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In vitro Shoot Tip Response to Encapsulation Dehydration Procedure for Cryopreservation of Philippine Taro [*Colocasia esculenta* (L.) Schott]

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Abstract **– This study was conducted to explore the feasibility of the encapsulation-dehydration cryopreservation procedure and provide the groundwork for establishing a cryopreservation protocol for routine application in germplasm conservation of Philippine taro. Shoot tips (2-3 mm) from 2-3 week old *in vitro* plantlets of VG-2 taro variety cultured on modified Murashige and Skoog (MS) medium were used. Initial experiments determined the effects of pretreatment (liquid MS with 0.15M or 0.3M sucrose), encapsulation (3% sodium alginate in MS solution and 0.1M calcium chloride in MS or distilled water), preculture (24h on MS with 0-1.2M sucrose) and dehydration with silica gel (3h or 4h) on tissue viability to ensure viable tissues before liquid nitrogen (LN) immersion. Results showed that only the high-sucrose preculture affected tissue viability in terms of reduced number of shoot-forming explants and delay in shoot initiation. In the encapsulation-dehydration experiments, the same pretreatment and encapsulation treatments as above, 12-24h preculture in MS with 0-1.0M sucrose, dehydration in silica gel for 3.5h (about 20% moisture content), 1-2d LN immersion, thawing in 40ºC water bath for 3 min, and regrowth in MS + 5% sucrose or MS + 3% sucrose + 0.2 mg/L kinetin + 0.2 mg/L benzyladenine, were tested. These treatments had no remarkable effect on post-thaw tissue survival. More than 80% of the shoot tips remained green after thawing. After 3d in the regrowth medium, 28-33% of the shoot tips remained green but there was no sign of shoot growth. Lack of regrowth indicates cryopreservation failure and this defines future research toward developing a successful protocol.**

Keywords **– Germplasm Conservation, Cryostorage, Cryoprotectant, Cryopreservation Failure.**

I. INTRODUCTION

Taro [*Colocasia esculenta* (L.) Schott] is an important food crop in developing countries and is considered as nature's healthiest health food (Lee, 1999). Major producers are Africa, Asia and the Pacific, with the Philippines ranking tenth (FAOSTAT, 2014). Because of its amazing health benefits and the increasing requirement of the food and health industries, market demand for taro is steadily growing worldwide. Continuing research on crop productivity improvement is critical to support the taro industry. Fundamental to increasing crop productivity is the development of new cultivars which heavily relies on germplasm collections (genebank). Genebanks are the lifeblood of breeding programs and support biodiversity maintenance, biotechnology, and development of climate-resilient agriculture (Harding, 1996). Unfortunately, genetic erosion and species extinction are occurring at an accelerated pace due to habitat destruction, alien species invasion and spread of agricultural systems characterized by genetic homogeneity which enhances genetic vulnerability to biotic and abiotic stresses (FAO, 2011).

Genebanks of plants that are vegetatively propagated, such as taro, are conventionally maintained in the field where serious problems persist including high maintenance costs, natural disasters, unfavorable climate and attacks by pests and diseases which can damage or wipe out some or entire collections. To minimize this problem, complementary collections can be maintained *in vitro*. *In vitro* genebank is an integral part of good genetic resources conservation program (Reed et al., 2001). At the Philippine Root Crops Research and Training Center (PhilRootcrops), an *in vitro* slow growth technique for taro germplasm conservation has been developed and can maintain taro collections for one year without subculture (Acedo et al., 2017). *In vitro* conservation requires material handling during storage, increasing labor and risks of losing collections due to human errors, contamination, and soma clonal variation, i.e. mutations that occur spontaneously in tissue culture, with a frequency that increases with repeated subculturing (Skirvin et al., 1994).

Cryopreservation or preservation in the frozen state (ultralow temperature of -196°C, i.e. the temperature of liquid nitrogen, LN), is the most effective and the only successful method for the safe and long-term storage of plant genetic resources especially vegetatively propagated species such as taro (Sakai, 2000; Engelmann, 2004; Gonzalez-Arnao and Engelmann, 2006). It has been shown to be less costly than field germplasm and other *in vitro* germplasm storage methods (Dulloo et al., 2009). At ultralow temperature, cell divisions and metabolic processes are arrested, thus, plant material can be preserved unchanged for unlimited time without danger of somaclonal variability and material loss due to human error (Engelmann, 2004; Panis and Lambardi, 2011). Based on accumulated radiation-induced DNA damage during cryogenic storage, maximum storage period has been estimated at 1,000 years. Moreover, cryogenic cultures are stored in a small volume, protected from contamination, and require a very limited maintenance. Cryopreservation is now used for a wide range of crops in both developed and developing countries.

