Jurnal Teknologi

Full Paper

SUCCESSFUL PRESERVATION OF BANANA EMBRYOGENIC CALLUS BY MINIMAL GROWTH AND CRYOPRESERVATION TECHNIQUE

Ika Roostika[a,d*], Fitri Damayanti[b], Witjaksono[c]

[a]Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture, Bogor 16111, Indonesia
[b]Universitas Indraprasta PGRI, Faculty of Math and Science,13760, Jakarta, Indonesia
[c]Research Center for Genetic Engineering, Research Organization for Life Sciences and Environment, National Research and Innovation Agency, Jl. Raya-Jakarta Km 46, Cibinong, Bogor 16911, Indonesia
[d]Research Center for Horticultural and Estate Crops, Research Organization for Agriculture and Food, National Research and Innovation Agency Jl. Raya-Jakarta Km 46, Cibinong, Bogor 16911, Indonesia

Article history
Received
12 February 2021
Received in revised form
1 July 2022
Accepted
4 July 2022
Published Online
21 August 2022

*Corresponding author
ikar004@brin.go.id

Graphical abstract

Abstract

This paper reports successful preservation methods by minimal growth and cryopreservation of banana embryogenic callus. The minimal growth and cryopreservation techniques can be applied for medium-term and long-term preservation. The embryogenic callus of banana cv Dwarf Parfitt was used as the plant material. Different kind of medium formulations, temperature, gelling agents, and ABA concentrations were tested in minimal growth study. The type of explants, dehydration periods, and preculture media were tested in cryopreservation study. The result showed that the use of WIC medium was better than BM2G for maintaining embryogenic callus. The application of 0.005 mg L^{-1} ABA combined with reduced temperature (15^{0}C) could preserve the cultures longer than 4 months with high survival rate (95%), high recovery rate (90%), and high number of recovered embryos indicating high embryogenic potential. For cryopreservation study, the best dehydration period was 20 minutes. The combined treatments of 150 g/l sucrose and 2 M glycerol at preculture step was the most suitable treatment for successful cryopreservation with 80% survival rates; and the recovered cultures maintained their somatic embryogenic capacity.

Keywords: Banana embryogenic callus, reduced temperature, ABA, vitrification, cryopreservation

1.0 INTRODUCTION

Banana is one of the most important commercial fruit in tropical countries such as Indonesia. To improve varieties, genetic diversity needs to be conserved. As cultivated bananas are vegetatively propagated and most of them are seedless, it is not an option to conserve them by using seeds. Field maintenance of germplasm is labor intensive and needs large area, high cost, yet it still faces many risks such as pest and disease. Thus, *in vitro* conservation is a valuable and potential alternative technique for preserving banana plant germplasm material.

There are three kinds of *in vitro* preservation methods: (1) preservation in growth media for short-term storage, (2) preservation by minimal growth technique for medium-term storage, and (3) cryopreservation for long-term storage [1]. Minimal growth preservation can be obtained by reducing temperature and light intensity [2, 3, 4], using osmotic regulator such as sucrose, sorbitol and mannitol at elevated concentration [3, 5], lessening essential factor such as medium strength [6], and using plant growth retardants such as paclobutrazol, cycocel, or ancymidol [3]. Cryopreservation is a potential method suitable for long-term conservation of plant genetic resources [5, 7] since they are stored in liquid nitrogen at -196 °C. By this method, the plant materials are brought to zero metabolisms and consequently the plant materials can be stored for a long period of time [8, 9]. By applying this technique, many samples (thousands) can be stored in one tank and they need not be sub-cultured intensively, and therefore it may reduce contamination risk, cost for labors, and time requires for maintenance. Moreover, the genetic stability of cryopreserved materials is guaranteed. Both minimal growth and cryopreservation have been applied routinely for preservation of banana shoots at International Transit Center (ITC) Belgium, Germany, Colombia, and Peru [10, 11].

Embryogenic callus of banana is usually maintained *in vitro* on solid medium and as suspension culture. Induction and maintainance of banana embryogenic cell suspension are laborious and time-consuming due to frequent subculture. Strosse *et al*. (2006) reported that it need about 22 months for obtaining ideal embryogenic callus and 34 months for obtaining embryogenic cell suspension [12]. Frequent subculture may also increase the frequency of somaclonal variation and eventually the complete loss of morphogenic potential. Thus, it is necessary to develop a simple method which can facilitate maintenance of the embryogenic callus under minimal growth condition and cryopreservation. Minimal growth condition is desirable for maintaining the cell suspensions for routine work, from which actively growing cultures can be retrieved readlily [13].

There is a report on maintenance of banana embryogenic cell by minimal growth method through temperature modification. The banana embryogenic callus can be maintained without sub-culturing at 8 °C for over 4 months [13]. The application of this low temperature has a consequent of high cost of electricity utilization. The use of plant growth inhibitor may be applicable and less expensive.

Several protocols have been published on cryopreservation of banana and plantain [14]. The study of cryopreservation of banana embryogenic cell suspension through slow freezing method has been reported. However, slow freezing required a programmable and expensive slow freezing equipment, high electricity cost and is a slow process. Meanwhile, there is an alternative tecnique, called vitrification which does not require that programable freezing equipment, and yet the dehydration process is quick [15, 16]. The efficacy of vitrification method for preservation of bananas has not been reported. Thus it is important to study and to establish a cryopreservation technique applicable to Indonesian banana germplasms in gene bank for supporting banana breeding program.

This paper reports on: (1) minimal growth technique of banana embryogenic callus for maintenance and medium-term storage without frequent subculture using abscisic acid (ABA) combined with low temperature, and (2) cryopreservation method of banana embryogenic callus for long-term storage by vitrification technique.

2.0 METHODOLOGY

The plant material used in this research was embryogenic callus of banana cultivar Dwarf Parfitt prepared at Research Center for Biology, The Indonesian Institute of Sciences. Two different preservation methods were tested in this study, i.e., minimal growth for medium-term preservation and cryopreservation for long-term preservation. The experiments were conducted at Tissue Culture Laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Agency for Agricultural Research and Development, Ministry of Agriculture, Indonesia.

