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Full Length Research Paper

Cryopreservation and plant regeneration of anther callus in Hevea by vitrification

Quan-Nan Zhou, Ai-Hua Sun, Zhe Li*, Yu-Wei Hua, Ze-Hai Jiang, Tian-Dai Huang, Xue-Mei Dai and Hua-Sun Huang*

Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences, State Center for Rubber Breeding, Danzhou 571737, China, Key Laboratory of Rubber Biology, Ministry of Agriculture, State Engineering and Technology Research Center for Key Tropical Crops, Danzhou 571737, China.

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Callus induced from anther of *Hevea brasiliensis* was successfully cryopreserved in liquid nitrogen (LN) by vitrification method and subsequently regenerated into plants. The effects of different preculture time, loading and dehydration duration on callus viability after cryopreservation were evaluated. The effective cryopreservation protocol involved preculturing on modified Murashige and Skoog (MS) medium containing 5 % sucrose (w/v) and 5% dimethyl sulfoxide (DMSO) (v/v) for 3 days, loading with 60% plant vitrification solution 2 (PVS2) for 20 min at 0℃ and dehydration with ice-cold PVS2 for 40 min. Dehydrated samples were directly immersed into LN, stored for 24 h and re-warmed in a water bath at 40℃. Using this protocol, *H. brasiliensis* callus showed 71.7% viability after cryopreservation. In conclusion, we developed a simple and effective method for the cryopreservation of *H. brasiliensis* callus, which allows long-term maintenance of valuable genotypes.

Key words: Callus, cryopreservation, *Hevea brasiliensis*, regeneration, vitrification.

INTRODUCTION

Rubber tree (*Hevea brasiliensis* Muell. Arg.) from tropical and subtropical countries supplies more than 30% of the elastomer market demand. Work under way in different rubber research institutes is focused on agricultural exploitation and on creating superior clones for rubber and wood production, and for disease resistance. The fact that rubber trees have long life cycle and cross-pollinated, makes the breeding of rubber trees more difficult. In recent years, the study on the tissue culture of *H. brasiliensis* Muell. Arg. has been developed (Nayanakantha and Seneviratne, 2007). Wang et al. (1980) successfully obtained the first *H. brasiliensis* plantlet from anther using tissue culture techniques.

Cryopreservation has become a very important tool for the long-term storage of plant germplasm with minimal space and maintenance (Sakai and Engelmann, 2007). At -196℃, metabolic processes and growth activity of cryopreserved cells are almost stopped (Stanwood, 1985; Benson, 2008). Theoretically, the plant materials are in a stable state for an infinite period of time, provided ice formation inside the cells is low enough to allow plant recovery.

Cryopreservation of *H. brasiliensis* has only been reported by a few researchers. Normah et al. (1986) found that embryonic axes could withstand cryopreservation after partial desiccation. Veisseire et al. (1993) successfully freeze embryogenic cell suspensions of one commercial clone. Subsequently, En*ge*lmann et al. (1997) developed two effective cryopreservation protocols for embryogenic callus of two commercial clones of *H. brasiliensis* but only somatic embryos not plantlets were established. Lardet et al. (2007) succeeded in obtaining the commercial clone plantlets after cyropreservation for *H. brasiliensis*. Their study mainly focused on the effect of different $CaCl_2$ concentrations in preculture medium.

*Corresponding author. E-mail: lizhecn@yahoo.cn and xjshhs@163.com. Tel: +86-898-23300585. Fax: +86-898-23300315.

2,4-D, 2,4-Dichlorophenoxyacetic acid; 6-BA, 6-Benzylaminopurine; DMSO, Dimethyl Sulfoxide; IAA, Indole-3-acetic acid; KT, Kinetin; LN, liquid nitrogen; MS, Murashige and Skoog (1962) medium; NAA, α-Naphthalene acetic acid; PVS2, plant vitrification solution 2; GA3, Gibberellic acid.

The experiments of Engelmann et al. (1997) and Lardet et al. (2007) both applied an average cooling rate of 0.2°C/min down to -40°C controlled by a programmable freezer or placed in -80°C freezer. The slow cooling procedure needs the use of expensive devices and a careful manipulation of plant materials and cooling rates.

