

Cryopreservation of papaya seeds cv. Sukma, Callina, and Caliso: Effect of loading treatment and immersion time in plant vitrification solution-2

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Abstract. Wardani FF, Efendi D, Dinarti D, Witono JR. 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: 71-78. Sukma, Callina, and Caliso are papaya cultivars released by the Center for Tropical Horticulture Studies, IPB University, Bogor, West Java, Indonesia. In general, papaya seeds cannot be stored for a long time with a conventional GenBank storage system, even though some of them consider as "orthodox" class of seed. Cryopreservation, storage at an ultra-low temperature (-196 °C) of liquid nitrogen, could be possible for long-term storage of papaya seeds. The experiment conducted to get the necessary of loading treatment and the best immersion time in PVS2 for cryopreservation of papaya seeds cv. Sukma, Callina, and Caliso, so they still had considerable viability. We conducted experiment as factorial in completely randomized design, with the first factor was loading treatment with two levels (with and without loading treatment), and the second factor was immersion time in PVS2 with three levels (15, 30 and 45 min). So, there was six treatment and we used 50 seeds for each treatment. Results showed that the three papaya cultivars gave different responses to the treatment before cryopreservation proved that papayas seed had different characteristics, depending on genotype. For papaya seeds cv. Sukma, loading treatment was not needed and the best immersion time in PVS2 was 15 min. The viability of papaya seeds cv. Callina was low, so we should try another treatment before cryopreservation. For papaya seeds cv. Caliso, loading treatment was needed and the best immersion time in PVS2 was 30 min.

Keywords: GenBank, liquid nitrogen, long-term storage, orthodox seed, ultra-low temperature

Abbreviations: DMSO: dimethyl sulfoxide, LS: loading treatment, PVS2: plant vitrification solution 2, MC: Moisture content, GP: Germination percentage, GR: Germination rate, VI: Vigor index, MGP: Maximum growth potential

INTRODUCTION

Papaya (*Carica papaya* L.) is one of tropical fruit native to Southern America and had been cultivated in Indonesia. Papaya fruits contain many nutrients such as carbohydrates, vitamin A, vitamin C, vitamin B-6, minerals (Na, Mg, Cu, Zn, Mn, Ca, P, K, Fe), protein, fat, and high in fiber (Silva et al. 2007). Papaya breeding has been carried out by the Center for Tropical Horticulture Studies-Bogor Agriculture University (PKHT-IPB) since 2000 and got 13 papaya cultivars (PKHT 2018). Some of the new papaya cultivars were Caliso for small-sized papaya (300-500 g), Callina for medium-sized papaya (800-1200 g) and Sukma for large-sized papaya (more than 1200 g).

Papaya propagation is often carried out by using seeds so continuous availability and proper seed storage management is needed. The seeds of some papaya cultivars can be orthodox, but their shelf life was relatively short compared to other orthodox seeds from the other plant

(Salomao and Mundin 2000). Therefore, seed storage method is needed that can prolong the storability of papaya seeds.

Cryopreservation is a storing method of plant material in liquid nitrogen (-196 °C) so the metabolism in cells, tissues, and organs become stopped. Therefore, plant material such as seeds can be stored for a long term without experiencing genetic changes or somaclonal variations (Hervani et al. 2016). The new cryopreservation method or vitrification is widely used method because it is easier to implement and the results are good for various species (Wang et al. 2008). For example, embryogenic culture of avocado recovered from cryopreservation through vitrification demonstrate normal growth, and somatic embryos can be recovered (Efendi and Litz 2003). Shoot-tips of *Byrsonima intermedia* A. Juss. that stored by cryopreservation with vitrification method, presented satisfactory regrowth level (67%) (Silva et al. 2013). Protocorm-like bodies (PLBs) of *Brassidium* Shooting Star

orchid were successfully cryopreserved using droplet-vitrification method (Rahmah et al. 2015).

