique is that only a single clonal plant can be propagated per bud. Consequently, a narrow genetic diversity exists amongst cultivated populations in the region. Moreover, this species has also been described as poor-seeding (Gordon-Gray et al., 1989; Salmina, 2017) which has two-fold negative effects. Firstly, cultivation through natural seed propagation is a serious challenge, therefore, current cultivation techniques are primarily through vegetative propagules derived from rhizomes with buds (Salmina, 2017). Secondly, a low seed set prevents seed banking as a long-term conservation strategy for this species. On the positive side, micropropagation can be considered as a tool for both ex situ conservation and bulk production of plants to replenish decimated populations. However, the success of micropropagation in any horticultural or conservation programme depends on developing efficient protocols for the different in vitro stages and ex vitro acclimatisation (Chaturvedi et al., 2007; Crouch et al., 1999; Jacob and Aravinthan, 2011).

In some parts of South Africa, micropropagated *S. aethiopicus* plantlets have been used to replenish nursery stocks that serve as source material for traditional healers and re-establishment programmes in protected areas (Berjak et al., 2011). This approach is based on direct organogenesis to produce in vitro shoots from rhizome buds (Berjak et al., 2011; Hannweg et al., 2016b; Kunene et al., 2018) as with micropropagation protocols established for other Zingerberaceae species (Kavyashree, 2009; Sathyagowri and Seran, 2011; Sevgin and Karatas, 2022). However, rhizomes are soil-associated organs and consequently have a high microbial load. Soil-associated microbial contaminants are extremely challenging to eliminate through conventional decontamination procedures and, as per the published protocols, they result in only a few microbial-free explants being transferred to the next in vitro stage (Hannweg et al., 2016b; Kunene et al., 2018). Subsequently, the yield of nursery stocks are very low. Furthermore, since *S. aethiopicus* is a poor-seeding species making seed banking an unsuitable option, the only technique for its long-term conservation is through cryopreservation of alternate explants such as in vitro-derived shoot apices (Pence, 2014). The requirements for successful cryopreservation are that the explant must be suitably small (0.5 – 1 mm length) to facilitate adequate tissue dehydration and cooling in liquid nitrogen without losing viability (Berjak et al., 2011; Engelmann, 2014; Pence, 2014; Wilms et al., 2020). Additionally, an efficient micropropagation protocol must exist for the regeneration of plants from the explants retrieved from cryostorage (Engelmann, 2014). An efficient reproducible micropropagation protocol for any plant or explant type can only be established by assessing different parameters for the in vitro stages of micropropagation (Chaturvedi et al., 2007; Rout et al., 2001; Sevgin and Karatas, 2022), and can only be considered successful when there is high survival of regenerated plants after acclimatisation. Previously established protocols on *S. aethiopicus* resulted in 3 – 4 plants from a rhizome bud (Hannweg et al., 2016b) while conventional propagation through separating rhizomes with sprouting buds yielded a single plant per rhizome bud (Salmina, 2017). Conventional vegetative propagation of this species also limits up-scaling production. Therefore, in the present study, our approach was to improve on the reported yields through a micropropagation protocol that used in vitro-derived shoot apices

(0.5 – 1 mm length) comprising a single leaf primordium and a small apical dome, and optimisation of plant growth regulators for the different in vitro stages. Such small-sized meristematic explants are reportedly devoid of microbial contaminants and are also suitably sized for cryopreservation (Engelmann, 2012; Varghese et al., 2009; Vujović et al., 2011).

## 2.2 Plant material and methods

### 2.2.1 Plant material

Dormant *Siphonochilus aethiopicus* rhizomes were collected from the eThekwini Municipal Medicinal Plant Nursery located at the Silverglen Nature Reserve in Durban, KwaZulu-Natal, South Africa (population A) and the Edakeni Muthi Futhi Trust Cultivation site at Eshowe, KwaZulu-Natal, South Africa (approximately 150 km north of Durban) (population B). A voucher specimen (accession number 0122671) from a wild population was previously lodged at the herbarium of the South African National Biodiversity Institute (SANBI) KwaZulu-Natal Herbarium (Durban). Mother plants for the current study were originally propagated vegetatively from this wild population. The dormant rhizomes were harvested from one-year-old mother plants propagated vegetatively through stored dormant rhizomes inserted into field beds in the previous growing season (September – December 2017 or 2018). Rhizomes with intact roots were removed from the ground immediately after leaf senescence in late winter (June – July 2018 [season 1] and 2019 [season 2]), placed into plastic bags and transported to the eThekwini Municipal Tissue Culture facility (EMTC) based at the Durban Botanic Gardens within 24 h of harvesting. In the laboratory, the rhizomes were rinsed under running tap water (15 – 20 min), air-dried overnight, dusted with Benlate® (Du Pont Agro Sciences, South Africa), and stored in open plastic trays (350 x 480 x 50 mm) (Calibre Plastics, South Africa), lined with brown craft paper (Croxley, South Africa), in single layers at 22 ± 2 °C in the dark until bud emergence was observed ±98 days (d) post-harvest during mid to late spring (late October to early November) for each harvest batch. These rhizomes were used for conventional vegetative propagation and explant preparation for in vitro culture.

### 2.2.2 Conventional vegetative propagation

Conventional vegetative propagation was performed by preparing a mixture of coarse river sand:potting medium (Gromor, South Africa) (1:1). The medium was placed into plastic plant pots (200 mm Ø, 320 mm in height, P C Plastics, South Africa), moistened with tap water, left for 24 h to drain the excess water, before inserting excised rhizome pieces with emerging buds.

The pots were maintained under tunnel-house conditions at the EMTC facility in late spring (October). Once shoot emergence was observed, each plant was watered (5 ml tap water) manually twice weekly and supplemented with a weekly half-strength foliar application (5 ml) of a water-soluble plant fertiliser (Multifeed®, AECI, South Africa).

### 2.2.3 Explant preparation

Whole rhizomes with emerging buds (Fig. 2.3 a) were rinsed under running tap water (30 min), then immersed in a 1% benzalkonium chloride solution (BAC20®, Chemlab Supplies, South Africa) (v:v) for 60 min, and rinsed again under running tap water (30 min). Emerging buds with approximately 10 – 15 $mm^3$ blocks of surrounding rhizome tissue were aseptically excised and transferred to sterile Petri dishes (Ø = 90 mm) lined with sterile filter paper (Ø = 90 mm) (Whatman™, no. 1) moistened with 1 ml sterile distilled water. These explants were then immersed in 70% ethanol (v:v) for 3 min and transferred to a laminar flow bench for further decontamination.

### 2.2.4 Decontamination of explants and in vitro culture establishment

Decontamination treatments were carried out for explants from population A material (harvested in 2018 upon leaf senescence). Three surface disinfectants, viz. a) 1% sodium hypochlorite (NaOCl) (v:v) (Unilever, South Africa) for 10 min, b) 1% calcium hypochlorite (CaOCl) (w:v) (Sigma-Aldrich, United States) for 10 min, or c) 0.5% sodium dichloroisocyanurate (NaDCC) (w:v) (Medi-Chlor®, South Africa) for 20 min were used as independent decontamination treatments or after a soak for 30 min in 0.2% Benlate® (w:v). One drop of Tween20® (Sigma-Aldrich, United States) per 100 ml was added to all the surface disinfectant solutions. The explants were rinsed three times with sterile distilled water (100 ml) after each treatment step. After the decontamination treatment, the explants were blotted with, and placed on, sterile tissue paper (Kimberly-Clark, South Africa) to remove excess water droplets. The intact rhizome tissue was trimmed off the emerging buds and single buds (5 – 8 mm in length) (Fig. 2.3 b) from each decontamination treatment were cultured on an inoculation medium consisting of Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal salts with vitamins, 0.09 M sucrose (Huletts, South Africa), 5 mg $l^{-1}$ 6-benzylaminopurine (BAP) (Sigma-Aldrich, United States) (Rout et al., 2001) and 4 g $l^{-1}$ Gelrite® (Duchefa Biochemie, Netherlands) (Hannweg et al., 2016) in glass culture tubes (100 mm x 25 mm, 40 ml) (Sigma-Aldrich, United States). The tubes were capped with polypropylene culture tube closures (Sigma-Aldrich, United States) and sealed with Parafilm® (Bemis Company Inc., United States). Each decontamination treatment consisted of five replicates of five emerging bud explants; these replicates were initiated 7 d apart, based on the rate of bud emergence from the stored rhizomes. The percentage of contaminant-free shoots that developed from the emerging rhizome buds, and those with bacterial or fungal contamination, were recorded after 42 d of culture. Cultures that appeared visually contaminant-free from all decontamination treatments were transferred to 523 medium (Viss et al., 1991) in Petri dishes (Ø = 90 mm) containing 10 g $l^{-1}$ sucrose, 8 g $l^{-1}$ casein hydrolysate (Sigma-Aldrich, United States), 4 g $l^{-1}$ yeast extract (Sigma-Aldrich, United States), 2 g $l^{-1}$ $KH_2PO_4$, 0.15 g $l^{-1}$ $MgSO_4.7H_2O$ (Associated Chemical Enterprises, South Africa) and solidified with 8 g $l^{-1}$ agar (Sigma-Aldrich, United States) for endogenous bacterial growth screening. Shoots were individually streaked across the 523 bacterial growth medium and then transferred to a fresh inoculation medium. These cultures were incubated, as described earlier, for 48 h. The cultures with visible bacterial growth were discarded. Healthy shoots, with no contamination, were continuously subcultured on the inoculation medium every 42 d until sufficient aseptic shoot cultures were available for subsequent experiments. The decontamination treatment with the highest contamination-free cultures and best shoot growth was thereafter applied to explants from population B (rhizomes harvested upon leaf senescence during 2018) and then from both populations A and B in the subsequent harvest season (2019).

### 2.2.5 In vitro culture conditions

All media used for this study were adjusted to pH 5.6 ± 0.2 using 1 M sodium hydroxide (NaOH) (Sigma-Aldrich, United States) or 1 M hydrochloric acid (HCl) (Sigma-Aldrich, United States) before autoclaving (Huxley, Taiwan) for 20 min at 120°C and 121 KPa. Cultures were maintained in a growth room under cool, white, fluorescent light (Osram, South Africa) at a photosynthetic photon flux density (PPFD) of 35 µmol $m^{-1}$ $s^{-1}$, and 16 h, 25°C day / 8 h, 22°C night temperatures.

### 2.2.6 Shoot multiplication

Once sufficient quantities of contaminant-free in vitro shoot cultures were obtained, the effects of different BAP concentrations on their multiplication were investigated. Single shoot apices (0.5 – 1 mm in length) forming at the base of the shoot clumps or between leaves of individual shoots were aseptically excised under a stereomicroscope (Leica, Germany) and individually transferred to culture tubes with 10 ml of a medium comprising basal MS salts and vitamins, 0.09 M sucrose, 4 g $l^{-1}$ Gelrite® and BAP (0, 1, 3, or 5 mg $l^{-1}$). Twenty-five shoot apices were used for each BAP concentration tested. The percentage of explants showing multiplication, the number of shoots formed per explant, the number of elongated shoots and the number of rooted shoots were determined 42 d after the multiplication experiments were initiated.

To further improve multiplication, comparisons were performed between a semi-solid culture system and a liquid temporary immersion-based bioreactor system; the medium used comprised basal MS salts and vitamins, 0.09 M sucrose and the BAP concentration was based on that which resulted in the earliest multiplication of shoots. The semi-solid medium (20 ml) was prepared in glass culture jars (120 ml) (Consol, South Africa) and solidified using 4 g $l^{-1}$ Gelrite® and the liquid medium (500 ml) was dispensed into Plantform® bioreactors (Plantform®, Sweden). The bioreactors were programmed to temporarily flush the explants for 10 min every 2 h (Welander et al., 2014). Air, used to displace the liquid medium to facilitate temporary immersion of the explants, was passed into each vessel through 0.25 µm filters (Plantform®, Sweden). Five shoot apices (0.5 – 1 mm length) were aseptically transferred to each culture vessel. For each culture system, three culture vessels were used and replicated five times. The percentage of explants with multiplied shoots and the number of shoots formed per explant were compared between the two culture systems after 42 d. Ultrastructural comparison at the cellular level between the two culture systems was performed, using the meristematic regions of the multiplied shoots under high resolution transmission electron microscopy (HRTEM). The culture system, which resulted in the highest shoot multiplication and explant survival and the best ultrastructural integrity indicated by the least deterioration of the cell ultrastructure, was selected for subsequent shoot elongation and rooting.

### 2.2.7 Shoot elongation and in vitro rooting

Four shoot elongation media were tested, viz. a) half-strength MS salts and vitamins, 15 g $l^{-1}$ sucrose, 4 g $l^{-1}$ Gelrite®; b) full MS basal salts and vitamins, 0.09 M sucrose, 4 g $l^{-1}$ Gelrite®; c) full MS basal salts and vitamins, 0.09 M sucrose, 0.1 mg $l^{-1}$ BAP, 4 g $l^{-1}$ Gelrite® and; d) full MS basal salts and vitamins, 0.09 M sucrose, 1 mg $l^{-1}$ BAP, 4 g $l^{-1}$ Gelrite®. The percentages of elongated and rooted shoots were determined after 28 d in the different media. Shoots that developed roots during elongation were transferred directly to acclimatisation conditions.

Elongated shoots cultured on full MS basal salts and vitamins, 0.09 M sucrose, 0.1 mg $l^{-1}$ BAP and 4 g $l^{-1}$ Gelrite® were selected to investigate in vitro rooting on five different rooting media, viz. a) half-strength MS basal salts and vitamins, 15 g $l^{-1}$ sucrose, 4 g $l^{-1}$ Gelrite®; b) full MS basal salts and vitamins, 0.09 M sucrose, 4 g $l^{-1}$ Gelrite®; c) full MS basal salts and vitamins, 0.09 M sucrose, 0.1 mg.$l^{-1}$ BAP, 1 mg $l^{-1}$ indole-3-acetic acid (IAA) (Sigma-Aldrich, United States), 4 g $l^{-1}$ Gelrite®; d) full MS basal salts and vitamins, 0.09 M sucrose, 1 mg $l^{-1}$ IAA, 1 g $l^{-1}$ activated charcoal (AC) (Sigma-Aldrich, United States), 4 g $l^{-1}$ Gelrite® and; e) full MS basal salts and vitamins, 0.09 M sucrose, 1 mg $l^{-1}$ IAA, 4 g $l^{-1}$ Gelrite® for 28 d. Following this, the percentage of shoots with roots and the total number of roots per shoot were determined.

### 2.2.8 Acclimatisation

Rooted plantlets from the elongation and in vitro rooting experiments were acclimatised in a 200 cavity polystyrene seedling tray (660 x 330 x 50 mm) containing peat:vermiculite (1:1; v/v) under a frost cover transmitting 95% light. The substrate was moistened with tap water and left overnight to drain the excess water before inserting the plantlets. The gelled medium was washed from the rooted plantlets under a slow stream of running tap water (4 – 5 min) and plantlets were individually inserted into the cavities of the seedling tray containing the substrate. The photoperiod for the acclimatisation phase was also maintained at a PPFD of 35 µmol $m^{-1}$ $s^{-1}$, and 16 h, 25°C day / 8 h, 22°C night temperatures provided by overhead cool white, fluorescent light (Sylvania, China) suspended approximately one metre (m) above the frost cover. The frost cover was suspended on galvanised steel hoops (Ø = 0.5 m), preventing direct contact of the cover cloth with the plantlets. Each plantlet was manually watered with 1 ml tap water biweekly and supplemented with a 1 ml weekly foliar application of full-strength Multifeed®. After 42 d, the percentages of plants from the different elongation and in vitro rooting treatments surviving acclimatisation were determined. The surviving plantlets were transferred upon acclimatisation to coarse river sand:potting medium (2:1; v/v) for further growth for 28 d in plastic pots and were maintained under the same tunnel-house conditions as the conventionally propagated plants. The transferred plants were watered twice weekly (5 ml) and supplemented with a weekly half-strength foliar application (5 ml) of Multifeed®. The yield of plants produced via the micropropagation protocol and the conventional vegetative propagation method were compared.

### 2.2.9 Photography and ultrastructural analysis

Photographs of plant cultures were captured with a Canon IXUS 190 digital camera (Canon, Japan), fitted with a 24 mm wide lens, 20.0 megapixels, or with the Leica application suite version 3.4.0 software (Leica Microsystems, Germany) supporting the digital camera for the Leica stereomicroscope (Leica, Germany).

The meristematic regions (apices) from the multiplied shoots (five each from the semi-solid and bioreactor culture systems) were fixed in a solution of 2.5% glutaraldehyde (v:v) (Agar Scientific, United Kingdom) in 0.1 M Sorensen phosphate (Agar Scientific, United Kingdom) buffer (pH 7.2) and stored at 4 °C until further processing. The fixed shoot apices were washed three times (5 min each) with phosphate buffer (0.1 M, pH 7.2). The apices were post-fixed in 0.5% osmium tetroxide (Agar Scientific, United Kingdom) for 1 h and washed again three times (5 min each) with phosphate buffer. The post-fixed apices were dehydrated in a graded acetone (Agar Scientific, United Kingdom) series (30, 50, 75 and 100%) and infiltrated with a low viscosity epoxy resin (1:1 mixture of resin [Agar Scientific, United Kingdom] and 100% acetone [Agar Scientific, United Kingdom]) (Spurr, 1969) for 4 h and then polymerised in fresh resin at 70 °C for 8 h. Ultrathin sections (100 nm) of shoot apices were made with a UC7 microtome (Leica, Germany), using glass (Agar Scientific, United Kingdom) knives and picked with uncoated copper grids (200 $\mu m^3$ mesh) (Agar Scientific, United Kingdom). Grids with resin-embedded sections were stained with aqueous uranyl acetate (Agar Scientific, United Kingdom) and lead citrate (Agar Scientific, United Kingdom) and used for ultrastructural studies. The resin-embedded sections on the copper grids were examined with a JOEL 2100 HRTEM (JOEL, Japan) at an accelerating voltage of 200 kV. Ultrastructural images were captured using the Gatan Digital Micrograph Imaging Suite Version 2 (Amatek, United Kingdom), and qualitative analyses of the images were performed.

### 2.2.10 Statistical analyses

Plant materials from the two cultivation sites and the two seasons were treated separately only during the decontamination and in vitro culture establishment stage. The data collected from both populations for the rest of the micropropagation stages were pooled since no significant differences were observed between the data sets collected.

The experiments were conducted in a randomised block design. All values were presented as mean ± standard error and were analysed using the Statistical Package for the Social Sciences (SPSS®, version 27; IBM, United States). The decontamination treatments, bud multiplication, shoot elongation and rooting treatments consisted of 25 explants (five replicates of five explants each), while the comparison of bioreactor and semi-solid culture systems consisted of 30 explants (six replicates of five shoot apices each). One-way Analysis of Variance (ANOVA) was used to test for significant differences amongst media formulations for each micropropagation stage followed by mean separation using Tukey's post-hoc test. Student T Test was used to compare bud multiplication in the two different culture systems. Where data did not meet the assumptions for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test), a $\log_{10}$ transformation was applied, and a Kruskal-Wallis H test was used. All differences were considered significant at the 0.05 significance level.

## 2.3 Results

### 2.3.1 Decontamination and inoculation

Rhizome buds (Fig. 2.3 b) treated with 0.5% NaDCC preceding a 0.2% Benlate® treatment and then inoculated in vitro produced the highest percentage (40 ± 0.0%) of decontaminated healthy in vitro shoots (Fig. 2.3 c); this was significantly higher than those achieved in all the other decontamination treatments (Fig 2.1). The Petri dishes with 523 medium, streaked with visually sterile shoots from this decontamination treatment did not show any bacterial growth after 48 h of incubation (data not shown). All the explants in the control were lost to fungal contamination (Fig 2.1). Fungal contamination of the bud explants was also significantly reduced with the NaDCC treatment that included a pre-treatment with 0.2% Benlate® (36 ± 7.5%) compared to the one without the fungicide (84 ± 4.0%). Significantly high bacterial contamination was recorded for explants treated only with 0.2% Benlate® (72 ± 8.0%) or treatment with 0.2% Benlate® and then 1% CaOCl (60 ± 0.0%) (Fig. 2.1).

There was no significant difference in the percentage of contaminant-free in vitro explants obtained after decontamination with 0.2% Benlate® (30 min) followed by a 0.5% NaDCC (20 min) and cultured in vitro, between population A rhizomes collected in the first season (A1; 40 ± 0.0%), rhizomes harvested from the same population in the second season (A2; 28 ± 5.0%) and rhizomes from population B in the first season (B1; 24 ± 5.0%) (Fig. 2.2). There was a significantly higher loss of explants to bacterial contamination for population B rhizomes harvested in the two seasons and for explants from population A rhizomes harvested in the second season compared with the loss of explants to bacterial contamination from population A rhizomes harvested in the first season (Fig 2.2). Interestingly, no fungal contamination was observed for the explants from population B rhizomes harvested in the second season (Fig. 2.2).

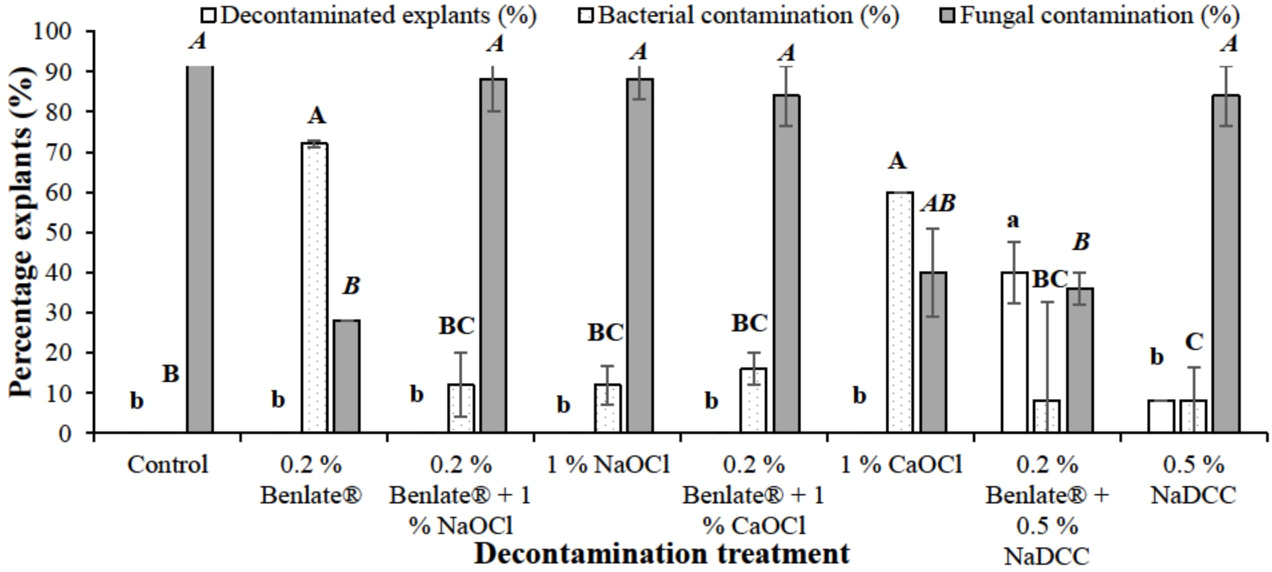


**Figure 2.1**: Uncontaminated and contaminated (±SE) rhizome bud explants of *Siphonocilus aethiopicus* from population A1 (Silverglen Nature Reserve) following different decontamination treatments. NaOCl = sodium hypochlorite (10 min), CaOCl = calcium hypochlorite (10 min), NaDCC = sodium dichloroisocyanurate (20 min). Benlate® was applied for 30 min. Different lowercase letters indicate significant differences in decontaminated explants (only two treatments). Different uppercase, and uppercase italicised letters, indicate significant differences in bacterial and fungal contamination, respectively (Kruskal-Wallis H Test, $p \leq 0.05$, $n = 25$).

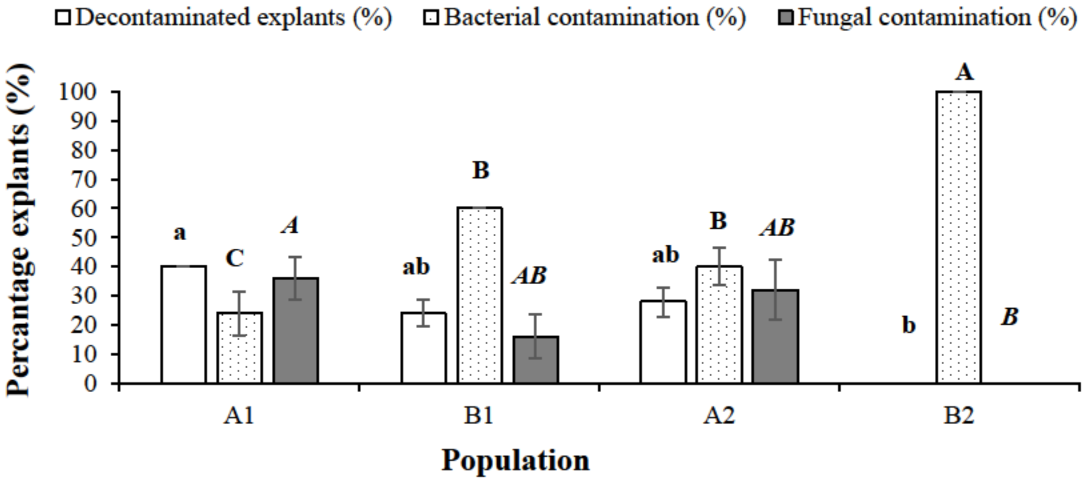


**Figure 2.2:** Uncontaminated and contaminated (±SE) rhizome bud explants of *Siphonocilus aethiopicus* from different populations. A1 and A2 = seasons 1 and 2 from Silverglen Nature Reserve, respectively, B1 and B2 = seasons 1 and 2 from Eshowe, respectively. All explants were decontaminated with Benlate® (30 min) and then sodium dichloroisocyanurate (20 min). Different lowercase letters indicate significant differences in decontaminated explants. Different uppercase, and uppercase italicised letters, indicate significant differences in bacterial and fungal contamination, respectively (Kruskal-Wallis H Test, $p \leq 0.05$, $n = 25$).

### 2.3.2 Shoot multiplication

#### *2.3.2.1 Comparison of BAP concentrations*

The in vitro-derived shoot apices (Fig. 2.3 d) comprising a single shoot primordium (Fig. 2.3 e) produced shoots on all the MS media with 0, 1, 3 or 5 mg $l^{-1}$ BAP and some multiplied buds elongated into shoots with leaf development greater than 1 mm (Fig. 2.3 f; Table 2.1). The earliest shoot multiplication occurred within 14 d of culture in explants on MS medium with 5 mg $l^{-1}$ BAP, while multiplication in other media was seen only after 28 d. For this reason, the 5 mg $l^{-1}$ BAP medium was used to compare micropropagation in different culture systems (see section 2.3.2.2). The number of shoots produced per explant was significantly higher in the BAP-supplemented media (4 buds per explant compared with the plant growth regulator (PGR)-free medium (2 buds per explant). The latter medium produced the highest percentage of elongated shoots greater than 1 mm per explant (96.7 ± 2.4 %), while the least elongated shoots (32.9 ± 3.5%) resulted from the medium with 5 mg $l^{-1}$ BAP (Table 2.1) after 42 d of culture.