Minimal Growth Preservation of Banana Embryogenic Callus

1. Preservation of Banana Embryogenic Callus in Liquid Media

Two different maintenance media were used in this experiment. The first medium was BM2G medium [13], consisted of MS salts (Murashige and Skoog, 1962) supplemented with (in mg L^{-1}): 2,4-D, 1; Biotin, 1; malt extract, 100; glutamin, 100; sugar, 45,000 [17]. Medium pH was adjusted to 5.7-5.8 prior to autoclaving. The second medium was WIC medium, developed by Witjaksono [personal communication]. This medium consisted of MS salts (Murashige and Skoog, 1962) supplemented with (in mg L^{-1}): myo-inositol, 100; pyridoxine HCL, 2; thiamine HCL, 4; nicotinic acid, 2;

Ca-pantothenat, 1; cysteine, 40; biotin, 1; glycine, 2; 2,4-D, 1; PVP (polyvinylpyrrolidone), 500; and sucrose, 30.000 [17]. Medium pH was adjusted to 5.7- 5.8 prior to autoclaving.

As much as 0.5 g embryogenic callus was used as the inoculum. Embryogenic callus was inoculated into 50 mL treatment media in 250 mL Erlenmeyer flasks. The treatment media consisted of WIC medium supplemented with ABA at concentration of 0, 0.001, 0.0025, and 0.005 mg L^{-1}. The cultures were maintained at 25°C on a rotary shaker with a rotating speed of 70 rpm, under dark condition. There were 2 replications for each treatment. At the end of preservation period, the cells were plated on WIC medium to recover embryogenic cell suspension and on REG medium to regenerate somatic embryos. The REG medium consisted of MS salts (Murashige and Skoog, 1962), vitamin, and (in mg L^{-1}, unless stated otherwise): myo-Inositol, 100; BA, 2; 20% liquid coconut endosperm; biotin, 2; sucrose, 30 [17]. Medium pH was adjusted to 5.7-5.8 before autoclaving. The percentage of browning, final fresh weight, percentage of recovered embryogenic cell suspension, and number of developed somatic embryos were observed. The percentage of browning was calculated by the number of embryogenic callus divided by the total number of embryogenic callus observed multiplied by 100%. The fresh weight of embryonic cell suspension was determined by weighing the settled cells in a sterile filter paper 15 minutes after double filtration with metal sieves of 100 µm and 62 µm pore size. The number of developed somatic embryos was determined by counting the number of white opaque somatic embryo per flask.

2. Preservation of Banana Embryogenic Callus on Semisolid Media

The effect of ABA preservation of embryogenic callus was also experimented on semi solid medium formulation of BM2G and WIC which were gelled with 2 kinds of gelling agents, i.e, 0.7 g L^{-1} agarose and 6 g L^{-1} agar. The medium was dispensed in glass jars. Unless stated otherwise, the inoculum for the experiments was masses of embryogenic callus with height of approximately 3 mm covering a circle label of 1 cm diameter (about 0.785 cm^2). The embryogenic callus cultures that were plated on the preservation media were incubated at temperature of 15 and 25 °C under dark condition for 4 months. There were two replications for each treatment.

After 4 months, the preserved embryogenic callus was replated on WIC and REG media devoid of ABA, both gelled with 6 g L^{-1} agar and maintained at temperature of 25 °C to assess its growth response. The responses observed included percentage of embryogenic callus survival, and recovery, and number of developed somatic embryos.

Cryopreservation of Banana Embyogenic Callus

Cryopreservation through vitrification method consists of stages. Stages of cryopreservation carried out in this work were [16]: preculture, *loading*, dehydration with cryoprotectant, freezing in liquid nitrogen, thawing, *unloading*, culture recovery, and regeneration.

There were two experiments in this cryopreservation study. The first experiment was intended to determine the optimal dehydration period. Dehydration is the most critical point during cryopreservation so that we optimized this step before doing all steps of cryopreservation work. The second experiment was aimed at studying the effect of preculture treatment to the survival and regeneration rate of cryopreserved embryogenic callus.

On the dehydration experiment, embryogenic callus was used as the inoculum. For dehydration treatments, embryogenic callus was subjected to cryoprotectant for 0, 5, 10, 20, and 40 minutes. The cryoprotectant was *Plant Vitrification Solution 2* (PVS2) (Sakai, 1990; Sakai *et al*., 2008) which consists of 30% glycerol, 15% ethylene glycol, 15% dimethylsufoxide, and 0.4 M (136.9 g L^{-1}) sucrose [18, 19] that were mixed in WIC liquid medium. After dehydration treatments, the embryogenic callus was unloaded with rinsing solution (RS) for 15 minutes to remove PVS2 solution that may be toxic to the embryogenic callus. The RS solution was composed of WIC medium containing 1.2 M (41 g L^{-1}) sucrose. The unloaded embryogenic callus was then dried up on filter paper and then plated on WIC semisolid medium and was incubated in the dark room with temperature of 20 °C for 2 weeks, for recovery. As a negative control, embryogenic callus pieces were not washed with RS solution before plating on WIC medium. To induce development of somatic embryos, the recovered embryogenic callus was subcultured on REG medium and incubated under 1000 lux light intensity with 16 hours photo period. The variables observed were the percentage of recovery and the total number of regenerated embryos. The recovered embryogenic callus was marked as organized round structure with yellowish color.

On the preculture experiment, two kinds of inocula were used; the early stage which contained small granular structures of yellowish color, and the late stage of embryogenic callus, which contained few greenish somatic embryos. The inocula were precultured for one day on WIC solid media containing sucrose at various concentrations as the treatments, i.e., 120, 150, 180, and 210 g L^{-1} with and without the addition of 2 M glycerol.

