
# DEVELOPMENT OF A CRYOPRESERVATION PROTOCOL FOR *IN VITRO* BUDS OF SOUTH AFRICAN SWEET POTATO ACCESSIONS

by

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**A thesis submitted in fulfilment of academic requirements for the Degree of Doctor of Philosophy**

in

**Plant Science**

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02 June 2025

## DECLARATION

I, Machoene Tshidi Manamela (Student Number: 715340) declare that this Thesis is my own, unaided work. It is submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

The data collection described in this thesis was conducted at the School of Animals, Plants and Environmental Sciences, University of Witwatersrand.

(Signature of candidate)

02 June 2025

## ABSTRACT

Cryopreservation improves and strengthens the conservation of sweet potato (*Ipomoea batatas*) accessions conserved in the field gene bank and tissue culture at the South African National Plant Genetic Resources Centre (NPGRC). Cryopreservation is usually achieved at -196°C in liquid nitrogen (LN) in which the metabolic functions of the material are halted. Field gene bank and tissue culture conservation methods are used for short to medium term conservation. Cryopreservation approaches need to be developed to effectively conserve sweet potato for long-term conservation because currently, there are no long-term conservation methods used.

The encapsulation-vitrification method using plant vitrification solution 2 (PVS2) is generally used for crop species, but the success varies depending on the cultivar. In this regard, modifications were needed to obtain successful cryopreservation of the sweet potato accessions of the South African NPGRC. It was believed that the encapsulation-vitrification method using PVS2 as a cryoprotectant would result in a higher percentage of regeneration compared with other cryopreservation methods. This study aimed to assess the buds' viability, water content and optimise the concentration and duration of exposure to cryoprotectants for successful cryopreservation, using the encapsulation-vitrification method.

The use of PVS2 resulted in no plant regeneration after retrieval from liquid nitrogen (LN) suggesting that it may have been toxic to these accessions. Therefore, it was important to understand the impact of the individual and combination components of PVS2 on the viability of the buds of sweet potato. The suitable concentration of combination components of PVS2 was PVS5% which resulted in 93% regeneration. However, the materials did not survive the cooling and rewarming processes indicating too higher water content to survive cooling and rewarming.

After treatment with preincubation, preculture, osmoprotection and PVS5% increased the water content from 26 to 72%. Swapping PVS5% with osmoprotection solution reduced the water content to 34%, but the regeneration was negatively impacted. Reducing exposure to the osmoprotection solution to 20 minutes resulted in 70–80% regeneration but did not provide sufficient dehydration for successful

cryopreservation. Modified Plant vitrification solution 3 (70%PVS3) applied sequentially with PVS5%, osmoprotection solution and physical dehydration, retained the regeneration rate (83–87%) and no crystallisation during cooling in LN. Recrystallisation occurred only during rewarming process in 1.2 M sucrose at 38–40°C. The combination treatment (PVS-PD) applied in this study exhibited potential in sweet potato cryopreservation and warrants further investigation to refine the rewarming technique following cryopreservation.

## DEDICATION

To God of my Ancestors

To myself

## ACKNOWLEDGEMENTS

I attribute my achievement in this research project to the unwavering tenacity provided in me by the God of my ancestors.

I would like to express my heartfelt thanks and gratitude to the following:

My beloved daughter, Itumeleng, your unwavering support and willingness to listen to my frustrations have given me the courage to face life's challenges. Your selflessness in taking on the role of deputy mother to your siblings, Thapelo and Malebo, has lightened my load and allowed me to focus on overcoming obstacles. The memories we have shared, from sibling rivalry to games, tears and laughter, continue to uplift me every day.

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I want to express my heartfelt appreciation to my mother, Reggy Manamela for providing unwavering support and having faith in my abilities to succeed till the very end. To my father, Jacob Manamela, you provided me with the background and wisdom that I can face anything without fear.

The people who have supported me throughout this journey are a blessing. Though I cannot name all of you, you know who you are. Thank you to my spiritual friends, well-wishers and colleagues at DALRRD and Wits University.

## RESEARCH OUTPUTS

### Conferences:

Machoene Tshidi Manamela and David J. Mycock. Determination of the optimal concentration of plant vitrification solution for shoot tip cryopreservation of South African sweet potato (*Ipomoea batatas*) accessions. 7th International Workshop on Desiccation Sensitivity and Tolerance across Life Forms (DESWORK 2016), Aquila Private Game Reserve, South Africa, 11 – 15th January 2016.

### Publications:

Responses of the buds of three South African sweet potato (*Ipomoea batatas*) accessions to different cryoprotectants. *CryoLetters* 40: 357-366

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| | | |
|---|---|---|
| BAP | - | Benzyl-Aminopurine |
| CBD | - | Conservation on Biological Diversity |
| DAFF | - | Department of Agriculture, Forestry and Fisheries |
| DALRRD | - | Department of Agriculture, Land Reform and Rural Development |
| DMSO | - | Dimethyl sulfoxide |
| EG | - | Ethylene Glycol |
| FAO | - | Food and Agriculture Organisation |
| G | - | Glycerol |
| ITPGRFA | - | International Treaty on Plant Genetic Resources for Food and Agriculture |
| MS | - | Murashige and Skoog (1962) minerals and vitamins |
| NPGRC | - | National Plant Genetic Resources Centre |
| LN | - | Liquid Nitrogen |
| LS | - | Loading Solution |
| LSD | - | Least Significant Difference |
| PGRFA | - | Plant Genetic Resources for Food and Agriculture |
| PVS2 | - | Plant Vitrification Solution 2 |
| PVS3 | - | Plant Vitrification Solution 3 |
| ROS | - | Reactive Oxygen Species |
| Second GPA | - | Second Global Plan of Action |

# ANNEXURES

## ANNEXURE 1.1. Plant species conserved in different institutes worldwide. (from Nagel *et al*., 2024)

**Supplemental Table S1**. Species cryopreserved worldwide

| Institute | Country | Common name | Species | Cryopreservation method | Explants | Total number | Number of cryopreserved accessions | Reference |
|---|---|---|---|---|---|---|---|---|
| Bioversity International, Leuven | Belgium | Banana | *Musa* spp. | Droplet vitrification | Shoot tips | 1700 | 1258 | B. Panis, personal communication, 2023 |
| Bioversity International, Leuven | Belgium | Stevia | *Stevia* spp. | Droplet vitrification | Shoot tips | 10 | 10 | B. Panis, personal communication, 2023 |
| Chinese Academy of Agricultural Sciences, Institute of Crop Sciences (CAAS/ICS) | China | Mulberry | *Morus* spp. | Dormant bud freezing | Dormant buds | 2064 | 3 | Zhang et al., 2014 |
| Chinese Academy of Agricultural Sciences, Institute of Crop Sciences (CAAS/ICS) | China | Banana | *Musa* spp. | Droplet vitrification | Shoot tips | 280 | 14 | Zhang et al., 2014 |
| Chinese Academy of Agricultural Sciences, Institute of Crop Sciences (CAAS/ICS) | China | Plum | *Prunus* spp. | Vitrification | Shoot tips | 969 | 6 | Zhang et al., 2014 |
| Chinese Academy of Agricultural Sciences, Institute of Crop Sciences (CAAS/ICS) | China | Peach | *Prunus* spp. | Desiccation | Pollen | 410 | | Zhang et al., 2014 |
| Chinese Academy of Agricultural Sciences, Institute of Crop Sciences (CAAS/ICS) | China | Wasabi | *Eutrema japonicum* | Encapsulation-dehydration | Shoot tips | | 1 | Zhang et al., 2014 |
| Chinese Academy of Agricultural Sciences, Institute of Crop Sciences (CAAS/ICS) | China | Potato | *Solanum tuberosum* | Droplet vitrification | Shoot tips | 1986 | 24 | Zhang et al., 2014 |
| Chinese Academy of Agricultural Sciences, Institute of Crop Sciences (CAAS/ICS) | China | Sweetpotato | *Ipomea spp.* | Droplet vitrification | Shoot tips | 1,136 | 5 | Zhang et al., 2014 |
| International Center for Tropical Agriculture (CIAT), Cali | Colombia | Cassava | *Manihot* spp. | Droplet vitrification | Shoot tips | | 200 | Panis et al., 2020 |
| Crop Research Institute (CRI), Prague | Czech Republic | Garlic | *Allium* spp. | Droplet vitrification | Shoot tips | 714 | 157 | Panis et al., 2020 |
| Crop Research Institute (CRI), Prague | Czech Republic | Potato | *Solanum tuberosum* | Droplet vitrification | Shoot tips | 2638 | 100 | Nagel et al., 2022 |
| AFOCEL, Paris | France | Elm | *Ulmus* ssp. | Dormant bud freezing | Dormant buds | | 440 | Niino & Arizaga, 2015 |
| INRAE, Institute for Genetics, Environment and Plant Protection (IGEPP) | France | Potato | *Solanum tuberosum* | Vitrification | Shoot tips | 12,120 | 100 | Nagel et al., 2022 |
| Julius Kühn-Institut (JKI), Institut für Züchtungsforschung an Obst, Dresden | Germany | Strawberry | *Fragaria* spp. | Vitrification | Shoot tips | 459 | 194 | Höfer & Hanke, 2017 |
| Julius Kühn-Institut (JKI), Institut für Züchtungsforschung an Obst, Dresden | Germany | Apple | *Malus* | Dormant bud freezing | Dormant buds | 202 | 180 | Höfer & Hanke, 2017 |

## ANNEXURE 1.1. Plant species conserved in different institutes worldwide. (from Nagel *et al*., 2024)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Julius Kühn-Institut (JKI), Institut für Züchtungsforschung an Obst, Dresden | Germany | Pear | *Pyrus* spp. | Dormant bud freezing | Dormant buds | | 35 | Höfer & Hanke, 2017 |
| Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben | Germany | Garlic and shallot | *Allium* spp. | Vitrification | Shoot tips | 519 | 213 | Nagel, personal communication, 202 |
| Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben | Germany | Mint | *Mentha* spp. | Droplet vitrification | Shoot tips | 181 | 157 | Nagel, personal communication, 202 |
| Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben | Germany | Potato | *Solanum tuberosum* | DMSO Droplet freezing, Droplet vitrification | Shoot tips | 3317 | 2060 | Nagel, personal communication, 202 |
| Tissue Culture and Cryopreservation Unit, National Bureau for Plant Genetic Resources (NBPGR) | India | Mulberry | *Morus* spp. | Dormant bud freezing | Dormant buds | | 329 | Niino & Arizaga, 2015 |
| Tissue Culture and Cryopreservatio, Unit,National Bureau for Plant Genetic Resources (NBPGR) | India | Citrus and other tropical fruits | *Citrus* spp. | Desiccation | Seeds, embryos, embryonic axes | | 6,650 | Malik & Chaudhury, 2019 |
| National Research Council, Institute of BioEconomy (CNR-IBE) | Italy | Citrus | *Citrus* spp. | Desiccation | Polyembryonic seeds | 24 | 40 | M. Lambardi, personal communication, 2023 |
| National Research Council, Institute of BioEconomy (CNR-IBE) | Italy | Apple | *Malus* | Dormant bud freezing | Dormant buds | 28 | 23 | M. Lambardi, personal communication, 2023 |
| Genebank Project, Genetic Resources Center, National Agriculture and Food Research Organization (NARO) | Japan | Mulberry | *Morus* spp. | Dormant bud freezing | Dormant buds | 1381 | 1236 | Niino & Arizaga, 2015 |
| Genebank Project, Genetic Resources Center, National Agriculture and Food Research Organization (NARO) | Japan | Potato | *Solanum tuberosum* | V-Cryo-Plate | Shoot tips | 1890 | 600 | Nagel et al., 2022 |
| International Institute of Tropical Agriculture (IITA) | Nigeria | Yam | *Dioscorea* spp. | Droplet vitrification | Shoot tips | 5839 | 27 | Panis et al., 2020 |
| International Potato Center (CIP), Lima | Peru | Sweetpotato | *Ipomea* spp. | Droplet vitrification | Shoot tips | 5328 | 385 | CIP. Cryopreservation Online, 2023 |
| International Potato Center (CIP), Lima | Peru | Potato | *Solanum tuberosum* | Droplet vitrification | Shoot tips | 4487 | 4338 | CIP. Cryopreservation Online, 2023 |
| N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) | Russia | Potato | *Solanum tuberosum* | Vitrification | Shoot tips | 8150 | 50 | Nagel et al., 2022 |
| National Agrobiodiversity Center (NAAS), RDA, Suwon | South Korea | Garlic | *Allium* spp. | Droplet vitrification | Shoot tips | 1200 | 1158 | Kim et al., 2012 |
| National Agrobiodiversity Center (NAAS), RDA, Suwon | South Korea | Potato | *Solanum tuberosum* | Droplet vitrification | Shoot tips | 1223 | 139 | Niino & Arizaga, 2015 |

## ANNEXURE 1.1. Plant species conserved in different institutes worldwide. (from Nagel *et al*., 2024)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| USDA-ARS, Ford Collins and Corvallis | USA | Citrus | *Citrus* spp. | Droplet vitrification, | Embryonic axes, dormant buds | 1372 | 451 | Volk et al., 2019 |
| USDA-ARS, Ford Collins and Corvallis | USA | Coffee | *Coffea arabica* | Desiccation, slow cooling | Seeds, shoot tips, embryonic axes | | 63 | Acker et al., 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Hazel | Corylus spp. | Dormant bud freezing Encapsulation-dehydration | Shoot tips, Embryonic axes, dormant buds | 798 | 5 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Bermuda grass | *Cynodon* spp. | Encapsulation-dehydration | Shoot tips | 175 | 33 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Strawberry | *Fragaria* spp. | Droplet vitrification | Shoot tips | 1382 | 280 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Hop | *Humulus lupunus* | Encapsulation-dehydration | Shoot tips | 387 | 90 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Sweetpotato | *Ipomea* spp. | Encapsulation-vitrification | Shoot tips | 776 | 166 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Apple | *Malus* | Dormant bud freezing | Dormant buds | 4280 | 2155 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Mint | *Mentha* spp. | Vitrification, Droplet vitrification, Encapsulation-dehydration | Dormant buds/shoot tips | 393 | 43 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Banana | *Musa* spp. | Droplet vitrification | Shoot tips | 160 | 22 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Prunus species | *Prunus* spp. | Dormant bud freezing | Dormant buds | 130 | 12 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Mountain mint | *Pycnanthemum* spp. | Encapsulation-dehydration | Shoot tips | 32 | 61 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Pears | *Pyrus* spp. | Droplet vitrification, Dormant bud freezing, slow cooling | Dormant buds/shoot tips | 1839 | 219 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Berries | *Ribes* | Droplet vitrification, Dormant bud freezing, Slow cooling | Dormant buds/shoot tips | 716 | 79 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Berries | *Rubus* | Droplet vitrification, Slow cooling | Dormant buds/shoot tips | 706 | 187 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Sugarcane | *Saccharum* spp. | Droplet vitrification | Shoot tips | 408 | 40 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Willow | *Salix* spp. | Dormant bud freezing | Dormant buds | 60 | 25 | Jenderek & Reed, 2017 |
| USDA-ARS, Ford Collins and Corvallis | USA | Potato | *Solanum tuberosum* | Droplet vitrification | Shoot tips | 1168 | 369 | Jenderek & Reed, 2017 |

# CHAPTER ONE: GENERAL INTRODUCTION AND LITERATURE REVIEW

Plant genetic resources for food and agriculture (PGRFA) such as sweet potato are a vital component of agro-biodiversity. These resources contribute to the raw genetic material of global food security for generating adaptive crops that can tolerate a range of abiotic and biotic factors (FAO, 2019).

The decline in sweet potato landrace production in South Africa is influenced by the fading interest of younger generations and a growing reliance on commercial varieties. On the other hand, activities for the maintenance of these crops were temporarily interrupted during the COVID-19 pandemic, which put these valuable resources at risk. These issues highlight the critical importance of improving and strengthening the conservation of landrace crops, such as sweet potato, for food security and future breeding purposes (FAO, 2023). Strengthening conservation strategies can be achieved through conservation methods such as cryopreservation and tissue culture, which is already applied to the National Plant Genetic Resources Centre (NPGRC) collection. Increasing the use of cryopreservation, which relies on the use of LN, can improve and strengthen the conservation of PGRFA which are important for food security and future breeding purposes.

Various laboratories have made progress in developing cryopreservation protocols for sweet potato as documented by Park and Kim (2015), Wilms *et al*. (2020) and Shevchenko *et al*. (2021). However, the South African NPGRC currently lacks a protocol for its sweet potato accessions. Furthermore, the varied responses of different genotypes to cryopreservation make it challenging to apply existing protocols to the entire sweet potato genotype (Park and Kim, 2015; Wilms *et al*., 2020; Shevchenko *et al*., 2021). This thesis aims to explore the use of vitrification-based techniques as a cryopreservation protocol for sweet potato accessions at the South African NPGRC.

## 1.1. Sweet Potato

*Ipomoea batatas* is a dicotyledonous root tuber crop belonging to the morning glory family (Convolvulaceae). Initially, it was described by Linnaeus as *Convolvulus batatas* in 1753 and later reclassified by Lamark in 1971 within the genus *Ipomoea* (Huaman, 1992). In this family, there are 60 genera and 1000 species. Sweet potato is a

hexaploid (2n=6x=90) (Huaman and Zhang, 1997; Ramanathan and Hermann, 2001) and the only cultivated, edible species in the family (Woolfe, 1992).

Historical evidence states that the sweet potato was first cultivated in the New World, specifically in the lowlands of Central or South America (Huaman, 1992). It is believed that indigenous communities in South America began growing this crop as early as 3,000 BC (Woolfe, 1992). It was then introduced to the rest of the Pacific Asia by the Polynesians (Zhang *et al*., 2004; Ladefoged *et al*., 2005). With time, it was brought to Europe and Asia, and eventually made its way to Africa by the 16th century (Allemann *et al*., 2004). Upon the Dutch colonisation of the Cape in 1652, sweet potato was introduced to South Africa (Bester and Louw, 1992).

Sweet potato is ranked the eighth most-produced crop globally, trailing behind wheat, rice, maize, potato, banana, soybean and cassava (FAO, 2023). Globally, China is the largest producer of sweet potato, followed by Malawi in Africa (FAO, 2023). In terms of cultivation and consumption, sweet potato is the third major food root crop after cassava and potato, in Eastern and Southern Africa. This highlights the crucial role sweet potato plays in food security and nutrition in these areas of Africa (Ewell and Mutuura, 2003; Parker *et al*., 2019).

There is a lot of morphological diversity in the species as colour of the stem and leaves can vary from green to purple (CIP, AVRDC, IBPGR, 1991; Laurie and Niederwieser, 2004). The petioles are between 50–300 mm long and the general leaf outline ranges from almost divided to round. The leaf margins are from deeply lobed to no lateral lobes (CIP, AVRDC, IBPGR, 1991).

The storage root shape and size range between curved or long irregular and round due to a range of environmental factors as well as the variety (Figure 1.1) (CIP, AVRDC, IBPGR, 1991; Woolfe, 1992). The storage root periderm is smooth and ranges from white to dark purple, with white to orange flesh colour in different varieties (Laurie and Niederwieser, 2004). The flowers are purple with white margins (Figure 1.1), resembling those of the morning glory (a horticultural member of the family), but flowering is dependent on environmental conditions (DAFF, 2011).
Due to its heterozygous nature, sweet potato is grown through vegetative propagation using stem cuttings, transplants and root sprouts from mother roots (Woolfe, 1992;

van den Berg and Laurie, 2004). It is thought that hexaploidy cultivated sweet potato varieties have formed from hybridization between early tetraploid and weedy diploid sweet potatoes (Huaman and Zhang, 1997; Ramanathan and Hermann, 2001).

Although sweet potato has a perennial life history, it is grown annually in moderate to slightly acidic and appropriately aerated (pH 5.6–5.8), sandy to sandy-loam soils (van den Berg and Laurie, 2004; DAFF, 2011). In a global context, sweet potato can be harvested between four to six months, depending on the variety and conducive temperature (Woolfe, 1992). Although it is sensitive to frost or cold weather below 15ºC (personal observation; DAFF, 2011), sweet potato can tolerate moderate drought, but yield can be lower if drought took place at the initial tuber development (DAFF, 2011; DALRRD, 2021).


**Figure 1.1.** Sweet potato leaves showing different shapes (a-c), purple petioles and vines (d) and different storage root shapes (e, f).

The extended storage root of sweet potato accumulates more nutritious components (fiber, vitamins, potassium, zinc, calcium) per unit volume than most other tuber crops, making them ideal for animal feed and human consumption (Ewell and Mutuura, 2003; Hossain *et al*., 2022). In comparison with other root crops, sweet potato also has lower fat content and protein, but contains minerals, carbohydrates and high quantities of secondary metabolites beneficial for health (Oke and Workneh, 2013, Woolfe, 1992). The high levels of β-carotene (which is a precursor of Vitamin A), contribute to human nutrition and health, particularly for children (Woolfe, 1992). Additionally, the purple-fleshed sweet potato tubers contain carotenoid pigments with protective antioxidants that have been suggested to have anti-inflammatory, anti-cancer and anti-diabetic potential (Oke *et al*., 1999 Motsa *et al*., 2016; Escobar-Puentes *et al*., 2022). Their leaves have been reported to reduce risk of cardiovascular diseases (Yamakawa and Yoshimoto *et al*., 2002; Escobar-Puentes *et al*., 2022).

The multiple uses of sweet potato have transformed the crop from simple “poor man’s crop” to an important crop (Oke and Workneh 2013). Some of these uses include being dried to make snacks, flakes and chips, pureed (for pie fillings, sauces, frozen patties and baby foods; Truong and Ramesh, 2010), pasta, confectionery, ice cream, tarts or other baking products; sweet potato flour and fries, noodles and as raw material for alcohol production (DAFF, 2011). As such for many communities in South Africa, consuming sweet potato helps to meet suggested dietary and nutritional requirements. However, sweet potato tuber processing in South Africa has traditionally been narrowed to eating fresh, boiling, roasting in oven or open fire or cooking in microwave oven (personal observation).

In South Africa, sweet potato can grow well with proper cultivation practices, making it a valuable crop with effective local and export markets. The primary production areas of sweet potato in South Africa are in the Limpopo province, the Nelspruit district of Mpumalanga, KwaZulu-Natal, Eastern Cape, Gauteng, Free State and the Western Cape provinces (DAFF, 2011; DALRRD, 2021). Sweet potato’s annual production reached about 81 000 tons (in 2020), with approximately 61 000 tons consumed locally in 2020 and the remainder exported to other countries such as the United Kingdom, Netherlands, Belgium, United Arab Emirates, Namibia, Botswana, Angola and France, and a small portion of the export to other African countries (DALRRD, 2021; FAO,

2023). These exports demonstrate that South Africa is self-sufficient in sweet potato production.

Sweet potato is a vital crop for small-holder and subsistence farmers in South Africa, especially in areas where it is commonly grown. It provides both food security and a source of income, especially since some South African varieties can be harvested in just three to six months (DAFF, 2014; DALRRD, 2021). Additionally, sweet potato is often regarded as a valuable money-making crop since it is typically ready for harvesting before other crops have fully matured (DAFF, 2011).

Due to climate change impacts that threaten food security, many farming communities have started relying on improved crops that grow faster with higher yields (Veteläinen *et al*., 2009; Maxted *et al.,* 2011). As such, there is a rising demand for new varieties adaptable to the expected changing environmental conditions to be developed (Veteläinen *et al*., 2009; Maxted *et al.,* 2011; Fatehi *et al*., 2021).

One challenge in the context of climate change is the difficulty of predicting which pests and pathogens will thrive due to shifting rainfall patterns and other environmental changes associated with climate change (FAO, 2019; Garrett *et al.*, 2006; Agarwal and Agarwal, 2007; Miranda *et al*., 2011; Chakraborty *et al*., 2020). To mitigate this risk, some reports (e.g., Veteläinen *et al*., 2009; Padulosi *et al.,* 2012) suggest that agricultural diversity, including landraces, can be used as a buffer against these unpredictable changes. As such, it is crucial to improve the conservation of agricultural diversity, especially PGRFA like sweet potato, given its vital role in promoting food security and reducing poverty. For this reason, NPGRCs have been established to safeguard these essential resources for the benefit of food and agriculture (Veteläinen *et al*., 2009; Padulosi *et al*., 2012).

### 1.2. Background of the National Plant Genetic Resources in South Africa

Several international conventions and agreements [International Treaty on Plant Genetic Resources for food and agriculture (ITPGRFA), Convention on Biological Diversity (CBD) and the Second Global Plan (Second GPA)] have documented the importance and necessity for conservation and sustainable use of PGRFA. All these encourage governments to ensure the conservation of PGRFA as they are a key

component in alleviating poverty and ensuring sustainable agriculture for future generations (FAO, 2019).

