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Seed and embryogenic callus cryopreservation of Thai rice (*Oryza sativa* L.) 'Hom Mali Daeng'

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Abstract Thai rice landraces are a valuable heritage that must be conserved. A suitable protocol for seed and callus cryopreservation of Hom Mali Daeng rice was investigated. Seed cryopreservation experiments were divided into three groups as room temperature storage, gradient freezing by storage at 0 ℃ for 30 min, -20 ℃ for 30 min before soaking in liquid nitrogen, and direct immersion in liquid nitrogen. All groups were stored for 1, 3 and 5 months before germination and planting. The highest germination percentage was recorded in the control group after 3 months of storage, while highest growth performance was found in the direct freezing protocol after 5 months of storage. The vitrification technique was used for callus cryopreservation. Calli were exposed to plant vitrification solutions PVS2 and PVS3 for 0, 20, 40 and 60 min before immersion in liquid nitrogen for 24 h. After thawing and regrowth, cryopreserved calli were cultured on MS medium with 1 mg/L 1-naphthaleneacetic acid and 3 mg/L 6-benzylaminopurine for 6 weeks. Results revealed that cryopreserved calli from PVS3 for 0 min treatment (immediately immersed in PVS3 before transfer into liquid nitrogen) and PVS2 for 40 min provided high survival percentages at 100% and 75%, respectively. The vitrification system used in this report demonstrated an alternate cryopreservation approach for Hom Mali Daeng rice seed and callus, which can be subsequently adapted to other rice varieties.

Keywords: Cryoprotectant, Germplasm preservation, Rice callus, Seed germination, Vitrification

Introduction

Rice is the second most widely cultivated cereal in the world after wheat, which belonging to the family Poaceae and has been cultivated for over 10,000 yr (Sasaki, 2001; Pazuki and Sohani, 2013). Currently, the worldwide rice demand is expected to rise due to world population is continuously growing (Mahathanaseth, 2014). Demand for rice production

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is increasing as rice consumers are increasing at the rate of 1.6 to 1.8% every year (Karthikeyan *et al*., 2009; Shobarani *et al*., 2010). Therefore, improvements in the quality and germplasm collection as genetic resources of rice cultivars are urgently needed. Although more than 17,000 rice cultivars have been recorded in Thailand since 1937 (Department of Agriculture Thailand, 2000) with only a few planted as commercial crops, but many rice cultivars are facing extinction. However, even though rice genetic conservation needs more input, but storage costs are high. Problems encountered in conservation of rice include seed quality degeneration, risk of destruction from diseases and pests and climate change. Therefore, rice germplasms both seed and callus cryopreservation are an alternative choice to solve those problems.

Plant cryopreservation involves the cooling and storage of vegetal structures such as plant cells, tissues or organs in liquid nitrogen (LN; -196 ℃) or liquid nitrogen vapor (-160 ℃). This approach ensures that samples remain viable after thawing, and allows for unlimited storage (Benelli, 2021). This advanced biotechnology is an effective alternative strategy for *ex situ* germplasm conservation to help overcome various constraints of traditional storage methods applied in both seed banks and clonal orchards (Paunescu 2009; Ruta *et al*., 2020). Cryopreserved cells and calli can preserve optimal cell line features (Meijer *et al*., 1991) and a source of protoplasts for plant improvements such as somatic hybridization in breeding programs (Olivares-Fuster *et al*., 2000) and genetic transformation. The ability of the surviving cells to regenerate plants and create protoplasts capable of genetic transformation was studied (Cornejo *et al*., 1995). Techniques used for rice cell and callus cryopreservation have included a simple one-step freezing procedure in Basmati 385 and Pusa Basmati 1 rice (Jain *et al*., 1996) and the encapsulation/dehydration technique in japonica variety Yerua P.A. rice (Marassi *et al*., 2006).

The cultivar Hom Mali Daeng is derived from KDML105R-PSL-E-14, which originated from Thai jasmine rice or KDML 105, the most popular commercial rice cultivar in Thailand. After many improvements, the Hom Mali Daeng cultivar shows an outstanding red seed coat that differs from KDML105. The Hom Mali Daeng cultivar has photoperiod sensitivity and can grow throughout the year, with resistance to brown planthopper and blast disease. The cooked rice is tender with aromatic features. Moreover, nutritional compositions are also high compared with other commercial rice cultivars. Therefore, this landrace cultivar is suitable for diabetes patients, especially type II diabetes because blood sugar (glucose) slightly increases after consumption (Rice Department of Thailand, 2019). Many plant cryopreservation reports have been published

but rice callus cryopreservation is poorly perceived and understood. Therefore, this research investigated Hom Mali Daeng rice to determine a suitable protocol for seed and callus cryopreservation to preserve rice genetics and biodiversity, and also to generate a model and database for further conservation of Thai rice landraces.

