Methods to assess the viability of cryopreserved *Jatropha curcas* L. seed germplasm

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ABSTRACT: *Jatropha curcas* L. is a plant species with many potential applications, especially medicinal uses (hypoglycemic, anti-inflammatory, haemostatic, healing, anti-tumor). The objective of this study was to test germination in moist paper rolls for whole seeds and *in vitro* for excised embryonic axes, in an attempt to identify the best method to assess the quality of *J. curcas* seed germplasm, cryopreserved with different water contents. The experimental sample with a 6.2% moisture content (MC) was divided in subsamples which were hydrated and dehydrated for 0 (control), 4, 8, 11 and 24h. The initial germination percentages were 63% for whole seeds and 81% for excised embryonic axes. After exposure to liquid nitrogen (LN), germination percentages were 48% (whole seeds) and 57% (excised embryonic axes). There was no significant difference between germination percentages in embryonic excised from seeds subjected or not subjected to freezing, with different MC. In contrast, there was a reduction of the whole seed germination percentage when exposed to LN (contrast = 0.17, standard error = 0.04, t = 4.09, p = 0.001) and not for the hydration and dehydration treatments. The methodology based on *in vitro* cultures of the embryonic axis isolated from seeds stored in LN with distinct MC values was more efficient than the standard germination test to evaluate the viability of *J. curcas* seeds before and after LN storage.

Keywords: seed, embryonic axis, cryopreservation, *Jatropha curcas*

RESUMO: Métodos para avaliar a viabilidade de germoplasma semente criopreservado de *Jatropha curcas* L. *Jatropha curcas* L., é uma espécie com várias aplicações potenciais, principalmente para usos medicinais (hipoglicemina, anti-inflamatório, homeostático, cicatrizante, antitumoral). O objetivo deste estudo foi testar a germinação em rolo de papel para sementes inteiras e *in vitro* para eixos embrionários excisados visando identificar o melhor método para avaliar a qualidade de germoplasma semente de *J. curcas*, criopreservado com diferentes teores de água. A amostra experimental com 6,2% de teor de água foi dividida em subamostras que foram hidratadas e desidratadas por 0 (controle), 4, 8, 11 e 24 h. Os percentuais de germinação inicial foram de 63% para sementes inteiras e de 81% para eixos embrionários excisados. Após exposição ao nitrogênio líquido (NL) os percentuais de germinação foram de 48% (sementes inteiras) e 57% (eixos embrionários). Não houve diferença significativa entre os percentuais de germinação de eixos embrionários excisados de sementes com diferentes umidades e submetidas ou não ao congelamento. Em contraste, houve redução de percentuais de germinação das sementes inteiras expostas ao NL (contraste = 0.17, erro padrão = 0.04, t = 4.09, p = 0.001), mas não aos tratamentos de hidratação e desidratação. A metodologia baseada em cultura de eixos embrionários *in vitro* isolados de sementes armazenadas em NL, com distintos teores de água foi mais eficiente que a germinação padrão para avaliar a viabilidade de sementes *J. curcas* antes e após a armazenamento em NL.

Palavras-chave: semente, eixo embrionário, criopreservação, *Jatropha curcas*

INTRODUCTION

Jatropha curcas L. is a species of flowering plant in the Euphorbiaceae family, native to the American tropics, most likely Mexico and Central America. It is cultivated in tropical and subtropical

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regions around the world due to its many potential applications, namely oil extraction for biofuel production, pest control, enzyme production, recovery eroded and arid soils and especially medicinal uses hypoglycemic, anti-inflammatory, haemostatic, healing, anti-tumor (Santos et al., 2008; Franco, 2013; Garba et al., 2013).

Therefore conservation of genetic resources of this species, particularly elite genotypes, in order to obtain recombinant hybrid is essential for genetic improvement. However, two aspects restrict their domestication, seed production is erratic due to heterozygous plants and the genetic variability in germplasm collections of the species is close (Singh et al., 2010; Sharmaa et al., 2011). Seeds with high lipid content have shown reduced lifespan under storage in genebanks when conventional conservation are adopted, -18ºC (Crane et al., 2003). The sensitivity of lipid seeds to storage conditions at low temperatures can be attributed possibly to hydrolytic and oxidative rancidity of fatty acids, resulting in loss of germination, viability and vigor (Chmielarz, 2009; Mbofung, 2012).

