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Research Article

Effect of Cryoprotectans and Cryopreservation on Physiological and Some Biochemical Changes of *Hopea odorata* Roxb. Seed

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ABSTRACT

Hopea odorata Roxb. is a forest plant from Dipterocarpaceae family that has important economic and ecological functions in the ecosystem. Generative propagation of *H. odorata* is limited because of its recalcitrant seed that cannot be stored for long periods at room temperature or even at low temperature. Cryopreservation is a seed storage technique that has the potential to prolong the shelf life of recalcitrant seeds. The aim of this study was to evaluate the effect of cryoprotectant and cryopreservation treatment on seed viability and biochemical change (electrolyte leakage, total malondialdehyde, total phenol) of *H. odorata* seeds. Fresh seeds of *H. odorata* were treated with two types of cryoprotectans namely PVS1 as non penetrating cryoprotectant and PVS2 as penetrating cryoprotectant, each type of cryoprotectant with four different concentrations (25, 50, 75 or 100%(w/v)) and four different immersion times (30, 60, 90 or 120 mins). Seeds were then stored in two different temperatures, at room temperature (28±2ºC) or in liquid nitrogen (-196±2ºC) for 24 hours to evaluate the cryoprotectant toxicity. The results showed that *H. odorata* seeds stored at room temperature and immersed either in 100%, 75% or 50% of PVS1 possess a higher viability as well as germination percentage, germination rate, vigour index and maximum growth potensial. In addition, they have lower value of electrolyte leakage, total malondialdehyde and total phenol compared to those seeds treated with PVS2. Meanwhile, both type of cryoprotectants and cryopreservation treatment in this study have not been able yet to increase seed viability of *H. odorata*. Cryopreservation treatments caused an increase in the total of malondialdehyde and electrolyte leakageas and these leads the inability of *H. odorata* seeds to germinate. PVS1 cryoprotectant seems to cause less toxic effects on *H. odorata* seeds but it can not prevent the negative impact of cryopreservation treatment.

INTRODUCTION

Hopea odorata Roxb. is a native forest plant in India, Bangladesh, Myanmar,Vietnam and several Indochina countries (Ly et al. 2017). *H. odorata* seedlings have a fast growth rate, reaching 30-40 cm at the age of 6-9 months

and are ready to be transplanted into the field. The diameter of its stem can reach 53 cm at the age of 25 years with a survival rate of almost 100% (Joker & Salazar 2000; Junaedi & Frianto 2012). Therefore, *H. odorata* is one of the species recommended for establishing Dipterocarpaceae forest (Weinland 1998), such as implemented in several countries like Malaysia, Sri Lanka, Vietnam and Cambodia (Chua et al. 2010; Ashton et al. 2011). On its economical value, *H. odorata* is widely used as a source of timber, gum or resin, and for construcion purposes (Orwa et al. 2009; Junaedi & Frianto 2012).

The high demand of wood and resin of *H. odorata* were not accompanied by the restoration process yet. Thirty to fifty percent of *H. odorata* population from three generations (± 300 years) continues to decline due to exploitation and habitat conversion for agriculture purposes. Declining population problem cause *H. odorata* to be categorized as plant species that are vulnerable to ecological extinction according to International Union for Conservation of Nature and Natural Resources (IUCN) (Ly et al. 2017). Propagation of *H. Odorata* currently is still limited to vegetative technique because of the seed storage problem. *H. odorata* flowers bear fruit only every one or two years, fruit ripening occurs for three months from anthesis and produces recalcitrant seeds (Sasaki 2008; Orwa et al. 2009).

Recalcitrant seeds are sensitive to desiccation and it differ to orthodox seeds which have desiccation tolerance. Recalcitrant seed normally cannot be stored for a long time under conditions that cause low moisture content of the seeds, such as high temperature, low humidity, tightness material container and direct light exposure. It is because they have active metabolism and high respiration rate under those conditions (Lodong et al. 2015). It has been reported that some recalcitrant seeds from family Dipterocarpaceae showed a decrease in their germination percentages if stored at room temperature (28±2C^{o}), for example *Vatica chinensis* decreased the germination percentage to 69.95% when stored at mositure content (MC) of 52.24% , *Hopea ponga* has germination percentage of 40% when stored at MC of 29%, and *Shorea seminis* has germination percentage of 30.11% when stored at MC of 49 % to 47%) (Sukesh & Chandrashekar 2011; Sukesh & Chandrashekar 2013; Zanzibar et al. 2019).

Cryopreservation is an alternative storage process for any biological constructs such as cell, tissue or organelles at very low temperatures (Jang et al. 2017). Cryopreservation procedure involves the utilization of liquid nitrogen to quickly deep freeze the cell, allowing suppresion of enzymatic activity, and metabolic activity will turn to stand still conditions trough out the storage periods until thawed (Kartha 1985; Radha et al. 2012). The most critical factor for the effectiveness of cryopreservation technique is the right choice of protective compounds that can prevent cells from chilling injury. Cryoprotectant is nonelectrolyte chemical used to reduce the amount of intracellular water and protect the cell for extracellular ice formation caused by very low temperatures (Joshi 2016). There are several cryoprotectants that

can be categorized according to the way of penetrating the cells, namely (a) small substances that are able to penetrate the cells through the cell wall and plasma membrane, (b) substances that are able to penetrate only through the cell wall and (c) substances that do not penetrate through the cell walls or the plasma membrane. Plant Vitrification Solution (PVS) and its modification for obtaining successfulness in cryopreservation has been reviewed by Zamecnik et al. (2021). Jitsopakul et al. (2012) reported that application of PVS2 solution as common cryoprotectan with 15 min loading treatment increase the germination percentage of cryopreserved seeds *Vanda tricolor*. Meanwhile, the effectiveness of cryopreservation techniques to maintain cell viability was also found in seed of *Percea americana* cv. Velvick. An exposure of this seeds into PVS2 and vitrification solution L (VSL) for 20 min caused a highest regrowth level after seeds were immersed into liquid nitrogen (O'Brien et al. 2021). Based on these findings, cryopreservation technique looks promising to be applied to recalcitrant *H. odorata* seeds, so that the seeds can be stored for a longer period without losing its viability. In addition, this kind of research has never been reported previously. The purpose of this study was to evaluate the effect of cryoprotectant treatment on seed viability and some biochemical change within the seed including electrolyte leakage, total malondialdehyde, total phenol of *H. odorata* seeds following storage at room temperature (28±2^{o}C) or liquid nitrogen (-196±2^{o}C).

MATERIALS AND METHODS

Materials

The experiment was held at Research Center for Plant Conservation and Botanical Gardens, Bogor and Plant Physiology Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta. The mature seeds of *H. odorata* were collected from Bogor Botanical Garden in December 2020. The seeds were classified as mature when 2/3 of the wings turned the color to dark brown and the pericarp or the fruit coat has changed as well from green to brown (Joker & Salazar 2000). The wings were removed for ease of the experiment.