Cryopreservation of biological tissues can be successful only if intracellular ice crystal formation is avoided, since this causes irreversible damage (cryoinjury) to cell membranes thus destroying their semi-permeability (Panis and Lambardi, 2011). Crystal formation, without an extreme reduction of cellular water, can only be prevented through vitrification, a physical process of transition of an aqueous solution into an amorphous and glassy (i.e. non-

crystalline) state (Sakai, 2000). Classical cryopreservation method involves controlled slow-freezing to concentrate the intracellular solution enough to vitrify upon transfer to LN (Withers and King, 1980). The protocol requires very expensive programmable freezer which is not practical in standard tissue culture laboratories. New cryopreservation techniques are simple and low-cost which make them far more suited in basic tissue culture laboratories. These techniques are vitrification-based procedures in which the critical step is the dehydration step prior to rapid freezing in LN and not the freezing step as in the classical methods. There are two approaches employed; use of vitrification solution (Langis et al., 1989) and dehydration (Fabre and Dereuddre, 1990). The effects of vitrification or dehydration treatments on survival of cryopreserved materials are usually evaluated after 24-48 hours from rapid freezing in LN. The vitrification solution protocol is very short and difficult to use for a large number of samples; the glasses formed are highly unstable and great care must be taken to prevent damaging glass relaxation and de-vitrification events upon re-warming; and the vitrification solution can be toxic to cells, so its application and removal must be precisely controlled to avoid cell damage and death (Zhao et al., 1999; Sakai and Engelmann, 2007).

The dehydration approach is a simpler technique and takes longer time to implement hence more time between steps than the vitrification solution-based approach (Zhao et al., 1999). It involves encapsulating plant tissues in alginate beads (which can contain nutrients) thus forming 'synthetic seeds' and rendering handling of explants very easy, before exposing to osmotic (high sucrose medium) and evaporative (silica gel or sterile air in laminar cabinet) dehydration (usually 20-30% moisture content), and subsequent immersion into LN for water molecules to vitrify (Fabre and Dereuddre, 1990; Engelmann, 2011). Alginate is the preferred encapsulation agent because of its polymeric inertness, easy manipulability, non-toxicity and its availability in large quantities. The alginate capsule protects the explants during the cryopreservation procedure, reduces the chemical toxicity or osmotic stress on the explants, and promotes regrowth after thawing (Wang et al., 2004; Sakai and Engelmann, 2007). The protocol has been applied to many plants but responses are species- and genotype-dependent (Malaurie et al., 1998; Mandal and Ahuja-Ghosh, 2007; Mandal and Dixit-Sharma, 2007).

The encapsulation-dehydration protocol is composed of seven steps; (1) pretreatment, (2) encapsulation, (3) preculture, (4) desiccation, (5) freezing and storage, (6) thawing and (7) regrowth (Gonzales-Arnao and Engelmann, 2006). The pretreatment step is a conditioning treatment before performing cryopreservation for explants to withstand freezing; this can be done by low temperature culture or culture on high sucrose medium. The encapsulation step involves suspending the explants in calcium free liquid basal medium supplemented with 3% sodium alginate and the mixture is dropped with a pipette into liquid culture medium containing a high concentration of calcium chloride (usually 0.1M), inducing polymerization of alginate in the presence of an elevated concentration of calcium thereby producing calcium alginate beads embedding the explants. The preculture step involves placing the beads containing the explants in Erlenmeyer flasks with liquid medium with high sucrose concentrations, usually between 0.50-1.25M for 16h to 7-10d, depending on the material. The high-sucrose preculture reduces the moisture content of the explants through an osmotic effect and also increases the concentration of internal solutes. The desiccation step involves dehydration either under the air current of a laminar flow cabinet or in sealed containers with dry silica gel. Dehydration under the laminar flow can produce different desiccation rates, depending on the air flow rate, the air temperature and humidity. By contrast, desiccation in air-tight containers with silica gel provides reproducible conditions from one experiment to the next and is thus highly recommended. After dehydration, beads are placed in cryotubes for rapid freezing by direct immersion in LN in storage tanks. Thawing can be done in a water bath at 40°C for 2-3 min. Regrowth takes place on standard semi-solid culture medium.