Vitrification is water transition process from liquid to amorphous or glassy phase to prevent ice crystal formation when freezing in the liquid nitrogen and thawing (warming) (Elliot et al. 2017). The main process in vitrification was done by cryoprotectant. Cryoprotectant is solution that protects cell from freezing and thawing (Vendrame et al. 2014). Plant Vitrification Solution 2 (PVS2) is cryoprotectant that has composition 30% glycerol, 15% ethylene glycol, and 15% DMSO on MS media with 0.4 M sucrose (Sakai et al. 1991). Some plants are sensitive to cryoprotectants. Therefore, to increase toleration to cryoprotectant, the plant material needed to be immersed in loading solution for 10-20 minutes before immersed in PVS2 (Kaczmarczyk et al. 2012). The composition of loading solution is 2 M glycerol on MS media with 0.4 M sucrose (Wang et al. 2008).

Some experiment about papaya seeds cryopreservation has reported by some researchers. Cryopreservation of papaya seeds that used Queensland genotype has been carried out by Azimi et al. (2005). Seeds with 10% moisture content being stored in liquid nitrogen had 48% germination percentage compared to the control. Papaya seeds from the cryopreservation seeds experienced 25% growth decline after planted on the field. Hervani et al. (2016) also have stored papaya seeds cv. Sukma using vitrification method. The results showed that papaya seeds which gave the best germination percentage, maximum growth potential and germination rate were seeds with 11-13% moisture content, removed sarcotesta and mesotesta, and immersed in PVS2 for 30 min. Germination percentage, maximum growth potential, and germination rate after cryopreservation were 38.4%, 38.4% and 2.2%/etmal, respectively. These results indicated that it is necessary to modify the cryopreservation method, so the stored papaya seeds have still high viability and able to grow well in the field. This experiment was preliminary experiment for seed cryopreservation of three papaya cultivars. The purpose was to obtain information about the necessity of loading treatment and the best immersion time in PVS2 for cryopreservation of papaya seeds cv. Sukma, Callina, and Caliso, so they still had considerable viability.

MATERIALS AND METHODS

Study area and materials

The experiment was done at the Center for Tropical Horticulture Studies (PKHT) Laboratory, Pasir Kuda experimental station, and Tajur experimental station of IPB University, Bogor, Indonesia.

The seeds used for this experiment were papaya seeds cv. Sukma, Callina, and Caliso. Those seeds extracted from fruit which were physiologically ripe with features of yellow color on the fruit skin as much as 25-49% (Suketi et al. 2010). Papaya fruit cv. Sukma and Caliso were taken from IPB Pasir Kuda experimental station, Bogor, while papaya fruit cv. Callina was taken from the Tajur experimental station, Bogor.

Procedures

Seeds extraction

Seeds that in the middle of the fruit were used in the experiment. The seeds were extracted by removing sarcotesta layer. Extraction began with immersing the papaya seeds with water for 48-72 h so the sarcotesta was easier to remove (Apriani 2017). After the sarcotesta was removed, the seeds were air-dried for 16 h to decrease the moisture content until 11-13% (Nurlovi 2004). After that, the mesotesta was removed in order to the seeds were stored better during cryopreservation (Hervani et al 2016). Hong et al (1996) stated also that the opened seed with exposed embryo had higher survival if stored in the liquid nitrogen.

Papaya seed cryopreservation procedure

Papaya seeds moisture content has been decreased and its mesotesta has been removed. Then the seeds were immersed in the loading solutions for 20 min. The composition of loading solution was 2 M glycerol which was dissolved in liquid MS medium with 0.4 M sucrose. Immersion time in the loading solution was 20 minutes (Kaczmarczyk et al. 2012). Seed without loading treatment was immersed in the sterile water for 20 min so the seeds had a similar condition with the other seeds with loading treatment. After that, the seeds were filtered and immersed in the plant vitrification solution-2 (PVS2) according to the immersion time in the treatment. PVS2 is cryoprotectant that had composition 30% glycerol, 15% ethylene glycol, and 15% DMSO on MS media with 0.4 M sucrose (Sakai et al. 1991). Seeds immersion in the cryoprotectant was carried out at a room with 25 °C temperature. Then, the seeds were put into 2 ml microtube and stored in liquid nitrogen for 24 h. The seeds were thawed at 40°C for 90-120 sec (Wang et al. 2005). After that, the seed was immersed in the liquid MS media for 30 min. Then, the seeds were germinated to observe the seed viability after cryopreservation. The seeds were sowed in plastic container with 1.9 L volume. The germination media was three stencil papers that have been moistened with sterile water. The seed viability test was done at room with 27-30 °C temperature and 37-49% RH.

