

## A method for seedling recovery in *Jatropha curcas* after cryogenic exposure of the seeds

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Received 25-X-2010. Corrected 10-V-2011. Accepted 09-VI-2011.

**Abstract:** Actually, the germplasm of *Jatropha* spp. is conserved as whole plants in field collections. Under this storage method, the genetic resources are exposed to disease, pest and natural hazards such as human error, drought and weather damage. Besides, field genebanks are costly to maintain and with important requirements of trained personnel. Thus, the development of efficient techniques to ensure its safe conservation and regeneration is therefore of paramount importance. In this work we describe a method for *Jatropha curcas* seeds cryoexposure and seedling recovery after thawed. In a first experiment, an efficient protocol for *in vitro* plant recovery was carried out using zygotic embryo or seeds with or without coat. In a second experiment, desiccated seeds with or without coat were exposed to liquid nitrogen and evaluated after cryoexposure. Germination percentages were variable among treatments, and seeds demonstrated tolerance to liquid nitrogen exposure under certain conditions. Seeds of *J. curcas* presented up to 99.6% germination after seed coat removal. Seeds with coat cultured *in vitro* did not germinate, and were 60% contaminated. The germination of the zygotic embryos was significantly higher in the ½ MS medium (93.1%) than in WPM medium (76.2%), but from zygotic embryo, abnormal seedlings reached up to 99%. Seeds with coat exposed to liquid nitrogen showed 60% germination in culture after coat removal with good plant growth, and seeds cryopreserved without coat presented 82% germination, but seedlings showed a reduced vigor and a significant increase in abnormal plants. Seeds cultured *in vitro* with coat did not germinate, independently of cryoexposure or not. This study reports the first successful *in vitro* seedling recovery methodology for *Jatropha curcas* seeds, after a cryopreservation treatment, and is recommended as an efficient procedure for *in vitro* plant recovery, when seeds are conserved in germplasm banks by low or cryo-temperatures. Rev. Biol. Trop. 60 (1): 473-482. Epub 2012 March 01.

**Key words:** *Jatropha* sp., *ex situ* conservation, cryopreservation, plant genetic resources, plant tissue culture, agroenergy, biodiesel.

Recently, biodiesel has become more attractive because of its environmental benefits and the fact that it is made from renewable resources. Considerable research has been done on vegetable oils as diesel fuel. *Jatropha curcas* (Linnaeus) is a hardy perennial shrub or tree thought to be native from Central America and possibly from Brazil (Carvalho *et al.* 2008). *Jatropha* is a large genus comprising more than 170 species that is widespread throughout the

tropical regions of the world (Deore & Johnson 2008). Most of these species are ornamental, except for *J. curcas* and *J. glandulifera*, which are oil-yielding species (Swarup 2004).

In fact, there is a growing interest in the cultivation of *J. curcas* for the production of oil as a fossil fuel substitute. The seeds of *J. curcas* contain at least 30-40% oil with a fatty acid pattern similar to that of edible oils (Gubitz *et al.* 1999). *Jatropha* oil contains linoleic acid

and oleic acid, which together account for up to 80% of the oil composition. Palmitic acid and stearic acid are other fatty acids present in this oil (Deore & Johnson 2008). The fact that the oil of *J. curcas* cannot be used for nutritional purpose without detoxification makes its use as an energy source for fuel production very attractive. Besides, this euphorbia is a drought-resistant plant which grows on wasteland and could easily be cultivated by low income farmers. Also, like all trees, it fixes atmospheric carbon (Openshaw 2000).

However, the full potential of *Jatropha* is far from being realized. The growth and management is poorly documented and it lacks an improved germplasm for high yielding. While *J. curcas* germplasm is being harvested all over the world with the purpose of crop improvement, little is known about the germination and storage behavior of *Jatropha* seeds in order to improve the breeding programmes (Carvalho *et al.* 2008). Actually, the germplasm of *Jatropha* spp. is conserved as whole plants in field collection. With this storage method, the genetic resources are exposed to disease, pest and natural hazards such as human error, drought, and weather damage. In addition, field genebanks are costly to maintain and trained personnel requirements are very important (Engelmann 1991). Thus, the development of efficient techniques to ensure its safe conservation is therefore of paramount importance.

Cryopreservation, i.e., the storage of biological material at ultra-low temperature, usually that of liquid nitrogen (-196°C, LN), is the only technique currently available to ensure the safe and cost-efficient long-term conservation of germplasm. At this temperature, all cellular divisions and metabolic processes are decreased at minimum limits and, the plant material can thus be stored for a theoretically unlimited period of time (Walters *et al.* 2004). Moreover, cultures are stored in a small volume, protected from contamination, and require a very limited maintenance (Engelmann 2004).

