RESEARCH NOTE

Cryopreservation of shoot-tips by droplet vitrification applicable to all taro (*Colocasia esculenta* var. *esculenta*) accessions

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Abstract The application of the droplet vitrification cryopreservation technique to taro accessions from a range of Asia Pacific countries is presented. The optimum protocol involves excision of about 0.8 mm shoot-tips from in vitro plants, 20–40 min PVS2 exposure at 0°C followed by rapid plunge into liquid nitrogen. Thawing was done at room temperature (25°C) and shoot-tips inoculated on MS medium with 0.1 M sucrose regenerated into plantlets 4–6 weeks later. This new droplet vitrification protocol improved the mean post-thaw regeneration rates to 73–100% from 21–30% obtained with the previous cryo-vial vitrification protocol.

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Taro (*Colocasia esculenta* var. *esculenta* (L.) Schott), a herbaceous, vegetatively propagated tuber crop, is a very important crop in the Pacific Island countries with great cultural, dietary and economical importance. Traditionally, taro germplasm has been conserved in situ and/or field genebanks and through an in vitro collection of 758 taro cultivars from Asia-Pacific countries at the Regional Germplasm Centre (RGC) of the Secretariat of the Pacific Community (SPC) in Suva, Fiji. Cryopreservation would be the most cost effective, secure and sustainable method of long term conservation of taro germplasm.

Thinh (1997) developed a tissue paper envelope vitrification protocol for Asian taro (*C. esculenta* var. *esculenta*) cultivars with 67–96% survival rates. Sant et al. (2006) applied this method for Pacific taro cultivars resulting in lower regeneration rates of 21–30%. Furthermore, this protocol was successful for only three out of eight cultivars investigated.

The droplet freezing method, first developed for cryopreservation of potato by Schäfer-Menuhr et al. (1997), is proving to be an efficient method across many species and gave the highest regeneration rates in comparative studies with different vitrification methods for yam (Leunufna and Keller 2003) and banana (Panis et al. 2005). This study attempts to verify that the droplet vitrification is an efficient and

easy to execute protocol resulting in high regeneration rates across a wide range of taro genotypes.

In vitro stock plants of taro (*C. esculenta* var. *esculenta*) were obtained from the taro collection maintained at the SPC RGC, Suva. It comprises of taro varieties from several countries in the Asia-Pacific region. Eighteen varieties were selected from the collection, representative for its diversity. Shoots were multiplied according to reported methods (Tuia 1997; Sant et al. 2006). When sufficient material was generated, the accessions were cultured in liquid Murashige and Skoog (1962) medium (MS) with no growth regulators for one month to reduce any carry-over effects of the plant growth regulators. From these plants, 2 cm explants, including some basal corm tissue and an adjoining length of leaf cluster base were excised and grown on 15 ml of MS in 100 ml glass jars (Cospak) for 3 months without subculture. The MS medium contained 3% sucrose and was solidified with 7.5 g l^{-1} Agar Type A (Sigma Chemical Co., Germany) at pH 5.8 ± 0.1. Cultures were incubated at 25°C under 30 µEm^{-2} s^{-1} photon flux density (cool white fluorescent lamps) with a photoperiod of 16/8 h day/night. After 3 months, 2 cm explants were sampled from these plants and conditioned on MS medium supplemented with 90 g l^{-1} (0.26 M) sucrose for 4–8 weeks.

Individual apical meristems were excised under a binocular microscope under aseptic conditions. Leaves were removed one by one with scalpel blade until the shoot-tip (ST) consisting of the apical dome covered with two young leaf primordia was isolated. This ST, 0.8–1.0 mm in size, was cut out with 1.0 mm cube leaf base (corm tissue) (Fig. 1a). The dissected ST was placed on solid MS medium containing 0.3 M sucrose until all STs were excised. The STs were immersed in a McCartney bottle containing 5 ml of filter sterilized loading solution that contained 2 M glycerol and 0.4 M sucrose dissolved in MS medium (pH 5.8) for 20 min at 25°C (Sakai et al. 1990).

After loading, the solution was replaced with 5 ml of chilled PVS2 solution. The filter sterilized PVS2 solution consisted of 30% (w/v) (3.26 M) glycerol, 15% (w/v) (2.42 M) ethylene glycol and 15% (w/v) (1.9 M) dimethylsulphoxide in liquid MS medium containing 0.4 M sucrose at pH 5.8 (Sakai et al. 1990). Just before the end of the PVS2 treatment time, STs were sucked up in a sterile disposable pipette and placed on a strip of sterile aluminium foil (5 × 20 mm) with a droplet of PVS2 solution. Five minutes prior to use, the strip was placed on a plastic Petri dish resting on top of a frozen cooling element to maintain the temperature around 0°C. After the PVS2 treatment, the aluminium strip was plunged directly into liquid nitrogen with a fine forceps until bubbling stopped then quickly transferred to a 2 ml cryo-vial filled with liquid nitrogen. Two replications with four to five shoot-tips per replication were executed for each length of PVS2 exposure. As a first step, shoot-tips of the cultivars CPUK and TNS were investigated for their PVS2 sensitivity at 0°C for exposure times of 5, 10, 20, 30, 40, 50, 60, 90 and 120 min. The resulting optimum PVS2 exposure times were further tested with 16 other cultivars (Table 1).