The main goal of *ex situ* conservation is ensuring more efficient and cost-effective germplasm storage and management. Among the operational activities of a genebank dehydration is carried out routinely to adjust the moisture content (MC) of samples in preparation for long term storage. Evaluation of sample quality before and after these desiccation treatments is essential. Generally, this evaluation is done by means of germination and viability tests. *In vitro* culture of embryos or embryonic axis can be an interesting alternative to evaluate germplasm quality. Independent of the specie, the beneficial cost and physiological integrity of conserving embryonic axis in cryogenic conditions should be evaluated before adopting this procedure.

Cryopreservation in liquid nitrogen (LN) has been an effective tool for the preservation of seeds with high levels of lipids, such as sunflower, cotton, soybean, peanut and castor (Almeida et al., 2010; José et al., 2010; Abreu et al., 2013; Lopes et al., 2013).

Freezing tolerance of *Jatropha curcas* seeds in LN was accessed previously using seeds with water contents ranging from 2.5 to 8% (Goldfarb et al., 2008; Salomão et al., 2011; Silva et al., 2011), embryos with 9.4% (Prada et al., 2015) and also of embryonic axes using the vitrification technique (Rocha et al., 2010).

The objective of the present study was identifying the best method to assess quality of cryopreserved *Jatropha curcas* seed germplasm at different moisture contents, testing seed germination in moist paper rolls and *in vitro* culture of excised embryonic axes.

MATERIALS AND METHODS

Plant material

Experimental sample consisted of equal number of seeds collected, when they started dispersal, from four different plants of *J. curcas* growing in the field collection of the Jatropha Germplasm Active Genebank maintained by Embrapa Agroenergia in Planaltina, Federal District, Brazil.

Seed moisture content adjustment

Seed initial MC was 6.2% (fresh weigh basis). In order to obtain seeds at different MC, two seed subsamples were placed in a single layer on the attached wire mesh of an acrylic germination box over 375 mL of pure distilled water or 375 mL of silica gel, respectively, for 0 (control), 4, 6, 8, 11 and 24h. After these treatments, the MC of excised embryonic axes was not measured, but the MC of whole seeds was measured by the oven-drying method in which seed samples were placed in an oven maintained at 103±2ºC for 17 hour, using three replications of 10 seeds (Brasil, 2009). The MC was calculated (fresh weight basis):

MC = Weight of fresh seeds - Weight of dry seeds x 100 / Weight of fresh seeds

Freezing in LN and thawing

Subsamples of whole seeds containing different MC, were packed in trifoliate aluminum foil envelopes, closed airtight by heat sealing and frozen by direct immersion in LN, at a cooling rate > - 200°C.min.$^{-1}$. Samples were stored in the liquid phase of LN for four months. Seeds were thawed slowly and for that they were removed from LN and allowed to equilibrate with room temperature (25 ± 2°C) for at least 4 hours.

Decontamination of seed and embryonic axes

Whole seeds (Figure 1A) were decontaminated prior to the germination tests by the immersion of seeds in a commercial solution of detergent [5%] for 10 min., followed by three rinses with tap water. Seeds were then immersed in a commercial solution of sodium hypochlorite (2.5% active chlorine) for 10 min. After this time seeds were brought to a laminar flow hood and rinsed three times with distilled water sterilized by autoclaving (121°C, 1,5 atm, 20 min.) for

removal of the product. To complement the decontamination of seeds, they were then placed in a water bath at 65°C for 8 min (Salomão et al., 2011). In order to soften the structures of the seeds and to allow excisions of the embryonic axes they were immersed for 24 h (Santos et al., 2002).

Viability assessment

Viability of each seed subsample was evaluated using standard germination test in moist germination paper rolls, with four replications of 25 seeds incubated at 25°C (12h photoperiod). For each treatment four replicates of 15 embryonic axes (Figure 1B) were cultivated individually in test tubes containing 10 ml of culture medium WPM supplemented with 3% activated charcoal (Lloyd & McCown, 1981). Embryonic axes were cultivated in growth room at 25°C, 30-40µmol.m^{-2}.s^{-1} light intensity (12h photoperiod). Daily seeds and embryonic axes were evaluated in order to count the normal seedlings (Figure 1C and D).