Observation variables

Observations were made based on the seed testing protocol on ISTA (2015), i.e. moisture content (MC), germination percentage (GP), germination rate (GR), vigor index (VI), and maximum growth potential (MGP). The duration of the viability test for papaya was 21 days (Nurlovi 2004). The test duration required to break dormancy before or during the test is not taken as part of the germination test period (ISTA 2015).

Measurement of moisture content was carried out before seeds cryopreservation. The method of measuring moisture content was carried out by oven with 103 °C for 16 hours (ISTA 2015). Moisture content can be calculated using the formula:

$$MC = \frac{M_2 - M_3}{M_2 - M_1} \times 100\%$$

Where M_1 = petri dish weight, M_2 = petri dish, and seed before drying weight, and M_3 = petri dish and seed after drying weight.

Germination percentage (GP) was the observed variable used to determine seed viability physiologically. GP was determined by counting the seeds that have germinated normally at the first count and final count. The time of the first count is approximate but must be sufficient to permit the seedlings to reach a stage of development which allows for accurate evaluation (ISTA 2015). The first count (KN I) germination observation was carried out at 14 days after sowing and the final count (KN II) at 21 days after sowing (Nurlovi 2004). The criteria for normal germination was the hypocotyl grows straight and healthy, cotyledons have been completely opened, accompanied by healthy shoots (ISTA 2015). Germination percentage can be calculated using the formula:

$$GP = \frac{\sum KN I + \sum KN II}{\text{Number of planted seed}} \times 100\%$$

Germination rate (GR) was observed by calculating the number of normal seedlings that appear every day from the first day to the last seedling observation, which was 21 days after sowing. Germination rate (%/etmal) was calculated by the formula:

$$GR = \sum_{i=1}^{21} d$$

Where d was percentage addition of normal seedlings per etmal (1 etmal = 24 h).

The vigor index (VI) was observed by calculating the number of normal seedlings in the first count germination observation (14 days after sowing). The vigor index (%) was calculated by the formula:

$$VI = \frac{\sum KN I}{\text{Total planted seed}} \times 100\%$$

Maximum growth potential (MGP) of seeds was obtained by calculating the number of seeds that germinate with germination criteria which were reviewed from physiological aspects. The seed germinated even though the new embryo only rise the radicle. MGP was calculated on the final count of germination observation (21 days after sowing) by the formula:

$$MGP = \frac{\text{number of germinated seed}}{\text{total planted seed}} \times 100\%$$

Data analysis

The experimental design used in the experiment was factorial in completely randomized design with 3 papaya cultivars, i.e. Sukma, Callina, and Caliso. Each cultivar was tested separately. The first factor was loading treatment with two levels (with loading treatment and without loading treatment). The second factor was immersion time in PVS2 with three levels (15, 30, and 45 min). The number of treatments in the experiment was six treatments with three replicates so total experimental unit was 18 unit.

The seeds used in each experimental unit were 50 seeds. The data were analyzed with F-test and Duncan's Multiple Range Test (DMRT) using Statistical Analysis Software (SAS) version 9.1 with α 5%.

RESULTS AND DISCUSSION

Cryopreservation of papaya seeds cv. Sukma

Loading treatment and immersion time in PVS2 gave significant effect on germination percentage, germination rate, vigor index, and maximum growth potential, while their interaction only gave significant effect on vigor index (Table 1). Interaction of loading treatment and immersion time in PVS2 showed that vigor index was decreased while the immersion time in PVS2 was increased for seed without loading treatment. Whereas for seed with loading treatment, the vigor index was similar for all immersion time in PVS2. The best vigor index was 25.33% obtained at seeds without loading treatment and 15 min immersion in PVS2 (Table 2).