**Table 2.1.** The effect of BAP concentrations on shoot multiplication and elongation of *S. aethiopicus* after 42 d.

| **BAP (mg $l^{-1}$)** | **No. of shoots/explant** | **Elongated shoots (%)** |
|---|---|---|
| 0 | $2 \pm 0.2^{b*}$ | $96.7 \pm 2.4^{a}$ |
| 1 | $4 \pm 0.4^{a}$ | $42.6 \pm 6.1^{b}$ |
| 3 | $4 \pm 0.5^{a}$ | $35.6 \pm 4.3^{b}$ |
| 5 | $4 \pm 0.4^{a}$ | $32.9 \pm 3.5^{b}$ |

BAP = 6-benzylaminopurine, d = days. Means (±SE) with different alphabets within columns are significantly different (Tukey's post hoc Test, $p \leq 0.05, n = 25$)

### *2.3.2.2 Comparison of semi-solid and temporary-immersion bioreactor culture systems*

There was no significant difference in the percentage of explants producing shoots, or in the number of shoots produced per explant, between the two culture systems (Table 2.2; Fig. 2.3 g and Fig. 2.3 i). However, the percentage of necrotic explants was significantly lower for the semi-solid system (16.6 ± 6.2%; Fig. 2.3 i) compared with the bioreactor system (62.5 ± 5.7%; Fig. 2.3 j). Explants in the bioreactor system produced significantly longer shoots (44.3 ± 5.5%) compared to those in the semi-solid system (25.9 ± 3.9%; Table 4). Since fewer explants were lost to necrosis in the semi-solid culture system, surviving shoot apices from this system were used for the elongation stage (see section 2.3.3).

At the ultrastructural level, differences were evident in the structure of the meristematic region of shoots multiplied in the semi-solid system (Fig. 2.4 a) and the bioreactor system (Fig. 2.4 e). In the former system, defined organelles, with intact cell membrane and cytomatrix were visible (Fig. 2.4 b - d). In contrast, in the latter culture system, large vacuoles were visible in addition to organelles being shifted towards the cell wall (Fig. 2.4 f). The meristematic region from necrotic explants from the bioreactor system had deteriorated nuclei, and chloroplasts could not be clearly distinguished (Fig 2.4 g); the cell membrane and cytoplasmic matrix integrity were also disrupted in this system (Fig 2.4 h).

**Table 2.2.** In vitro shoot apex production, shoot multiplication, shoot elongation and explant necrosis in *S. aethiopicus* in semi-solid and bioreactor culture systems.

| Culture system | Explants producing shoots (%) | No. of shoots/ explant | Elongated shoots/explant (%) | Necrotic explants (%) |
|---|---|---|---|---|
| Semi-solid | 76.7 ± 12.0$^{a}$ | 5 ± 0.4$^{a}$ | 25.9 ± 3.9$^{b}$ | 16.6 ± 6.2$^{b}$ |
| Bioreactor | 73.3 ± 12.3$^{a}$ | 4 ± 0.5$^{a}$ | 44.3 ± 5.5$^{a}$ | 62.5 ± 15.7$^{a}$ |

Means (±SE) with different alphabets within columns are significantly different (Student T Test, $p \leq 0.05$, $n = 30$).

### 2.3.3 Elongation

Since the bioreactor culture system resulted in high explant loss compared to the semi-solid culture system, only multiplied shoots from the semi-solid culture system were used for elongation on half strength, full strength MS medium or full strength MS medium supplemented with 0.1 or 1 mg $l^{-1}$ BAP for 28 d. In the elongation phase the individual shoots elongated (Table 2.3; Fig. 2.3 n), and some produced an additional shoot (Table 2.3; Fig. 2.3 m). Full strength MS basal medium and supplemented with 1 mg $l^{-1}$ BAP significantly reduced shoot elongation which was also not significantly different to those produced on half strength MS basal medium (60.1 ± 6.6%) (Table 2.3). A small percentage of shoots (data not shown) also developed a single root (Fig. 2.3 k).

**Table 2.3.** The effect of basal salt strength or BAP concentrations on shoots produced per explant and elongated shoots per explant in semi-solid culture of *S. aethiopicus*.

| Treatment | Shoots/explant | Elongated shoots (%) |
|---|---|---|
| 0.5 MS | 0$^{b}$ | 60.1 ± 6.6$^{ab}$ |
| MS | 1 ± 0.2$^{ab}$ | 49.2 ± 6.7$^{b}$ |
| MS + 0.1 mg $l^{-1}$ BAP | 1 ± 0.3$^{a}$ | 82.1 ± 4.7$^{a}$ |
| MS + 1 mg $l^{-1}$ BAP | 1 ± 0.3$^{a}$ | 50.4 ± 7.8$^{b}$ |

BAP = 6-benzylaminopurine. Means (±SE) with different alphabets within columns are significantly different (Tukey's post hoc Test, $p \leq 0.05$, $n = 25$).

### 2.3.4 In vitro rooting and acclimatisation of rooted plantlets

Since a large proportion of explants elongated (82.1 ± 4.7%) in the MS basal medium with 0.1 mg $l^{-1}$ BAP, these shoots were transferred to different rooting media (Table 2.4) for 28 d to promote in vitro rhizogenesis. The shoots produced roots on all the tested media and the root number ranged from 1 to 3 amongst the media. The shoots continued to multiply on all the media; those cultured on MS medium supplemented with 1 mg l$^{-1}$ IAA and 0.1 mg l$^{-1}$ BAP produced the highest number of shoots per explant, and rhizome development was also evident (Fig. 2.3 n). All the rooted plantlets transferred from half-strength MS medium or from MS basal medium supplemented with 1 mg l$^{-1}$ IAA and 0.1 mg l$^{-1}$ BAP were successfully acclimatised (Fig. 2.3 o) to ex vitro conditions. However, only plantlets from the latter medium survived upon transfer from the 200 cavity polystyrene seedling trays to a coarse sand and potting medium substrate in plastic pots under tunnel-house conditions for a further 28 d (Fig. 2.3 p).

**Table 2.4.** The effect of IAA with BAP or activated charcoal on in vitro rooting of elongated shoots and plantlet acclimatisation in *S. aethiopicus*

| **Treatment** | **Rooted shoots (%)** | **Number of roots/shoot** | **Number of new shoots/explant** | **Plantlets acclimatised (%)** |
|---|---|---|---|---|
| 0.5 MS | 92 ± 4.9$^{a}$ | 3 ± 0.6$^{a}$ | 2 ± 0.2$^{b}$ | 100 ± 0$^{a}$ |
| MS | 88 ± 0$^{a}$ | 1 ± 0.2$^{b}$ | 2 ± 0.3$^{b}$ | 30 ± 7.8$^{b}$ |
| MS + 1 mg l$^{-1}$ IAA | 84 ± 16$^{a}$ | 2 ± 0.2$^{ab}$ | 1.0$^{b}$ | 0$^{c}$ |
| MS + 0.1 mg l$^{-1}$ BAP + 1 mg l$^{-1}$ IAA | 88 ± 12$^{a}$ | 2 ± 0.2$^{ab}$ | 4 ± 0.6$^{a}$ | 100 ± 0$^{a}$ |
| MS + 1 g l$^{-1}$ activated charcoal + 1 mg l$^{-1}$ IAA | 84 ± 16$^{a}$ | 2 ± 0.2$^{ab}$ | 2 ± 0.2$^{b}$ | 24 ± 7.8$^{b}$ |

IAA = indole-3-acetic acid, BAP = 6-benzylaminopurine. Means (±SE) with different alphabets within columns are significantly different (Tukey's post hoc Test and Kruskal-Wallis H test for log$_{10}$ transformed percentage data, $p \leq 0.05, n = 25$).

### 2.3.5 Plant yield from the improved micropropagation protocol

Using in vitro-derived shoot apices yielded 13 new plants from each healthy sterile explant after 322 d compared with vegetative propagation (Fig. 2.5), which yielded a single plant per rhizome bud after 238 d (two plants per rhizome) (Table 2.5). Only decontaminated healthy explants (23 per 100 rhizome buds) recovered after decontamination for the two populations harvested from the two subsequent seasons (Table 2.5) were used to determine the yield achieved using the micropropagation protocol performed in this study.

**Table 2.5.** *Siphonochilus aethiopicus* micropropagation and vegetative propagation using the same number of rhizomes with emerging buds.

| **Propagation stage** | **Vegetative propagation** | | **Micropropagation** | | |
|---|---|---|---|---|---|
| | No. of days | No. of recovered parts or plants | No. of days | No. of recovered parts or plants | Example from Figures |
| Bud emergence per rhizome at dormancy breaking | ±98 | 2 | ±98 | 2 | Fig. 2.3 a |
| Rhizomes | | 50 | | 50 | |
| Separated rhizome pieces with emerging buds | | 100 | | 100 | Fig. 2.3 b |
| Recovered contaminant free explants | - | - | 42 | 23 | Fig. 2.3 c |
| Clean in vitro cultures | - | - | 42 | 69 | |
| Shoots multiplied from excised shoot apices | - | - | 42 | 345 | Fig. 2.3 g |
| Elongated shoots | | | 28 | 345 | Fig. 2.3 l |
| Rooted shoots | - | - | 28 | 345 | Fig. 2.3 n |
| Acclimatised plants | - | - | 42 | 345 | Fig. 2.3 o |
| Mature plants | 140 | 100 | - | 304 | Fig. 2.3 p |
| Plant yield/initial explant | | | | 13 | |
| **Total** | **238** | **100** | **322** | **304** | |

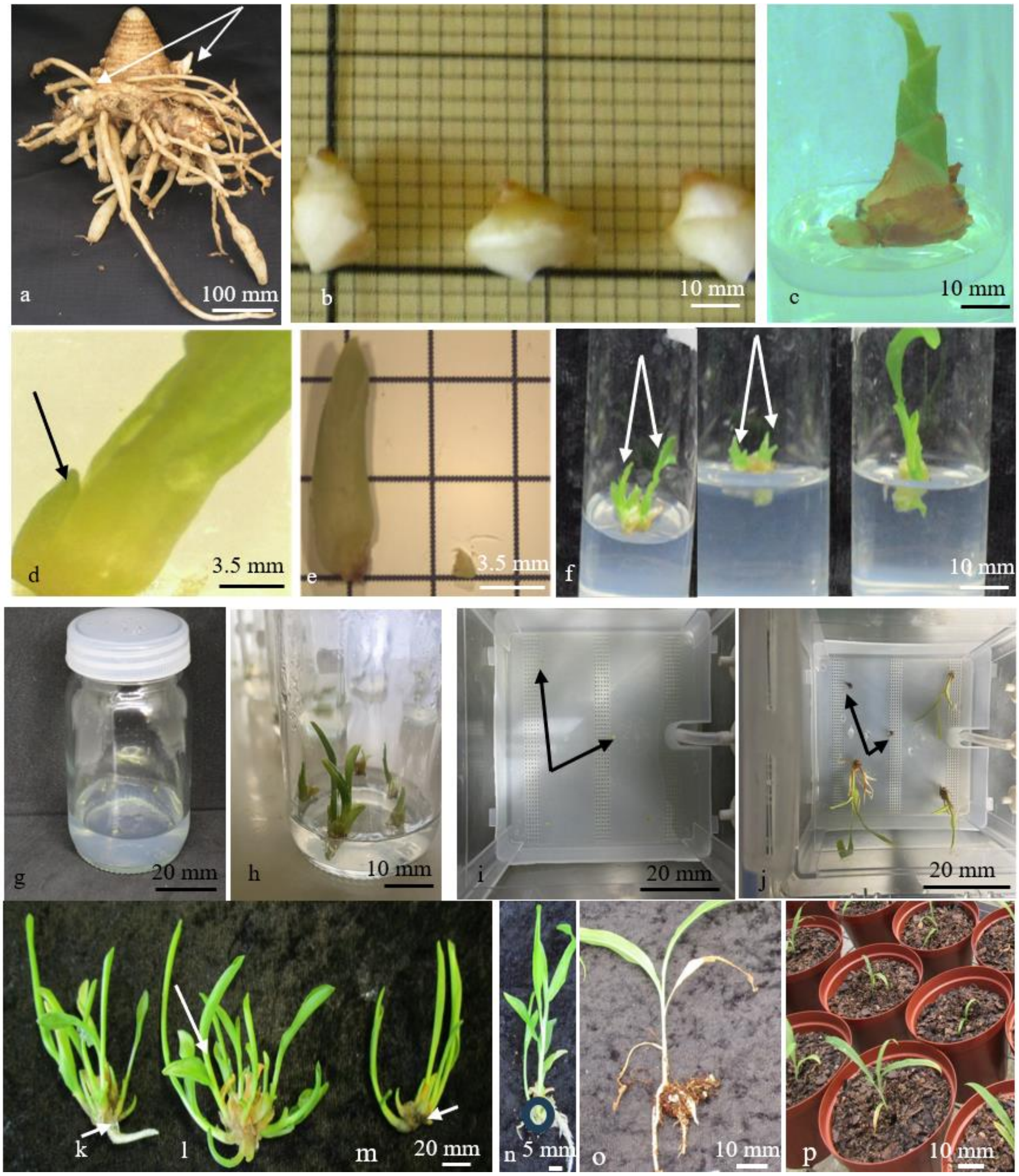


**Figure 2.3.** Micropropagtion stages in *S. aethiopicus*. A single rhizome and arrows showing the relative size and distribution of emerging buds (a); rhizome bud explants with excess rhizome tissue trimmed from the point of attachment (b); in vitro healthy shoot development from bud explant (c); the arrow shows the position of an axillary shoot apex on an in vitro developed shoot (d); the relative size of an excised shoot apex (right) to separated axillary shoots used in other *S. aethiopicus* micropropagation protocols (left) (e); bud shoot multiplication and arrows showing shoot elongation in the presence of 5 mg $l^{-1}$ BAP (left), 3 mg $l^{-1}$ BAP (center) and PGR-free medium (right) (f); the relative size and position of shoot

tip apices (g) and multiplied shoots (h) for the semi-solid culture system; the relative size and position of shoot tip apices (indicated by arrows) (i) and multiplied shoots and necrotic explants (indicated by arrows) (j) for the Plantform® bioreactor culture system; elongated shoots with an arrow showing a single root (k); shoot elongation with leaf development (indicated by an arrow) (l); or axillary bud formation (indicated by an arrow) (m) after elongation for 28 d; in vitro rooted plantlets with a circle indicating early rhizome development (n); acclimatised plantlet removed from seedling trays (o) and acclimatised plants transferred to a coarse sand and potting mix substrate in plastic pots (p).

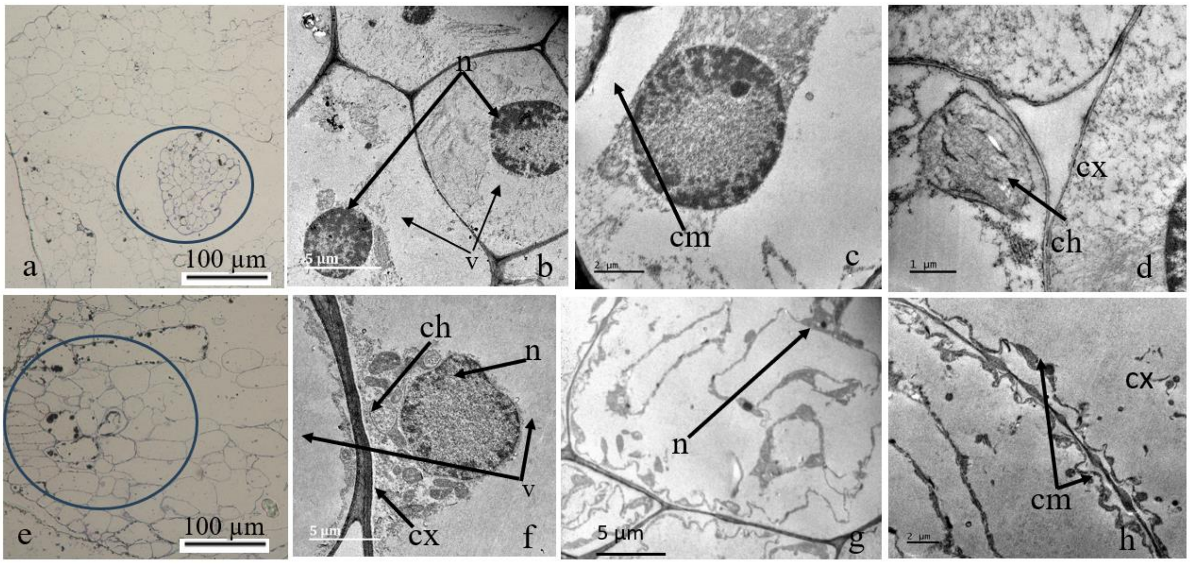
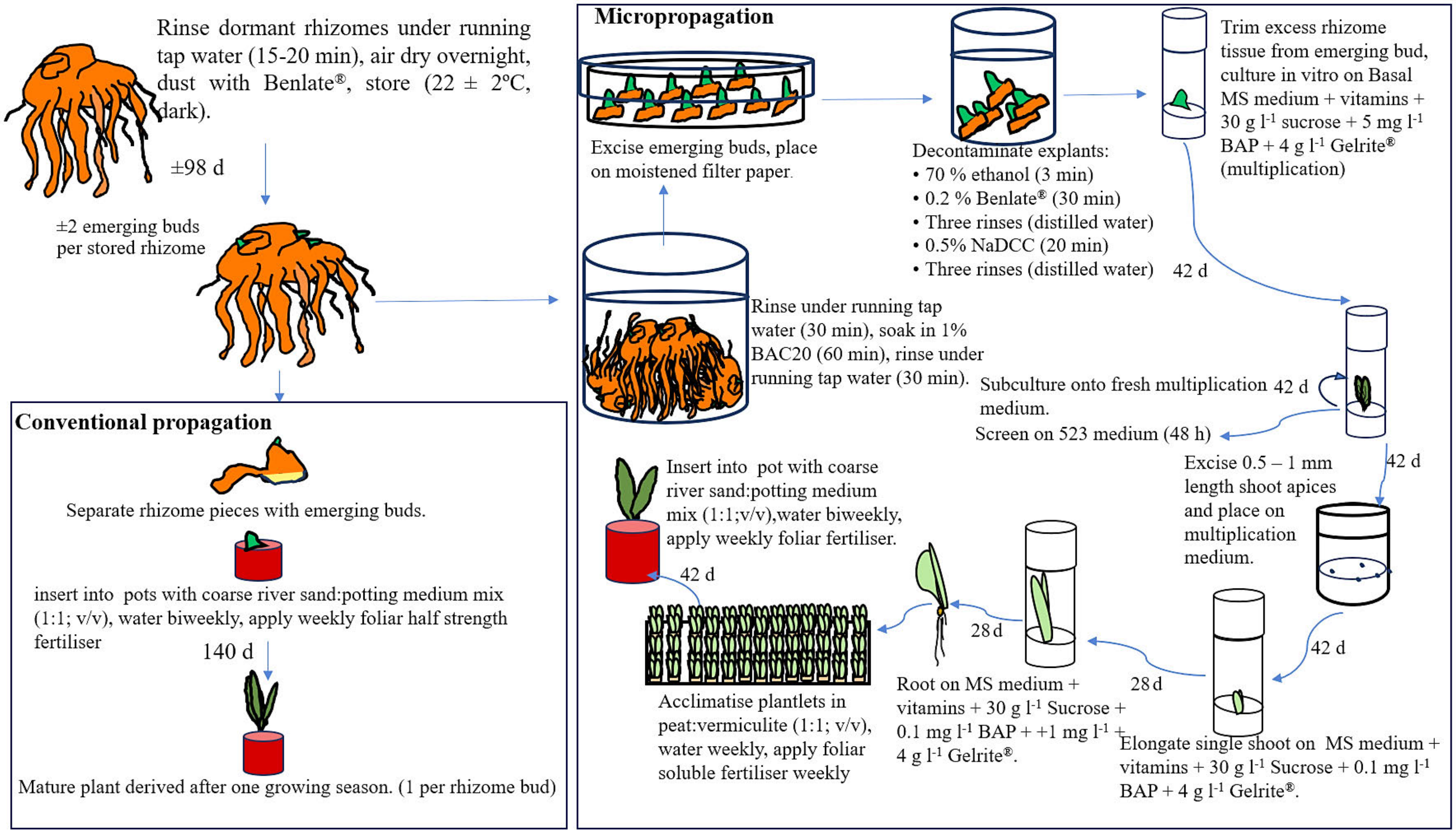


**Figure 2.4:** Ultrastructure of the meristematic region of in vitro multiplied shoot apices from the two culture systems. Top row: meristematic region of shoot apex on semi-solid medium (a) with distinctive nuclear (n) development (b), intact cell membrane (cm) (c), and chloroplast (ch) and cytoplasmic matrix (cx) (d); Bottom row: meristematic region of shoot apex from bioreactor system (e) with large vacuoles (v), chloroplast (ch), nuclei (n) and cytoplasmic matrix (cx) (f). The meristematic region of a necrotic shoot apex from the semi-solid culture system with deterioration of nuclei (n) and other (unidentifiable) organelles (g). The meristematic region of a necrotic shoot apex from the bioreactor system accompanied by deterioration of the cell membrane (cm) and cytoplasmic matrix (cx) (h).


Rinse dormant rhizomes under running tap water (15-20 min), air dry overnight, dust with Benlate®, store (22 ± 2°C, dark).

±2 emerging buds per stored rhizome

**Conventional propagation**

Separate rhizome pieces with emerging buds.

insert into pots with coarse river sand:potting medium mix (1:1; v/v), water biweekly, apply weekly foliar half strength fertiliser

Mature plant derived after one growing season. (1 per rhizome bud)

**Micropropagation**

Rinse under running tap water (30 min), soak in 1% BAC20 (60 min), rinse under running tap water (30 min).

Excise emerging buds, place on moistened filter paper.

Decontaminate explants:
- 70 % ethanol (3 min)
- 0.2 % Benlate® (30 min)
- Three rinses (distilled water)
- 0.5% NaDCC (20 min)
- Three rinses (distilled water)

Trim excess rhizome tissue from emerging bud, culture in vitro on Basal MS medium + vitamins + 30 g $l^{-1}$ sucrose + 5 mg $l^{-1}$ BAP + 4 g $l^{-1}$ Gelrite® (multiplication)

Subculture onto fresh multiplication medium.

Screen on 523 medium (48 h)

Excise 0.5 – 1 mm length shoot apices and place on multiplication medium.

Elongate single shoot on MS medium + vitamins + 30 g $l^{-1}$ Sucrose + 0.1 mg $l^{-1}$ BAP + 4 g $l^{-1}$ Gelrite®.

Root on MS medium + vitamins + 30 g $l^{-1}$ Sucrose + 0.1 mg $l^{-1}$ BAP + +1 mg $l^{-1}$ + 4 g $l^{-1}$ Gelrite®.

Acclimatise plantlets in peat:vermiculite (1:1; v/v), water weekly, apply foliar soluble fertiliser weekly

Insert into pot with coarse river sand:potting medium mix (1:1;v/v),water biweekly, apply weekly foliar fertiliser.

**Figure 2.5:** A diagrammatic comparison of the processes of vegetative propagation and micropropagation of *S. aethiopicus*. BAP = 6-benzylaminopurine; IAA = indole-3-acetic acid; MS = Murashige and Skoog basal salt medium; NaDCC = sodium dichloroisocyanurate

## 2.4 Discussion

Given the endangered status of *S. aethiopicus*, efforts on micropropagation have been undertaken previously (Ngwenya et al., 2010; Hannweg et al., 2016; Kunene et al., 2018). Hannweg et al. (2018) used rhizome bud explants to develop and multiply shoots through direct organogenesis in vitro and a similar approach has been reported by Kunene et al. (2018). Additionally, Ngwenya et al. (2010) followed indirect somatic embryogenesis or direct organogenesis depending on the explant used. However, in all three studies large explants (rhizome buds, leaf or root sections) were used. The present study focussed on obtaining in vitro shoot apices of small size (0.5 – 1 mm) from decontaminated rhizome buds, to promote adequate decontamination and multiply shoots; such small shoot apices would also be amenable for cryostorage (Engelmann, 2012; Varghese et al., 2009; Vujović et al., 2011). Moreover, it was necessary to improve on the published micropropagation protocols to improve reported acclimatisation success (Hannweg et al. – 87%) and increase reported plant yield per explant (Hannweg et al. – 3 to 4 plants per explant; or Kunene et al. – 3 plantlets per explant). This would also ensure that plants could be successfully regenerated following retrieval of the shoot apices from cryostorage efforts.

*S. aethiopicus* rhizomes are dormant during winter (Geldenhuys, 2007) during which time they can be harvested and stored; they resprout in spring when dormancy has broken. Such seasonal, and other environmental factors influence dormancy breaking in geophytes (Zhao et al., 2022) and waiting until spring, when emerging buds are visible on the rhizomes, has been reported to improve vegetative propagation of *S. aethiopicus* (Salmina, 2017). In the present study, the seasonally-induced dormancy breaking of rhizomes was considered, therefore, only emerging buds from stored rhizomes (±98 d after harvest) were used during mid-to-late spring as the source of explants for in vitro culture initiation. In two of the previous studies on *S. aethiopicus* (Hannweg et al., 2016; Kunene et al., 2018), rhizome bud explants were excised, disinfected and cultured in vitro immediately upon harvesting rhizomes.

Contamination of rhizome-derived explants has been reported as a major impediment to the successful establishment of in vitro cultures (Chaturvedi et al., 2007; Hannweg et al., 2016; Kunene et al., 2018) owing to rhizome exposure to various soil pathogens. In this study, the rhizomes with emerging buds were pretreated with Benlate® prior to storage in an attempt to reduce the microbial load on the explants at the time of bud excision. The active compound in Benlate® is carbendazim, and it functions as a broad-spectrum systemic fungicide (Elslahi et al., 2014). The scarcity of rhizomes of *S. aethiopicus* limited the number of attempted decontamination treatments in this study to three widely-used chlorine-based surface disinfectants (Srivastava et al., 2010; Oyebanji et al., 2011) viz., CaOCl, NaOCl and NaDCC. The multi-step decontamination procedure which included a broad-spectrum fungicide treatment followed by treatment with NaDCC was the most effective in eliminating microbial contaminants for one population for one season (A1) but resulted in only 40% decontaminated healthy buds (Fig. 2.1). The same decontamination treatment was then applied to material harvested for the same population, but from a second season and another cultivated population (B) for the same two harvesting seasons. They responded differently to the decontamination treatment; there were significant differences in the loss of explants to both bacterial and fungal contaminants. This is not uncommon as field conditions and microbial loads vary for different cultivation sites and seasons (Reed and Tanprasert, 1995; Kidus and Teka, 2020).