In comparison, vitrification is a simple cryopreservation procedure which does not required the use of expensive cooling equipments. By vitrification, detrimental intracellular ice formation is avoided by using a highly concentrated cryoprotective solution to dehydrate and penetrate cells (Sakai and Engelmann, 2007). The plant vitrification solution 2 (PVS2) removed cellular water, changed cellular freezing behavior and limited ice crystallization in mint and garlic shoot tips during cryoprotection (Volk and Walters, 2006).

However, the solution was also lethal with extended exposure times (Volk et al., 2006). Until now, the vitrification method has been successfully used in many species (Volk and Caspersen, 2007; Turner et al., 2001; Fahy et al., 2004). However, there is no report of it being applied to the callus of *H. brasiliensis* anther.

The optimal preculture time, loading and dehydration duration are the keys of producing a high level of survival and successful cryopreservation by vitrification. In this study, we investigated the effects of preculture time, loading and dehydration duration on the viability of *H. brasiliensis* callus after cryopreservation. A simple vitrification protocol for cryopreservation of *H. brasiliensis* callus was established with a commercial clone.

MATERIALS AND METHODS

The callus regenerated from *H. brasiliensis* Reyan 7-33-97 anthers, a line cultivated in the experimental farm of Rubber Research Institute, Chinese Academy of Tropical Agriculture Sciences was used. The induction of the callus was performed using a modified method of Wang et al. (1980). The anthers were cultured on MS medium supplemented with 1.5 mg l^{-1} 2, 4-dichlorophenoxyacetic acid (2,4-D), 1.5 mg l^{-1} Kinetin (KT) and 1.5 mg l^{-1} α-naphthalene acetic acid (NAA) and incubated at 28 ± 2°C under darkness for 4 weeks. The callus was transferred into fresh medium every 4 weeks under the same culture conditions.

Three experiments were conducted to evaluate the effects of different preculture period, loading time in 60% PVS2 and dehydration time in ice-cold PVS2.

Different preculture time

Callus was transferred into the preculture medium [modified Murashige and Skoog (1962) medium with sucrose (5% w/v), dimethyl sulfoxide (DMSO) (5% v/v)] and cultured for 0, 1, 2, 3, 4, 5 and 7 days. Ten (10) calli were transferred into 1.8 ml cryotube, respectively. They were then loaded with 60% PVS2 at room temperature for 20 min, treated with ice-cold PVS2 for 40 min, and immersed directly into liquid nitrogen (LN) for 24 h (Sakai et al., 1990). The callus was then recovered from cryopreservation, warmed in a water-bath by thermostat at 40°C and then plant regeneration was carried out according to the modified methods of Wang et al. (1980).

Different loading time

Callus precultured for 3 days was loaded with 60% PVS2 at room temperature for 10, 15, 20, 30 and 40 min and then treated with ice-cold PVS2 for 40 min, and immersed directly into LN for 24 h. The callus was recovered from cryopreservation as described previously.

Different dehydration duration

The callus precultured for 3 days was loaded with 60% PVS2 at room temperature for 20 min and treated with ice-cold PVS2 for 10, 20, 30, 40 and 60 min, respectively. Subsequently, the callus was rapidly immersed in LN for 24 h and then recovered from cryopreservation as described previously.

Regrowth assessment and plant regeneration

In all experiments, about 10 to 20 calli were used for each treatment and the experiments were repeated three times. The regrowth rate of the calli was expressed relative to the number of cryopreserved ones. The viability of the calli after cryopreservation was assessed by visual observation of growth.

Callus precultured for 3 days were loaded with 60% PVS2 at room temperature for 20 min, treated with ice-cold PVS2 for 40 min and immersed directly into LN for 24 h. After thawing, the callus was resumed and then transferred into the embryo-induction medium which comprised Murashige and Skoog (MS) medium supplemented with 1.0 mg l^{-1} 6-benzylamino purine (6-BA), 3.0 mg l^{-1} KT, 0.2 mg l^{-1} NAA and 0.05 mg l^{-1} gibberellic acid (GA_3) and cultured in darkness at 28 ± 2°C for 4 weeks. Mature somatic embryos were transplanted into test tubes containing 30 ml MS medium supplemented with 0.5 mg l^{-1} KT, 0.2 mg l^{-1} indole-3-acetic acid (IAA), 0.3 mg l^{-1} GA_3 and incubated in a growth room under the conditions of 28 ± 2°C, humidity (60/70%), 12-h light:12-h dark photoperiod and transferred into fresh medium every 4 weeks. About 8 weeks later, the plantlets were obtained in the test tubes.