Germination percentage, germination rate, and maximum growth potential were significant at single factor (Figure 3). At loading treatment factor, seeds without loading treatment had the highest germination percentage (37.11%), germination rate (2.37%/etmal), and maximum growth potential (49.53%). At immersion time in PVS2 factor, seed with the highest germination percentage (30.33%), and maximum growth potential (41.33%) was seeds that 15 min immersed in PVS2 but did not significant with 30 min immersion. The highest germination rate was 2.17%/etmal, obtained by 15 min immersed in PVS2 (Table 2). Those results were better than Hervani et al. (2016) experiment results. Hervani et al. (2016) stated that papaya seeds cv. Sukma with 11-13% moisture content, removed sarcotesta and mesotesta, 30 min immersed in PVS2 before cryopreservation, had germination percentage, germination rate, and maximum growth potential 38.39%, 2.24 %/etmal, and 38.39%, respectively. Immersed in PVS2 for 15 min gave more maximum growth potential than immersed in PVS2 for 30 min. So, the best immersion time in PVS2 for papaya seeds cv. Sukma was 15 min. The optimal immersion time in PVS2 (15 min for papaya seeds cv. Sukma) could increase the solute concentration in the cell and protect the cell from crystal ice formation (Kaczmarczyk et al. 2012).

Table 1. The F-test results of germination percentage (GP), germination rate (GR), vigor index (VI), and maximum growth potential (MGP) on the treatment of loading (LS), immersion time in PVS2 and their interaction for papaya seeds cv. Sukma

Source of variation	P-value			
	GP	GR	VI	MGP
LS	0.00*	0.00*	0.00*	0.00*
PVS2	0.01*	0.00*	0.03*	0.00*
LS*PVS2	0.19 ^{ns}	0.40 ^{ns}	0.01*	0.09 ^{ns}

Note: LS = loading treatment, PVS2 = plant vitrification solution-2, * = treatment gave significant effect on observed variables ($\alpha=5\%$), ^{ns} = treatment gave non-significant effect on observed variables ($\alpha=5\%$).

Compared to initial viability, the seeds viability after cryopreservation was decreased. Seeds had initial germination percentage, germination rate, vigor index, and maximum growth potential were 81.11%, 2.48 %/etmal, 37.78%, and 85.56%, respectively (Table 2). The viability was decreased because every living cell that store in the liquid nitrogen would be damage and the result from this research showed that loading and immersion in PVS2 treatment could reduce it.

Data analysis stated that papaya seeds cv. Sukma had decreased germination percentage, germination rate, vigor index, and maximum growth potential while the immersion time in PVS2 increased (Table 2 and Figure 1). These showed that immersion in PVS2, initially, protected cell from damage during cryopreservation, but also caused the cell damage. Immersion in PVS2 for 15 min could protect the cell during cryopreservation because of vitrification (Gonzalez-Arnan et al. 2007). Vitrification is the freeze-avoidance mechanism that enables hydrated cells, tissues, and organs to withstand exposure to the temperature of liquid nitrogen. Vitrification defined as the transition of the liquid phase to an amorphous glassy solid at the glass transition temperature. The glass may contribute to preventing tissue collapse, solute concentration, and pH alterations during dehydration. Vitrification is achieved by direct immersion in liquid nitrogen of samples which have been dehydrated at a non-freezing temperature. The vitrified state is achieved in systems that become sufficiently concentrated after a drastic desiccation process and that are cooled sufficiently rapidly so that the increase in cellular viscosity inhibits molecular rearrangement of water into a crystalline pattern. As cooling progresses, the viscosity of intracellular solutes increases to the point where translational molecular motion is essentially halted and the solution becomes a glass. The resultant solid retains the random molecular arrangement of a liquid, but has the mechanical properties of a solid.

PVS2 could protect cell from damage because PVS2 dehydrated intracellular water and changed it with PVS2

solution. The intracellular solute would be solid and prevent crystal ice formation when stored in the liquid nitrogen. Hervani et al. (2016) stated that papaya seeds cv. Sukma cells that immerse in cryoprotectant had cell wall more tightly and the intracellular water was exchanged by the cryoprotectant (thick solution). The seeds cell wall after cryopreservation were more stretch and some were damaged. Vendrame et al. (2014) stated the similar report that cryoprotectant exchange the intracellular water of orchid seeds (orthodox seeds), so the membrane content is more solid and make amorphous or vitreous forming when storage in the liquid nitrogen. There was some membrane content that did not make amorphous form so the ice crystal forming and cell damage.

