Scientific Article

Cryoprotectant solutions in star orchid seeds and bamboo orchid conservation in liquid nitrogen[1]

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ABSTRACT

An alternative for the conservation of orchid species threatened with extinction is the seeds preservation for long periods by cryopreservation. This work aimed to evaluate the influence of cryogenic solutions on the orchids *Epidendrum radicans* and *Arundina bambusifolia* seeds cryopreservation in liquid nitrogen. Seven treatments were performed: 0.4 M sucrose; 2 M glycerol; PVS1; PVS2; PVS3; PVS2 + 1% phloroglucinol and the control. After liquid nitrogen removal, half of the seeds were subjected to tetrazolium test, and another half was *in vitro* cultured. After 30 days seed germination was evaluated. There was no significant difference between *A. bambusifolia* and *E. radicans* in tetrazolium test and seeds germination. On the other hand, it was observed that in *A. bambusifolia* tetrazolium and seed germination tests the solutions of PVS1, PVS2, PVS2 + 1% phloroglucinol showed the best results. *E. radicans* seeds with 4% water content cryopreservation does not require the use of cryoprotectant. On the other hand, cryoprotectant´s use resulted in positive effect in maintenance and viability preservation of *A. bambusifolia* seeds. PVS1, PVS2 and PVS2 + 1% phloroglucinol showed best seeds survival rates after cryopreservation in liquid nitrogen.

Keywords: *Epidendrum radicans*, *Arundina bambusifolia*, Orchidaceae, tetrazolium, seed bank.

RESUMO

Soluções crioprotetoras na conservação de sementes de orquídea estrela e orquídea bambu em nitrogênio líquido

Uma alternativa para a conservação de espécies de orquídeas ameaçadas de extinção é a preservação por longos períodos de tempo das sementes por criopreservação. O objetivo deste trabalho foi avaliar a influência de soluções criogênicas na criopreservação de sementes das orquídeas *Epidendrum radicans* e *Arundina bambusifolia* em nitrogênio liquído. Foram realizados sete tratamentos: sacarose 0,4 M; glicerol 2 M; PVS1; PVS2; PVS3; PVS2 com 1% de floroglucinol e o controle. Após a retirada do nitrogênio líquido, metade das sementes foi submetida ao teste de tetrazólio, e outra metade foi cultivado in vitro. Após 30 dias foi avaliada a germinação das sementes. No teste de tetrazólio e na avaliação da germinação das sementes de *A. bambusifolia*, não houve diferença significativa entre os tratamentos de *E. radicans*. Por outro lado, no teste de tetrazólio e no teste de germinação das sementes de *A. bambusifolia*, observou-se que as soluções de PVS1, PVS2 e PVS2 + 1% de floroglucinol apresentaram os melhores resultados. A criopreservação de sementes de *E. radicans* com 4% de teor de água não requer o uso de crioprotetores. Por outro lado, a utilização dos crioprotetores apresentou efeito positivo na manutenção e preservação da viabilidade de sementes de *A. bambusifolia*, sendo que a utilização das soluções de PVS1, PVS2 e PVS2 acrescentado de 1% floroglucinol, apresentam melhores taxas de sobrevivência das sementes após criopreservação em nitrogênio líquido.

Palavras-chave: *Epidendrum radicans*, *Arundina bambusifolia*, Orchidaceae, tetrazólio, banco de sementes.

1. INTRODUCTION

Orchids are one of the most cultivated ornamental plants due to their importance to floriculture. They have between 800 and 1,000 genus and 20,000 to 25,000 species, which make this family one of the biggest plant families (MENINI NETO, 2010).

Many species have been extinct, and others are in risk of extinction mainly because of the agricultural area expansion and its consequent destruction of the plant's habitats. With science and biotechnology improvement, it is possible to find ways to preserve orchids' species. Among the methods used for germplasm preservation, cryopreservation stands out for its low cost and because it shows low risk of preserved material losses (VIEIRA, 2000).

Cryopreservation is a method which basic principle is to reduce temperature to extremely low values (-196 °C) aiming to decrease cellular metabolism, with material maximum conservation and quality, without losing vigor, in such a way that the material can retake its growth after liquid nitrogen storage (PEGG, 2007).

For the success of cryopreservation process, it is essential to add specific substances that provide cryoprotective action

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for the cell during temperature reduction. These substances, known as cryoprotectants agents, are fundamental to satisfactory results in cryopreservation, and, therefore, they are recommended to decrease or avoid injuries caused by the low temperatures (VATJA et al., 2007).

The simplest way to preserve genetic material is through seeds. Seeds banks allow a large range of genetic material preservation in small places for long periods (SANTOS, 2000).