Methods

Cryopreservation Procedure

H. odorata seeds were immersed in two different types of cryoprotectants, the first was Plant Vitrification Solution Number 1 (PVS1) which consists of mannitol and the second was Plant Vitrification Solution 2 (PVS2) which consists of DMSO 15% (w/v), ethylene glycol 15% (w/v) and glycerol 30% (w/v) in 0.4 M sucrose. For each type of cryoprotectant four different concentrations were prepared namely 25%, 50%, 75% or 100% (w/v). The seeds were immersed in each type of cryoprotectant for 30 mins, 60 mins, 90 mins or 120 mins. For control, seeds were immersed in distilled water and put in cryo-tubes. Both control and seeds treated with cryoprotectant were then stored at room temperature (28±2^{o}C) or subjected to liquid nitrogen (-

196±2°C) for 24 hours. Seeds that have been subjected to liquid nitrogen were thawed with warm water (40°C) for 3 minutes. Seeds were rinsed with distilled water to remove any residual cryoprotectant. Subsequently, seeds were sown in polybags (15 cm diameter, volume ± 4.420 cm^3) containing sterile sand (90% sand grains that passed a ± 5 mm sieve) and placed in a greenhouse with average air temperature of 30.5°C and 53% of humidity, 147x100 Lux of light intensity, and 74% of soil moisture (average condition at midday). The germination tests were carried out using three replications of 25 seeds. The other biochemical tests were performed using three replicates as well.

Physiological and Biochemical Observation Variables

Observation variables were divided into physiological variables (moisture content, germination test) and biochemical variables (electrolyte leakage, total malondialdehyde, total phenol). Normal seedling category and germination test were carried out according to procedure of seed testing protocol of International Rules for Seed Testing (ISTA) 2015 and 2018.

Moisture content was calculated using the formula :

$$MC = \frac{M2 - M3}{M2 - M1} \times 100\%$$

Where, M1 = weight of petridish; M2 = weight of petridish and seed before drying; M3 = weight of petridish and seed after drying.

Germination percentage was calculated using the formula :

$$GP = \frac{first\ count + final\ count}{Number\ of\ germinated\ seeds} \times 100\%$$

Where, the first count was determined at 7 days after sowing (DAS) and the final count was determined at 12 DAS.

Germination rate was calculated using the formula :

$$GR = \sum_{i=1}^{12} d$$

Where, *d* is additional percentage of normal seedling/etmal (1 etmal = 24 hours).

Vigor index was calculated using the formula :

$$VI = \frac{\sum seedlings\ of\ first\ count}{total\ planted\ seed} \times 100\%$$

Maximum growth potential was calculated using the formula :

$$MGP = \frac{\sum germinated\ seed}{\sum planted\ seed} \times 100\%$$

Total Malondialdehyde was determined using 1 gr of *H. odorata* seed extract according to method explained by Zhang and Huang (2013) and calculated using the formula:

$$MDA = [6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}] \times 5$$

Where, A_{532} = absorbance of 532 nm, A_{600} = absorbance of 600 nm, A_{450} = absorbance of 450 nm.

Total phenol was determined using Folin-Ciocalteu method and total phenol was determined using a UV-Vis spectrophotometer at 765 nm and three times reading were performed to obtain the variations of absorbance value.

Percentage of electrolyte leakage was measured using a method explained in Stewart and Bewley (1980) and calculated using the formula :

$$C = \frac{Cl}{Ch} \times 100\%$$

Where, C = percentage of electrolyte leakage, Cl = Leachate or first conductivity, Ch = Homogenate or final conductivity.

Data Analysis

Data analysis was performed using the Analysis of Varince (ANOVA) test an.d continued with the Duncan's Multiple Range Test (DMRT). Data were calculated using Statistical Product and Service Solutions (SPSS) software version 16.0 with $\alpha = 5\%$.

RESULTS AND DISCUSSION

Effect of cryoprotectant on *H. odorata* seed stored at room temperature

Results showed that different types and concentrations of cryoprotectant had a significant effect on physiological and biochemical variables ($p<0.05$) (Table 1). Meanwhile, immersion time variations did not show any significant effect on moisture content, germination percentage and germination rate. Non-significantly immersion time variation (I) on the observed variables has implications for its interaction with other treatments variation (T-C / C-I / T -C-I). This indicates that different duration of immersion in this study gave the same effect on *H.odorata* seeds stored at room temperature regardless of the type of cryoprotectants.

Table 1. Analysis of variance for the effect of cryoprotectans, concentration, and immersion time on the variable observations and its interactions.

Source of variation	P-value							
	MC	GP	GR	VI	MGP	EL	MDA	PH
Type (T)	.000*	.000*	.000*	.000*	.000*	.000*	.000*	.000*
Concentration (C)	.001*	.000*	.000*	.000*	.000*	.000*	.000*	.000*
Immersion time (I)	.182ns	.095ns	.412ns	.002*	.000*	.000*	.000*	.000*
T*C	.056 ns	.000*	.000*	.000*	.000*	.000*	.000*	.000*
T*IT	.010*	.449ns	.183ns	.000*	.000*	.000*	.000*	.000*
C*IT	.997ns	.790ns	.307ns	.286ns	.000*	.000*	.000*	.000*
T*C*IT	1.00ns	.652ns	.232ns	.894 ns	.000*	.000*	.010*	.000*

Note : * = treatment gave significant effect on observed variables at $\alpha=5\%$. ns = treatment gave non-significant effect on observed variablesat $\alpha=5\%$. Type (PVS1 and PVS2). Concentration (25%, 50%, 75%, 100%). Immersion time (30, 60, 90 or 120 min). MC: Mouisture Content, GP: Germination Percentage, GR: Germination Rate, VI: Vigour Index, MGP: Maximum Growth Potensial, EL: Electrolyte Leakage, MDA: Total Malondialdehyde, PH: Total Phenol. (Rohmah 2021).

Chemical compound of DMSO (dimethyl sulfoxide, Me_2SO) as a component of PVS2 was reported to be toxic to cell membranes, especially when used at room temperature (Aronen et al. 1999; Bettoni et al. 2019). In the study of damar (*Agathis damara*) seeds, it has been reported that high germination rate (84,67%) was obtained from seeds that have been subjected to vitrification for one hour without any cryoprotectant. Cryoprotectant normally used to increase seed viability of recalcitrant or intermediate seeds. However, the application of cryoprotectant and vitrification for one hour lowered seed viability of damar (Djam'an et al. 2006). The effectiveness of cryoprotectant and duration required to immerse the seed into cryoprotectant seems different and it depends on the type of seed cultivar and pre-cryopreservation treatment such as the loading treatment (Wardani et al. 2019; O'Brien et al. 2021).