Immersion more than 15 min (overexposure) to PSV2 may cause damage to the cells owing to the toxic nature of the PVS2 or excessive dehydration (Kaczmarczyk et al. 2012). Vendrame et al. (2014) stated that cryoprotectant (PVS2) was toxic that caused osmotic stress, cell death, and cell morphogenetic. Some of papaya seeds cv. Sukma was death, and was expected caused by excessive dehydration so it could not germinate.

Table 2. Interaction of loading (LS) treatment and immersion time in PVS2 on vigor index (VI, %) of papaya seeds cv. Sukma after cryopreservation

Treatment	Immersion time in PVS2 (min)		
	15	30	45
Without loading treatment	25.33a	16.00b	6.67c
With loading treatment	5.33c	2.67c	1.33c

Note: The similar letter in the table indicates that the treatment gives a nonsignificant effect at DMRT test ($\alpha=5\%$). Papaya seeds cv. Sukma without loading and immersion in PVS2 treatment had germination percentage, germination rate, vigor index, and maximum growth potential 0%. The initial germination percentage, initial germination rate, initial vigor index, and initial growth maximum potential papaya seeds cv. Sukma were 81.11%, 2.48 %/etmal, 37.78%, and 85.56%, respectively.

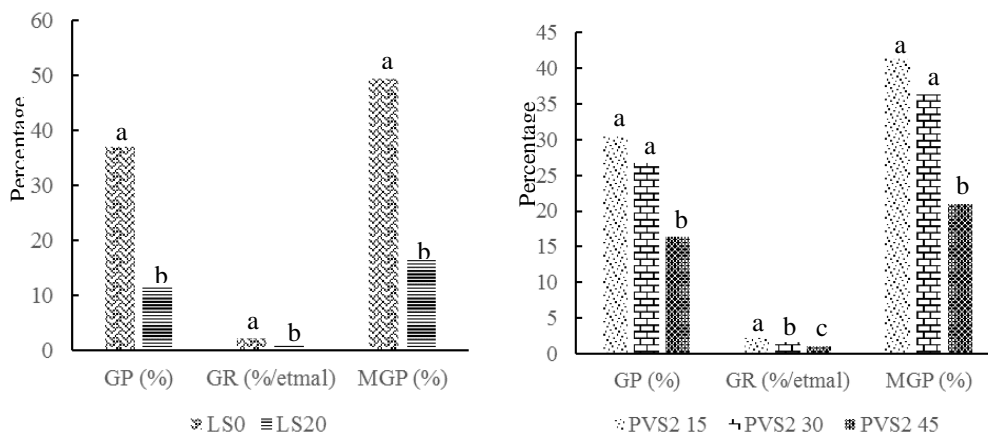


Figure 1. Effect of loading treatment (*left*) and immersion time in PVS2 (*right*) on germination percentage (GP), germination rate (GR), and maximum growth potential (MGP) papaya seed cv. Sukma after cryopreservation

Table 3. The F-test results of germination percentage (GP), germination rate (GR), vigor index (VI), and maximum growth potential (MGP) on the treatment of loading (LS), immersion time in PVS2 and their interaction for papaya seeds cv. Callina

Source of variation	P-value			
	GP	GR	VI	MGP
LS	0.24 ^{ns}	0.20 ^{ns}	0.40 ^{ns}	0.32 ^{ns}
PVS2	0.13 ^{ns}	0.13 ^{ns}	0.09 ^{ns}	0.16 ^{ns}
LS*PVS2	0.14 ^{ns}	0.12 ^{ns}	0.09 ^{ns}	0.11 ^{ns}

Note: LS = loading treatment, PVS2 = plant vitrification solution-2, * = treatment gave significant effect on observed variables ($\alpha=5\%$), ^{ns} = treatment gave non-significant effect on observed variables ($\alpha=5\%$)

Table 4. Germination percentage (GP), germination rate (GR), vigor index (VI), and maximum growth Potential (MGP) of papaya seeds cv. Callina after cryopreservation

Treatment	Observed variables			
	GP (%)	GR (%/etmal)	VI (%)	MGP (%)
Initial seeds viability	84.67	6.13	51.33	86.67
Loading treatment				
Without loading treatment	1.78a	0.15a	1.33a	3.11a
With loading treatment	2.89a	0.26a	2.00a	4.89a
Immersion time in PVS2				
15 min	3.67a	0.33a	3.00a	6.33a
30 min	2.00a	0.17a	1.00a	3.67a
45 min	1.33a	0.13a	1.00a	2.00a
Without loading and immersion in PVS2 treatment	0.00	0.00	0.00	0.00

Note: The similar letter in a similar column indicates that the treatment gives a nonsignificant effect at DMRT test ($\alpha 5\%$).

