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Cryopreservation of seeds of barbatimão with different water contents

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ABSTRACT

Barbatimão [*Stryphnodendron adstringens* (Mart.) Coville], is a tree with predominant occurrence in the Cerrado regions in the Brazilian States. Its bark is rich in tannins which is used in folk medicine due to its astringent function. In recent years, the indiscriminate exploitation of barbatimão tree led to a decrease in bark production of this medicinal plant. The aim of this study was to induce calluses from cotyledon segments and determine the weight of fresh and dry matter and the levels of phenols the induced calluses. For callus induction, cotyledon segments were inoculated in a medium with different combinations of growth regulators: 2,4-D × TDZ (0, 0.5, 1.0 and 2.0 mg L^{-1}) and kinetin × picloran (0; 0.5, 1.0 and 2.0 mg L^{-1}). Cultures were incubated in the dark and at a temperature of 27 ± 2 ° C. At 60 days of subculture the fresh and dry weight and the levels of total phenols were evaluated. The best results obtained for the calluses fresh weight with the combination (2,4-D × TDZ) were: 0.5 mg L^{-1} of 2,4-D associated with 0.5, 1.0 and 2.0 mg L^{-1} concentrations of TDZ. For dry matter values the best combination was: 0.5 and 2.0 mg L^{-1} TDZ in the absence of 2,4-D. For the (Kinetin × Picloram) combination, the best results for callus induction were: 2 mg L^{-1} of kinetin and 0.5 mg L^{-1} of Picloram, with an average of 0.2 g of fresh weight and 0.02 g of dry weight. The results indicated that higher levels of total phenols were found in calluses induced on a medium supplemented with 1.0 mg L^{-1} of 2,4-D, 2.0 mg L^{-1} of kinetin and 1.0 mg L^{-1} of Picloram. A negative effect for TDZ regarding the total phenol levels when combined with 2,4-D was noted. The tests performed in this study, where different growth regulators were used for barbatimão callus induction in order to increase phenol concentrations, did not present satisfactory results. Although a low concentration of phenolic compounds was obtained, it is important to note that the culture medium may be optimized for large scale *in vitro* production of this compound.

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INTRODUCTION

The barbatimão [Stryphnodendron adstringens (Mart.) Coville] is a typical plant specie of brazilian cerrado biome, which belongs to Fabaceae family, presents wide distribution and is used in the popular medicine, mainly because of its astringents and cicatrizing properties (Minatel *et al*., 2010), besides its use as anti-inflammatory agent (Lima *et al*, 1998). These uses are directly associated to high content of tannins found in the suber, around 20-30%. However, according to Almeida *et al*. (1998) the domestic bark production of S. adstringens has been decreasing due to the indiscriminate production by the leather tanning industries.

Latest studies also evidenced that because of large quantity of tannins in this plant specie is possible producing adhesive used in the plywood pasting (Almeida *et al*., 2010; Goulart *et al*., 2012). The disordered extraction of plant bark, which occurs regardless the plant size, have been causing depletion of this genetic resource in wild populations. Thus, guidelines are necessary to preserve this plant specie such as development of sustainable management techniques, domestication programs and recomposition of native forests (Borges Filho & Felfili, 2003) as well as the establishment of storage alternatives of plant genetic resources for long term, such as cryopreservation (Silva *et al*., 2013).

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The conservation of a plant specie may be through seedbanks, which constitute a widely used conservation model of plant genetic resources ex situ (Li & Pritchard, 2009). In general, the storage of various plant species is using seed with low content of water, under low temperature, what allows seed conservation for decades (Vásques-Yanes & Orozco-Segovia, 1993). However, studies about seed cryopreservation of various ecologically and economically important plant species are increasingly evident, considering that this technique aims to preserve plant genetic resources for an undetermined time, with no loss of viability, with relatively low cost and in an environment that enables the preservation of physical and biological integrity of the material (Wu *et al*., 2013; Engelmann, 2004).

However, cryopreservation is a promising technique for the storage of plant genetic resources for prolonged periods (Panis *et al*., 2005; Sakai & Engelmann, 2007; Skrlep *et al*., 2008, Silva *et al*., 2013), and consists on conserving the biological material under ultra-low temperature into liquid nitrogen at -196oC, where cellular division and metabolical processes are drastically reduced, allowing storage with no structural changes (Engelmann, 2011).

Several techniques have been used for cryopreservation of different plant parts such as somatic and zygotic embryos, meristems, callus and buds. Seeds are most appropriate for specie conservation by being the most organized systems. However, when they have high content of water, cannot be cryopreserved because of the risk of ice crystal formation and, consequently, the death of material under liquid nitrogen.