For the germination test using whole seeds it was considered as a normal seedling those that presented a root system including a central pivoting radicle and four peripheral roots, hypocotyls with white-green color and cotyledons partially enclosed by endosperm (Figure 1C). For embryonic axis grown *in vitro* it was established that normal seedlings were those showing a root system consisting of a central pivoting radicle and four lateral roots, hypocotyls of

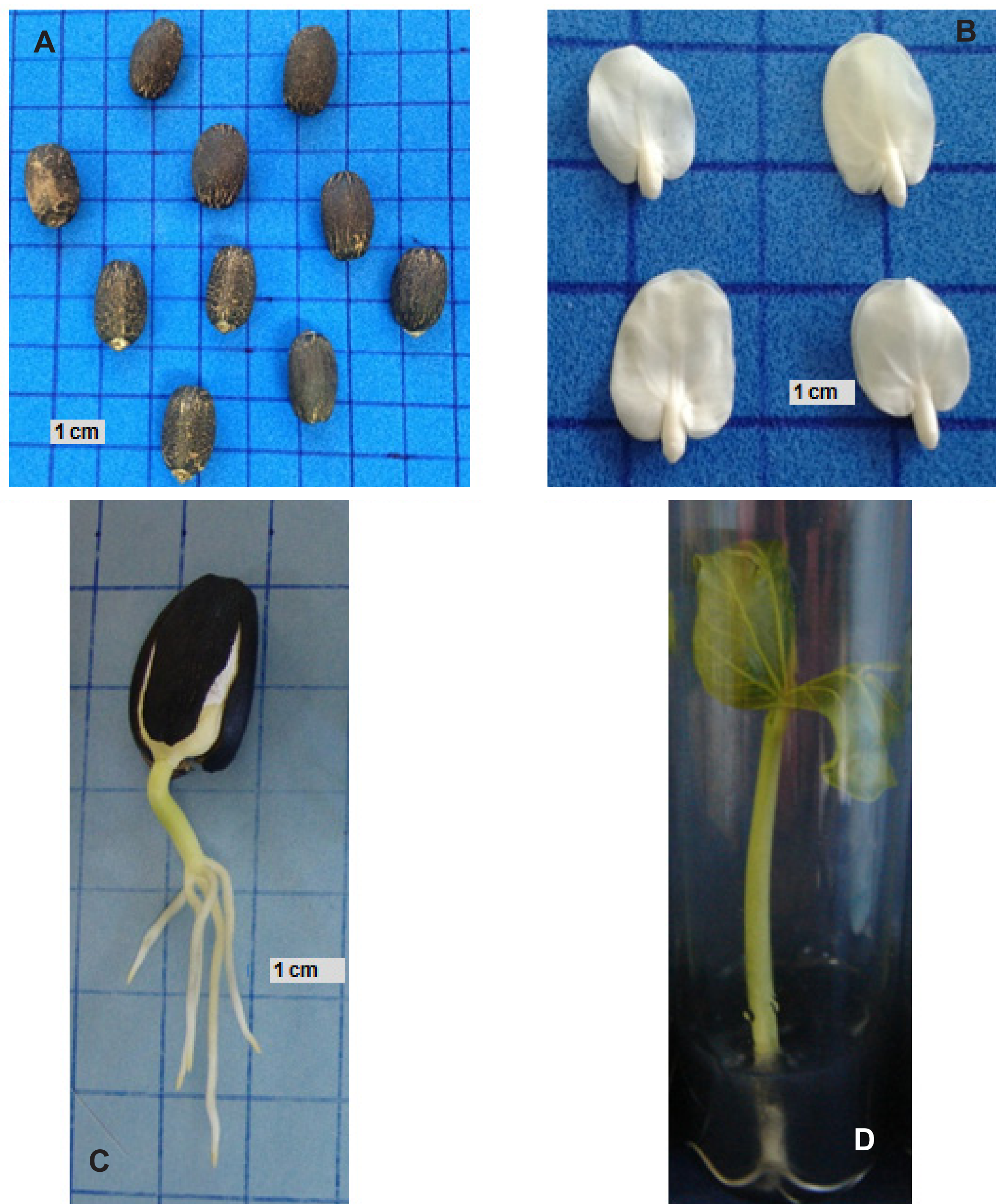

FIGURE 1. (A) Whole seeds, (B) excised embryonic axes, (C) and (D) normal seedlings of *Jatropha curcas* L.

white-green color with expanded cotyledons (Figure 1D) (Nunes et al., 2009).