After preculture treatment, the embryogenic callus was put into 1.8 mL cryotube and then subjected to three different stages of cryopreservation to evaluate the efficacy of the treatment: 1) the embryogenic callus was plated directly to WIC medium for recovery (preculture); 2) the embryogenic callus was subjected to fresh cold PVS2 cryoprotectant to dehydrate them for 20 minutes, then plated them onto WIC medium for recovery (dehydration); 3) the embryogenic callus was subjected to fresh cold PVS2 cryoprotectant to dehydrate them for 20 minutes, then together with its cryotube containers were plunged directly to a Dewar flask containing of liquid nitrogen (-196 °C) (freezing in

liquid N2. After storage overnight, the embryogenic callus was rapidly thawed by stirring the cryotubes in warm water of 40 °C for about 1 minute. Directly after thawing, the PVS2 solution was removed from the cryotubes and replaced with the RS solution as an unloading step for 15 minutes at room temperature. The thawed embryogenic callus was taken from the RS solution, dried on filter paper and then plated on semisolid WIC media in 90 mm Petri dishes for regrowth for 2 weeks and placed in the dark. The recovered embryogenic callus was then sub-cultured to WIC and REG regeneration media to develop somatic embryos. They were incubated under 1000 lux light intensity with 16 hours photo period. The survival percentage of embryogenic callus and the total number of developed somatic embryos were observed. The regrowth embryogenic callus was indicated by the appearance embryogenic callus new growth or greenish somatic embryos.

3.0 RESULTS AND DISCUSSION

Minimal Growth Preservation of Banana Embryogenic Callus

1. Preservation of Banana Embryogenic Callus in Liquid Media

Embryogenic callus cultured in BM2G liquid media containing various concentrations of ABA resulted in complete loss of survival capacity since the embryogenic callus turned brown after 2 weeks preservation period (Figure 1A, Figure 2A-D). This indicated that the media were not suitable for preservation. Browning is an indicator of oxidative reaction occurred in stressed cells. Stressed cells are known to release quinone which might be inhibitory and resulted in growth cessation and death of the embryogenic callus. A better response was obtained when the WIC liquid media was used for maintaining embryogenic callus. Mostly, embryogenic cell suspension could proliferate and survive until 4 months preservation period (Figure 1B, Figure 2E-H). The inclusion of PVP in the WIC medium may contribute to the prevention browning. Cai *et al*. (2020) found that addition of 0.5 gL^{-1} reduced browning to 95% in tissue cultures of herbaceous peony 'Festival Maxima' [20].

Treatment of ABA in the liquid culture medium did not improve in regard to either browning or fresh weight for both medium formulations. This result indicated that ABA was not necessary for preserving banana embryogenic cell suspension. Unfortunately, the survived embryogenic callus in liquid WIC medium failed to be recovered and regenerated on WIC and REG respectively. Therefore, we used semisolid media for the following experiments to improve the success of minimal growth preservation of banana embryogenic callus.

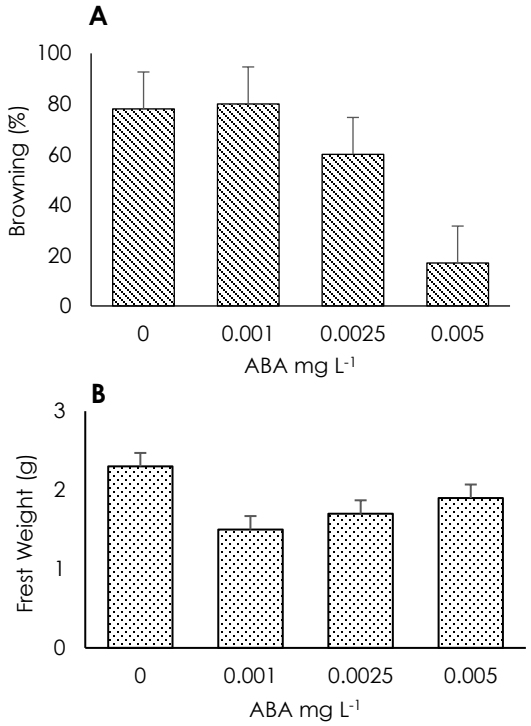

Figure 1 Preservation of banana embryogenic callus cv Dwarf Parfitt in liquid media with addition of various concentration of ABA. (A) the embryogenic callus underwent severe browning in BM2G media with various ABA concentrations; (B) the embryogenic callus proliferated and the fresh weight gain maintained in WIC medium after 4 months preservation period

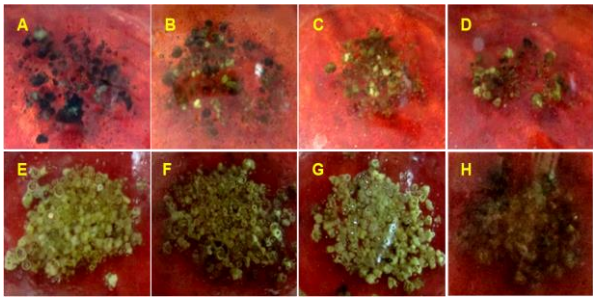

Figure 2 The growth of banana embryogenic callus cv Dwarf Parfitt in BM2G liquid media after 2 weeks (upper) and in WIC liquid media after 4 months preservation period (lower) with different concentration of ABA (mg L^{-1}): 0 (A, E), 0.001 (B, F), 0.0025 (C, G), and 0.005 (D, H)

2. Preservation of Banana Embryogenic Callus in Semisolid Media

Upon transfer to BM2G preservation media, the embryogenic callus turned brown. The incident of browning tent to be higher with the use of agarose than the use of agar, however, not significantly different with different in temperature, whether 15 or 25 °C. Browning of embryogenic callus tent not to be affected by increasing concentration of ABA in the

medium except when the it was cultured in Agar (Figure 3A).