Following established conventions and agreements, the South African NPGRC currently operating within the Department of Agriculture, Land Reform and Rural Development (DALRRD) has been entrusted with the responsibility of implementing the Second GPA. With its 18 priority activities, the Second GPA focuses on the importance of preserving and expanding *ex-situ* conservation of germplasm (FAO, 2011).

The NPGRC in South Africa has developed a National Plan to ensure the conservation and sustainable use of PGRFA. Among the activities outlined in the national plan, is the need to conduct research on conservation protocols for significant vegetatively propagated species to South African agriculture. In light of this, it is essential to investigate conservation protocols for sweet potato, as one of the fundamental crops for food security crops, to strengthen and enhance its conservation. The conservation methods currently used at the South African NPGRC are detailed below.

### *1.2.1. In-situ conservation*

*In-situ* conservation of plant species is defined as conservation in the natural environment where they developed their distinguishing characteristics (Maxted *et al*., 1997; CBD, 1992). This approach can be accomplished by the setting up of genetic reserves (e.g. national parks) or on-farm. Jarvis *et al.* (2000) highlighted that on-farm conservation is critical for the ongoing process of evolution and adaptation of crop plants in their environment. In this regard, the NPGRC of South Africa is currently applying on-farm conservation, multiplication and encouraging communities to develop their own seed banks in different areas of the country. This approach inspires subsistence farmers to continue growing landraces on their farms as another approach to *in-situ* conservation. Thus, apart from ensuring food security, the crop germplasm is conserved and allowed to evolve (Tao, 2003).

Reed *et al*. (2004) emphasised that there is no single perfect solution for conserving plant species. While *in-situ* conservation methods have their advantages, they are not without drawbacks. These drawbacks include limited access for plant breeders to the genetic material being conserved; genetic erosion due to unforeseen circumstances

like natural disasters, war, social and economic change. These factors may impede on-farm biodiversity conservation over time, ultimately impacting food security and nutrition (Jarvis *et al.*, 2000; FAO, 2022). Unfortunately, the South African NPGRC is facing similar challenges including, limited access to germplasm due to changes in rain patterns, which lead to genetic erosion of previously conserved material. Furthermore, the loss of interest and commitment by traditional farmers' descendants has also been acknowledged (pers. comms. South African subsistence farmer communities).

A comprehensive conservation approach for plant genetic resources involves a blend of *in-situ* and *ex-situ* conservation methods. This approach, according to Reed *et al*. (2004), ensures adequate duplicates of accessions in both field and *ex-situ* and if required, in the *in-vitro* condition.

### *1.2.2. Ex-situ conservation*

*Ex-situ* conservation entails plant germplasm storage out of their natural environment to ensure their longevity, viability, and availability (Cohen *et al*., 1991; CBD, 1992; Maxted *et al*., 1997). This type of conservation uses a variety of techniques for different plant materials, such as cold storage of seeds or propagules and *in-vitro* (tissue culture and cryopreservation).

Throughout agricultural evolution, humans began collecting seeds from plants with the most desired traits for planting the following year, a practice now known as selective breeding (Maxted *et al*., 1997; Keller, 2005). Over the years, this led to the identification of specific traits which resulted in breeding specific plant varieties to obtain desired traits such as drought tolerance, disease resistance, better taste and higher yield (Bidinger, 1992; Dhillon *et al*., 2003; Keller *et al*., 2005).

#### *1.2.2.1 Seed conservation*

*Traditional Seed Storage*

Farmers have applied various traditional storage practices to avoid loss of seeds due to animals and microorganisms, as well as to maintain the quality of seeds. A few of these practices include storage in sealed containers or calabashes, clay-baked jars, bottle gourds, closed granaries of ash, herbs, sand, salt, cow dung or oil. Such practices vary between countries and communities (Almekinders *et al*., 1994; Li and

Wu, 1996; Karthikeyan *et al*., 2009; Matsa and Mukoni, 2013). The most common way for storing maize, millet and sorghum is the hanging of husks on the roofs of places that use firewood, then the smoke from firewood serves as a treatment of seeds against infestation by insects and microorganisms (personal observation).

According to a study conducted by Matsa and Mukoni (2013) in Zimbabwe, it was found that treating seeds before storage is crucial. They found that when the seeds of melons and pumpkins that were sun- and air-dried instead of being treated with the smoke, were spoiled. The study also noted that pumpkin and watermelon seeds treated with smoke, could be stored for up to a year or until the next planting season, without pest infestation. Similarly, beans, groundnuts and peas treated with smoke can be stored with their natural coverings until the next planting season.

*Conventional seed storage*

As noted by Harrington (1960), seed is the most convenient way of storing and distributing plant germplasm. Over time, gene banks have evolved to preserve seed viability and conserve genetic diversity in limited space (Hong and Ellis, 1996).

Gene banks are widely used for *ex-situ* conservation by drying and storing seeds at low temperatures and low relative humidity (Fassil and Engels, 1997). However, this method is only suitable for seed-producing species that can withstand storage at low seed water contents of 5% or less (Hong and Ellis, 1996). Such seeds (orthodox seeds) naturally lose water to the atmosphere at the prevailing relative humidity as they mature, a process known as maturation drying, which allows them to remain viable for decades under proper storage conditions of low temperatures and low relative humidities (Ellis *et al*., 1990).

The successful storage of germplasm depends on the species' physiological characteristics, the quality of the material being stored and the chosen storage method (Villabos and Engelmann, 1995). At the South African NPGRC, the base collections of orthodox seed are stored for the long-term below 0°C usually at -18–20°C whilst the active collection accessions are stored at 4°C to maintain at least 65% viability for 10-20 years (FAO, 2014).

Base collections are used merely to regenerate active collections. The active collection is used for distribution to areas that need restoration and rehabilitation in damaged/destroyed crop production areas, in particular back to the areas where they were initially collected. Moreover, active collections can also be important for utilisation and exchange purposes as well as monitoring and evaluation (Qhobela and Marandu, 2009; FAO, 2014).

According to Engelmann (2004), most crops produce orthodox seeds. However, some crops do not produce seeds, have sterile genotypes, are highly heterozygous (not true-to-type) (Radha *et al.*, 2012), or have large seeds. Such species require other approaches to propagation and storage e.g., vegetative propagation (Villabos and Engelmann, 1995). As an example, collections of sweet potato can be conserved as clones in field gene banks, under glasshouse conditions, *in-vitro* plantlets in the laboratory or by cryopreservation in LN (Reed *et al*., 2004).

#### *1.2.2.2. Field gene banks*

Field gene banks are used for conservation of the germplasm that cannot be easily conserved as seed or when the crop is propagated vegetatively. The South African NPGRC uses shade houses as field gene banks. Shade houses are structures that provide an ideal climate for promoting plant growth by allowing the required air, moisture and sunlight to pass through their shade nets. Field gene bank entails the continual growth of plant materials and necessitates continuous maintenance in the field (Reed *et al*., 2004). Field gene banks are mainly used to conserve vegetatively propagated crops such as sweet potato, banana, cassava and tropical and temperate fruit tree species (Engelmann *et al.,* 2003). This method provides easy access to the collection for research and utilisation purposes. e.g., characterisation and evaluation. Furthermore, continual evolution and natural selection can occur in the long-term (Engelmann *et al.,* 2003; González-Benito *et al*., 2004). The disadvantage of this method is that the collection may be at risk of environmental conditions, pests and diseases.

The South African NPGRC maintains live collections of sweet potato, taro, cassava and medicinal plants in shade houses to ensure their conservation. However, this method has certain limitations, as these collections are vulnerable to natural disasters,

attacks by microorganisms and animal consumption. In fact, during characterisation of sweet potato, it was found that rodents had eaten some of the tubers in the shade house. Additionally, sweet potato is sensitive to frost and need to be harvested and stored to avoid damage and loss. It must then be replanted in the following growing season for continuous maintenance. These activities can be labour intensive and may result in accession mix-ups or loss (Keller *et al*. 2013). To address these challenges, the collection is duplicated in tissue culture, as recommended by gene bank standards (FAO, 2014).

#### *1.2.2.3. In-vitro conservation*

*In-vitro* conservation is a highly advanced and specialised form of biotechnology used to preserve vegetatively propagated and recalcitrant species (Benson, 2008a). There are two main methods of *in-vitro* conservation; tissue culture for short- to medium-term and cryopreservation for long-term storage (Reed *et al*., 2004). These methods require specialized facilities that are expensive to maintain, including clean rooms with adequate lighting, electricity, cooling systems, liquid nitrogen (LN) and ventilation (Engelmann, 2004).

#### *(i) Tissue culture*

Tissue culture has numerous applications including micropropagation, conservation, production of secondary metabolites, germplasm exchange, plant collection, crop improvement and research (Ramanathan and Hermann, 2001; Reed *et al*., 2004). Tissue culture involves propagating plant explants i.e., organs, cells or tissues isolated from the mother plant material. These explants are grown on an artificial medium, dispensed in test tubes or Petri-dishes, containing a mixture of micro and macro elements, amino acids, vitamins, carbohydrates, growth regulators and gelling agent (excluding liquid cultures which do not require gelling agent) (George, 1993). These essential nutrients are required for complete plant regrowth. A sterile environment in a laminar flow hood is required for this process (Rao, 2004). The culture vessels containing the media and explants are then placed in the growth chamber for proper growth and development of the explant.

Tissue culture is made possible through two biological concepts i.e., plasticity and totipotency. Plasticity allows parent plants to adapt their metabolism, growth and

development to suit their environment. Totipotency refers to the ability of isolated totipotent plant tissues to regenerate complete plants given the proper stimuli, expressing the total genetic potential of the parent plant (Engelmann, 2004; Rao, 2004; Reed *et al*., 2004).

In tissue culture, explants can undergo two forms of growth i.e., differentiated and undifferentiated growth. Differentiated growth leads to the development of organs like shoots, roots and embryos; while undifferentiated growth results in the formation of callus - a cluster of actively dividing cells that have not yet specialized (George, 1993).

There are various advantages and disadvantages associated with tissue culture (Table 1.1). Tissue culture offers the distinct advantage of eliminating diseases (Reed *et al*., 2004). For example, Alam *et al*. (2013) reported the elimination of diseases from sweet potato plants in their study, using the tissue culture method. Furthermore, disease-free material is produced under aseptic conditions in tissue culture (Vir and Kumar, 2021). In their study, Laurie *et al*. (2016) reported success in using tissue culture technology together with a disease testing scheme, to produce disease-free sweet potato material which was supplied to producers in South Africa for food security. However, some pathogens can be difficult to eradicate, which require other procedures such as meristem culture, thermotherapy, chemotherapy and cryotherapy (Wang *et al*., 2009; Benson *et al*., 2011; Vir and Kumar, 2021).

*Types of media*

Different plant species have unique requirements, so there are several types of media with different compositions, such as Murashige and Skoog (MS), Nitsch and Nitsch, Gamborg's B5, N6, and Driver and Kuniyuku walnut. MS is the most commonly used because its composition of macronutrients, micronutrients, vitamins and organics is highly suitable for majority of plant species, with the ability to promote increased plant growth and root formation (Kumar and Reddy, 2011; Sá *et al*., 2018).

*Contamination*

Contamination poses a major challenge in tissue culture; hence proper sterilisation of the explant and the laboratory equipment are crucial for successful tissue culture. It is important to consider the type and quality of chemicals used, as impurities can lead to contamination and hinder explant growth (George *et al*., 2008; Phillips and Garda,

2019). Additionally, the choice of explant (such as leaf, petiole, internode, stem, and age) should be based on the plant species and reason for tissue culture use (Reed *et al*., 2004).

**Table 1.1.** Advantages and disadvantages of tissue culture technique (compiled from George, 1993; De Klerk, 2007; Benson, 2008a)

| Advantages | Disadvantages |
|---|---|
| No interference from environmental factors | Failure to reproduce due to contamination, browning of the media, humidity of the vessel |
| Independent on the season | Hyperhydricity and stunted growth |
| Mass-production of plants in a short period | Genetic instability |
| Production of disease-free plants | Labour intensive |
| Require little space | Propagated plants may be less resilient when exposed to the outside environment |
| Conservation of endangered species, recalcitrant species and vegetatively propagated plants | Advanced technique requiring knowledge and practice |
| Study of plant physiology | Expensive to set up and maintain due to specialised equipment |
| Genetic modification of plants | |

*Humidity and temperature*

Maintaining ideal humidity levels is also vital, as high humidity increases the risk of fungal infection while low humidity dries out the culture. Similarly, the intensity of light should be carefully regulated to prevent bleaching or etiolation of the material. It is crucial to establish the specific light requirements for each genus, species, variety and cultivar to achieve successful storage (Engelmann, 1997; Ramanathan and Hermann, 2001; Reed *et al*., 2004).

For successful tissue culture conservation, it is essential to provide each plant species with the appropriate temperature during storage. Some plant species can withstand colder temperatures ranging between 0–5°C, while others are more sensitive and need temperatures between 15–22°C. For instance, Keller *et al*. (2006) found that *Allium* species at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) thrived in tissue culture at a temperature range of 2–10°C. However, the sweet potato

collection at the NPGRC was found to tolerate only 15–27°C, as exposure to <15°C *in-vitro* resulted in stunted growth (data not shown).

*Uses of tissue culture method*

*Collection of samples*

Some plant species may produce non-viable seeds, while others have large, fleshy seeds that are not likely to remain viable during transportation, especially if the collecting site is remote from the gene bank. Tissue culture method is therefore used to collect such species. Depending on the species and the purpose for collecting, various parts of the plant such as leaves, nodal segments, shoot tips, embryos, roots and flower buds can be collected.

The collection process involves selecting the plant material, surface sterilisation with disinfectants such as sodium hypochlorite and 70% ethanol and washing off the sterilising solution. Following that, damaged tissues of the plant material are cut off, then the plant material is inoculated by placing the plant material into the culture vessel containing nutrient medium. The vessels are then transported to the gene bank and processed in the laminar flow cabinet to prevent contamination. The plant material is removed from the culture vessel and further sterilised then inoculated into the nutrient medium, followed by storage in the gene bank (Pence *et al*., 2002; Rao, 2004; Reed *et al*., 2004). Tissue culture collecting methods have been developed for a variety of species, including oil palm, banana, forage grass, coffee, grapes, coconut and citrus species (Assy Bah *et al*., 1987; Withers and Engelmann, 1997).

*Exchange of germplasm*

Another benefit of tissue culture is the opportunity for countries to exchange germplasm, which is facilitated by more flexible quarantine restrictions. This can enhance genetic diversity and promote agricultural innovation (Rao, 2004; Reed *et al*., 2004; Benson *et al*., 2011).

*Conservation and micropropagation*

Tissue culture is an effective method for plant conservation, which allows for short- to medium-term storage i.e., several months up to a few years, known as *in-vitro* active gene banking. There is also the slow-growth conservation in which the environmental conditions and the culture medium are modified. This results in the increased interval

between subcultures (Engels and Ebert, 2021). The most usual methods applied to achieve slow growth are physical and chemical/nutrient growth limitations.

Physical growth limitations are applied through the reduction in temperature and/light intensity, minimal supply of nutritional elements or alteration of the gaseous environment. Additionally, the type and volume of culture container and type of culture tube closure are adjusted. The limitation of growth using chemicals involves the inclusion of osmotic or other growth retardants such as mannitol, abscisic acid, chlorophonium chloride, polyethylene glycol and dianuazide (Engelmann, 1997; Rao, 2004; Reed, 1999; Benson *et al*., 2011).

These growth limitations can trigger somaclonal variation, callus formation, ploidy instability and epigenetic change mediated through DNA methylation (Harding, 2004; Benson *et al.*, 2011). Somaclonal variation is defined as the general term for all the genetic variability encountered in plants. Variation usually occurs during the dedifferentiation phase and characterises plants regenerated from callus, protoplasts, leaf pieces and petioles. However, somaclonal variations are not common in plants regenerated from meristems, shoot tips and axillary buds (Kaviani 2011; Kaczmarczyk *et al*., 2012). Axillary buds were used in this study, as such the implications associated with somaclonal variation should be limited.

Tissue culture material involves repeated sub-culturing, which includes transferring specimens to fresh medium (Rao, 2004). Sweet potato internode cuttings can take up to four weeks to develop into complete plantlets with roots before sub-culturing. Depending on the number of accessions to be sub-cultured, this method may be expensive and subject to loss due to handling (human) error, contamination and/or somaclonal variation and mislabelling (Engelmann, 1991; Rao, 2004; Charoensub *et al*., 2004, FAO, 2014). Another use of tissue culture is micropropagation ─ a technique used to generate numerous plantlets in a controlled environment on growth media (Chaundhury *et al*., 2003).

The National Plan developed by the NPGRC of South Africa stipulates the strengthening of research on conservation protocols applied to the PGRFA. In this regard, the conservation of sweet potato collection needs to be strengthened by duplication in all of the available conservation methods. It is therefore important to

develop complementary safety duplicate techniques for long-term conservation such as cryopreservation, as recommended by the gene bank standards (FAO, 2014). This is especially true for vegetatively propagated species such as sweet potato, that have traditionally been maintained in field gene banks and tissue culture at the South African NPGRC. It is worth noting that *in-vitro* collection must serve as a back-up option, not as a replacement of field collections, as this is also essential for the secure conservation of genetic diversity (Engels and Ebert, 2021).

***(ii) Cryopreservation***

Based on the limitations and risks associated with field and tissue culture conservation methods, cryopreservation is considered the only long-term conservation method for *in vitro* material. 'Cryo' is derived from the Greek word '*kruos*' meaning icy cold/ frost; and cryopreservation is a method in which biological tissues are stored at sub-zero temperatures for long periods (Chaundhury *et al.,* 2003; Mandal *et al.,* 2003). This is usually achieved in LN at -196°C and/or nitrogen vapour from -160 to -190°C. At this temperature, the metabolic functions of the plant cells and tissues are virtually halted (Towill and Jarret, 1992, Sakai, 2007, Benson *et al*., 2011; Panis *et al*., 2020), for indefinite periods. Thereafter, the explants are regenerated when required (Engelmann, 2004; Penmetcha and Arali, 2011).

Plant species require their unique temperature optimum for proper growth and development. Most plant material cannot be frozen without affecting cell viability. This is due to injury associated with intracellular water transformation from the liquid to the crystalline state. The growing ice in the extracellular spaces of the cells causes the intracellular water to be lost. This further dehydrates the cell causing freezing injury (Benson, 2008b). However, some plants survive winter without injury due to antifreeze (specific carbohydrates and proteins) accumulation, which they secrete into their apoplast to inhibit growth of extracellular ice and/or accumulate in the cytomatrix (Griffith and Yaish, 2004).

Similarly, animal species such as wood frogs, fish and some insects (Constanzo *et al*., 2015), that can withstand freezing temperatures produce ice nucleators that trigger extracellular ice formation (Moffatt *et al*., 2006). This process causes water to be drawn from cells and initiates extracellular ice formation. However, not all intracellular water is withdrawn. This is due to the accumulation of low molecular weight solutes,

which increase the osmotic potential and stabilise the structure of cells, membranes and proteins. As a result, residual water remains, allowing these organisms to function even after the freeze-thaw cycle (Moffatt *et al*., 2006; Popova *et al*., 2023).

Plants are limited in their ability to adapt to unexpected changes in their environment, such as extreme temperatures, water scarcity and pathogenic threats (Mahajan and Tuteja, 2005). Thus, for plant species that can tolerate low temperatures, mother plants undergo cold conditioning at 1–4°C for several days before explants are taken for cryopreservation. However, for tropical species like sweet potatoes, which require temperatures between 21 and 29°C for optimal yields, cold conditioning is not possible (van den Berg and Laurie, 2004; DAFF, 2011). Some varieties of sweet potato can tolerate temperatures as low as 15°C and as high as 35°C when planted in the field.

Withdrawing water from the plant tissues should prevent growth, but some tissues continue to grow even in a heavily reduced amount of water. Cooling is therefore an alternative method that makes water unavailable to metabolic processes, allowing for successful preservation (Gonzalez-Arnao *et al*., 2008; Keller *et al*., 2008).

When water is exposed to extremely low temperatures, it freezes and forms a solid. This solid state inhibits molecular movements, which ultimately leads to the cessation of biochemical reactions and diffusion. Consequently, the cells can be maintained at low temperatures without compromising their viability, thereby enabling them to be preserved indefinitely in a state of suspension, thus cryopreservation. For this to happen, cells must be sufficiently dehydrated to prevent crystallisation from causing damage to the membrane structure (Panis, 2019; Engelmann, 2004; Griffith and Yaish, 2004). However, to achieve successful cryopreservation, it is crucial to have regeneration systems in place to ensure the complete recovery of plants after thawing (Al-Bahrany and Al-Khayri, 2012).

*Benefits of cryopreservation*

Cryopreservation offers several benefits such as low maintenance and limited space usage (Engelmann, 2004), preventing microbial growth during storage and preventing physiological deterioration resulting from metabolic inactivity in tissues (Engelmann, 1997; Gonzalez-Arnao *et al*., 2008; Keller *et al*., 2008). For *in-vitro* tissues, the material

can be stored for extended periods without frequent sub-culturing, decreasing the likelihood of somaclonal variation.

It is worth noting, however, that the initial costs associated with cryopreservation can be costly due to expensive equipment. Nevertheless, ongoing maintenance is typically limited to weekly monitoring and replenishment of LN as required in cryo dewars (Reed, 2004). Moreover, once the material is successfully stored in cryopreservation, there is a reduced risk of contamination (Engelmann, 2004; Kaczmarczyk *et al*., 2012).

*Factors affecting the success of cryopreservation*

The success of cryopreservation largely depends on the water content of the sample to be cryopreserved (Kaczmarczyk *et al*., 2012). It is important to remove water from the cell while maintaining enough to sustain it (Gonzales-Arnao, 2008). To avoid lethal ice formation, samples to be cryopreserved must be dehydrated to a moisture content that will not cause freezing injury (Berjak, 1996; Engelmann, 2004), but also prevent desiccation damage.

Ice crystals occupy a greater volume in the cell due to their lower density in comparison with water (Benson, 2008b; Kim and Popova, 2023). As more water is converted to ice, intracellular organelles experience increased pressure, leading to higher solute concentration in the unsolidified liquid and ultimately causing cell damage (Benson, 2008b; Kaviani, 2011).

The potential for successful cryopreservation depends on explant sensitivity to dehydration; this can be achieved either by physical drying (e.g. air-flow equilibration drying, flash-dry) or by treatment chemicals referred to as cryoprotectants. As highlighted above, the damage that may result to cells during cryopreservation is often attributed to perturbations of their water content.

*Cryoprotectants*

In most cases for vegetatively propagated plants, cryoprotectants are employed before exposure to LN temperatures (Engelmann, 2004). There are two kinds of cryoprotectants based on their ability to cross the plasmalemma. These are the penetrating cryoprotectants that can cross the plasmalemma and enter cells (e.g. dimethyl sulphoxide [DMSO]; glycerol; ethylene glycol) and the non-penetrating

cryoprotectants that remain outside cells but play a role in osmotic pressure (e.g. propylene glycol; polyvinylpyrrolidone) (Panis and Lambardi, 2005; Wowk, 2007; Benson, 2008a).

Once inside cells, penetrating cryoprotectants impede intracellular ice crystallisation by forming hydrogen bonds with water molecules. As a result, ice crystal formation is reduced/prevented by vitrification (glass-like), where water solidifies without expanding and crystalising (Mazur, 2004; Benson, 2008b). By remaining outside the cell non-penetrating agents draw free water out of the cell, causing intracellular dehydration. As a result, the amount of freezable water present in tissues decreases (Panis and Lambardi, 2005). Together with penetrating agents, they reduce ice crystal formation during cooling and rewarming (Volk and Walters, 2006; Penmetcha and Arali, 2011).

The commonly used cryoprotectants are DMSO, glycerol, mannitol, sucrose, sorbitol and polyethylene glycol, alone or in combination (Sakai and Engelmann, 2007). The kind of plant being treated determines the type of cryoprotectant. One of the most frequently used is DMSO, but it is cytotoxic at high concentrations. Gaspar *et al*. (2002) reported that the stress caused by the toxicity of DMSO can be mild, but if exposure is protracted, it can result in physiological changes or even death.