Regardless of immersion time of *Hopea odorata* seeds in the cryoprotectants, both PVS1 and PVS2 cryoprotectants with concentrations of 25%, 50%, 75% or 100% were generally able to reduce seeds moisture content above the critical moisture content value (lethal MC) of *H. odorata* (Figure 1). All treatment combinations significantly reduce the MC of *H. odorata* seeds compared to those of control that showed moisture content value of seeds about 36,89 - 45,31%. According to Orwa et al. (2009), the lethal MC for *H. odorata* seed is 33% at 35°C, in which seeds will die within five days under that condition due to dehydration. Immersing seeds in cryoprotectants before vitrification is important to prevent seeds from mechanical damage due to intracellular fluid crystallization. However, type of cryoprotectants, concentrations, and immersion time must be determined so that it may not cause the water content of the seeds decreased below the lethal MC, especially for recalcitrant seeds (Berjak & Pammenter 2013). The results of this study were in line with the finding that application of 35% cryoprotectant effectively reduce the moisture content of Sugi (*Crypometria japonica*) seeds and increase its germination. It was also reported that application of PVS2 for 2 hours was significantly able to maintain the critical

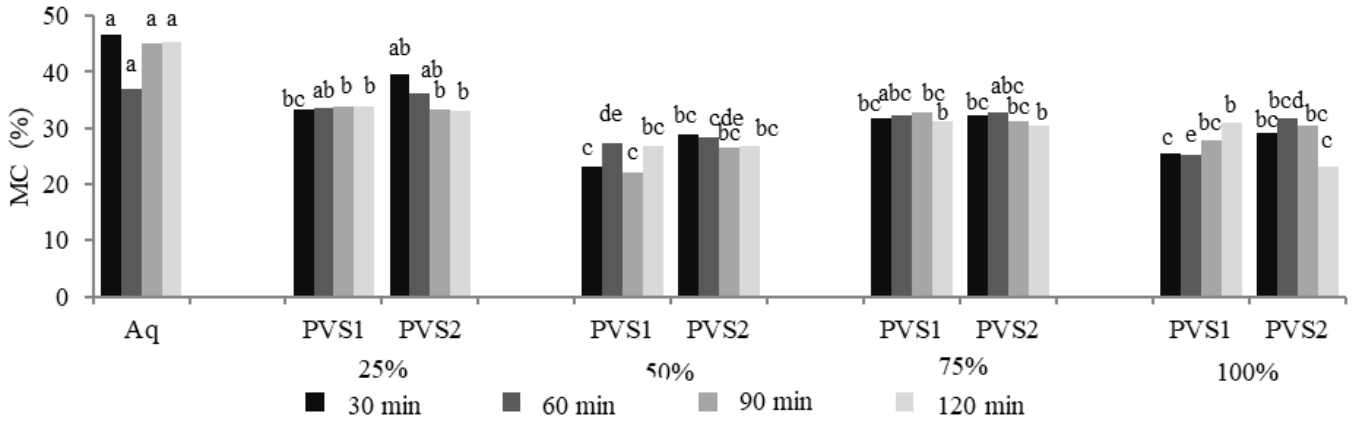

Figure 1. Interaction of cryoprotectant types, concentrations and immersion times on percentage of moisture content (MC) of *H.odorata* seed stored at room temperature. Note: Aq= Aquadest. The similar letter above each column indicates that the treatment gave a nonsignificant effect based on DMRT test (α=5%). (Rohmah 2021)

moisture content of *Hibiscus sabdariffa* seeds (14.28%) compared to control (10.71%) or vitrification at a temperature of -5ºC (11.53%) (Kim et al. 2009; Suhendra et al. 2014).

Figure 2 showed that PVS2 treatment for *H. odotara* seeds at all concentrations and immersion time resulted in the germination percentage (GP) less than 50% compared to those treated with cryoprotectant PVS1 or control. Decreasing value of seed GP can be caused by seed moisture content that below the critical limit. However, seed moisture content of *H. odorata* presented in Figure 1 showed that the PVS2 application was generally able to maintain the seeds moisture content that is appropriate to keep seed viability (38.58% to 26.56%). The fact that seed germination value is low suggests that there might be a toxic effects of PVS2 cryoprotectant.