Papaya seeds cv. Sukma was orthodox seeds because still could germinate while stored in $-20\text{ }^{\circ}\text{C}$ for three months but did not germinate after one year (Wulandari 2007, Oktaviani 2012). So, seed cryopreservation with the optimal treatment could be expected to prolong the storage period of papaya seeds cv. Sukma. The optimum treatment for papaya seeds cv. Sukma was desiccation until 11-13%, removed sarcotesta and mesotesta, immersed in PVS2 for 15 min, direct immerse in liquid nitrogen, thawing at $40\text{ }^{\circ}\text{C}$ for 90-120 sec, immersed in liquid MS media for 30 min, and viability test. The other orthodox seeds could be cryopreserved with the other treatment. Chmielarz (2010) reported that desiccated seed of *Alnus glutinosa* L. (orthodox seeds) with 8-13% moisture control after stored in liquid nitrogen had similar viability with desiccated seed that store in $-3\text{ }^{\circ}\text{C}$. Galdiano et al. (2012) also reported that Dendrobium seeds that dehydrated with PVS2 and 1% phloroglucinol for 60 minutes at $0\text{ }^{\circ}\text{C}$ could store in the liquid nitrogen. These seeds had 79% germination percentage and could grow normally.

Cryopreservation of papaya seeds cv. Callina

At papaya seeds cv. Callina, loading treatment, immersion time in PVS2, and their interaction did not give significant effect on germination percentage, germination

rate, vigor index, and maximum growth potential after cryopreservation (Table 3). Data analysis showed that the viability of papaya seeds cv. Callina were low after cryopreservation even though the initial seeds viability was high (Table 4). These were caused by crystal ice formation in the seed cells while freezing and thawing (warming) procedures. Crystallization during freezing is a complex process that comprises a number of critical steps: nucleation, growth of crystals and/or recrystallization, which are considered the main elements affecting survival of cells subjected to cryopreservation (Gonzalez-Arno et al. 2007).

Gonzalez-Arno et al. (2007) stated that the formation of ice crystals detrimental to cellular integrity can also take place during warming procedure. A potential cause of injury is the use of a low warming rate to attain room temperature. Under slow warming conditions, there is a tendency for large crystals to grow at the expense of small ones, or for devitrification (ice crystal formation) to take place, when unstable glasses have been formed during cooling. Unstable glasses are obtained during rapid immersion in liquid nitrogen of little concentrated samples; such glasses are considered metastable, because devitrification can occur upon rewarming, returning to either a liquid or crystalline state (Gonzalez-Arno et al. 2007). So, devitrification could be prevented by fast thawing/warming at $40\text{ }^{\circ}\text{C}$ for 1-2 min.

According to Oktaviani (2012), papaya seeds cv. Callina were orthodox seed but for cryopreservation need different treatment from Sukma. Callina had low viability after cryopreservation with similar treatment with Sukma. So, to increase the viability, it should try another treatment. Chmielarz (2009) stated that the key problem in the cryopreservation of orthodox seeds was the identification of their optimum (safe) moisture content. Chmielarz (2009) suggested that the safe range of moisture content of orthodox seeds preserved in liquid nitrogen was 3.8–11%. It was consistent with Azimi et al. (2005) that decreased the seed moisture content until 10% and stored directly in the liquid nitrogen for papaya seeds cv. Queensland (orthodox seeds). The result showed that the seeds could germinate with 48% germination percentage. The similar treatment also used for papaya seeds cv. Formosa and Mamaohinzo by Obisesan et al. (2005). Obisesan et al. (2005) decreased the seed moisture content until 6.5-7% and stored directly in the liquid nitrogen. The seeds could germinate with 50-56% germination percentage. According to all literature, papaya seeds var. Callina could be cryopreserved only with desiccation. The moisture content could decrease until 6.5-10% before store directly in the liquid nitrogen.