The most common penetrating cryoprotectant solution is PVS2 consisting of 30% glycerol (w/v), 15% (w/v) ethylene glycol and 15% (w/v) DMSO and 0.4 M sucrose (Sakai *et al*., 1990). The high concentration of cryoprotectants in this combination is thought to cause the vitrification of freezable water in the cell (Engelmann, 2000; Sharma *et al*., 2006). This should, in turn, maintain a stable condition of the biological material during preservation (Burke, 1986 and Fahy *et al*., 1984 in Gonzalez-Arnao *et al*., 2008). However, pre-treatment with cryoprotectants alone (PVS2), does not guarantee protection against the freezing temperatures of LN. In vitrification-based methods, it is thus necessary to attain osmotic tolerance to dehydration by PVS2. As such, this mitigates the damaging effects during dehydration (Keller *et al*., 2008; Zámečník *et al*., 2012), as well as rewarming of the cryopreserved material at rates that avoid ice crystal damage.

*Pre-treatments*

Pre-treatment with sucrose and glycerol (loading solution) before PVS2 treatment can improve osmotic tolerance. Subsequently, this would improve dehydration tolerance and condition the cells to tolerate the stress of treatment with PVS2 as well as that imposed by the cooling and thawing procedures (Kaviani, 2011). However, there is a need to carefully observe and adjust exposure time to pre-treatment solution since lengthy or reduced times were reported to lower the recovery of shoot tips after cryopreservation (Hirai and Sakai, 2003; Hamalgyi *et al*., 2010; Chen *et al*., 2018).

*Types of explants*

Various types of plant organs, cells and tissues can be cryopreserved. For instance, shoot tips from *in-vitro* cultures, seeds and isolated somatic embryos, embryonic axes, bulbils, buds and pollen (Hirai and Sakai, 2003; ZámečnÍk *et al*., 2012; Vollmer *et al*., 2021). Cryopreservation can also be applied to leaf square-bearing adventitious buds (Chen *et al*., 2018), stem disk-bearing adventitious buds (Wang *et al*., 2020), microtubers (Uchendu *et al*., 2016) and rhizome buds (Carmona-Martin *et al*., 2018).

Due to their high water content and temperature gradient, it's almost impossible to cryopreserve large seeds (Rao, 2004). In such cases, isolating the embryos from the seeds can resolve this issue, but requires tissue culture for plant regeneration (Fang *et al*., 2004; Kim *et al*., 2006; Berjak and Pammenter, 2008). Additionally, the embryonic axis requires fast drying of the samples to appropriately low water contents (Berjak and Pammenter, 2008). However, in cases where explants are shoot apices, preculture on medium with elevated sucrose content is generally employed, before further dehydration (e.g. Hirai and Sakai, 2003). Cell suspension cultures and calli can survive substantial water loss. However, they are not suitable for germplasm conservation due to their inability to remain true-to-type – an essential requirement for germplasm conservation (Radha *et al*., 2012).

Depending on the species, it could take several weeks for small, cryopreserved samples like shoot apices/meristems to re-establish shoot cultures after retrieval from LN. For tree species, this process could take even longer, with several months required to produce micropropagated plants that can be safely transferred to the field (Kaczmaczyk *et al*., 2012).

The best organs for cryostorage of vegetatively propagated species such as potato, sweet potato, banana, strawberry, sugarcane and cassava are the shoot tips or meristems. These can be obtained from the *in-vitro* plant material since they can produce virus-free plants with fewer mutations. Additionally, they are easier to recover as compared with other organs (Scowcroft, 1984; Kaczmaczyk *et al*., 2012) and have inherent genetic stability (Padayachee *et al*., 2008; Normah *et al*., 2019), which is a fundamental requirement of conservation. In this regard, Kaczmarczyk *et al*. (2012), noted that the well-organised structure of apical meristems typically leads to immediate shoot formation after cryopreservation. This is due to the small size and unique cellular makeup of meristematic cells, which have high cytoplasmic content and thin cell walls, making them more resilient to cooling in LN than larger, more vacuolated cells (Engelmann, 1991; Bajaj, 1995). It is thus, imperative to carefully select the type of tissue for freezing to achieve positive cryoconservation.

*Explant stress and damage*

The process of cryopreservation can be stressful to biological material, starting with the extraction/excision of the explant, followed by dehydration procedures and the sudden temperature change (Uchendu *et al*., 2013). Isolation of the explant can result in wounding, which can lead to chemical changes such as the release of phenolic compounds, reactive oxygen species, alkaloids and flavonoids. Additionally, cell wall modification can also occur (Davis, 1987; Ren *et al*., 2021). In some cases, plants like sweet potato will produce callus as a means of protection against contaminants and other stresses (Kaity *et al*., 2008). However, it's important to note that callus formation on areas that are not at the shoot base, should be avoided during the regeneration of cryopreserved material, as it can be linked to somaclonal variation and genetic instability (Hirai and Sakai, 1999; Kaity *et al*., 2008).

The response of plants to stress can be initiated by stress-related damage, such as membrane loss or rupture (Gaspar *et al*., 2002). Additionally, the use of cryoprotectants for osmotic dehydration treatment can cause further stress due to their toxic nature, eventually leading to physiological alterations or even cell death (De Klerk 2007; Zámečník *et al*., 2021).

According to Mycock *et al*. (1995), the success of cryopreservation can be credited to the optimisation of variables such as type and size of explants, type and concentration of cryoprotectant and the rate of cooling and rewarming. Additionally, the response of plants to stress caused by cooling in LN is influenced by developmental age and genotype. This is due to some plants being more or less sensitive to stress at specific stages of development.

To take into account the varying capacities of temperate and tropical plants to tolerate dehydration and freezing temperatures, various techniques have been devised for cryopreserving plant species. Reed (2004) categorizes cryopreservation methods into three types of cooling: slow cooling, rapid cooling and ultra-rapid cooling.

*Slow-cooling / controlled- cooling/two-step cooling*

The slow-cooling method is a conventional cryopreservation technique also known as freeze-dehydration, involving the use of cryoprotectants like sugars and PEG on explants. Programmable freezers or devices like "Mr. Frosty" Nalgene ® (Sigma-Aldrich) are used to gradually cool the samples to about -40°C at a rate of <1°C/min before plunging them into LN (Engelmann, 2011; Panis, 2019).

This method causes extracellular ice to form before intracellular ice (Volk and Walters, 2006), leading to dehydration due to an osmotic imbalance (Engelmann, 1997; Benson, 2008b). The remaining water in cells and tissues becomes vitrified during cooling, with little or no intracellular ice crystal formation, if the correct water levels are achieved (Engelmann, 1997; Mazur, 2004; Mahajan and Tuteja, 2005). After rewarming the cryopreserved samples at 35–40°C, removal of the cryoprotectant and restoration of osmolarity to physiological levels, the samples are transferred to a specific recovery medium (Kaczmaczyk *et al*., 2012; Gonzalez-Arnao *et al*., 2008).

Dehydration of plant samples via the cooling process may not be suitable for shoot tips and meristems of some species, due to the extreme rates of dehydration, causing cell volume reduction, which leads to breakage of plasmodesmata and possible cellular death (Engelmann, 1997; Danso and Ford-Lloyd, 2011; Panis, 2019). Therefore, for shoot tips and meristems, rapid and ultra-rapid cooling methods are used.

*Rapid and ultra-rapid cooling*

Rapid cooling involves freezing samples at 200─1000°C/min to prevent ice crystal formation that can damage cells. However, some ice may still form depending on the cooling rate and cryoporeservation. Ultra-rapid cooling takes this further, reaching 10,000─30,000°C/min, aiming to completely eliminate ice formation and achieve a glass-like solid state (Panis, 2019; Nagel *et al*., 2024).

These two methods involve the direct immersion of a cryovial/cryotube containing biological material into LN. The rate at which the sample is transferred to ultra-low temperatures can be an important factor in post-freezing recovery (Benson, 1994; Panis, 2019). Rapid cooling has proven highly successful for cooling encapsulated shoot tips, meristems and embryos of several species (e.g., banana, sweet potato, potato), if they have been pre-treated to tolerate cryoprotection (Engelmann, 2004; Panis, 2019). However, it is worth noting that ice crystallization can occur upon rewarming if the rates are not fast enough to bypass the crystallization phase (Panis, 2019).

There are variations of the rapid and ultra-rapid cooling methods that can be used in single or combination i.e., pregrowth, dehydration/desiccation, vitrification as well as encapsulation as an extra step for handling the material.

*Pregrowth.* This involves culturing the biological material to be cryopreserved in a single or combination of cryoprotectants before rapid cooling in LN. For example, culturing somatic embryos of sweet potato and shoot tips of banana in high concentrations of sucrose, allowed for subsequent successful cryopreservation (Panis *et al*., 1996; Blakesley *et al*., 1995; Engelmann and Dussert, 2012).

*Dehydration/desiccation*. In this method, the biological material is dehydrated using several different methods. For example, material is exposed to air passing through the laminar flow hood, or is equilibrium dried to specific relative humidities, or dried in a desiccator containing activated silica gel down to a suitable water content before plunging into LN (Kim *et al*., 2006; Panis, 2019). This method is often used to dry zygotic embryos and embryogenic tissues from seeds. Rewarming is done in sterile air at room temperature, followed by plating explants on culture media. Berjak *et al*. (1989) introduced flash drying ─ an ultra-rapid drying that uses a high-velocity stream

of air. This technique quickly reduces the water content of materials to levels that minimise the risk of desiccation injury, but appropriate for cryogen exposure. By efficiently lowering the water content without compromising the material's integrity, flash drying enhances the overall quality and performance of the material for successful cryopreservation.

*Vitrification.* Vitrification is when glasses instead of damaging ice crystals are formed within the cell as they are cooled in LN. This is achieved by the treatment of the explant with a highly concentrated viscous cryoprotectant mixture such as PVS2 or PVS3. These mixtures withdraw freezable water from the explants, which then form metastable glass upon cooling in LN (Fahy *et al*., 1984; Wang *et al*., 2021). High concentrations of soluble compounds and fast freezing rates are required to achieve vitrification. The glassy state prevents the collapse of tissues, alterations of solute concentration and pH. This glassy state halts all chemical reactions requiring diffusion.

*Encapsulation.* This involves the suspension of the explant in a culture medium containing sodium alginate (2─3%, species dependent) and sucrose (0.08─0.4 M, species dependent) without calcium, then pipetted into a liquid medium containing calcium chloride. Polymerisation happens when the drop enclosing the explant comes into contact with calcium chloride and forms a bead in which the explant is enclosed (Hirai and Sakai, 1999; Kulus *et al*., 2015). Encapsulation supports and protects the explants against pre-treatments which could be harmful, yet needed to guarantee survival after cooling. Additionally, it is worth noting that without encapsulation, biological material such as shoot tips may be difficult to handle; as a result, damage may occur (personal observation).

*Pregrowth-desiccation/dehydration*. This method combines pregrowth and dehydration methods in which the explants are treated with cryoprotectants, followed by desiccation in the laminar flow or silica gel, then frozen in LN. After LN exposure, explants are rewarmed in a water bath at 40°C, then regenerated on a recovery medium (Hazubska-Przybyl, *et al*., 2013; Agrawal *et al*., 2019).

*Encapsulation-Dehydration.* In the encapsulation-dehydration method, the explant is first encapsulated and dehydrated (as above), placed into a cryotube, then slowly frozen or plunged directly into LN. Rewarming is done by removing the cryotubes from

LN and placing them in a water bath at 38─40°C, followed by plating on the recovery medium (Sakai, 2000; Hirai and Sakai, 2003). Similarly with embryogenic tissues, there is no need to extract the explants from their alginate bead for recovery (Hirai and Sakai, 2003; Sharma *et al*., 2006; Asmah *et al*., 2011). However, in certain cases, manual extraction of the biological tissue from the bead can facilitate faster regrowth after cryopreservation (data not shown). The first report on the successful use of this method was by Fabre and Dereuddre (1990) using shoot tips of potato. This method has been reported (Pennycook and Towill, 2001), who obtained 67% survival in their study on sweet potato.

*Encapsulation-vitrification.* This method combines the advantage of easy handling of the encapsulated explants and dehydration by cryoprotectants. It follows the same encapsulation procedure above, followed by treatment of the explants with 0.3 M sucrose and loading solution usually consisting of 2 M glycerol and sucrose ranging between 0.4-1.6 M. The loading solution provides osmotic protection against the toxic effect of PVS2 or PVS3. The treated explants are transferred to cryotubes containing PVS2 or PVS3, then exposed rapidly to LN. Rewarming is done by placing the cryotube in a water bath at 38–40°C for two minutes or directly into the unloading solution. Thereafter, the shoot tips are rinsed with 1.2 M sucrose and cultured on recovery medium. This approach was reported by Hirai and Sakai (2003), where they obtained 80% shoot formation (sweet potato shoot tips) in comparison with encapsulation-dehydration (63%) (Sharma *et al*., 2006).

It should be noted, however, that the toxicity of the concentrated vitrification solutions is a significant drawback of this approach. Biological material (shoot tips) of crops such as sweet potato is very sensitive to vitrification solutions. As such, conditioning of biological material, temperature adjustment as well as stepwise increment of these solutions may be required. For example, cold hardening of the biological material or application of vitrification solutions at 0°C may assist in overcoming the toxicity of these solutions (Panis, 2019). Additionally, stepwise treatment with PVS2 as reported by Towill and Jarret (1992) in their study, improved the tolerance of shoot tips of sweet potato to the toxic effect of PVS2.

*Droplet-vitrification.* This approach, derived from Kartha *et al*. (1992), is achieved by treating the explant with loading solution, followed by cryoprotectants (PVS2 or PVS3). Before the end of the treatment, the explant is placed within a droplet of cryoprotectant on a piece of aluminium foil, immersed into LN, then placed in a cryovial already present in the LN. Rewarming is done rapidly by removing the foil from the cryotube and exposing the explant that was within the foil directly to the 1.2 M sucrose solution at room temperature to remove the cryoprotectant, followed by regeneration on medium (Pennycook and Towill, 2000; Panis *et al*., 2005; Sakai and Engelmann, 2007; Halmagyi *et al*., 2010; Pawlowska and Szewczyk-Teranek, 2014; Wang *et al*., 2020).

The use of foil provides a direct contact of the explant with the LN and the rewarming solution, which, according to Panis *et al*. (2005), increases the cooling and warming rates in comparison with methods using cryovials. In their study, Pennycook and Towill (2000), reported 62% recovery of shoot tips of sweet potato cryopreserved using this method without callus formation.

*Cryoplate methods*

These are the methods that use welled aluminium plates that fit in a 2 ml cryotube. In the cryoplate method, shoot tips are trapped inside the wells of the cryoplate containing sodium alginate, then polymerised by pouring calcium chloride solution dropwise into the wells. Thus, the shoot tip adheres to the cryoplate throughout the procedure (Niino *et al*., 2019). After polymerisation the following cryoplate procedures are applied:

*Vitrification-cryoplate* (*V-cryoplate*). The v-cryoplate was developed by Yamamoto *et al*. (2011). In this method, the cryoplate procedure mentioned above is applied, then the shoot tips attached to the cryoplate are treated with osmoprotection solution, followed by dehydration with PVS2. After PVS2 treatment, the cryoplates with shoot tips are transferred to the cryotube and plunged directly into LN. Rewarming is done by removing the cryoplate from the cryotube and immersion in 1.2 M sucrose solution, then transferring the shoot tips to the regrowth medium (Yamamoto *et al*., 2011; Niino *et al*., 2019; Vujović *et al*., 2024). This method has been reported successful for potato and sugarcane (Yamamoto *et al*., 2015).

*Dehydration-cryoplate.* This method, developed by Niino *et al*. (2013), is similar to the v-cryoplate procedure. The difference lies in that the osmoprotected shoot tips are dehydrated in the air-current of the laminar flow cabinet, followed by transfer of cryoplate to cryotube, then plunged directly into LN. Rewarming is done the same as v-cryoplate. A few reports (Yamamoto *et al*., 2015; Rafique *et al*., 2016; Tanaka *et al*., 2021; Vujović *et al*., 2024), have reported successful application of this method to potato, strawberry, sugarcane and *Allium* spp.

*Cryomesh*. This method is similar to the v-cryoplate method, differing in the use of cryo-mesh instead of a cryo-plate. A mesh strip made of stainless steel was developed by Funnekotter *et al*. (2017). Shoot tips are trapped inside the mesh containing sodium alginate, then polymerised with calcium chloride. Then the shoot tips attached to the cryomesh are treated with an osmoprotection solution, followed by dehydration with PVS2. After PVS2 treatment, the cryo-mesh with shoot tips is transferred to the cryotube and plunged directly into LN. Rewarming is done by removing the cryomesh from the cryotube and immersing it in 1.2 M sucrose solution, then transferring the shoot tips to the regrowth medium. Funnekotter *et al*. (2017) obtained 83% regrowth of kangaroo paw (*Anigozanthos viridis*) and Lukić *et al*. (2022) >90% on *Arabidopsis taliana* by applying this method.

### 1.3. Application of cryopreservation in various institutes

The foundation of cryopreservation was laid in 1948 when scientists (Polge *et al*., 1949) accidentally discovered that glycerol could protect fowl spermatozoa during freezing. This breakthrough led to further research on cryoprotectants like dimethyl sulfoxide (DMSO), which became widely used in the 1950s and 1960s.

The concept of cryopreservation for plant diversity was formulated by Sakai in 1956 when he found that parenchyma cells located in the cortex of mulberry (*Morus alba*), could withstand exposure to extreme cold, including temperatures as low as -30°C. This method was also successfully applied to twigs of poplar (*Populus sieboldii*) in 1960 by Sakai. Since then, there has been remarkable advancements in the development of cryopreservation techniques for food crops, horticultural plants, wild species and forests species from various tropical and temperate regions (Wang *et al*., 2012; Annexure 1 (from Nagel *et al*., 2024).

Some examples of crops and the major institutes applying cryopreservation method on a larger scale are presented in Annexure 1 (from Nagel *et al*., 2024). Only 10% of these crops are listed in the International Treaty on Plant Genetic Resources for food and agriculture (ITPGRFA).

### 1.4. The importance of cryopreservation of sweet potato

Various reports on the development of cryopreservation methods for sweet potato have been published (Pennycook and Towill, 2000; Hirai and Sakai, 2003; Park and Sue, 2015, Wilms *et al*., 2020; Shevchenko *et al*., 2021). Many of these are limited to reporting on survival and not complete regeneration the cryopreserved material, or to only small selections of cultivars being evaluated (Wilms *et al*., 2020). For cryopreservation of sweet potato conserved at the NPGRC of South Africa, it is believed that the encapsulation-vitrification method would be the most suitable as it resulted in higher percentage of survival and regeneration.

### 1.5. Rationale for the study

As a vegetatively propagated crop, sweet potato collections are conserved for short – medium term in field gene bank and tissue culture. However, as mentioned earlier, the limitation of using these methods require cryopreservation as an advanced, improved and strengthened method that would complement the current methods. In implementing the National Plan, the NPGRC of South Africa identified a gap in the conservation methods for sweet potato collection conserved in the field gene bank and tissue culture. Therefore, there is a need to strengthen the conservation of valuable landraces such as sweet potato for food security and breeding purposes.

Significant progress has been made in the field of cryopreservation techniques and its application to different materials (cells, protoplasts, meristems, embryos etc.). In view of the significance of cryopreservation for sweet potato, the NPGRC of South Africa has established a cryo-storage unit to cryopreserve the genetic resources of several crops, including sweet potato, taro, cassava, temperate and tropical fruit germplasm. Currently, sweet potato is the main focus at the NPGRC of South Africa.

Several cryopreservation methods have been published with varying levels of survival/regeneration for different cultivars of sweet potato (Wilms *et al*., 2020; Shevchenko *et al*., 2021). As indicated by Vollmer *et al*. (2014), some crops may react

differently to the same method, making its applicability to all of the genotypes difficult. For example, using the droplet-vitrification method, they obtained recovery ranging between 1.7–66% of shoot potato shoot tips. Similarly, Wilms *et al*., (2020) obtained varying levels between 9.5 and 83% recovery of shoot tips of sweet potato. This indicates the possibility that the methods that have been reported successful in cryopreserving shoot tips of sweet potato, may need modifications to obtain successful cryopreservation of the sweet potato accessions of the South African NPGRC.

### 1.6. Aims and Objectives

The present study aimed at developing and standardising a cryopreservation protocol for sweet potato accessions at the South African NPGRC. This would contribute towards long-term conservation of landrace food crops as well as ensuring food security and availability of traditional crop varieties for future breeding programmes.

From the cryopreservation methods discussed earlier, encapsulation-vitrification devised by Hirai and Sakai (2003) and further evaluated by Shevchenko *et al*. (2021) with some modifications, have been selected for application and assessment on buds of sweet potato conserved at the NPGRC of South Africa. This was because those authors obtained 80–95% recovery in their studies. It was therefore considered possible to develop a successful cryopreservation method using encapsulation-vitrification with modifications where necessary. The research objectives were:

**1.6.1.** To assess the responses of the buds of three South African sweet potato (*Ipomoea batatas*) accessions to different cryoprotectants.

**1.6.2.** To quantify the water content of the buds of three South African sweet potato (*Ipomoea batatas*) after treatment with cryoprotectants.

**1.6.3.** To optimise the concentration and durations of cryoprotectants for successful cryopreservation of buds of three South African sweet potato (*Ipomoea batatas*) accessions.

## CHAPTER TWO: RESPONSES OF THE BUDS OF THREE SOUTH AFRICAN SWEET POTATO (*Ipomoea batatas*) ACCESSIONS TO DIFFERENT CRYOPROTECTANTS

### 2.1. Introduction

Sweet potato (*Ipomoea batatas* L.) is one of the crops collected National Plant Genetic Resources Centre (NPGRC) from subsistence farmers in different areas of South Africa. The crop is maintained as live collections in field gene banks and *in-vitro* (tissue culture) at the NPGRC, Roodeplaat, South Africa. Sweet potato is primarily grown for its storage roots (tubers), which are rich in carbohydrates, dietary fibre and beta-carotene (DAFF, 2014). In South Africa, sweet potato is mainly grown by small holder farmers in almost all the provinces, with Limpopo as the major production area, followed by Mpumalanga, Kwa-Zulu Natal and Western Cape (DAFF, 2014).

There has been an increasing concern that important food crop landraces, such as those of sweet potato in South Africa, are gradually being replaced by improved varieties. This threatens their availability for future breeding programmes and food security. In this regard, the FAO has promoted the establishment of conservation centre such as the NPGRC as *ex-situ* safety facilities, to maintain and protect agro-biodiversity and food crops, thus ensuring food security as well as preserving valuable genes for future breeding purposes (FAO, 2014).

At the NPGRC of South Africa, sweet potato, taro, cassava landraces and a range of indigenous medicinal plants are maintained as live collections in shade and glass houses. Sweet potato accessions are also maintained in tissue culture at 25°C as a complementary method to the shade and glass house storage. Maintaining tissue culture involves the ongoing process of transferring the cultured cells or tissues to fresh growth medium every four to eight weeks. This process requires more work and attention to detail, as there is a risk of losing the accessions due to contamination as with other conservation methods (Berjak *et al*., 1999; Engelmann, 2004; Kim *et al*., 2006).

At the South African NPGRC, duplicates of sweet potato accessions in cryo-storage are yet to be established. Consequently, and in line with the gene bank standards developed by the FAO (2014), the South African NPGRC is currently in the process of developing a cryopreservation protocol for the sweet potato accessions maintained in

tissue culture. The success of cryopreservation primarily lies in the water content of the sample to be preserved (Berjak *et al*., 1999; Engelmann, 2004). For most vegetatively propagated plants, the amount of freezable water is regulated using cryoprotectants before exposure of the explants to liquid nitrogen (LN) (Engelmann, 2004). The significant amount of research in this area has emphasised that it is important to determine the optimal water content, cryoprotectant type and concentration for successful cryopreservation. This would in turn prevent the formation of intracellular ice crystals (e.g. Mycock *et al*., 1995; Benson, 2008; Kaviani, 2011). For example, the study of Mycock *et al*. (1995), reported the best recovery of the axillary buds of cassava when cryoprotectants were applied prior to physical dehydration and LN exposure.

Commonly used cryoprotectants are dimethyl sulphoxide (DMSO), glycerol, ethylene glycol, sucrose and these are used alone or in combination (Sakai and Engelmann, 2008). The kind of explant to be cryopreserved also influences the type of cryoprotectant used and consequently, all of these factors require optimisation depending on the species and even genotype (Hirai and Sakai, 2003; Chen *et al*., 2010; Hamalgyi *et al*., 2010). For cryopreservation of shoot apical meristems of vegetatively propagated plant species such as sweet potato, combinations of cryoprotectants have often been more successful than when the compounds are used alone (Hirai and Sakai, 2003; Al-Bahrany *et al*., 2012).