In taro, cryopreservation by the vitrification technique has been successfully applied (Takagi et al., 1997; Sant et al, 2006, 2008). Information on encapsulation-dehydration technique for taro cryopreservation is very limited. Hong and Yin (2013) claimed their work to be the first report on the use of an encapsulation dehydration method for the successful cryopreservation of *in vitro*-grown shoot tips of Chinese genuine red bud taro cv. Hongyayu. Shoot tips or meristems are recommended for germplasm conservation to avoid the production of variants associated with shoot regeneration from callus (Skirvin et al., 1994).

In the Philippines, there has been no attempt to adapt the cryopreservation procedures for taro and other root crops. This research would be a pioneering activity to evaluate the feasibility of the encapsulation-dehydration technique for taro cryopreservation and provide the groundwork in establishing a workable cryopreservation protocol for routine application for Philippine taro germplasm conservation.

II. Materials and Methods

A. Plant Material

The plant materials used were the *in vitro* plantlets of VG-2 taro variety grown at PhilRootcrops Tissue Culture Laboratory. The plantlets were micro propagated using quarter shoot tip explants cultured on modified Murashige and Skoog (1962) medium (MS) following the protocol established earlier (Acedo et al, 2018). Shoot tips of about 2-3 mm were aseptically excised from 2-3 week old plantlets in a laminar flow cabinet for the cryopreservation experiments.

B. Bead Production and Standardizing Dehydration

Beads were produced by immersing the shoot tips in 3% sodium alginate (Sigma) in MS solution and aseptically pipetting each shoot tip for dispensing as drops in 0.1M calcium chloride ($CaCl_2$) (Unilab) solution in MS solution or distilled water in 50 ml flask. The formed beads were

allowed to stand for 20 minutes with gentle shaking. Bead moisture content was standardized following the procedure of Gonzales-Arnao and Engelmann (2006) who reported the problem of reproducibility of dehydration results using laminar air flow. To determine the time needed to desiccate the beads to desired moisture content, standard desiccation curve was established. Silica gel at 20g and 30g was used as desiccant per 250 mL glass bottle with 10 beads per bottle. Three replications were used with 8 bottles per replicate. The beads were placed on sterilized filter paper and placed inside the bottle with silica gel (LabChem) which was then closed tightly. Weighing of samples was done every 30 minutes. In succeeding trials, only 20g silica gel was used comparing the desiccation rate of beads formed using sodium alginate in MS medium and $CaCl_2$ in distilled water with beads produced using only distilled water as solvent. Moisture content (MC) of the beads was calculated as percentage of the initial fresh weight using the following formula: MCn = [(FW0 – DW)/FWn] x 100, where: MCn = percent moisture content of beads after a given dehydration period; FW0 = fresh weight of beads before dehydration; FWn = fresh weight of beads after a given dehydration period; and DW = weight of beads after oven drying (80°C) to constant weight. In addition to MC, physical characteristics of the beads produced (uniformity in shape and transparency) were noted.

C. Pre-LN Immersion Experiments

The encapsulation-dehydration method had seven steps – pretreatment, encapsulation, preculture, dehydration, liquid nitrogen (LN) immersion, thawing, and regrowth (Figure 1). Initially, the effects of pretreatment (liquid MS with 0.15M or 0.3M sucrose), encapsulation (3% sodium alginate in MS solution and 0.1M $CaCl_2$ in MS or distilled water), preculture (1d on MS with 0-1.2M sucrose) and dehydration (3h or 4h) on tissue viability were determined to ensure viable tissues for LN immersion. The treatments tested for each stage were as follows:

Pretreatment: 3h or 12h dark pretreatment with liquid MS+0.15M or 0.3M sucrose medium; freshly dissected shoot tips as control. The pH of the medium was adjusted to 5.8 before dispensing into 50 ml Erlenmeyer flasks at 10 ml/flask.