Statistical analysis

The experimental design is four treatments with four replications / treatment (whole seeds and embryonic axes). The comparison between treatments was done by analysis of deviancies (ANODEV) using generalized linear models with normal distribution and logarithmic link function to check if the variables whole seed or embryonic axis, hydration or dehydration and freezing or not effect on the percentage of germination of whole seeds and embryonic axes. When there was significant in the variables were compared by orthogonal contrasts in the model that had the lowest AIC (McCullagh & Nelder, 1989).The correlation was made through the scatter and Pearson's correlation between MC and percentages of whole seed and embryonic axis germination.

RESULTS

It was observed a significant effect of the variables tested (whole seed, embryonic axis, hydration, dehydration and freezing or not) on the germination rates of whole seeds and embryonic axes. The linear model that best fit to the data (AIC = 1392.32) is described in Table 1.

The control germination percentages were 63% for whole seeds, with a 6.2% MC, and 81% for excised embryonic axes. After exposure to LN, it was observed a significant reduction in germination percentages, 48% (whole seeds) and 57% (excised embryonic axes). The reduction in germination values, probably, may have been attributed to fungal contamination. The seedlings obtained were not considered normal, due to infection (Figure 2 A and B).

At the end of hydration and dehydration treatments whole seeds achieved 9.2% and 4.3% MC, respectively (Table 2). Whole seeds with 9.2% or 4.3% MC reached 62% germination, values similar to the controls (Figures 3A and B). In contrast, seeds subjected to those treatments and subsequently exposed to LN showed a significantly lower germination percentages, 7% (9.2% MC) e 15% (4.3% MC) (Figures 4A and B). In this case, the average time for obtaining normal seedlings was six days after seeding whole seeds hydrated or dehydrated and control. This time went to eight days for the whole seeds submitted to freezing.

Embryonic axes excised from hydrated or desiccated whole seeds presented high germination percentages, with the exception of embryonic axes excised from seeds with 9.2% MC, which showed 56% germination (Figures 3A and B). After freezing, there were no significant differences in germination percentages between embryonic axes excised from whole seeds with 8.0% MC. (98%), 8.5% MC (100%) and 9.2 MC (94%). The higher germination percentage was obtained for embryonic axes excised from whole seeds dehydrated with 5.5% MC

TABLE 1. Analysis of deviancies (ANODEV) obtained from the normal model with logarithmic link function (P = 0.05 level of significance)

Variables	Sum of squares	Degrees of freedom	F	P value
Whole seed Embryonic axes	113486	1	334.4555	<0.00001
Hydration Dehydration	1443	1	04.2514	0.04088
+LN -LN	5532	1	16.3038	0.00008

TABLE 2. Moisture contents of whole seeds of *Jatropha curcas* L. hydrated and dehydrated over different periods.

Treatment periods (hours)	Moisture content (%)	
	hydration	dehydration
0	6,2	6,2
4	6,8	5,8
6	7,9	5,8
8	8,0	5,5
11	8,5	4,9
24	9,2	4,3

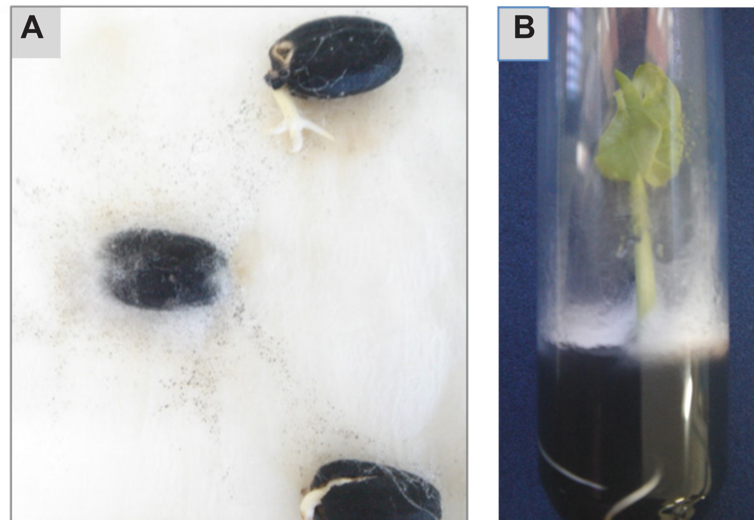

FIGURE 2. Fungal contamination of whole seed (A) and embryonic axis (B) of *Jatropha curcas* L.