Cryopreservation of papaya seeds cv. Caliso

Loading treatment and immersion time in PVS2 gave significant effect on germination percentage, germination rate, vigor index, and maximum growth potential, while the interaction gave significant effect on germination percentage, germination rate, and maximum growth potential (Table 5). At seed without loading treatment,

germination percentage, germination rate, and growth maximum potential were similar on the all immersion time in PVS2. At the seed with loading treatment, the highest germination percentage (60.00%), germination rate (4.35%), and maximum growth potential (64.17%) were on 30 min immersed in PVS2, and decreased on more or less than 30 min (Table 6). Vigor index only significant on single factor. The best vigor index was obtained on seed with loading treatment. Immersion time that gave the best vigor index was 30 min but did not significant with 15 min (Figure 2).

Seed without loading and immersion in PVS2 treatment did not germinate after cryopreservation, because the seed cells did not protect by cryoprotectant. Compared to the initial viability, the viability after cryopreservation was decreased. The initial germination percentage, germination rate, vigor index, and growth maximum potential were 92.18%, 5.04 %/etmal, 25.67%, dan 96.80%, respectively (Table 6).

So, the papaya seeds cv. Caliso could be cryopreserved with some treatment, i.e. desiccation until 11-13%, removed sarcotesta and mesotesta, loading treatment in the loading solution (liquid MS + 2 M glycerol + 0.4 M sucrose) for 20 min, immersed in PVS2 for 30 min, direct immerse in liquid nitrogen, thawing at 40 °C for 90-120 sec, immersed in liquid MS media for 30 min, and viability test. These result consistent with Ashmore et al. (2009) that papaya seeds cv. Solo and Queensland could be cryopreserved with decreased moisture content to 5% and gave pre-culture in medium with 2 mM GA₃ for 15 min. This treatment made papaya seeds cv. Solo and Queensland could germinate more than 80%. It showed that some papaya seeds from another cultivar need to treat more before cryopreservation (need different treatment from Sukma and Callina).

Table 5. The F-test results of germination percentage (GP), germination rate (GR), vigor index (VI), and maximum growth potential (MGP) on the treatment of loading (LS), immersion time in PVS2 and their interaction for papaya seeds cv. Callina

Source of variation	P-value			
	GP	GR	VI	MGP
LS	0.00*	0.00*	0.02*	0.00*
PVS2	0.00*	0.00*	0.03*	0.01*
LS*PVS2	0.04*	0.03*	0.07 ^{ns}	0.04*

Note: LS = loading treatment, PVS2 = plant vitrification solution-2, * = treatment gave significant effect on observed variables ($\alpha=5\%$), ^{ns} = treatment gave non-significant effect on observed variables ($\alpha=5\%$)

Table 6. Interaction of loading (LS) treatment and immersion time in PVS2 on germination percentage (GP), germination rate (GR), and maximum growth potential (MGP) of Caliso papaya seeds after cryopreservation

Treatment	Immersion time in PVS2 (min)		
	15	30	45
Germination percentage (%)			
Without loading treatment	12.50c	30.00bc	27.50bc
With loading treatment	40.00b	60.00a	30.00bc
Germination rate (%/etmal)			
Without loading treatment	0.67c	2.23b	1.73bc
With loading treatment	2.77b	4.35a	1.93b
Maximum growth potential (%)			
Without loading treatment	16.67b	34.17b	33.33b
With loading treatment	46.67b	64.17a	37.50b

Note: The similar letter in a similar variable indicates that the treatment gives a nonsignificant effect at DMRT test ($\alpha=5\%$). Papaya seeds cv. Caliso without loading and immersion in PVS2 treatment had germination percentage, germination rate, vigor index, and maximum growth potential 0%. The initial germination percentage, initial germination rate, initial vigor index, and initial growth maximum potential papaya seeds cv. Sukma were 92.18%, 5.04 %/etmal, 25.67%, dan 96.80%, respectively.