Encapsulation: First trial involved encapsulation of shoot tips in 3% sodium alginate in MS solution and dispensed as drops in 0.1M $CaCl_2$ in distilled water with 12h dark pretreatment with liquid MS+0.15M or 0.3M sucrose medium; freshly dissected shoot tips with and without encapsulation and pretreatment as controls; second trial involved encapsulation of shoot tips in 3% sodium alginate dissolved in MS solution and dispensed as drops in 0.1M $CaCl_2$ in distilled water or MS solution with 12h dark pretreatment with liquid MS+0.15M or 0.3M sucrose medium. The shoot tips grew with and without encapsulation (Figure 2).

Preculture: From the encapsulation experiments above, the shoot tips in beads were precultured for 1d on liquid MS alone or MS with 0.25M, 0.5M, 0.75M or 1.0M sucrose. The second trial used MS alone or MS with 0.75M, 1.0M or 1.2M sucrose. More than one day preculture resulted in growth of shoots with tips coming out of the beads.

Fig. 1. Encapsulation-dehydration procedure from shoot tip explant preparation to culture in regrowth medium.

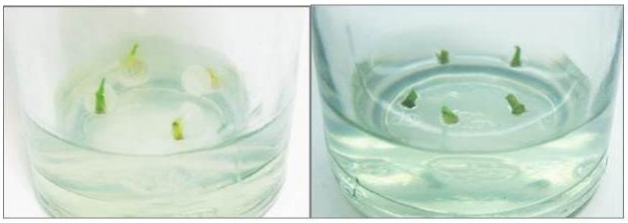

Fig. 2. Shoot tip growth with and without encapsulation.

Dehydration: This was done using silica gel in airtight 250 mL glass bottles for 3h or 4h. The experiments were repeated twice.

All treatments in the above experiments were replicated three times with 20-30 shoot tips per replicate. After imposing the different pretreatment, encapsulation, preculture and dehydration treatments, the shoot tips were cultured on solid MS medium with 0.15M sucrose, placed in culture shelves illuminated with white fluorescent tubes (Philips) at a room temperature of about 25±2°C, and monitored for shoot and root development.

D. Encapsulation-Dehydration Experiments

A series of experiments applying the whole 7-step encapsulation-dehydration procedure was conducted. Experiment 1 tested the following treatments as described above for pretreatment to dehydration steps:

Pretreatment: MS medium+0.3M sucrose and MS medium+0.15M sucrose, 12h dark

Encapsulation: 3% sodium alginate and 0.1M $CaCl_2$ in MS or distilled water
Preculture : MS with 0-1.0M sucrose, 24 h
Dehydration : 3.5h in silica gel to about 20% MC
LN immersion : 1d
Thawing : 3 min in 40°C water bath
Regrowth : MS with 0.15M sucrose

After dehydration, the beads were transferred into the 1.5 ml cryogenic vials (Biologix). Ten dehydrated beads with shoot tip tissues were placed in each cryovial. The cryovials were tightly closed before immersion in LN contained in Chengdu Golden Phoenix Liquid Nitrogen Biological tank. After LN storage, the cryovials were removed from the tank and immediately transferred to a water bath of 40°C for 3 minutes. Thawing for 2 min was observed to result in opaque appearance of the beads indicating that the beads were still partially frozen in contrast to thawing for 3 min which resulted in clear beads similar to their appearance before LN immersion. After thawing, the shoot tip tissues were aseptically removed from the beads and immediately inoculated into the regrowth medium. The cultures were then placed in culture shelves illuminated with white fluorescent tubes (Philips) in 25±2°C room and observed for growth and physical changes. The treatments were replicated three times with 30 shoot tips per replicate.

In the second experiment, pretreatment with liquid or solid MS+0.3M sucrose medium, preculture in MS+1.0M sucrose for 12h under dark condition, and two regrowth medium, MS+5% sucrose and MS+3% sucrose+0.2 mg/L kinetin (Ki)+0.2 mg/L benzyladenine (BA), were tested. Ki and BA are cytokinins commonly used in *in vitro* culture to promote shoot growth. LN storage was done for 2d. Other procedures were the same as that in Experiment 1. All treatments were replicated three times with 30 shoot tips per replicate. For all experiments, at least two trials were done, with the second trial serving to confirm the results of the first trial.