Statistical analysis

All experiments described here were repeated three times. The data were analyzed by one-way analysis of variance (ANOVA) on the statistical package of statistical analysis system (SAS) program (Version 9.0). Significant differences between means were assessed by Duncan's test at $P = 0.05$.

RESULTS

Evaluation of different preculture time

The length of the preculture time was critical to the viability of *H. brasiliensis* callus after cryopreservation. As shown in Figure 1, with the prolonging of preculture time, the viability of callus dramatically increased and reached a maximum at 3 days, followed by decline after 3 days. A significant difference was observed when precultured for 3 day compared with 0, 1, 2, 4, 5 or 7 days. However, no significant difference was examined when precultured for 2 or 4 days.

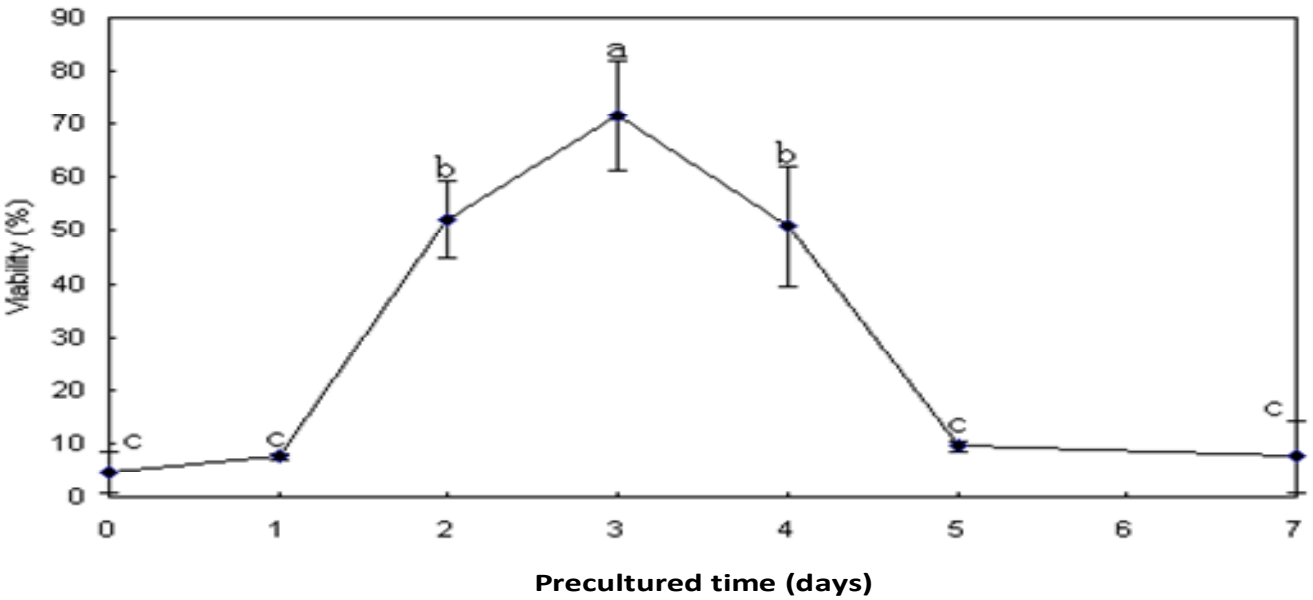

Figure 1. Effect of preculture time on the viability of *Hevea brasiliensis* callus after cryopreservation. Callus were precultured for 0, 1, 2, 3, 4, 5 and 7 days, respectively loaded with 60% PVS2 for 20 min, dehydrated with ice-cold PVS2 for 40 min, immersed in LN and bathed in 40°C water. Data are presented as mean values ± standard error. Values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test). Each value is the mean of three replicates. The bars represent standard errors.