Rapid thawing was executed by removing the aluminium strip from liquid nitrogen with fine forceps and quickly plunging in 5 ml of filter sterilized unloading solution (liquid MS medium with 1.2 M sucrose at pH 5.8) (Sakai et al. 1990), contained in 15 ml Petri dish, for 15 min at 25°C.

The STs were then plated on two layers of sterile filter paper placed on solid MS with 0.3 M sucrose. These plates were cultured overnight in the dark at 25°C. The following day, the STs were transferred to plates containing solid MS with 0.1 M sucrose without the filter paper. The cultures were maintained in the dark for five days and then transferred to dim light (3.5 µEm^{-2} s^{-1}) for 10 days before exposure to normal culture conditions as stated above. STs growing into plantlets with proper leaves and roots 4–6 weeks following cryopreservation were scored as regeneration (Fig. 1b, c). Statistical analysis was performed with STATISTICA for Windows 5.1 (StatSoft Inc., Tulsa, USA).

All PVS2 sensitivity trials, even after 120 min of PVS2 exposure, gave 100% recovery for the cultivars CPUK and TNS (data not presented), indicating these taro cultivars' low sensitivity towards dehydration with PVS2. The optimal PVS2 exposure range for successful vitrification of shoot-tips of two cultivars was 20–40 min (Fig. 2).

According to Tukeys HSD test ($P < 0.05$) there were no significant differences in post-thaw regeneration rates for 20, 30 and 40 min PVS2 exposure times for any of the cultivars. Some genotype specific responses were observed as cultivars such as PNG
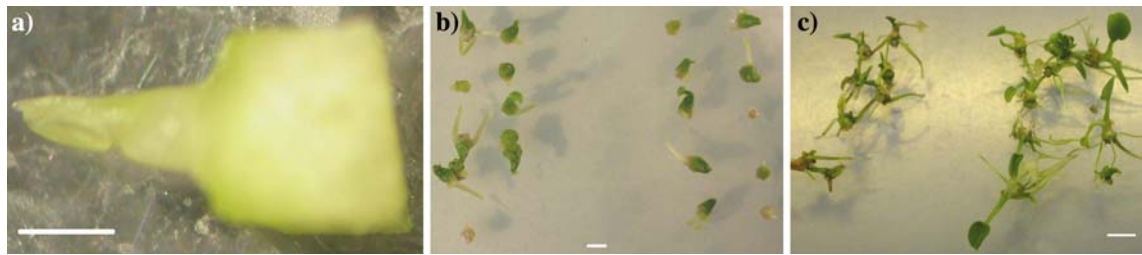
Fig. 1 (**a**) Dissected shoot-tip of cultivar CPUK consisting of meristematic dome and first two leaf primodia (Bar = 0.5 mm). Growth of cryopreserved shoot-tips after (**b**) 2 weeks and (**c**) 5 weeks of thawing (the STs in the two rows on the left were exposed to 20 min PVS2 and those on the right were exposed to 30 min) (Bar = 1 cm)

Table 1 Regeneration rates of 18 taro cultivars from the Asia-Pacific region after droplet vitrification

Country	Cultivar	Length of PVS2 exposure (min)			Average
		20	30	40	
Fiji	TNS	100	90	60	83.3ab
	Hybrid	90	90	70	83.3ab
Cook Islands	CPUK	100	100	90	96.7ab
PNG	PNG 129	75	87.5	100	87.5ab
	PNG 17	100	100	100	100a
	PNG 165	70	77.5	80	75.8b
	PNG 05	70	77.5	100	82.5ab
	PNG 46	90	100	100	96.7ab
	PNG 103	77.5	100	87.5	88.3ab
Niue	E399	100	100	70	90ab
	Nu 23	90	70	90	83.3ab
Vanuatu	VAN 38	80	90	100	90ab
	VAN 77	100	100	100	100a
Samoa	SAM 10	100	80	90	90ab
Solomon Islands	SI 83	90	90	100	93.3ab
New Caledonia	NC 16	87.5	90	90	89.2ab
Indonesia	IND 23	77.5	90	87.5	85ab
Malaysia	MAL 09	77.5	67.5	75	73.3b
	Average	87.5	88.9	88.3	88.2

Regeneration rates are means of two independent experiments each containing four to five shoot-tips. Values followed by the same letter are not significantly different according to Tukeys HSD test ($P < 0.05$) after arcsin transformation

165 and MAL 09 had significantly lower post-thaw regeneration rates than PNG 17 and VAN 77. Although there are genotypic differences in regeneration percentages, even the lowest rate of 73.3% is sufficient for practical purposes of in vitro conservation of germplasm.