E. Experimental Design

The different experiments were conducted in Completely Randomized Design (CRD) with three replications per treatment. The results were analyzed by performing analysis of variance (ANOVA) and comparison of means by the Least Significance Difference Test (LSD) at 5% level using the MSTAT statistical package version 2.0.0 (Michigan State University, USA).

III. Results and Discussion

A. Dehydration Standard

The beads formed in 3% sodium alginate and 0.1M $CaCl_2$ in distilled water were more uniform in shape and more transparent than the beads produced in 3% sodium alginate and 0.1M $CaCl_2$ in MS. Dehydration of the beads in 30g silica gel was expectedly faster than in 20g silica gel (Figure 3A). However, MC reduction time differed only by about 30 min. Because of this and the higher expense in using 30g silica gel, subsequent standardization trials focused on the use of 20g silica gel. The beads

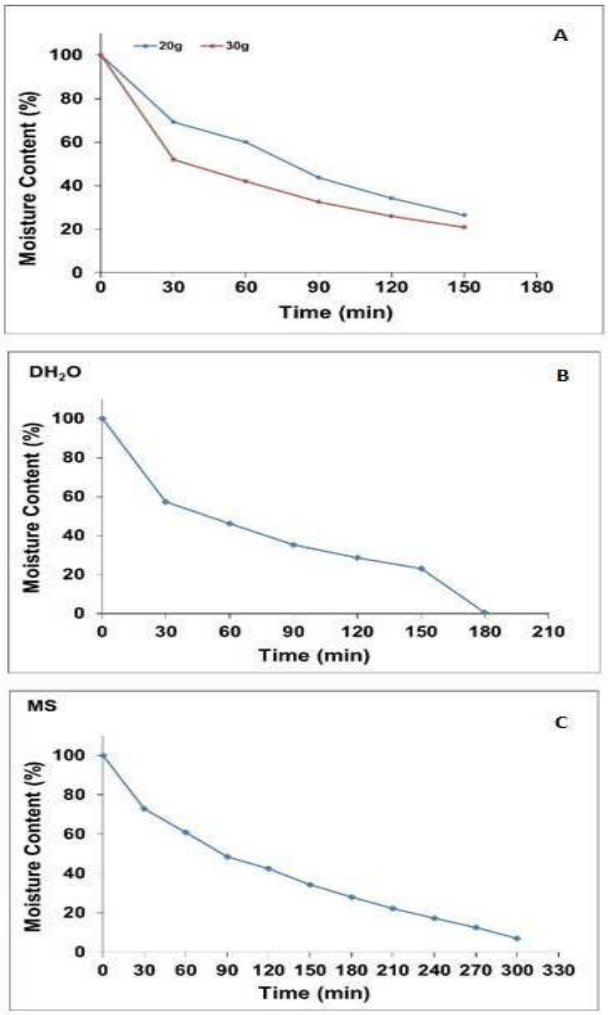

Fig. 3. Dessication curve of beads dehydrated using 20g or 30g silica gel (A) and beads produced using 0.1M $CaCl_2$ in distilled water (B) or MS (C) and dehydrated in 20g silica gel. (Values are average of two trials.)

formed in 3% sodium alginate and 0.1M $CaCl_2$ in distilled water dried faster in 20g silica gel compared to the beads produced in 3% sodium alginate and 0.1M $CaCl_2$ in MS, with the former reaching 20% MC after about 2.5h or one hour earlier than the latter (Figure 3B-C). This was anticipated as the solutes in MS (i.e. 0.1M $CaCl_2$ in MS) can increase the water holding capacity of the beads. The encapsulation of explants in calcium alginate beads allows the subsequent application of very drastic treatments including preculture with high sucrose concentrations and desiccation to low moisture contents; both are highly damaging or lethal to cells (Engelmann, 2011).

B. Tissue Viability at Pre-LN Immersion Stage

Pretreatment with MS added with0.15M or 0.3M sucrose for 3h or 12h did not affect tissue viability (Table 1). The shoot tips remained highly viable regardless of pretreatment conditions and responded in the same manner as the freshly dissected shoot tips. All shoot tip explants from all treatments developed shoot in about 2.5-3.5d. However, only 53-80% of the total number of shoot tip explants developed roots in about 7-11d. No significant differences among treatments were obtained.