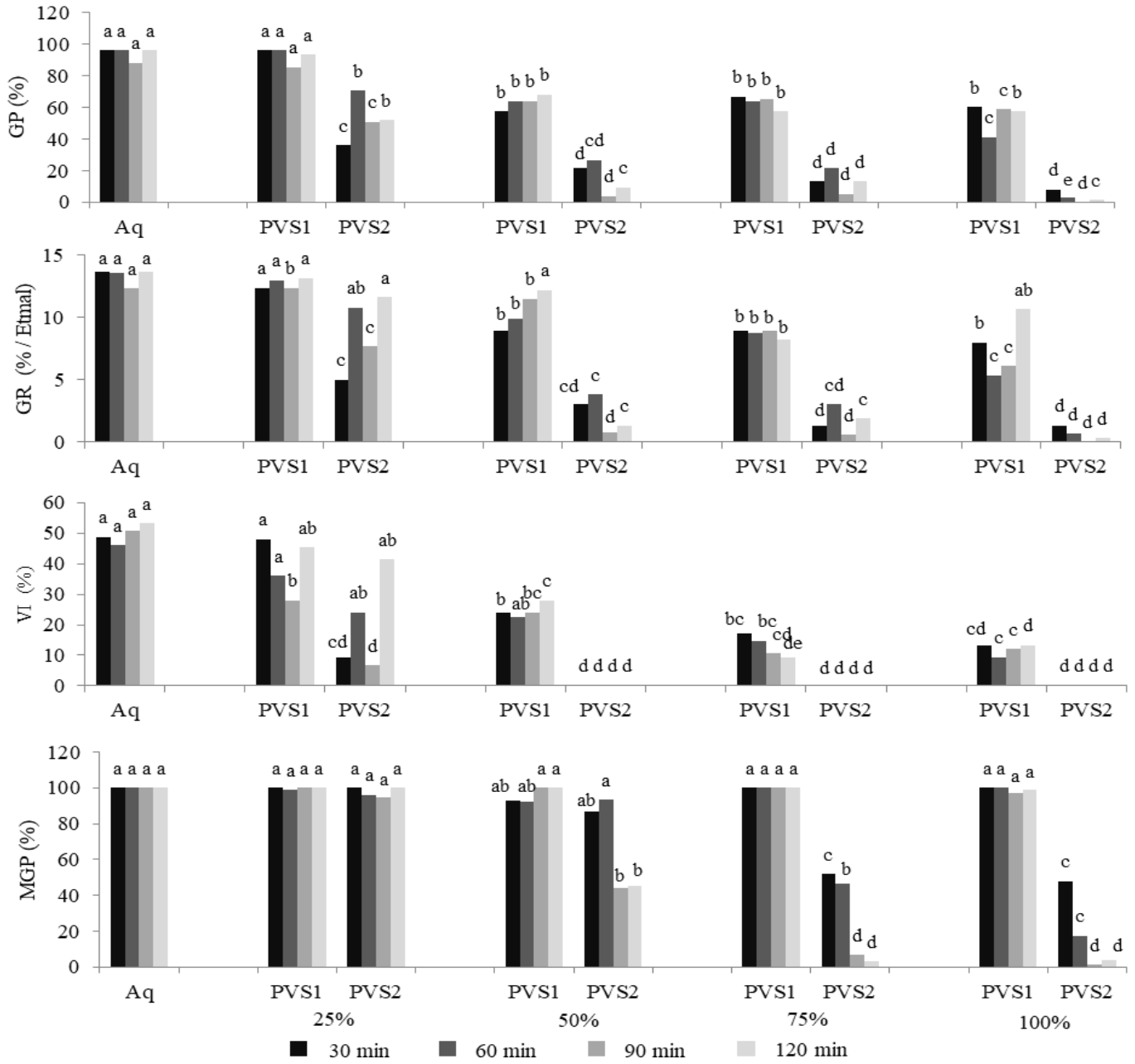

Figure 2. Interaction of cryoprotectant types, concentrations and immersion times on percentage of germination percentage (GP), germination rate (GR), vigor index (VI), and Maximum Growth Potential (MGP) of *H.odorata* seed at room temperature. Note: Aq= Aquadest;Etmal = 24 h. The similar letter in the graphic indicates that the treatment gives a nonsignificant effect at DMRT test (α=5%). (Rohmah 2021)

The results showed that PVS2 treatment decreased the average of germination rate percentage (GR), vigor index (VI), and Maximum Growth Potential (MGP) compared to those of PVS1 treatment (GR: 5.34 - 16.38%; VI: 9.33 - 48.0%; MGP: 92 - 100%) (Figure 2). *H. odorata* seeds that were immersed in PVS2 showed very small VI percentage (0% - 4%), except at concentration of 25% and immersion time of 120 mins (41.33%), but in general the value was lower than PVS1 treatment or controls. Treatment of 75% PVS2 with immersion time 90 mins (6.7%) and 120 mins (30.7%) was significantly decrease the percentage of MGP of *H. odorata* seed compared to PVS1 in the same concentration and immersion time (MGP: 100%). *H. odorata* seeds treated with PVS2 of 50% for 90 min or 120 min showed a decline in MGP, whereas the higher concentration of PVS2 (75% or 100%) start lowering the percentage of MGP at 30 min immersion. These results indicate that PVS2 treatment with a concentration of more than 50% with immersion time more than 60 mins caused inhibition for germination of *H. odorata* seeds. Meanwhile, the percentage of VI after PVS2 treatmentis were lower than PVS1,this result confirmed that *H. odorata* seeds can successfully germinate but not able to grow in the normal category of ISTA (2018). It was also reported that application of PVS2 on shoots tissue of *Pimpinella pruatjan* increased survival to 90% when applied before explants were stored in liquid nitrogen, whereas after freezing with liquid nitrogen the percentage of survival was only 40%) (Roostika et al. 2013). Best (2015), reported that PVS2 is dangerous for the structure of phospholipid bilayer, causing a decrease in the permeability function or turgidity of the cell membrane. The effectiveness of PVS2 could be increased by the addition of melatonin or vitamin C to reduce the effects of toxicity and accumulation of oxidative compounds due to low temperature stress. Burritt (2012), reported that addition of reactive oxygen species (ROS) binders in cryoprotectants will improve plant tissue adaptation mechanisms.

Results presented in Figure 3 showed that treatments of PVS1 or PVS2 at concentrations of 25%, 50%, 75% or 100% with 30 minutes immersion time did caused any significant difference on electrolyte leakage (EL) percentage compared to control, but there was a tendency for a slight increase in electrolyte leakage in those seeds treated with longer immersion time of PVS1 or PVS2. The increasing percentage of EL after seeds were immersed into croprotectants might be due to cryoprotectant concentration that was lower compared to the intracellular fluid, and it promotes hypotonic conditions for cells. Under hypotonic conditions, the solutes in the seeds will move to the extra cellular which in this experimental unit is a cryoprotectant compound with a lower concentration.

Total malondialdehyde (MDA) of *H. odorata* seeds that were immersed into 25% PVS1 for 30 mins showed a lower value (5,110 nmol g^{-1} FW) than immersion into 50% PVS1 (5,211 nmol g^{-1} FW), 75% PVS1 (5,379 nmol g^{-1} FW) or 100% PVS1 (5.451 nmol g^{-1} FW). In addition, total MDA increased concomitant with longer immersion time even when the concentration of