Cryoprotectants are categorised based on their ability to cross the cell membrane, either penetrating or non-penetrating (Panis and Lambardi, 2005; Benson, 2008). In this regard, one of the most common combination cryoprotectant solutions is Plant Vitrification Solution (PVS2) comprising 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO (all penetrating cryoprotectants) supplemented with 0.4 M sucrose (non-penetrating cryoprotectant) (Sakai, 1990).

The highly concentrated chemicals of PVS2 cause the freezable water in the cell to vitrify (Engelmann, 2000; Sharma *et al*., 2006). PVS2 has an overall molarity of 7.8 M. This has been found to be toxic to plant tissues of some tropical plant species when applied at higher temperatures, with DMSO as the most toxic component (Kim *et al*., 2006; Park and Sue, 2015; Volk *et al*., 2014). Thus, exposure time and temperature of PVS2 are critical for the success of cryopreservation (Kim *et al*., 2006; Park and

Sue, 2015, Volk *et al*., 2014). For example, some reports have indicated that exposure of explants to PVS2 for prolonged periods (more than 30 minutes), caused damage and lowered the recovery after cryopreservation (Pennycook and Towill, 2001; Charoensub *et al*., 2004; Kim *et al*., 2006; Yi *et al*., 2016).

Despite this toxicity, PVS2 remains an effective cryoprotective solution for shoot tips of numerous species (Volk *et al*., 2014), including sweet potato. In this regard, Pennycook and Towill (2000) and Hirai and Sakai (2003), reported 80% shoot formation of shoot tips of a limited number of sweet potato varieties cryoprotected with PVS2. For example, Hirai and Sakai (2003), reported 80% shoot formation on three sweet potato varieties. However, the tested sweet potato varieties have been shown to be sensitive to direct exposure to the vitrification solution, due to its osmotic stress and dehydration intolerance (Hirai and Sakai, 2003; Park and Sue, 2015). To accommodate this sensitivity, the protocols outlined by those authors pre-treat the plant materials with increasing concentrations of the cryoprotectants in a series of distinct steps. The propagules are then exposed to the PVS2 and thereafter exposed to LN.

The study presented here describes the application of the Hirai and Sakai cryoprotocol for sweet potato to three South African accessions and details modifications to that protocol.

## 2.2. Materials and methods

### *2.2.1. Plant material*

Nodal segments of approximately 4 mm were obtained from *in-vitro* plantlets of sweet potato (*Ipomoea batatas* L.) accessions (AP130, AP134 and AP156) from the South African NPGRC. The material was established on full strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 0.5 $mgl^{-1}$ gibberellic acid ($GA_3$) adjusted to pH 5.7 for four weeks in test tubes in a growth room (16 hour light / 8 hour dark hour photoperiod at 25°C). The plantlets were used in the experiments after four weeks of plating. The nodal segments containing one shoot tip or axillary bud were dissected into 3 mm segments and plated under the same conditions as described above for 10 days to achieve uniform growth. For consistency, the word ‘bud’ will be used to represent both shoot tips and axillary

buds. Buds (1–2 mm) with three or four leaf primordia (Figure 2.1) were isolated using a dissecting microscope.

### *2.2.2. Treatments*

All treatments were conducted at room temperature unless otherwise stated.

#### *2.2.2.1. Cryopreservation of buds of sweet potato using the protocol developed by Hirai and Sakai (2003) using PVS2*

The protocol of Hirai and Sakai (2003) is divided into several steps and was used for the preparation of sweet potato buds for cryopreservation.

*Control*

Non-encapsulated buds (n = 10) were plated on recovery medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 1 $mgl^{-1}$ $GA_3$ and 0.5 $mgl^{-1}$ BAP in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C).

*Encapsulation*

Encapsulation was achieved by sucking each bud into 3% low viscosity alginic acid using a pipette, then dripping them into 0.1 M $CaCl_2$ in liquid MS medium supplemented with 0.4 M sucrose to form beads. Beads were left to polymerise in the calcium solution for 20 minutes (Figure 2.2). Encapsulated buds (n = 10) were plated on the recovery medium following the same conditions as the control experiment.

*Preincubation and Preculture*

Encapsulated buds (beads) were transferred to preincubation solution (liquid MS containing 30 $gl^{-1}$ sucrose (10 beads:40 ml) and placed on a rotary shaker at 90 rpm for 24 hours. The beads were then precultured in liquid MS containing 0.3 M sucrose (10 beads:40 ml) and placed on a rotary shaker at 90 rpm for 16 hours. After the treatments, the beads were plated on the recovery medium following the same conditions as the control experiment.

**Figure 2.1.** Sweet potato apical bud dissected from nodal segments cultured on sweet potato medium for 10 days. Bar represents 1 mm

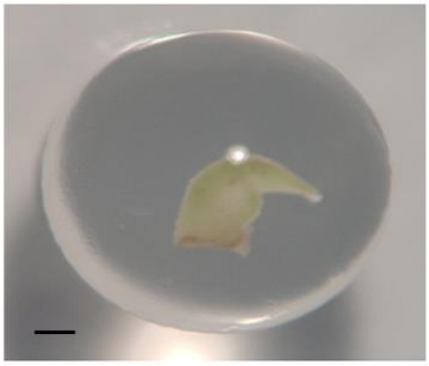

**Figure 2.2.** Encapsulated bud of sweet potato dissected from nodal segment cultured for 10 days on sweet potato growth medium. Bar represents 1 mm

*Osmoprotection (loading solution)*

Precultured beads were then osmoprotected in liquid MS containing 1.6 M sucrose and 2 M glycerol for three hours on a rotary shaker at 60 rpm. After the treatment, the beads were plated on the recovery medium following the same conditions as the control experiment.

*PVS2 and Cooling*

Osmoprotected beads (10 beads:20 ml) were treated with PVS2 (liquid MS medium supplemented with 30% glycerol (w/v), 15% (w/v) ethylene glycol and 15% (w/v) DMSO and 0.4 M sucrose) for one hour on a rotary shaker at 60 rpm. One set (n = 10) of beads were plated on the recovery medium following the same conditions as the control experiment. Another set (n = 10) was then transferred to fresh PVS2 (0.5 ml) held in 2 ml cryovials (10 beads per vial) secured in the cryocanes and plunged directly into LN for one hour.

*Rewarming and Regeneration*

Rewarming was done by placing the cryovial still secured in the cryocane into 38°C water for two minutes. PVS2 was then drained from the cryovial and replaced twice at 10 minutes intervals with 1 ml unloading solution (liquid MS containing 1.2 M sucrose). The beads were blotted dry and plated on a recovery medium and incubated for seven days under the same conditions as the control experiment.

*Viability*

The beads from the control experiment and treatments were transferred from the recovery medium to a normal sweet potato medium (full strength (MS) medium supplemented with 30 $gl^{-1}$ sucrose, 0.5 $mgl^{-1}$ $GA_3$ and 2.21 $gl^{-1}$ gelrite. Shoot regeneration was recorded as a total percentage of the shoot tips forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

### *2.2.2.2. Treatments of the encapsulated buds with DMSO, Ethylene glycol and Glycerol (individually and in combination)*

Cryoprotectants are generally applied either warm at 25°C or cold at 0°C (Benson, 2008a; Volk and Caspersen, 2017). Given that sweet potato is cold sensitive, these treatments were applied at room temperature (Hirai and Sakai, 2003; Park and Sue, 2015).

*Control*

Encapsulated buds (n = 10) were plated on recovery medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 0.5 $mgl^{-1}$ $GA_3$ in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C). All treatments were conducted at room temperature.

*(i) Treatment of the buds with individual cryoprotectants*

*DMSO, ethylene glycol and glycerol*

Encapsulated buds (beads) (n = 30 per accession) were treated in solutions (10 beads: 20 ml) comprising liquid MS medium, 0.4 M sucrose and individual concentrations (5, 10 and 15%) of DMSO and ethylene glycol; and (5, 10, 20, 30 and 40%) of glycerol on a rotary shaker (60 rpm) for 30 and 60 minutes at room temperature (Table 2.1). However, glycerol treatment was for 30 minutes taking into consideration the low viability rate after DMSO and ethylene glycol for 60 minutes. The material was then plated as the control experiment.

**Table 2.1.** The different concentrations of individual and combination components of PVS (measurement: weight/volume) containing 0.4 M sucrose used for the treatment of the encapsulated buds of three South African accessions (AP130, AP134 and AP156) of sweet potato for 30 and 60 minutes (excluding glycerol).

| Individual treatments | Combination treatments |
|---|---|
| 1. DMSO: 5, 10 and 15%<br>2. Ethylene glycol: 5, 10 and 15%<br>3. Glycerol: 5, 10, 20, 30, and 40% | 1. 5% Glycerol + 5% DMSO + 5% Ethylene glycol<br>2. 10% Glycerol + 10% DMSO + 10% Ethylene glycol<br>3. 20% Glycerol + 10% DMSO + 10% Ethylene glycol<br>4. 10% DMSO + 10% Ethylene glycol<br>5. 10% DMSO + 15% Ethylene glycol<br>6. 15% DMSO + 15% Ethylene glycol |

*Viability*

Shoot regeneration was recorded as a total percentage of the plated shoot tips forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*(ii) Treatment of the buds with combination cryoprotectants*

Encapsulated buds (n = 30 per accession) were exposed to combinations of each of the cryoprotectants for 30 minutes on a rotary shaker (60 rpm). The ratio of explants to solution was 10 beads:20 ml (Table 2.1). The material was blotted dry and then plated under the same conditions as the control experiment.

*Viability*

Shoot regeneration was recorded as a total percentage of the shoot tips forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*2.2.2.3. Modified cryopreservation protocol by Hirai and Sakai (2003) with PVS5% as the cryoprotectant solution and cooling*

This experiment used PVS5% (5% (w/v) DMSO, 5% (w/v) Ethylene glycol, 5% (w/v) glycerol in liquid MS medium supplemented with 0.4 M sucrose instead of PVS2 according to Hirai and Sakai (2003). All treatments were conducted at room temperature.

*Control*

Encapsulated buds (n = 10) were plated on recovery medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 1 $mgl^{-1}$ $GA_3$ in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C). All treatments were conducted at room temperature.

*Preincubation and Preculture*

Encapsulated buds (beads) were transferred to preincubation solution (liquid MS containing 30 $gl^{-1}$ sucrose (10 beads:40 ml)) and placed on a rotary shaker at 90 rpm for 24 hours. The beads were then precultured in liquid MS containing 0.3 M sucrose (10 beads:40 ml) and placed on a rotary shaker at 90 rpm for 16 hours. After the treatment, the beads were plated on the recovery medium under the same conditions as the control experiment.

*Osmoprotection (loading solution)*

Precultured beads were then osmoprotected in liquid MS containing 1.6 M sucrose and 2 M glycerol for three hours on a rotary shaker at 60 rpm. After the treatment, the beads were plated on the recovery medium as with the control experiment.

*PVS5% and Cooling*

Osmoprotected beads (10 beads:20 ml) were treated with PVS5% (liquid MS medium supplemented with 5% glycerol (w/v), 5% (w/v) ethylene glycol and 5% (w/v) DMSO and 0.4 M sucrose) for one hour on a rotary shaker at 60 rpm. A set of 10 beads was plated on the recovery medium as the control experiment. Another set of 10 beads was transferred to fresh PVS5% (0.5 ml) held in 2 ml cryovials (10 beads per vial) secured in the cryocanes and plunged directly into LN for one hour.

*Rewarming and Regeneration*

Rewarming was done by placing the cryovial still secured in the cryocane into 38°C water for two minutes. PVS5% was then drained from the cryovial and replaced twice at 10 minutes intervals with 1 ml unloading solution (liquid MS containing 1.2 M sucrose). The beads were blotted dry and plated on recovery medium and incubated for seven days under the same conditions as above.

*Viability*

The beads from the control experiment and treatments were transferred from the recovery medium to a normal sweet potato medium (full strength (MS) medium supplemented with 30 $gl^{-1}$ sucrose, 0.5 $mgl^{-1}$ $GA_3$ and 2.21 $gl^{-1}$ gelrite. Shoot regeneration was recorded as a total percentage of the shoot tips forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*Analyses*

Experiments were repeated three times. An analysis of variance (ANOVA) in GENSTAT was used to calculate means to compare plant viability among the treatments. The data are presented as percentages and the least significant difference (LSD) was calculated at $P<0.05$. MS Excel (Microsoft Office 2007) with add-ins statistical program was used to generate graphs.

## 2.3. Results and Discussions

### *2.3.1. Cryopreservation of buds of sweet potato using the PVS2 protocol developed by Hirai and Sakai (2003)*

*Control and Encapsulation*

In this study, the viability of non-encapsulated (control) and encapsulated buds of all the tested accessions (AP130, AP134 and AP156) of sweet potato was 100% with no significant difference, indicated by the letter ‘a’ in the result means (Figure 2.3). Encapsulation had no impact on the viability of the buds. The shoot protruded from the bead seven days after plating on the recovery medium (Figure 2.4).

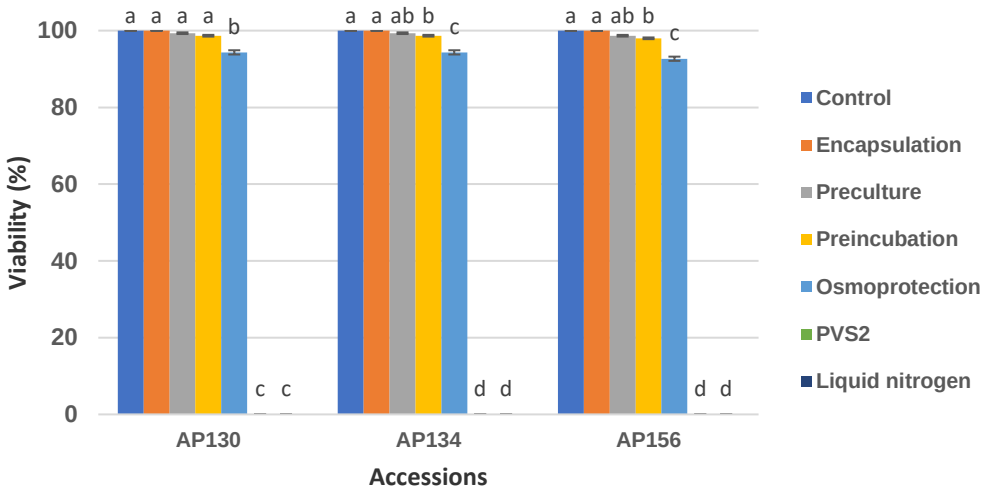


**Figure 2.3.** Viability of buds of three South African sweet potato accessions (AP130, AP134 and AP156) after encapsulation, preincubation, preculture, osmoprotection, PVS2 and LN exposure. Non-treated buds acted as the control. The least significant difference at P = 0.005 (LSD 5%) indicates difference between the treatments. Different letters within the accession indicate significant difference in the treatments.

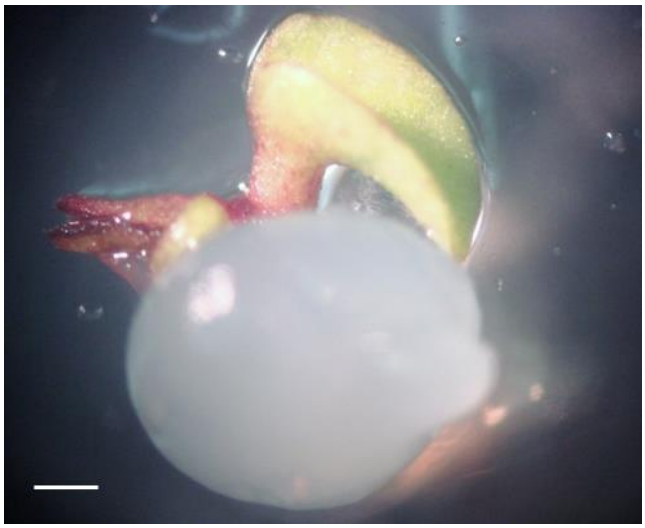

**Figure 2.4.** An encapsulated (without pretreatments) shoot of sweet potato protruded from the bead seven days after plating on the recovery medium. Scale bar = 1 mm.

*Preincubation and Preculture*

These treatments had no impact on the viability of the material with the regeneration percentages ranging from 98–99% (Figure 2.3). There was no significant difference within and between the preincubation and preculture treatments in all the accessions

(indicated by the letter ‘a’ in the result means). The duration of treatment of the buds in the preincubation solution for 24 hours did not impact the viability of the buds. Similarly, Hirai and Sakai (2003) and Shevchenko *et al*. (2021), obtained 100% regrowth of the buds of sweet potato after the preincubation treatment. According to Hirai and Sakai (2003), treatment with the preculture solution for 16 hours also did not impact the viability of the buds of sweet potato.

*Osmoprotection, PVS2 and Cooling*

The treatment with osmoprotection, PVS2 and cooling in LN differed significantly with the control, encapsulation, preincubation and preculture treatments across the accessions. There was a significant difference (AP130: $F = 6936.78$, $df = 6$, $P<0.001$; AP134: $F = 16185.81$, $df = 6$, $P<0.001$ and AP156: $F = 7202.73$, $df = 6$, $P<0.001$), after the treatment with osmoprotection, resulting in 92–95% regrowth for all the accessions (Figure 2.3). One would have expected lower viability due to the highly concentrated nature of the osmoprotection solution compared with the preincubation and preculture solutions. Nonetheless, the viability of the sweet potato buds of the three accessions (AP130, AP134 and AP156) was not impacted after treatment with the osmoprotection solution.

In all the accessions, there was no growth after the PVS2 treatment and after the cooling and rewarming treatments (Figure 2.3). The beads turned cloudy during cooling and rewarming. The buds were green immediately after rewarming, but turned pale and black after two days on the recovery medium. Similarly, Pettinelli *et al*. (2012) and Park and Kim (2015) also reported no survival after treatment with PVS2 in their study on guinea hen weed and sweet potato.

Applying the cryopreservation protocol developed by Hirai and Sakai (2003) to the sweet potato accessions maintained at the South African NPGRC, resulted in no plant regeneration after retrieval from LN. This indicates that PVS2 may have been toxic to the buds of the tested sweet potato accessions.

Wilms *et al*. (2020) reported shoot development after treatment of the buds with PVS2 at 0°C. As explained earlier (Chapter One), exposing sweet potato explants to <15°C in tissue culture, resulted in stunted growth indicating cold sensitivity of certain varieties. In support, Takagi *et al*. (2018) also mentioned the difficulty of cold-

hardening sweet potato in their study. Other work (Park and Sue, 2015; Yi *et al*., 2016; Shevchenko *et al*., 2021) reported shoot regrowth after applying PVS2 at room temperature. Based on these reports, the application of PVS2 at 0°C was not taken into consideration.

Reducing the time of exposure of the buds of sweet potato to PVS2 for 30 minutes did not produce shoot formation (data not shown). Further reduction of exposure time of the buds to less than 30 minutes was not explored as some publications (Pennycook and Towill, 2000; Park and Sue, 2015), reported low survival rates as well as callus formation.

Due to the high concentration and toxic nature of PVS2, other studies reduced the concentration of PVS2 to 20–80% and/or preconditioning the buds to a higher concentration of full-strength PVS2 at room temperature (Towill and Jarret, 1992; Park and Kim, 2015; Shevchenko *et al*., 2021; Lee *et al*., 2023).

Based on the results obtained in this study, it was necessary to better understand the impact of the PVS components on the buds of sweet potato of South African provenance. The subsequent experiments assessed the response of the sweet potato buds to the different concentrations of DMSO, ethylene glycol and glycerol individually and in combination. These investigations would indicate the specific component and concentration that contributed to the high level of toxicity of PVS2 that resulted in the loss of viability of buds of sweet potato tested in this study.

### *2.3.2. Treatments of the encapsulated buds with DMSO, Ethylene glycol and Glycerol (individually and in combination)*

*(i) Treatment of the buds with individual cryoprotectants*

*DMSO*

Like the control, exposure of all three accessions to 5% DMSO did not impact viability. (Table 2.2). These results agree with the study of Salaj *et al*. (2022) who reported satisfactory regrowth after using 5% DMSO as the cryoprotectant. Volk and Caspersen (2017) reported DMSO to be permeable to the plasma membrane of the buds of sweet potato within 30 minutes exposure. Similarly, Kreckel *et al*. (2023) reiterated the ability of DMSO to enter the cell within the first five minutes exposure. Once inside the cell,

DMSO increases the osmotic pressure, causing expansion of the meristematic region and eventually the entire shoot tip.

When the material was treated with 10% DMSO (both 30 and 60 minute exposure) the viability dropped to between 75–93% (Table 2.2). A significant difference was observed between accessions AP134 ($F = 42.77$, $df = 3$, $P < 0.001$) and AP130 ($F = 11.00$, $df = 3$, $P = 0.007$) AP156 ($F = 19.57$, $df = 3$, $P = 0.002$) for both 30 and 60 minutes exposure with 91–93% and 75–84%, respectively. Likewise, Park and Kim (2015) also indicated 10% as the highest concentration of DMSO to be tolerable to sweet potato at room temperature.

Treatment of the buds with 15% DMSO for 30 and 60 minutes exposure resulted in 69–84% and 55–66% regeneration of the buds, respectively. There was a significant difference between accessions AP134 ($F = 60.55$, $df = 3$, $P<0.001$) and AP130 ($F = 40.20$, $df = 3$, $P<0.001$), AP156 ($F = 40,11$, $df = 3$, $P<0.001$) for the 30 minutes exposure in 15% DMSO, in which accession AP134 obtained 84% regeneration. However, the growth was stunted and slow and some buds did not develop into complete plantlets with roots. Longer exposure (60 minutes) of accession AP134 to the DMSO significantly reduced viability and indeed, all the accessions were negatively impacted by this concentration.

Kreckel *et al*. (2023), observed that DMSO was not uniformly distributed across and throughout the shoot tip. Some areas of the shoot tip had accumulated more than the 15% that was initially applied. Prolonged exposure of buds to DMSO at higher concentrations ultimately resulted in loss of viability of the treated buds (Table 2.2). In support, Bekheet *et al*. (2020), noted that longer exposures can impact the survival and viability of the biological material due to osmotic shock and severe cellular plasmolysis leading to death of the material. In this regard, Yi *et al*. (2016) ultimately resorted to removing DMSO from the cryoprotectant solution to protect buds from its toxicity. It has been stated that DMSO enhances the permeability of the cell membranes and promotes cell protection during dehydration (Ma *et al*., 2023). Therefore, the length of the buds to DMSO as a cryoprotectant is of utmost importance to the integrity of cell membrane.

**Table 2.2.** The viability of encapsulated buds of three different sweet potato accessions AP130, AP134, and AP156. The buds were treated with varying concentrations (5%, 10%, and 15%) of DMSO in liquid MS supplemented with 0.4 M sucrose. The treatments were for 30 and 60 minutes in each concentration. The material was not cooled in LN. The least significant difference (LSD 5%) at p<0.005 indicates the difference between the treatments. Values followed by the same letter (a, b or c) are not significantly different.

| Accession | Treatment time | Viability (%) | | | | LSD | F pr |
|---|---|---|---|---|---|---|---|
| | | Concentrations | | | | | |
| | | Control | 5 | 10 | 15 | | |
| **AP130** | 30 minutes | 100[a] | 100[a] | 82[b] | 69[c] | 1.201 | <0.001 |
| **AP134** | | 100[a] | 100[a] | 93[a] | 84[b] | 1.153 | 0.007 |
| **AP156** | | 100[a] | 100[a] | 84[b] | 73[c] | 1.523 | 0.002 |
| | 60 minutes | | | | | | |
| **AP130** | | 100[a] | 100[a] | 75[b] | 51[c] | 1.483 | <0.001 |
| **AP134** | | 100[a] | 100[a] | 91[a] | 66[c] | 1.290 | <0.001 |
| **AP156** | | 100[a] | 100[a] | 75[b] | 55[c] | 1.762 | <0.001 |

*Ethylene glycol*

When exposed to 5% and 10% ethylene glycol for both 30 and 60 minutes all three accessions of sweet potato fully regenerated (Table 2.3) with no significant difference (indicated by the letter 'a' in the result means). Like 5 and 10%, there was no significant difference when the buds of accession AP134 were treated for 30 minutes (98% viability) with 15% ethylene glycol. As observed with the DMSO treatments, accession AP134 was better able to tolerate ethylene glycol treatment (Table 2.3) in comparison with accessions AP130 and AP156.