Similarly, the different encapsulation treatments (use of 0.1M $CaCl_2$ in MS or distilled water, with 12h pretreatment in MS with 0.15-0.3M sucrose) did not affect tissue viability as 87-97% of the shoot tips formed shoots in 2.5-3.6d and 53-67% of the shoot tips developed roots in 10.2-14.3d (Table 1). Treatment differences in shoot and root growth were not statistically significant. In a separate experiment that used only 0.1M $CaCl_2$ in distilled water during encapsulation with the same pretreatment condition as above, all shoot tips developed shoots in 2.9-3.2d and 46-67% of the shoot tips also developed roots in 8.7-9.5d. These responses were statistically comparable to the responses of freshly dissected shoot tips with or without encapsulation (results not shown).

Table 1. Shoot and root growth of taro shoot tips on MS+0.15M sucrose after imposing the different pretreatment and encapsulation treatments.

Step / Treatments	Shoot growth		Root growth	
	% of total no. of samples	Days to first sign of growth	% of total no. of samples	Days to first sign of growth
Pretreatment step				
MS+0.15M sucrose, 3h	100	3.5	80	8.7
MS+0.15M sucrose, 12h	100	3.4	53	7.9
MS+0.3M sucrose, 3h	100	3.5	60	6.9
MS+0.3M sucrose, 12h	100	2.5	60	7.9
Control (freshly dissected)	100	2.5	67	11.3
Encapsulation step (with $CaCl_2$ in MS or distilled water)				
With 12h 0.15M sucrose pretreatment				
Encapsulation used $CaCl_2$ in d. water	87	2.5	60	11.2
Encapsulation used $CaCl_2$ in MS	97	3.4	53	12.6
With 12h 0.3M sucrose pretreatment				
Encapsulation used $CaCl_2$ in d. water	90	3.6	57	10.2
Encapsulation used $CaCl_2$ in MS	93	3.1	57	14.3

No significant treatment differences were obtained based on ANOVA.

Preculture in MS alone or with 0.25-1.0M sucrose resulted in 93-100% of the shoot tips showing shoot growth (results not shown). The follow-up experiment confirmed this response to MS with 0-1.0M sucrose (Table 2). However, when 1.2M sucrose was used, shoot-forming explants significantly decreased. These results were obtained in shoot tips subjected to 12h pretreatment in MS with 0.15M sucrose. When the 12h pretreatment in MS with 0.3M sucrose was used, sucrose at 0.75-1.2M markedly decreased the number of shoot forming explants relative to that of MS alone. In both pretreatment conditions, preculture in MS with 0.75-1.2M sucrose significantly delayed shoot development to 6-10d in contrast to the preculture in MS alone which initiated shoot in 2-3d. The effects of the three preculture sucrose levels (0.75, 1.0 and 1.2M) did not differ significantly.

Dehydration for 3-4h had no remarkable effect on shoot growth regardless of the preculture condition (MS alone or with 0.75-1.2M sucrose) (Table 2). The percentage shoot

Table 2. Shoot growth of taro shoot tips on MS+0.15M sucrose after imposing the different preculture and dehydration treatments.