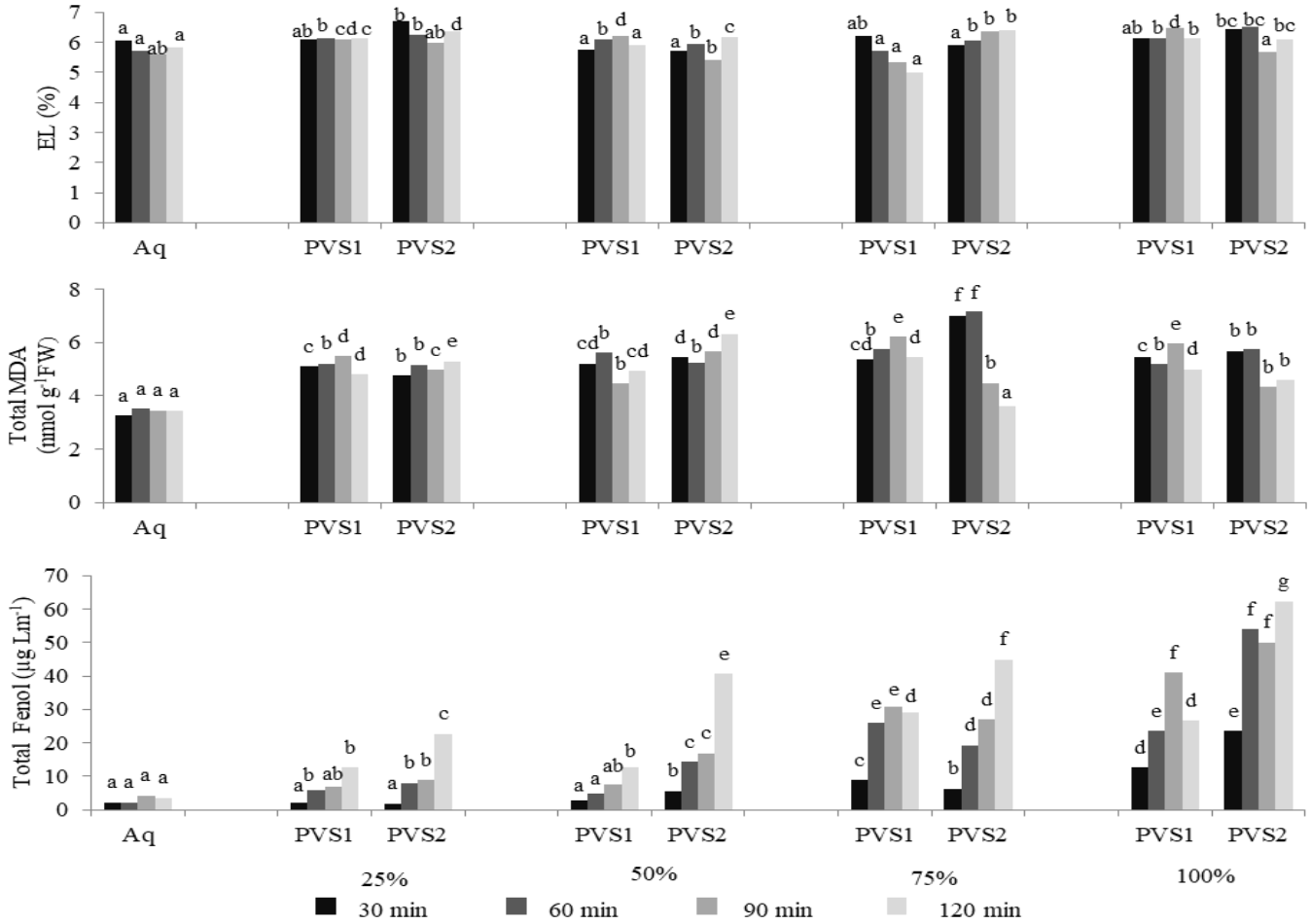

Figure 3. Interaction of cryoprotectant types, concentrations and immersion times on percentage of electrolyte leakage (EL), total malondialdehyde (MDA), and total phenol (PH)at room temperature. Note: Aq= Aquadest. The similar letter in the graphic indicates that the treatment gives a nonsignificant effect at DMRT test (α=5%). (Rohmah 2021)

PVS1 used was 25%. The similar pattern of increasing total MDA was also seen in the PVS2 treatment. Overall, both PVS1 and PVS2 treatments caused an increased in total MDA compared to control. Regarding to the germination percentage, application of PVS1 (25%) had similar value with control, however greater concentration of PVS1 or PVS2 tend to reduce the germination percentage of *H. odorata*. This results indicate that PVS1 of 25% was not toxic for *H. odorata* seeds and has a prospect to be used as a cryoprotectant. Malondialdehyde is a reactive compound in cell membranes resulting from the denaturation of polyunsaturated fatty acids (PUFA) due to biotic and abiotic stresses from the extracellular environment. An excess of MDA accumulation can damage the property of cell membrane, such as its selective permeability function (Olvera-Carrillo et al. 2011).

The effect of cryoprotectant on total phenol (FE) is presented in Figure 3. *H. odorata* seeds that were immersed into PVS1 of 25% or 50 % for 30 min, 60 min or 90 min still has relatively similar total phenol content compared to control. Whereas greater PVS1 concentration or PVS2 caused an increased in total phenol content within the seeds. According to Ma et al. (2016), excessive phenol accumulation in plant tissues may indicate oxidative stress that could be due to environmental stress. Phenol as a non-enzymatic

antioxidant group will suppress lipid peroxidation products such as malondialdehyde, proline or group of *reactive oxygen species* (ROS). In this experiment, a reduction of total MDA that concomitant with an increase in total phenol was found in *H.odorata* seeds treated with PVS2 of 75% or 100% and immersion time of 90 minutes or 120 minutes. However, germination percentage, germination index, vigor index and maximum growth potential of *H,odorata* seeds were not supported by those treatments.

Cryoprotectant and Cryopreservation Treatment of *H. odorata* Seed (-196±2ºC)

Variations of cryoprotectants, concentrations, immersion times and their interactions did not significantly affect the moisture content (MC) of cryopreserved seeds, except for the interaction between types of cryoprotectans and immersion times (T-I). Cryoprotectant immersion times only affected the value of total MDA and total phenol (Table 2). Results of analysis of variance did not show any significant effect of the immersion time on germination variables (GP, GR, VI, MGP) of *H.odorata* seeds following cryopreservation. All seeds were become necrosis and could not germinate. Therefore, the results of Duncan's test of germination variables after cryopreservation were not presented.

Table 2. Analysis of variance for effect of cryoprotectans types, consentration, and immersion time on the observations variables and its interactions following cryopreservation.

Source of variation	P-value			
	MC	EL	MDA	PH
Type (T)	.476^{tn}	.720^{tn}	.000*	.000*
Concentration (C)	.112*	.571^{tn}	.000*	.000*
Immersion time (I)	.845^{tn}	.232^{tn}	.000*	.000*
T*C	.158^{tn}	.640^{tn}	.000*	.000*
T*IT	.608^{tn}	.320^{n}	.000*	.000*
C*IT	.943^{tn}	.380^{tn}	.010*	.001*
T*C*IT	1.00^{tn}	.616^{tn}	.009*	.000*

Note : * = treatment gave significant effect on observed variables at α=5%. ^{ns} = treatment gave non-significant effect on observed variablesat α=5%. Type (PVS1 and PVS2). Concentration (25%, 50%, 75%, 100%). Immersion time (30, 60, 90 and 120 min). MC: Mouisture Content, EL: Electrolyte Leakage, MDA: Total Malondialdehyde, PH: Total Phenol. (Rohmah 2021)

Figure 4 showed that variations in type of cryoprotectans, concentrations and immersion times mostly did not caused any significant difference on MC of *H. odorata* seeds following cryopreservation compared to control. A slight increase of MC was found in *H.odorata* seeds treated with 25% of PVS1 or 25% PVS2 with immersion times of 30 mins or 60 mins