This study shows a dramatic increase in average post-thaw regeneration rates (73–100%) compared to the previously reported paper-envelope/cryo-vial method that was applied to the same cultivars

(21–30%) (Sant et al. 2006). A possible reason for this could be the ultra-rapid cooling rate of 130°C/min obtained with PVS2 droplet on aluminium strip compared to cryo-vial freezing cooling rates of about 6°C/min (Towill and Bonnart 2003).

A second reason for the enhanced success of this new method could be the exposure of shoot-tips to PVS2 at 0°C instead of 25°C. Most injury effects caused by the dehydration process are reduced or eliminated by the loading step and optimizing PVS2

Fig. 2 Post-thaw
regeneration of shoot-tips of
taro cultivars CPUK and
TNS (%). In vitro shoot-tip
donor plants were grown on
solid MS + 90 g l^{-1} sucrose
medium for 7 weeks; shoot-
tips dissected and
cryopreserved the same day.
Each data point is the mean
of two replicates with five
shoot-tips per replicate
($n = 10$). Vertical bars
represent standard error of
regeneration
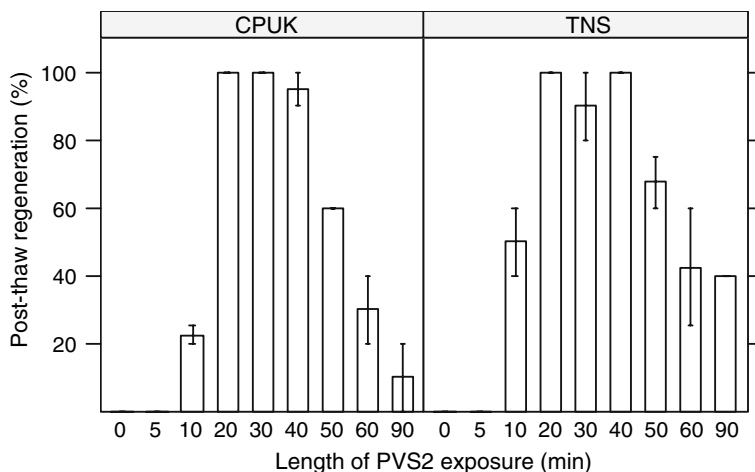
exposure at 0°C (Sakai et al 1990). PVS2 exposure at
0°C allows good recovery for a range of exposure
times as opposed to the critical exposure time of
12 min at 25°C (Sant et al. 2006). Not only PVS2
exposure at 0°C gives better replication of results, but
also a broad range of optimum PVS2 exposure time
offers the practical advantage of cryopreserving
larger numbers of shoot-tips in a day. This was also
observed for banana where optimal length of PVS2
treatment was 30–50 min at 0°C (Panis et al. 2005).

The droplet vitrification method used in this study
is based on the one developed for banana meristems
(Panis et al. 2005). Panis et al. (2005) found an
increase of 42% regeneration for banana shoot-tips
from four genomic groups cryopreserved by droplet
vitrification compared to cryo-vial vitrification. They
were also able to cryopreserve cultivars that were
previously considered recalcitrant. Our results vali-
date these findings as droplet vitrification method has
considerably improved plant regeneration rates in a
range of taro cultivars from 21–30% in cryo-vial
vitrification (Sant et al. 2006) to 73–100% in droplet
vitrification method (Fig. 2 and Table 1). Further-
more, only three of the eight cultivars investigated
could be successfully cryopreserved with the former
method (Sant et al. 2006), whereas all 18 cultivars
investigated for droplet vitrification in this study had
high post-thaw regeneration rates.

Cryopreservation survival of different cultivars of
the same species can depict genotypic dependence, as
reported for papaya (Wang et al. 2005), banana (Thinh
1997; Panis et al. 2005), black spruce (Touchell et al.
2002), sugar beet (Vandenbussche et al. 2000), and
taro (Takagi et al. 1997; Sant et al. 2006) and seen in

this study (Table 1). Despite these differences the
droplet vitrification protocol is applicable to a wide
range of taro cultivars of different origins as the lowest
regeneration rate recorded is also high enough for
cryopreservation purposes. The fact that droplet vitri-
fication method has a broad optimal PVS2 exposure
range (20–40 min), is equally applicable across vari-
ous genotypes and is easy to execute, enhances its
chances of being an efficient and reliable protocol
under different laboratory settings.

With this study, we clearly demonstrate that
pacific taro (*C. esculenta* var. *esculenta*) cultivars
can be successfully cryopreserved using the droplet
vitrification method.

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