Although 2 g $l^{-1}$ CAPTAB® (active ingredient: captab at 500 g $kg^{-1}$) fungicide (2 h), followed by 1% CaOCl (w/v) for 15, 20 or 30 min (Hannweg et al., 2016) and 3.5% NaOCl (w/v) (15 min) (Kunene et al., 2018) had been previously used for surface decontamination of the rhizomes, this is the first report on the efficacy of NaDCC in removing microbial contaminants from *S. aethiopicus* rhizome bud explants. The chlorine in NaDCC is available in an organic form at pH 7 and its success in eliminating culturable contaminants has been previously reported (Parkinson et al., 1996; Neidz and Bausher, 2002; Webster and Mitchell, 2003). Regardless of the chlorine-based disinfectants used, their decontamination activity is due to free chlorine in the form of hypochlorous acid (HOCl) formed when the disinfectant is diluted in water (Parkinson et al., 1996). NaDCC hydrolyses in water to form HOCl and sodium cyanurate ($C_3N_3O_3H_2Na$) which is easily biodegradable (Phong et al., 2018; Shetty and Narasimhan, 2021). Since NaDCC solutions are acidic and its action is pH-dependent, the free available chlorine exists not only as hypochlorous acid, but also as hypochlorite ions. The ratio of hypochlorous acid to hypochlorite ions increases rapidly with a decrease in pH; thus the former acts as the predominant active species required to bring about the necessary decontamination action (Coates, 1996). The chlorine, partially stored as chlorinated isocyanurates, is slowly released upon exposure to changes in pH, commonly encountered during exposure to high microbial load (Shetty and Narasimhan, 2021). Screening for covert bacterial contaminants on 523 media (Viss et al., 1991), as done in this study, was an effective non-destructive technique to select microbial free explants for subsequent stages of micropropagation. The use of this screening technique is especially important when limited explants are available for in vitro initiation (Attree and Sheffield, 1986; Viss et al., 1991; Thomas, 2004; Moreno-Vázquez et al., 2014), as was the case for *S. aethiopicus*.

Although rhizome buds have been used for micropropagation on semi-solid medium in previous studies (Hannweg et al., 2016; Kunene et al., 2018), the present study was the first to use shoot apices of small size (0.5 – 1 mm) comprising a single apical dome, from in vitro-derived shoots initiated from rhizome buds. In efforts to improve acclimatisation success and increase plant yield, this study considered different BAP concentrations and two types of culture systems (semi-solid and temporary immersion bioreactor) for shoot multiplication. BAP is a synthetic cytokinin that is most widely used to induce shoot formation in rhizomatous geophytes compared to kinetin, another synthetic cytokinin (Seran, 2013; Sevgin and Karatas, 2022). One successful example of a close relative to *S. aethiopicus* is *Zingiber officinale* Rosc. for which BAP resulted in shoot multiplication in a semi-solid-based culture system with 97% regeneration (Yamuna et al., 2007; Ramirez and Zuniga, 2019). Rout et al. (2001) reported on the successful high bud multiplication rates from *Z. officinale* meristem explants in the presence of high BAP (6 mg $l^{-1}$). In the present study, shoot apices of *S. aethiopicus* cultured on MS medium supplemented with 5 mg $l^{-1}$ BAP multiplied the earliest and produced five shoots per in vitro-derived shoot apex in the semi-solid system compared with Hannweg et al. (2016) who reported that 2 mg $l^{-1}$ BAP resulted in 3 to 4 shoots/explant; or Kunene et al. (2018) who reported 2 to 3 shoots/explant using 1 mg $l^{-1}$ BAP.

One of the major limitations of semi-solid culture is the requirement of regular sub-cultures (every 28 to 42 d) due to nutrient depletion from the relatively small volumes of the media utilised; this can reduce multiplication and is also accompanied by increasing running and labour costs. Therefore, temporary immersion semi-automated systems, using Plantform® bioreactors, were tested and compared with semi-solid culture in the present study. In bioreactor systems, the culture vessels are self-contained sterile environments that capitalise on liquid nutrient or liquid/air inflow and outflow systems and are designed for intensive and scaled-up cultures; they also allow close monitoring and control over the micro-environmental conditions. Cultures in a temporary immersion-based system have improved contact with the nutritive media (Watt, 2012; Welander et al., 2014; De Carlo et al., 2021). Several studies have reported on the increased multiplication rate in temporary immersion systems compared to semi-solid culture systems (De Carlo et al., 2021; Uma et al., 2021; Murthy et al., 2023). However, in this study, there was no significant difference in the shoot multiplication rate for the two culture systems. Moreover, the bioreactor system resulted in significantly higher necrotic explants (62.5 ± 15.7%) compared to the semi-solid system (16.6 ± 6.2%). At the ultrastructural level, meristematic cells of the necrotic explants in the bioreactors showed deterioration (high degree of vacuolisation, damaged cell membranes, reduced organelle development) usually observed in hyperhydric cells (Kevers et al., 2004; Polivanova and Bedarev, 2022). Hyperhydricity is an in vitro stress-induced response (Kevers et al., 2004; Polivanova and Bedarev, 2022) which can lead to poor plant growth, ex vitro acclimatisation or necrosis. Hyperhydricity can be the result of long exposure to a liquid medium and a high concentration of cytokinin in the confined vessel (Polivanova and Bedarev, 2022). In this study, the bioreactors were programmed to flush the shoot apices with a liquid medium for 10 min every 2 h. Similar immersion time, also in Plantform® bioreactors, was used to increase multiplication from shoot explants of *Digitalis lutea* L. x *purpurea* L., *Echinacea purpurea* (L.) Muench, *Rubus idaeus* L.  (Welander et al., 2014) and *Salvia viridis* L. (Grzegorczyk-Karolak et al., 2022). However, the explants used in those studies were approximately ten times (10 mm in length) larger than the explants (0.5 – 1 mm in length) used in the present study. Uma et al. (2021) developed a novel TIB (temporary immersion bioreactor) system using the banana cultivar, Rasthali AAB—Silk, to successfully improve the multiplication rate for in vitro-derived bud explants produced in a semi-solid medium and then cultured in the TIB which used a 2 min immersion frequency every 6 h. However, in the present study, the minute explants (0.5 – 1 mm) were exposed to both a higher duration (10 min) and frequency (2 h) of immersion in the liquid media which subsequently led to a high rate of necrosis. Therefore, more research is needed to overcome the deteriorative ultrastructural changes of the explants observed in the bioreactors. Despite the high loss of explants and buds to necrosis, the semi-solid and bioreactor culture systems showed no significant difference in shoot multiplication success (76.7 ± 12.00% and 73.3 ± 12.3%, respectively), but the bioreactor system had a higher percentage of elongated shoots (44.3 ± 5.5%). Therefore, if the deteriorative response can be minimised, then it would be possible to improve on the multiplication rate and scale-up capacity of the bioreactors too. Factors to investigate should include changing the immersion frequency and duration, adjusting the number of explants per culture vessel, or changing the bioreactor type.

Shoots formed in the semi-solid culture system were selected for further elongation since high loss of explants or multiplied shoots to necrosis was recorded for shoot multiplication in the bioreactor system (62.5 ± 15.7%); however, only a small proportion (25.9 ± 3.9%) of shoots elongated to more than 10 mm in the semi-solid system, hence it was necessary to include an elongation step in the micropropagation process. Transferring the multiplied axillary buds to MS medium with reduced BAP (0.1 mg $l^{-1}$) resulted in the longest shoots after 28 d. Kunene et al. (2018) also reported that low BAP concentrations (0 – 0.5 mg $l^{-1}$) successfully elongated *S. aethiopicus* shoots as did Srivastava et al. (2012) for the banana cultivar, Dwarf Cavendish. When the elongation medium was supplemented with 1 mg $l^{-1}$ IAA, it resulted in a high percentage of rooted shoots along with high shoot multiplication and development of rhizomes after 28 d (Table 2.4). IAA is an auxin that is widely used in micropropagation protocols to promote in vitro rooting (Kavyashree, 2009; Rout et al., 2001) and has been successful for *S. aethiopicus* (Hannweg et al., 2016; Kunene et al., 2018). Rooted shoots were successfully acclimatised ex vitro (100%; 42 d) and continued to develop further upon transfer to a coarse sand and potting mix substrate in a tunnel-house (28 d).

The improved micropropagation protocol reported here is favourable for mass multiplication and plant regeneration of *S. aethiopicus* using shoot apices derived from rhizome buds in vitro. Although the time to regenerate *S. aethiopicus* plants is longer (322 d) than producing plants through conventional vegetative propagation via the separation of rhizome pieces with emerging buds (218 d), the yield was increased in the former approach. Our protocol yielded 13 plants per explant, 3 to 7 times more, when compared to 4 plants per bud produced in a previous micropropagation study (Hannweg et al., 2016) and with a single plant per bud via the conventional propagation method (Salmina, 2017). The present protocol also led to all rooted shoots being successfully acclimatised using a peat:vermiculite (1:1, v/v) substrate and survival of all acclimatised plantlets transplanted to a mixture of coarse river sand and potting medium, as opposed to 87% acclimatisation in a pine bark:coarse sand (1:1, v/v) substrate in the study by Hannweg et al. (2016). Our optimised approach offers a promising and reproducible option for the large-scale propagation of *S. aethiopicus*, furthermore, the small-sized shoot apices offer a suitable explant type of conservation through cryopreservation.

## 2.5 Conclusion and future recommendations

This study confirmed that *S. aethiopicus* plants can be generated from in vitro-derived shoot apices that are sufficiently small (0.5 – 1 mm length). Plant growth regulators were optimised at the various micropropagation stages. Following shoot multiplication on semi-solid culture, an intervening elongation period followed by in vitro rooting led to successful acclimatisation and a higher plant yield per shoot apex. This protocol also shows potential as a viable recovery method for the successful regrowth of small shoot apices following cryopreservation, providing an effective approach for conserving the critically endangered species, *S. aethiopicus*.

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# Chapter Three: Vitrification-based cryopreservation of in vitro shoot apices of *Siphonochilus aethiopicus*

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## Abstract

*Siphonochilus aethiopicus* is a sought-after medicinal plant that plays a significant role in primary healthcare, generates income and is used in cultural practices in African rural communities. Unfortunately, unsustainable harvesting has driven this species to extinction in the wild, and it is now classified as critically endangered in KwaZulu-Natal, South Africa. Poor seed production not only complicates seed propagation, but also poses a challenge for long-term conservation efforts through seed banking. This necessitates an alternate long-term ex situ conservation strategy for this species. Therefore, this study aimed to establish a cryopreservation protocol for *S. aethiopicus* using in vitro-derived shoot apices. The shoot apices were dehydrated by culturing on sucrose-enriched medium followed by cryoprotection and cooling. Water content, viability and the survival of explants, and the effect of each sequential cryo-preparative procedure at a subcellular level were compared prior to cooling. The dehydrated shoot apices were subjected to three methods of cooling: direct cooling in liquid nitrogen, vitrification using Plant Vitrification Solution 2 (PVS2) and droplet-vitrification using PVS2 and followed by immersion in liquid nitrogen. Shoot apices cultured on Murashige and Skoog basal salt and vitamin medium with 0.09 M sucrose and 3 g $l^{-1}$ Gelrite® for 48 h and then on the same medium but with 0.3 M sucrose in the dark for 24 h, followed by sequential dehydration in cryoprotection mixture (10 min), loading solution (5 min) and then in PVS2 (10 min), showed the highest viability and survival of explants (58.33 ± 0.07%) prior to cooling. Droplet-vitrification of the apices in LN for 24 h following the sequential dehydration process, gave 66.67% regrowth from the cryostored apices following thawing, rehydration and recovery. The successful recovery of shoot apices using droplet-vitrification and the regeneration of the plantlets from the recovered shoot apices represents the initial steps towards the long-term conservation of *S. aethiopicus*, although further research is necessary to, firstly, assess if the major phytocompound, siphinochilone found in rhizomes and roots of *Siphonochilus aethiopicus* is present in plants recovered from cryopreservation, and secondly, further studies need to focus on enhancing explant recovery and regeneration rates.

**Keywords:** ex situ conservation; droplet-vitrification; PVS2; geophytes; Zingerberaceae

## 3.1 Introduction

In developing countries such as South Africa, approximately 80% of the population depend on plant-derived traditional medicines for primary healthcare because of their affordability and cultural acceptance (Maroyi, 2013). *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt is a rhizomatous perennial geophyte and one of the top ten most sought-after plants in traditional African medicinal practices (Lötter et al., 2022). It holds significant cultural importance and possesses substantial socio-economic value. However, unsustainable harvesting has caused the species to become extinct in the wild in KwaZulu-Natal and to be listed as critically endangered in South Africa (Lötter et al., 2022). This necessitates an urgency for the long-term ex situ conservation of this species as recommended for species that are extinct in the wild (Pence, 2014; Niino and Arizaga, 2015).

Seed banking is considered the most efficient long-term ex situ strategy for plant biodiversity conservation (Engelmann, 2011; Pence, 2014) but is unsuitable for poor-seeding species such as *S. aethiopicus.* It is a species that produces few or no flowers and, if produced, are relatively short-lived. This poses a problem for sexual reproduction, seed production and perpetuation of the species. Additionally, in those rare instances when flowers are produced, pollinated and fertilised to produce seeds, the seeds can take up to a year to germinate (Adebayo et al., 2021). Therefore, seed banking has a low chance of success, precluding this approach as a viable long-term germplasm conservation strategy for *S. aethiopicus*. This then threatens the future availability of this important medicinal plant and calls for alternate conservation approaches.

Cryopreservation of plant tissues, vegetative parts or embryos is reportedly an efficient ex situ conservation technique for plants that cannot be conserved through conventional seed banking (Engelmann, 2014; Reed, 2017; Kundu et al., 2018). This technique is based on the storage of tissues at ultra-low temperatures in liquid nitrogen (LN) at -196$^{o}$C or sometimes in its vapour phase (-150$^{o}$C) (Reed, 2017; Kundu et al., 2018). When any tissue is cryopreserved, biochemical, metabolic, and cellular activities are arrested and long-term tissue conservation is possible. Over the past 50 years, plant biotechnologists have developed and tested several cryopreservation techniques to preserve plant material (Kundu et al., 2018; Benelli, 2021;Amankwaah et al., 2023). One of the landmark studies in this field was by Sakai et al. (1990) who developed a vitrification-based technique to preserve plant tissue at ultra-low temperatures.

Vitrification-based cryopreservation techniques aim to prevent ice crystal formation in the plant cells during the preservation stages. Crystallisation of water in the plant cells is prevented by first replacing the water in the explant with cryoprotecting substances prior to cooling in LN. When cooled at a suitable cooling rate, the cryoprotective solutions solidify into a metastable, amorphous glass without undergoing crystallisation (Fahy et al., 1984). Sequential dehydration of tissues in cryoprotective solutions until final immersion in a Plant Vitrification Solution (PVS) before cooling in LN, has been the most successful cryopreservation approach to many plant species globally. Vitrification-based techniques reduce the water content within the cells to avoid injury from cellular ice formation when cooled to low temperatures (-196°C) during immersion in LN. Vitrification thus reduces or prevents the cellular damaging processes associated with water freezing in and around the cells (Luyet, 1937).

In vitro-derived explants are commonly used for cryopreservation when seeds are unavailable or not viable (Pence 2014). However, the explant must be small enough (0.5 – 1 mm in length) to allow for rapid dehydration before cooling; moreover, an efficient micropropagation protocol should be available to generate explants for cryopreservation and to regenerate plants from the cryopreserved shoot apices. Shoot apices of in vitro-grown cultures are considered ideal for cryopreservation, as they are sufficiently small, are composed of organised meristematic cells (Engelmann, 2014; Wilms et al., 2020), and they are also already sterile, not having been exposed to the environment. The plantlets developed from these apices also have high genetic stability (Hajari et al., 2011). In vitro-derived shoot apices have been used for successfully cryopreserving geophytic perennial rhizomatous species (Yamuna et al., 2007; Preetha et al., 2021) . However, there are no reports of successful cryopreservation of *S. aethiopicus* to date. Therefore, this study aimed at developing a cryopreservation protocol using in vitro-derived shoot apices for the critically endangered, *S. aethiopicus*.

## 3.2 Plant material and methods

### 3.2.1 Plant material

Plant material of *S. aethiopicus* was collected from the eThekwini Municipal Medicinal Plant Nursery located at the Silverglen Nature Reserve (Durban, KwaZulu Natal, South Africa). The rhizomes were harvested in late winter (July 2020) and stored for ±98 d (September – December 2020) after harvest until dormancy was broken. Buds emerging from these rhizomes were used to initiate in vitro cultures.

### 3.2.2 In vitro culture establishment

The in vitro cultures were established and maintained on Murashige and Skoog (MS; Murashige and Skoog, 1962) basal salts and vitamins with a 0.09 M sucrose concentration (Huletts, South Africa), supplemented with 5 mg $l^{-1}$ 6-benzylaminopurine (BAP) (Sigma-Aldrich, United States) and 4 g $l^{-1}$ Gelrite® (Duchefa Biochemie, Netherlands) (multiplication medium), as per the micropropagation protocol developed in Chapter Two. These stock cultures were subcultured onto the same medium every 42 d and maintained under cool white, fluorescent light (Sylvania, China) at a photosynthetic photon flux density (PPFD) of 35 µmol $m^{-1}s^{-1}$, 16 hours (h) light at 25 ± 2°C/ and 8 h dark at 22 ± 2°C photoperiod.

All media used for this study were adjusted to pH 5.6 ± 0.2 using 1 M sodium hydroxide (NaOH) (Sigma-Aldrich, United States) or 1 M hydrochloric acid (HCl) (Sigma-Aldrich, United States) before autoclaving for 20 min at 120 °C and 121 KPa (Huxley, Taiwan). The media (10 ml) for all cryopreservation experiments were dispensed in sterile Petri dishes (Ø = 90 mm) (Carbi, South Africa). The Petri dishes with explants inserted into the medium were sealed with Parafilm® (Bemis Company Inc., United States) and maintained under similar conditions as stock cultures, unless otherwise stated.

### 3.2.3 Excision of shoot apices

Shoot apices (Fig. 3.8 a) were excised from developed axillary buds of stock cultures, using a binocular stereomicroscope (Leica, Germany). The leaves were removed, and the shoot apices comprising only a single shoot primordium (0.5 – 1 mm) were excised from the base of the shoots; these shoot apices were used for all experiments in this study.

### 3.2.4 Pretreatment of shoot apices

#### *3.2.4.1 Preculture*

Excised shoot apices were cultured on MS basal salt mixture with 0.3, 0.4 or 0.5 M sucrose concentrations and supplemented with 4 g $l^{-1}$ Gelrite® for 24, 48 or 72 h in the dark at 25 ± 2ºC and thereafter transferred to similar medium and culture conditions as the stock cultures. In an attempt to improve the preculture treatment, a sequential preculture treatment was tested, involving a 48 h preculture on 0.09 M sucrose medium under mother stock culture conditions, followed by the most effective preculture treatment identified in the first step, in terms of the highest percentage of explants producing new shoots and the highest number of buds per explant. For the control treatment, shoot apices were directly placed onto MS (Murashige and Skoog, 1962) basal salts and vitamins medium containing 0.09 M sucrose, 5 mg $l^{-1}$ BAP, and 4 g $l^{-1}$ Gelrite®. The gravimetric water content (WC) of the untreated shoot apices and shoot apices following the preculture treatments, the rate of multiplication per explant, the rate of necrotic multiplied buds per explant, and the percentage of explants surviving or showing necrosis were used to judge the best preculture treatment to optimise further steps of the sequential dehydration procedures.

#### *3.2.4.2 Cryoprotection*

The precultured shoot apices were cryoprotected for 10, 20 or 40 minutes (min) in a cryoprotection mixture (CPM). The CPM comprised MS basal salt mixture with a 0.09 M sucrose concentration, 5% (w:v) dimethylsulphoxide (DMSO) (Sigma-Aldrich, United States), and 5% (w:v) glycerol (MINEMA Chemicals, South Africa). The DMSO and glycerol were filtered using a microfilter (Ø = 25 mm and 0.2 µm) (Pall Cooperation, United States). The shoot apices treated with CPM were blotted dry on sterile filter paper (Whatman™ No. 1, Sigma-Aldrich, United States) and cultured on multiplication medium in Petri dishes under similar culture conditions as the stock cultures. Shoot apices from the CPM treatment with the highest percentage of explant survival and bud multiplication and the lowest bud necrosis was used for vitrification treatments.

#### *3.2.4.3 Vitrification*

The precultured and cryoprotected shoot apices were treated with a loading solution (LS) comprising MS basal salt mixture, 0.4 M sucrose and 2 M glycerol (filter sterilised) at pH 5.7 ± 0.2 for 5 or 10 min. The treated shoot apices were blotted dry on sterile filter paper and cultured on multiplication medium in Petri dishes under similar culture conditions as the stock cultures. The LS treatment showing the highest percentage of survival and regrowth of explants, was used in the succeeding steps.

Subsequent to the loading treatment, the shoot apices were treated with Plant Vitrification Solution (PVS) 2 (10 shoot apices in 1 ml PVS2) for 10 min. The PVS2 solution comprised MS basal salt mixture and a 0.4 M sucrose concentration supplemented with, 30% glycerol (w:v), 15% ethylene glycol (w:v) and 15% DMSO (w:v) at pH 5.7 ± 0.2 (DMSO, ethylene glycol and glycerol were filter sterilised into the autoclaved MS basal salt + sucrose mixture).

### 3.2.5 Cooling of shoot apices

The pretreated shoot apices were subjected to three cooling methods – rapid cooling by direct plunging in LN, vitrification, and droplet-vitrification both prior to immersion in LN. For rapid cooling, the precultured and cryoprotected shoot apices were transferred into polypropylene cryovials (Sigma-Aldrich, United States) (10 per cryovial) which were mounted on aluminium cryocanes (Sigma-Aldrich, United States) and plunged into a Dewar (Schorn Cryogenics, South Africa) containing LN. For vitrification, ten shoot apices treated with PVS2 were suspended in 1 ml fresh PVS2 per cryovial. The cryovials were then mounted onto cryocanes before plunging into LN. For droplet-vitrification, ten shoot apices were suspended individually in droplets (2 µl) of fresh PVS2 on a 20 x 10 mm aluminium strip; the foil strips carrying the droplet-vitrified shoots were cooled in LN before transferring to individual cryovials (Sigma-Aldrich, United States) mounted on cryocanes and plunged into LN. In all three methods, the shoot apices were stored in LN for 24 h prior to thawing, rehydration and recovery.

### 3.2.6 Thawing, rehydration and recovery

Thawing, unloading and recovery were carried out as described by Varghese et al. (2009). After 24 h, the cryovials containing the shoot apices that were directly plunged into LN were removed from LN and rewarmed in a 40°C water bath for 1 min, rehydrated and transferred to a recovery medium under partial light conditions. Partial light was provided by switching off the room light and reducing the light in the laminar flow bench to the lowest light intensity. The cryovials with vitrified shoot apices were rewarmed in a water bath at 40°C for 1 min. Subsequently, the vitrification solution from the cryovials was drained and replaced twice with a 2 ml unloading (rehydrating) solution comprising MS basal salt medium with a 1.2 M sucrose concentration at 10 min intervals. Droplet-vitrified shoot apices (in 2 µL PVS2 droplets) were transferred from cryovials to the 2 ml unloading solution warmed to 40°C for 10 min. This step was repeated with a fresh solution for an additional 10 min. The rewarmed and rehydrated shoot apices were placed on sterile filter paper suspended on MS basal salt mixture with vitamins, 0.09 M sucrose, 5 mg $l^{-1}$ BAP and 4 g $l^{-1}$ Gelrite® in Petri dishes (Ø = 90mm) and incubated in the dark overnight (24 h). For dark incubation, all Petri dishes with the thawed and rehydrated cryopreserved explants were wrapped in aluminium foil. The rehydrated shoot apices that remained green were removed from the filter paper and transferred to fresh medium (Petri dishes) and incubated in the dark for a further seven days (until shoot apices remained green) then in partial light conditions for another seven days. Partial light conditions were provided by wrapping the Petri dishes in a layer of white tissue paper. The shoot apices were transferred from partial light conditions to the same photoperiod as the stock cultures and screened regularly for regrowth and necrosis. The regeneration percentage was recorded as the percentage of the meristems producing normal green shoots upon transfer to the regeneration medium 90 d after retrieval from LN.

### 3.2.7 Regrowth of cryopreserved explants

The regenerated shoots from the cryopreservation method that had the highest percentage of survival and shoot development were transferred to an elongation medium containing full MS basal salts and vitamins, 0.09 M sucrose, 0.1 mg $l^{-1}$ BAP and 4 g $l^{-1}$ Gelrite® (28 d) and then rooted in vitro on full MS basal salts and vitamins, 0.09 M sucrose, 0.1 mg.$l^{-1}$ BAP, 1 mg $l^{-1}$ indole-3-acetic acid (IAA) and 4 g $l^{-1}$ Gelrite® (28 d) (refer to Chapter Two, Material and methods, elongation and in vitro rooting). The individual recovered explants were transferred to the respective medium (10 ml) prepared in glass culture tubes (100 mm x 25 mm, 40 ml) (Sigma-Aldrich, United States). The tubes were capped with polypropylene culture tube closures (Sigma-Aldrich, United States) and sealed with Parafilm®.

### 3.2.8 Gravimetric water content

Five shoot apices were placed in individual aluminium foil boats (Ø = 5 mm) to record the gravimetric WC using a six-decimal place balance (Sartorius, Germany). This was determined immediately after excision, preculture and after each stage of the dehydration treatments. All treated shoot apices were blotted dry on filter paper before measuring their fresh weights (g). The shoot apices were oven-dried at 80°C for 48 h, and the final WC was expressed on a dry mass basis (g $H_2O$ $g^{-1}$ dry weight [DW]).

### 3.2.9 Photography and ultrastructural analysis

Photographs of the cultures were captured with a Canon IXUS 190 digital camera (Canon, Japan), fitted with a 24 mm wide lens, 20.0 megapixels, or with the Leica application suite version 3.4.0 software (Leica Microsystems, Germany) supporting the digital camera for the Leica stereomicroscope (Leica, Germany).

For ultrastructural studies, ten shoot apices from each of the best preculture, cryoprotection, loading and vitrification treatments were fixed in a solution of 2.5% glutaraldehyde (v:v) (Agar Scientific, United Kingdom) in 0.1 M Sorensen phosphate (Agar Scientific, United Kingdom) buffer (pH 7.2) and stored at 4 °C until further processing. The fixed shoot apices were washed three times (5 min each) with phosphate buffer (0.1 M, pH 7.2). The apices were post-fixed in 0.5% osmium tetroxide (Agar Scientific, United Kingdom) for 1 h and washed again three times (5 min each) with phosphate buffer. The post-fixed apices were dehydrated in a graded acetone (Agar Scientific, United Kingdom) series (30, 50, 75, and 100%) and infiltrated with a low viscosity epoxy resin (1:1 mixture of resin [Agar Scientific, United Kingdom] and 100% acetone [Agar Scientific, United Kingdom]) for 4 h and then polymerised in fresh resin at 70 °C for 8 h. Ultrathin sections (100 mm) of shoot apices were made with a UC7 microtome (Leica, Germany), using glass knives (Agar Scientific, United Kingdom) and picked with uncoated copper grids (200 $\mu m^3$ mesh) (Agar Scientific, United Kingdom). Grids with resin embedded sections were stained with aqueous uranyl acetate (Agar Scientific, United Kingdom) and lead citrate (Agar Scientific, United Kingdom) and then examined with a JOEL 2100 HRTEM (JOEL, Japan) at an accelerating voltage of 200 kV. Ultrastructural images were captured using the Gatan Digital Micrograph Imaging Suite Version 2 (AMATEK, United Kingdom), and qualitative analyses of the images were carried out.