Materials and methods

Seed cryopreservation

Mature seeds of Hom Mali Daeng rice were collected from Kalasin Province, Thailand. Healthy seeds were used as explants for the seed storage experiment. Rice seeds were incubated using a hot air oven at 45 °C for 48 h that reduced weight by 9%. Incubated seeds were divided into three groups as Group1: room temperature storage (control group), Group 2: gradient freezing by storage at 0 ℃ for 30 min then -20 ℃ for 30 min before soaking in liquid nitrogen, and Group 3: direct immersion in liquid nitrogen. All three groups were stored for 1, 3 and 5 months. Cryotubes containing cryopreserved seeds were thawed in a water bath at 38±2 °C for 2 min. Results of the control and the other two groups were recorded in two phases as immediately after freezing (0 d) for seed viability using 2,3,5-triphenyl tetrazolium chloride (TTC), and 14 days after germination. Germination percentage, root length, stem length, fresh weight and dry weight of all treatments were recorded. All three storage periods (1, 3 and 5 months) had 4 replicates of 50 seeds each. Some growth characteristics of 14-day-old rice seedlings at the initiation step (0 months) and after germination from cryopreserved seed after 1, 3 and 5 months of storage were compared in terms of changing percentage as shown in Equation 1:

Changing percentage = (Treatment value / initiation value) x 100 (1)

Callus cryopreservation

Dehusked seeds were surface sterilized using 20% (v/v) sodium hypochlorite (Clorox) supplemented with 2-3 drops of Tween 20 for 20 min, with shaking before rinsing three times with sterile distilled water. For callus initiation, sterilized seeds were cultured on Murashige and Skoog (MS) 1962 medium with 2 mg/L 2,4-D for 4 weeks under 25 ±2 ^{o}C with a light flux density of 40 mol/m^{2}/s at 25±2 ^{o}C (16/8 h light/dark). Calli derived from seed were precultured on liquid MS medium with 0.4 M

sucrose for 24 h before loading in liquid MS medium supplemented with 2 M glycerol and 0.4 M sucrose for 20 min. After that, calli were exposed in plant vitrification solutions as PVS2 (30% glycerol + 15% DMSO + 15% EG + 13.7% sucrose (Sakai *et al*., 1990), and PVS3 (50% glycerol + 50% sucrose (Nishizawa *et al*., 1993) for 0, 20, 40 and 60 min before immersion in liquid nitrogen for 24 h. Cryotubes containing cryopreserved calli were then thawed in a water bath at 38±2 °C for 2 min. The cryopreserved calli were soaked in liquid MS medium with 1.2 M sucrose for 20 min and transferred for regrowth on MS medium with 1 mg/L NAA (1-naphthaleneacetic acid) and 3 mg/L BAP (6-benzylaminopurine) for 6 weeks. Viability percentages of cryopreserved calli were recorded using the TTC assay. Each treatment consisted of 3 replicates of 4 calli.

Data analysis

Seed cryopreservation data were analyzed to determine the impact of two factors as preservation period and preservation process by two-way ANOVA. Data for callus cryopreservation were analyzed by one-way ANOVA using the SPSS program. Growth behavior relations were investigated using correlation coefficients between interesting pairs of growth features at phenotypic levels based on Searle (1961) and Singh *et al*. (2018) as shown in Equation 2:

$$\text{Phenotypic correlation coefficients (rp)} = \frac{\text{cov.XP (p)}}{\sqrt{var.Xp.var.Yp}} \quad (2)$$

where cov.XY (p) indicates phenotypic covariance between characteristics X and Y, and var.X (p) and var.Y (p) indicate variance for phenotypic levels of characteristics X and Y, respectively. Data were analyzed using the SPSS program.

Results

Seed cryopreservation

After storage for 1, 3 and 5 months, the preservation period and preservation process affected seed germination and seedling growth of Hom Mali Daeng rice. Seed germination percentage after 1 month of storage was lower than after 3 months. For the control group, 5 months of storage presented the highest germination percentage, stem length, root length and fresh and dry weight. All values were significantly different from 1 month

of storage (Figure 1). Positive TTC examination results of germinated seeds were found in all treatments, including the control (Figure 2).