According to Crowe et al. (1987), structures that accumulate sugar as seeds show tolerance to cryopreservation for most angiosperms. However, orchid seeds have lipid stock, which inhibit cryopreservation. Water removal through cryoprotectants that cause cytoplasm and membrane vitrification is one of the alternatives to handle this problem. For this reason, these substances are fundamental in cryopreservation.

Orchid seeds cryopreservation results have shown great fluctuation. Results vary from 95% for *Dendrobium candidum* seeds when vitrification solution (PVS2) is used to 0% for *Vanda coerulea* Griff. *ex* Lindl seeds also through vitrification process, which shows the difference between species using different cryopreservation methods (PRITCHARD, 1985; THAMMASIRI and SOAMKUL, 2007).

In view of these considerations, this work aimed to evaluate the effect of cryoprotectants solutions in the cryopreservation of *Epidendrum radicans* and *Arundina bambusifolia* seeds.

2. MATERIAL AND METHODS

Epidendrum radicans and *Arundina bambusifolia* seeds were obtained through artificial pollination of cultivated plants in greenhouse. Orchids were pollinated two days after flower opening. Capsules formed were collected when matured and dissected by the use of scalpel to remove seeds.

Initially seeds physiologic quality was evaluated through tetrazolium test and determined water content, following recommendations in Regras para Análise de Sementes (BRASIL, 2009).

Initial water content was obtained through gravitropic method through a stove at 105 °C, which is based on seed water weight that was removed during stay in stove for 24 hours. Arithmetic media of four seeds subsamples with 0,02g was calculated and results were obtained according to equation below:

$$\%\,(H) = \frac{100\,(W - w)}{W - t}$$

In which:

H - humidity rate in humid basis percentage;

W - initial weight, recipient and its cover weight plus humid seed weight;

w - final weight, recipient and its cover weight plus dry seed weight;

t - Tare, recipient and its cover weight.

Considering tetrazolium test, seeds were placed in a cryotube and distilled water was added, being kept for 24 h at 25 °C in BOD (germination chamber). Following, water was removed, and it was added tetrazolium salt solution at 1%, and seeds were maintained in BOD in the absence of light for 24 hours, at 30 °C. After that, viable seed percentage was evaluated with Motic Plus Images 2.0 software. Empty seeds, i.e. seeds without embryo, were not considered for viable seeds percentage determination.

Treatments evaluated were: sucrose 0.4 M; glycerol 2 M; PVS1 (19% glycerol, 13% ethylene glycol, 6% dymethil sulphoxide, 0,5M sorbitol); PVS2 (30% glycerol, 15% ethylene glycol, 15% dymethil sulphoxide, 0.4 M sucrose); PVS3 (50% glycerol + 50% sucrose); PVS2 with 1% phloroglucinol and the control.

Each treatment was constituted by 50 mg of seeds that were placed in cryotubes containing 2 mL of one of the different solutions according to treatment. Seeds remained in contact with sucrose and glycerol solutions for 20 min in ambient temperature (25 °C), meanwhile seeds in vitrificant solutions PVS1, PVS2, PVS3 and PVS2 + 1% phloroglucinol remained for 20 minutes under 0 °C, for subsequent immersion in liquid nitrogen (LN, -196 °C).

After 15 days of freezing, seeds were submitted to fast thawing in water bath at 40 °C, for 1.5 minutes. Cryoprotectants removal was realized with a Pasteur pipette. Subsequently, a sucrose solution 1M was added for 20 min, being removed 0.5 mL of it and added a sucrose solution 0.1M for extra 5 min; this last step was repeated twice.

Half of the seeds were submitted to tetrazolium test to evaluate its viability. The other half was germinated in MS medium (MURASHIGE and SKOOG, 1962) modified with half macronutrients concentration. MS medium was autoclaved at 105 kg cm^{-2} pressure and 121 °C, for 30 min (FARIA et al., 2012) and distributed in sterilized Petri dishes under laminar flow (20 mL $dish^{-1}$).

Before seeding, seeds were disinfected in sodium hypochlorite 0.5% for 20 min, in laminar flow and, after that, washed three times with autoclaved distilled water. Seeding took place in modified MS medium with macronutrients half concentration and the material was taken to growth room with 25 °C temperature, 16 h photoperiod and 120 µmol $s^{-1}m^{-2}$ luminosity from fluorescent lamps. 10 dishes were seeded for each treatment. After 30 days protocorm formation rate was determined.

Trial design was completely randomized, considering that, seven treatments and 10 replications were performed for each orchid species, with a dish as experimental unity. Data was submitted to variance analysis and medias were compared through Tukey test at 5% probability.

3. RESULTS AND DISCUSSION

Arundina bambusifolia and *Epidendrum radicans* seeds used in this experiment showed, respectively, 82% and 79% of viable seeds (Figure 1) and 7.1% and 4% water content before liquid nitrogen cryopreservation process.