### 3.2.10 Statistical analyses

The experiments were conducted in a randomised block design. Ten axillary shoot apices (replicated three times) were used for each successive step of the pretreatment, cryoprotection, loading, vitrification and cooling experiments. The percentage of shoot apices surviving, necrotic apices, and number of axillary apices produced per explant or number of necrotic apices per explant with regrowth or complete necrosis on MS basal salt medium with 5 mg $l^{-1}$ BAP and 4 g $l^{-1}$ Gelrite® were recorded after preculture, each step of the dehydration procedures and after thawing, rehydration and recovery after cooling for each replicate treatment. Statistical analyses of data were performed using the Statistical Package for the Social Sciences

(SPSS®) Version 29 software package for Windows (IBM, United States). All values were presented as mean ± standard error. One-way analysis of variance (ANOVA) was used to test for differences in the different dehydration stages. Means and percentage means for all treatments were further separated using the Tukey's post hoc Test and tested for normality using Shapiro-Wilk's test. Where data did not meet the assumptions for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test), a $\log_{10}$ transformation was applied, and a Kruskal-Wallis H test was used and when two treatments were compared, the Student T-test was used. All differences were considered significant at the 0.05 significance level.

## 3.3 Results

### 3.3.1 Pretreatment of shoot apices

*3.3.1.1 Sucrose preculture*

Preculture of shoot apices on media with the different sucrose concentrations (0.3, 0.4 or 0.5 M) for 24, 48 or 72 h significantly influenced the number of buds produced per explant (Fig 3.1) and the percentage of necrotic explants and explants that produced buds (Fig 3.2) 42 d after applying the different preculture treatments. Control explants and the explants precultured on 0.3 M sucrose (24 h) produced 2 ± 0.2 buds, 0.3 M (72 h) precultured explants produced 3 ± 0.4 buds per explant (Fig. 3.1). Only one bud was produced in the rest of the treatments.

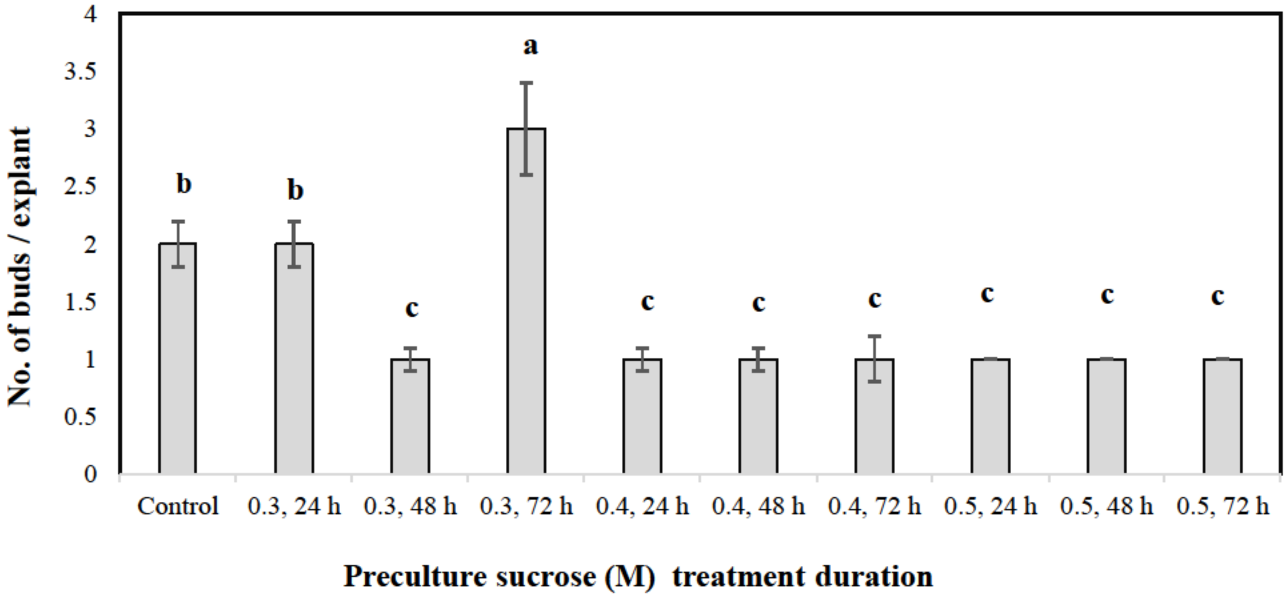


**Figure 3.1:** Effects of different sucrose preculture concentrations and exposure durations in the dark on the number of buds produced per explant. Columns with different alphabets denote significant differences in the number of multiplied buds/explant. The error bars represent standard error (Tukey's post hoc Test, $p \leq 0.05, n = 10$).

The percentage of necrotic explants (Fig. 3.8 b) was significantly lower in the control (8.33 ± 4.77 %) and explants precultured on 0.3 M sucrose for 24 h (16.67 ± 7.60 %) (Fig. 3.2) compared with the higher percentage of explants lost to necrosis for the rest of the preculture treatments (56.67 -100 %) (Fig. 3.2). The percentages of explants that produced buds in the control for which no preculture treatment was applied, and in the 0.3 M (24 h) sucrose preculture treatment, were significantly higher with 53.33 ± 9.54 % and 23.33 ± 7.60 %, respectively, than any of the 0.5 M sucrose preculture treatments (Fig. 3.2). All the shoot apices precultured on media with 0.5 M sucrose did not produce shoots (Fig. 3.2; Fig. 3.8 b). The lower sucrose preculture treatments (0.3 M) and the 0.4 M (48 h) had similar percentages of explants with multiplication to the control (Fig. 3.2).

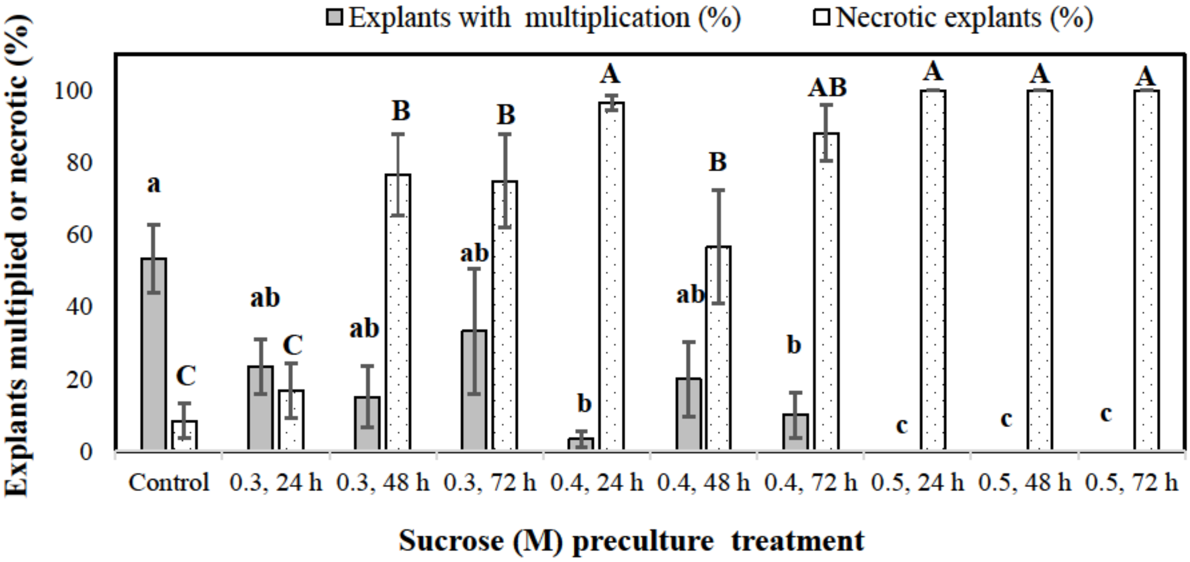


**Figure 3.2**: Effect of sucrose preculture concentrations and dark exposure durations on percentage explants with bud multiplication and necrosis. Columns with different alphabets denote significant differences (uppercase alphabets for percentage necrotic explants and lowercase alphabets for percentage explants with bud multiplication). The error bars represent standard error (Kruskal-Wallis H Test, *p ≤ 0.05, n = 10*).

Bud multiplication rates after a 0.3 M sucrose preculture (24 h, dark) following a 0.09 M sucrose preculture (48 h) (2 ± 0.1) was not different to the number of buds produced per untreated control explant (2 ± 0.1), nor to the number of buds produced per 0.3 M (24 h, dark) precultured explant (2 ± 0.1) (Fig. 3.3). However, the 0.3 M sucrose preculture (24 h, dark) following a 48 h 0.09 M sucrose preculture significantly increased the % explants producing buds (50 ± 0.6 %) in comparison to applying the 0.3 M sucrose (24 h, dark) preculture treatment alone (23 ± 0.7 %) (Fig. 3.4). The % explants producing buds for the sequential preculture treatment was also not different to the control explants (53 ± 0.8 %) or to the 0.09 M sucrose preculture (48 h) treatment (53 ± 0.9 %) (Fig. 3.4). There was also no difference in the % necrosis across all the treatments and the control (8 – 20 %) (Fig. 3.4).

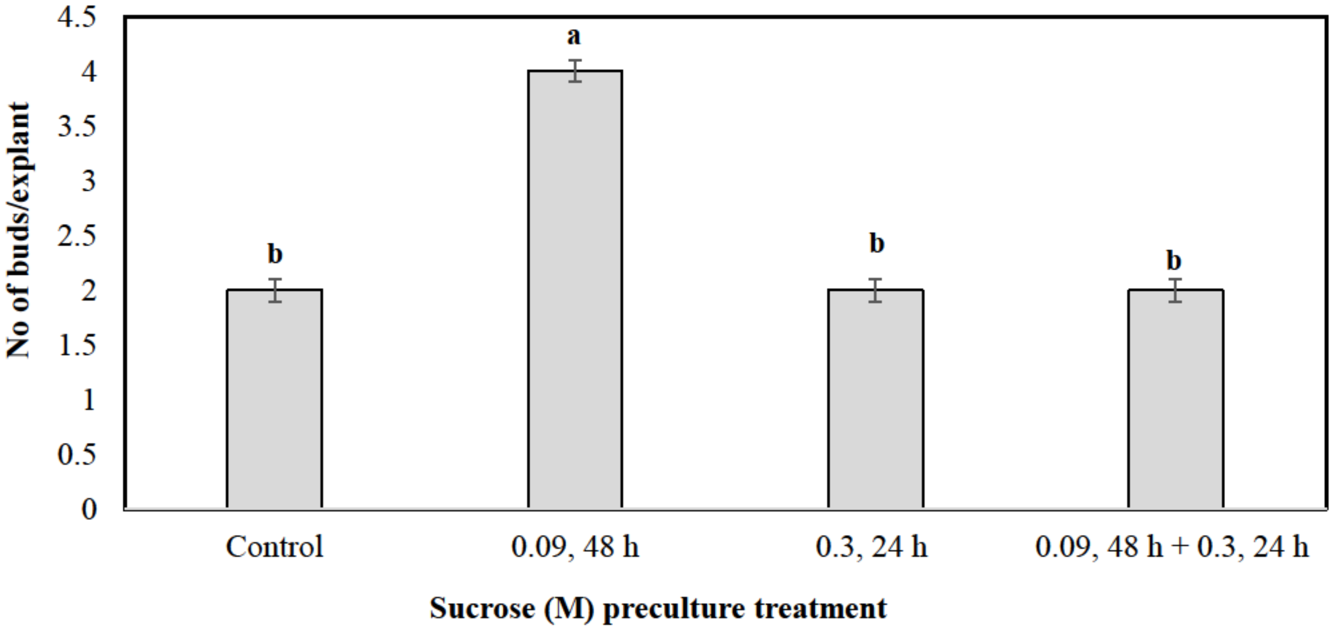


**Figure 3.3:** Effects of sequential preculture with two sucrose concentrations and exposure duration on the number of buds produced per explant. The 24 h treatments were performed in the dark. Columns with different alphabets denote significant differences in the number of multiplied buds/explant. The error bars represent standard error (Tukey's post hoc Test, $p \leq 0.05, n = 10$).

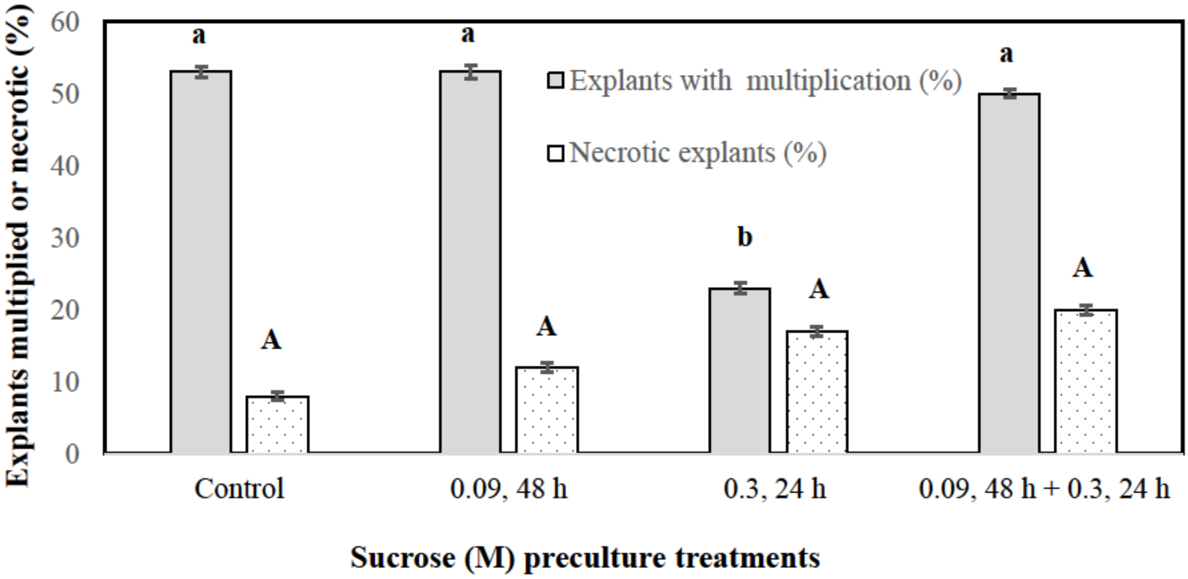


**Figure 3.4:** Effects of sequential preculture with two sucrose concentrations and exposure durations on the percentage of explants with multiplication or necrosis. The 24 h treatments were performed in the dark. Columns with different alphabets denote significant differences (uppercase alphabets for percentage necrotic explants and lowercase alphabets for percentage explants with bud multiplication). The error bars represent standard error (Kruskal-Wallis H Test, $p \leq 0.05, n = 10$).

In the thin, stained sections of shoot apices of the control, dense meristematic regions were apparent with prominent nuclei (Fig. 3.9 a); similar to this were the sections of shoot apices cultured on media with 0.09 M (48 h) followed by 0.3 M (24 h, in dark) sucrose (Fig 3.9 b). However, at an ultrastructural level, cellular integrity of the meristematic region was better in the control (Fig. 3.9 f - h) compared with those from the other sucrose preculture treatments (Fig. 3.9 i - j). The meristematic cells from the control shoot apices showed distinctive cell structure and integrity (Fig. 3.9 f), with developed and defined outer and inner membranes of the nuclear envelope, intact cell wall and cell membrane (Fig. 3.9 g), with distinct thylakoid stacking in the chloroplasts and developing mitochondrion (Fig. 3.9 h). However, meristematic cells from the sucrose precultured explants were shaped irregularly, had numerous plastids, chloroplasts with large starch bodies, and irregularly shaped vacuoles with cytoplasmic invaginations and extracellular water (Fig. 3.9 i) accompanied by withdrawal of the cell membrane from the intact cell wall (Fig. 3.9 j).

### *3.3.1.2 Effect of the cryoprotective mixture (CPM)*

There was no significant difference in the number of buds produced per explant for CPM treatments administered for 10 or 40 min (Fig. 3.5). However, all tested CPM treatment durations (10, 20 or 40 min) resulted in a similar % of explants producing buds (Fig. 3.6) but there were significantly more necrotic explants (72 ± 10 %) when the CPM treatment was administered for 40 min, as opposed to 10 min (38 ± 5 %) and 20 min (52 ± 6 %) (Fig. 3.6).

Sections of the meristematic region from shoot apices cryoprotected for 10 min immediately after CPM treatment exhibited well-defined meristematic cells with distinct nuclear staining and subcellular features characteristic of active growth. These features included irregularly shaped cells with defined nuclei surrounded by a nuclear envelope (Fig. 3.9 c) and vacuoles with cytoplasmic invagination (Fig 3.9 k). Additionally, plastid formation and withdrawal of the cell membrane from the cell wall were observed after the CPM (10 min) treatment (Fig 3.9 l).

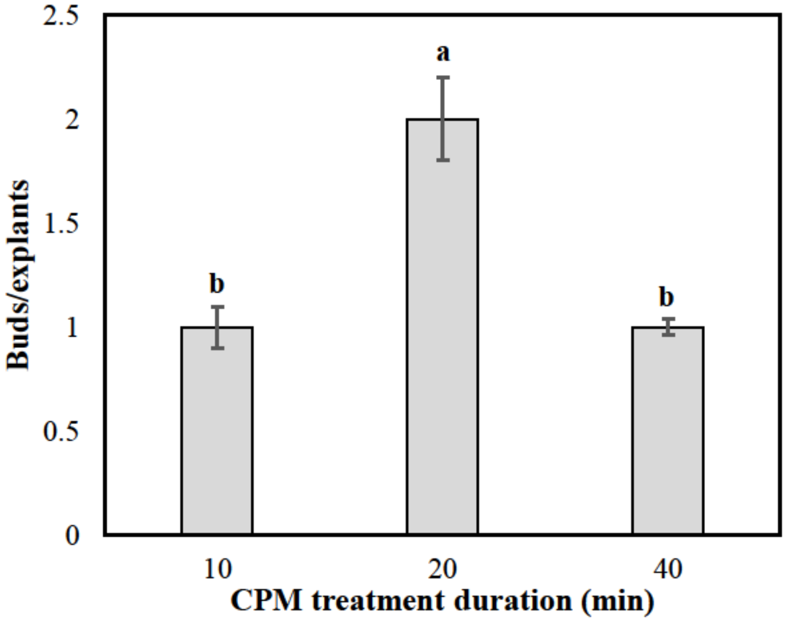


**Figure 3.5:** Effect of CPM treatment durations on the number of buds produced per explant. Columns with different alphabets denote significant differences in the number of multiplied buds/explant. The error bars represent standard error (Tukey's post hoc Test, $p \leq 0.05$, $n = 10$). CPM = Cryoprotection Mixture.

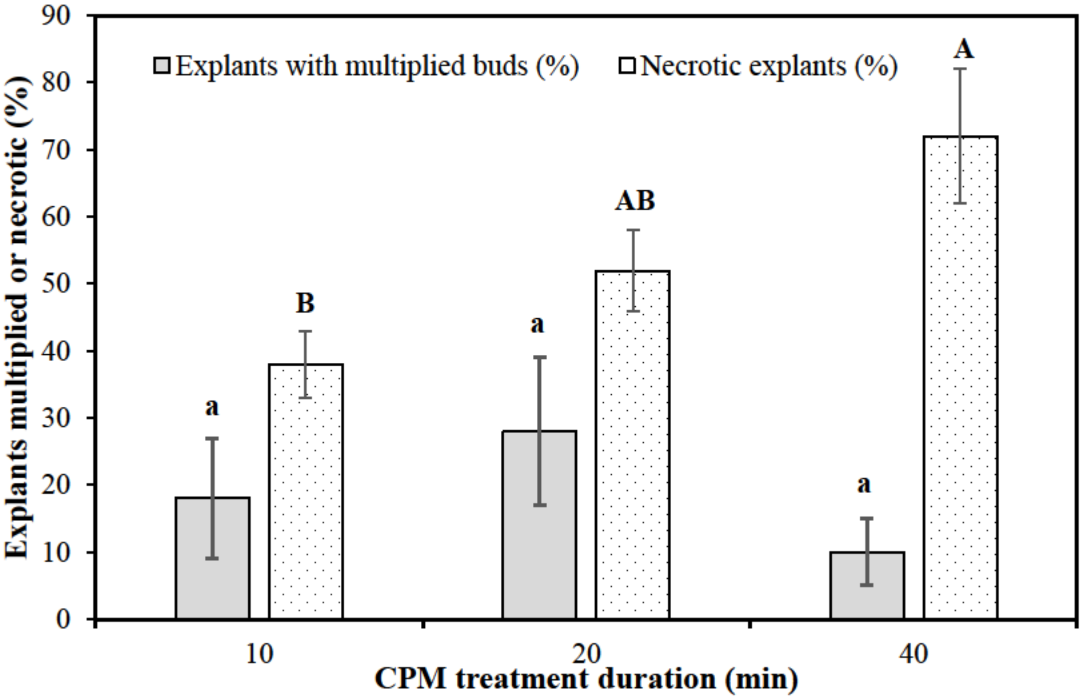


**Figure 3.6:** Effect of CPM durations on percentage explants with bud multiplication and necrosis. Columns with different alphabets denote significant differences (uppercase alphabets for percentage necrotic explants and lowercase alphabets for percentage explants with bud multiplication). The error bars represent standard error (Kruskal-Wallis H Test, $p \leq 0.05$, $n = 10$). CPM = Cryoprotection Mixture.

### *3.3.1.3 Effect of loading solution*

The initial development by the greening and swelling of the LS-treated explants was observed from 50 d after treatment (Fig. 3.8 d), 8 d later than explant development and bud multiplication were first observed in the precultured explants or in the CPM-treated explants (42 d). Further explant elongation and development of new leaves were observed in the shoot apices treated with LS (5 or 10 min) only after another 40 d of culture (Figure 3.8 h). The percentage of explants with regrowth was significantly higher for the 5 min LS treatment (68.33 ± 6.54%) compared to the explants treated for 10 min with LS (35.00 ± 4.28%) (Fig. 3.7).

The meristematic region of the shoot apices treated with LS (5 min) was dense, with some dead cells present in the outer region (Fig 3.9 d). At the ultrastructural level, plastid development and no segregation of the nucleus from the nuclear envelope or from the rest of the cytoplasmic matrix were some of the features observed in the cells from the dense meristematic region of shoot apices treated in LS for 5 min (Fig. 3.9 m).

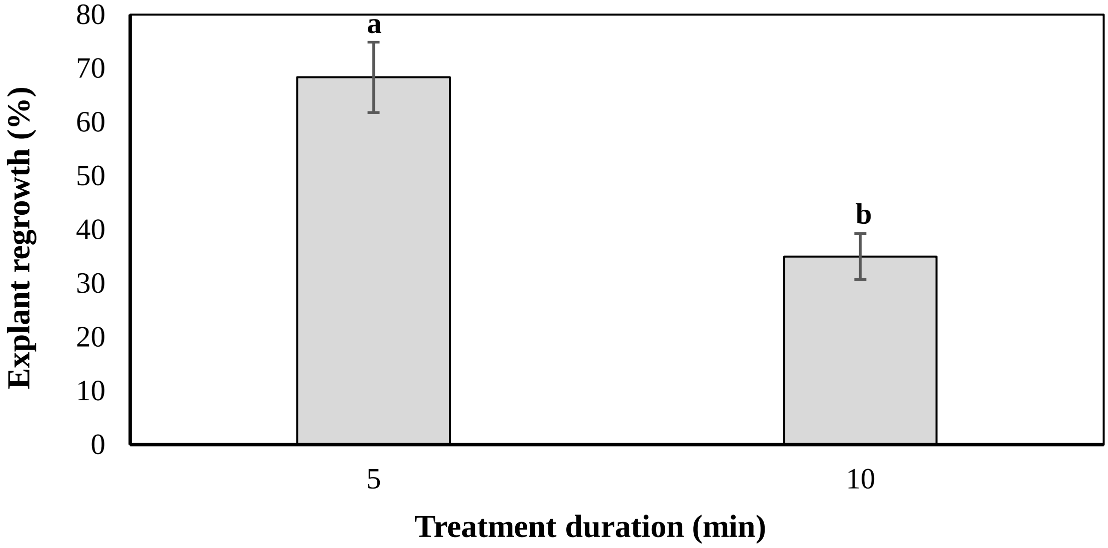


**Figure 3.7:** Effect of LS treatment durations on regrowth of cryoprotected *S. aethiopicus* shoot apices. Columns with different alphabets denote significant differences in the percentage of shoot apices with regrowth. The error bar represents the standard error (Student T Test, $p \leq 0.05$, $n = 10$). LS = Loading Solution.

*3.3.1.4 Effect of Plant Vitrification Solution 2 (PVS2)*

The survival and regrowth of the PVS2 (10 min)-treated explants (58.33 ± 0.07%) (Fig. 3.8 d) and necrosis (41.67 ± 3.07 %) were recorded after 90 d of culture. Similar to the LS (5 min) treatment, swelling of the PVS2-treated explants was observed at ±50 d from initial treatment, accompanied by further elongation and development of shoots ±40 d later. At the microscopic level, selective cell death of the outer cell layers in the shoot apex was observed and only a few underlying meristematic cells maintained their integrity and exhibited visibly stained nuclei (Fig. 3.9 e). However, control shoot apices displayed no such cell death or nuclear staining (Fig. 3.9 a). An ultrastructural overview of the same meristematic cells from this region of the PVS2 (10 min)-treated shoot apices showed the withdrawal of the cell membrane from the cell wall and irregular cell shape accompanied by extensive cytoplasmic invagination in the vacuoles (Fig. 3.9 n); additionally, lipid bodies and plastids were also observed in those cells (Fig 3.9 o).