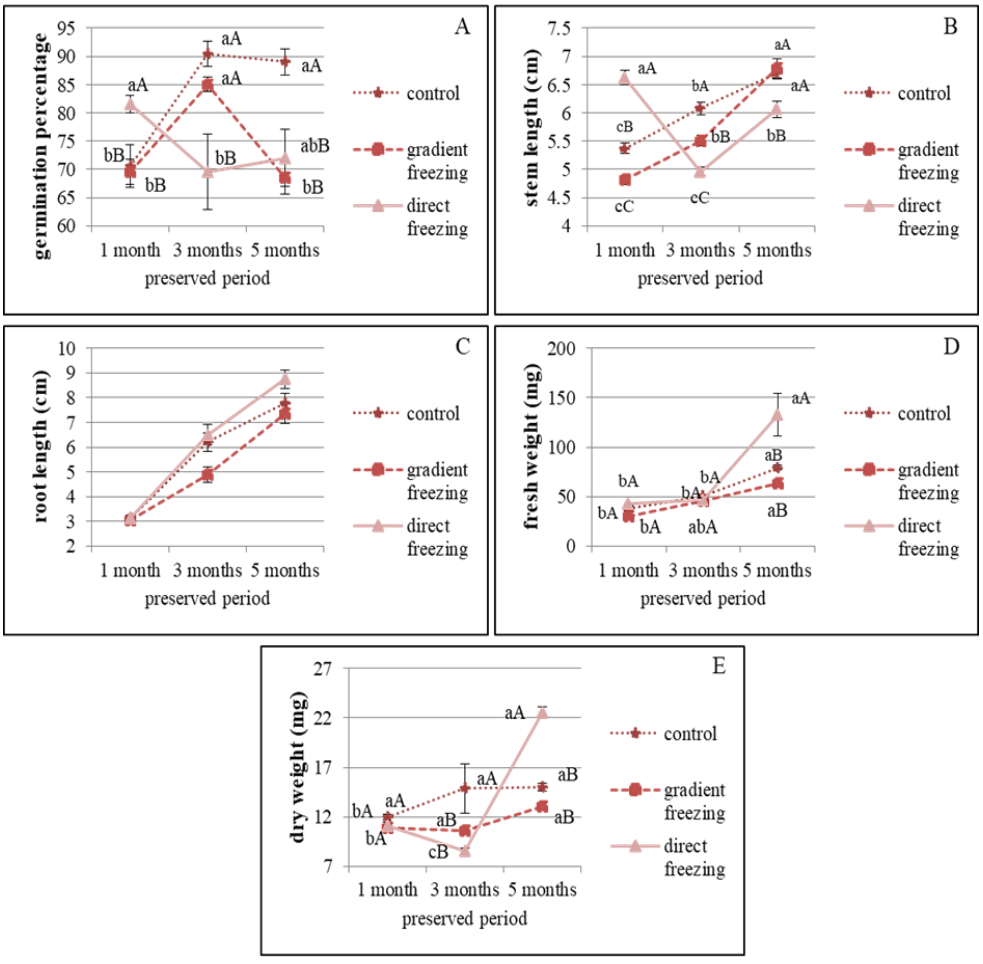

Figure 1. Growth of Hom Mali Daeng rice seedlings after 1, 3 and 5 months of storage: (A) percentage germination, (B) stem length, (C) root length, (D) fresh weight and (E) dry weight 14 days after planting. Mean ± SE followed by different letters are significantly different by Fisher's Least Significant Difference (LSD) ($p < 0.05$). Results were submitted to two-way ANOVA; simple main effect. The same lowercase letter in a line denotes the simple main effect of the preserved period, while the same uppercase letter in a column denotes the simple main effect of the preserved process