Step / Treatments	% of total no. of samples	Days to first sign of growth
Preculture stage (24h)		
With 12h 0.15M sucrose pretreatment; encapsulation used $CaCl_2$ in MS	*	**
MS alone	100a	2.0b
MS+0.75M sucrose	78a	9.9a
MS+1.0M sucrose	78a	6.1a
MS+1.2M sucrose	67ab	7.0a
With 12h 0.3M sucrose pretreatment; encapsulation used $CaCl_2$ in MS		
MS alone	100a	2.7b
MS+0.75M sucrose	56b	7.0a
MS+1.0M sucrose	22c	6.0a
MS+1.2M sucrose	67ab	9.3a
Dehydration stage (12h 0.3M sucrose pretreatment; encapsulation used $CaCl_2$ in MS)	ns	*
3h in silica gel		
Preculture in MS alone, 24h	46	2.6b
Preculture in MS+0.75M sucrose, 24h	59	4.3a
Preculture in MS+1.0M sucrose, 24h	59	3.6a
Preculture in MS+1.2M sucrose, 24h	46	3.8a
4h in silica gel		
Preculture in MS alone, 24h	52	4.7a
Preculture in MS+0.75M sucrose, 24h	61	3.8a
Preculture in MS+1.0M sucrose, 24h	48	3.9a
Preculture in MS+1.2M sucrose, 24h	75	3.8a
Dehydration stage (preculture in MS+1.0M sucrose for 24h)	ns	ns
3h in silica gel		
With 12h 0.15M sucrose pretreatment; encapsulation used $CaCl_2$ in d. water	35	4.8
With 12h 0.15M sucrose pretreatment; encapsulation used $CaCl_2$ in MS	54	4.5
With 12h 0.3M sucrose pretreatment; encapsulation used $CaCl_2$ in d. water	54	3.6
With 12h 0.3M sucrose pretreatment; encapsulation used $CaCl_2$ in MS	44	5.2
4h in silica gel		
With 12h 0.15M sucrose pretreatment; encapsulation used $CaCl_2$ in d. water	50	5.5
With 12h 0.15M sucrose pretreatment; encapsulation used $CaCl_2$ in MS	52	4.6
With 12h 0.3M sucrose pretreatment; encapsulation used $CaCl_2$ in d. water	64	4.7
With 12h 0.3M sucrose pretreatment; encapsulation used $CaCl_2$ in MS	74	3.9

*ns-not significant; *-significant at 5% level; **-significant at 1% level; Means in a column per experiment having the same letter are not significantly different based on LSD, 5%.*

forming explants varied from 46-75% while the number of days to shoot formation, from 2.6-4.7d. In another experiment that used only MS+1.0M sucrose as preculture treatment, dehydration for 3-4h had also no pronounced effect on shoot growth regardless of pretreatment and encapsulation conditions. Shoot forming explants varied from 35-74% while the number of days to shoot formation, from 3.6-5.5d; treatment differences were not significant.

High-sucrose preculture and dehydration are highly damaging to tissues especially if not encapsulated but both are necessary steps in cryopreservation to enable the tissues to survive during LN immersion (Engelmann, 2011). The results of the present study showed that only the high-sucrose preculture (0.75-1.2M sucrose) had significant damaging effect on tissue viability. However, it did not result in complete loss of viability as some shoot tips were able to survive and later form shoots. Thus, in

the succeeding encapsulation-dehydration experiments, 12-24h preculture in 0.75-1.0M sucrose was tested for their effect on post-LN survival of shoot tip explants.

C. Encapsulation-Dehydration Experiments

The different pretreatment, encapsulation and preculture conditions tested in experiment 1 yielded negative result as no shoot tip explant survived during post-thaw regrowth. Although the explants appeared green after thawing, the shoot tips started to turn gray after one day in the regrowth medium and after 2 days, all shoot tips turned gray or brown with no observable growth. In the second experiment, pretreatment in liquid or solid MS+0.3M sucrose medium and the regrowth medium had no remarkable effect on post-thaw tissue survival. More than 80% of the shoot tips remained green (Figure 4). After 3d culture in either MS+5% sucrose or MS+3% sucrose+0.2 mg/L Ki+0.2 mg/L BA, 28-33% of the shoot tips remained green. However, no signs of growth were noted.

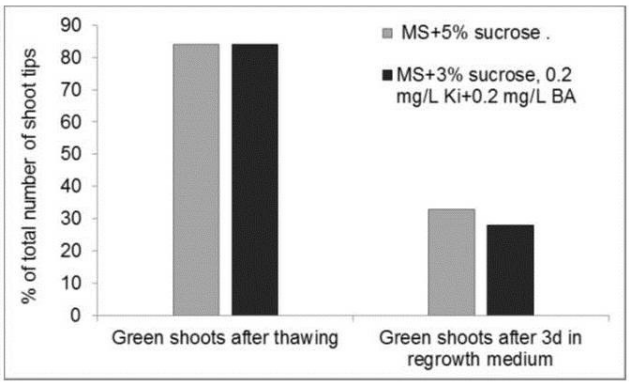

Fig. 4. Shoot tips remaining green right after LN immersion and after 3d culture in regrowth medium of MS+5% sucrose or MS+3% sucrose+0.2 mg/L Ki+0.2 mg/L BA. Values are average of two trials.