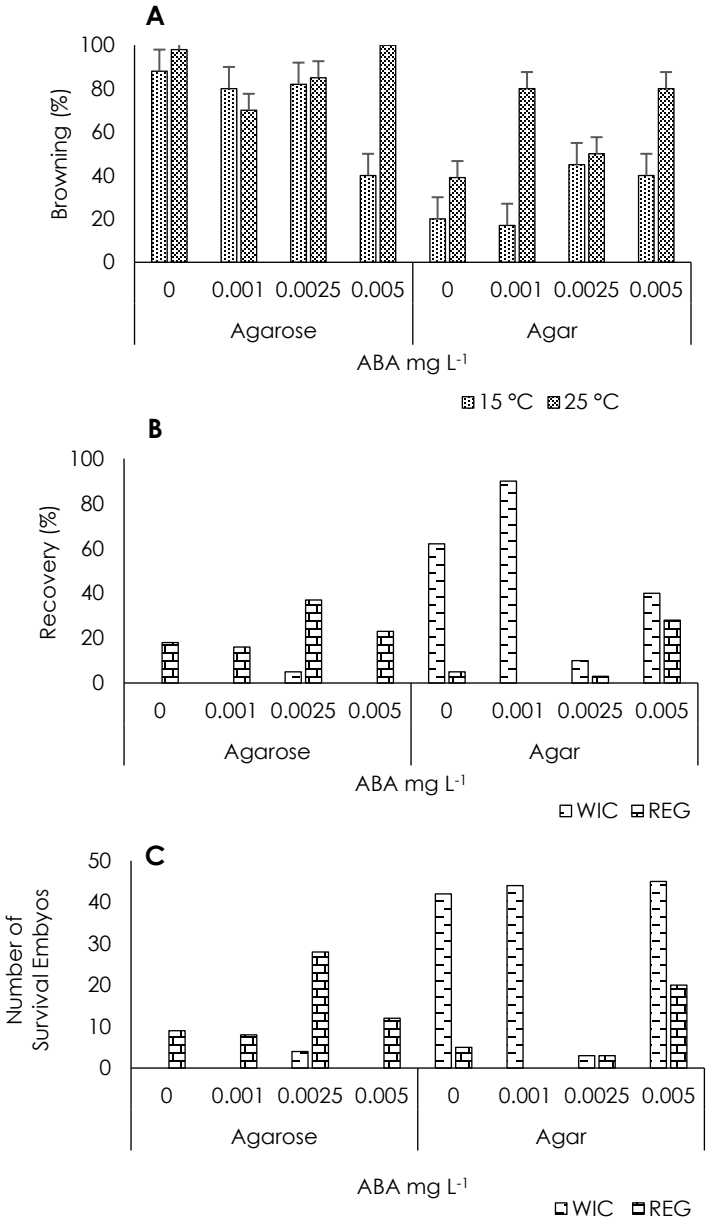

Figure 3 Preservation of banana embryogenic callus cv Dwarf Parfitt on BM2G semisolid media and subsequent growth and development. (A) the effect of ABA and temperature to the browning level; (B) the recovery rate after 4 months preservation; (C) the number of embryo developed after 5 weeks inoculated on recovery (WIC) and regeneration media (REG)

Regardless of the type of gelling agent used, gel strength increased with gelling agent concentration and was critical to the maturation response. High gel strength was associated with reduced water availability from the medium to the cultures [21]. The recovery of embryogenic callus after preservation treatment of this medium formulation were significantly affected by formulation of recovery medium, gelling agent and treatment of ABA, and the best recovery of embryogenic callus up to 80% was reached at ABA concentration 0,001 mg L^{-1}, on WIC medium and gelled with agar (Figure 3B). Similar response was observed for somatic embryo development where WIC medium gelled with agar were superior than REG medium and agarose, but with no clear pattern on the effect of ABA (Figure 3C). Similarly, when the embryogenic callus was preserved in the WIC semi solid medium, agar was superior as gelling agent than the agarose, while increasing concentration of ABA tent to decrease the incident of browning (Figure 4A). The recovery of embryogenic callus was also significantly higher on WIC medium gelled with agar, but with addition of ABA at concentration of 0.0025-0.005 mg L^{-1}. In this media, somatic embryo development was also the best (Figure 4C, Figure 5).

Apparently WIC based medium was more suitable than REG media for recovering embryogenic callus after preservation. The highest recovery rate (about 90% survival and more than 40 developed somatic embryos) was obtained from WIC medium supplemented with 0.001 mg L^{-1} ABA. This WIC medium (with addition of ABA) could preserve embryogenic callus longer than 4 months. Beside of the longer preservation period, the medium could increase survival rate of preserved cultures and subsequent recovery rate. The best treatment was 0.005 mg L^{-1} ABA, which resulted in more than 90% survival, 95% recovery, and 45 developed somatic embryos. Kulkarni and Ganapathi (2009) reported that banana embryogenic callus could be maintained over 4 months by using low temperature at 8 °C [13]. As illustrated in Figure 4 and 5, the embryogenic callus from that treatment could proliferate as friable callus and regenerate into somatic embryos. According to Hoekstra *et al.* [19], ABA caused somatic embryos to suspend the growth, exhibit low rates of respiration, and maintain elevated sucrose content. It means that by reducing cellular metabolism, ABA may maintain high sugar content which contributes to the stability of membrane, protein, and cytoplasmic glassy matrix during preservation. Generally somatic embryos cannot be conserved for long periods due to precocious germination, even at low temperature [23]. However, the application of ABA can artificially induce the quiescent state in somatic embryos, similar to the dormancy state in zygotic embryos, and promote short- to medium-term conservation [24]. Rai *et al.* [25] stated that ABA is often used in tissue culture systems to promote somatic embryogenesis and enhance somatic embryo quality by increasing desiccation tolerance and preventing precocious germination.