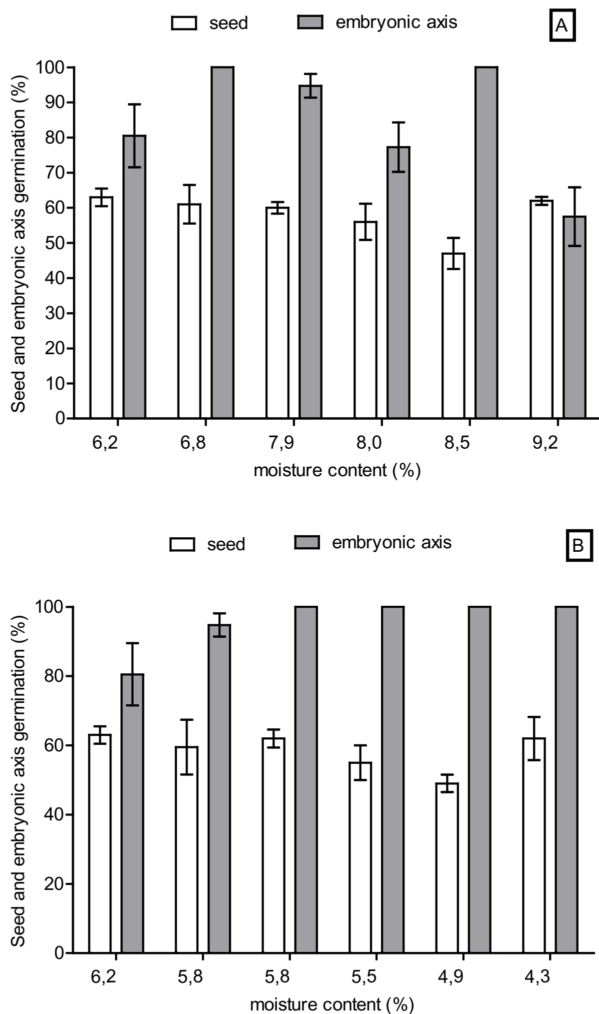

FIGURE 3. Whole seed and embryonic axis germination percentages of *Jatropha curcas* L. subjected to hydration (A) and dehydration (B). Every column represents the mean of four replicates and the error bars indicate standard deviation.