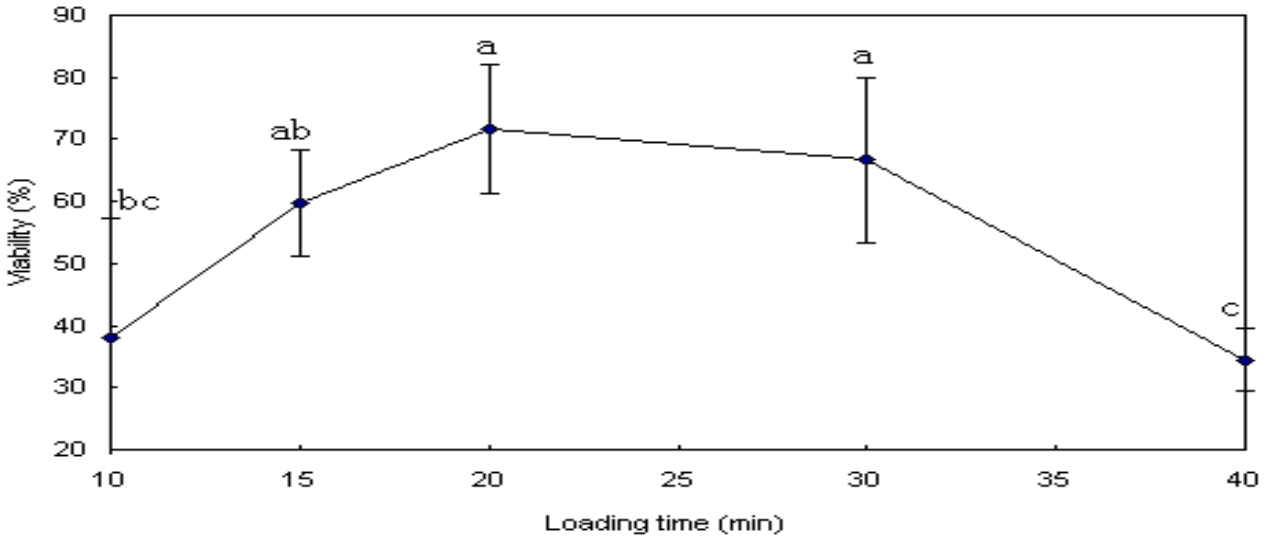

Figure 2. Effect of loading time on the viability of *Hevea brasiliensis* callus after cryopreservation. Callus were precultured with 3 days, loaded with 60% PVS2 for 10, 15, 20, 30 and 40 min, respectively, dehydrated with ice-cold concentrated PVS2 for 40min, immersed in LN and bathed in 40°C water. Data are presented as mean values ± standard error. Values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test). Each value is the mean of three replicates. The bars represent standard errors.

Optimization of loading and dehydration duration

The length of the loading and dehydration duration was also very important to the viability of *H. brasiliensis* callus after cryopreservation. As shown in Figure 2, the viability of the callus increased considerably when callus precultured for 3 days was loaded with 60% PVS2 at room temperature between 15 and 30 min and reached the highest (about 70%) at 20 min. Nevertheless, a significantly lower viability was examined when callus was loaded for 10 or 40 min. Figure 3 shows the effect of different dehydration duration on the viability of *H. brasiliensis* callus after cryopre-