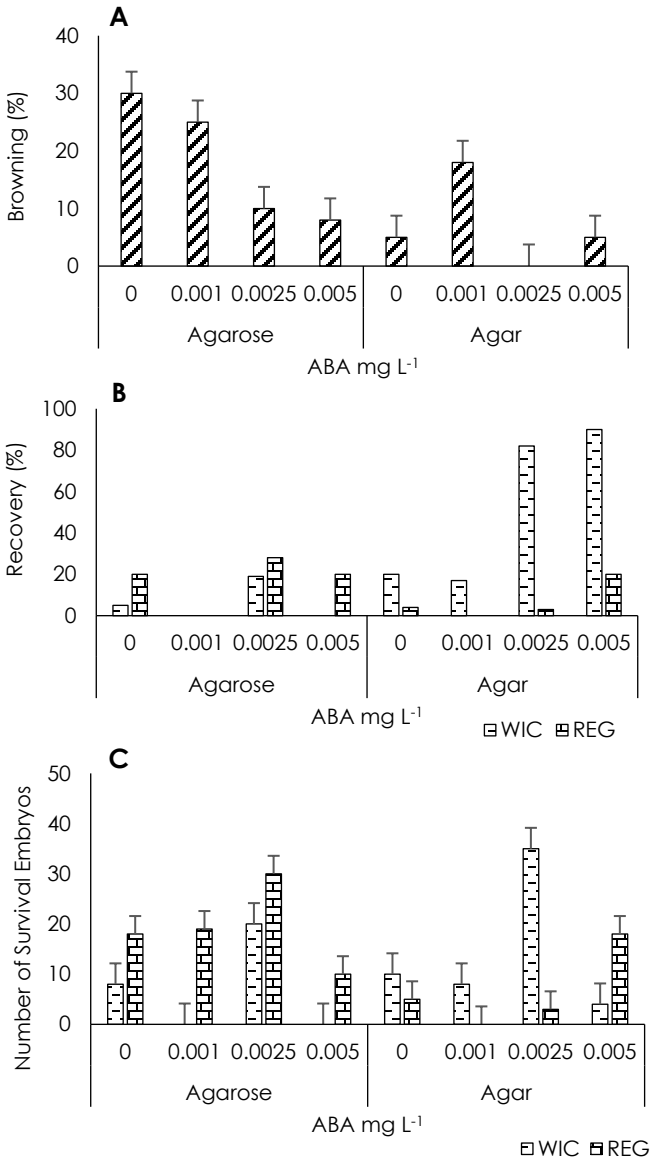

Figure 4 Preservation of banana embryogenic callus cv Dwarf Parfitt on WIC semisolid media and subsequent growth and development. (A) the effect of ABA and gelling agent to the browning level; (B) the recovery rate after 4 months preservation; (C) the number of embryo developed after 5 weeks inoculated on recovery (WIC) and regeneration media (REG)

After preservation, there was no phenotypical different between the plantlets derived from the control and ABA treated embryogenic callus (Figure 6). It indicates ABA does not cause any change in the morphological character of somatic embryos and their subsequent developments, i.e., shoot development/plant conversion.

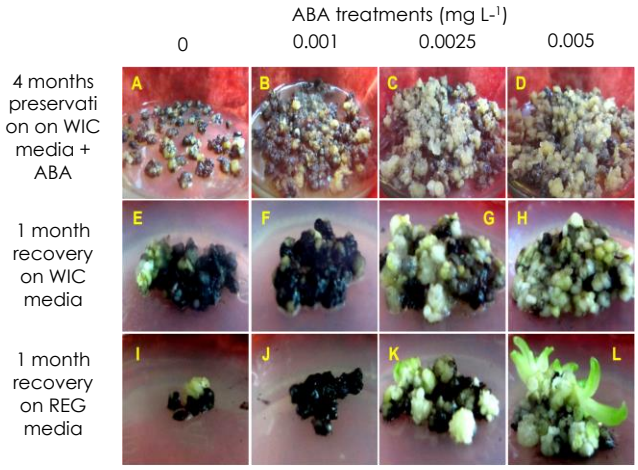

Figure 5 Growth and development of banana embryogenic callus cv Dwarf Parfitt on WIC semisolid agar preservation media after 4 months at 15 ◦C (A-D), and subsequent growth and development after recovery on WIC medium (E-H) and on REG medium after 1 month (I-L)

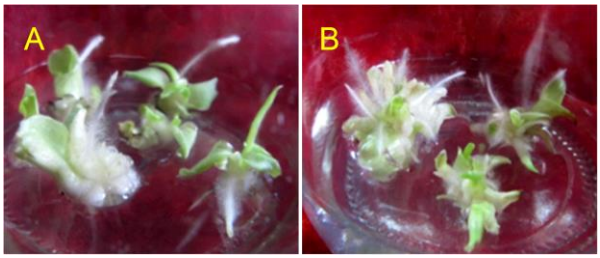

Figure 6 Plantlet development from banana embryogenic callus cv. Dwarf Parfitt on REG regeneration medium (2 months) after preservation by minimal growth technique, in which the preservation medium containing ABA. (A) the medium without ABA (as the control); (B) the medium had ABA

Cryopreservation

1. The Optimization of Dehydration

Cryopreservation of banana embryogenic callus by using slow freezing has been reported by Panis (2009) [23]. However, vitrification technique is the method of choice since it is simpler and applicable. Vitrification-based protocols involve some degree of dehydration before freezing by exposure of samples to highly concentrated solution of cryoprotectant to remove most of freezable water [15]. Dehydration is the most critical point during cryopreservation, therefore it should be optimized before proceeding to all steps of cryopreservation work [16]. Our results showed that dehydration period from 0-40 minutes in dehydration solution PSV2 did not significantly affected the recovery of embryogenic callus after 2 month, but the its recovery after 1 month decreased significantly at 40 minutes (Figure 7).

The rinsing of PSV2 during unloading step with RS solution inhibited the recovery of embryogenic callus after 1 month, but the recovery increased significantly

after 2 months. The process of recovery probably takes some time before the embryogenic cultures stably regrew. The development of somatic embryos from the dehydrated-recovered embryogenic callus were also significantly affected by period of dehydration, but in any specific pattern. Nonetheless, dehydration period of 20 minutes resulted in the highest number of somatic embryo development (Figure 7). Some of the developed embryos were able to convert to shoots, regardless of the previous dehydration treatment (Figure 8).

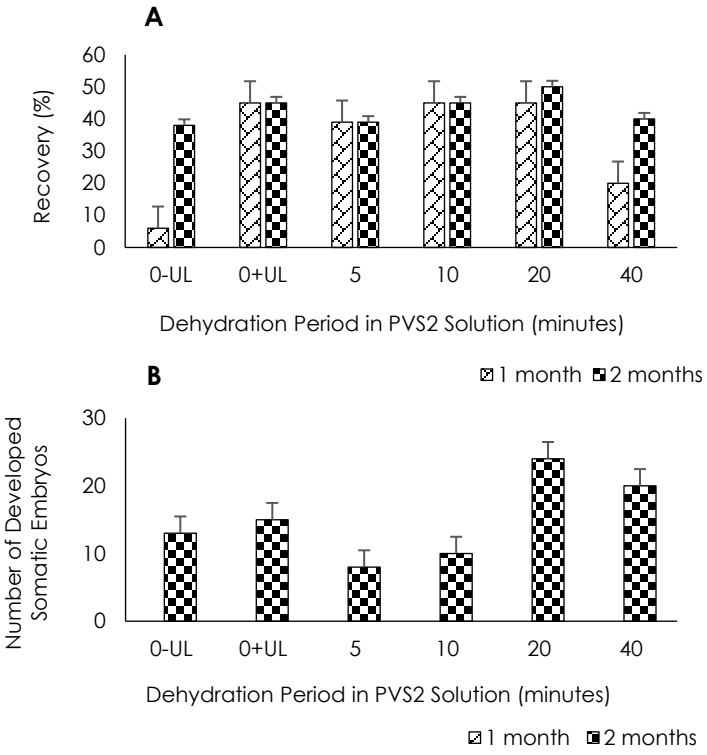

Figure 7 The effect of length of dehydration treatment with PVS2 solution to the growth of banana embryogenic callus cv Dwarf Parfitt planted on REG regeneration medium. (A) percent recovery of embryogenic callus; (B) number of somatic embryo developed. 0-UL = the embryogenic callus was not rinsed with RS solution before plating in REG medium. 0+UL = embryogenic callus was rinsed with RS solution before plating the same as with the treatment of 5-40 minutes dehydration