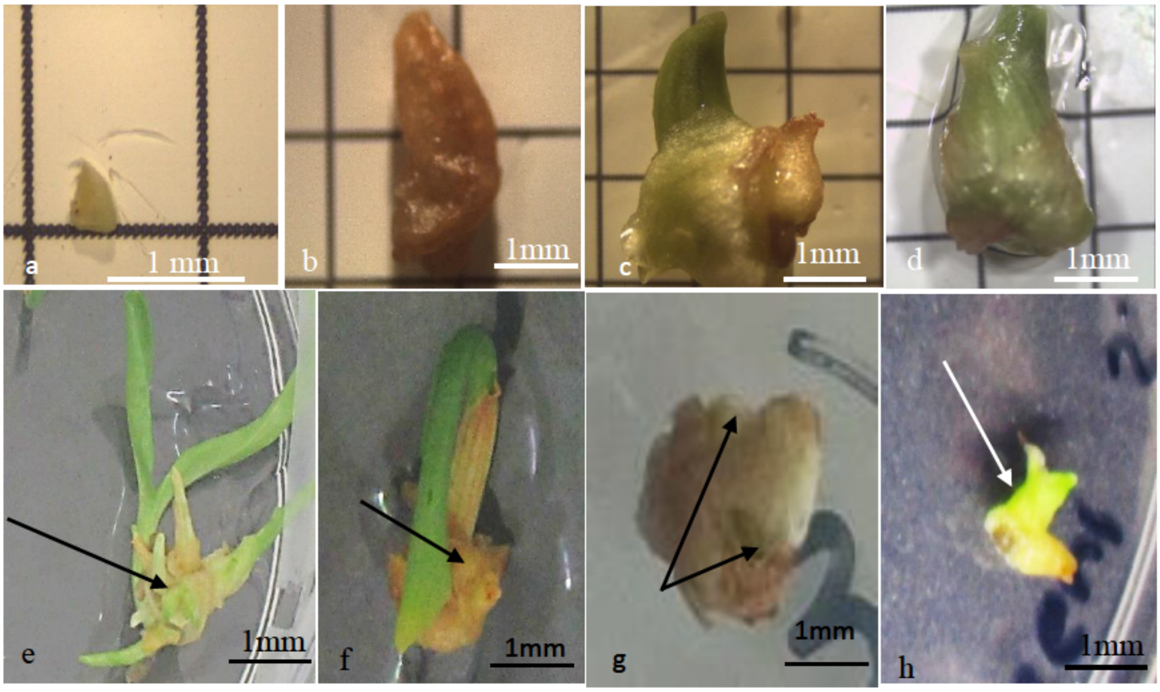


**Figure 3.8:** The effect of sequential dehydration on the development of in vitro-derived shoot apices. First row – the morphology of a representative explant (axillary shoot apex) selected for all cryopreservation studies (a); necrotic explant from 0.5 M preculture treatments (b); surviving explants with new axillary buds (c); or explants that regrew only (d). Second row – control explants with visible elongation of the excised shoot apex and multiplication (42 d) (indicated by an arrow) (e); explant precultured on 0.09 M (48 h) and 0.3 M sucrose medium (24 h dark) with multiplied axillary shoot buds (indicated by arrow (f); CPM (10 min)-treated explants with visible multiplication of axillary shoot buds indicated by arrows (g); and LS (5 min)-treated explants with regrowth and elongation of the shoot apex indicated by an arrow (90 d) (h).

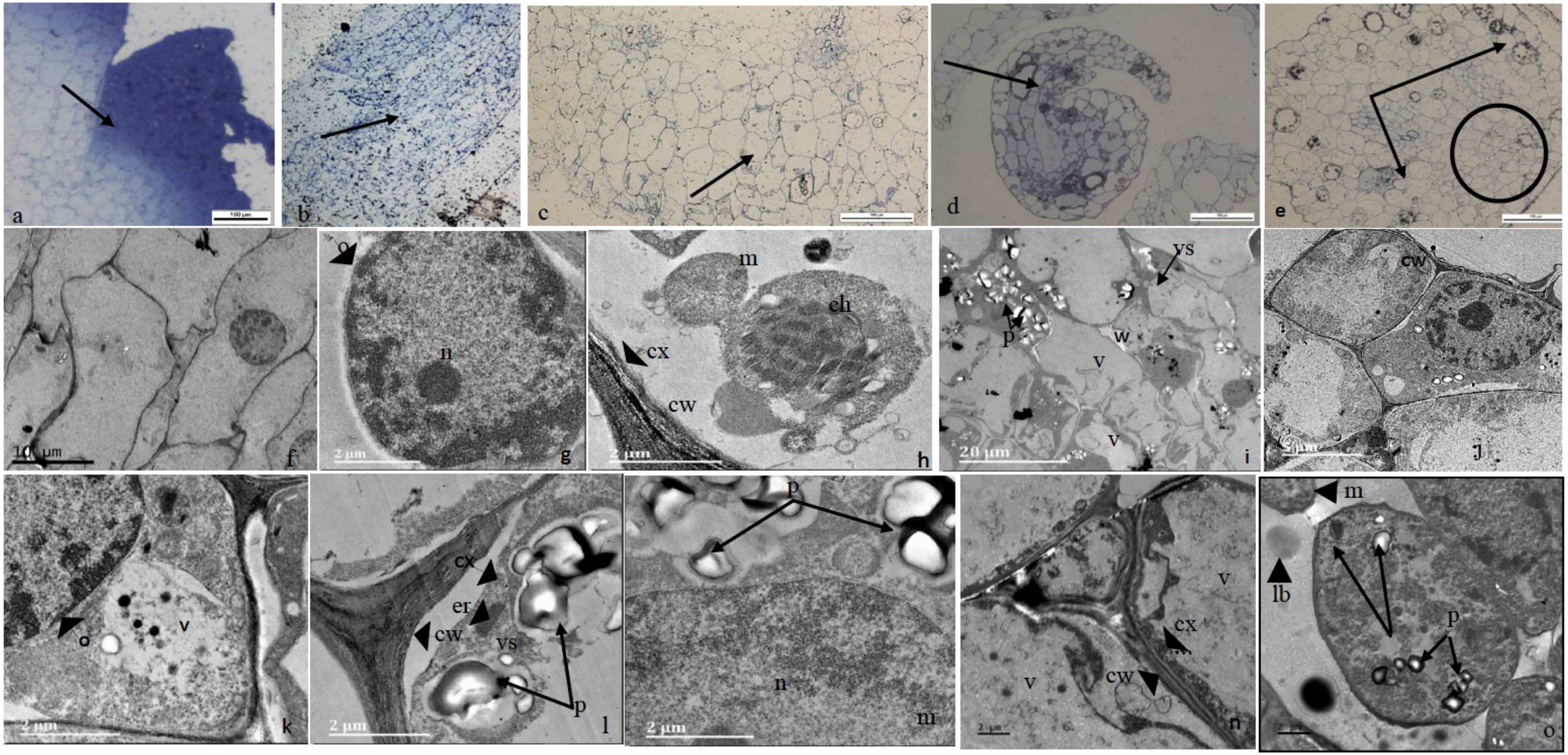
#### *3.3.1.5 An evaluation of the WC of explants after sequential dehydration*

Shoot apices in the control had the most water (28.04 ± 9.76 g $H_2O$ $g^{-1}$ DW) (Table 3.1). Significant reductions in water occurred in all the dehydration treatments and ranged from 0.92 ± 0.36 to 11.81 ± 1.80 g $H_2O$ $g^{-1}$ DW in the order of increasing exposure to solutes.

**Table 3.1:** A comparison of gravimetric water content (WC) for in vitro *S. aethiopicus* shoot apices after each dehydration step.

| **Dehydration procedure** | **WC (g $H_2O$ $g^{-1}$dry weight)** |
|---|---|
| Control | $28.04 \pm 9.77^{a}$ |
| 0.09 M sucrose medium (48 h) | $11.81 \pm 1.80^{b}$ |
| 0.09 M sucrose medium (48 h) + 0.3 M sucrose medium (24 h dark) | $10.44 \pm 2.77^{b}$ |
| 0.09 M sucrose medium (48 h) + 0.3 M sucrose medium (24 dark) + CPM (10 min) | $3.35 \pm 0.52^{bc}$ |
| 0.09 M sucrose medium (48 h) + 0.3 M sucrose medium (24 dark) + CPM (10 min) + LS (5 min) | $1.85 \pm 0.44^{bc}$ |
| 0.09 M sucrose medium (48 h) + 0.3 M sucrose medium (24 dark) + CPM (10 min) + LS (5 min) + PVS2 (10 min) | $0.92 \pm 0.36^{c}$ |

Means (± SE) with different alphabets within columns are significantly different (Tukey's post hoc Test, $p \leq 0.05, n = 5$).


a
b
c
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cw
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**Figure 3.9:** The effect of sequential dehydration on cell ultrastructure of shoot apices. Top row – longitudinal sections of shoot apex in the control with an arrow showing the meristematic region especially the nucleus (a); sucrose-precultured shoot apex showing the meristematic region (arrow) (b); CPM (10 min)-treated shoot apex with the meristematic cells (arrow) (c); LS (5 min)-treated shoot apex showing the meristematic region (arrow) (d); PVS2 (10 min)-treated shoot apex showing distinct cell death (arrow) and dense central meristematic region (circled) (e). Middle row (ultrastructure) – meristematic cells from a shoot apex in the control showing cell structure (f); nucleus with intact outer and inner membranes (g); chloroplast, cell wall, cell membrane and mitochondrion (h); ultrastructural overview of the sucrose-precultured shoot apex (i-j). Bottom row (ultrastructure) – overview of meristematic cells from a CPM (10 min)-treated shoot apex (k); a further magnified view of the same region from k (l); overview of meristematic cells from a LS (5 min)-treated shoot apex (m); overview of meristematic cells from a PVS2 (10 min)-treatment (n-o). CPM = cryoprotection mixture, ch = chloroplast, cm = cell membrane, cw = cell wall, cx = cytoplasmic matrix, lb = lipid body, m = mitochondrion, n = nucleus, o = nuclear envelope, p = plastid, PVS = Plant Vitrification Solution, th = thylakoid stacking, v = vacuole, w = water.

### 3.3.2 The effect of cooling on recovery and regrowth

Ninety days after retrieval from LN, thawing, rehydration and recovery, the regrowth of cryostored shoot apices that were droplet-vitrified was significantly higher (66.67 ± 16.67%) than shoot apices rapidly cooled in LN (13.33 ± 8.82%) or vitrified shoot apices (0%). (Fig. 3.10). The droplet-vitrified shoot apices remained green upon transfer to the recovery medium for 7 d under dark conditions and then upon transfer for a further 7 d in partial light conditions (Fig. 3.11 a). Vitrified shoot apices cooled and stored in LN remained green directly after rewarming, rehydration, dark recovery and partial light conditions. However, when transferred to normal light conditions, these green apices stopped growing and turned brown and necrotic within 90 d (Fig 3.11 f).

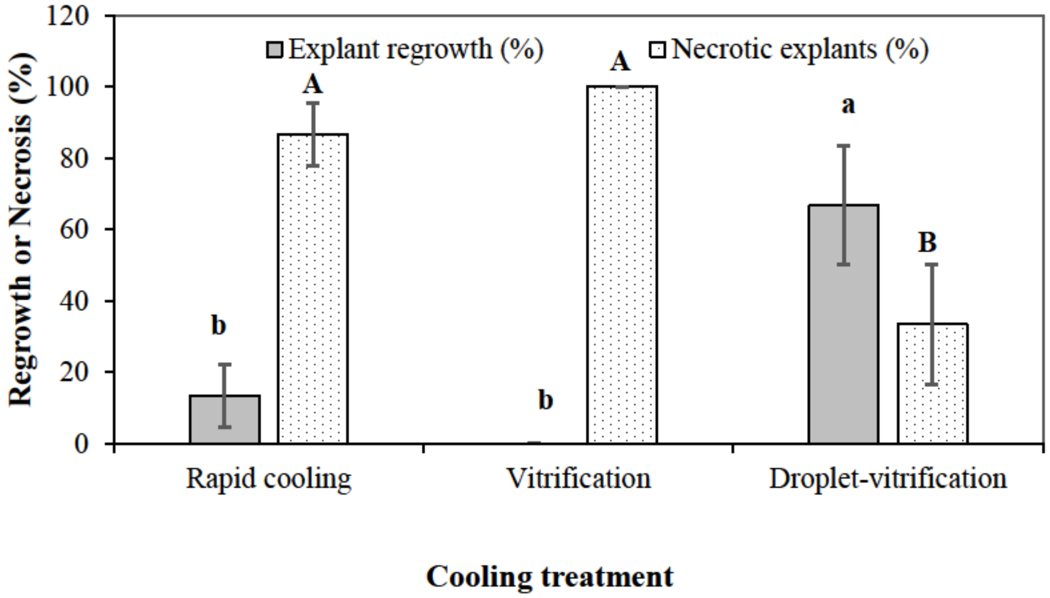

**Figure 3.10:** Effect of cryopreservation methods on *S. aethiopicus* shoot apices. Different alphabets denote significant differences in the percentage of regrowth (lowercase) and necrotic (uppercase) explants. The error bars represent standard error (Kruskal-Wallis H Test, $p \leq 0.05$, n = 10).

After 42 d following transfer from partial light conditions, browning of the droplet-vitrified shoot apices was observed (Fig. 3.11c); however, 18 d later, swelling of the base of the shoot apices and separation of the thin, brown layer and the emergence of a shoot tip (Fig. 3.11 d) were observed. The regrowth of rapidly cooled shoot apices was initially with callus formation at the base of the apex, followed by elongation of the tip (Fig. 3.11 e); no further elongation or development of the shoot apices (Fig 3.11 f) were seen in this treatment 90 d after retrieval from LN. The shoot apices following rapid cooling in LN (90 d recovery) elongated only, with no further development observed (Fig. 3.11 g).

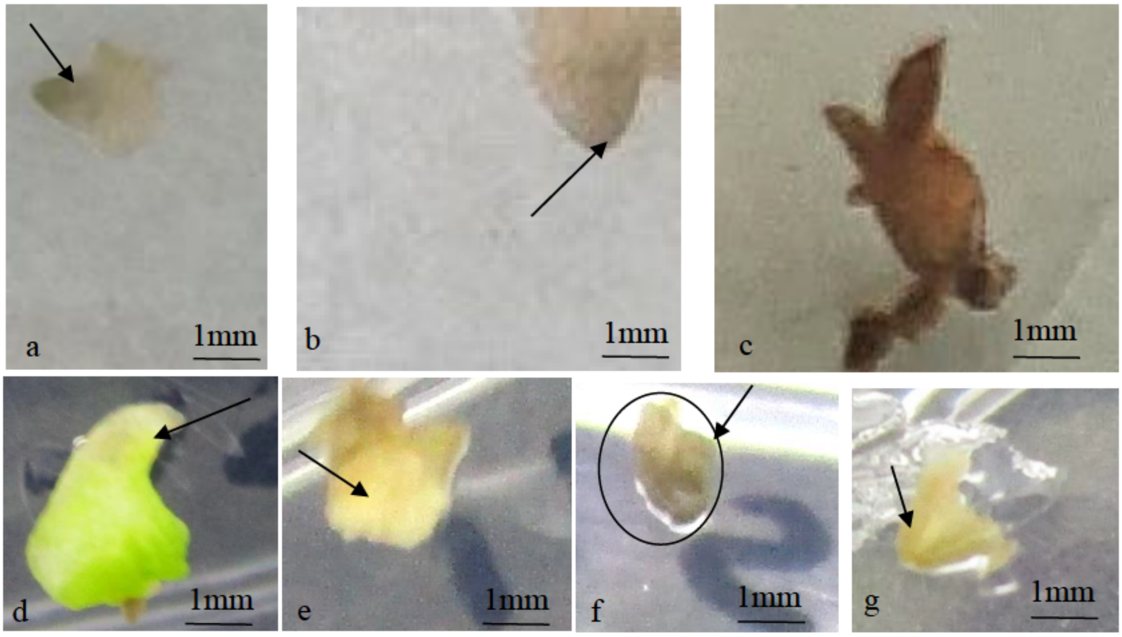

**Figure 3.11:** Shoot apices retrieved from LN, following thawing, rehydration and culture on regeneration medium. Top row – droplet-vitrified shoot apex following direct plunging with arrow indicating green meristematic cells (a), necrotic shoot apex following rapid cooling (b); necrotic shoot apex following vitrification (c). Bottom row – regrowth (arrow) in droplet-vitrified shoot apex (90 d) (d); shoot apex following rapid cooling (50 d recovery) with callus development (arrow) at the base and elongation of the shoot tip (e); necrotic vitrified shoot apex cooled in LN (90 d recovery) (f); shoot apex following rapid cooling (90 d recovery) with elongation of the shoot tip with no further development (g).

### 3.3.3 Regeneration

The droplet-vitrified shoot apices regenerated into single shoots (66.67 ± 16.67 %) upon transfer to a recovery medium after 90 d of culture; 33.33 ± 16.67 % of the droplet-vitrified apices showed necrosis. The shoot apices without any treatment (control), transferred directly onto the regeneration medium showed regeneration (produced 2 more shoots per explant) 42 d after culture (Fig. 3.1) and had a lower rate of explant loss to necrosis (8.33 %) (Fig. 3.2). Thereafter, explants that developed shoots, were transferred to an elongation medium (28 d) followed by in vitro rooting medium (28 d), to generate plantlets. Plantlets from droplet-vitrified cryo-recovered shoot apices developed a single shoot and root (Fig. 3.12 a); however, plantlets regenerated from the control explants were more robust with regard to their shoot and root development (Fig. 3.12 b). The cryopreservation protocol developed for the shoot apices is represented in Fig. 3.13.

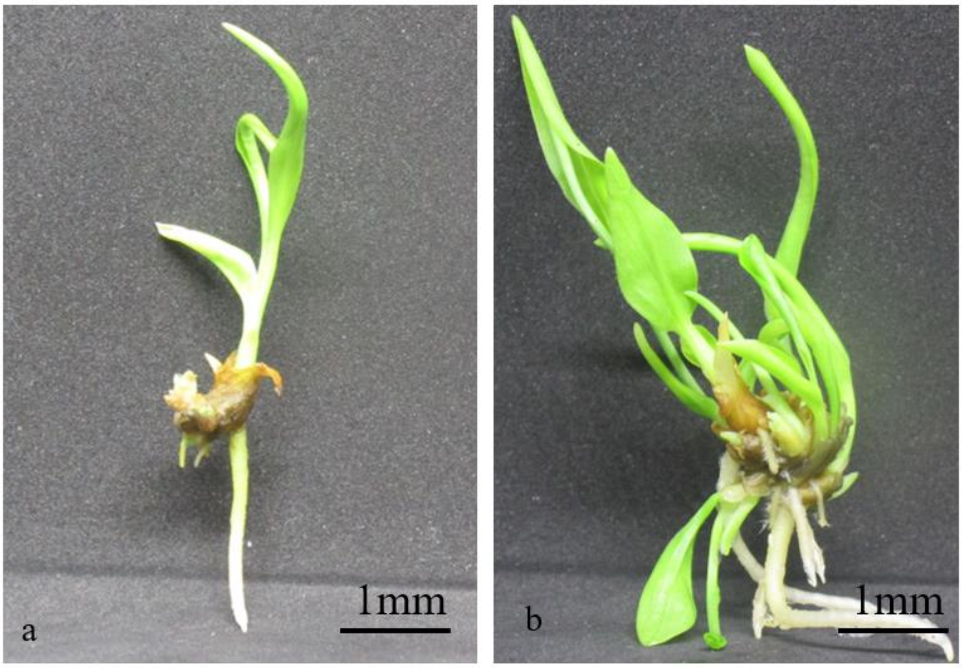

**Figure 3.12:** Plantlet regenerated from droplet-vitrified cryopreserved explants (a); plantlet regenerated in the control (b).

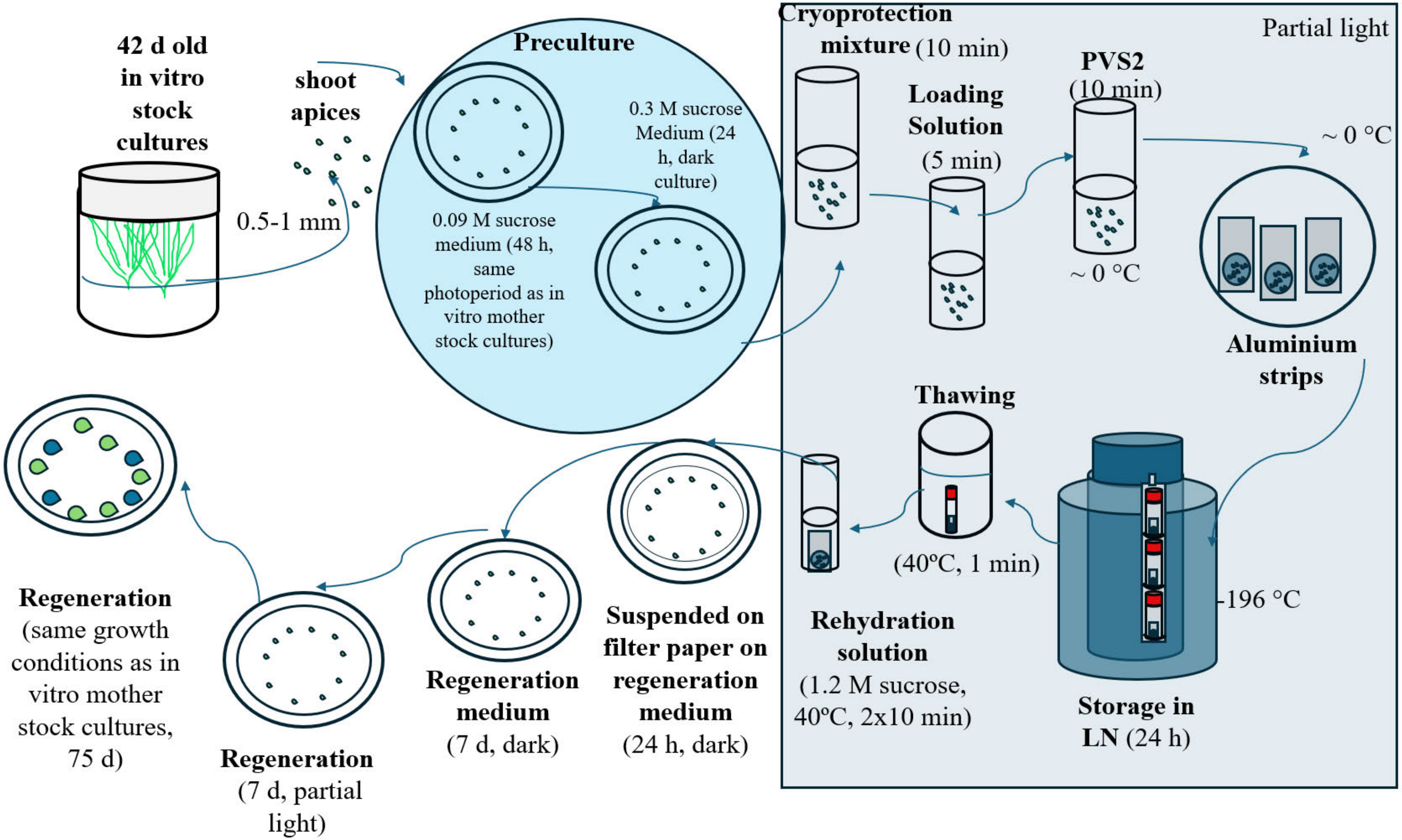

**Figure 3.13:** Stepwise cryopreservation process for *S. aethiopicus* using the droplet-vitrification method and PVS2 as the vitrification solution. PVS = Plant Vitrification Solution.

## 3.4 Discussion

This study presents the first insight into long-term conservation of the critically endangered species, *S. aethiopicus*, through droplet-vitrification-based cryopreservation. This technique has been used in long-term germplasm conservation of a variety of unrelated species (Hajari et al., 2011; Kalaiselvi et al., 2017; Kundu et al., 2018) and specifically for storing in vitro-derived explants of many geophytes (Pawlowska and Szewczyk-Taranek, 2014; Wilms et al., 2020; Yamuna et al., 2007). The key steps that need to be considered when developing such a protocol as discussed by Engelmann (2014) include: preculture of the explants on a medium containing high concentrations of sugars or sugar alcohols to improve tolerance to dehydration and subsequent freezing in LN, cryoprotection to avoid cryoinjury associated with freezing in LN, osmoprotection with a loading solution prior to PVS2 treatment, followed by rapid cooling. As such, this study investigated the preculture of shoot apices using sucrose, cryoprotection in MS salts supplemented with DMSO and glycerol, and osmoprotection in MS salts containing sucrose and glycerol prior to direct plunging in LN (rapid cooling) or vitrifying in PVS2 or PVS2 using the droplet-vitrification approach. All preparative steps were performed on in vitro-derived shoot apices which were then subjected to cryopreservation experiments.

Shoot apices consist of actively dividing homogenous groups of meristematic cells and have hence been amenable for cryopreservation of several species (Lee et al., 2021; Maślanka and Szewczyk, 2021; Niino and Arizaga, 2015; Quain and Berjak, 2012; Sen-Rong and Ming-Hua, 2013). This is because meristematic cells contain few vacuoles and have a high nucleo-cytoplasmic ratio (Engelmann, 2012), making them more desiccation tolerant; desiccation tolerance of explants stored in LN is necessary for their successful recovery and survival after retrieval from cryostorage. In this study, too, at the ultrastructural level, the explants (shoot apices) comprised meristematic regions of cells with low vacuole formation and prominent nucleus development (Fig. 3.9 m). Shoot apices are also the preferred explants for cryopreservation due to a lower chance of somaclonal variation in their organised tissues as there is a lower chance of natural mutations because the tissue is young and also not yet exposed to negative environmental conditions (Engelmann, 2012; Panis and Lambardi, 2006). Avoiding somaclonal variation is beneficial in cases where true-to-type cloning is desired (Engelmann, 2014), for example, in genotypes that yield high biomass or compounds of interest. In both cases, these are applicable to *S. aethiopicus*, as it is primarily recognised as a medicinal species with the highly active siphonochilone produced in its rhizomes and roots. In this study too, *S.*

*aethiopicus* in vitro-derived axillary shoot apices (0.5 – 1 mm in length) were used as explants for cryopreservation, and more so since the shoot apices of a closely related rhizomatous species, *Zingiber officinale* Rosc. had been successfully cryopreserved (Yamuna et al., 2007). Similar success has been reported using shoot apices of the tuberous *Ipomoea batatas* (L.) (Wilms et al., 2020).

In addition to the explant type, the physiological status of the stock (mother) plants, and applicability of a regeneration (micropropagation) protocol are also critical parameters that influence the recovery of explants from cryopreservation (Engelmann, 2012). An improved regeneration protocol (compared to that in the literature) for in vitro-derived shoot apices was established in Chapter Two. Shoot apices used in this study were sourced from in vitro cultures of *S. aethiopicus* to ensure a steady supply of explants devoid of endophytic contaminants. The shoot apices were obtained from 42 d old shoot cultures (Chapter Two), subjected to three subcultures onto fresh medium 42 d apart for the different cryopreservation experimental stages to ensure uniformity across all experiments. Although uniform subculturing of parent stock cultures was performed to retrieve shoot apices for all cryopreservation experiments in this study, the plant cultures were maintained under in vitro conditions over six months. Wilms et al. (2020) reported that the age of parent stock cultures influenced the recovery rate of cryopreserved explants; hence, in this study, too, there is a possibility that the age of the cultures influenced the recovery rate of cryopreserved explants, nonetheless more than 60% of droplet-vitrified shoot apices survived post thawing and recovery.