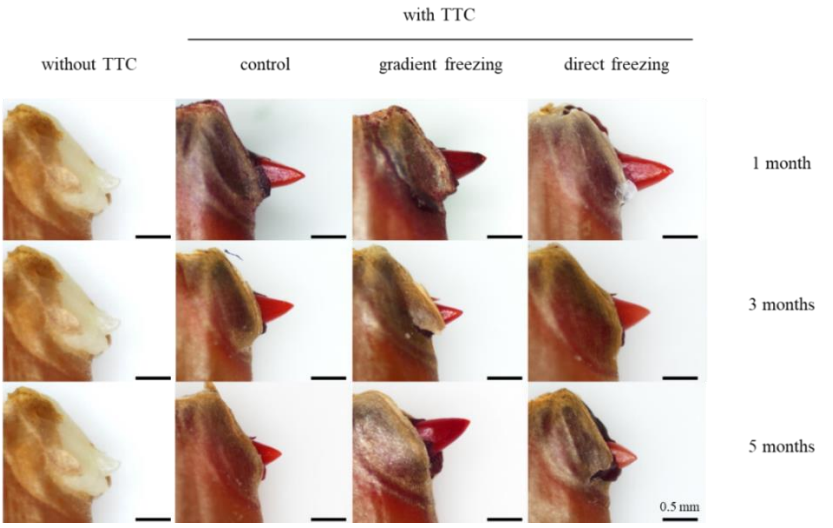

Figure 2. Viability test of cryopreserved germinated seeds by TTC assay after storage in liquid nitrogen for 1, 3 and 5 months.

Cryopreserved seeds were germinated after storage for 1, 3 and 5 months. The germination percentages, stem length, root length, fresh weight and dry weight of 14-day-old seedlings were compared with the control set at the initiation step (0 months). Almost all percentage changes were significantly different (Table 1). Five months of storage showed the highest percentage changes, especially for stem length in the control group (without freezing) and gradient freezing, including fresh and dry weight of direct freezing.

Table 1. Comparison of growth characteristics of 14-day-old rice seedlings at initiation step (0 months) and after germination from cryopreserved seeds after storage for 1, 3 and 5 months.

		Percentage change from initiation								
Characteristic	**0 M**	**Control**			**Gradient freezing**			**Direct freezing**		
		1 M	3 M	5 M	1 M	3 M	5 M	1 M	3 M	5 M
GP (%)	97.5 ± 1.77	83.11	92.82	91.28 *	71.28	87.17	70.25	83.58 *	71.28 *	73.84 *
SL (cm)	6.2 ± 0.71	85.50	96.81 *	106.84	76.59 *	87.73 *	107.96 *	105.41 *	78.98 *	96.49 *
RL (cm)	9.0 ± 2.54	34.28 *	68.35 *	85.66 *	33.51 *	53.80 *	81.14 *	34.39 *	71.77 *	96.47
FW (mg)	130.0 ± 0.01	29.25 *	39.23 *	61.06 *	23.36 *	35.28 *	48.38 *	32.69 *	35.74 *	102.06
DW (mg)	20.0 ± 0.00	59.80 *	74.45 *	75.00 *	54.60 *	42.60 *	65.20 *	55.65 *	42.60 *	112.85

* Significant difference from initiation step at $p < 0.05$; GP = germination percentage; SL = stem length; RL = root length; FW = fresh weight; DW = dry weight; M = month

Five growth characteristics of the control and the two treatments were calculated for correlation coefficient estimation (Figure 3). Germination percentage after 1 month of storage showed both positive and negative correlation with other characteristics of the control, gradient and direct freezing groups. Stem length after 1 month of storage negatively correlated with root length, while fresh weight had a significantly positive correlation with dry weight. All growth characteristics after 3 months of storage showed positive correlation with each other, except for the correlation estimation of the direct freezing treatment. After storage for 5 months, germination percentage exhibited positive correlation with all characteristics in the control treatment but showed negative correlation with all characteristics in the gradient freezing treatment and root length in the direct treatment. This result proved that after 5 months of storage, seed directly immersed in liquid nitrogen or the direct freezing protocol presented higher germination percentage, root length, fresh weight and dry weight than the control and the gradient freezing method.

1 month of storage												
	Control				**Gradient freezing**				**Direct freezing**			
Trait	GP	SL	RL	FW	GP	SL	RL	FW	GP	SL	RL	FW
DW	0.357	-0.055	-0.117	0.592**	-0.982*	0.051	0.059	0.672**	-0.080	0.320*	-0.034	0.615**
GP		-0.870	-0.512	-0.221		-0.121	-0.524	0.448		0.761*	0.485	0.726*
SL			-0.366	0.162			-0.224	0.120			-0.054	0.696**
RL				-0.340*				0.054				0.108
3 months of storage												
DW	0.012	0.028	0.085	0.105	0.186	0.265*	0.347*	0.540**	-0.101	0.151	0.213	0.150
GP		0.308	0.701	0.770*		0.613	0.732*	0.783*		-0.008	-0.588	0.249
SL			0.261*	0.725**			0.310*	0.613**			0.142	0.397**
RL				0.509**				0.572**				0.533**
5 months of storage												
DW	0.201	0.631**	0.184	0.868**	-0.573	0.387**	0.069	0.797**	0.199	0.667**	0.200	0.436**
GP		0.821*	0.828*	0.846*		-0.363	-0.398	-0.069		0.283	-0.013	0.313
SL			0.596**	0.838**			0.588**	0.732**			0.570**	0.243*
RL				0.739**				0.647**				0.359**