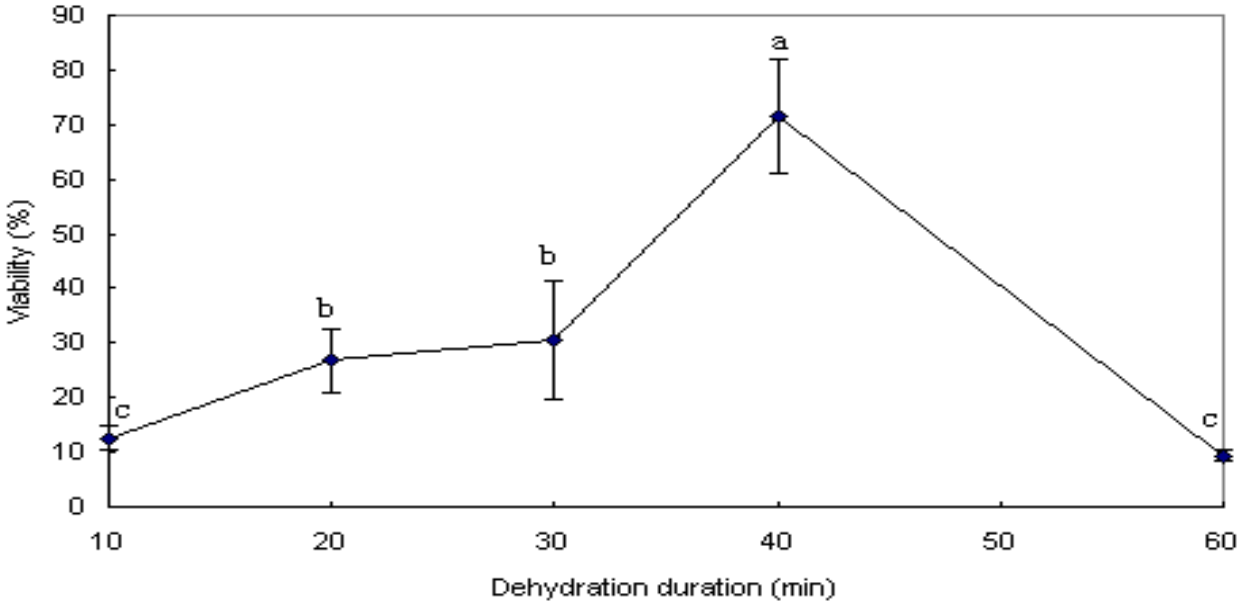

Figure 3. Effect of dehydration duration on the viability of *Hevea brasiliensis* callus after cryopreservation. Callus were precultured with 3 days, loaded with 60% PVS2 for 20 min, dehydrated with ice-cold concentrated PVS2 for 10, 20, 30, 40 and 60 min respectively, immersed in LN and bathed in 40°C water. Data are presented as mean values ± standard error. Values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test). Each value is the mean of three replicates. The bars represent standard errors.

servation. The viability of the callus largely increased with dehydration duration prolonging and reached a maximum when treated with ice-cold PVS2 for 40 min and then significantly decreased at 60 min. The viability of callus treated with PVS2 for 20 or 30 min was not significantly different.

Plant regeneration

After thawing, the callus stored in LN for 24 h showed a 2 to 3 days lag phase and recovered completely and grew into somatic embryos after 40 days of incubation. The embryos were induced into regenerated plantlets (Figure 4).

DISCUSSION

In this study, we reports for the first time the successful cryopreservation of *H. brasiliensis* anther callus using a simple vitrification protocol. The effective cryopreservation protocol involved preculturing on modified MS medium containing 5% sucrose (w/v) and 5% DMSO (v/v) for 3 days, loading with 60% PVS2 for 20 min at 0°C and dehydration with ice-cold PVS2 for 40 min before dehydrated callus was directly plunged into LN, stored for 24 h and re-warmed in a water bath at 40°C.

Studies of several groups found that preculture had significant influences on the survival rate of plant material after cryopreservation (Charoensub et al., 1999, Lambardi et al., 2000). Preculture could induce the stress hardening and enhance the freeze tolerance and survival rates (Göldner et al., 1991, Takagi et al., 1997). It was reported that preculture before cryopreservation on a medium with or without calcium was necessary for post-thaw callus growth recovery (Kohmura et al., 1992, Lardet et al., 2007). In this experiment, it was observed that the viability of *H. brasiliensis* callus after cryopreservation reached the highest when precultured for 3 days. Preculturing for 0, 1 or 2 days all resulted in the lower viability, which may be due to an insufficient acquisition of desiccation tolerance or cryoprotection (Turner et al., 2001b). Preculturing for longer than 3 days also showed the lower viability, this was probably attributed to the tissue growth and changes in physiological condition. Using *Picea mariana* (black spruce) embryogenic cultures, Touchell et al. (2002) obtained the highest survival by preculturing embryogenic masses for 2 days followed by incubation in PVS2 solution at 0°C for 30 min and plunging directly into LN.

In many species, preculture appears to be insufficient to produce a high level of survival by vitrification. Direct exposure to highly concentrated vitrification solutions is toxic. Therefore, samples cryopreserved by vitrification need to be loaded with a cryoprotective solution (Sakai and Engelmann, 2007). In the present study, the viability of *H. brasiliensis* callus after cryopreservation reached a maximum when loaded with 60% PVS2 for 20 min at room temperature. In *Anigozanthos viridis*, the optimal loading time was also 20 min at room temperature (Turner et al., 2001a), while in *Asparagus officinalis* L., the time was 10