Studies about relation between content of humidity and the storage time, in different plant species, show that there is no a specific value of humidity, but a range of acceptable values for cryopreservation. Therefore, it recommends humidity content less than 10% for seed conservation, according to Kermode & Finch-Savage (2002). Through this work, we aimed to study the seed germination process of S. adstringens with different water contents, cryopreserved for different storage periods.

MATERIALS AND METHODS

Plant specimen:

S. adstringens ripe fruits were harvested before dehiscence, from plants of wild population located in the Ijací city, southern region of Minas Gerais, Brazil. Subsequently, seeds were manually extracted and, those which showed physical integrity were selected and transferred for glass flask and stored in the refrigerator at 4oC for 30 days.

Characterization of seed content of water:

The initial content of water of the seeds was determined using three samples of 3 g of seed, approximately 40 seeds, which were weighed and put up in perforated 10×10 cm aluminium foil, and placed into a hothouse at 105±3ºC for 72 hours. Thereafter, seeds were cooled into a dryer with constant air circulation at 25oC for 15 minutes and, then, were reweighed. The percentage of content of water was determined based on the fresh weigh, as outlined in Brasil (1992).

$$CW(\%wb) = \frac{IW-FW}{IW} \times 100\%$$; where: CW = content of water in wet basis (%); IW = initial weight of the sample (g); and FW = final weight of the sample (g).

After determining the initial content of water, another two seed lots were dried on silica gel for different times until obtaining content of water around 6 and 9%. Were used 11×11×3.5 cm acrylic boxes Gerbox-type containing 100 g of silica gel. Seeds were uniformly put in boxes, in single layer on a Petri dish, placed on the silica gel. Boxes were closed and, then, sealed with plastic parafilm and kept under 25±2ºC.

Content of water of 6% and 9% were obtained after 24 and 17 hours, respectively, with record of weight loss of seeds during drying. The fresh mass of the samples, corresponding to desired content of water were calculated using the following equation described in Cromarty *et al*. (1985).

$$SW_f = SW_i \times \frac{(100-CW_i)}{(100-CW_f)}$$; where: SWf = sample weight after drying (g); SWi = sample weight before drying (g); CWi = content of water before drying (%); and CWf = content of water after drying (%).

Cryopreservation of seeds:

Seeds with 6, 9 and 12% of content of water were packed in aluminium cylindrical tubes and immersed in liquid nitrogen for 1, 30, 60 or 90 days before reheating. Then, seeds were rapidly reheated by immersion in distilled water at 30oC for 90 seconds before tests of physiological quality, namely, the germination percentage, samples vigour and content of DNA.

Germination test:

All cryopreserved seeds, control included, were scarified into 95% sulphuric acid for 60 minutes and, then, washed in water (Martins & Nakagawa, 2008). Working in a laminar flow hood, these seeds were sterilized in 70% alcohol for around one minute and, then, transferred to a disinfectant solution of 2% sodium hypochlorite, during five minutes. Then, seeds were washed through three rinses into sterile distilled water to remove excessive disinfectant, and placed on dishes containing MS medium (Murashige & Skoog, 1962), added 10 g.l-1 of sucrose, 7 g.l-1 of water and pH adjusted to 5.8. Incubation was in a room, under constant 36 µmol m-2.s-1 photons irradiation, for 16 hours photoperiod at 25±2oC. Were used 10 replications with 8 seeds for each treatment, these represented by seeds cryopreserved or not, with 6, 9 or 12% of content of water. The evaluation of germination was performed 4 days after inoculation, considering all seeds with radicle approximately 20 mm long.

Test of vigour:

The seeds vigour was assessed measuring the aerial part length and seedlings dry weight. The aerial part length was determined 50 days after seeds inoculation, measuring from transition region between roots-stem to the stem apex. Were also used 10 replications with 8 seeds for each treatment, these represented by seeds cryopreserved or not, with 6, 9 or 12% of content of water.

The seedlings dry weight was determined after packed in paper bags, and dried at 80±3ºC for 72 hours, after which samples were cooled into a dryer for 15 minutes and, the dry weight was determined using electronic 0.0001 g precision scale.

Analysis of DNA content:

The DNA content of the samples was assessed by flow cytometry kit, using folioles obtained from plants in-vitro after 50 cultivation days. Were used three samples for each treatment from seeds with 6, 9 or 12% of content of water, stored in liquid nitrogen for 24 hours, and seedling from seeds not cryopreserved. The control consisted of seeds with the same water contents, but not immersed in liquid nitrogen.