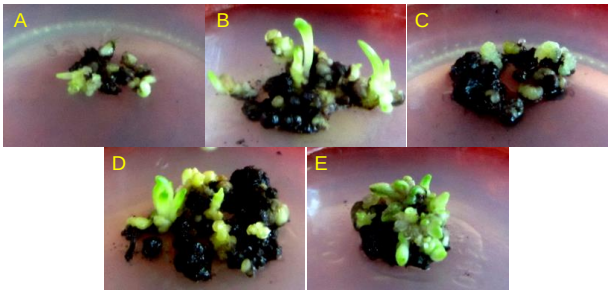

Figure 8 Shoot development from somatic embryos on REG regeneration media 1 month after recovery of embryogenic callus from dehydration treatment with PVS2 solution: for 0 minute (A), 5 minutes (B), 10 minutes (C), 20 minutes (D), and 40 minutes (E)

2. The Optimization of Preculture

Preculture treatments were evaluated by the embryogeic callus recovery and subsequent somatic embryo development as a measure of embryogenic potential. Generally, after preculture treatment prior to dehydration and freezing step, the growth of the embryogenic cultures fell into tree different pattern, i.e., whitish, yellowish, and blackish granular structures during the early of observation (1−2 weeks). The white color indicated cell death after dehydration treatment, while yellowish color indicated the new growth of embryogenic callus. According to Panis (2009), there were four type of explant reactions to freezing, i.e., one of them was white structure resulting from an immediate death of the tissue without blackening. As shown at Figure 9 (C-D), there was not any regeneration from the whitish embryogenic callus after freezing and recovery step during cryopreservation. Some of the embryogenic cultures turned black after dehydration with PVS2 solution and freezing with LN, but new growth as spots of granular and semi compact structures and proliferating callus developed (Table 1. Figure 9). The embryogenic callus could develop into somatic embryos after sub-culturing onto REG media (Figure 10).

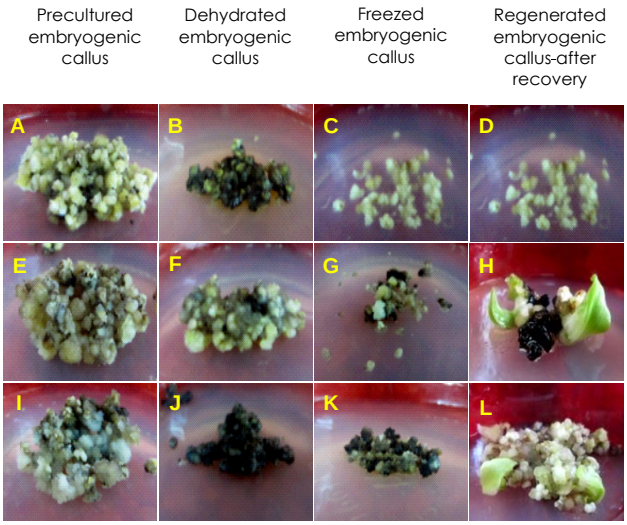

Figure 9 Recovery of banana embryogenic callus cv Dwarf Parfitt on WIC medium (1 month) after preculture only (A, E, I), preculture and dehydration (B, F, J), and preculture, dehydration followed by freezing (C, G, K), and subsequent development somatic embryos and plantlets on REG medium (D, H, L). A−D: The early stage of embryogenic callus was treated with 150 g/l sucrose, E−H: the late stage embryogenic callus was treated with 150 g/l sucrose and I−L: with 150 g/l sucrose and 2 M glycerol

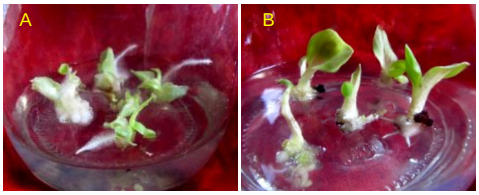

Figure 10 Morphology of the regenerated plantlets of banana cv. Dwarf Parfitt on REG regeneration medium (2 months) before (A) and after (B) cryopreservation of embryogenic callus by vitrification technique

As shown in Table 1, the late stage embryogenic callus yielded more friable and proliferable embryogenic callus after dehydration and freezing than that of the early stage of embryogenic callus. As water content in the late stage embryogenic callus might be lower than that in the early stage embryogenic callus, the former might be more resistant to dehydration during cryopreservation when exposed to high osmoticum and super-low temperature in liquid nitrogen. Ishikawa *et al.* (2006) reported that the late exponential phase or early stationary phase proved best for survival after cryopreservation [27]. This is contrary to the classical concept which stated that cells in exponential growth phase are generally more tolerant to freezing than those in linear or stationary phase [24]. The culture growth phase issue may be species dependent.

Preculture treatment with sucrose and glycerol also affected the increase of inoculum viability explants post-dehydration (up to 90%) and freezing in liquid nitrogen (up to 80%). Zhu *et al*. (2006) reported that preculture by using sucrose could support regeneration of the banana culture post-cryopreservation by increasing the content of various sugars (glucose, fructose, sucrose, raffinose, inositol, and sorbitol) and total fatty acids, as well as changing the ratio of sterols [29]. Sugar is an important osmoticum and affects the reduction of freezing point, maintaining the membrane bilayers, and stabilize proteins during freezing, whereas sterols is one of the constituent components of cell membranes and play an important role in the stabilization and cell membrane permeability. In addition, Gamez-Pastrana *et al*. (2011) reported that preculture can reduce nucleation temperature of ice crystals formation during freezing [30]. This indicates that preculture treatment can significantly increase the success of cryopreservation.