Although research on the development of conservation and cryopreservation techniques have been conducted with seeds of numerous

plant species (Walters *et al.* 2004, Chmielarz 2009 a,b), up to now, there is no report in a methodology for seedling regeneration of *Jatropha* species after treatments of cryopreservation. This paper presents the first successful report on seedlings recovery after cryopreservation of *Jatropha curcas* seeds.

## MATERIALS AND METHODS

Mature seeds of *Jatropha curcas* were obtained from growers of Janaúba City (Minas Gerais, Brazil) (15°48'35" S - 43°18'28" W at 516m in elevation), and stored at temperature of 27±2°C until use. Seeds were weighted and oven-dried at 105°C for 24h to constant weight. The initial seed moisture content was estimated using three replicates of 10 seeds.

**In vitro germination procedures:** In order to develop an efficient protocol for in vitro plant recovery after cryopreservation process, treatments were carried out as the following procedure: after being washed in running tap water for five min, the seeds were surface sterilized by soaking in 70% (v/v) ethanol for three min, and 2.5% (w/v) sodium hypochlorite for 20 min. Seeds were rinsed three times with sterile distilled. For *in vitro* germination, three treatments including seeds with (1) or without (2) coat and zygotic embryos (3) were evaluated. For seeds without coat, before the inoculation on culture media, a second superficial sterilization was realized in 70% (v/v) ethanol for one min, and 1.25% (w/v) sodium hypochlorite for 15min, following by rinsing three times in sterile distilled water. Zygotic embryo isolation was performed under sterile conditions, in a laminar flow hood, with the help of surgical blades and tweezers.

The explants were placed into test tubes (25x150mm) on MS (Murashige & Skoog 1962) with the macro- and micronutrients at half-strength (½ MS), and WPM (Wood Plant Medium, Lloyd & McCown 1980) medium, without plant growth regulation. All media contained 2% (w/v) sucrose, 0.6% agar and 0.03% (w/v) activated charcoal. The media

were adjusted to pH 5.8 before being autoclaved at 121°C for 15min. The explants were cultured under 12h light/12h darkness photoperiod (50µmol/m<sup>2</sup>s light intensity provided by daylight white fluorescent tubes) and 26±2°C. Seed germination *in vitro*, development of seedlings (plant height, number of roots) and culture contamination were determined weekly for 21 days.

This experiment was conducted as completely randomized design with five replications and 20 seeds/replicate.

**Cryoexposure of seeds:** A lot composed of 300 seeds was divided in five treatments (60 seeds each), being the two first considered as control (no exposure to LN). Before cryopreservation, seeds were hermetically sealed in laminated aluminum foil packets (15 seeds per packet), and placed in cryotanks to be exposed directly into LN. After 24h of storage in LN, seeds were thawed at room temperature (22-25°C) for 24h and germinated.

The germination test-control and cryopreserved seeds -were carried out on ½ MS medium, with 0.03% (w/v) activated charcoal, as the following treatments: 1) whole seeds (with the coat) sterilized in 70% (v/v) ethanol for 3min and 2.5% (w/v) sodium hypochlorite for 20min; 2) whole seeds (without the coat), the same procedure of treatment one was followed, but after seed coat was removed, a new sterilization was made to the seed endosperm with 70% (v/v) ethanol and 1.25% (w/v) sodium hypochlorite for 15min, followed by rising three times in sterile distilled water (control); 3) cryoexposure of seeds after seed coat removal, following the sterilization of endosperm after thawed; 4) cryoexposure of seeds with coat, following the superficial sterilization of the seed after thawed, and 5) cryoexposure of seeds with coat, following by tegument removed and superficial sterilization of the tegument after thawed.

All cultures were maintained in the same conditions used for *in vitro* germination procedure. In this experiment, fifteen seeds were

used per treatment, and the experiment was replicated four times.

The following parameters were assessed: germination (%), shoot height (cm), fresh (FW) and dry (DW) matter weight of aerial, roots and whole plants (g). Germination was determined for all treatments every seven days for 21 days, and it was considered when the radicle protruded at least 3mm. Seedling emergence was estimated to observe abnormal seedlings formation. The plant height was considered as the distance between the medium surface and the insertion of the last visible leaf sheath. After 21 days the roots and aerial parts of five plants per treatment, selected at random, were used to measure fresh and dry matter weight (at 65°C, until constant weight). After a month in culture, the remaining seedlings were transferred to the greenhouse into pots with commercial substrate (Plantmax®), and observed over a period of up to three months for any phenotypic variation.