Were used around 20-30 mg of seedlings folioles and leaf fragments of the external reference standard (Pisum sativum) for determination of the DNA content. Samples were triturated on Petri dishes containing 1.0 ml of frozen LB01 extraction buffer (15 mM Tris, 2.0 mM Na2EDTA, 0.5 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.5) for nucleus release, as outlined in Dolezel *et al*. (1989). The nucleus suspension was aspirated through two gauze layers, with the help of plastic pipette and filtered through 50 µm mesh. Nucleus were stained adding 25 µl of 1.0 mg/1.0 ml of propidium iodide.

The content of DNA was estimated in picograms (pg) by using the following formula:

$$\text{Sample, 2C DNA} = = \frac{\text{Peak Channel G1 of the sample}}{\text{Peak Channel G1 of } P.sativum} \times \text{2C content of standard DNA (9.09pg)}$$

Analyzes were carried out in the Facscalibur cytometer (BD, Biosciences, San Jose, CA, USA), and histograms were obtained through Cell Quest software (Becton Dickinson Company, San Jose, CA, USA).

Experimental Design and Statistical Analysis:

The experiment was performed in completely randomized design. Data were subjected to Analysis of Variance (ANOVA), and means of germination and vigour were compared by Tukey test at 5% significance level using SISVAR (Ferreira, 2011). The analysis of DNA content was carried out using WinMDI 2.8 software.

RESULTS AND DISCUSSION

The germination of seeds was not affected by different water contents. The average of radicular protrusion was around 87.8%, regardless exposition or not to liquid nitrogen (LN). In similar conditions, Castro *et al*. (2001) cryopreserved seeds of urucum (*Bixa orella* L.) with around 6 to 12% of content of water, and found no significant differences for germination.

The least aerial part length (APL) and the least dry weight (DW) were found on seedlings obtained from seeds with 12% of initial content of water, cryopreserveed or not. Only seeds with 9% of initial content of water resulted in seedlings with APL statistically different in the comparison among exposed or not exposed seeds to LN treatment. In this case, the control showed the best results. Seeds with 6% of initial content of water resulted in normal seedlings, with no statistical differences of APL among cryopreserved and not preserved seeds. Seedlings obtained from seeds with 6% or 9% of initial content of water showed the highest DW when seeds were not cryopreserved. Among cryopreserved seeds, samples with 6% and 9% of content of water resulted in

seedlings with averages of APL and DW statistically greater than those from seeds with 12% (Table 1). After germination, seedlings were normal in appearance (Figure 1).

Fig. 1: Seedlings obtained from seeds with 6% (A), 9% (B) and 12% (C) of content of water before cryopreservation.

Results of APL and DW obtained in this study showed that the 12% content of water has negative effect in the initial growing of seedlings in vitro (Table 1). A high content of water, however, may cause damages during cooling and formation of ice crystals from free water present into cells. Then, by the seeds humidity be one of the main factors affecting cryopreservation (Walters *et al*., 2004), these problems are part of limitations for the cryopreservation of various tissues, since may cause irreversible cellular injuries, according to Engelmann (2011). Therefore, for orthodox seeds it recommends content of water less than 10% but, the favourable humidity for freezing differ among plant species.

Table 1: Means of aerial part length (APL) and dry weight (DW) of seedlings obtained in vitro from seeds with different water contents, not stored (-LN) or stored in liquid nitrogen (+LN) for 24 hours.

Water contents of seeds (%)	APL (mm)		DW (g)	
	-NL	+NL	-NL	+NL
6	109,4 aA	101,1 aA	0,038 bA	0,033 aB
9	113,7 aA	103,7 aB	0,043 aA	0,032 aB
12	94,3 bA	84,5 bA	0,027 cA	0,026 bA
	CV (%) = 11,0		VC (%) = 9,2	

Averages followed by the same capital letter in the row, for each APL or DW, and small letter in the column do not differ one another by Tukey test at 5% of significance level

Tresena *et al*. (2010), for cryopreservation of seeds of yellow ipe (*Tabebuia chrysotrica*), found that the germination percentage and seeds vigour was greater as greater was the content of water. In this case, content of water around 6% and 8% did not change the vigour of cryopreserved seeds of *Jatropha curcas*. In fact, the least vigour was for seeds with high content of water (Goldfarb *et al*., 2008).

In relation to the content of DNA, samples of isolated nucleus from leaves of the control and from leaves of plants obtained from seeds with different water contents, and cryopreserved for 24 hours, did not show statistical differences after storage in liquid nitrogen (Figure 2). These indicate that there is no risk of loss of genetic content of the cryopreserved material, regardless the initial content of water into seeds. Hirano *et al*. (2005) used the flow cytometry to examine the ploidy level on seedlings of a specie of orchid (*Bletilla striata*) submitted to cryopreservation. Then, they found the same peak of DNA content. Therefore, assessments of genetic and molecular stability after freezing are essential if the cryopreservation is used as the mean to store plant germplasm, according to Larkin & Scowcroft (1981).