A significant difference (F = 75.00, df = 3, P<0.001) was observed when buds of accession AP156 were treated with ethylene glycol at 15% concentration for 60 minutes, resulting in 66% viability. Accessions AP130 and AP134 produced 78 and 87% regeneration of the buds, respectively (Table 2.3) after treatment with 15% ethylene glycol for 60 minutes of exposure. Rostovtseva *et al*. (2022) emphasised that ethylene glycol forms a very viscous supercooled mass which forms a glassy substance when solidified. Although less toxic, at high concentrations, ethylene glycol

can impact the viability of the cells (Pegg, 2007; Zamecnik *et al*., 2021) as observed in this study. Based on the viability rates of 66–98% after treatment of buds of the three tested accessions in 15% ethylene glycol for 30 and 60 minutes exposure in this study, ethylene glycol is less toxic than DMSO.

**Table 2.3.** The viability of encapsulated buds of three different sweet potato accessions AP130, AP134, and AP156. The buds were treated with varying concentrations (5%, 10%, and 15%) of ethylene glycol in liquid MS supplemented with 0.4 M sucrose. The treatments were for 30 and 60 minutes in each concentration. The material was not cooled in LN. The least significant difference (LSD 5%) at P<0.005 indicates the difference between the treatments. Values followed by the same letter (a, b or c) are not significantly different.

| Accession | Treatment time | Viability (%) | | | | LSD | F pr |
|---|---|---|---|---|---|---|---|
| | | Concentrations | | | | | |
| | | Control | 5 | 10 | 15 | | |
| | 30 minutes | | | | | | |
| **AP130** | | 100[a] | 100[a] | 100[a] | 82[b] | 0.5767 | <0.001 |
| **AP134** | | 100[a] | 100[a] | 100[a] | 98[a] | 0.5767 | 0.455 |
| **AP156** | | 100[a] | 100[a] | 100[a] | 82[b] | 0.5767 | <0.001 |
| | 60 minutes | | | | | | |
| **AP130** | | 100[a] | 100[a] | 100[a] | 78[b] | 1.526 | 0.004 |
| **AP134** | | 100[a] | 100[a] | 100[a] | 87[b] | 0.999 | 0.006 |
| **AP156** | | 100[a] | 100[a] | 100[a] | 66[c] | 0.999 | <0.001 |

The treatment of the encapsulated buds with DMSO and ethylene glycol for 60 minutes resulted in reduced viability, particularly at high concentrations as indicated in Tables 2.2 and 2.3. Additionally, abnormal growth was observed. This led to the decision to only test the viability of the buds after treatment with glycerol for 30 minutes.

*Glycerol*

Exposure of the buds to 5, 10 and 20% glycerol produced 96-100% viability (Table 2.4). There was a significant difference (AP130: F = 43.00, df = 5, P<0.001; AP134: F = 83.39, df = 5, P<0.001; AP 156: F = 75.99, df = 5, P<0.001) between material treated

with 30 and 40% glycerol. PVS2 developed by Sakai *et al*. (1990), used 30% glycerol which produced approximately 80% survival of sweet potato buds (Hirai and Sakai, 2003). In contrast, 30 and 40% glycerol produced 55 and 22% viability of buds in this study respectively (Table 2.4). These results are supported by Volk and Walters (2006) and Volk and Caspersen (2017), in their studies on garlic and mint. They stated that glycerol caused plasmolysis during cryopreservation. This was due to the high molecular weight and viscosity of glycerol which resulted in the slow penetration across the cell membrane (Kim *et al*. (2009).

In all the treatments used in this study, accession AP134 was the most robust in its response to this vitrification solution (or its individual components), as higher survival and regeneration percentages of buds were achieved in comparison with accessions AP130 and AP156. The sensitivity of the buds of sweet potato to dehydration and osmotic shock caused by the high concentrations of each of the components of PVS2 was also demonstrated. In contrast to the previous studies (Sakai, 2000; Hirai and Sakai, 2003; Reyes *et al*., 2017) that employed PVS2 as a cryoprotectant, the South African accessions were sensitive to 15% DMSO and ethylene glycol as well as 30% glycerol. Consequently, the 30 minutes exposure to the cryoprotectants used in this study was considered suitable as buds of approximately 1-2 mm were used.

**Table 2.4.** Viability of buds of sweet potato accessions AP130, AP134 and AP156 after treatment with 5, 10, 20, 30 and 40% glycerol supplemented with 0.4 M sucrose in liquid MS for 30 minutes (60 minutes not tested). The material was not cooled in LN. The least significant difference at P<0.005 (LSD 5%) indicates the difference between the treatments. Values followed by the same letter (a, b or c) are not significantly different.

| Accession | Viability (%) | | | | | | LSD | F pr |
|---|---|---|---|---|---|---|---|---|
| | Concentrations | | | | | | | |
| | Control | 5 | 10 | 20 | 30 | 40 | | |
| **AP130** | 100$^{a}$ | 100$^{a}$ | 96$^{a}$ | 92$^{a}$ | 45$^{b}$ | 22$^{c}$ | 2.341 | <0.001 |
| **AP134** | 100$^{a}$ | 100$^{a}$ | 100$^{a}$ | 98$^{a}$ | 55$^{b}$ | 27$^{c}$ | 1.616 | <0.001 |
| **AP156** | 100$^{a}$ | 100$^{a}$ | 98$^{a}$ | 95$^{a}$ | 47$^{b}$ | 25$^{c}$ | 1.819 | <0.001 |

*(ii) Treatment of the buds with combination cryoprotectants*

When combined, 15% DMSO and ethylene glycol were found to have an impact on the survival of the encapsulated buds of the South African accessions. Although it has been reported that the toxic effect of individual components of PVS2 can be reduced by using these components in combination (Malabadi and Nataraja, 2006), this was not the case in this study (Figure 2.5).

Volk *et al*. (2014) and numerous other authors (Bajaj, 1995; Park and Kim, 2015; Berjak *et al*., 2019) have shown that glycerol damage (plasmolysis) to the biological tissues is exacerbated by the addition of ethylene glycol and DMSO. This damage happens due to the ability of DMSO to decrease membrane thickness which improves the diffusion of glycerol into the cell. Consequently, as more glycerol enters the intracellular spaces of the cell, the cell becomes plasmolysed leading to damage and death in severe cases. On average, the highest viability (100%) of the buds in this study were obtained in the treatment with combination concentrations of 5% of each of the components (Figure 2.5) viz. PVS5%. All the treated buds of sweet potato regenerated into complete plantlets within six weeks of plating on MS medium (Figure 2.6).

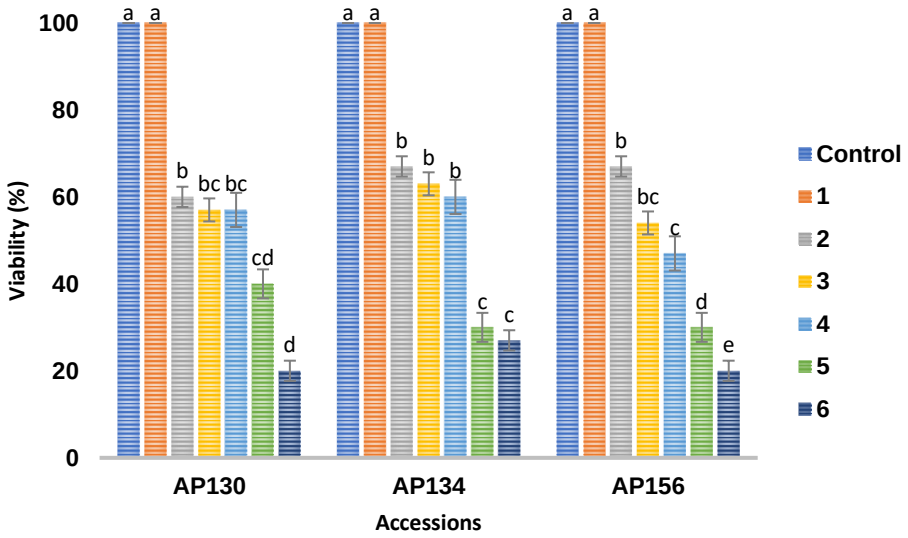


**Figure 2.5.** Viability of buds of three South African sweet potato accessions (AP130, AP 134 and AP156) after non-treatment (control) and treatment with various combinations of the cryoprotectants DMSO, ethylene glycoland glycerol. **1**. 5% glycerol + 5% DMSO + 5% ethylene glycol. **2**. 10% glycerol + 10% DMSO + 10% ethylene glycol **3**. 20% glycerol + 10% DMSO + 10% ethylene glycol **4**. 10% DMSO + 10% ethylene glycol **5**. 10% DMSO + 15% ethylene glycol and **6**. 15% DMSO + 15% ethylene glycol.

There was no significant difference between the control and PVS5% (indicated by the letter 'a' in the result means). A significant difference (AP130: $F = 76.23$, $df = 6$, $P<0.001$; AP134: $F = 71.29$, $df = 6$, $P<0.001$; AP 156: $F = 185.42$, $df = 6$, $P<0.001$), was observed across all the accessions in treatments 4–6 (Figure 2.5), in which 10-15% DMSO and ethylene glycol were used in combination, supporting the deleterious effects of combining other cryoprotectants with glycerol.

Like the earlier results obtained in the treatment of encapsulated buds with individual components of PVS, 10–15% concentration of DMSO and ethylene glycol impacted the viability of the buds. Interestingly, accession AP134 which showed higher survival when exposed to the cryoprotectants on their own (Tables 2.2 and 2.3), showed intolerance to the chemicals when combined. In this regard, the viability of buds in treatments 5 (10% DMSO and 15% ethylene glycol) and 6 (15% DMSO and ethylene glycol) dropped to 25 and 15%, respectively (Figure 2.5).

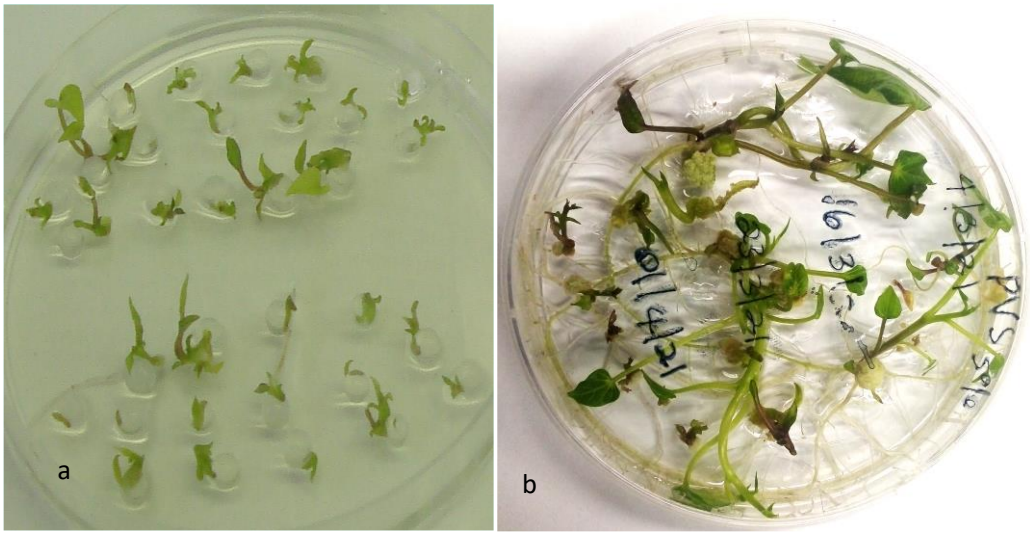


**Figure 2.6.** Shoots developed from the encapsulated buds of sweet potato treated with PVS5% supplemented with 0.4 M sucrose in liquid MS seven (a) and 30 (b) days after plating on MS medium.

Other advances and modifications of PVS2 have included alterations of the exposure time to between five and forty minutes, in which 0–85% survival were produced (Pennycook and Towill, 2000; Sakai, 2000; Hamalgyi *et al*., 2010; Reyes *et al*., 2017; Tanaka *et al*., 2018; Bettoni *et al*., 2019; Wilms *et al*., 2020). In line with those reports, the viability of the buds of the South African sweet potato accessions exposed to PVS2 for only 30 minutes in a previous study (data not shown) was lost. Additionally, exposure of the buds to individual components of PVS2 at concentrations above 10%, impacted their survival and regeneration (Tables 2.2 and 2.3).

While components of PVS2 are effective as cryoprotectants on their own, exposure of the buds of sweet potato in this study to higher concentrations of these components resulted in loss of viability. Even ethylene glycol and glycerol in concentrations of 15 and 30% had moderate toxic effects. These results agree with Halmagyi *et al*. (2010), Chen *et al*. (2011), Park and Kim (2015) and Shevchenko *et al*. (2021), who reported the toxicity of PVS components at higher concentrations (15–30%) on plant tissues.
In other studies (Rao, 2004; Park and Kim, 2015; Tanaka *et al*., 2018; Wilms *et al*., 2020), buds were precultured and preconditioned with loading solution before treatment with PVS2, to alleviate its harmful effects. Although higher survival was obtained, Pettinelli *et al*. (2012) reported that the regrowth originated from callus,

which is not desirable in germplasm conservation. Nonetheless, sweet potato buds of the accessions maintained at the South African NPGRC responded negatively when directly exposed to 100% PVS2 at room temperature, despite the modifications with the pre-treatments.

### *2.3.3 Modified cryopreservation protocol by Hirai and Sakai (2003) with PVS5% as the cryoprotectant solution and cooling*

The pre-treatment of the buds with the different concentrations of sucrose (30 $gl^{-1}$, 0.3 M and 1.6 M) according to the protocol developed by Hirai and Sakai (2003), had little to no impact on viability which ranged between 93 and 100% (Figure 2.7). All those that survived regenerated into complete plantlets with vigorous root development. Furthermore, the high regeneration rate of 93% obtained using a 5% concentration of the combined components (PVS5%), presented the potential of developing an effective cryoprotectant solution for successful regeneration of the buds of the sweet potato accessions. However, further exposure of the buds of all three sweet potato accessions to LN resulted in the loss of viability (Figure 2.7). As mentioned earlier, one of the factors affecting the success of cryopreservation is the water content.

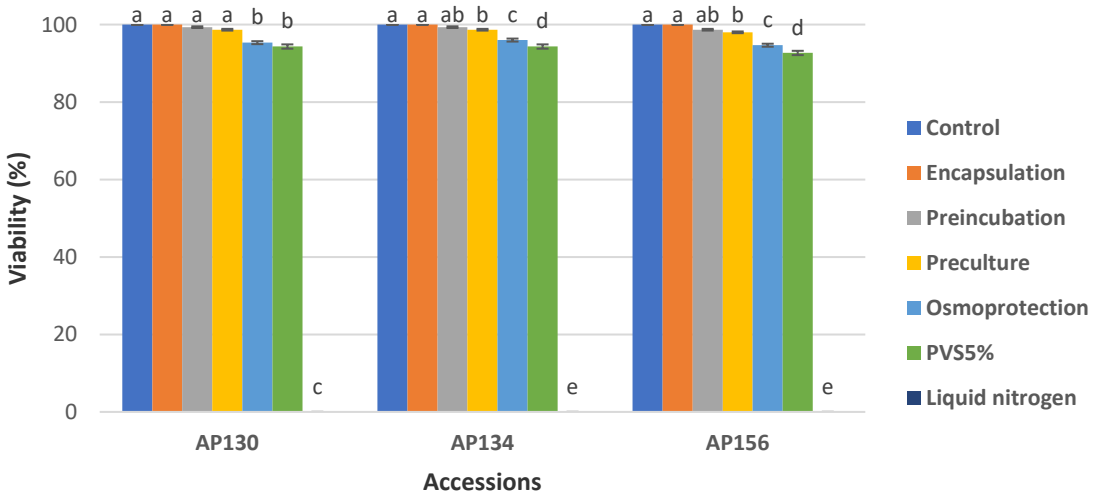


**Figure 2.7.** Viability of buds of three South African sweet potato accessions (AP130, AP 134 and AP156) after non-treatment (control) and treatment with encapsulation, preincubation, preculture, osmoprotection, PVS5% and LN exposure. The least significant difference at P<0.005 (LSD 5%) indicates the difference between the treatments. Different letters within the accession indicate significant differences in the treatments.

The lack of viability obtained in this study might be attributed to the water remaining within the buds of the tested accessions. When temperatures drop in LN, water molecules in plant tissues arrange into crystals that continue to grow, eventually damaging the membrane structure (Panis, 2019). As such, any freezable water in the sample can form ice crystals when exposed to LN. The whitening of beads in this study indicated ice crystallisation, suggesting that the buds were not sufficiently dehydrated when exposed to LN. It is therefore, important to determine the optimal water content of the buds of sweet potato for successful cryopreservation (Benson, 2008; Kaviani, 2011).

### 2.4. Conclusions and Recommendations

The success of cryopreservation is best judged by the viability level of the biological material after retrieval from the LN. As observed in this and other studies the concentration of and exposure time to PVS2 is species and genotype dependent. Due to the high concentration and toxic nature of PVS2, other scientists (Shevchenko *et al*., 2021; Wilms *et al*., 2020), resorted to reducing the strength of PVS2 to 20–80% and/or preconditioning the buds to the higher concentration of full strength PVS2. The lack of viability from buds treated with PVS2 in this study indicated the sensitivity of the South African accessions to the high concentrations of components of PVS2. The application of PVS5% as a cryoprotectant resulted in 93% regeneration of the buds of all three accessions tested in this study. This indicated that PVS5% may present the potential of being a successful cryoprotectant for the buds of sweet potato accessions maintained at the South African NPGRC. However, as the materials did not survive the cooling and rewarming processes PVS5% may have not provided sufficient dehydration effect due to its low concentration. As such the material may have been at too higher water contents to survive cooling and rewarming. Thus, the water content of the buds of sweet potato accessions needed to be assessed for further evaluation of PVS5% as the cryoprotectant for the development of a successful cryopreservation protocol.

# CHAPTER THREE: ASSESSMENT OF THE WATER CONTENT OF THE ENCAPSULATED BUDS OF SOUTH AFRICAN SWEET POTATO (*Ipomoea batatas*) ACCESSIONS AFTER TREATMENT WITH DIFFERENT CRYOPROTECTANTS

## 3.1. Introduction

The water content in the plant tissue to be cryopreserved is considered the principal factor that can affect the success of cryopreservation, as it regulates the amount of ice crystal formation within that sample (Kaczmarczyk *et al*., 2012). To ensure optimal outcome, it is important to carefully dehydrate the sample to a water content that will avoid freezing injury (Berjak, 1996; Engelmann, 2004), while also preventing desiccation damage (Pammenter *et al*., 2002). Achieving the delicate balance of removing water from the cell while maintaining viability is key to successful cryopreservation (Gonzales-Arnao *et al*., 2008).

Cryoprotectants are in most cases, applied to dehydrate the tissues before exposure to LN temperatures (Engelmann, 2004). As reported in the study of the responses of buds of sweet potato accessions to cryoprotectants (Chapter Two), lower concentrations of these cryoprotectants (PVS5%) may not sufficiently dehydrate the buds to a water content that would avoid ice crystal formation during cooling. This was observed when the beads turned white during cooling resulting in the lack of viability of the tested buds.

Reed (1999) highlighted that when insufficiently dehydrated tissues are exposed to low temperatures like those of LN, they can cause ice crystals to take up more space within the cell because they have a lower density compared with water. This increase in ice formation can cause pressure on intracellular organelles, leading to an increase in solute concentration and ultimately cell damage (Benson, 2008). However, too low a water content can also disrupt biological activities before cooling. Therefore, it is crucial to determine the optimal water content that can prevent crystallisation and promote vitrification in LN (Benson, 2008; Kaviani, 2011; Popova *et al*., 2023).

While trying to sufficiently dehydrate the cells and avoid a wide range of injuries due to ice-crystallisation, too much dehydration has been reported (Volk and Walters, 2006), to cause irreversible lipid phase transition in the plasma membrane. This can result in the loss of integrity and flexibility. Additionally, other vital functionalities such

as destabilization of macromolecule structures, increased cytoplasm viscosity and concentration of potentially toxic electrolytes in cytoplasm have been mentioned (Kim *et al*., 2005; Benson, 2008; Volk and Walters, 2006; Kim and Popova, 2023).

For successful cryopreservation of vegetative propagules of crops such as sweet potato, taro, grapevine and cassava, a water content level of 18─35% on a fresh weight basis (FWB) has been proposed to be optimal (Wang *et al*., 2000; Pennycook and Towill, 2001; Benelli *et al*., 2013; Hong *et al*., 2014; Feng *et al*., 2011). Depending on the species/organ/tissue, in this range there is usually no freezable water bound to cell membranes and macromolecules, crucial in maintaining their functionality (Kim *et al*., 2009; Feng *et al*., 2011).

For example, Kim *et al*., (2009) obtained 80% regrowth when the water content of garlic shoot tips in their study was reduced to 33%. Similarly, Engelmann *et al*. (2008) alluded to the 30% water content in their study on cassava, which resulted in better regrowth compared with >30%. Although Charoensub *et al*. (2004) reported 70% regrowth of cassava shoot tips at 20% water content compared with Engelmann *et al*. (2008), it still fell within the specified range of 18–35%.

Some plant species such as apple and *Allium sativum* have been reported to survive and regrow after cooling at a water content of 37–40% (Bilavcik *et al*., 2019). At this higher water content, crystallisation can still take place in tissues during cooling and after rewarming, but may not impact the survival of the tissues as indicated by Kim *et al.* (2006 and 2009). This is due to freeze-induced dehydration where the water in the extracellular spaces freezes and turns into ice crystals. As a result, the cell contents become more concentrated, leading to an increase in cell volume. Eventually, the cells can transition directly to an amorphous solid state as they are cooled in LN (Bilavcik *et al*., 2019).

The reports on the regrowth of vegetative propagules at different water content levels mentioned earlier suggest that each crop has specific water content requirements to sustain the functionality of its tissues. In this study (Chapter Two), using PVS5% as a cryoprotectant led to a 93% viability of buds in all three sweet potato accessions that were tested. However, when the buds were exposed to the cooling and rewarming processes, their viability was lost. It was presumed that the PVS5% may have not

provided sufficient dehydration effect due to its low concentration. As such, the resultant water content of the buds may have been too high to survive cooling in LN. It was therefore, deemed necessary to determine the water content of the buds of sweet potato after each step of cryopreservation. If the water content is high, the beads will be treated with PVS5% before osmoprotection and then cooled in LN. This process will facilitate a more thorough assessment of PVS5% as the cryoprotectant for the development of an efficient cryopreservation protocol.

## 3.2. Materials and Methods

### *3.2.1 Plant materials*

Nodal segments of approximately 4 mm were obtained from *in-vitro* plantlets of sweet potato (*Ipomoea batatas* L.) from the South African NPGRC. The material was established on full strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 0.5 $mgl^{-1}$ gibberellic acid ($GA_3$) adjusted to pH 5.7 for four weeks in test tubes in a growth room (16 hour light / 8 hour dark (photoperiod) at 25°C). The plantlets were used in the experiments after four weeks of plating. The nodal segments containing one shoot tip or axillary bud were dissected into 3 mm segments and plated under the same conditions as described above for 10 days to achieve uniform growth. Buds (1—2 mm) with three or four leaf primordia were isolated using a dissecting microscope.

Encapsulation was achieved by sucking each bud into 3% low viscosity alginic acid using a pipette and then dripping them into 0.1 M $CaCl_2$ in liquid MS medium supplemented with 0.4 M sucrose to form beads. Beads were left to polymerise in the calcium solution for 20 minutes.

### *3.2.2. Treatments*

#### *3.2.2.1. Determination of water content and regeneration of the encapsulated buds of sweet potato accessions after treatment with preincubation, preculture, osmoprotection and PVS5% (before swapping)*

*Control*

Beads (n = 10) were plated on normal sweet potato medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 0.5 $mgl^{-1}$ $GA_3$ in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C).

*Treatments*

A set of beads (n = 10 per treatment) was treated as outlined in Table 3.1 and Figure 3.1, then regenerated on the normal sweet potato regeneration medium following the same conditions as the control experiments. Another set of beads was dried in the oven to determine the water content of the beads after each treatment i.e. preincubation, preculture, osmoprotection and PVS5% (Table 3.1 and Figure 3.1).