The experiment was repeated twice, and all data were subjected to analyses of variance (ANOVA) and comparisons of means were made with Tukey's multiple comparison Test at 5%.

## RESULTS

Seeds of *J. curcas* after coat removal presented 99.6% and 98.3% germination at the end of 21 days of *in vitro* culture, on ½ MS and WPM media, respectively. Significant differences were observed between seeds and zygotic embryos. Zygotic embryos present lower germination in the WPM, compared with those of the ½ MS medium, with germination percentages of 76.2% and 93.1%, respectively. Seeds with coat on the ½ MS and WPM media did not germinate, and contamination reached 62.7% and 67.3% in both media, respectively, demonstrating a need for larger studies to improve the sterilization method (Table 1).

Seeds without coat germinated on WPM resulted in higher growth of the seedlings, with an average height of 7.7cm after 21 days of *in vitro* growth. However, seeds in the same conditions, germinated on ½ MS medium,

TABLE 1  
*In vitro* germination (%) and contamination (%) from cryopreserved (24h) *Jatropha curcas* seeds after 7, 14 and 21 days of culture

Medium	Explant	Germination (%)			Contamination (%)		
		Days			Days		
		7	14	21	7	14	21
1/2 MS	(+) coat	0.0bA	0.0cA	0.0cA	52.0	62.7	62.7
	(-) coat	76.2aB	99.6aA	99.6aA	0.0	0.0	0.0
	ZE	93.1aA	93.1abA	93.1abA	0.0	0.0	0.0
WPM	(+) coat	0.0bA	0.0cA	0.0cA	60.5	67.3	67.3
	(-) coat	82.2aB	98.3aA	98.3aA	0.0	0.0	0.0
	ZE	76.2aA	76.2bA	76.2bA	0.0	0.0	0.0

Means following by different letters, small letters within a column, and capital letters along rows, within each variable evaluated, differ for the Tukey's test ( $p \leq 0.05$ ).

(+) teg: seeds with coat; (-) teg: seeds without coat; ZE: zygotic embryo.

showed lower growth, presenting an average height of 5.6cm after 21 days. In relation to the number of roots formed after 21 days of seedling growth, no significant differences were observed between the types of explants used or between the culture media tested (Table 2).

A relatively high level was observed for the formation of abnormal seedlings, with atrophied cotyledonary leaves, low vigor and delayed root development (Fig. 1). Seeds cultured in WPM and 1/2 MS medium after coat removal formed on average 20.8% and 33.9%, of abnormal seedlings respectively. For zygotic

embryos, the percentage of abnormal seedlings reached up to 99.6% (Table 2). However, with the exception of seedlings originating from zygotic embryos which did not revert the characteristics of abnormality, all the seedlings of the other treatments, germinated from seeds returned to normal development after acclimatization.

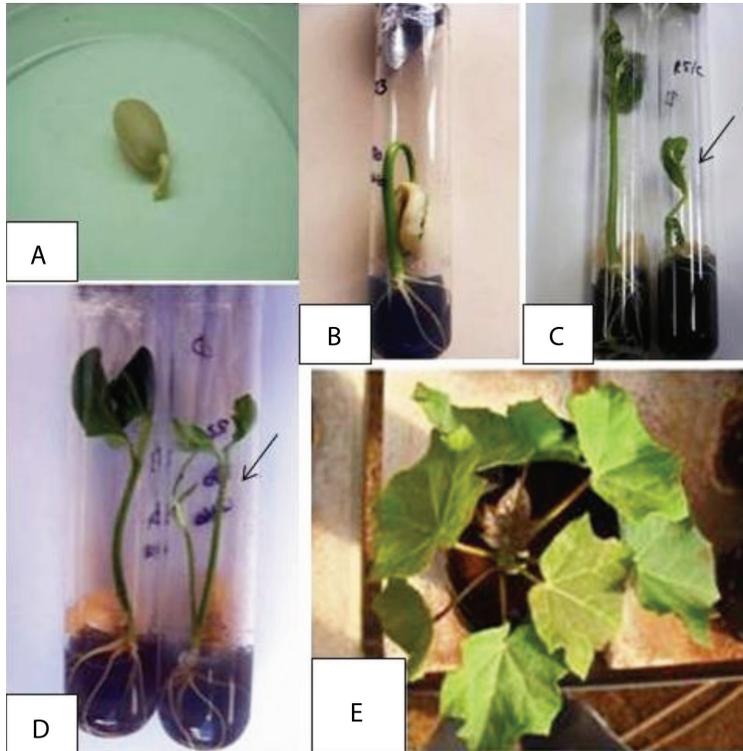
The initial moisture content of cryopreserved seeds was 9.8% with germination values of 82.4% in the treatment where the seeds were stored and germinated without coat (Table 3). Seeds cultured *in vitro* with coat did not