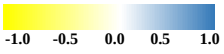

Figure 3. Estimates of phenotypic correlation coefficients between 5 traits in rice germination and seedling growth of rice after cryopreservation for 1, 3 and 5 months. * Significant correlation at $p < 0.05$, ** Significant correlation at $p < 0.001$. (GP = germination percentage, SL = stem length, RL = root length, FW = fresh weight, DW = dry weight)

Callus cryopreservation

Cryopreserved calli of PVS3 for 0 min and PVS2 for 40 min treatments provided high survival percentages of 100% and 75%, respectively (Figure 4). Positive TTC examinations of calli were presented

in PVS2 at 40 min treatment and PVS3 at 0 and 40 min treatments (Figure 4).

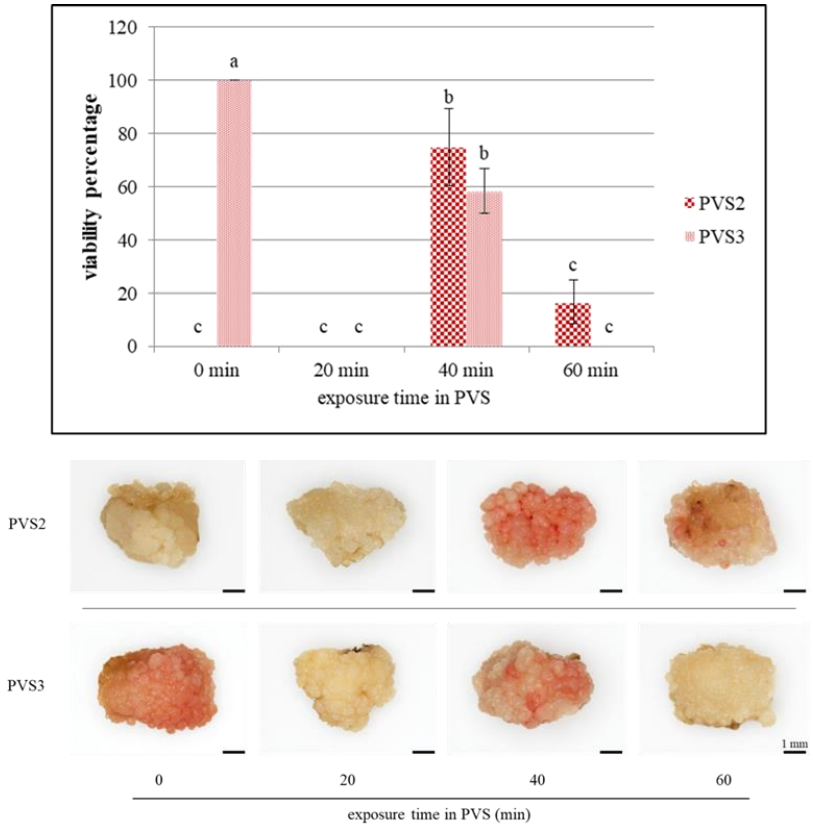

Figure 4. Percentage viability of cryopreserved calli after storage in liquid nitrogen using the vitrification technique and TTC assay of cryopreserved calli after treatment in PVS2 and PVS3 at various times before storage in liquid nitrogen. Mean ± SE followed by the different letter are significantly different by Duncan's Multiple Range Test ($p < 0.05$).