Observation on the morphology plantlets derived from the control and those of cryopreservation treatments showed no different (Figure 10). However, the growth of the plantlets of control treatment were slower than the plantlets derived from cryopreservation treatment. Similar result was achieved by Georget *et al.* (2009) where the number of somatic embryos from cryopreservation treatments of banana (cv. Grand Naine) cell suspension was higher than that of control [31].

By using cryopreservation technique, embryogenic callus can be preserved and recovered and therefore would be available without often doing culture initiation that is difficult and take a long time. As reported by Panis [14], the ability to produce somatic embryos remains intact and embryogenic cell suspension could again be re-established even from 15 years-cryopreserved embryogenic callus.

Table 1 The recovery rate of banana embryogenic callus cv Dwarf Parfitt of early and late stage on WIC medium 1 month after preculture and subsequent treatment, i.e., preculture only, preculture treatment followed by dehydration only, and preculture treatment followed by dehydration and freezing during cryopreservation by vitrification technique

Type of inoculum	1-day preculture treatment		Embryogenic Callus Recovery (%) after			Additional observation after cryopreservation
	Sucrose (g/l)	Glycerol (M)	Preculture	Preculture, Dehydration	Preculture, Dehydration, Freezing	
The early stage embryogenic callus	120	0	95	75	0	-
	150	0	100	30	0	-
	180	0	50	10	50	-
	210	0	80	5	0	-
	120	2	40	60	70	1 spot
	150	2	40	30	70	2 spots
	180	2	95	50	80	6 spots
	210	2	30	40	30	1 spot
The late stage embryogenic callus	120	0	70	90	0	-
	150	0	80	80	70	14 spots, semi compact
	180	0	60	60	70	11 spots, semi compact
	210	0	80	10	40	3 spots, semi browning
	120	2	70	10	0	-
	150	2	60	40	80	10 spots, friable, proliferable
	180	2	80	80	0	-
	210	2	80	50	50	3 spots, friable

4.0 CONCLUSION

The use WIC medium was better than BM2G medium for maintaining morphogenetic capacity of embryogenic callus of banana cv. Dwarf Parfitt. Minimal growth technique could preserve the embryogenic callus longer than 4 months. The combined treatment of 0.005 mg L^{-1} ABA and low temperature of 15^0C provided 95% survival, 90% recovery rate, and no loss of embryogenic potential. The cryopreservation of the late stages of embryogenic callus that included dehydration period of 20 minutes, and preculture step with 150 g L^{-1} sucrose and 2 M glycerol was the most suitable treatments for successful cryopreservation with 80% survival rate.

Acknowledgement

The research was partly funded by Indonesia Toray for Science and Technology Foundation through Science and Technology Research Grant year of 2010. Funding from Indonesian Institute of Sciences (LIPI) through Competitive Research Grant 2004-2007, 2008-2010 to Witjaksono was highly appreciated. All authors are the main contributors to this work.

References

[1] Mariska, I., Suwarno. and Damardjati, D. S. 1996. Pengembangan Konservasi *In Vitro* sebagai Salah Satu Bentuk Pelestarian Plasma Nutfah di dalam Bank Gen. *Seminar Penyusunan Konsep Pelestarian Ex Situ Plasma Nutfah Pertanian.*

[2] Hu, C. Y. and Wang, P. J. 1983. Meristem, Shoot Tip, and Bud Culture. *In* D.A. Evans, W.R. Sharp, P.V. Amiroto, and Y. Yamada (*Eds*.). Handbook of Plant Cell Culture Vol. I. Techniques for Propagation and Breeding. McMilan Publishing, New York. 177-227.

[3] Withers, L. A. 1985. Cryopreservation and Storage of Germplasm. *In* D. A. Dixon (*Ed*.). Plant Cell Culture. IRL Press, Washington. 169-190.

[4] Keller, E. R. J., Senula, A., Leunufna, S. and Grube, M. 2006. Slow Growth Storage and Cryopreservation-Tools to Facilitate Germplasm Maintenance of Vegetatively Propagated Crops in Living Plant Collections. *International Journal of Refrigeration*. 29: 411-417. DOI: https:/doi.org/10.1016/j.ijrefrig.2005.07.012.

[5] Bessembinder, J. J. E., Staritsky, G. and Zandvoort, E. A. 1993. Long Term *in Vitro* Storage of *Colocasia Esculenta* under Minimal Growth Conditions. *Plant Cell Tissue and Organ Culture*. 33: 121-127. DOI: https://doi.org/10.1007/BF01983224.

[6] Desbrunais, A. B., Noirot, A. and Charrier, A. 1992. Slow Growth *In Vitro* Conservation of Coffee (*Coffea* spp.). *Plant Cell Tissue and Organ Culture*. 31: 105-110. DOI: https://doi.org/10.1007/BF00037693.

[7] Bajaj, Y. P. S. 1979. Technology and Prospects of Cryopreservation of Germplasm. *Euphytica*. 28: 267-285. DOI: https://doi.org/10.1007/BF00056584.

[8] [8] Bhojwani, S. S. and Razdan, M. K. 1983. *Plant Tissue Culture. Theory and Practise.* Elsevier. Amsterdam. New York. 502.

[9] Ashmore, S. E. 1997. Status Report on the Development and Application of *In Vitro* Techniques for the Conservation and Use of Plant Genetic Resources. IPGRI (International Plant Genetic Resources Institute). Rome. Italy. 67.

[10] Leunufna, S. 2004. Improvement of the *in vitro* Maintenance and Cryopreservation of Yams (*Dioscorea* spp.). Dissertation Martin-Luther-Universitat Halle-Wittenberg. Berlin. Unpublished. 12.

[11] Leunufna, S. 2007. Cryopreservation Technique for Conservation of Plant Germplasm: Its Possible Use in Indonesia. *Journal Agrobiogen*. 3(2): 80-88. DOI: https://doi.org/10.21082/jbio.v3n2.2007.p80-88.