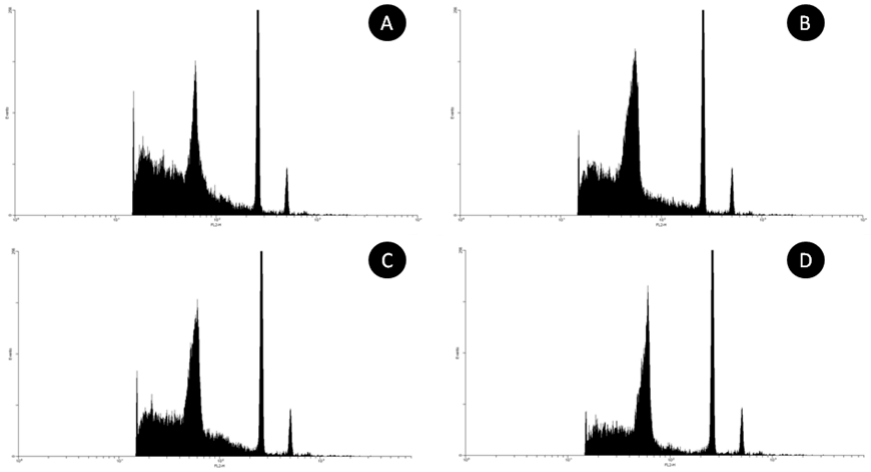

Fig. 2: Histograms of nuclear DNA preparations of S. adstringens seedlings in vitro. First peak: (A) folioles of seedlings obtained from seeds not immersed in LN; (B) folioles of seedlings obtained from seeds with 6% of content of water immersed in LN; (C) folioles of seedlings obtained from seeds with 9% of content of water immersed in LN; (D) folioles of seedlings obtained from seeds with 12% of content of water immersed in LN. Second peak: is the G1 phase of pea (Pisum sativum L.) used as standard.

Seeds with different water contents (6, 9 and 12%) and stored in LN for different periods (30, 60 and 90 days) did not show statistically significant differences in germination, with overall average around 89.8%. Again, seeds with 6% and 9% content of water resulted in seedlings with high APL (Table 2).

Table 2: Mean values of the aerial part length (mm) of seedlings in vitro, obtained from seeds with different content of water, submitted to different storage periods in liquid nitrogen

	Storage periods (Days)		
Water contents of seeds (%)	30	60	90
6	87,38 a	101,56 a	89,56 ab
9	91,38 a	107,02 a	95,92 a
12	73,48 b	86,74 b	82,06 b
CV (%) = 12,0			

Averages followed by the same capital letter in the row, for each APL or DW, and small letter in the column do not differ one another by Tukey test at 5% of significance level

In other hand, it was found different results among the storage periods. Seeds with 6% and 9% of content of water and stored for 30 and 90 days showed the highest DW. After 60 days, seedlings obtained from seeds with 9% of content of water showed differences statistically significant, with the highest average of DW (Table 3). Almeida *et al*. (2002) found different results for ricinus (*Ricinus communis*), with faster reduction of germination percentage and DW of seedlings obtained from seeds with 12% of content of water than in seedlings obtained from seeds with 4, 6, 8 and 10%.

Table 3: Mean values of dry weight (g) of seedlings submitted to different storage periods in liquid nitrogen.

	Storage periods (Days)		
Water contents of seeds (%)	30	60	90
6	0,0359 a	0,0306 b	0,0312 ab
9	0,0403 a	0,0359 a	0,0365 a
12	0,0209 b	0,0227 c	0,0309 b
CV (%) = 16,0			

As médias seguidas das mesmas letras nas colunas não diferem estatisticamente pelo teste deTukey, ao nível de 5% de probabilidade.

Although in the present work we did not compare averages for tests of germination and seeds vigour submitted to different storage periods in liquid nitrogen, we realized that averages did not vary too much. This, suggested that different storage periods did not greatly affect neither germination nor seed vigour of the barbatimão.

Goldfarb *et al*. (2010), by analyzing seeds of *Jatropha curcas*, without considering the initial content of water, they found that after exposition in liquid nitrogen for 90 days, the germination percentage remained stable. Therefore, according to Santos (2004), cryopreservation of biological material in liquid nitrogen may ensure the conservation for long period, since, in very low temperature, the cellular metabolism is so low, and the biological deterioration is almost paralyzed.

Conclusions:

The cryopreservation may be used as a strategy to conservation of seeds of barbatimão, and must be performed using seeds containing content of water between 6% and 9%, before storage in liquid nitrogen.

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