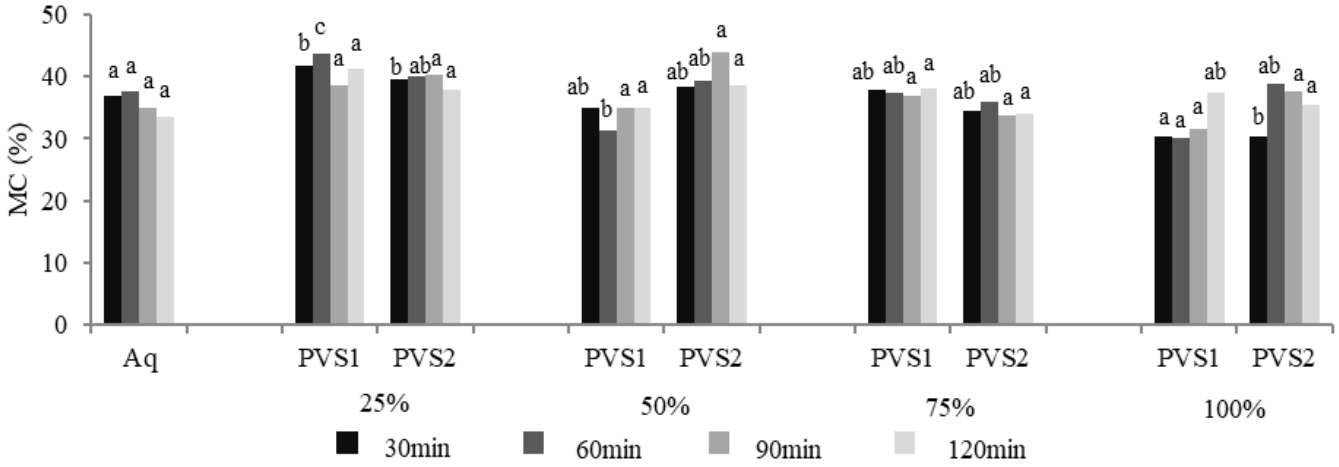

Figure 4. Interaction of cryoprotectant types, concentrations and immersion times on percentage of moisture content (MC) of *H.odorata* seed following croyopreservation. Note: Aq= Aquadest. The similar letter in the graphic indicates that the treatment gives a nonsignificant effect at DMRT test (α=5%). (Rohmah 2021)

(41.58% and 39.66% respectively). According to Yan et al. (2014) an increase of MC following cryopreservation might be due to immersion treatment that was carried out previously, in which the solvent of cryoprotectant moves into the seed tissue.

All variation of cryoprotectants and immersion times of *H. odorata* seeds that followed by cryopreservation did not caused any significant difference effect on the electrolyte leakage (EL) compared to the control (Figure 5). This results indicate that both PVS1 or PVS2 did work effectively as a seed protector of cell membrane damage that may occurred following low temperature treatment . The increase of electrolyte leakage after cryopreservation is an indicator that normally used to determine condition of cell membranes. Ntuli et al. (2011) reported that the amount electrolyte leakage will increase if the cell membrane is mechanically damaged due to ice crystals or suffer from physiological damage due to accumulation of oxidative compounds in response to chilling injury. The research report of Pukacki and Juszczyk (2015) stated that cryopreservation *Acer pseudoplantanus* as recalcitrant seeds had electrolyte leakage accumulation more than 50% and indicated a breakdown of cell membrane integrity.

Results presented in Figure 5 also showed that variation of immersion times and cryopreservation tend to increase the total malondialdehyde (MDA) of *H.odorata* seeds. Total MDA in *H. odorata* seeds treated with PVS2 and followed with liquid nitrogen treatment had a higher value (5.767-12.905 nmol g^{-1} FW) than those seeds subjected to liquid nitrogen but treated with PVS1 (4.927-8.898 nmol g^{-1}FW) regardless of its concentration and time of immersion. The total MDA in the H. odorata seeds treated with PVS2 and continued with cryopreservation was significantly higher than control (immersion into distilled water: 4,372-5,792 nmol g^{-1} FW). This result indicates that PVS2 which was initially used to minimize damage of cryopreservation actually increases the risk of deterioration and seed viability of *H. odorata.*

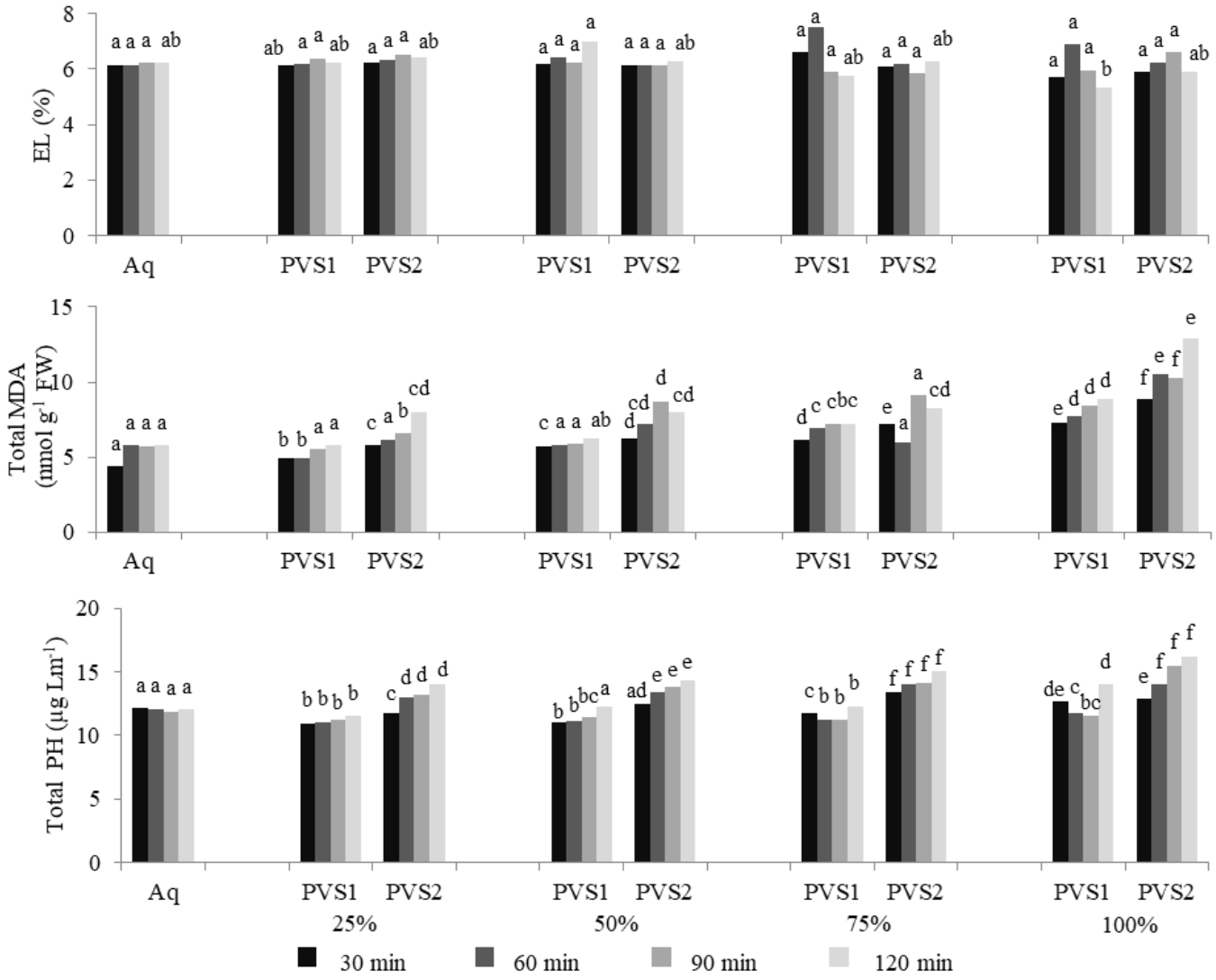

Figure 5. Interaction of cryoprotectant types, concentrations and immersion times on percentage of electrolyte leakage (EL), total malondialdehyde (MDA), and total phenol (PH) after cryopreservation. Note: Aq= Aquadest. The similar letter in the graphic indicates that the treatment gives a nonsignificant effect at DMRT test (α=5%). (Rohmah 2021)