**Table 3.1.** Treatments, concentrations, duration and volumes used to assess the water contents and viability of buds of sweet potato accessions using PVS5% as a cryoprotectant after osmoprotection solution (before swapping).

| Treatment | Concentrations | Duration | Volumes |
|---|---|---|---|
| Control | – | – | – |
| Preincubation | liquid MS supplemented with 30 $gl^{-1}$ sucrose | 24 hours | 10:40 ml |
| Preculture | liquid MS supplemented with 0.3 M sucrose | 16 hours | 10:40 ml |
| Osmoprotection | liquid MS supplemented with 1.6 M sucrose and 2 M glycerol | 3 hours | 10:20 ml |
| Plant Vitrification Solution 5% | liquid MS supplemented with 5% DMSO, ethylene glycol, glycerol and 0.4 M sucrose | 30 minutes | 10:20 ml |

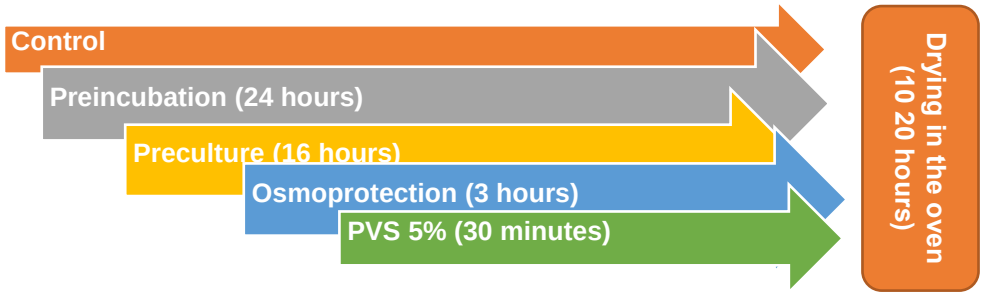


**Figure 3.1.** Flow chart of the experimental procedure. First, the fresh weight (FW) of the beads was recorded before treatment with preincubation solution. Following that the beads were dried in the oven to determine the dry weight (DW). Another set of beads was plated on the normal sweet potato regeneration media. After the control treatment, beads were sequentially treated with preincubation, preculture, osmoprotection and PVS5% (before swapping). FW was recorded after each treatment before drying the beads in the oven for 10, 15, 17 and 20 hours.

*Drying*

The drying oven was preheated to 80°C for 24 hours before drying the beads. Silica gel was placed on Petri-dishes and left in the oven overnight to be activated.

Aluminium boats without beads were carefully weighed and recorded. Subsequently, the beads were placed on the weighed aluminium boats and their weights were recorded to determine the fresh weight before placing them in the oven. Following that, the boats containing beads were placed on Petri-dishes containing silica gel (already in the oven) and dried for various durations (10, 15, 17, 19, and 20 hours).

*Water content determination*

Water content determination procedures were adopted from the International Seed Testing Association. The water content of the beads was determined after drying in the oven at 80°C for 17 hours. This duration was determined by recording weight until constant weight was attained. At the end of the 17 hour drying period, the Petri-dishes containing silica gel and boats were taken out of the oven and covered immediately until they cooled to room temperature. The boats were then weighed to determine the dry weight. Experiments were repeated three times (n = 10). For water content determination in the beads, data on fresh weight basis (FWB) collected from the experiments were expressed as:

$$\% \text{ Water content} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

*Viability*

Viability was recorded as a total percentage of the buds forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*3.2.2.2. Determination of water content and regeneration of encapsulated buds of sweet potato accessions after treatment with preincubation, preculture, PVS5% and osmoprotection (after swapping)*

Refer to 3.2.1 for plant material and encapsulation.

*Control*

Beads (n = 10) were plated on normal sweet potato medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 0.5 $mgl^{-1}$ $GA_3$ in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C).

*Treatments*

A set of beads (n = 10 per treatment) was treated as per Table 3.2 and Figure 3.2, and regenerated on the normal sweet potato regeneration medium following the same

conditions as the control experiments. In this experiment, the beads were first treated with PVS5% before osmoprotection (after swapping—Table 3.2 and Figure 3.2). The total osmolarity of PVS5% is 2.8 M and the osmoprotection solution is 3.6 M.

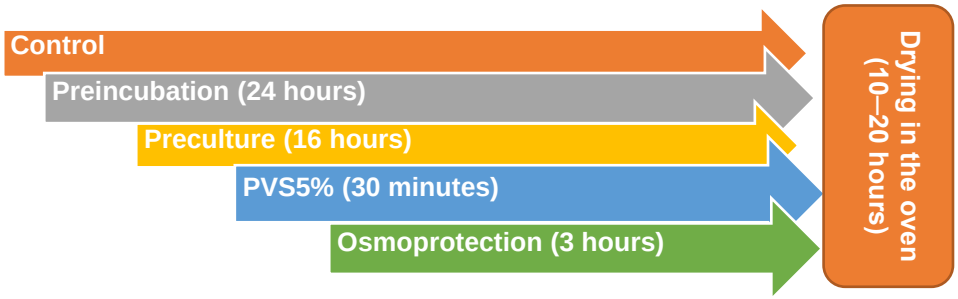


**Figure 3.2.** Flow chart of the experimental procedure. First, the fresh weight (FW) of the beads was recorded (Control) before treatment with preincubation solution. Following that the beads were dried in the oven to determine the dry weight (DW). Another set of beads was plated on the normal sweet potato regeneration media. After the control treatment, beads were sequentially treated with preincubation, preculture, PVS5% and osmoprotection (after swapping). FW was recorded after each treatment before drying the beads in the oven for 17 hours.

**Table 3.2.** Treatments, concentrations, duration and volumes used to assess the water contents and viability of buds of sweet potato accessions using PVS5% before osmoprotection as a cryoprotectant for cryopreservation. The beads were cooled in LN.

| Treatment | Concentrations | Duration | Volumes |
|---|---|---|---|
| Control | — | — | — |
| Preincubation | liquid MS supplemented with 30 $gl^{-1}$ sucrose | 24 hours | 10:40 ml |
| Preculture | liquid MS supplemented with 0.3 M sucrose | 16 hours | 10:40 ml |
| Plant Vitrification Solution 5% | liquid MS supplemented with 5% DMSO, ethylene glycol, glycerol and 0.4 M sucrose | 30 minutes | 10:20 ml |
| Osmoprotection | liquid MS supplemented with 1.6 M sucrose and 2 M glycerol | 20 minutes | 10:20 ml |

Treating the beads with osmoprotection solution and PVS5% (before swapping: Table 3.1, Figure 3.1) led to an increase in water content. It is possible that applying PVS5% before the osmoprotection solution could reduce the water content. In this regard,

another set (n = 10) of encapsulated buds was dried in the oven to determine the water content.

*Drying, water content determination and viability*

Refer to 3.2.2.1.

### *3.2.2.3. Viability of buds of sweet potato accessions after treatment with osmoprotection for different times*

*Control*

Beads (n = 10) were plated on normal sweet potato medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 0.5 $mgl^{-1}$ $GA_3$ in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C).

*Osmoprotection*

Beads were treated with an osmoprotection solution for 20, 25 and 30 (minutes), then one, two and three (hours). The beads were blotted dry and plated on the normal sweet potato medium under the same condition as the control.

*Viability*

Viability was recorded as a total percentage of the buds forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

### *3.2.2.4. Evaluation of PVS5% as a cryoprotectant followed by osmoprotection solution for cryopreservation of the buds of three sweet potato accessions (AP130, AP134 and AP156)*

Refer to 3.2.1 for plant materials and encapsulation.

*Control*

Beads (n =10) were plated on recovery medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 1 $mgl^{-1}$ $GA_3$ and 0.5 $mgl^{-1}$ BAP in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C).

*Preincubation, Preculture, PVS5%, Osmoprotection and Cooling in LN*

Beads (n = 10 per treatment) were treated as per Table 3.2 and placed on a rotary shaker at 90 rpm (preincubation and preculture) and 60 rpm (PVS5% and osmoprotection). After each treatment, the beads were plated on the recovery medium following the same conditions as the control experiment. One set (n = 10) of beads

was plated on the recovery medium following the same conditions as the control experiment. Another set (n = 10) was transferred to a fresh 2 ml osmoprotection solution held in cryovials (10 beads per vial) secured in the cryocanes and plunged directly into LN for one hour.

*Rewarming and Regeneration*

Rewarming was done by placing the cryovial still secured in the cryocane into 38°C water for two minutes. The osmoprotection solution was then drained from the cryovial and replaced twice at 10 minutes intervals with 1 ml unloading solution (liquid MS containing 1.2 M sucrose). The beads were blotted dry and plated on a recovery medium and incubated for seven days under the same conditions as the control experiment.

*Viability*

The beads from the control experiment and treatments were transferred from the recovery medium to a normal sweet potato medium (full strength (MS) medium supplemented with 30 $gl^{-1}$ sucrose, 0.5 $mgl^{-1}$ $GA_3$ and 2.21 $gl^{-1}$ gelrite. Shoot regeneration was recorded as a total percentage of the shoot tips forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*Analyses*

Experiments were repeated three times. An analysis of variance (ANOVA) in GENSTAT was used to calculate means to compare plant viability among the three treatments. The data are presented as percentages and the least significant difference (LSD) was calculated at $P<0.005$. MS Excel (Microsoft Office 2007) with add-ins statistical program was used to generate graphs.

## 3.3. Results and Discussion

### *3.3.1. Determination of water content and viability of the encapsulated buds of sweet potato accessions after treatment with preincubation, preculture, osmoprotection solutions and PVS5% (before swapping)*

The impact of the cryoprotectants on the water content and viability of buds of sweet potato is shown in Table 3.3. The initial water content of the buds was 94% on a FWB. After treatment with preincubation and preculture solutions, the water content

gradually decreased to 92 and 86%, respectively. A significant rapid decline from 86 to 26% was observed after treatment with an osmoprotection solution (Table 3.3). This indicated that water was withdrawn from the cells and tissues of the buds and replaced by the cryoprotectants in the osmoprotection solution. In contrast, when the buds were treated with PVS5%, the water content increased to 72%, indicating that water that was removed by the osmoprotection solution re-entered the cell and increased its water content.

The increase in water content observed after the treatment with PVS5% was presumably the reason for the loss of viability observed after exposure of the buds to LN as mentioned earlier (Chapter Two). When water re-entered the cell during treatment with PVS5%, the free water in the cell increased providing provision for the intercellular movement of water molecules (Griffith and Yaish, 2004; Jahed *et al*., 2023). Consequently, when subjected to LN, the material experienced negative osmotic pressure that led to the movement of water from the cell interior to the extracellular spaces, ultimately decreasing cell volume. Moreover, upon cooling and subsequent rewarming, the beads exhibited cloudiness. This mechanism can trigger the formation of vesicles ─ small membrane-bound sacs that can merge with the plasma membrane and modify its surface area. Upon rewarming, the re-entry of water into the cell can result in its rupture before fully restoring its original volume (Ambroise *et al*., 2020).

The viability of the buds after the treatment with these cryoprotectants was not highly impacted as it ranged between 94 and 100% (Table 3.3.). However, successful cryopreservation relies on the reduction of free water in cells to prevent ice crystal formation during cooling and rewarming. This was not the case as the water content increased from 26 to 72% in this study. This increase in water content prompted the suggestion of swapping the last two treatments i.e. applying the PVS5% solution before the osmoprotection solution. Given the collected data this would reduce the water content to between 18–20%, which has been reported to be optimal for the successful cryopreservation of crops like sweet potato (Pennycook and Towill, 2001; Hong and Yin, 2013; Benelli *et al*., 2013; Feng *et al*., 2011).

*3.3.2. Determination of water content and regeneration of encapsulated buds of sweet potato accessions after treatment with preincubation, preculture, PVS5% and osmoprotection (after swapping)*

Like the results obtained before swapping, a reduction in water content was observed with an increase in the concentration of the cryoprotectants. A significant difference (df = 4; P<0.001) was observed in the water content of the buds across the treatments (Table 3.3). A reduction in water content from 92 to 86% after treatments with preincubation and preculture solutions was observed. A decline from 79 to 34% was observed when buds were treated with PVS5% and then the osmoprotection solution. Although this decline in the water content is required and necessary for the development of a successful cryopreservation protocol, the viability of the buds was however, impacted.

**Table 3.3.** Water content and regeneration (%) of buds of South African sweet potato accessions after non-treatment (control) and treatment with encapsulation, preincubation, preculture, osmoprotection and PVS5%, before and after swapping osmoprotection with PVS5%. The least significant difference at P<0.005 (LSD 5%) indicates the difference between the treatments. Different letters indicate significant differences in the treatments.

| **Treatment (BS)** | **WC (%) (BS)** | **Regeneration (%) (BS)** | | **Treatment (AS)** | **WC (%) (AS)** | **Regeneration (%) (AS)** |
|---|---|---|---|---|---|---|
| Control | 94$^{a}$ | 100$^{a}$ | | Control | 94.33$^{a}$ | 100$^{a}$ |
| Preincubation | 92.33$^{b}$ | 99.33$^{ab}$ | | Preincubation | 92.00$^{b}$ | 99$^{ab}$ |
| Preculture | 86.0$^{0c}$ | 98.67$^{b}$ | | Preculture | 86.00$^{c}$ | 98.9$^{b}$ |
| Osmo | 26.33$^{e}$ | 96.00$^{c}$ | | PVS5% | 79.33$^{d}$ | 94$^{c}$ |
| PVS5% | 72.33$^{d}$ | 94.33$^{d}$ | | Osmo | 33.67$^{e}$ | 26.25$^{d}$ |
| LSD | 1.993 | 1.146 | | LSD | 1.993 | 1.993 |
| F-pr | <0.001 | <0.001 | | F-pr | <0.001 | <0.001 |
| *WC=water content; BS=before swapping; AS=after swapping; Osmo=Osmoprotection; PVS5%=Plant Vitrification Solution 5% | | | | | | |

The treatment of the buds with an osmoprotection solution for three hours showed a decline in the viability from 94 to 26% (Table 3.3.). The decline in the viability may have been due to the prolonged exposure of the buds to glycerol, which has been reported to cause damage (plasmolysis) to the biological tissues when DMSO and ethylene glycerol are added (Bajaj, 1995; Volk *et al*. 2014). The ability of DMSO to reduce membrane thickness enhances the diffusion of glycerol into the cell.

Consequently, as more glycerol enters the intracellular spaces of the cell, the cell becomes plasmolysed leading to damage and death in severe cases. This observation aligns with the study of Wilms *et al*. (2020), who reported 20% viability of shoot tips of sweet potato after treatment with osmoprotection solution for six hours and slower growth after three hour treatment. These results prompted the need to determine the optimal duration of exposure of the buds to the osmoprotection solution.

### *3.3.3. Regeneration of the encapsulated buds of sweet potato accessions after treatment with osmoprotection solution at different times*

There was a decline in the viability of sweet potato buds as the time of exposure to the osmoprotection solution increased from 20 minutes to three hours (Figure 3.3). There was a significant difference ($F = 1401.76$; $df = 6$; $P<0.001$) among all the durations which resulted in 86%, 82, 78% and 60% viability, respectively. Further treatments for two and three hours dropped the viability to 40 and 22%, respectively.

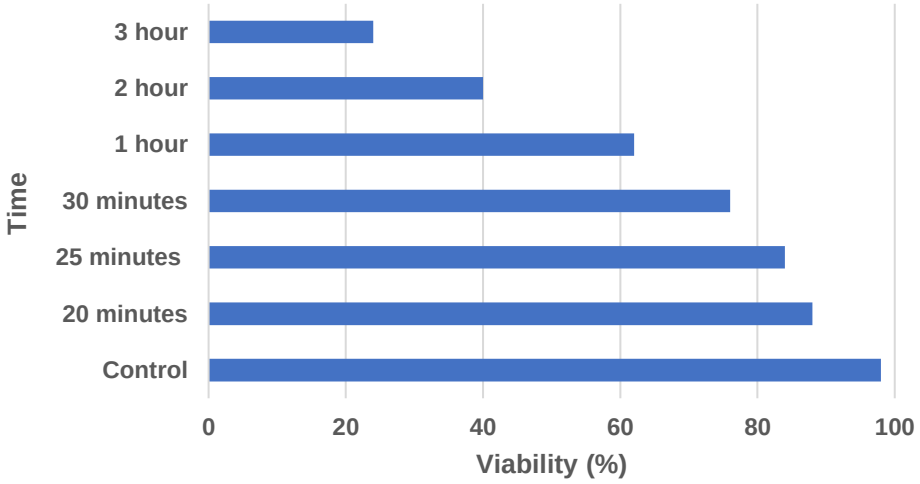


**Figure 3.3.** Viability of buds of the South African sweet potato accessions after non-treatment (control) and treatment with osmoprotection solution for different times.

While treatment of the buds with an osmoprotection solution has been considered necessary for decreasing the toxic effects of the subsequent vitrification solution, prolonged exposure of more than 20 minutes has induced toxicity. This was the case with *Citrus madurensis* (Cho *et al*., 2002), in which a decline in viability was observed after treatment with an osmoprotection solution for 30 minutes. On the contrary, Panis *et al*. (2005) reported good regeneration rates after seven hour treatment in their study

on banana shoot tips. Based on these observations 20 minutes was selected for further evaluation with other steps of the cryopreservation protocol.

### *3.3.4. Evaluation of PVS5% as a cryoprotectant followed by osmoprotection solution for cryopreservation of the buds of three sweet potato accessions (AP130, AP134 and AP156)*

As with other experiments, there was no significant difference between the control, preincubation and preculture solution treatments. A significant difference was observed ($F = 3572.50$, $df = 6$, $P<0.001$) when buds were further treated with PVS5% in which the regeneration rate ranged from 85–96%. Further treatment with the osmoprotection solution for 20 minutes caused a decline in the regeneration to between 70 and 80%. When the buds were exposed to LN, only 1% survival was observed, and the beads turned white (Figure 3.4).

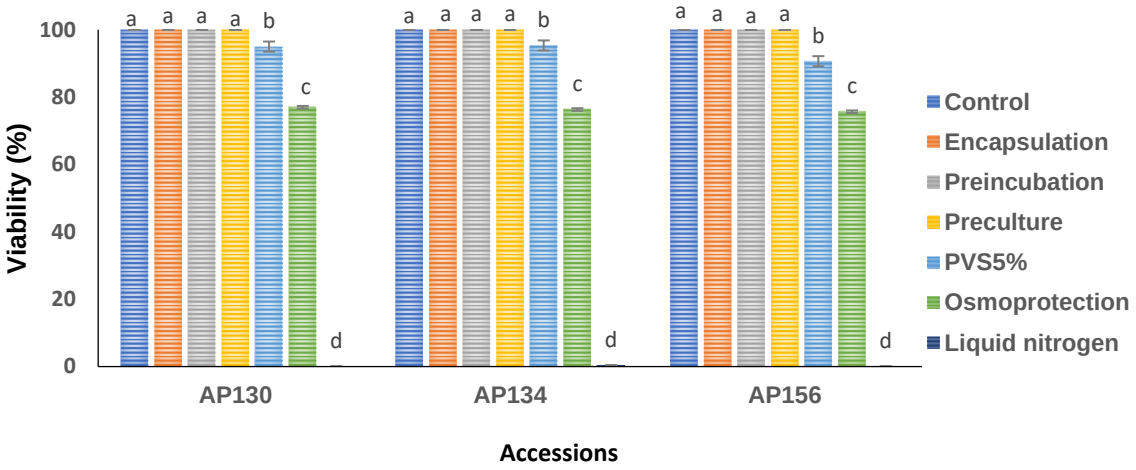


**Figure 3.4.** Viability of buds of three South African sweet potato accessions (AP130, AP134 and AP156) after non-treatment (control) and treatment with encapsulation, preincubation, preculture, PVS5%, osmoprotection and LN exposure. The least significant difference at $P<0.005$ (LSD 5%) indicates the difference between the treatments. Different letters within the accession indicate significant differences in the treatments.

The findings suggest that the PVS5% and osmoprotection solution have the potential to effectively act as cryoprotectants for cooling the buds in LN. It is possible that the DMSO in the PVS5% facilitated the diffusion of glycerol into the cells, leading to sample dehydration. However, the resulting water content of 26% may have been relatively high, potentially causing ice crystallisation during cooling in LN. Subsequent repetitions of the experiment did not yield any growth. After treating the buds with the

osmoprotection solution for more than 20 minutes, their viability was reduced. As a result, it may be necessary to proceed with additional dehydration measures.

Li *et al*. (2018) and Ambroise *et al*. (2020) noted that when samples are insufficiently dehydrated and exposed to normal freezing conditions, free water migrates from intracellular spaces to extracellular spaces. In this context, the buds in the current study were subjected to LN conditions, it is therefore, possible that a similar phenomenon occurred. This reaction is due to the difference in osmotic pressure, causing the cell wall to collapse and rupture due to the influx of water. Although the ultrastructural changes in the cells of the buds were not investigated in this study, the ice crystal formation observed when the osmoprotected buds were exposed to LN, indicated that freeze-induced dehydration may have taken place.

### 3.4. Conclusions and Recommendations

A successful cryopreservation protocol requires the maintenance of a low level of water content that would prevent ice crystals from grouping during cooling and ensure recovery after rewarming (Benson, 2008b). Cryoprotectants have been applied for the osmotic dehydration of plant tissues.

Treatment of the buds of sweet potato accessions with PVS5% for 30 minutes as reported in Chapter Two, had no detrimental impact. Further treatment of the buds with the osmoprotection solution for 20 minutes resulted in 70─80% regeneration, but did not provide sufficient dehydration to induce vitrification and prevent ice crystallisation during cooling and rewarming. Some reports mentioned the use of Plant Vitrification Solution (PVS3) containing 50% sucrose and 50% glycerol (weight/volume) (Nishizawa *et al*., 2003) instead of PVS2, as it does not contain DMSO which has been proven toxic when applied in higher concentrations (30% w/v). The study of Shevchenko *et al*. (2021) on sweet potato, reported 90% viability using physical drying in the lamina flow or silica gel. Additionally, direct cooling in LN and rewarming without the cryovial have been reported to increase the rates of cooling and rewarming (Park and Sue, 2015).

The lack of regeneration after cooling the osmoprotected buds indicated that the osmotic dehydration using PVS5% and osmoprotection may not have been sufficient.

This prompted the following questions and ideas to assist in improving the dehydration of the buds without causing damage:

- ✓ Replace the osmoprotection solution with PVS3.
- ✓ Treat the buds with stepwise increments of concentrations of cryoprotectants.
- ✓ Combine osmotic dehydration of the buds with physical dehydration.
- ✓ Compare the use of cryovial and cryomesh to cool the buds in LN.

Investigating these recommendations may contribute to the development of a successful cryopreservation protocol for the buds of sweet potato maintained at the NPGRC of South Africa.

# CHAPTER FOUR: OPTIMISATION OF THE CONCENTRATION AND DURATIONS OF CRYOPROTECTANTS FOR SUCCESSFUL CRYOPRESERVATION OF THE BUDS OF SOUTH AFRICAN SWEET POTATO (*Ipomoea batatas*) ACCESSIONS

## 4.1. Introduction

The use of Plant Vitrification Solution 3 (PVS3) developed by Nishizawa *et al*. (1993) for osmotic dehydration of samples before cooling, has been reported in various studies (Volk *et al*., 2006; Shevchenko *et al*., 2021). PVS3 is less toxic than PVS2 as it does not contain DMSO which has been proven toxic when applied in higher concentrations (30% w/v). PVS3 contains (50% w/v each) glycerol (penetrating) and sucrose (non-penetrating), consequently having a high dehydration strength and high glass-forming properties (Nishizawa *et al.*, 1993).

According to the previous studies (Volk *et al*., 2006; Teixeira da Siva, 2015; Park and Sue, 2015; Noor *et al.*, 2021), the successful application of PVS3 has been reported for several plant species, including sweet potato (Park and Sue, 2015; Texieira da Siva, 2015; Noor *et al.*, 2021). As reported by Senula and Nagel (2021) and Lee *et al*. (2023), PVS3 has been shown to induce vitrification at lower concentrations (80─85% PVS3) and with shorter exposure times (five minutes) (Nishizawa *et al*., 2003; Senula and Nagel, 2021; Lee *et al*., 2023) in comparison with 100% PVS3. This observation was supported by Volk and Caspersen (2017) in their study on sweet potato. Although success has been reported for sweet potato, the protocols do not apply to a wide range of its genotypes. Some studies reached 66% regeneration (Park and Sue, 2015) and others 2% (Vollmer *et al*., 2014). Overall, the use of PVS3 in cryopreservation is a promising technique that can be further optimised to improve regeneration rates.

In some cases, the physical dehydration method is employed to reduce the water content of the sample before cooling. There are numerous methods of physical dehydration of cryosamples but common procedures for *in vitro* materials are the use of the air-flow current in the laminar air flow or dehydration over activated silica gel (Matsumoto, 2017). However, the air-flow method can be difficult to regulate and reciprocate due to the variations in temperature and air-flow velocity of the laminar air-flow chamber. Dehydration using silica gel is more reproducible and thus recommended (Benson *et al*., 2008; Niino *et al.*, 2019). Like the vitrification method,

physical dehydration causes water to migrate from the intracellular to the extracellular spaces from where it is evaporated thereby reducing the overall water content of the sample to be cryopreserved (Ambroise *et al*., 2020).