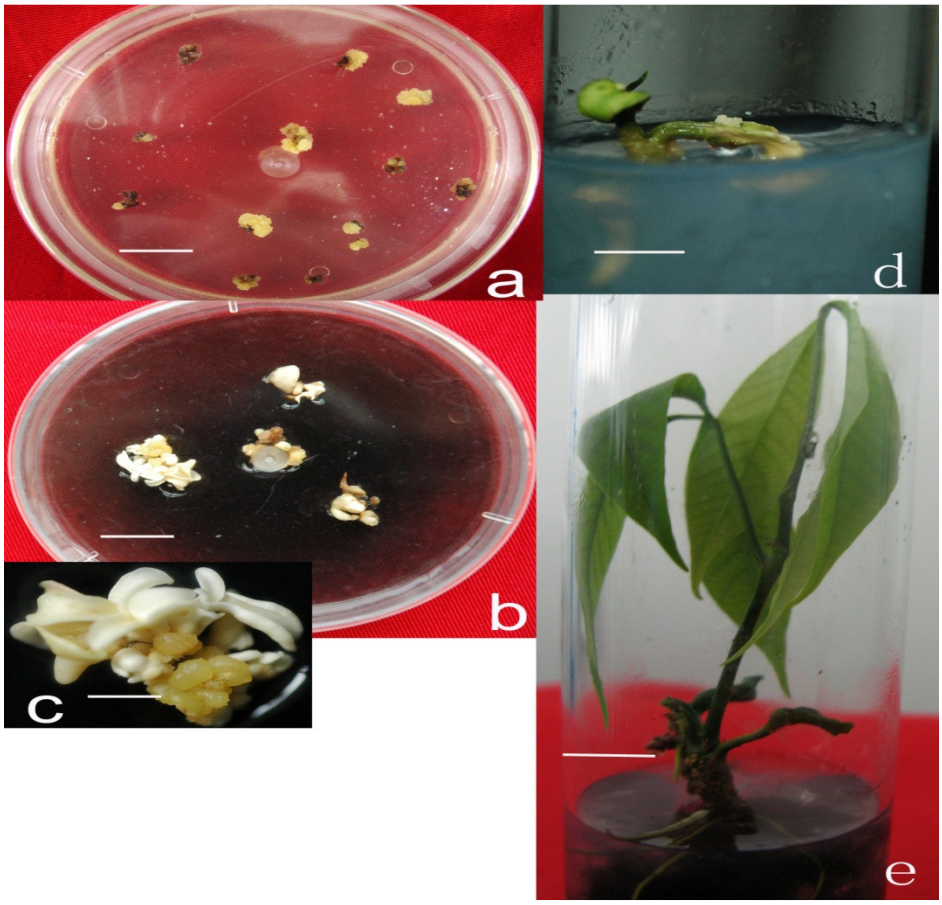

Figure 4. Plant regeneration of *Hevea brasiliensis* callus after cryopreservation. (a) Callus developed after cryopreservation and culturing for 20 days. (b) Somatic embryos developed from callus after cryopreservation and culturing for 60 days. (c) The amplified embryos. (d) (e) Plantlets obtained from somatic embryos. Scale bars represent 1 (a, b, d, and e) and 0.2 cm (c), respectively.

min (Nishizawa et al., 1993).

The dehydration duration is also very important to produce high regrowth rate of the callus after cryopreservation by vitrification. In the experiment, the highest viability of *H. brasiliensis* callus after cryopreservation was obtained when exposed to PVS2 solution for 40 min. The dehydration duration shorter than 40 min showed the lower viability which may be due to inadequate desiccated callus. The dehydration duration longer than 40 min also resulted in lower viability, most likely attributed to the injury from chemical toxicity or excessive desiccation (Sakai et al., 2007; Touchell et al., 2002). In sweet potato and black spruce, the optimal exposure time to PVS2 solution was reported to be 60 and 30 min at 25℃, respectively (Hirai and Sakai, 2003; Touchell et al., 2002).

In the present study, it was shown that the cell recovery of cryopreserved callus delayed by 2 to 3 days compared to non-cryopreserved ones. A similar observation was reported in lychee (Xie et al., 2008). The callus developed after cryopreservation was transferred into proliferation medium for 40 days which were longer than the non-cryopreserved callus, then transferred into embryo-induction medium, and the plantlets were observed in the cuvettes after 60 days. The plantlets had no phenotypic difference with those derived from non-cryopreserved callus.

In conclusion, this experiment successfully obtained the plantlets of the line of large-scale cultivated *H. brasiliensis* Reyan 7-33-97 anther callus after cryopreservation. The method that the callus immersed in LN directly from room temperature is simpler and more convenient than CIRAD that achieved an average cooling rate of 0.2℃/min down to -40℃ controlled by a programmable freezer (Engelmann et al., 1997; Lardet et al., 2007).

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