Vendrame et al. (2014) stated that PVS2 at high concentrations promoted osmotic stress and cell death. O'Brien et al. (2021) reported that PVS2 at high concentrations (100%) with an immersion time of 30-40 mins caused damage to meristem cells and decreased generative capacity of avocado (*Persea americana*) shoot tip cells. Wang et al. (2014) suggested that DMSO in PVS2 could be replaced with glycerol to prevent the osmotic shock.

Total phenol of *H. odorata* seeds following cryopreservation increased in both PVS1 and PVS2 treatments regardless of the duration of immersion in those cryoprotectants. According to Galluzzi et al. (2013) an increase in the amount of total phenol as a group of non-enzymatic antioxidant compounds is a normal response of seed tissue that face oxidative stress due to the extreme temperature of liquid nitrogen. Phenol is regulated to suppress the accumulation of oxidative compounds such as $O2^{\bullet-}$ and H_2O_2 or products of lipid peroxidation processes such as malondialdehyde, proline and acrolein that cause damage to plant tissues (Narayanan et al. 2015).

CONCLUSION

From the results and discussion it can be inferred that immersion of *H. odorata* seed into Plant Vitrivication Solution Number 1 (PVS1) at room temperature resulted in higher percentage of physiological variables and lower values of biochemical variables compared to those immersed into Plant Vitrivication Solution Number 2 (PVS2) at higher concentration (50% - 100%). PVS1 and PVS2 of 25% did not caused significant difference in physiological variables. Meanwhile, combination of cryoprotectant and cryopreservation treatments were not able to increase the viability of *H. odorata* seeds. Cryopreservation treatment increased the amount of total malondialdehyde and total phenol content in *H. odorata* seeds. Based on this result , it seems that PVS1 is kind of cryoprotectant that had less toxic effect on *H. odorata* seeds but still can not prevent the negative impact of cryopreservation treatment. Further research still important to be conducted to obtain appropriate cryopreservation storage techniques for H. odorata seeds, such as encapsulation in alginate capsules to form artificial seeds, which can be combined with dehydration using PVS1.

AUTHORS CONTRIBUTION

L.A.R. collected plant samples, carried out experiments, analyzed the data, and wrote the manuscript. F.F.W. and A.H.W. help in data collection and analysis. D.L. and K.D. designed the research, supervised all the processes laboratory analysis, and edited the manuscript.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

REFERENCES

Aronen, T.S. et al., 1999. Genetic fidelity of cryopreserved embryogenic cultures of open- pollinated *Abies cephalonica*. *Plant Science*, 142(2), pp.163-172. doi: 10.1016/S0168-9452(98)00244-1.

Ashton, M.S., Singhakumara, B. & Gunatilleke, N., 2011. Sustainable forest management for mixed-dipterocarp forests : acase study metadata of the book that will be visualized online. *TROPICAL*,8, pp 193-213. doi:10.1007/978-3-642-19986-8_13.

Berjak, P. & Pammenter, N.W., 2013. Implications of the lack of desiccation tolerance in recalcitrant seeds. *Frontiers in Plant Science*, 4, pp.1-10. doi: 10.3389/fpls.2013.00478.

Best, B.P., 2015. Cryoprotectant toxicity: facts, issues, and questions. *Rejuvenation Research*, 18(5), pp.422-436. doi: 10.1089/rej.2014.1656.

Bettoni, J.C. et al., 2019. Cryopreservation of 12 vitis species using apical shoot tips derived from plants grown in vitro. *HortScience*, 54(6), pp.976 -981. doi: 10.21273/HORTSCI13958-19.

Burritt, D.J., 2012. Proline and the cryopreservation of plant tissues: functions and practical applications. In*Current frontiers in cryopreservation*. London, UK: IntechOpen.doi: 10.5772/2528.

Chua, L.S.L. et al., 2010. Research Pamphlet No. 129Malaysia plant red list Peninsular Malaysian Dipterocarpaceae,Forest Research Institute Malaysia.

Djam'an, D.F., Priadi, D. & Sudarmanowati, E., 2006. Cryopreservation of Dammar (*Agathis damara* Salisb.) seeds in liquid nitrogen. *Biodiversitas Journal of Biological Diversity*, 7(2), pp.164–167. doi: 10.13057/biodiv/ d070215.

Galluzzi, L. et al., 2013. *Cell Senescence: methods and protocols*, Totowa, N.J.: Humana Press. doi: 10.1007/978-1-62703-239-1.

ISTA. International Rules for Seed Testing. 2015. International Seed Testing Association, Bassersdorf, Switzerland.

ISTA. International Rules for Seed Testing. 2018. International Seed Testing Association, Bassersdorf, Switzerland.

Jang, T.H. et al., 2017. Cryopreservation and its clinical applications. *Integrative Medicine Research*, 6(7), pp.12-18. doi: 10.1016/ j.imr.2016.12.001.

Jitsopakul, N. et al., 2012. Effect of cryopreservation on seed germination and protocorm development of *Vanda tricolor*. *ScienceAsia*, 38(3), pp.244 -249. doi: 10.2306/scienceasia1513-1874.2012.38.244.

Joker, D.F.S.C. & Salazar, C.R., 2000. Seed leaflet King. *Danida Forest Seed Centre*, 35(17), pp.1-2.

Joshi, A.J., 2016. A review and Application of Cryoprotectant: The Science of Cryonics. *PharmaTutor*, 4(1), pp.12-18.

Junaedi, A. & Frianto, D., 2012. Kualitasbibit Merawan (Hopea odorata Roxb.) asal KOFFCO system pada berbagai umur. *Jurnal Penelitian Hutan dan Konservasi Alam*, 9(3), pp.265-274. doi: 10.20886/ jphka.2012.9.3.265-274.