Discussion

Germination percentage of the direct freezing protocol after 5 months of storage did not significantly correlate with the other growth factors, while growth performance was higher than gradient freezing and the control treatments. Germination percentage of gradient freezing after 5 months of storage showed negative correlation with all other growth characteristics, indicating that cryopreservation impacted seed germination and seedling growth, and concurring with Acosta *et al*. (2020). Liquid

nitrogen treatment caused slow plant growth (Arguedas *et al*., 2018; Pereira *et al*., 2019), while the germination percentages of gradient freezing and direct freezing were significantly lower than the control group after 3 and 5 months of storage. Root length and fresh and dry weight of the direct freezing method were significantly higher than gradient freezing and the control group, indicating that the direct freezing protocol was suitable for Hom Mali Daeng seed preservation. This result concurred with Bekheet *et al*. (2020) and Lanuinla (2020) who found that cryopreservation using liquid nitrogen preserved plants by stopping all metabolic processes in plant cells, resulting in delayed cell deterioration. Chemical and biological reactions in living cells are greatly reduced, if not completely stopped at cryopreserved temperatures, accomplishing long-term preservation of numerous biological samples (Steponkus *et al*., 1990; Chang and Zhao, 2021). However, germination performance after cryopreservation involves other factors such as seed quality, storage period and storage temperature.

Cryopreserved calli using the vitrification technique were cultured on MS medium containing 1 mg/L NAA and 3 mg/L BAP. This result concurred with Kim *et al*. (2009) who found that PVS3 solution was suitable for garlic and chrysanthemum. Moreover, PVS3 was found to be a more suitable cryoprotectant solution for *Satureja spicigera* callus after recovery from liquid nitrogen than PVS2 solution (Ghaffarzadeh-Namazi *et al*., 2017). Our results differed from previous studies that determined PVS2 as appropriate for callus cryopreservation of wild crocus species (*Crocus hyemalis* and *Crocus moabiticus*) (Baghdadi *et al*., 2011) and globe artichoke (*Cynara scolymus* L.) (Bekheet *et al*., 2020). However, higher doses of DMSO in PVS2 solution can harm the plasma membrane (Salas-Leiva and Dupre, 2011). Potential toxicity of the PVS2 solution also jeopardized plant cell viability, and the exposure period was determined as a critical parameter that must be optimized (Bekheet *et al*., 2020).

Cryopreserved calli in this study showed viability signals but their growth did not continue after recovery. This occurrence may result from the low temperature effect, whereby plant cell growth or tissue metabolism cease at extremely low temperatures. Plant cells and tissues required more time for adaptation after recovery from soaking in liquid nitrogen, concurring with Zhou *et al*. (2012) who determined that cell recovery by callus of rubber tree (*Hevea brasiliensis* Muell. Arg.) after cryopreservation was delayed by 2 to 3 days compared to the control (non-cryopreserved callus). Vitrification-based cryopreservation accomplishes storage by increasing cell viscosity with numerous stressors that cause reactive oxygen species (ROS) (Ren *et al*., 2013) with accumulation resulting in cell death (Ren *et al*., 2020). Moreover, the preculture process is crucial in plant

cryopreservation because this stimulates proline accumulation, which causes stress tolerance (Kulus and Zalewska, 2014). High concentration of cryoprotectants or long preculture may impact dehydration efficiency, resulting in inappropriate withered cells or intracellular ice crystal formation during cryopreservation, causing physical damage within plant cells (Ninagawa *et al*., 2016). Ice crystal formation, growth, and recrystallization are lethal to cryopreserved samples, posing major challenges and limiting cryopreservation efficiency (Pegg, 2010; Bissoyi *et al*., 2014; Chang and Zhao, 2021). This unsuitable protocol can be resolved by decreasing sucrose concentration from 0.4 M to 0.3 M (Huang *et al*., 2018), with increased preculture duration (Ghaffarzadeh-Namazi *et al*., 2017). Moreover, supplementation of single-wall carbon nanotubes (SWCNTs) in the plant vitrification solution generated survival rate improvement of *Agapanthus praecox* embryogenic callus after cryopreservation (Ren *et al*., 2020).

In conclusion, results from this study identified a suitable protocol that was beneficial for Hom Mali Daeng rice cryopreservation. After 5 months of preservation, the direct immersion in liquid nitrogen protocol was suitable for Hom Mali Daeng rice seed cryopreservation. This protocol provided higher germination percentage, root length, fresh weight and dry weight than the control and gradient freezing seed protocols. The PVS3 for 0 min treatment (immediate immersion in PVS3 before transfer into liquid nitrogen) provided the highest survival percentage of cryopreserved callus. The vitrification system used in this report demonstrated an alternate cryopreservation approach for Hom Mali Daeng rice seed and callus. However, a more effective procedure and longer regrowth periods should be considered in the future. Our findings can be used as a springboard to build a more effective cryopreservation strategy for Hom Mali Daeng rice that can then be adapted and applied to other rice varieties.

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