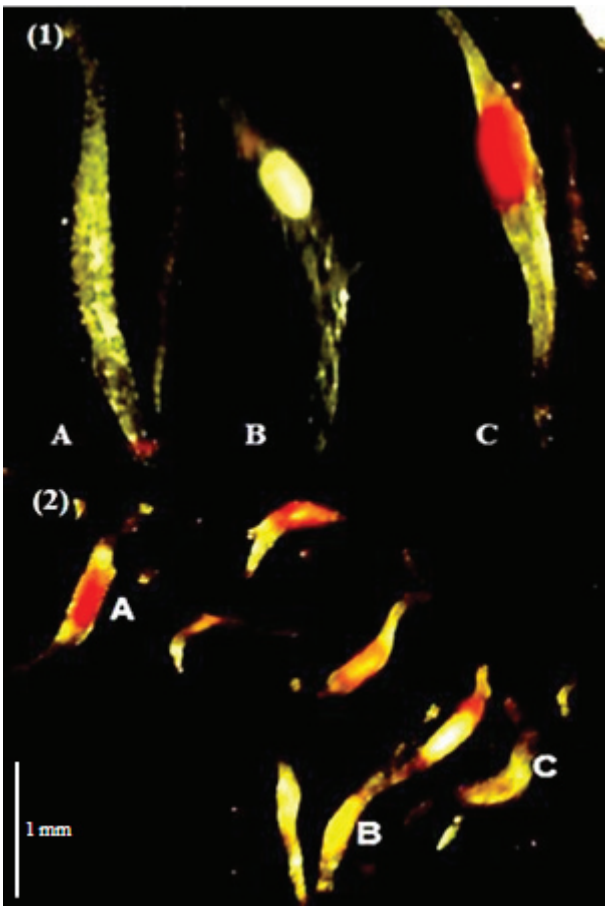

Figure 1. *Epindendrum radicans* (1) and *Arundina bambusifolia* (2) seeds cryopreserved in liquid nitrogen and submitted to tetrazolium test: seed without embryo (A), inviable seed (B) and viable seed (C).

Tetrazolium test (Figure 1) allowed differing seeds from analyzed treatments in terms of physiologic quality and viability. *A. bambusifolia* seeds showed higher viability rate after liquid nitrogen freezing when in PVS2 + 1% phloroglucinol, with 65% of viable seeds, although there was no statistical difference between PVS1 (55%), PVS2 (58%) and PVS3 (53%) (Table 1).

Table 1. Viability by tetrazolium test of *Epidendrum radicans* and *Arundina bambusifolia* seeds treated with cryoprotectant and submitted to cryopreservation in liquid nitrogen for 15 days.

Treatments	Tetrazolium (%)	
	Epidendrum radicans	*Arundina bambusifolia*
Control	77 a	50 bc
Glycerol	58 b	47 bc
Sucrose	60 b	43 c
PVS1	74 a	55 abc
PVS2	74 a	58 ab
PVS3	77 a	53 bc
PVS2 + 1% phloroglucinol	77 a	65 a
CV (%)	14.36	13.42

Means followed by the same lower-case letter in the column do not differ from Tukey test 5% of probability.

On the other hand, *E. radicans* seeds in control, PVS1, PVS2, PVS3 and PVS2 + 1% phloroglucinol treatments showed higher viability rates, reaching 77, 74, 74, 77 and 77% of viable seeds, respectively, while glycerol and sucrose treatments resulted in lower values, 58 and 60%, respectively, of viable seeds.

Seeds low viability observed in glycerol and sucrose treatment is probably related to the formation of an ambient outside the cell that allowed cellular dehydration. However, as higher cellular dehydration level is unknown for these species, excessive dehydration could have happened, which may have interfered in cellular metabolism irreversibly, reducing seed viability.

Related to the higher seed viability in PVS2 + 1% phloroglucinol, Galdiano Junior et al. (2012) observed that treatments using PVS2 + 1% phloroglucinol and PVS2 + 1% phloroglucinol + supercool X1000 showed higher percentage of viable seeds of a *Dendrobium* hybrid after cryopreservation.

Related to *A. bambusifolia* protocorm formation (Figure 2), results were similar to the ones obtained in tetrazolium test and PVS2 + 1% phloroglucinol treatment promoted higher protocorm formation percentage (61%), although it did not differ statistically from PVS1 (55%) and PVS2 (54%).