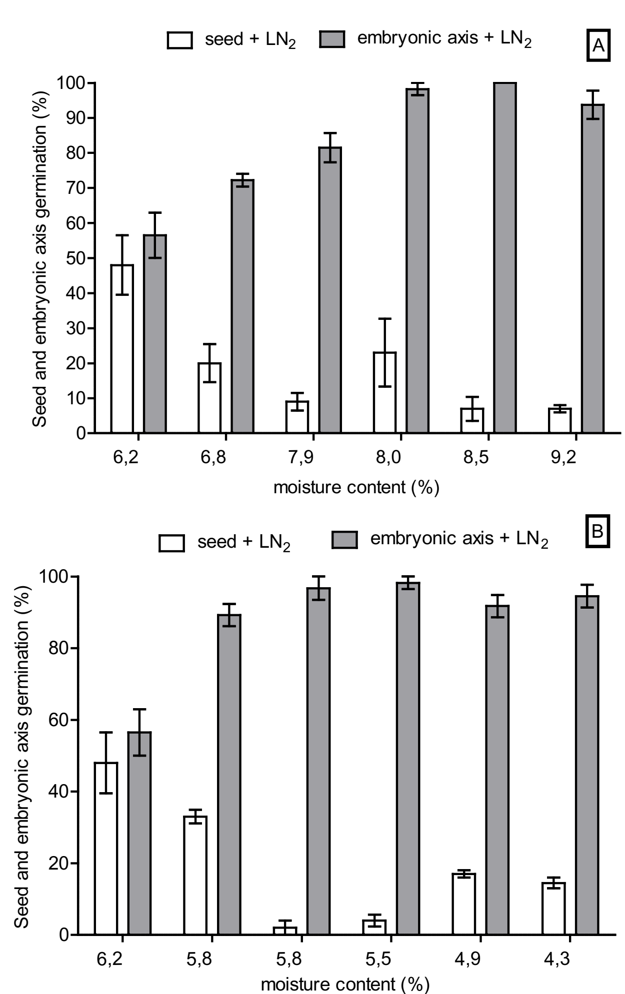

FIGURE 4. Whole seed and embryonic axis germination percentages of *Jatropha curcas* L. subjected to hydration and in liquid nitrogen (LN) exposure (A) and dehydration and in liquid nitrogen (LN) exposure (B). Every column represents the mean of four replicates and the error bars indicate standard deviation.

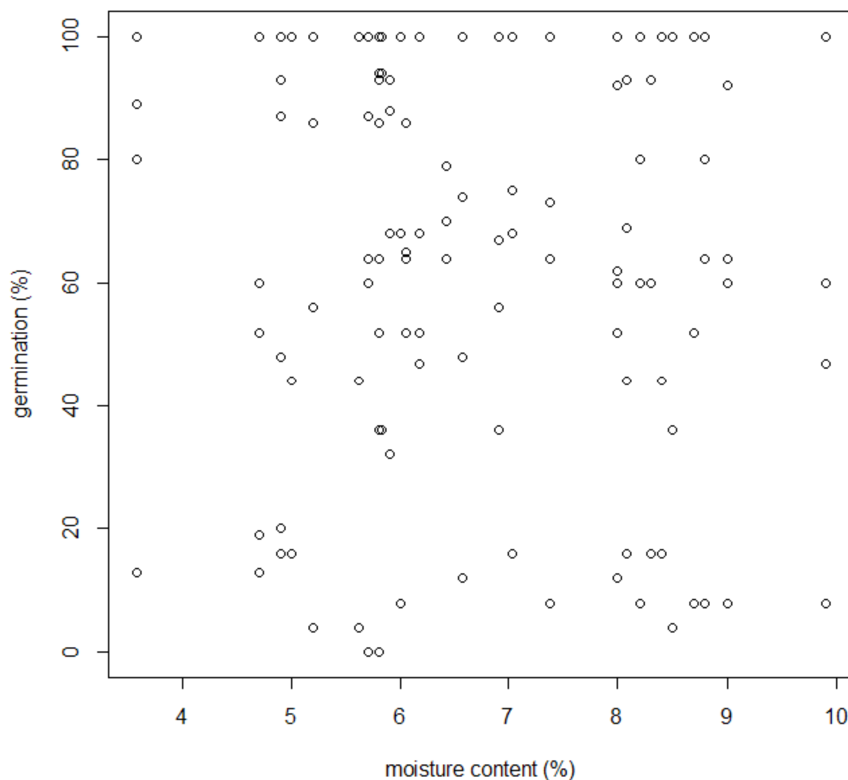

FIGURE 5. Spread between moisture content and germination percentages of whole seeds and excised embryonic axes of *Jatropha curcas* L. (Pearson's correlation=-0.07).

after freezing (Figures 4A and B). Embryonic axes produced normal seedlings after five days of culture.

There were no significant differences between germination percentages of embryonic axes excised from frozen or not frozen seeds, regardless of different MC. In contrast, there was a significant reduction on the germination percentages of whole seed exposed to LN (contrast = 0.17, standard error = 0.04, t = 4.09, p = 0.001).

Comparing the variables by orthogonal contrasts it was possible to verify that the average germination percentage was higher in embryonic axes than whole seeds (contrast = 0.90, standard error = 0.05971746, t = 9.15, p <0.0001) (Figures 3 and 4). The pattern of relationship between MC and the percentage of germination of whole seeds and embryonic axes was completely random, Pearson's correlation = -0.07 (Figure 5).

