Towards Development of a Cryopreservation Protocol for Germplasm of *Podocarpus henkelii*

by

Lubaina Essack

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Preface

The experimental work described in this dissertation was carried out in the School of Life Sciences at the University of KwaZulu-Natal (Westville), Durban, under the supervision of Professor Patricia Berjak and Professor Norman W. Pammenter.

These studies represent original work by the author, and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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L. Essack

January 2012

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Abstract

The trees belonging to the genus *Podocarpus*, of which only four species are native to South Africa, are renowned for their superior quality timber. Prior to 1880, *Podocarpus henkelii*, together with *P. falcatus* and *P. latifolius*, played a significant role in the development of the country as they were heavily utilised as timber trees for the building of dwellings, furniture and other necessary items. Due to this over-exploitation in the timber trade, all the *Podocarpus* species in South Africa have been afforded a 'Protected' status on the IUCN red data list of species that are either threatened or in danger of extinction. However, despite the obvious need to conserve the threatened genetic diversity of these species, few attempts (aside from *in vitro* micropropagation) have been made to explore *ex situ Podocarpus* germplasm conservation in the long-term. Consequently, the primary aim of this study was to establish a protocol for the long-term conservation of germplasm of *Podocarpus henkelii* Stapf ex Dallim. Jacks.

The seeds of *Podocarpus henkelii* exhibit recalcitrant behaviour and can therefore not be stored in conventional seed banks. This has necessitated the investigation of alternative methods of germplasm conservation with a focus on cryopreservation which is presently considered the most reliable, efficient and cost-effective means of storing the genetic resources of recalcitrant-seeded species for prolonged periods. The first objective of this study was to investigate the effect of slow (two-step) and ultra-rapid cooling on the post-thaw survival of variously treated *P. henkelii* embryos. The results of this investigation revealed that the rate of cooling employed had a significant effect on explant viability as none of the precultured, cryoprotected embryos that were slowly cooled survived cryostorage while some of the preconditioned embryos responded to ultra-rapid cooling (i.e. 36% shoot production and 88% callus formation). For ultra-rapid cooling, it was found that flash-drying prior to cooling was a prerequisite for survival as osmotic dehydration alone did not effectively prepare the tissues for the stresses imposed during cryostorage. Furthermore, for those flash drying intervals that yielded positive results, preconditioning explants with 10% glycerol proved the most effective pre-cooling treatment. However, due to the low recovery numbers after ultra-rapid cooling, a third cryopreservation technique i.e. cryogenic vitrification, was investigated.

For cooling by vitrification, data obtained from preliminary experiments showed that precultured explants needed to be initially loaded with 18% sucrose (w/v) + 14% glycerol (v/v) for 20 min and subsequently immersed in Plant Vitrification Solution 3 (PVS3) at 0°C for 10 min prior to cooling. However, relatively low success was achieved for *P. henkelii* embryos cooled by vitrification as the highest post-cooling survival obtained was only 20% germination, 27% shoot formation and 37% callus formation.

Due to the low post-thaw survival obtained despite the rigorous manipulations employed in the development of the slow cooling, ultra-rapid cooling and vitrification protocols, it was decided that an alternative explant should be investigated for the conservation of *P. henkelii* germplasm. The explant of choice was adventitious buds induced to form on, and subsequently excised from, mature *P. henkelii* embryos. The first objective was to develop a suitable protocol for the induction of adventitious buds on *P. henkelii* embryos. The medium that induced in the highest percentage of embryos (85%) to form adventitious buds consisted of Douglas-fir cotyledon revised (DCR) basal medium supplemented with 30 g L^{-1} sucrose, 0.05 mg L^{-1} NAA, 0.5 mg L^{-1} BA and 6 g L^{-1} agar. This medium also resulted in the highest average number of buds formed per embryo (i.e. 35 ± 3 buds per embryo). Once the adventitious bud induction medium was developed, it was necessary to optimise the size of adventitious bud clumps to be used as explants for cryopreservation. Three bud clump sizes were investigated: *ca* 3, 5 and 10 buds per clump. However, none of the bud clumps survived excision from the mother-tissue despite the investigation of three different types of bud-break media. The resultant tissue mortality is suggested to have occurred because the adventitious bud clumps were excised prior to bud break and shoot development which could have exacerbated excision-related cellular and sub-cellular damage**.**

It was therefore decided that attempts should be made to induce adventitious buds directly on *P. henkelii* embryos post-cooling, thereby eliminating the possibility of potentially lethal excision-related damage. The protocols that yielded the best results after ultra-rapid cooling and cooling by vitrification were used in this experiment. For ultra-rapid cooling, embryos were first cryoprotected with 5% followed by 10% glycerol for 1 h in each and subsequently flash dried for 30 min prior to immersion in nitrogen slush. For cooling by vitrification, embryos that were first precultured on 0.3

M sucrose for 1 d were loaded with 10% glycerol + 14% sucrose (LS4). The loaded explants were then immersed in ice-cold PVS3 and maintained on ice for 10 min prior to cryostorage. The effect of each pretreatment (either independently or in combination) on adventitious bud production pre-cooling was also investigated. For both protocols the various pretreatments decreased not only the capacity of the embryos to form buds but also the average number of buds formed per embryo (i.e. 7 ± 2 buds per embryo and 14 ± 2 buds per embryo were formed on treated embryos prior to ultra-rapid cooling and cooling by vitrification, respectively). Thus, it was predicted that even if the percentage of cryopreserved embryos forming buds was minimal, the number of possible plantlets that could be regenerated from adventitious buds per cryopreserved explant would compensate for the low recovery of embryos post-cooling. However, none of the embryos that were cryopreserved by either ultra-rapid cooling or by vitrification formed adventitious buds after eight weeks in culture.

The very restricted success achieved in this study despite the investigation of three cryopreservation techniques and two different explants only serves to reinforce the difficulties associated with the conservation of recalcitrant germplasm. The large size and structural complexity of *P. henkelii* embryos, coupled with their high water content post-shedding, are just some of the characteristics to which their intractability to the manipulations involved in the development of a successful cryopreservation protocol could be attributed. For future investigations, development of adventitious buds produced on cryopreserved root segments (as opposed to entire roots), and/or use of seedling meristems as explants which might be amenable to cryopreservation are suggested as possible avenues for the long-term conservation of *P. henkelii* genetic diversity.

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List of Abbreviations

BA	Benzyladenine
BAP	6-benzylaminopurine
°C	degrees Celsius
CaMg	calcium magnesium
CaOCl	calcium hypochlorite
comm.	communication
d	day(s)
DCR	Douglas-fir cotyledon revised medium
dmb	dry mass basis
DMSO	dimethylsulphoxide
FDA	fluorescein diacetate
g	gram(s)
gly	glycerol
h	hour(s)
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2iP	isopentyl adenine
K	kinetin
L	litre(s)
LN	liquid nitrogen
LS	loading solution
M	molar
m	metres
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mol	mole(s)
MS	Murashige and Skoog (1962) nutrient formulation
NAA	α-naphthaleneacetic acid
NaOCl	sodium hypochlorite
no.	number

%	percent
PE	phosphatidylethanolamine
pers.	personal
PGR	plant growth regulator
pH	hydrogen ion content
pt.	point
PVS	Plant Vitrification Solution
ROS	Reactive Oxygen Species
s	second(s)
SD	standard deviation
sucr.	sucrose
TDZ	thidiazuron
T_g	glass transition temperature
T_m	exothermic melting point
TTZ	triphenyl tetrazolium chloride
µ	micro
µE	microeinstein
v	volume
vs.	versus
w	weight
WPM	Woody Plant Medium