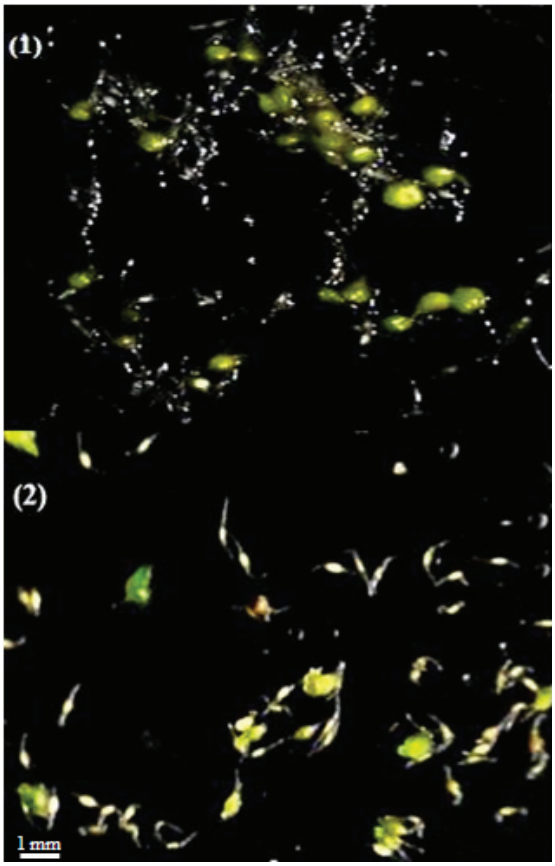

Figure 2. *Epindendrum radicans* (1) and *Arundina bambusifolia* (2) protocorms formation, after 30 days of seeding in media culture modified MS with half concentration of salts.

Similar results were obtained in *Dendrobium nobile* protocorms, in which PVS2 + 1% phloroglucinol resulted in better results, with 68% of protocorms survival (VENDRAME and FARIA, 2011). *E. radicans* protocorms formation percentage did not differ statistically between treatments (Table 2).

Table 2. Germination percentage in MS medium of *Epidendrum radicans* e *Arundina bambusifolia* seeds treated with cryoprotectant and submitted to cryopreservation in liquid nitrogen for 15 days.

Treatments	Germination (%)	
	Epidendrum radicans	***Arundina bambusifolia***
Control	52[ns]	39 cd
Glycerol	57	29 d
Sucrose	58	42 c
PVS1	69	55 ab
PVS2	55	54 ab
PVS3	62	47 bc
PVS2 + 1% de floroglucinol	65	61 a
CV (%)	13.17	16.61

[ns] Means do not show significant difference. Means followed by the same lower-case letter in the column does not differ from Tukey test 5% of probability.

According to Nikishina et al. (2007), seeds germination rate after exposure to liquid nitrogen is dependent on species and can be lower or higher than control, and cryoprotectant efficiency varies in function of the structure (cell or tissue) to be cryopreserved (FULLER and PAYNTER, 2004), the cryoprotectant type, concentration and time of exposure used before cryopreservation itself. It was noticed that, for both species, germination percentage was lower in control when compared the cryoprotectants usage and only for *A. bambusifolia* they were efficient in cryopreservation process.

Results indicate that the use of vitrificants solutions for *E. radicans* were not necessary since no statistical difference were observed between control and treatments regarding seeds germination (Table 2). On the other hand, cryoprotectants usage for *A. bambusifolia* was necessary. Probably this difference between species is related to water content in seeds, since the greater the amount of intracellular water, the better are the conditions to form ice crystal, and then, protectant solutions became efficient.

Studies show that the ideal humidity content in seeds to cryopreservation process succeed can vary according to genus and species. *Doritis pulcherrima* seeds with 31% humidity were preserved in liquid nitrogen, unsuccessfully, because germination percentage was zero after thawing, as well as three *Dendrobium* cultivars in which seeds with 9% humidity, putted directly in liquid nitrogen (-196 °C), did not germinate after thawing. On the other hand, 88% of viability was observed in *Dendrobium candidum* seeds after cryopreservation in liquid nitrogen, with 12% to 19% water content (THAMMASIRI, 2000; VENDRAME et al., 2007; WANG et al., 1998). This result reinforces the hypothesis that there is no need to use cryoprotectants for *E. radicans* due to its seed low water content, since it was of 4% before cryopreservation.

Glycerol treatment caused the smallest protocorm formation rate for *A. bambusifolia*, not statistically differing from control (no cryoprotectant) (Table 2). This result may be related to the fact that glycerol showed greater toxicity degree and smaller penetration in cells (DALIMATA and GRAHAM, 1997) between used cryoprotectants. This also explains the smallest protocorm formation frequency in PVS3 solution compared to others PVSs, as that is formulated with 50% (v/v) glycerol. This high concentration may cause cellular intoxication.

4. CONCLUSIONS

This study showed that, for the cryopreservation of native orchid seeds *Epidendrum radicans*, with 4% water content, it is not necessary the use of cryoprotectants.

Cryoprotectants showed positive effect in maintenance and viability preservation of *Arundina bambusifolia* seeds. Using PVS1, PVS2 and PVS2 plus 1% phloroglucinol it was possible to obtain better seeds survival rates after cryopreservation in liquid nitrogen.

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