The successful application of the dehydration method has been reported for cassava, sweet potato, yam and taro (Engelmann *et al*., 1995; Mandal *et al*., 1996; Pennycook and Towill, 2001; Gupta *et al*., 2013; Sen-Rong *et al*., 2013). It has been emphasised (Bettoni *et al*., 2021), that physical dehydration is a less time-sensitive and straightforward method than chemical-based vitrification procedures. However, its success depends on the extent of dehydration and the remaining water content in the sample before cooling. As with the vitrification procedure, the duration of dehydration needs to be determined before cooling to prevent damage due to excessive dehydration (Benson *et al*., 2008; Bettoni *et al*., 2021).

When cooling dehydrated material, various objects such as cryovial, foil, cryomesh, or cryoplate can be used (Pennycook and Towill, 2001; Hirai and Sakai, 2003; Yamamoto *et al*., 2015; Funnekotter *et al*., 2017). However, this study will focus on the use of cryovial and cryomesh. In cryomesh, materials are held in a mesh that contains sodium alginate and then polymerized with calcium chloride (Funnekoter *et al*., 2017). The shoot tips attached to the cryomesh are then treated with an osmoprotection solution, followed by dehydration with a cryoprotectant, then plunged directly into LN. In contrast, when using cryovial, the materials are transferred into a cryovial attached to a cryocane and then plunged into LN (Bettoni *et al*., 2021).

Effective rewarming of cryopreserved samples is crucial in all cryopreservation procedures, regardless of the explant type (Bettoni *et al*., 2021). During rewarming, the materials undergo devitrification (loss of the glassy state), which may lead to the development of lethal ice crystals (Benson, 2008). To prevent recrystallisation, the samples must be quickly rewarmed between 25°C (room temperature) and 42°C, depending on the protocol. However, determining the ideal rewarming temperature and duration is a critical factor in this step. In some protocols, the entire cryovial comprising the explants is immersed directly into a warm water bath (38–42°C) for 2–10 minutes (Park and Kim, 2015; Bettoni *et al*., 2021; Kaviani and Kulus, 2022). In other protocols, beads in foils/cryomesh/cryoplates are removed from the cryovial and

directly placed in a warm (25–42°C) unloading solution for 2–10 minutes (Funnekotter *et al*., 2017; Guo *et al*., 2024).

The lack of regeneration after cooling the osmoprotected sweet potato buds indicated that the osmotic dehydration using PVS5% and osmoprotection solution may have not been sufficient (Chapter Three). To improve the efficiency and regeneration rate of buds of sweet potato after cryopreservation, stepwise increments of concentrations of cryoprotectants with the incorporation of PVS3 were recommended in this study. This approach aimed to gradually increase the concentration of cryoprotectant in a way that allowed the buds to adapt to the solution and adjust to the osmotic changes. In turn, this would minimise cellular damage during the cryopreservation process. Additionally, a combination of osmotic and physical dehydration may also assist in improving the viability rate after cryopreservation.

While physical dehydration can be reproducible and less time-sensitive than the vitrification procedure, its success depends on the extent of dehydration and the remaining water content in the sample before cooling. On the other hand, osmotic dehydration can reduce the water content of the sample by allowing water to migrate from the intracellular to the extracellular spaces. By using a combination of these two methods and incorporating PVS3, it may be possible to improve the efficiency and regeneration rate of buds of sweet potato after cryopreservation.

Another factor to consider can be wounding of the plant material during the isolation process. In response to this wounding, chemical changes such as the release of phenolic compounds, reactive oxygen species, alkaloids and flavonoids, as well as cell wall modification in the plant material take place (Davis, 1987; Ren *et al*., 2021). For this study, wound healing response was considered for the improvement of recovery of the plant material before exposure to cryoprotectant.

## 4.2. Materials and Methods

### *4.2.1 Plant materials*

Nodal segments of approximately 4 mm were obtained from *in-vitro* plantlets of sweet potato (*Ipomoea batatas* L.) from the South African NPGRC. The material was established on full strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 0.5 $mgl^{-1}$ gibberellic acid

($GA_3$) adjusted to pH 5.7 for four weeks in test tubes in a growth room (16 hour light / 8 hour dark (photoperiod) at 25°C). The plantlets were used in the experiments after four weeks of plating. The nodal segments containing one shoot tip or axillary bud were dissected into 3 mm segments and plated under the same conditions as described above for 10 days to achieve uniform growth. Buds (1-2 mm) with three or four leaf primordia were isolated using a dissecting microscope.

A set of buds (n = 10) was encapsulated without being left on the medium for wound healing. Another set of buds (n = 10) was plated on the normal sweet potato medium following the same conditions as described above. These buds were left on the medium for two days to allow their cells to respond to the wounding caused by the cutting equipment during isolation. At the end of two days, the buds were encapsulated following the procedure below.

Encapsulation was achieved by sucking each bud into 3% low viscosity alginic acid using a pipette and then dripping them into 0.1 M $CaCl_2$ in liquid MS medium supplemented with 0.4 M sucrose to form beads. Beads were left to polymerise in the calcium solution for 20 minutes.

### *4.2.2. Treatments*

*4.2.2.1. Treatment of the beads with PVS3 for different durations*

*Control*

Beads (n = 10) (with and without wound healing) were plated on normal sweet potato medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite and 0.5 $mgl^{-1}$ $GA_3$ in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C).

*Treatment*

Beads (n = 10─with and without wound healing) (10 beads:20 ml) were treated with PVS3 for 30, 45 and 60 minutes, then plated on the normal sweet potato medium following the same conditions as the control experiment.

*Viability*

Viability was recorded as a total percentage of the buds forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*4.2.2.2 Treatment of encapsulated buds with stepwise increment of cryoprotectants (PVS5%, Osmo and 70%PVS3) with and without LN*

In this experiment, wound healing was applied before encapsulation based on the observation from the experiment in 4.2.2.1. It was observed that the growth of the buds that were allowed to respond to wound healing was better compared with no wound healing. Additionally, more callus was observed on the buds with no wound healing.

*Control*

Beads (n = 10) were plated on sweet potato recovery medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite; 1 $mgl^{-1}$ $GA_3$ and 0.5 $mgl^{-1}$ BAP in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C).

*Treatment*

*(i) Cryoprotectants C1: Treatment of the beads with PVS5% (30 min), Osmo (15 min), 70%PVS3 (10 min) and LN*

The encapsulated buds (beads) (n = 10 per accession) were treated with a stepwise increment of cryoprotectants (PVS5% followed by Osmo, then 70%PVS3) (Figure 4.1). After the treatment, one set (n = 10) of beads was plated on the recovery medium following the same conditions as the control experiment.


**Figure 4.1**. Flowchart of the experimental procedure. The encapsulated buds (beads) of sweet potato accessions (AP130, AP134 and AP156) were sequentially treated with PVS5% for 30 minutes, Osmoprotection (Osmo) solution for 15 minutes and 70%PVS3 for 10 minutes. The beads were cooled in liquid nitrogen (LN) for one hour.

*Cooling*

After treatment with 70%PVS3 a set of beads was transferred to cryomesh (10 beads per cryomesh) and suspended directly in LN, then transferred to cryovial (already in LN) without the lid, secured on cryocane for one hour. Another set of beads was transferred to a cryovial (10 beads per vial) secured on a cryocane and placed in LN

(Figure 4.1). Unlike the cryomesh procedure, the cryovial already containing the beads was plunged directly into LN for one hour.

*Rewarming and regeneration*

Rewarming of the beads in the cryomesh was done by removing the cryomesh and placing it directly into 1 ml unloading solution (liquid MS containing 1.2 M sucrose) at room temperature and 38°C–40°C for 10 minutes. The beads were then blotted dry and plated on recovery medium and incubated for seven days under the same conditions as the control.

Rewarming of the beads in the cryovials was done by placing the cryovial still secured in the cryocane into 38°C–40°C water for two minutes. Then the beads in the cryovial were washed with 1 ml unloading solution (liquid MS containing 1.2 M sucrose) at room temperature for 10 minutes. The beads were blotted dry and plated on recovery medium and incubated for seven days under the same conditions as the control.

*Viability*

The beads from the control experiment and treatments were transferred from the recovery medium to a normal sweet potato medium (full strength (MS) medium supplemented with 30 $gl^{-1}$ sucrose, 0.5 $mgl^{-1}$ $GA_3$ and 2.21 $gl^{-1}$ gelrite. Shoot regeneration was recorded as a total percentage of the shoot tips forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*(ii) Cryoprotectants C2: Treatment of the beads with PVS5% (30 min), Osmo (15 min) and 70%PVS3 (15 min)*

The encapsulate buds (beads) (n = 10 per accession) were treated with a stepwise increment of cryoprotectants (PVS5% followed by Osmo, then 70%PVS3) (Figure 4.2). The treated beads were not exposed to LN. After the treatment, the beads (n = 10) were plated on the recovery medium following the same conditions as the control experiment.


**Figure 4.2**. Flowchart of the experimental procedure. The encapsulated buds (beads) of sweet potato accessions (AP130, AP134 and AP156) were sequentially treated with PVS5% for 30 minutes, Osmoprotection (Osmo) solution for 15 minutes and 70%PVS3 for 15 minutes. The beads were not cooled in liquid nitrogen (LN).

*Viability*

Viability was recorded as a total percentage of the buds forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

#### *4.2.2.3 Treatment of encapsulated buds with stepwise increment of cryoprotectants (PVS5%, Osmo and 70%PVS3) followed by physical dehydration and LN*

*Control*

The encapsulated buds (beads) (n = 10) were plated on sweet potato recovery medium (MS medium with 30 $gl^{-1}$ sucrose; 2.21 $gl^{-1}$ gelrite; 1 $mgl^{-1}$ $GA_3$ and 0.5 $mgl^{-1}$ BAP in Petri dishes, adjusted to pH 5.7 (16 hour light / 8 hour dark (photoperiod) at 25°C).

*Treatment*

Beads (n = 10 per accession) were exposed to osmotic and physical dehydration using a stepwise increment of cryoprotectants for different durations. The physical dehydration process involved placing the beads on activated silica gel on a Petri dish, which was subsequently placed in a desiccator for the specified period.

*(i) Cryoprotectants C3: Treatment of the beads with PVS5% (30 min), Osmo (15 min), 70%PVS3 (10 min), Physical dehydration (10 min) and LN*

Beads (n = 10 per accession) were treated with a stepwise increment of cryoprotectants (PVS5% followed by Osmo, then 70%PVS3). After the treatments, the beads were dehydrated for 10 minutes (Figure 4.3). After the dehydration, one set (n = 10) of beads was plated on the recovery medium following the same conditions as the control experiment.


**Figure 4.3**. Flowchart of the experimental procedure. The encapsulated buds (beads) of sweet potato accessions (AP130, AP134 and AP156) were treated with PVS5% for 30 minutes, followed by Osmoprotection (Osmo) solution for 15 minutes, then 70%PVS3 for 15 minutes. After 70%PVS3 treatment the beads were subjected to physical dehydration in a desiccator for 10 minutes. The beads were then cooled in liquid nitrogen (LN) for one hour.

*Cooling*

Another set of beads was transferred to cryomesh (10 beads per cryomesh) and suspended directly in LN, then transferred to cryovial without the lid secured on cryocane into LN for one hour.

*Rewarming and regeneration*

Rewarming of the beads in the cryomesh was done by removing the cryomesh and placing it directly into 1 ml unloading solution (liquid MS containing 1.2 M sucrose) at room temperature, 38°C–40°C for 10 minutes. The beads were then blotted dry and plated on recovery medium and incubated for seven days under the same conditions as the control.

*Viability*

The beads from the control experiment and treatments were transferred from the recovery medium to a normal sweet potato medium (full strength (MS) medium supplemented with 30 $gl^{-1}$ sucrose, 0.5 $mgl^{-1}$ $GA_3$ and 2.21 $gl^{-1}$ gelrite. Shoot regeneration was recorded as a total percentage of the shoot tips forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*(ii) Cryoprotectants C4 (PVS-PD): Treatment of the beads with PVS5% (30 min), Osmo (10 min), 70%PVS3 (10 min), Physical dehydration (10 min) and LN*

Beads (n = 10 per accession) were treated with a stepwise increment of cryoprotectants (PVS5% followed by Osmo, then 70%PVS3). After the treatments,

the beads were dehydrated for 10 minutes (Figure 4.4). After the dehydration, one set (n = 10) of beads was plated on the recovery medium following the same conditions as the control experiment.


**Figure 4.4**. Flowchart of the experimental procedure. The encapsulated buds (beads) of sweet potato accessions (AP130, AP134 and AP156) were treated with PVS5% for 30 minutes, followed by Osmoprotection (Osmo) solution for 10 minutes, then 70%PVS3 for 10 minutes. After 70%PVS3 treatment the beads were subjected to physical dehydration in a desiccator for 10 minutes. The beads were then cooled in liquid nitrogen (LN) for one hour.

*Cooling*

Another set of beads was transferred to cryomesh (10 beads per cryomesh) and suspended directly in LN, then transferred to cryovial without the lid secured on cryocane into LN for one hour.

*Rewarming and regeneration*

Rewarming of the beads in the cryovials was done by placing the cryovial still secured in the cryocane into 38°C–40°C water for two minutes. Then the beads in the cryovial were washed with 1 ml unloading solution (liquid MS containing 1.2 M sucrose) for 10 minutes. The beads were blotted dry and plated on recovery medium and incubated for seven days under the same conditions as the control.

Rewarming of the beads in the cryomesh was done by removing the cryomesh and placing it directly into 1 ml unloading solution (liquid MS containing 1.2 M sucrose) at room temperature, 38°C–40°C for 10 minutes. The beads were then blotted dry and plated on recovery medium and incubated for seven days under the same conditions as the control.

*Viability*

The beads from the control experiment and treatments were transferred from the recovery medium to a normal sweet potato medium (full strength (MS) medium

supplemented with 30 $gl^{-1}$ sucrose, 0.5 $mgl^{-1}$ $GA_3$ and 2.21 $gl^{-1}$ gelrite. Shoot regeneration was recorded as a total percentage of the shoot tips forming normal shoots 30 days after plating. A total of 10 beads were treated in each of the three replicates per treatment.

*Analyses*

Experiments were repeated three times. An analysis of variance (ANOVA) in GENSTAT was used to calculate means to compare plant viability among the three treatments. The data are presented as percentages and the least significant difference (LSD) was calculated at $P < 0.005$. MS Excel (Microsoft Office 2007) with add-ins statistical program was used to generate graphs.

## 4.3. Results and Discussion

### *4.3.1. Treatment of buds of sweet potato with PVS3 for different durations*

The viability of untreated (control) buds of all the tested accessions (AP130, AP134 and AP156) of sweet potato was 99─100% (Figure 4.5).

*30 and 45 minutes*

Treatment of the buds with PVS3 for 30 and 45 minutes of exposure resulted in 73-83% and 71─81% regrowth, respectively. There was a significant difference ($df = 3$, $P < 0.001$) between accessions AP156 and AP130, AP134 with the 30 minutes of exposure in PVS3, in which accession AP130 obtained 73% regrowth of the buds. Another significant difference ($df = 3$, $P < 0.001$) was observed between accessions AP130 and AP134, AP156 in which 71% regrowth of buds of accession AP156 was obtained. Although regrowth was observed, most of it originated from callus and some buds showed signs of hyperhydricity (Figure 4.6). Some buds remained green without further expansion and root development 30 days after plating on MS medium.

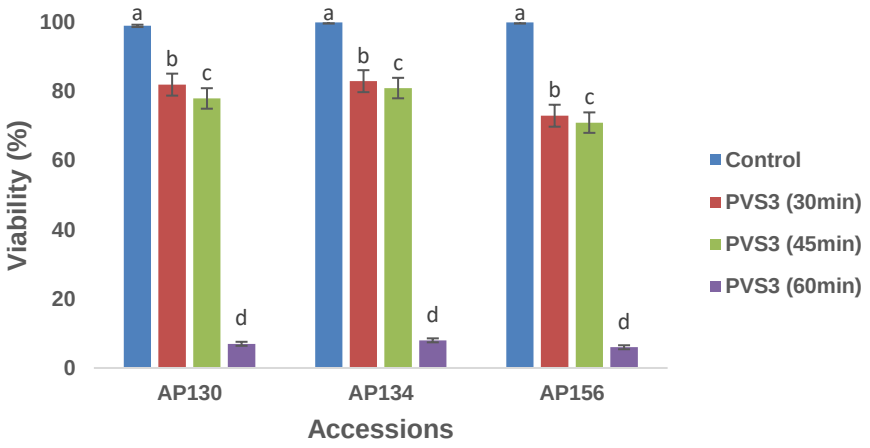


**Figure 4.5.** Viability of buds of South African sweet potato accessions (AP130, AP134 and AP156) after non-treatment (control) and treatment with PVS3 for 30, 45 and 60 minutes. The least significant difference at P<0.005 (LSD 5%) indicates the difference between the treatments. Different letters indicate significant differences in the treatments.

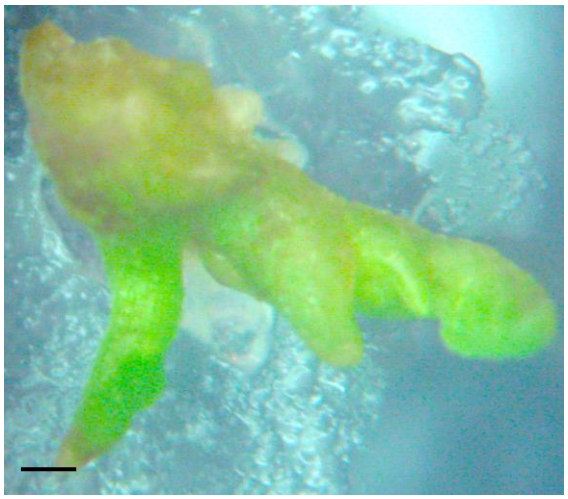

**Figure 4.6.** Shoot tip regenerated from the encapsulated bud of sweet potato showing signs of hyperhydricity 30 days after plating on MS medium. The bud was treated with PVS5% (30 minutes), Osmoprotection (15 minutes), 70%PVS3 (15 minutes) and physical dehydration (10 minutes). Scale bar = 1 mm

*60 minutes*

The treatment of the buds with PVS3 for 60 minutes showed a decline in the regeneration from 100-6% (Figure 4.5.). This observation agreed with the study of Park and Kim (2015) and Shevchenko *et al*. (2021) on sweet potato, in which a decline in regeneration was obtained after treatment with PVS3 for 60 minutes (19% regeneration). It has been mentioned (Volk and Caspersen, 2017), that PVS3 induces

plasmolysis within five minutes of exposure and cells can remain plasmolysed for three hours. The buds of sweet potato investigated in this study may have been sensitive to the chemical dehydration of PVS3 for 60 minutes of exposure.

The application of PVS3 on sweet potato has shown promising results in improving the cryopreservation of sweet potato buds. However, longer exposures have resulted in a decline in viability as shown by the study of Noor *et al*. (2021). To address this, some studies resorted to modifying PVS3 to improve its effectiveness in cryopreservation. For example, Kim *et al*. (2009), Shevchenko *et al*. (2021) and Lee *et al*. (2023), have explored adjusting the concentration of PVS3 to between 30 and 90%. Other adjustments included reducing exposure time to 10 minutes (Noor *et al*., 2021). But this did not improve the overall success rate of cryopreservation. Further research is needed to optimise the concentration and duration of exposure to PVS3 to achieve consistent success across a wider range of sweet potato genotypes. Based on the results obtained in this study, it was deemed necessary to modify PVS3 by reducing its concentration to 70% as well as applying stepwise increments of concentrations of the cryoprotectant solution.

### *4.3.2 Treatment of encapsulated buds with stepwise increment of cryoprotectants (PVS5%, Osmo and 70%PVS3) with (+LN) and without (-LN)*

Treatment of sweet potato buds with PVS5% for 30 minutes followed by immersion in osmoprotection solution had no impact on their viability (Chapter Three). Notably, the application of PVS5% for 30 minutes, followed by PVS3 for 20 minutes, resulted in the lower viability of sweet potato buds (data not shown). Similarly, treatment with stepwise increment of cryoprotectants (PVS5% for 30 minutes followed by osmoprotection for 20 minutes, then PVS3 for 10 minutes) did not impact the viability rate (data not shown). The total molarity of PVS3 is 6.8 M, osmoprotection solution 3.6 M and PVS5%, 2.4 M. It was then suggested that treatment of the buds with 6.8 M immediately after 3.6 M may have imposed an osmotic shock on the buds of sweet potato.

The adjustment of PVS3 to 70% in this study, was based on the results obtained in other studies in which >50% regeneration was reported (Shevchenko *et al*., 2021; Lee *et al*., 2021; Lee *et al*. 2023). In their study on sweet potato, Shevchenko *et al*. (2021) obtained 51% regeneration after exposing shoot tips to 88%PVS3. That finding

provided the idea to reduce the concentration of PVS3 to 70% (with a molarity of 4.8 M) thereby reducing the osmotic shock between solutions and possibly improve the regeneration rate after cryopreservation. Thus, the subsequent investigation applied PVS5%, osmoprotection and 70%PVS3 for 10 and 15 minutes of exposure.

*(i) Cryoprotectants C1: Treatment of the beads with PVS5% (30 min), Osmo (15 min), 70%PVS3 (10 min) and LN*

The buds of sweet potato exhibited a high viability range of 92 to 98% when subjected to treatment C1, which involved a stepwise treatment in PVS5%, osmoprotection solution and 70%PVS3 for varying durations (Table 4.1). All the viable plantlets regenerated into complete plantlets throughout the accessions. Such a high viability range suggested a strong potential for regrowth in subsequent experiments involving LN.

*Cooling and Rewarming*

Upon cooling the beads inside the cryovials turned white. In contrast, the beads within the cryomesh did not turn entirely white during cooling. This phenomenon was documented by Park and Kim (2015) in their study on sweet potato, where they found that cryomesh facilitated direct contact of the buds with LN and minimise the total thermal mass (Zhan *et al*., 2021), resulting in a more rapid cooling rate. Likewise, Guo *et al*. (2024) observed an accelerated cooling rate with the use of cryomesh over cryovials.

After being warmed in a 1.2 M sucrose solution, the beads appeared cloudy. Although the buds were initially green, they eventually turned pale and black after two days on the recovery medium. This lack of growth could be due to the presence of water within the tested accessions' buds. The whitening of the beads in our study indicated ice crystallization, which suggests that the buds were not dehydrated enough when exposed to LN. These results warranted an extended duration of 70%PVS3 to 15 minutes (C2) in the subsequent experiments. Additionally, cryomesh was used instead of cryovial due to the higher rates of cooling and rewarming

**Table 4.1.** Viability of buds of three South African sweet potato accessions (AP130, AP134 and AP156). The buds were treated with sequential increments of cryoprotectants (C1, C2, C3 and C4 (PVS-PD) for different durations. After these treatments, the buds were plated on the sweet potato recovery media. The least significant difference at P<0.005 (LSD 5%) indicates the difference between the treatments. The percentage of accessions that remained viable is represented by (V) and those regenerated into complete plantlets are represented by (R).Values followed by the same letter are not significantly different.

| **Accession** | **Viability (V) and Regeneration (R) (%)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Concentrations** | | | | | | |
| | **C1** | **C2** | | **C3** | | **C4** (PVS-PD) | |
| | V/R | V | R | V | R | V | R |
| **AP130** | 92$^{c}$ | 77$^{b}$ | 35$^{b}$ | 93$^{c}$ | 43$^{c}$ | 97$^{a}$ | 87$^{a}$ |
| **AP134** | 98$^{a}$ | 51$^{c}$ | 28$^{c}$ | 95$^{b}$ | 50$^{b}$ | 96$^{a}$ | 86$^{a}$ |
| **AP156** | 95$^{b}$ | 82$^{a}$ | 38$^{a}$ | 97$^{a}$ | 54$^{a}$ | 94$^{b}$ | 83$^{b}$ |

*(ii) Cryoprotectants C2: Treatment of the beads with PVS5% (30 minutes) followed by osmoprotection (15 minutes) then 70%PVS3 (15 minutes)*

The treatment of the buds with 70%PVS3 for 15 minutes of exposure (C2) led to a decline in the viability of sweet potato buds, ranging between 51 and 82%. However, 20–38% of these viable buds regenerated into complete plants. These results may have been due to an extended duration of the beads in 70%PVS3, from 10 to 15 minutes. A regeneration rate of 38% prior to cooling was inadequate to support successful cryopreservation. Across all accessions, a significant difference (df = 3, P <0.001) was observed, indicating that genotypes respond differently to cryoprotectants (Figure 4.3). As stated by Sakai and Engelmann (2007), exposing tissues to a highly concentrated solution leads to plasmolysis in the meristematic cells, producing concentrated spherical protoplasm. However, excessive exposure can result in irreversible plasmolysis and cell destruction (Lang *et al*., 2014). To further reduce the water content of sweet potato buds, physical dehydration with silica gel was introduced as a response to this observation.