[12] Strosse, H., Schoofs, H., Panis, B., Andre, E., Reyniers, K. and Swennen, R. 2006. Development of Embryogenic Cell Suspensions from Shoot Meristematic Tissue in Bananas and Plantains (*Musa* spp.). *Plant Science*. 170: 104-112. DOI: https:/doi.org/10.1016/j.plantsci.2005.08.007.

[13] Kulkarni, V. M. and Ganapathi, T. R. 2009. A Simple Procedure for Slow Growth Maintenance of Banana (*Musa* spp.) Embryogenic Cell Suspension Culture at Low Temperature. *Current Science*. 96(10): 1372-1377.

[14] Panis, B., Piette, B. and Swennen, R. 2005. Droplet Vitrification of Apical Meristems: A Acryopreservation Protocol to All *Musaceae*. *Plant Science*. 168: 45-55. DOI: https:/doi.org/10.1016/j.plantsci.2004.07.022.

[15] Engelmann, F. 2000. Importance of Cryopreservation for the Conservation of Plant Genetic Resources. *In*: Engelmann F, Takagi H (Eds). Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application, IPGRI, Rome. 8-20.

[16] Roostika, I. and Mariska, I. 2003. Pemanfaatan Teknik Kriopreservasi dalam Penyimpanan Plasma Nutfah Tanaman. *Buletin Plasma Nutfah*. 9(2): 10-18.

[17] Murashige, T. and Skoog, F. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Plant Physiology*. 15: 473-497. DOI: https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

[18] Sakai, A., Kobayashi, S. and Oiyama, I. E. 1990. Cryopreservation of Nucellar Cells of Navel Orange (*Citrus sinensis* Osb. var. brasiliensis Tanaka) by Vitrification. *Plant Cell Reports*. 9(10): 33-57.

[19] Sakai, P. A., Hirai, D. and Niino, T. 2008. Development of PVS-Based Vitrification and Encapsulation–Vitrification Protocols. In *Plant Cryopreservation: A Practical Guide*; Springer: Singapore. 33-57.

[20] Cai, X., Wei, H., Liu. C., Ren, X., Thi, L. T. and Jeong, B. R. 2020. Synergistic Effect of NaCl Pretreatment and PVP on Browning Suppression and Callus Induction from Petal Explants of *Paeonia lactiflora* Pall 'Festival Maxima'. *Plants*. 9(346): 1-13. DOI: https://doi.org/10.3390/plants9030346.

[21] Klimaszewska, K., Bernier-Cardou, M., Cyr, D. R. and Sutton, B. C. S. 2000. Influence of Gelling Agents on Culture Medium Gel Strength, Water Availability, Tissue Water Potential, and Maturation Response in Embryogenic Cultures of *Pinus Strobus* L. *In Vitro Cell. Dev. Biol-Plant*. 36: 279-286. DOI: https://doi.org/10.1007/s11627-000-0051-1.

[22] Hoekstra, F. A., Golovina, E. A., Tetteroo, F. A. A. and Wolkers, W. F. 2001. Induction of Desiccation Tolerance in Plant Somatic Embryos: How Exclusive Is The Protective Role of Sugar? *Cryobiology*. 43: 140-150. DOI: http://doi.org/10.1006/cryo.2001.2358.

[23] Choi, Y. E. and Jeong, J. H. 2002. Dormancy Induction of Somatic Embryos of Siberian Ginseng by High Sucrose Concentrations Enhances the Conservation of Hydrated Artificial Seeds and Dehydration Resistance. *Plant Cell Rep*. 20: 1112–1116. DOI: https://doi.org/10.1007/s00299-002-0455-y.

[24] Zimmermann, J. L. 1993. Somatic Embryogenesis: A Model for Early Development in Higher Plants. *Plant Cell*. 5: 1411-1423. DOI: http://doi.org/10.1105/tpc.5.10.1411.

[25] Rai, M. K., Shekhawat, N. S., Harish, A. K., Gupta, M., Phulwaria, K., Ram. and Jaiswal, U. 2011. The Role of

Abscisic Acid in Plant Tissue Culture: A Review of Recent Progress. *Plant Cell Tiss Organ Cult*. 106: 179-190. DOI: http://doi.org/10.1007/s11240-011-9923-9.

[26] Panis, B. 2009. Cryopreservation of *Musa* Germplasm. 2nd Edition. Technical guidelines No. 9. F. Engelmann and E. Benson (*Eds.*). Bioversity International. Montpellier-France. 48p.

[27] Ishikawa, M., Suzuki, M., Nakamura, T., Kishimoto, T., Robertson, A. J. and Gusta, L. V. 2006. Effect of Growth Phase on Survival of Bromegrass Suspesnsion Cells Following Cryopreservation and Biotic Stresses. *Annals of Botanny*. 97(3): 453-459. DOI: https://doi.org/:10.1093/aob/mcj049.

[28] Yoshida, S., Hattanda, Y. and Suyama, T. 1993. Variations in Chilling Sensivity of Suspension Cultures Cell of Mung Bean (*Vigna radiata* (L.) Wilczek) During the Growth Cycle. *Plant Cell Physiology*. 34: 673-679. DOI: https://doi.org/10.1007/BF00029997.

[29] Zhu, G-Y., Geuns, J. M. C., Dussert, S., Swennen, R. and Panis, B. 2006. Change in Sugar, Sterol and Fatty Acid Composition in Banana Meristems Caused by Sucrose-Induced Acclimation and Its Effects on Cryopreservation. *Physiologia Plantarum*. 128: 80-94. DOI: https://doi.org/10.1111/j.1399-3054.2006.00713.x.

[30] Gamez-Pastrana, R., Gonzalez-Arnao, M. T. and Martinez-Ocampo, Y. 2011. Thermal Events in Calcium Alginate Beads During Encapsulation Dehydration and Encapsulation-Vitrification Protocols. In: B. Panis and P. Lynch (Eds). *Proceedings of The First Symposium on Cryopreservation in Horticultural Species*. ISHS Comission Molecular Biology and In Vitro Culture. Belgium. 47-54. DOI: https://doi.org/10.17660/ActaHortic.2011.908.3

[31] Georget. F., Engelmann, G. F., Domergue, R. and Côte, F. 2009. Morpho-Histological Study of Banana (*Musa* Spp. Cv. Grande Naine [Aaa]) Cell Suspensions During Cryopreservation and Regeneration. *Cryo Letters*. 30(6): 398-407.