In many plant cryopreservation protocols (Yamuna et al., 2007; Varghese et al., 2009; Chen et al., 2011; Hajari et al., 2011; Sen-Rong and Ming-Hua, 2013; Engelmann, 2014; Wilms et al., 2020; Maślanka and Szewczyk, 2020), preculture of explants (shoot apices) in high sucrose medium in the dark reportedly increase the regeneration potential of the cryopreserved explants. In this study, the shoot apices were precultured on media with three different sucrose concentrations (0.3, 0.4 and 0.5 M) for three incubation periods (24, 48, 72 h). Although there was no significant difference in the multiplication rate of the explants precultured on 0.3 M sucrose (24 h dark) and the control, there was a significant drop in the WC of the shoot apices (explants selected for cryopreservation) in the former. Reducing the water content of the explants prior to cryopreservation is necessary to minimise ice crystal formation during cryopreservation, which causes lethal damage at a cellular level and prevents the successful recovery of cryostored explants. Yamuna et al. (2007) also used 0.3 M sucrose (72 h) as a preculture treatment for successful vitrification-based cryopreservation of *Z. officinale* shoot apices. In the present study, including a 0.09 M sucrose (48 h) preculture of the excised apices prior to the 0.3 M sucrose (24 h dark) preculture improved multiplication without any significant change in the WC of the apices, compared with those precultured on a 0.3 M sucrose medium only. Further, the ultrastructure of the meristematic cells in the shoot apices from the sequential sucrose preculture treatments (0.09 M [48 h] and then on 0.3 M [24 h dark] sucrose media) remained intact, and similar to the meristematic region of shoot apices in the control, as reported in sucrose-precultured *Solanum tuberosum* L. (potato) shoot tips (Kaczmarczyk et al., 2008). The results in this study also support a previous study on the ultrastructural changes of *Garcinia cowa* shoot tips precultured for 24 h on a 0.3 M sucrose medium, where dehydration tolerance of the shoot tips to PVS2 was enhanced (Yap et al., 2011). According to those authors, sucrose preculture strengthened the plasma membrane by thickening its inner lining and, therefore, promoted active transport of sucrose into the cell, resulting in the formation of soluble carbohydrate accumulation. In this study, a similar observation was made in the meristematic region of the shoot apices precultured on 0.09 M (48 h) and then on 0.3 M (24 h dark) sucrose media. Furthermore, distinctive separation of the plasma membrane from the cell wall with associated thickening, the accumulation of carbohydrates (starch granules) in the newly formed plastids, as well as the presence of irregularly shaped vacuoles containing cytoplasmic invaginations were observed at an ultrastructural level in the current study, similar to that reported by Kaczmarczyk et al. (2008).

The precultured shoot apices of *S. aethiopicus* were cryoprotected for 10, 20 or 40 min. A 10 min CPM treatment resulted in the least loss of explants to necrosis with no significant change in the multiplication rate, compared with longer durations of CPM treatments. During cryoprotection, the samples are treated with cryoprotectants to avoid cryoinjury during storage in LN. The CPM interacts with and alters water distribution inside and outside the cells and dehydrates them. These substances increase the stability of the plasma membrane, lowering the freezing point and increasing the viscosity of the cytosol and, at the same time, protecting the cells from injury during freezing (Sershen et al., 2012; Teixeira et al., 2013). Although the explants (shoot apices) were precultured on a medium with a high concentration of the non-penetrating cryoprotectant (sucrose at 0.3 M for 24 h in the dark), this preculture treatment was not sufficiently adequate to reduce the WC to below 1 g $H_2O$ $g^{-1}$dry weight (the most desirable water content for freezing in LN; Nadarajan and Pritchard, 2014). Including a CPM treatment which comprised a combination of penetrative cryoprotectants in a basal MS salt solution with a high sucrose concentration, was shown to be effective in further reducing the water content in other species (Engelmann, 2014; Quain and Berjak, 2012; Varghese et al., 2009). Yamuna et al. (2007) included a CPM treatment comprising 5% DMSO and 5% glycerol for 20 min at room temperature to develop a droplet-vitrification-based cryopreservation protocol for *Z. officinale* that resulted in 80% recovery and regrowth of cryopreserved explants. Some vitrification-based cryopreservation protocols omit CPM treatment when higher concentrations of sucrose are used in the preculture medium (Wilms et al., 2020). However, in contrast to Wilms et al. (2020), the higher concentration of sucrose (0.5 M) in the preculture medium negatively impacted the survival of the *S. aethiopicus* explants, hence, lower concentrations of sucrose were used. The CPM treatment reduced the WC of the precultured shoot apices but the WC of the explants was still not sufficiently low (1 g $H_2O$ $g^{-1}$DW) for them to be effectively cryostored. Nonetheless, the changes associated with the CPM treatment observed at an ultrastructural level provided further evidence that the meristematic cells in the explant experienced dehydration.

Although cryoprotection can improve the efficiency of the vitrification process, PVS2 is often reported to be toxic to the explants due to its high osmotic concentration (Kalaiselvi et al., 2017; Pawlowska and Szewczyk-Taranek, 2014; Teixeira et al., 2013). The cryoprotected shoot apices (CPM for 10 min) in the present study were osmoprotected for 5 or 10 min with a loading solution. Multiplication of the shoot apices (explants) was observed during preculture and cryoprotection. However, upon transfer to the LS, multiplication ceased but shoot development continued. Treatment with LS delayed regeneration of shoots from the explants (90 d after, treatment) compared to the ones that were cryoprotected only, where regeneration and multiplication were observed 42 d after treatment. Although the LS is important to resist the toxic effects of the vitrification solution, it is also toxic to the explant upon prolonged exposure (Wilms et al., 2020). In this study, although regrowth of the explants treated with LS was observed, multiplication was inhibited. The 10 min exposure to LS was more toxic than the 5 min exposure since significantly reduced regrowth was observed in the former. Nonetheless, the LS treatment contributed to a further decrease in the WC of the explants. Although dehydration of explants is a key parameter for successful recovery of cryostored explants, for some species, rapid dehydration to WC below 1 g $H_2O$ $g^{-1}$dry weight can cause irreversible ultrastructural damage of the meristematic cells and hence, a sequential, slow decrease in WC is necessary. The findings in this study are in line with reports of LS treatments in other crop species (Wilms et al., 2020; Yamuna et al., 2007). Ultrastructural changes of the explants treated with the LS also reflected its toxic effect, including high cytoplasmic invaginations and withdrawal of the cell membrane from the cell wall, and regions of cell degeneration in the outer regions of the cells.

In vitrification-based cryopreservation methods, PVS2 is widely used for many unrelated plant species (Hajari et al., 2011; Yap et al., 2011; Martinez-montero et al., 2012; Antony et al., 2013; Sen-Rong and Ming-Hua, 2013; Wilms et al., 2020), as well as for *Z. officinale* (Yamuna et al., 2007), a close relative of *S. aethiopicus*. The role of the PVS, a highly viscous solution, is to penetrate the cell and remove its free water so that when it is cooled in LN, it induces a glassy state within the cell preventing damaging ice crystal formation during freezing. In this study, PVS2 treatment for 10 min reduced cell water content to 0.92 ± 0.36 g $H_2O$ $g^{-1}$ DW which is considered ideal for successful cryostorage of shoot apices (Varghese et al., 2009) and prevention of ice crystallisation. However, a similar trend in the development of shoot apices was seen for the LS and PVS2 treatment in that shoot development was only visualised 90 d after initial treatment without multiplication. The viscosity within the cell of the PVS2 treated explants was seen in the ultrastructural images as dense regions in the cytoplasmic matrix. In this study, only one vitrification solution, PVS2, was considered since it is the most widely used, however, it may be useful to consider the effects of other vitrification solutions such as PVS3, in future experiments.

The shoot apices were either rapidly cooled by plunging directly in LN or cooled through vitrification (explants immersed in 1 ml of PVS2) or via droplet-vitrification (shoot apices suspended in PVS2 droplets on a piece of aluminium foil and then plunged in LN). Once the *S. aethiopicus* shoot apices were retrieved from LN after 24 h of cryostorage, they were thawed and recovered according to the procedures used by Varghese et al. (2009) for *Trichelia emetica* shoot tips. Rehydration (after unloading where PVS2 was used) of the explants was carried out by gradually decreasing the sucrose concentration to avoid the toxic effect of rapid rehydration. Following rehydration, the shoot apices were gradually exposed to light and finally to stock culture conditions to reduce the risk of cell deterioration during recovery, if exposed immediately to high light intensity. Gradual rehydration of the cryopreserved explants was performed since Popova et al. (2023) emphasised the importance of sequential rehydration and regeneration procedures as a mechanism to reduce the induced stress cryopreserved explants endure during the cryopreparative dehydration procedures, during cryopreservation and then again during the cryorecovery. Earlier, Kaczmarczyk et al. (2008) and then Yap et al. (2011) showed that when vitrified shoot tips were retrieved from LN, gradually rehydrated and subjected to gradual increase in light intensity during recovery, cell ultrastructure of recovered samples were similar to the ultrastructure of meristematic cells from the control shoot tips. In this study, ultrastructural examination of the droplet-vitrified explants retrieved from LN was not pursued, however, the regrowth (66.67 ± 16.67%, 90 d after retrieval) from droplet-vitrification is evidence of successful cryopreservation. Further, all the surviving cryostored explants following droplet-vitrification, developed into plantlets in vitro.

## 3.5 Conclusion and future recommendations

Axillary shoot apices derived in vitro from the critically endangered *Siphonochilus aethiopicus* were successfully cryopreserved using droplet-vitrification. This study marks the first successful application of cryopreservation techniques for long-term germplasm conservation of this species.  The cryopreparative procedures and immersion in LN can take up to 72 h after initial excision of the axillary shoot tips. Nonetheless, the droplet-vitrification-based approach, with a recovery rate of 66.67 ± 16.67% explants surviving cryostorage, was the most effective of the three methods tested for *S. aethiopicus*.  Optimisation of certain steps of the protocol, such as the use of different plant vitrification solutions, different cooling rates (ultrarapid and two-step cooling), thawing, rehydration and recovery procedures could further reduce the recovery period and increase the recovery percentage of explants following cryopreservation. Further studies are required to the point of acclimatisation followed by growth for at least one season prior to rhizome dormancy; chemical analyses can be then performed to confirm the presence and concentration of siphonochilone in plants recovered from cryostorage.

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# Chapter Four: Evaluation of biomass accumulation and quantification of siphonochilone in rhizomes and roots of micropropagated and conventionally propagated *Siphonochilus aethiopicus*

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## Abstract

The increasing demand for plant-derived pharmacologically active compounds has necessitated sustainable alternative sources and conservation of medicinal plant species in South Africa. The purpose of the present study was to compare the morphological characteristics, biomass accumulation and siphonochilone content of the rhizomes and roots of micropropagated and conventionally propagated plants of the critically endangered medicinal species, *Siphonochilus aethiopicus*. Using hexane extracts, Nuclear Magnetic Resonance Spectroscopy was used to elucidate the structure of siphonochilone, while Gas Chromatography-Mass spectrometry was used to quantify it. The mature two-year-old post-acclimatised micropropagated plants produced rhizomes (35.80 ± 6.11 g) and roots (15.70 ± 2.00 g) with higher biomasses compared to conventionally propagated plants (3.73 ± 2.48 g and 1.14 ± 0,22 g, respectively). The root siphonochilone yield in the former was also significantly higher in different harvest batches (0.03120 ± 0.1109 to 0.4498 ± 0.1081 g $g^{-1}$extract) than in the latter (0.0435 ± 0.0136 g $g^{-1}$extract). Further, there were no significant differences in siphonochilone yield in rhizomes from the two-year-old micropropagated plants compared to that from field-cultivated plants. This study has shown that it is possible for micropropagated plants to produce greater biomass and more siphonochilone than conventionally grown plants, which, in turn, provides a means of alleviating the demands on natural populations. The results from this study can be further used to explore the in vitro synthesis and extraction of siphonochilone for the pharmaceutical industry.

**Keywords:** GC-MS; hexane extract; NMR; siphonochilone; rhizomes

## 4.1 Introduction

Plant-derived compounds have been integral in supporting the healthcare needs of many rural communities worldwide (World Health Organisation, 2019); have been shown to alleviate symptoms of some deadly human diseases without the deleterious effect of allopathic drugs (Khan and Ahmad, 2019; Mordeniz, 2019; Adebayo et al., 2021). However, sustaining the demand for plant-based drugs has become increasingly challenging because of the depletion of medicinal plants in the wild through habitat destruction, climate change and over-harvesting (Xego et al., 2016; Khan and Ahmad, 2019). There is, therefore, an urgent need to consider sustainable alternate sources of plant-derived products and effective conservation approaches for pharmaceutically valuable species.

Micropropagation is a widely applied biotechnological approach in which medicinal plants of interest can be propagated on a mass scale through in vitro culture (Kavyashree, 2009; Kaya et al., 2018). This approach can ensure sustainable sources of pharmaceutical products and also doubles as a conservation tool (Bairu et al., 2007; Amoo and van Staden, 2013) for species that are vulnerable or endangered. One such plant is *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt, for which an improved micropropagation protocol (from that currently in the literature) was established in Chapter Two. This species is critically endangered in South Africa (Lötter et al., 2022) and is listed as one of the top ten most sought-after species for African traditional medicine (Ozioma and Okaka, 2019). The rhizomes and tuberous roots are popularly used in the treatment of flu-like symptoms and coughs. The rhizomes are also chewed by females to alleviate abdominal pain during menstruation (van Wyk, 2002; Van Wyk et al., 2008; Ndhlala et al., 2011). Its use in pharmaceutical products and as an antimalarial treatment was prompted by the isolation of the novel furanoterpenoid (4aα,5β,8α)-3,5,8a-trimethyl4,4a,9-tetrahydo-naphtho[2,3-b]-furan-8(5H)-one, commonly known as siphonochilone (Viljoen et al., 2002), from the rhizomes (Holzapfel et al., 2002; Viljoen et al., 2002) and roots (Viljoen et al., 2002). The antiplasmodic and anti-inflammatory activity of siphonochilone has also been confirmed (Light, 2002). These findings, together with the successful isolation of siphonochilone from rhizomes and roots, have led to an increase in the demand for this species.

Despite *S. aethiopicus* being widely distributed across Africa in the past, overharvesting has resulted in its critically endangered status in its natural area of distribution in South Africa (Lötter, et al., 2022). Although micropropagation protocols were developed for *S. aethiopicus* prior to the present study (Hannweg et al., 2016; Kunene et al., 2018), those studies did not investigate the presence of siphonochilone in the clonal materials, hence, field-cultivated populations remain the primary source of material for both traditional medicinal practices and phytocompounds in pharmaceutical products. There was an urgent need, therefore, to determine whether micropropagated plants of *S. aethiopicus* could offer an alternate, sustainable source of siphonochilone. The main aim of the present study then, was to evaluate the presence of, and quantify, siphonochilone in roots and rhizomes of acclimatised plants of *S. aethiopicus* following the micropropagation protocol established in Chapter Two. Conventionally propagated plants were used as a means of yield comparisons. This study further assessed morphology and explored the biomass accumulation in rhizomes and roots derived from both methods of propagation.

## 4.2 Plant materials and methods

### 4.2.1 Plant materials

Dormant rhizomes (with intact roots) from S. *aethiopicus* were harvested after shoot senescence (in July 2019) from field mother plants maintained at the eThekwini Municipal Medicinal Plant Nursery, Silverglen Nature Reserve, Durban, KwaZulu Natal, South Africa. They were transported in clear plastic bags to the eThekwini Municipal Tissue Culture (EMTC) facility based at the Durban Botanic Gardens. They were washed under running tap water for an hour and then air-dried for 24 h. One batch of the dormant rhizomes was used to optimise the chemical extraction procedure and to isolate siphonochilone, which was subsequently used as a standard in this study (harvest batch 1; Fig. 4.1). A second batch of rhizomes was utilised as a source of starting material for the different propagation methods following storage.

The rhizomes were dusted with Benlate® (Du Pont Agro Sciences, South Africa) before storage in open plastic trays (350 x 480 x 50 mm) (Calibre Plastics, South Africa), lined with brown craft paper (Croxley, South Africa), in single layers at 22 ± 2°C in the dark for ±98 d. Once dormancy was broken and bud emergence was observed, they were then prepared for the different propagation methods.

### 4.2.2 Methods

#### *4.2.2.1 Conventional propagation*

Conventional vegetative propagation was done on coarse river sand:potting medium (Gromor, South Africa) (1:1[v/v]) mix in plastic plant pots ([200 mm Ø, 320 mm in height]; P C Plastics, South Africa); the medium was moistened with tap water and left for 24 h to drain excess water, before inserting the rhizome pieces with emerging buds. The pots were maintained under tunnel-house conditions at the EMTC in late spring (October). The tunnel-house cover comprised a black shade cloth transmitting 60% light and a 100 µm thick tunnel-house plastic. Only once shoot emergence was observed were the plants watered twice weekly and supplemented weekly with half-strength foliar application of a water-soluble plant fertiliser (Multifeed®, AECI, South Africa). Water and fertiliser application were discontinued upon the onset of leaf senescence (harvest batch 2, Fig. 4.1).

#### *4.2.2.2 Micropropagation*

In vitro-derived shoot apices of *S. aethiopicus* were regenerated into plantlets and acclimatised according to the protocol established in Chapter Two. Following acclimatisation, half of the ex vitro plants were transferred to similar conditions as the vegetatively propagated tunnel-grown plants, and the same fertiliser and watering regimes were followed until leaf senescence. Plant material from this experiment was harvested for two successive dormancy seasons (harvest batches 3a and 3b, Fig. 4.1).

The other half of the ex vitro plants were transferred to 0.5 l potting bags (Gundell, South Africa) containing a similar substrate as the tunnel-grown plants. They were transferred to a shade-house (covered with a black shade cloth transmitting 60% light) at the Medicinal Plant Nursery, following which they were harvested for one dormancy season under the shade cloth (harvest batch 4a) and then upon transfer to the same field conditions as the mother plants for a second dormancy season (harvest batch 4b) (Fig. 4.1).

#### *4.2.2.3 Harvesting*

Rhizomes and roots harvested for chemical analysis were from the following harvest batches:

1. Field-grown mother stock plants (control; harvest batch 1).
2. Vegetatively propagated plants under tunnel-house conditions (harvest batch 2).
3. Micropropagated plants transferred to tunnel-house (harvested for two successive dormancy seasons, harvest batches 3a and 3b, respectively).
4. Micropropagated plants transferred to shade-house (a), then transferred to the same field as mother stock plants, and then harvested in the second dormancy season (b) (harvest batch 4).

Harvest batch 1 was used to optimise the chemical analysis procedure following which all further harvested batches were analysed using the optimised protocol. For each harvest batch, following complete leaf senescence, three dormant rhizomes with intact roots were randomly harvested (Fig. 4.1) for biomass (dry mass) and chemical analyses. All the harvested plant material was first washed under running tap water (20 ± 5 min) to remove soil particles, air dried at ambient temperature (24 h) to remove excess water and then stored in open plastic trays lined with a double sheet of paper towel for one week in the dark at the EMTC laboratory before visual observations of the root and rhizomes morphologies were made and further processing was carried out.

A diagrammatic representation of the different propagation methods used, and harvest batches used for chemical analyses are illustrated in Fig 4.1.

#### *4.2.2.4 Processing*

Each batch of harvested rhizomes with roots still intact was transported in brown paper bags (Croxley, South Africa) to the University of KwaZulu-Natal (UKZN) (Westville campus, Durban, KwaZulu-Natal, South Africa). They were separated into roots and rhizomes using carbon steel no. 3 surgical blades (Healthease®, Neomedic, South Africa), then cut into small pieces and separately freeze-dried for 48 h at -60 °C and $10^{-3}$ mbar in a benchtop EF4 Modulyo bench-top freeze dryer (Edwards, England). Thereafter, the dry weight (g) of the rhizomes and roots were measured using a four-place digital balance (Mettler-Toledo, United States).

#### *4.2.2.5 Chemical extraction*

Chemical extraction of siphonochilone was adapted from Light (2002). The freeze-dried material was crushed to a fine powder using a pestle and mortar (Fisherbrand™, Fisher Scientific, United Kingdom), weighed and suspended in hexane (hx) (Sigma-Aldrich, United States) in a ratio of 1 g dried material:5 ml hx in 250 ml borosilicate Erlenmeyer flasks (Pyrex®, DWK Life Sciences, UK), sealed with Parafilm® (Bemis Company Inc., United States) and placed on a benchtop shaker (Labcon, South Africa) for 48 h at 100 rpm. The solvent containing the crude extracts were then filtered under vacuum using Whatman™ No. 1 filter paper and transferred to glass jars (Consol, South Africa), which were left open in a fumehood overnight for solvent evaporation. The residue on the filter paper was re-suspended in ethyl acetate (ea) (Merck, Germany) and extracted precisely as the hx extract.

### *4.2.2.6 Isolation of the standard and NMR spectroscopy*

Only the crude hx extracts from the rhizomes of the parent stock were used to perform column chromatography (cc) to isolate siphonochilone, which was then used as the standard throughout the study. The cc separation of the crude extract was carried out using Silica 60, 0.063 – 0.2 mm (Macherey-Nagel, Germany) as the stationary phase and hexane:ethyl acetate step gradients (on the same column and extract) as the mobile phase. Eighteen fractions of 10 ml were collected for each hx:ea solvent ratios of 9.5:0.5 (fraction 1), 9:1 (fractions 2 – 6), 8:2 (fractions 7 and 8), 7.5:2.5 (fraction 9), 7:3 (fractions 10 and 11), 6.5:3.5 (fractions 12 and 13), 6:4 (fractions14 and 15), 5:5 (fraction 16) and 0:10 (fractions 17 and 18). The fractions were collected in pre-weighed glass vials (60 ml, 20 mm Ø) (Sigma-Aldrich, United States) and left uncovered (24 h) in a fume hood to evaporate the solvent and the weight (g) of each fraction was thereafter gravimetrically determined.

Each fraction was suspended in the solvent chloroform-d ($CDCl_3$) (Sigma-Aldrich, United States) to perform Nuclear Magnetic Resonance (NMR) Spectroscopy. Siphonochilone in fraction 5, $^1$H NMR and $^{13}$C NMR spectra were recorded with a 400 MHz Varian Unity Inova spectrometer (Spectris Scientific, Australia) at the School of Chemistry and Physics, UKZN.

### *4.2.2.7 Thin layer chromatography (TLC)*

Thin Layer Chromatography (TLC) was performed to qualitatively validate the presence of siphonochilone in the isolated extracts of all harvested batches. Silica gel containing a fluorescent indicator on aluminium-backed plates ($60F_{254}$) (Merck, Germany) was used as the stationary phase for TLC. First, the solvent ratio of the mobile phase was standardised using a rhizome hx extract from *S. aethiopicus* parent stock. Three different solvent ratios of hexane:ethyl acetate (9:1, 8.5:1.5 and 6:4) were tested. The separation of the hx extract on the TLC plates (100 mm x 100 mm) for the different solvent ratios was compared. Rhizome hx extracts (0.01 - 0.02 mg) were suspended in hexane:dichloromethane (Sigma-Aldrich, United States) (1:1[v/v]) and spotted 20 mm apart, 10 mm from the bottom of the TLC plates. The plates were developed in an enclosed rectangular glass chamber containing the different solvent ratios and then stained in a methanol:sulphuric acid (9:1) solution for 5 min. The TLC plates were gently heated using a hot air gun (Bosch, Germany) and compared for visibility and separation of the spotted sample on the plate. The solvent gradient that yielded the best TLC separation of the original plant (control batch) chemical extract was used to separate the chemical extracts from the other plant materials. The TLC separation was performed to qualitatively confirm the presence of siphonochilone in these extracts, using the isolated standard as a reference.

### *4.2.2.8 Gas Chromatography-Mass Spectrometry (GC-MS) analysis*

To optimise the efficiency of the Gas Chromatography-Mass Spectrometry (GC-MS) analysis for this study, two methods were compared using a GCMS-QP 2010SE instrument (Shimadzu, Japan), and the isolated standard. In the first method, the standard isolated from the field-grown mother stock was analysed under the conditions described by Viljoen et al. (2002). The instrument was fitted with a HP-Innowax FSC column (60 m x 0.25 mm Ø, with 0.25 µm film thickness) and helium (He) was used as the carrier gas (0.8 ml min$^{-1}$). The GC-MS oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C min$^{-1}$, then held constant at 220 °C for 10 min and programmed to 240 °C at a rate of 1°C min$^{-1}$. The split ratio was adjusted to 50:1. The injector temperature was set at 250 °C. A sample ionisation energy of 70 eV was used and the mass range was from 35 to 425m/z. The sample passed through the column in 2 h.

In the second method, the GC-MS instrument was fitted with a HP-5MS capillary column (0.25 µm film thickness and 30 m [length] x 0.25 µm [inner Ø]) (Shimadzu, Japan) for analysis of the standard and He was also used as the carrier gas at 7.5 kPa, with an oven temperature of 60 °C (2 min) to 300 °C (30 min) increased at a rate of 4 °C min$^{-1}$. GC-MS is usually performed using a small quantity of the crude extract (1000 ppm), therefore, a 2 µl sample was manually injected at an injection temperature of 300 °C with a split ratio of 1:10 for detection. The sample passed through the column in 1 h. Using an HP-5MS column with an injection temperature of 300 °C and 60 min injection time reduced the time required for analysis, thereby improving the efficiency of the method. The data were captured using the GC-MS workstation solution software (Shimadzu, Japan).

### *4.2.2.9 Liquid Chromatography-Mass Spectrometry (LCMS) analysis*

To quantify the molecular mass of the furanoterpenoid in the isolated standard, an LC-MS instrument (Shimadzu, Japan) was used according to the method described by Lategan et al. (2009). Acetonitrile (ACN) (Sigma-Aldrich, United States) was used as the mobile phase and was passed through the instrument without a column.

#### *4.2.2.10 Photography*

Images of visual morphological differences amongst the different harvest batches in this study were captured with a Canon IXUS 190 digital camera (Canon, Japan), fitted with a 24 mm wide lens and at 20.0 megapixels.

#### *4.2.2.11 Data recording and statistical analyses*

For all the analyses carried out in this study, samples were randomly harvested from each propagation experiment or developmental stage post-acclimatisation of the micropropagated plants. Siphonochilone was identified from the different GC-MS chromatograms by comparing the retention times of the peaks and their respective fragmentation patterns to that of the reference sample. The relative amount of the compound in all the extracts was then computed by comparing the area under the peak of the total area. Root and rhizome biomasses, extract yield and siphonochilone concentrations were compared for significant differences. Statistical analyses of all the data were performed using the Statistical Package for the Social Sciences (SPSS®) Version 29 software package for Windows (IBM, United States). All values were presented as mean ± standard error of three replicates. One-way analysis of variance (ANOVA) was used to test for differences in the yield of siphonochilone from the plant material from each of the micropropagation stages and among the propagation methods. Means for all analyses of variance were further separated using Tukey's post-hoc test; all differences were considered significant at $P \leq 0.05$.

## 4.3 Results

### 4.3.1 Biomass of roots and rhizomes

Differences were observed in the root and rhizome development amongst the harvest batches (1, 2, 3a, 3b, 4a and 4b) (Fig. 4.1). Visual morphological (Fig. 4.2) and biomass differences were observed in both the roots and the rhizomes in the control (harvest batch 1) and in the micropropagated plants derived from the first dormancy season for both harvest batches 3a (Fig. 4.2 e) and 4a (Fig. 4.2 f). However, this changed after an additional year, in that there was no significant biomass difference in the rhizomes harvested from batches 3b and 4b (Table 4.1; Fig. 4.2 c - d). On the other hand, harvest batch 4b produced heavier root biomass (15.70 ± 2.00 g) compared to the control (harvest batch 1, 1.14 ± 0.22 g) and the two micropropagated batches after one season post-acclimatisation (harvest batches 3a and 4a); these produced roots with biomasses of 0.17 ± 0.04 and 2.56 ± 0.57 g, respectively (Table 4.1). Harvest batch 4b also produced heavier rhizomes (35.80 ± 6.11 g) compared with the rhizomes from the other five harvest batches (Table 4.2).