*4.3.3 Treatment of encapsulated buds with stepwise increment of cryoprotectants (PVS5%, Osmo and 70%PVS3) followed by physical dehydration with LN exposure*

*(i) Cryoprotectants C3: Treatment of the beads with PVS5% (30 minutes) followed by osmoprotection (15 minutes) then 70%PVS3 (10 minutes) and physical dehydration (10 minutes) then LN exposure*

In C3, the duration of exposure of the encapsulated buds to 70%PVS3 was reduced to 10 minutes, with incorporation of physical dehydration for 10 minutes. After being treated with C3, the sweet potato buds exhibited 93–97% viability. This retention of viability was consistent across all treated accessions. However, only 43–54% of these viable buds successfully regenerated into complete plants (Figure 4.8.). The rest remained green without further development (Figure 4.7.). These results were higher than those obtained in C2 treatment (38%), indicating that further modification of the duration in the treatments may improve the regeneration rate of the encapsulated buds of sweet potato.

Physical dehydration causes the migration of water from the intracellular to the extracellular spaces and as a result, cell volume decreases. This has been reported (Benson *et al*., 2008; Bettoni *et al*., 2021), to result in an increased solute concentration within the cell. This action results in the infolding of the plasma membrane forming endocytic vesicles, leading to the plasma membrane losing the surface area during extreme dehydration (Ambroise *et al*., 2020; Bettoni *et al*., 2021). The incorporation of physical dehydration in this case, was necessary to further reduce the amount of water in the encapsulated buds of sweet potato.

**Figure 4.7.** Plantlets regenerated from the buds of South African sweet potato accessions 30 days after plating on MS medium. The buds were treated with PVS5% (30 minutes) followed by osmoprotection (15 minutes) and 70%PVS3 (15 minutes).

**Figure 4.8.** Plantlets regenerated from the buds of sweet potato 30 days after plating on MS medium. The buds were treated with PVS5% (30 minutes) followed by osmoprotection (15 minutes) then 70%PVS3 (15 minutes) and physical dehydration (10 minutes).

*Cooling and Rewarming*

Notably in this experiment, there was no crystallisation observed during the cooling process, as the beads remained translucent. This lack of crystallisation indicated that the buds had been sufficiently dehydrated prior to exposure to LN. Upon rewarming in 1.2 M sucrose at room temperature and 38°C, the beads turned cloudy. During rewarming, the melted extracellular water re-enters the cell, causing the cell to rupture before regaining its original volume (Ambroise *et al*., 2020). Eventually, the cell wall collapses and ruptures due to the influx of water leading to cell death (Yamada *et al*., 2002). This may have been the case in this study. It was therefore suggested that the treatment duration of the osmoprotection solution be reduced to 10 minutes to further improve the regeneration rate.

*(ii) Cryoprotectants C4 (PVS-PD): Treatment of the beads with PVS5% (30 minutes) followed by osmoprotection (10 minutes) then 70%PVS3 (10 minutes) and physical dehydration (10 minutes) with LN exposure*

An improved regeneration rate (83–87%) was observed when buds of sweet potato were treated with C4 (PVS-PD), in comparison with C3 treatment which resulted in a maximum of 54% regeneration. In the C4 treatment, the duration of the beads of sweet potato in the osmoprotection solution was reduced from 15 to 10 minutes (Table 4.1). These results suggested that PVS-PD had the potential for further development of the cryopreservation protocol.

*Cooling and Rewarming*

There was no ice crystallisation during cooling. The beads remained clear without any sign of cloudiness. It has been reported earlier in this study that the avoidance of ice crystallisation during cooling relies in the amount of water in the samples to be cryopreserved (Berjak, 1996; Engelmann, 2004). Lack of cloudiness observed in this study indicated that the buds dehydrated to a moisture content that will not cause freezing injury.

Recrystallisation was observed during rewarming in 1.2 M sucrose at room temperature and 38°C. The buds were green immediately after rewarming, but turned pale and brown two days after plating on the recovery medium. Kaczmarczyk *et al*. (2008) in their study on *potato*, found that ice recrystallisation is possible during rewarming and that uneven distribution of cryoprotectants in different parts of shoot tips can lead to ice crystal formation and cell death. Additionally, rewarming can result in oxidative stress due to the increased level of Reactive Oxygen Species (ROS) in the mitochondria. Oxidative stress is a major contributor to cryo-injury (Berjak and Pammenter, 2014; Funnekotter *et al*., 2017).

To prevent ice recrystallisation within cells, high rewarming speeds are recommended to ensure a rapid transition from glass to liquid phase. Bettoni *et al*. (2021) and Fahy and Wowk (2021), also noted that ice crystals can occur below the freezing point and can damage cell membranes and plant tissue structures if rewarming is not rapid enough. However, other studies have shown that survival and regeneration can still occur in the leaf primordial tissues and meristematic region of shoot tips despite ice recrystallisation during rewarming (Barraco *et al*., 2014; Volk and Caspersen, 2017). It is therefore suspected that the rewarming rates may have not been fast enough to prevent ice recrystallisation during rewarming. Additionally, temperature of the unloading solution may have also affected the rewarming rates.

## 4.4. Conclusions and Recommendations

Although previous studies have suggested that PVS3 is less toxic than PVS2 (Nishizawa *et al.*, 1993; Senula and Nagel, 2021; Lee *et al*., 2023), this study found that the buds of sweet potato accessions were sensitive to the original PVS3. To improve regeneration rates after cryopreservation, it has been suggested to test multiple combinations and factors (Kim and Popova *et al.*, 2023), or manipulate current

protocols and cryoprotectants (Benson, 2008). Therefore, in this study, the original PVS3 was modified to 70% to reduce its toxic effect. Additionally, a step-wise treatment of the buds with PVS5%, osmoprotection solution and 70%PVS3 sequentially, was implemented and resulted in the regeneration rate of the encapsulated buds of sweet potato after treatment with C1 and C2 (both without physical dehydration) to 98 and 38%, respectively. However, further physical dehydration of the buds with silica gel was necessary to further reduce the amount of water in the encapsulated buds of sweet potato and prevent ice crystallisation during cooling and rewarming.

The application of physical dehydration following the sequential increment of cryoprotectants (PVS5%, Osmo and 70%PVS3) improved the regeneration rates. Treatment of the encapsulated buds of sweet potato with C3 and C4 (PVS-PD) resulted in the regeneration rates of 54 and 87%, respectively. The lack of crystallisation during cooling of the beads treated with PVS-PD, suggested that PVS-PD has the potential for further development of a successful cryopreservation protocol for sweet potato.

Although recrystallisation occurred during rewarming of the beads in 1.2 M sucrose at room temperature and 38°C, the combination treatment applied in this study showed some potential that requires further investigation to optimise the rewarming aspects after cooling. As maintained by Kaviani (2011) and Benson (2008), determining the appropriate rewarming solution, duration and temperature are crucial for successful cryopreservation. Some studies (Barraco *et al*., 2014; Volk and Caspersen, 2017), reported that some cells can survive the recrystallisation that can occur during rewarming. Future studies are required to optimise the rewarming process as well as taking into account the recovery media composition to improve the survival and regeneration of the cryopreserved material. In view of the results discussed above, treatment C4 (PVS-PD) will be used to further develop cryopreservation protocol for sweet potato accessions conserved in the South African NPGRC.

# CHAPTER FIVE: GENERAL DISCUSSION, CONCLUSION AND FUTURE PERSFECTIVE

## Discussion

The department of Agriculture, Land Reform and Rural Development (DALRRD), through the South African National Plant Genetic Resources Centre (NPGRC) developed a National Plan for conservation of genetic resources. One of the objectives of the plan was to strengthen conservation protocols for vegetatively propagated crop species of importance to South African agriculture. One of these crops is sweet potato (*Ipomoea batatas*) as it plays a significant role in food security and nutrition in most areas of Africa, including South Africa. The conservation methods currently applied at the South African NPGRC are field and tissue culture methods. However, these methods are for the short-medium term. Cryopreservation is a long-term conservation method that can help conserve landrace food crops for future breeding programs, ensuring the availability of traditional crop varieties and food security through time.

Numerous publications on the cryopreservation methods available for cryopreservation of vegetatively propagated species such as sweet potato may lead one to believe that these methods are ready for implementation. However, this is not the case due to varying levels of success which provide evidence that no uniform method applies to various genotypes of the same species. These varying levels of success have resulted in a collection of diverse approaches and cryopreservation procedures that can be further adjusted to specific plant materials or individual genotypes.

For cryopreservation of sweet potato conserved at the NPGRC of South Africa, it was believed that the encapsulation-vitrification method would be the most suitable as it resulted in a higher percentage (80%) of survival and regeneration. However, this was not the case (Chapter Two). Thus, supporting the evidence that, damage accumulating during the different steps of the cryopreservation protocol often leads to reduced regrowth, even when the apparent optimised protocol was applied (Popova *et al*., 2023).

A successful cryopreservation protocol relies on the withdrawal of free water from the cells/tissues/organs of the cryopropagule, subsequently depressing the freezing point

and the concurrent increase in the glass transition temperature (*Tg*). In most cases, cryoprotectants such as those found in PVS2 and PVS3 have been used to withdraw and replace water in the sample. As observed in this and other studies the concentration of, and exposure time to PVS2 is species and indeed genotype-dependent (Pettinelli *et al*., 2012; Park and Sue, 2015). The lack of viability from buds treated with PVS2 in this study (Chapter Two) indicated the sensitivity of the South African accessions to the high concentrations of PVS2. Exposing sweet potato explants to <15°C in tissue culture resulted in stunted growth indicating its cold sensitivity. In support, Takagi *et al*. (2018) also mentioned the difficulty of cold-hardening sweet potato in their study. Other publications (Park and Sue, 2015; Yi *et al*., 2016; Shevchenko *et al*., 2021) reported shoot regrowth after applying PVS2 at room temperature. Based on these reports, the application of PVS2 at 0$^{o}$C was not taken into consideration.

Due to the high concentration and toxic nature of PVS2, other studies reduced the strength of PVS2 to 20─80% and/or preconditioned the buds to a higher concentration of full-strength PVS2 (Park and Sue, 2015; Shevchenko *et al*., 2021; Lee *et al*., 2023). Similarly, this study reduced the concentration of PVS2 to 5% and its application as a cryoprotectant resulted in 93% regeneration of the control buds of all three tested accessions (Chapter Two). This indicated that PVS5% may present the potential of being a successful cryoprotectant for the buds of sweet potato accessions maintained at the South African NPGRC. However, when exposed to LN, the viability of the buds was lost (Chapter Two). Despite the potential of PVS5% as a cryoprotectant, it was suspected that PVS5% may have not provided sufficient dehydration due to the lower concentration of its components (5%; 2.4 M). It was proposed that the water content of the buds of sweet potato accessions be assessed for further evaluation of PVS5%, as the cryoprotectant for the development of a successful cryopreservation protocol.

In Chapter Three of this study, the effect of cryoprotectants on water content and regeneration of sweet potato buds was shown (Table 3.1). PVS5% treatment caused an increase in water content from 26─72%, leading to loss of viability when the material was exposed to LN. It is a fact that the success of cryopreservation depends on maintaining low water content to prevent ice crystal formation during cooling and ensuring recovery after rewarming. The increase in water content observed after the

treatment with PVS5% was presumably the reason for the loss of viability observed after exposure of the buds to LN (Chapter Two). When water re-entered the cell during treatment with PVS5%, the free water levels in the cell increased providing provision for the intercellular movement of water molecules (Griffith and Yaish, 2004; Jaheed *et al*., 2023). As a result, when this material was exposed to LN, the negative osmotic pressure drove water from the cell interior to the extracellular spaces, thus reducing cell volume. The fact that the beads turned cloudy upon cooling and rewarming was indicative of lethal ice crystal formation. This increase in water content prompted the suggestion of swapping the last two treatments, i.e. applying the PVS5% solution before the osmoprotection solution. This was based on the overall molarity of the two solutions.

Like the results obtained before swapping, a reduction in water content was observed with an increase in the concentration of the cryoprotectants (Chapter Three). A decline from 79 to 34% was observed when buds were treated with PVS5% and then the osmoprotection solution. Although this decline in the water content is required and necessary for the development of a successful cryopreservation protocol, the regeneration of the buds was however, impacted. The treatment of the buds with an osmoprotection solution for three hours showed a decline in the regeneration from 94 to 26% (Table 3.1.). Based on these results, it was deemed necessary to determine the optimal duration of exposure on the buds to the osmoprotection solution.

It was proven in this study that prolonged exposure of more than 20 minutes can induce toxicity (Chapter Three). Based on these observations 20 minutes (viability 86%) was selected for further evaluation with other steps of the cryopreservation protocol. When the osmoprotected buds were exposed to LN, they turned white during cooling and rewarming processes, evidence that ice crystallization had taken place. Only 1% survival was observed. This indicated that osmoprotection solution at 20 minutes with a water content of 26%, may have not sufficiently dehydrated the buds to the desired water content despite its total molarity of 3.6. When the experiment was repeated, no growth was observed. The application of PVS prior to osmoprotection has not been widely reported in other publications, which considered osmoprotection solution necessary for decreasing the toxic effects of the subsequent vitrification solution. PVS5% has a lower concentration of DMSO (5% weight/volume), which

effectively decreases the thickness of the cell membrane. Consequently, glycerol can more easily penetrate the cell, causing dehydration and a decrease (optimal level) in water content within the cell. This study proved that PVS5% can be applied prior to osmoprotection solutions, however, a duration of more than 20 minutes can result in reduced viability.

According to certain reports (Nishizawa *et al*., 2003; Volk *et al*., 2006; Shevchenko *et al*., 2021) there has been a preference for the use of PVS3 (as opposed to PVS2). This is due to the absence of DMSO, a toxic substance that has demonstrated harmful effects when used in higher concentrations (≥30% w/v). It was recommended to incorporate PVS3, apply step-wise increments of cryoprotectants then follow up with a physical drying method.

Like other studies (Volk *et al*., 2006; Shevchenko *et al*., 2021; Lee *et al*., 2023), this study (Chapter Four) contributed to the evidence that the treatment of the buds with PVS3 for 60 minutes results in a decline in the regeneration from 100─6% (Table4.1.). This study proposed that, the buds of sweet potato investigated in this study may have been sensitive to the chemical dehydration of PVS3 for 60 minutes of exposure. Volk and Caspersen (2017) mentioned that PVS3 induces plasmolysis within five minutes of exposure and cells remain plasmolysed for three hours. This may have been the case in this study. To address this, some studies resorted to modifying PVS3 to improve its effectiveness in cryopreservation. For example, Kim *et al*. (2009), Shevchenko *et al*. (2021) and Lee *et al*. (2023), have explored adjusting the concentration of PVS3 to between 30 and 90%. Other adjustments included reducing exposure time to 10 minutes (Noor *et al*., 2021) but this did not improve the overall success rate of cryopreservation.

Further research was needed to optimise the concentration and duration of exposure to PVS3, to achieve consistent success across a wider range of sweet potato genotypes. The subsequent experiments investigated the use of 70%PVS3 (modified PVS3) as well as applying stepwise increments of concentrations of cryoprotectants. PVS3 (70%) has a molarity of 4.8 M, which may reduce the osmotic shock between solutions and possibly improve the regeneration rate after cryopreservation. Therefore in the subsequent investigations, this study treated the buds of sweet potato with PVS5%, osmoprotection solution and 70%PVS3 for 10 and 15 minutes of exposure

(Table 4.1). The results showed a high viability range of 92 to 98% when subjected to a stepwise treatment in PVS5%, osmoprotection solution and 70%PVS3 for varying durations (C1). This study showed that the modified PVS2 and PVS3 can be applied in the same protocol and result in the regrowth of the buds of sweet potato. This finding has not been reported in other publications. Despite the extreme dehydration observed in the buds after treatment with C1, there was no growth detected after exposure to LN. The beads, which were not entirely white during cooling, turned cloudy upon rewarming in 1.2 M sucrose. The buds were green when initially plated but eventually turned pale and black after two days on the recovery medium.

This lack of growth may be attributed to the presence of water within the tested accessions' buds. As the temperature decreases in LN, water molecules in plant tissues form crystals that continue to grow, eventually causing damage to the membrane structure (Panis, 2019). Thus, any freezable water in the sample can form ice crystals upon exposure to LN. The whitening of the beads in this study indicated ice crystallisation, suggesting that the buds were not sufficiently dehydrated when exposed to LN.

Further experiments treated buds with PVS5%, osmoprotection solution and 70%PVS3, followed by dehydration with silica gel for 10 minutes (C3; Chapter Four). Following these experiments, the sweet potato buds exhibited 93-97% viability. This increase in viability was consistent across all treated accessions. However, only 43–54% of these viable buds successfully regenerated into complete plants (Figure 4.7.). The rest remained green without further expansion (Figure 4.8.). During physical dehydration, water moves from the intracellular to the extracellular spaces, leading to a reduced cell volume. This can result in the plasma membrane forming endocytic vesicles and losing the surface area (Ambroise *et al*., 2020; Bettoni *et al*., 2021). Reintroducing water during rewarming can cause the cell to burst before returning to its original size (Ambroise *et al*., 2020). To enhance the regeneration rate of sweet potato buds, it was suggested in the study to decrease the treatment duration of the osmoprotection solution to 10 minutes.

An improved regeneration rate (83–87%) was observed when buds of sweet potato were treated with C4 (PSV-PD), in which the duration in the osmoprotection solution was reduced to 10 minutes (Chapter Four). Nonetheless, viability was impacted after

cryopreservation. There was no ice crystallisation during cooling. The beads remained clear without any sign of cloudiness. Surprisingly, recrystallisation was observed during rewarming in 1.2 M sucrose. The buds were green when initially plated but eventually turned pale and black after two days on the recovery medium.

Several factors may be attributed to recrystallisation that was observed during rewarming process. One of these factors may be the rate of rewarming which was not fast enough to bypass ice formation. Kaviani *et al.* (2011) advised high rewarming rates that ensure a rapid transition from glass to liquid phase without ice recrystallisation within the cells. Other factors may be the concentration and composition of the rewarming solution, duration and temperature of the rewarming solution. In this study, recrystallisation was observed during rewarming in 1.2 M sucrose. The beads were green immediately after rewarming but turned pale and brown a day later. Kaczmarczyk *et al*. (2008) in their study on *Solanum,* found that ice recrystallisation is possible during rewarming. Moreover, the uneven distribution of cryoprotectants in different parts of shoot tips can lead to ice crystal formation and cell death. Additionally, rewarming can cause oxidative stress due to increased production of Reactive Oxygen Species (ROS) in the mitochondria. Oxidative stress is a major contributor to cryo-injury (Berjak and Pammenter, 2014; Funnekotter *et al*., 2017).

To prevent ice recrystallisation within cells, high rewarming speeds are recommended to ensure a rapid transition from glass to liquid phase. Bettoni *et al*. (2021) and Fahy *et al*. (2021), also noted that ice crystals can occur below the freezing point. This can damage cell membranes and plant tissue structures if rewarming is not rapid enough. However, other studies have shown that, survival and regeneration can still occur in the leaf primordial tissues and meristematic region of shoot tips, despite ice recrystallisation during rewarming (Zyk *et al*., 2008; Barraco *et al*., 2014; Volk and Caspersen, 2017). It is important to note that, cryoprotective procedures can vary greatly among different plant species due to differences in cell structure and composition.

## Conclusion

This study proved that treatment of the buds of sweet potato with a step-wise increase of cryoprotectants, followed by physical dehydration was beneficial to successful regrowth. Preincubation and preculture treatments in this study were omitted. And, this

proved that these pretreatments were not necessary in preconditioning the material to the subsequent treatment with cryoprotectants; the regenerate percentage (>80%) was obtained. Although recrystallisation occurred during rewarming process in 1.2 M sucrose at 38─40°C, the combination treatment applied in this study exhibited the potential and warrants further investigation to refine the rewarming technique following cryopreservation.

As recommended by Kaviani (2011) and Benson (2008), determining the appropriate rewarming solution, duration and temperature are critical to achieving successful cryopreservation. Additionally, Popova *et al*. (2023), emphasised the importance of rewarming the cryopreserved buds with a step-wise increase of concentration of the rewarming solution. The recovery conditions are also important, i.e. lighting intensity, dark or light, recovery medium with or without plant growth regulators (Popova *et al*., 2023).

The findings of this study have made significant contributions to the field of cryopreservation, particularly in improving the understanding of the process. These include:

- ✓ The discovery that plating sweet potato buds on a normal medium for two days to allow wound healing response before treatment with cryoprotectants, can significantly improve regeneration rates. Surprisingly, this finding has not been widely reported in other publications. Most publications (Hirai and Sakai, 2003; Wilms *et al*., 2020; Shevchenko *et al*., 2021), applied the cryopreservation procedures immediately after the excision of the buds.

  The cryopreservation process can be a challenging experience for biological material. It starts with the extraction of the explant, followed by dehydration procedures and sudden temperature changes, which can all lead to stress (Uchendu *et al*., 2013). Isolating the explant can cause wounding, which results in chemical changes such as the release of phenolic compounds, ROS, alkaloids and flavonoids, as well as modifications to the cell wall (Howe, 2004). Some plants, like sweet potato, develop calluses in response to wounding, which can protect against contaminants and other factors (Kaity *et al*., 2008). During the regeneration of cryopreserved material, it is crucial to avoid callus formation as it may lead to

somaclonal variation, genetic instability and ultimately cause genetic instability (Hirai and Sakai, 1999; Kaity *et al*., 2008).

- ✓ The success of cryopreservation relies on the resulting water content in the explant before exposure to LN. Both chemical and physical dehydration methods have been employed to remove water from the explant before cryopreservation. The development of a modified plant vitrification solution with a 5% (PVS5%) concentration in this study has resulted in improved regeneration rates of sweet potato buds. The experiments preceding the application of PVS play a crucial role in dehydrating the plant material to prepare it for the extremely cold temperature of LN. However, with this PVS5% concentration, it was not necessary to apply the preincubation and preculture pre-treatments as mentioned in other publications (Hirai and Sakai, 2003; Park and Kim, 2015; Wilms *et al*., 2020). And this has not impacted the viability of the buds of sweet potato accessions investigated in this study. Achieving higher regeneration rates of the explants after treatment with PVS5% is essential to minimise stress on the material during cryopreservation. Therefore, it is imperative that all preceding treatments result in higher viability rates as was the case in this study.

- ✓ The demonstration that modified cryoprotectants (PVS5% and 70% PVS3) can be applied sequentially in the same protocol to achieve high regeneration rates (>80%). Most studies have focused on the use of either PVS2 or PVS3, but not their combination in one protocol.

- ✓ The demonstration that the encapsulation-vitrification-dehydration protocol in which the bud is encapsulated and chemically dehydrated with both modified PVS2 and PVS3, followed by physical dehydration with silica gel, can provide successful regrowth percentages, in preparation for cryopreservation. This concept has not been reported in other publications; instead, the material to be cryopreserved was encapsulated, treated with osmoprotection solution then followed by chemical dehydration with either PVS2 or PVS3. Although the encapsulation-vitrification-dehydration protocol developed in this study has not provided regrowth after cooling. It has however, provided sufficient dehydration and offers the potential for future enhancements and optimisation. Several factors can cause recrystallisation during rewarming.

**Future perspectives**

The highest regeneration rate (87%) of encapsulated sweet potato buds was obtained after treatment with PVS-PD (C4) and there was no ice crystallisation observed during cooling of the material. It is recommended that this treatment be used in further development of cryopreservation protocol for sweet potato accessions conserved at the South African NPGRC.

It is recommended that the rewarming processes be explored to enhance the understanding of recrystallisation in cryopreserved samples, particularly during the rewarming stage. Future research should focus on studying and optimising the rewarming solution, temperature and duration of the rewarming solution to improve the rewarming rates, recovery condition and the composition of the recovery medium. This could further enhance knowledge and understanding of cryopreservation. This, in turn, would lead to an overall improvement in the regeneration rate of cryopreserved tissues. The successful development of this protocol would strengthen conservation efforts for crucial food crops in South Africa, aligning with the need to address the impact of climate change on food security.

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