DISCUSSION

The best methodology to evaluate seed germplasm following storage in LN must be one that allows full expression of the germination potential of the seed sample. However, many internal and external factors can affect germination capacity of the seed. Factors such as origin, quality and integrity of seeds can interfere both in germination and in vigour from lots of *J. curcas* (Brenha et al., 2012; Loureiro et al., 2013). The seed sample used in this study was composed of seeds of four different plants, which probably varied as to their physiological maturity stage or phytosanitary condition, and even their oil content. Possibly due to these factors, or the interaction between them, the highest initial germination percentage of whole seeds was 63%. In contrast, *in vitro* the germination percentages of embryonic axis excised from these seeds was on average 81%.

Physiological and phytosanitary status of the plant material can also have a detrimental effect on its survival after freezing in LN, as was observed with embryos of other oleaginous species (Engelmann et al., 1995; Michalak et al., 2013). The decrease in germination percentages observed for whole seeds (48%) and embryonic axis (57%) of *Jatropha* after exposure to LN was probably a result of a poor sanitary state of the seeds or a random contamination in this sample. Whole seeds and embryonic axes showed extensive fungal contamination during the germination test (Figure 2). So the reduction in germination percentages might have been caused more by contamination rather than by freezing damage.

There is no consensus regarding the most favorable MC interval which allows cryopreservation of whole seeds of *Jatropha*. Significant decline in

germination percentages of these seeds occurred when were frozen with ≤ 6.4% MC (Silva et al., 2011). In contrast, seeds cryopreserved with MC between 4 and 8% had no significant reduction in their germination percentages and vigour (Goldfarb et al. 2008 and 2010). In this later study, it was observed that regardless of the MC, germination percentages of embryonic axis excised from cryopreserved seeds was significantly superior to the percentages obtained with germination of whole seeds. Embryonic axis excised from seeds cryopreserved at 4.3; 4.9; 5.5; 5.8; 8.0; 8.5 or 9.2% MC and cultured *in vitro* showed germination percentages superior to 90%. The high survival rate of excised embryonic axes of *Jatropha* seeds, after freezing has been evidenced for species with lipid seeds such as peanuts (Normah & Makeen 2008). The freezing process did not interfere on the time required for embryonic axis to grow into normal seedlings, which was typically 5 days. In contrast, whole seeds in the control treatment required 6 to 8 days to germinate and produce a normal seedling. In this case, it seems that embryonic axes were protected against injuries by the surrounding structures during freezing and thawing activities. Additionally, it is possible that whole seeds required more time to partially repair destabilizing events caused by cryopreservation process.

The chemical composition of lipid seed determines its longevity (Balešević-Tubic et al., 2010). Generally, lipid seeds are tolerant of dehydration, but may be sensitive to freezing in LN (Bhowmik et al., 2011). Besides, lipid seeds containing unsaturated fatty acids in its composition may have the negative effect of the transition phase during freezing attenuated (Chmielarz 2009). However, cryopreservation has been indicated with efficient conservation method for lipid and not long-lived seeds (Michalak et al., 2013).

The lipid constitution of *Jatropha* seeds includes high concentrations of unsaturated fatty acids such as oleic acid and linoleic acid (Furquim et al. 2014). The possible damage occurring in the endosperm of the seeds did not reach the embryonic axis, as observed in other species with lipid seeds (Dussert 1997; Normah & Makeen, 2008; Lopes et al., 2013). In addition, as observed by Rocha et al (2010), undifferentiated cells on the embryonic axis of *Jatropha* are less prone to suffer damage due to freezing in LN.

As reported by Halmagyi & Pinker (2014), germination percentage of embryonic axes excised from cryopreserved *J. curcas* seed was 95%, while the percentage of germination was 64% for zygotic embryonic axis cryopreserved using droplets vitrification. It is proposed, therefore, that for the management of collections of *J. curcas* seed germplasm is considered the assessment by *in vitro* culture of embryonic axes. The results obtained on the present work are in agreement with their observations, since cryopreserved whole seeds and subsequent excision and *in vitro* culture of embryonic axes was more efficient and allowed best germination percentages. Likewise, *in vitro* germination was also superior to standard germination test to evaluate viability of control (not frozen) embryonic axes.

The methodology reported here is simple, efficient and cost effective and can therefore be applied on a routine basis for long term storage and management of seed germplasm of *J. curcas* in cryogenic genebanks.

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