Lack of regrowth indicates cryopreservation failure which could be due to insufficient or excessive cryoprotectant application (Niino et al., 2013; Niino and Arizaga, 2015). The present study applied cryoprotectant treatment through high-sucrose preculture (0.75-1.0M sucrose for 12-24h) and dehydration to 20% MC before LN immersion. It has been generally recommended that in the encapsulation-dehydration method, preculture in high sucrose level of 0.50-1.25M for 16h to 10d and dehydration to 20-30% MC can be used (Fabre and Dereuddre, 1990; Gonzales-Arnao and Engelmann, 2006; Engelmann, 2011). Sucrose is the most commonly used to induce cryotolerance. Halmagyi et al (2005) successfully demonstrated increased tolerance to cryopreservation by applying 0.5M sucrose preculture for 24h at 24°C. Folgado et al (2014) demonstrated as well an improved recovery of meristems following an elevated sucrose treatment (0.3M) during the 2 weeks of cold-hardening of mother plants. The encapsulation-dehydration technique developed for Chinese taro used 0.75M sucrose and 18-19% MC to induce cryotolerance (Hong and Yin, 2013). Sucrose has also been used as cryoprotectant in other crops, such as yam, sugarcane and *Capparis spinosa* (Malaurie et al., 1998; Gonzales-Arnao et al., 199; Mandal and Aguha-Ghosh, 2007; Mandal and Dixit-Sharma, 2007; Shatnawi, 2011). Preculture on sucrose-enriched medium increased the concentrations of sugar, starch and proline in the shoot tips, enhancing the stability of membranes under conditions of severe dehydration (Matsumoto and Sakai, 2003; Kaczmarczyk et al., 2011). However, Panta et al (2015) found that sucrose pretreatment had no positive effect on post-thaw survival in potato shoot tips. They added that the application of cryoprotectant alone often does not provide enough protection against lethal cryo-damage. Berjak et al (1995) attributed cryopreservation failure (no shoot growth) in *Quercus robur* to deranged intracellular organization as a result of over-rigorous surface sterilization and prolonged dehydration period which, though not lethal in themselves, act synergistically and lethally with freezing stress. Other researchers implicated non-cryogenic factors in cryopreservation failure, including physiological factors such as loss of antioxidant defense (Harding et al., 2009) and programmed cell death (Baust et al., 2001; Baust, 2002).

The present study represents the first work in adapting cryopreservation in Philippine taro conservation and much remains to be done to develop a viable protocol. Further works are needed to optimize the encapsulation-dehydration technique and/or develop more efficient cryopreservation protocol. Other cryoprotective or osmoprotective treatments can be explored in future research. Post-thaw survival of shoot tips depends on many factors such as subculture conditions, size of the shoot tips and their location on the plantlet axis, sucrose concentration of the preculture medium, preculture time, dehydration, cooling and warming, and unloading step (Kim et al., 2006; Yoon et al., 2006). Future works could also test newer techniques, such as increasing the cooling and warming rates which usually result in little or no crystallization and high regrowth after rewarming (Niino et al., 2013). The use of capped cryotubes for immersion into LN and retrieval from LN has cooling and warming rates of about 100–200°C/min and about 80–120°C/min, respectively, which are far less than new methods and this has a great impact on regrowth of cryopreserved materials.

Responses to cryopreservation are strongly genotype- and species-specific, which could limit the use of one cryopreservation protocol to large collections (Niino and Arizaga, 2015). It is therefore crucial to optimize the protocol for different genotypes as well as to make a uniform, healthy and robust shoot tips able to tolerate cryopreservation procedures.

IV. Conclusion

The different pretreatment, encapsulation and dehydration conditions did not markedly affect tissue viability prior to LN immersion. Only the high-sucrose preculture affected tissue viability by reducing the number of shoot-forming explants and delaying shoot initiation but it did not result in total loss of viability of tissues. After LN storage and thawing and subsequent regrowth for 3d, some shoot tips remained green but there was no sign of shoot growth. Lack of regrowth indicates cryopreservation

failure and future research should delve into other factors to improve cryotolerance and increase post-thaw survival in order to develop a successful protocol. It is important that the cryopreservation protocol is simple and suitable for large scale application to Philippine taro germplasm.

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