### 4.3.2 Biochemical analyses

Siphonochilone in the control rhizomes (harvest batch 1) was elucidated using $^{13}$C (Fig. 4.3) and $^{1}$H (Fig. 4.4) NMR spectroscopy using the fifth cc fraction, and appeared as a pale yellow solid in the hx extract when the hexane:ethyl acetate solvent ratio of 9:1 was used as the mobile phase. This fraction was then used as the standard for the TLC and the GC-MS analyses for all the sample extracts. Further, the siphonochilone ($C_{15}H_{18}O_4$) molecular weight in the standard (294.950) was confirmed using LC-MS analysis of the same fraction between 290 – 300 m/z (Fig. 4.5).

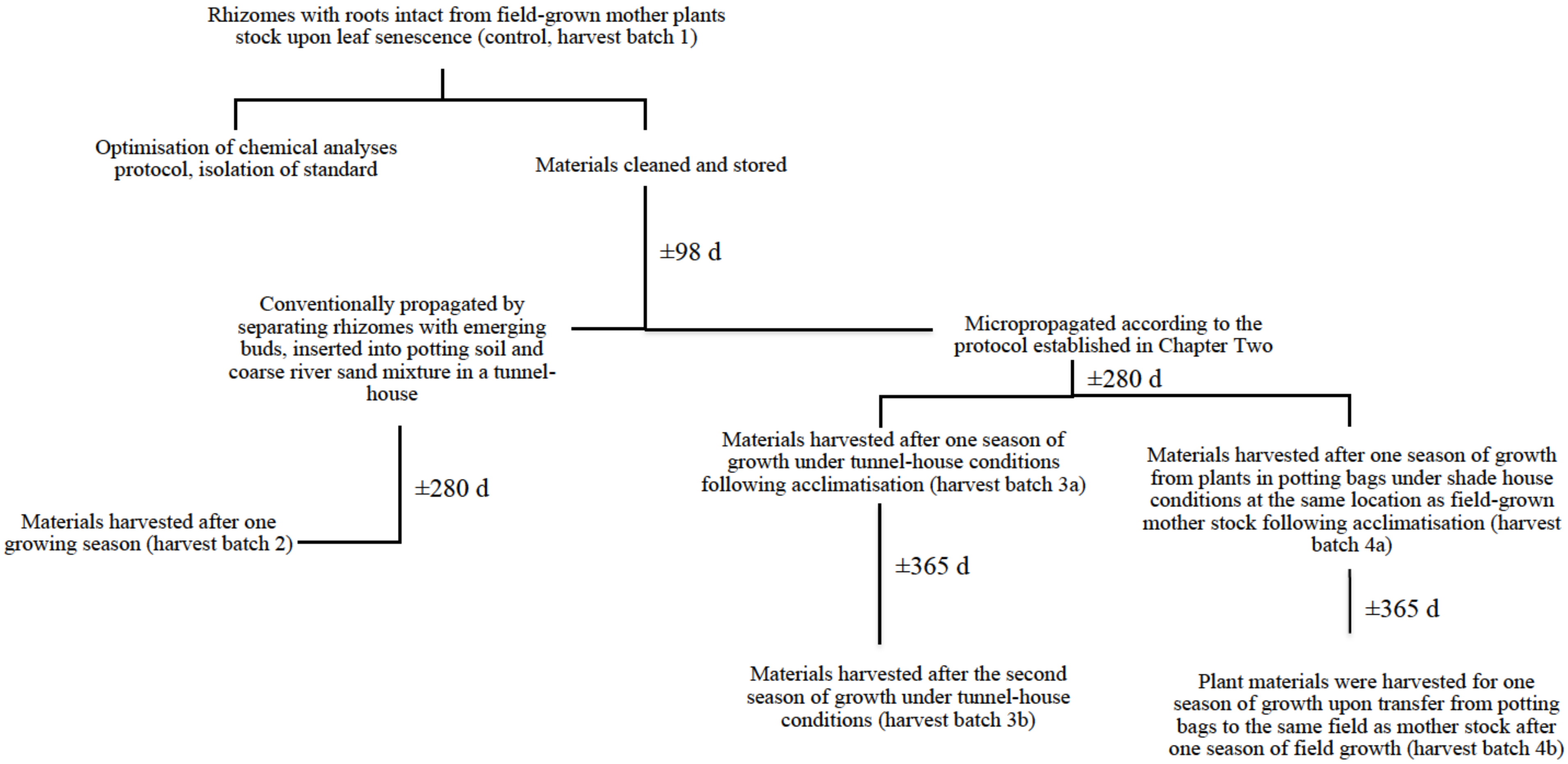

**Figure 4.1:** Diagrammatic representation of the different propagation methods used and harvesting of batches for chemical analyses. d = days

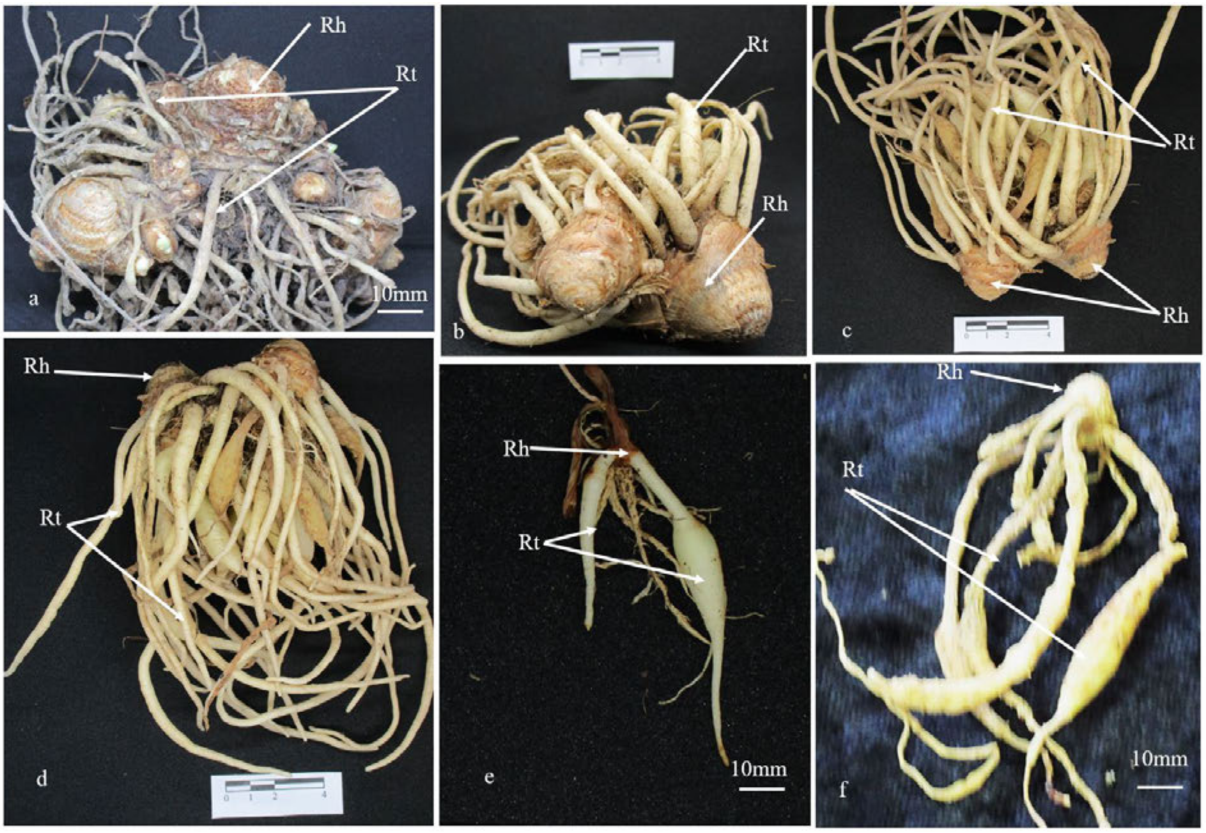

**Figure 4.2:** *S. aethiopicus* rhizomes (Rh) with intact roots (Rt) harvested upon shoot senescence from different harvest batches: field-grown mother stock (control, harvest batch 1) (a); conventionally propagated tunnel-grown (harvest batch 2) (b); micropropagated plants after two seasons in a tunnel-house (harvest batch 3b) (c); or transferred from shadehouse to the field for a second season following acclimatisation (harvest batch 4b) (d); acclimatised micropropagated plants after one season under tunnel-house conditions (harvest batch 3a) (e) and micropropagated plants grown for one season in a shade-house (harvest batch 4a) (f).

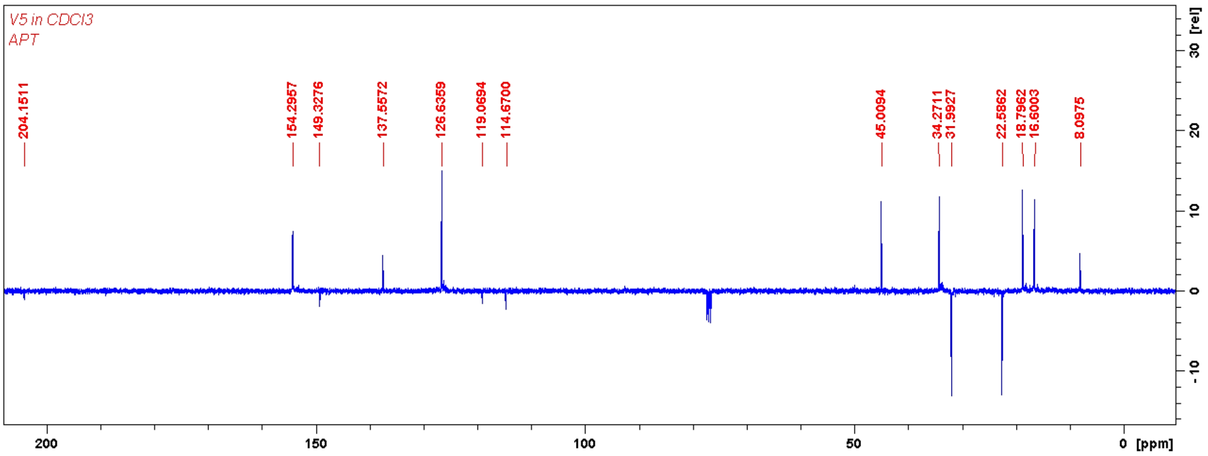

**Figure 4.3:** $^{13}$C NMR spectrum of siphonochilone isolated from rhizomes of the mother stock (control) of *S. aethiopicus*.

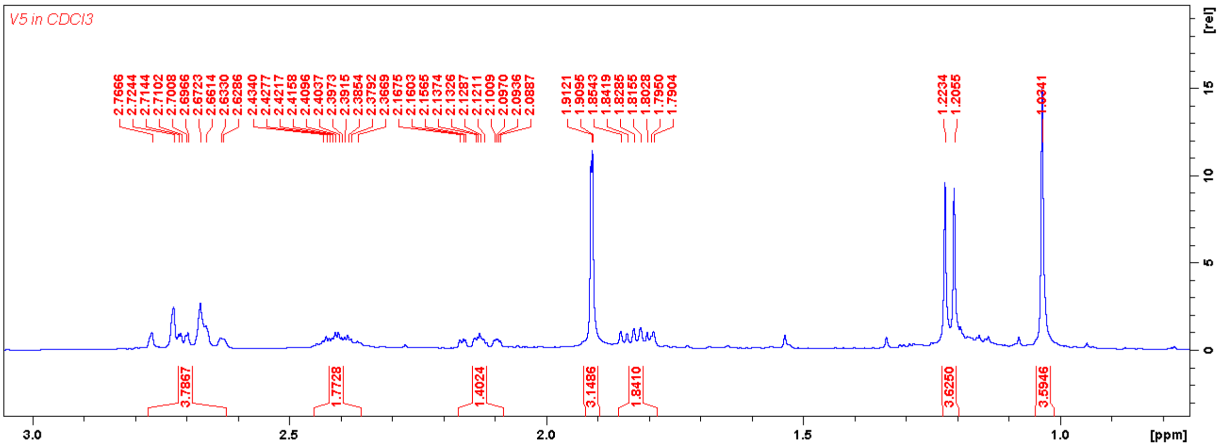

**Figure 4.4:** The full $^{1}$H NMR spectrum of siphonochilone isolated from rhizomes of the mother stock (control) of *S. aethiopicus*.

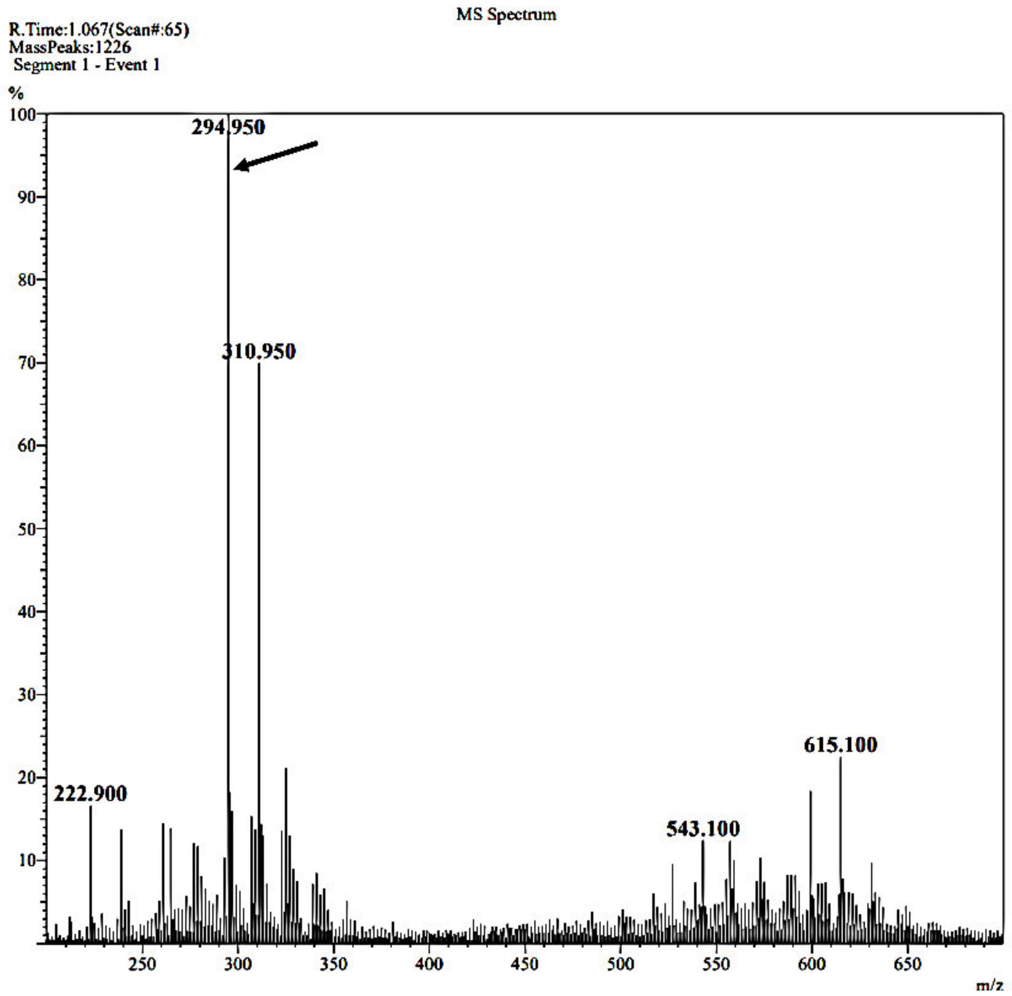

**Figure 4.5:** LC-MS chromatogram showing the molecular mass peak of siphonochilone (indicated by arrow).

Although there was TLC separation for the hexane:ethyl acetate ratios of 9:1 and 6:4 (Fig. 4.6 a and 4.6 b, respectively) for the hx extracts from the mother stock (control; harvest batch 1), the separation was most distinctive for the mobile phase with hexane:ethyl acetate in a 8.5:1.5 ratio (Fig. 4.6 c). There was no siphonochilone present in the TLC separation of the ea extract. Therefore, the chemical extraction for all subsequent plant materials were processed using hx (48 h) only, and the presence of siphonochilone was validated using the pure siphonochilone isolated from the fifth cc fraction of the control grown rhizomes as the standard.

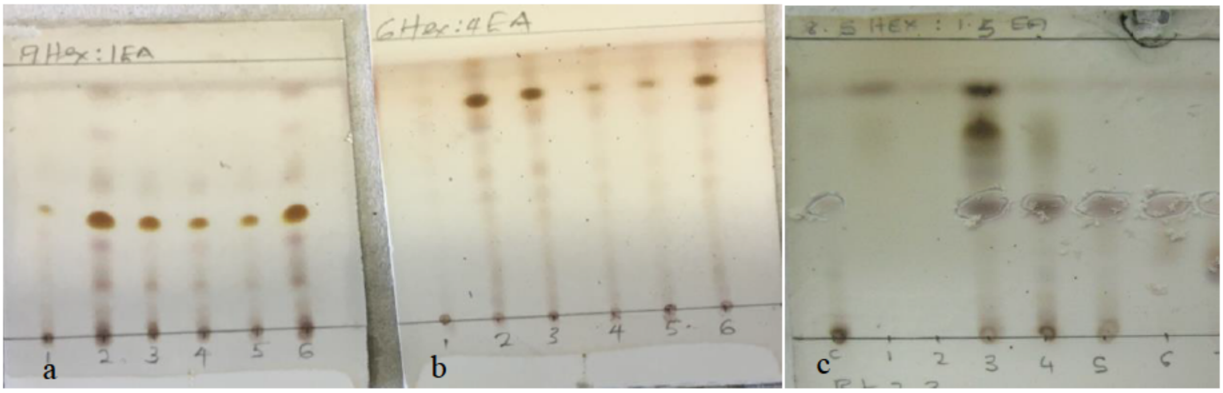

**Figure 4.6:** Representative TLC plates for separation of mother stock hx extracts for three different ratios of hx:ea as the mobile phase. The hx:ea ratios used were 9:1(a), 6:4 (b) and 8.5:1.5 (c).

Following the GC-MS analysis according to the method described by Viljoen et al. (2002), with an HP-Innowax column and an injection temperature of 250 °C, the peak retention time (Rt) for siphonochilone was between 50.6 and 50.7 min (Fig. 4.7). However, the Rt was between 23.4 and 24.4 min using the HP-5MS column and an injection temperature of 300 °C (Fig. 4.8). The GC-MS analyses for the hx extracts for all the harvested plant material was thereafter carried out using the HP-5MS column and an injection temperature of 300 °C.

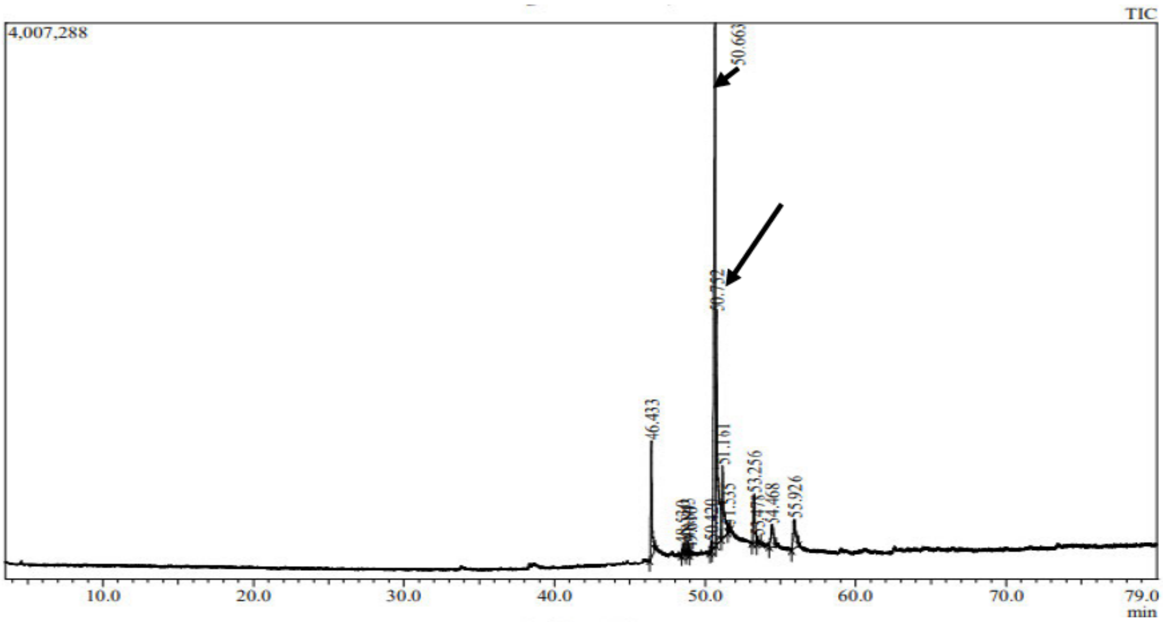

**Figure 4.7:** GC-MS chromatogram with peak retention time (Rt) between 50.6 and 50.7 min (arrows) for siphonochilone isolated through cc from the hx extract of mother stock rhizomes, using an HP-Innowax column and injection temperature of 250 °C.

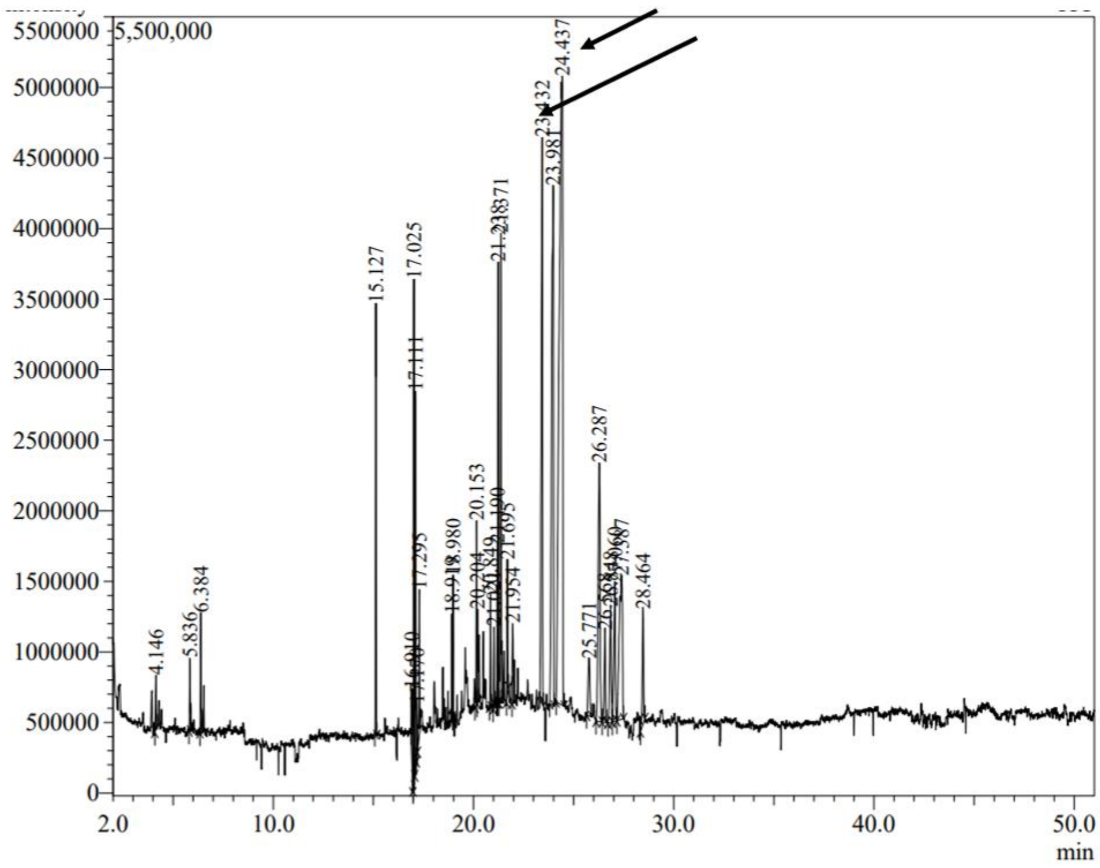

**Figure 4.8:** GC-MS chromatogram with peak retention time (Rt) between 23.4 and 24.4 min (arrows) for siphonochilone isolated through cc from the hx extract of mother stock rhizomes, using an HP-5MS column and injection temperature of 300 °C.

When the organs were extracted with hx, the amount of extract derived was the same on a g g $^{-1}$ basis (Tables 4.1 and 4.2). However, the siphonochilone yields differed. Roots in harvest batches 3b (tunnel, 0.4498 ± 0.0140 g g$^{-1}$ extract) and 4b (field, 0.13120 ± 0.1109 g g$^{-1}$ extract) (micropropagated and grown for two seasons) had more siphonochilone than those in batches 1, 3a and 4a (Table 4.1, 0.0435 ± 0.0136, 0.0052 ± 0.0136 and 0.0324 ± 0.0140 g g$^{-1}$ extract, respectively). Siphonochilone content in rhizomes demonstrated a very different trend i.e. rhizomes in all harvest batches (0.0596 ± 0.0220 to 0.7797 ± 0.1592 g g$^{-1}$ extract) outperformed those in batch 3a (0.0006 ± 0.0000 g g$^{-1}$ extract) (Table 4.2).

**Table 4.1:** *Siphonochilus aethiopicus* root biomasses, and yields of hexane extract and siphonochilone amongst different harvest batches.

| **Harvest batch** | **Biomass (g)** | **Hexane extract yield (g $g^{-1}$ biomass)** | **Siphonochilone yield (g $g^{-1}$ extract mass)** |
|---|---|---|---|
| 1 | $1.14 \pm 0.22^{b}$ | $0.09 \pm 0.02^{a}$ | $0.0435 \pm 0.0136^{b}$ |
| 2 | $9.04 \pm 3.92^{ab}$ | $0.03 \pm 0.01^{a}$ | $0.3187 \pm 0.0700^{ab}$ |
| 3a | $0.17 \pm 0.04^{b}$ | $0.26 \pm 0.22^{a}$ | $0.0052 \pm 0.0013^{c}$ |
| 4a | $2.56 \pm 0.57^{b}$ | $0.04 \pm 0.01^{a}$ | $0.0324 \pm 0.0140^{b}$ |
| 3b | $8.37 \pm 1.45^{ab}$ | $0.06 \pm 0.01^{a}$ | $0.4498 \pm 0.1081^{a}$ |
| 4b | $15.70 \pm 2.00^{a}$ | $0.05 \pm 0.00^{a}$ | $0.13120 \pm 0.1109^{a}$ |

1 = Field-grown mother stock plants (control); 2 = Vegetatively propagated plants under tunnel-house conditions; 3a = Micropropagated plants transferred to tunnel-house after one dormancy season following acclimatisation; 3b = Micropropagated plants transferred to tunnel-house after two dormancy seasons following acclimatisation; 4a = Micropropagated plants transferred to a shade-house in the same field as the mother stock plants; 4b = Micropropagated plants transferred to a shade-house in the same field as the mother stock plants and then transferred to the same field as mother stock plants and then harvested in the second dormancy season. Means (± SE) with different alphabets within columns are significantly different (Tukey's post hoc test, $p \leq 0.05, n = 3$).