Kartha, K.K., 1985. Meristem Culture and Germplasm Preservation. In *Cryopreservation of plant cells and organs*. Florida: Cue Press, pp. 116-134.

Kim, D.H. et al., 2009. Effects of cryoprotectants and post-storage priming on seed germination of Sugi (*Cryptomeriajaponica* D. Don). *Silvae Genetica*, 58(4), pp.162-168. doi: 10.1515/sg-2009-0021.

Lodong, O. et al., 2015. Peranan kemasan dan media simpan terhadap ketahanan viabilitas dan vigor benih Nangka (*Artocarpus heterophyllus* Lamk) kultivar Tulo-5 selama penyimpanan. *Agrotekbis*, 3(3), pp.303-315.

Ly, et al., V. 2017. *Hopea odorata.* The IUCN red list of threatened species2017 viewed 5 Mei 2020, from https://doi.org/10.2305% 2FIUCN.UK.2017-3.RLTS.T32305A2813234.en.

Ma, D. et al., 2016. Accumulation of phenolic compounds and expression profiles of phenolic acid biosynthesis-related genes in developing grains of white, purple, and red wheat. *Frontiers in Plant Science,*7, pp.1-11.doi: 10.3389/fpls.2016.00528.

Narayanan, S. et al., 2015. Wheat leaf lipids during heat stress: I. high day and night temperatures result in major lipid alterations. *Plant Cell Environment,* 39(4), pp.787-803. doi: 10.1111/pce.12649.

Ntuli, T.M. et al., 2011. Increased drying rate lowers the critical water content for survival in embryonic axes of english Oak (*Quercus robur* L.) seeds F. *Journal of Integrative Plant Biology*, 53(4), pp.270-280. doi: 10.1111/j.1744-7909.2010.01016.x.

O'Brien, C. et al., 2020. First report on cryopreservation of mature shoot tips of two avocado (*Persea americana* Mill.) rootstocks. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 144(1), pp.103-113. doi: 10.1007/S11240-020-01861-Y.

Olvera-Carrillo, Y., Luis Reyes, J. & Covarrubias, A.A., 2011. Late embryogenesis abundant proteins Versatile players in the plant adaptation to water limiting environments. *Plant Signaling & Behavior,* 6, pp.586-589. doi: 10.4161/psb.6.4.15042.

Orwa, C.et al., 2009. Agroforestree Database: A Tree Reference and Selection Guide Version 4.0, in World Agroforestry, viewed 21 November 2020, from http://apps.worldagroforestry.org/treedb/AFTPDFS/Hopea_odorata.PDF

Pukacki, P.M. & Juszczyk, K., 2015. Desiccation sensitivity and cryopreservation of the embryonic axes of the seeds of two Acer species. *Trees*,29, pp.385-396. doi: 10.1007/s00468-014-1118-7.

Radha, R.K., Decruse, W.S. & Krishnan, P.N., 2012. 21 Plant Cryopreservation. In *Current frontiers in cryopreservation*. London, UK: IntechOpen.doi: 10.5772/35919.

Rohmah, L.A. 2021. Uji Daya Simpan Benih Merawan (*Hopea odorata* Roxb.) dan Evaluasi Peningkatan Viabilitasnya Melalui Teknik Kriopreservasi. Thesis Program Magister. Program Studi Biologi Tropika. Fakultas Biologi. Universitas Gadjah Mada. Yogyakarta

Roostika, I. 2013. Optimasi dan evaluasi metode kriopreservasi Purwoceng. *Jurnal Littri,* 19(3), pp.147-157. doi: 10.21082/jlittri.v19n3.2013.147-157.

Sasaki, S., 2008. Physiological characteristics of tropical rain forest tree species: a basis for the development of silvicultural technology. *Proceedings of the Japan Academy. Series B, Physical and biological sciences*, 84(2), pp.31–57. doi: 10.2183/PJAB.84.31.

Stewart, R.R.C. & Bewley, J.D., 1980. Lipid peroxidation associated with accelerated aging of soybean axes. *Plant Physiology,*65, pp.245-248. doi:1104/pp.65.2.245.

Suhendra, D., Haryati, H. & Siregar, L.A., 2014. Pengaruh metode strafikasi suhu rendah, krioprotektan dan kriopreservasi terhadap viabilitas benih Rosela (*Hibiscus sabdariffa* L.). *Jurnal Agroekoteknologi Universitas Sumatera Utara*, 2(4), pp.1511–1517. doi: 10.32734/jaet.v2i4.8451.

Sukesh & Chandrashekar, K.R. 2011. Effect of temperature on viability and biochemical changes during storage of recalcitrant seeds of *Hopea ponga* (Dennst.) Mabberly : an endemic species of Western Ghats. *Research Journal Seed Science,*4, pp.106-116. doi: 10.3923/rjss.2011.106.116.

Sukesh & Chandrashekar, K.R., 2013. Effect of temperature on viability and biochemical changes during storage of recalcitrant seeds of Vatica chinensis L. *International Journal of Botany*, 9(2), pp.73-79. doi: 10.3923/ijb.2013.73.79.

Vendrame, W. et al., 2014. Review orchid cryopreservation. *Agrotec Science Journal,* 38(3), pp.213-229. doi: 10.1590/S1413-70542014000300001.

Wang, B. et al., 2014. Potential applications of cryogenic technologies to plant genetic improvement and pathogen eradication. *Biotechnology Advances*, 32(3), pp.583–595. doi: 10.1016/J.BIOTECHADV.2014.03.003.

Wardani, F.F. et al., 2019. Cryopreservation of papaya seeds cv. Sukma, Callina, and Caliso: Effect of loading treatment and immersion time in plant vitrification solution-2. *Nusantara Bioscience*, 11(1), pp.71-78. doi: 10.13057/nusbiosci/n110112.

Weinland, G. 1998. Chapter 9 plantations. In *A review of Dipterocarps taxonomy, ecology and silviculture.* Bogor: CIFOR, pp.151-186.

Yan, Q. et al., 2014. Cryopreservation strategies for pomelo seeds from Xishuangbanna, South China. *Seed Science and Technology*, 42(2), pp.202–213. doi: 10.15258/sst.2014.42.2.09.

Zanzibar, M.et al., 2019. Teknik penyimpanan benih Meranti Balau (*Shorea seminis* (de Vriese) Sloot). *Jurnal Perbenihan Tanaman Hutan*, 7(2), pp.113-125.

Zamecnik, J. et al, 2021. Vitrification solutions for plant cryopreservation: modification and properties. *Plants*, 10(12), pp.1-17. doi:10.3390/plants10122623

Zhang, Z. & Huang, R., 2013. Analysis of malondialdehyde, chlorophyll proline, soluble sugar, and glutathione content in Arabidopsis seedling. *BIO-PROTOCOL*, 3(14). doi: 10.21769/BIOPROTOC.817.