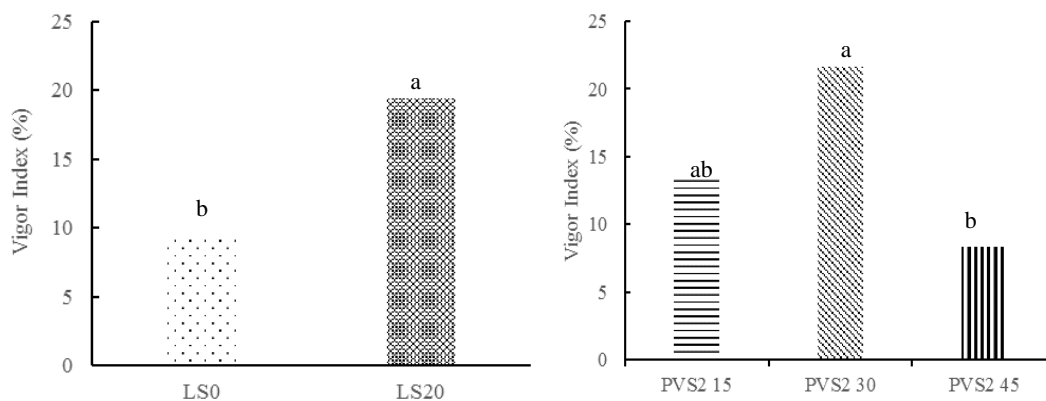


Figure 2. Effect of loading treatment (*left*) and immersion time in PVS2 (*right*) on vigor index of Caliso papaya seed after cryopreservation

Papaya seeds var. Caliso needs loading treatment in order to the cells could adapt well while stored in the liquid nitrogen. Loading solution could increase the dehydration tolerance on sensitive cryoprotectant plants (intolerance to dehydration and osmosis stress) (Vendrame et al. 2014). Loading treatment increased the membrane cell permeability in order to cryoprotectant could enter the membrane cell (Vendrame et al. 2014). The increasing permeability also made dehydration happened more slowly so prevent irreversible plasmolysis (Roostika et al 2007). The permeability of membrane cell increased with the increasing of osmosis concentration in the cell. Furthermore, compared to Sukma, Caliso needs more immersion time in PVS2, i.e., 30 min, because loading treatment made the permeability of membrane increased, so the vitrification required more time (dehydration more slowly).

The seed characteristic of papaya seeds cv. Caliso did not know yet. But, according to t-test ($\alpha=5\%$) that compared the viability after cryopreservation between Caliso, Sukma, and Callina, papaya seeds cv. Caliso had a similar characteristic with papaya seeds cv. Sukma ($p>5\%$). Nevertheless, papaya seeds cv. Caliso needed different treatment to get good viability after cryopreservation. Papaya seeds cv. Sukma did not need loading treatment but Caliso needed it, and the immersion time in PVS2 was 15 min for Sukma and 30 min for Caliso.

According to Elliot et al. (2017), plant material could survive after cryopreservation because of the optimal cellular dehydration and the intracellular ice formation (IIF) level. The optimal cellular dehydration (in the presence of cryoprotectant/PVS2), which could be achieved by allowing regulated extracellular ice crystal growth during controlled cooling. The bound water compartment is associated with structural organization of membranes and organelles, and polymeric components such as proteins. As dehydrative freezing progresses to impinge on restricted water, cell structures can become unstable leading to irreversible injury under some slow cooling conditions. The second factor observed at higher cooling rates was related to intracellular ice formation (IIF), with the incidence of injury due to IIF increasing with increasing cooling rate. The balance between these two factors results in a maximal survival for a particular cell type across a limited cooling rate profile.

From the experiment, we concluded that papaya seeds cv. Sukma, Callina, and Caliso had different responses when stored by cryopreservation. For papaya seeds cv. Sukma, seeds without loading treatment and 15 min immersion in PVS2 were the best treatment that obtained the best viability. For papaya seeds cv. Callina, we should to try another treatment to get high viability after cryopreservation. For papaya seeds cv. Caliso, seeds with loading treatment and 30 min immersion in PVS2 were the best treatment that obtained the best viability.

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