**Table 4.2:** *Siphonochilus aethiopicus* rhizome biomasses, and yields of h**e**xane extract and siphonochilone amongst different harvest batches.

| **Harvested batch** | **Biomass (Dry mass) (g)** | **Hexane extract yield (g g$^{-1}$ dry mass)** | **Siphonochilone yield (g g$^{-1}$extract mass)** |
|---|---|---|---|
| 1 | 3.73 ± 2.48$^{bc}$ | 0.03 ± 0.02$^{a}$ | 0.0596 ± 0.0220$^{a}$ |
| 2 | 11.52 ± 1.27$^{bc}$ | 0.03 ± 0.01$^{a}$ | 0.3524 ± 0.0460$^{a}$ |
| 3a | 1.17 ± 0.04$^{bc}$ | 0.03 ± 0.00$^{a}$ | 0.0006 ± 0.0000$^{b}$ |
| 4a | 0.74 ± 0.26$^{c}$ | 0.03 ± 0.02$^{a}$ | 0.0160 ± 0.0061$^{a}$ |
| 3b | 13.53 ± 1.13$^{b}$ | 0.06 ± 0.03$^{a}$ | 0.7797 ± 0.1592$^{a}$ |
| 4b | 35.80 ± 6.11$^{a}$ | 0.02 ± 0.01$^{a}$ | 0.7334 ± 0.4462$^{a}$ |

1 = Field-grown mother stock plants (control); 2 = Vegetatively propagated plants under tunnel-house conditions; 3a = Micropropagated plants transferred to tunnel-house after one dormancy season following acclimatisation; 3b = Micropropagated plants transferred to tunnel-house after two dormancy seasons following acclimatisation; 4a = Micropropagated plants transferred to a shade-house in the same field as the mother stock plants; 4b = Micropropagated plants transferred to a shade-house in the same field as the mother stock plants and then transferred to the same field as mother stock plants and then harvested in the second dormancy season. Means (± SE) with different alphabets within columns are significantly different (Tukey's post hoc test, $p \leq 0.05$, $n = 3$).

## 4.4 Discussion

In this study, the chemical extraction, isolation and confirmation (qualitative and quantitative using TLC and GC-MS, respectively) of the presence of the phytocompound siphonochilone from dormant rhizomes and roots of *S. aethiopicus* was optimised. The study is also the first of its kind to compare visual morphological differences and biomass accumulation in rhizomes and roots, as well as the yield of siphonochilone from field-grown, tunnel-house propagated and micropropagated plants of *S. aethiopicus*, all originating from the same stock of mother plants. Micropropagated plants were harvested at two maturity stages (one and two years) post-acclimatisation. In perennial geophytic species such as *S. aethiopicus*, the accumulation of biomass, phytocompounds and other metabolites in the storage organs such as rhizomes and tuberous roots have been reported to be the highest upon the onset of dormancy (Khan and Ahmad, 2019). Hence, in this study, the rhizomes and roots were harvested upon dormancy to assess the biomass accumulated and  siphonochilone contents.

The morphological characteristics such as shape, size and general physical appearance of a plant organ provide an initial indication of the development and health of the plant in general. This study revealed visual differences in terms of the size of both the rhizomes and tuberous roots amongst the harvest batches, which increased as the micropropagated plants matured. The increase in sizes of the roots and rhizomes was further confirmed quantitatively by comparing the biomass of the roots (Table 4.1) and the rhizomes (Table 4.2) from the micropropagated plants upon dormancy at the different maturity stages post acclimatisation. Root and rhizome biomasses for the micropropagated plants after field growth (harvest batch 4b) were 15.70 ± 2.00 g (Table 4.1) and 35.80 ± 6.11 g (Table 4.2), respectively, higher than those resulting from field growth (harvest batch 1) with root and rhizome biomasses of 1.14 ± 0.22 g (Table 4.1) and 3.73 ± 2.48 (Table 4.2), respectively. Salmina (2017) reported on the effect of different irrigation regimes on biomass accumulation for the dry mass of different plant parts, including rhizomes and roots of field-cultivated *S. aethiopicus* where the well-irrigated plants had higher fresh and dry rhizome and root biomasses. Further, nutrient application and soil moisture content (Ashraf and Harris, 2013), together with other biotic and abiotic stresses, influence the morphological and physiological status of plants. In this study, the mature micropropagated plants were first grown in vitro on a nutrient-rich medium comprised of MS nutrients (Murashige and Skoog, 1962), supplemented with carbohydrates (sucrose), vitamins and plant growth regulators (PGRs); and then acclimatised under high humidity, provided with a consistent (weekly) supply of foliar water-soluble fertiliser then watered biweekly post acclimatisation under shade-house conditions before being transferred to field conditions. These regulated parameters would have improved the biomass accumulation in the storage organs of the micropropagated plants. The physiological characteristics, such as the accumulation of biomass in plant storage organs, are increased to improve the ability of a plant to adapt to changes in environmental conditions (Zhang et al., 2020). In this study too, the micropropagated plants were subjected to different environmental conditions during their development, firstly grown under in vitro conditions, then acclimatised under high humidity before being maintained under shade-house conditions and finally being transferred to the field (harvest batch 4b).

Rhizomes are the primary storage organ in perennial geophytes in the Zingerberaceae family; these storage organs accumulate biomass, nutrient reserves and phytocompounds during the onset of dormancy (Sevgin and Karatas, 2022). To adapt to the transition from controlled tunnel-house conditions to the field conditions, these plants (harvest batch 4b) accumulated higher biomass and stored nutrients and more phytocompounds in the rhizomes during the onset of dormancy, in comparison to rhizomes from the field-grown control plants (harvest batch 1) or to mature micropropagated plants maintained under tunnel-house conditions (harvest batch 3b). This was possible since mature micropropagated plants maintained under tunnel-house conditions (harvest batch 3b) were exposed to controlled growing conditions, water and fertiliser application was halted upon the onset of leaf senescence compared to the field-grown micropropagated plants that were exposed to one season of growth under field conditions, also another stress-related change in environmental conditions. These results validate that micropropagation improves biomass accumulation in roots and rhizomes of *S. aethiopicus* as they mature. Similarly, Balachandran et al. (1990) also showed increased rhizome development and biomass accumulation of micropropagated *Curcuma* spp. and *Zingiber officinale* Rosc. post acclimatisation as the plants matured, two other perennial geophyte Zingerberaceae relatives of *S. aethiopicus*.

To investigate siphonochilone accumulation, harvesting and analysis occurred during the dormancy season, immediately after acclimatisation and a year later for micropropagated plants. Further, the higher biomass accumulation in the roots of the mature micropropagated plants (Table 4.1; harvest batches 3b and 4b), resulted in an increase in siphonochilone yield in both tunnel-grown and field-grown micropropagated plants. However, tunnel-grown micropropagated plants produced the highest rhizome siphonochilone yield (0.7797 ± 0.1592 g $g^{-1}$ extract) (Table 4.2). The siphonochilone yield in the roots of micropropagated plants increased significantly over time (Table 4.1). Micropropagated plants in the tunnel-house (harvest batch 3b) and field-transferred plants (harvest batch 4b) harvested during the second dormancy season produced roots with significantly higher siphonochilone yields compared to the yield in the roots of field-grown control plants (harvest batch 1). Although the tunnel-house harvest batch (3b) was maintained under controlled growing conditions, nutrient and irrigation seized upon the onset of leaf senescence, prior to complete dormancy. The rapid change in controlled fertilizer and irrigation regimes changed the environmental conditions the mature micropropagated plants were exposed to under tunnel-house conditions (harvest batch 3b) which stimulated an increased accumulation of phytocompounds in the tuberous roots. The micropropagated plants transferred to the field (harvest batch 4b) were also exposed to a different environmental stress, controlled growing conditions under shade-house conditions then exposed for one growing season to field conditions with unpredictable changes in environmental conditions. In both circumstances the imposed stresses induced a higher accumulation in biomass and phytocompound accumulation in the tuberous roots as well.

In this study chemical extraction, isolation, confirmation of siphonochilone presence and its concentration in the different harvest batches were carried out according to modification to previously reported methods by Holzapfel et al. (2002), Light (2002) Viljoen et al. (2002) and Lategan et al. (2009). In all four previous studies, field-cultivated plant material was used to document siphonochilone presence. Field-cultivated material was also used in the present study, but analyses were additionally performed on tunnel-house grown and micropropagated plants. In the present study, the field cultivated material (harvest batch 1) was first used to modify the chemical extraction, confirmation of siphonochilone methods and isolate a standard to evaluate the presence and compare the concentration of siphonochilone to the other harvest batches.

All plant material for chemical analysis was first freeze-dried (48 h) before processing for chemical extraction procedures. Light (2002) dried the harvested rhizomes at 50°C, Holzapfel et al. (2002) used steam distillation for one hour to obtain a pale yellow semi-crystalline mixture from the fleshy fresh crushed roots of several *S. aethiopicus* plants, Viljoen et al. (2002) hydrodistilled fresh roots and rhizomes for 3 h to yield a yellow oil and Lategan et al. (2009) used freeze-dried rhizomes in a powdered form to carry out chemical extraction procedures. The benefits of freeze-drying plant material that produce highly volatile phytocompounds, is to avoid loss of the volatile compound through evaporation when dried using heat and to ensure consistent unform drying rates of the plant material. Therefore, the freeze-drying method was used in this study. The rhizome hexane extract (48 h) of the freeze-dried control batch (harvest batch 1), was separated using the column chromatography method described by Light (2002), to isolate pure siphonochilone that was then used as a standard throughout the study for both TLC (qualitative) and GC-MS (qualitative) analysis. The chemical structure of this pure compound was confirmed using NMR-spectroscopy, which was also used by Holzapfel et al. (2002) to identify this furanoterpenoid from *S. aethiopicus* rhizomes, and the molecular weight confirmed using LC-MS (Lategan et al. 2009).

In this study, GC-MS was used to quantify the volatile phytocompound siphonochilone in all the harvest batches. GC-MS is a popular analytical technique used to identify volatile long chain hydrocarbon-based phytocompounds in various plant parts (Bansal et al., 2024; Bodede et al., 2022), GC-MS was also used to first quantify siphonochilone concentration in *S. aethiopicus* roots and rhizomes by Viljoen et al. (2002). Hence, in this study GC-MS analysis was used to quantify the siphonochilone yield in all harvest batches. Two different GC-MS methods were first tested on the siphonochilone standard to reduce the time required for analysis, thereby improving the efficiency of the method. The first method tested was described by Viljoen et al. (2002), using an HP-Innowax FSC column in which the sample passed through the GC-MS column in 2 h under an injector temperature of 250 °C. In the second method, the GC-MS instrument was fitted with a HP-5MS capillary column at an injection temperature of 300 °C; the sample passed through the column in 1 h. The retention time (Rt) for the siphonochilone standard was quantified at 50.6 – 50.7 min with the former method and at 23.4 – 24.4 min with the latter. Using the latter method reduced the time required for analysis, thereby improving the efficiency of the method. Therefore, the latter method was selected to assess the siphonochilone concentration in all hexane extracts isolated from the different harvest batches. Siphonochilone yield in the extracts of field-cultivated plants was 0.0596 ± 0.0220 g $g^{-1}$ in rhizome extract and 0.0435 ± 0.0136 4 g $g^{-1}$ in root extract, and siphonochilone yield in the mature micropropagated plant root extracts were 0.13120 ± 0.1109 – 0.4498 ± 0.1081 g $g^{-1}$ extract and in the rhizome extract were 0.7334 ± 0.4462 – 0.7797 ± 0.1592 g $g^{-1}$ extract (harvest batches 4b – 3b). Viljoen et al. (2002) reported a siphonochilone yield of 0.20 g $g^{-1}$ extract in rhizomes and roots. There are different biotic and abiotic factors that have been reported to influence phytocompound concentrations in the same species, geographic localities of the collected plant material, different biochemical analysis procedures used or age of the collected plant material (Khan and Ahmad, 2019) as observed in this study. The geographic locality of the collected plant material for this study differed to the geographical localities for the plant material collected for all the previous studies. In this study, a chemical extraction procedure using hexane (48 h) was performed for the plant material from the different harvest batches, this differed to the extraction procedure performed for each of the earlier studies (Holzapfel et al., 2002; Light, 2002; Viljoen et al.; 2002; Lategan et al., 2009). The maturity of the plant material for each study was not provided. Maturity of the plant material is a key factor to consider when comparing the yield of siphonochilone. The siphonochilone yield in this study differed to the concentration provided in the earlier study by Viljoen et al. (2002).

This investigation substantiated the production of siphonochilone within the root and rhizome systems of *S. aethiopicus* propagated via micropropagation techniques. Furthermore, the study demonstrated that mature micropropagated plants exhibited enhanced siphonochilone accumulation compared to those derived from conventional propagation methods. Micropropagation, a well-established biotechnological approach (as detailed in Chapter Two), not only facilitates the large-scale production of *S. aethiopicus* but also amplifies the yield of siphonochilone, a major photocompound in the plant's rhizomes and roots. This advancement provides a sustainable alternative to harvesting from dwindling natural populations, thereby mitigating the pressure on this critically endangered species while simultaneously supporting its continued use in African traditional medicinal practices. The observed increase in siphonochilone yield in mature micropropagated plants is directly correlated with the progressive augmentation of root and rhizome biomass over successive growing seasons. Recognizing the escalating demand for siphonochilone within the pharmaceutical sector, the exploration of in vitro liquid culture systems presents another avenue for further enhancing siphonochilone synthesis and extraction efficiency, offering another valuable biotechnological tool to optimise the production of this valuable phytocompound.

## 4.5 Conclusion and Future Recommendations

This study is the first to compare the morphology, biomass accumulation and siphonochilone content in rhizomes and roots harvested from micropropagated plants with those of conventionally propagated plants of the critically endangered species, *S. aethiopicus*. Biomass accumulation in dormant rhizomes and roots harvested from mature micropropagated plants post-acclimatisation was significantly higher than in the field-grown or conventionally propagated plants. This study also showed that rhizomes and roots from micropropagated plants accumulated more siphonochilone than conventionally grown *S. aethiopicus,* thus paving the way for sustainable mass production of this important phytocompound. Although micropropagation of *S. aethiopicus* has the potential for sustainable mass production of siphonochilone, further studies are necessary to evaluate the efficacy and bioactivity of this phytocompound isolated from the micropropagated plants. The results and optimised biochemical analysis technique in this study can be used in future studies to explore the potential to increase the rate of synthesis and extraction of siphonochilone through in vitro liquid culture systems.

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# Chapter Five: General Conclusions and Recommendations for Future Research

## 5.1 Conclusions

Global plant biodiversity is declining rapidly due to factors like climate change, habitat destruction and overexploitation. This loss is particularly concerning for medicinal plants, including the endangered *Siphonochilus aethiopicus*, an important species in South African traditional medicine. To address this issue, a combination of in situ and ex situ conservation strategies, such as habitat protection and seed banking, is necessary to ensure the long-term survival of these valuable plants. The rhizomes and roots of *S. aethiopicus* are harvested for a wide range of therapeutic and religious practices. The major active phytocompound, first isolated from *S. aethiopicus* rhizomes, is the furanoterpernoid, siphonochilone. It has documented anti-plasmodial and anti-inflammatory properties, further encouraging its application and benefits in the pharmaceutical industry. The root and rhizome extracts are also added to commercial health beverages. Considering the medicinal value and the endangered status of this species, the present study set out to investigate effective strategies for its long-term conservation through the improvement of published micropropagation protocols and the establishment of cryostorage protocols.

The Global Strategy for Plant Conservation (GSPC) provides an overall framework to address plant conservation through various measures at both global and national levels. Although an ecosystem approach (in situ) to biodiversity conservation is the primary objective of the Convention on Biodiversity (CBD), improved long-term conservation of plant biological diversity requires, when necessary, ex situ measures, the protection of species outside their natural habitat in botanic gardens, and cultivated in protected areas, nurseries or seed banks. Habitat degradation deters in situ conservation for many species, including *S. aethiopicus.* Ex situ conservation thus is the alternative method, however, the space and labour required to maintain field collections are limited. Moreover, conservation through seed banking is not feasible for poor-seeding species in instances when mainly non-viable seeds are produced; such challenges exist for *S. aethiopicus,* earning it an ‘exceptional’ species status. Such species can be targeted for biotechnological approaches to germplasm conservation as performed in the present study. This was studied in two ways; first (objective one in Chapter Two) through improving on micropropagation protocols published in the literature for the species by using more suitable explants (small-sized shoot apices), and second (objective two in Chapter Three), through the cryopreservation of those shoot apices. The former also complemented the latter since an efficient protocol for propagating explants recovered from cryostorage is necessary for a successful cryopreservation protocol. Furthermore, it was deemed important to investigate that siphonochilone was present in the rhizomes and roots of the micropropagated clones (objective three in Chapter Four) as clonal populations could then be used as alternate sources of medicinal treatments, thereby relieving the pressure on natural populations somewhat.

In this study, *S. aethiopicus* plants were regenerated from in vitro-derived shoot apices. (Chapter Two). The full protocol spanned 322 days (d) which included harvest (±98 d) to in vitro culture establishment (42 d), bud culture to generate sufficient numbers of small (0.5 - 1 mm) axillary shoot apices (42 d), multiplication of those small shoot apices (42 d), then their elongation (28 d), rooting (28 d), and acclimatisation (42 d), followed by another 42 d to reach the stage of rhizome development. Despite the long duration of the protocol, it improved the yield of regenerated plants and rhizome biomass, compared with those reported in the literature (Ngwenya et al., 2010; Hannweg et al., 2016; Kunene et al., 2018). The micropropagation protocol optimised in this study, evaluated different parameters for the different micropropagation stages until post acclimatisation. During in vitro initiation, three different sodium hypochlorite surface disinfectants were tested in conjunction with a systemic fungicide treatment on emerging rhizome bud explants to test their efficacy in removing bacterial and fungal contaminants from the rhizome bud explants. Since some covert bacterial inoculum can remain undetected during initiation but can proliferate upon transfer to different micropropagation stages, the visually clean explants after in vitro initiation were screened on bacterial screening medium (Thomas, 2004) using a non-invasive screening technique to avoid any further loss of explants. This was an effective screening step that can be widely applied by propagators as no bacterial contamination was detected in any of the subsequent micropropagation stages. Small shoot apices (0.5 – 1 mm in length) were isolated from in vitro generated mother stock cultures for the multiplication stage, for which different BAP concentrations were assessed for the best multiplication rate, and the best BAP concentration (5 mg $l^{-1}$) was further assessed under two different culture systems; a semi-solid and a temporary-immersion-based culture system. The shoot apices used to induce multiplication were also excised from deep under the layers of leaves of the in vitro mother stock plantlet, providing additional sterility of the explants and avoiding the transfer of covert microbial contaminants to the subsequent micropropagation stages to yield sterile explants. A preceding elongation stage for the multiplied buds produced from the shoot apices in the presence of a reduced BAP concentration (0.1 mg $l^{-1}$) was needed to achieve 100 % survival of in vitro rooted plantlets during acclimatisation. In vitro rooting in the presence of BAP (0.1 mg $l^{-1}$) and IAA (1 mg $l^{-1}$), also induced rhizome development and successful acclimatisation and post acclimatisation of all plantlets. Conventional vegetative propagation has the potential to produce one plant from one emerging rhizome bud in 238 days but the improved *S. aethiopicus* micropropagation protocol in this study has the potential to produce 13 plants in 84 more days from one emerging rhizome bud. The plant yield in this study was much higher than conventional vegetative propagation (a single plant per bud) or previously established micropropagation protocols (3 – 4 plants per bud). Therefore, the improved protocol demonstrated in this study provides an improved means of mass-producing *S. aethiopicus* plant material for breeders, healers, nurseries and so forth. This can reduce the collection of plants from the wild, thereby reducing pressure on sparse, endangered populations. Furthermore, the protocol can provide sufficient numbers of acclimatised plants to be reintroduced into their natural habitat to augment the restoration of declining populations.

The micropropagation protocol reported here also contributes significantly to the ex situ conservation of *S. aethiopicus*. The protocol provides a source of sterile shoot apices, the germplasm of which can be successfully preserved through cryopreservation (Chapter Three). Despite the preferred parts for cryopreservation being seeds or zygotic embryos (Engelmann, 2014; Hubel and Skubitz, 2017; Pence et al., 2020) the use of in vitro-derived shoot apices as alternate explants for cryopreservation is particularly useful for poor seeding species such as *S. aethiopicus*. As cryopreservation entails genetic resources to be stored at ultralow temperatures (at -196°C in liquid nitrogen), virtually all cellular activities remain suspended (until thawing and recovery), thereby promoting long-term germplasm conservation. Prolonged culture durations and exposure to plant growth regulators can cause in vitro cells to mutate during cell division, causing somaclonal variation. This is undesirable when true-to-type clones are required, particularly in the case of important or high-biomass/phytocompound-yielding genotypes. Considering the medicinal significance of *S. aethiopicus* and its sought-after siphonochilone, it would be advantageous to implement conservation and micropropagation protocols that will minimise genetic variation in the regenerated clones. Hence, in this study, shoot apices were used to regenerate plants. By virtue of their undifferentiated meristems, shoot apices are less likely to accumulate mutations (or undergo somaclonal variation) compared to other plant tissues (Panis and Lambardi, 2006; George et al., 2008, Panis et al., 2020), leading to genetic stability. Furthermore, the reported protocol showed that shoot apices underwent direct organogenesis, bypassing the callus formation stage and reducing the risk of genetic changes that might occur (Engelmann, 2014; Hubel and Skubitz, 2017; Pence et al., 2020). The genetic stability of regenerated clones can be investigated in further studies using the appropriate molecular analyses. Shoot apices (which contain meristems) do not only provide alternate explants for in vitro multiplication, but also offer the option of synthetic seed production that can be explored further.

Vitrification-based cryopreservation protocols have been amongst the most successful of cryopreservation techniques. This is a process whereby the cytosols of cells and meristematic tissue are osmotically dehydrated stepwise in a highly concentrated vitrification solution without causing injury and transforms into a stable glass state when cooled in LN. Droplet-vitrification of organised plant tissues such as axillary shoot apices have been widely used to cryopreserve a wide range of unrelated species as it is a simple and time-efficient method. Further parameters necessitating consideration for successful cryopreservation include, but are not limited to, the type of explants, preculture conditions, cryoprotectants used, and the cooling rates. In the present study, the shoot apices were dehydrated by transferring the explants onto sucrose-enriched medium followed by cryoprotection and cooling. Preculture of the shoot apices on MS basal salt and vitamin medium with 0.09 M sucrose and 3 g $l^{-1}$ Gelrite® for 48 h and then on MS basal salt medium with 0.3 M sucrose and 3 g $l^{-1}$ Gelrite® in the dark for 24 h, followed by sequential dehydration in cryoprotection mixture (10 min), loading solution (5 min) and then in PVS2 (10 min), ensured the explants were slowly dehydrated to the desired WC for cryopreservation while still ensuring their viability. Using the droplet-vitrification method, which has not been reported before, makes this a pioneering work in the long-term germplasm conservation of *S. aethiopicus*. A recovery rate of 66.67 ± 16.67% was achieved, but this can be improved in future research. To improve the recovery rate from cryostorage, other plant vitrification solutions (e.g. PVS3) should be tested for their vitrification effects before cryostorage and other sucrose concentrations can also be examined to sequentially rehydrate cryorecovered shoot apices. In the present study, plants recovered from cryostored explants have entered the acclimatisation stage and will be maintained at the EMTC tunnel house as part of their base collection. Rhizome development is expected to occur several weeks later and then the onset of dormancy, around mid-June to early July 2025. Siphonochilone content analysis can only be performed upon dormancy to enable meaningful comparisons with the mother plants. Nonetheless, herbaria and botanic gardens have a starting point for the long-term preservation of this important species.

The third objective, as mentioned in Chapter Four, was to quantify root and rhizome biomasses and siphonochilone content using GC-MS in micropropagated plants, and then compare these with their parent/stock plants and those propagated conventionally. GC-MS was successfully used to analyse siphonochilone in the crude extract using hexane. Prior to quantifying the content of siphonchilone in the micropropagated plants, the solvent extraction procedure for the preparation of crude extracts was optimised. The solvent concentrations of the mobile phase in TLC were standardised to ensure an effective qualitative method to confirm the presence of the active compound. Siphonochilone was isolated from field-collected plants using column chromatography, the structure confirmed using NMR, and the molecular weight using LC-MS; this was used as the standard throughout the biochemical analyses of all the samples collected. The GC-MS procedure for quantitative measurement of siphonochilone was optimised, employing the field-collected plants to isolate the standard. This part of the study confirmed that dormant rhizomes and roots harvested from mature micropropagated post-acclimatised plants had an improved biomass compared to conventionally propagated plants because the micropropagated plants were first maintained under favourable controlled growth conditions, timed irrigation and fertiliser feeds. This part of the study provided the first quantitative comparison of siphonochilone yields in micropropagated plants at various stages of maturity to those of conventionally grown plants. In the context of this study, these results show that rhizomes and roots from mature micropropagated plants accumulated more biomass and siphonochilone than conventionally grown *S. aethiopicus*, which further advances sustainable and mass production of this important phytocompound. Additionally, the optimised solvent extraction and GC-MS methods significantly enhance the efficiency and reduce the time required for siphonochilone crude extract preparation and analysis, enabling streamlined future investigations.

## 5.2 Recommendations for Future Research

Droplet-vitrification was the most effective method for cryopreserving *S. aethiopicus* in this study, however, future research to optimise the cryopreperative procedures and the sequential rehydration procedures should be conducted to further improve recovery rates from cryostorage since a 66.67 ± 16.67% recovery rate from cryostorage was achieved currently. In addition, other commercially available vitrification solutions such as PVS3 or PVS4 could be examined for their vitrification effects.

To assess the long-term physiological and biochemical effects of cryopreservation on *S. aethiopicus*, further studies are needed in dormant rhizomes of post-acclimatised plantlets regenerated from cryopreserved explants Furthermore, investigations are needed to compare the presence, yield and bioactivity of siphonochilone in rhizomes and roots of post-acclimatised plants regenerated from cryopreserved explants with those of micropropagated plants. These analyses can utilise the method as established in Chapter Four for determining siphonochilone content.

Finally, micropropagated *S. aethiopicus* rhizomes and tuberous roots exhibit optimal siphonochilone yields upon maturity, a process that can extend to two years. Therefore, other speedier approaches such as cell suspension cultures can be considered for siphonochilone production at the pharmaceutical level. However, for the production of fully grown plants in cases of population restoration efforts for example, liquid culture systems such as bioreactors can be investigated in greater detail for the improvement of shoot multiplication rates, and hence, improved plant yields.

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