TABLE 2  
*In vitro* plant height (cm), number of roots (per plant) and abnormal plants (%) from cryopreserved (24h) *Jatropha curcas* seeds after 7, 14 and 21 days of culture

Medium	Explant	Plant height (cm)			Root number (per plant)			Abnormal plants (%)		
		Days			Days			Days		
		7	14	21	7	14	21	7	14	21
MS 1/2	(+) coat	0.0cA	0.0cA	0.0cA	0.0cA	0.0bA	0.0bA	0.0aA	0.0cA	0.0cA
	(-) coat	2.5aB	3.3bB	5.6bA	3.8abB	4.3aAB	4.7aA	0.0aB	33.9aA	33.9bA
	ZE	0.9bcB	1.3cB	1.6cA	3.4bB	4.2aA	4.2aA	0.0aB	0.0cB	99.6aA
WPM	(+) coat	0.0cA	0.0cA	0.0cA	0.0cA	0.0bA	0.0bA	0.0a	0.0c	0.0c
	(-) coat	2.2abC	5.8aB	7.7aA	4.6aA	4.6aA	4.6aA	0.0aB	10.9bA	20.8bA
	ZE	0.7bcA	1.1cA	1.5cA	3.3bB	4.3aAB	4.5aA	0.0aB	0.0cB	97.5aA

Means following by different letters, small letters within a column, and capital letters along rows, within each variable evaluated, differ for the Tukey's test ( $p \leq 0.05$ ).

(+) teg: seeds with coat; (-) teg: seeds without coat; ZE: zygotic embryo.



**Fig. 1.** Seed germination, seedlings development and plant growth after cryopreservation. (A) Germinating seed with the coat removed after 3 days. Bar: 1.4cm. (B) Seedling development after 7 days. Bar: 1.6cm. (C, D) Details of normal and abnormal seedlings (arrows) after 21 days. Bars: 2.1 and 1.7cm, respectively. (E) Plant growth in greenhouse after four months. Bar: 7.5cm.

TABLE 3  
*In vitro* germination (%), contamination (%) and seedling height (cm) from cryopreserved (24h) *Jatropha curcas* seeds after 7, 14 and 21 days of culture

LN Treatment	<i>In vitro</i> culture	Germination (%)			Contamination (%)			Plant height (cm)		
		Days			Days			Days		
		7	14	21	7	14	21	7	14	21
(+) coat (+) LN	(+) coat	0.0bA	0.0cA	0.0cA	68.4aA	75.6aA	77.3aA	-	-	-
(+) coat (+) LN	(-) coat	46.5aA	56.4bA	60.6bA	0.0bA	1.7bA	1.7bA	3.6aB	6.2aA	7.3aA
(-) coat (+) LN	(-) coat	41.5aB	76.7abA	82.4abA	0.0bA	0.0bA	0.0bA	1.5aB	2.8bAB	3.9bA
(+) coat (-) LN	(+) coat	0.0bA	0.0cA	0.0cA	55.5aA	62.9aA	62.9aA	-	-	-
(+) coat (-) LN	(-) coat	52.5aB	88.4aA	90.6aA	0.0bA	0.6bA	0.6bA	2.1aB	6.6aA	8.4aA

Means following by different letters, small letters within a column, and capital letters along rows, within each variable evaluated, differ for the Tukey's test ( $p \leq 0.05$ ).

(+) coat: seeds with coat; (-) coat: seeds without coat; (+) LN: exposure to liquid nitrogen; (-) LN: non exposure to liquid nitrogen.

germinate, and there were no significant differences between these and the control (-LN). However, seeds placed to germinate after removal of the coat (control) presented germination around 91%. In these plants, after 21 days of germination the occurrence of 10% of abnormal plants was observed, with low vigor, atrophied cotyledonary leaves and delayed root development. The percentage of contaminated seeds submitted or not to cryogenic conditions were 77.3% and 62.9%, respectively for those in which the coat was left intact at the moment of germination, showing that the sterilization for seeds with coat was not efficient. On the other hand, it was observed that the percentage of contaminated seeds immersed or not in liquid nitrogen, after removal of the coat, was less than 2% (Table 3).

Seeds cryopreserved with coat and then cultured in vitro without coat, presented around 60% germination after 21 days of in vitro culture. This is significantly lower than that observed for the control treatment and for seeds cryopreserved without the coat (82%, no statistical difference in relation to the control treatment). However, as described previously, contamination was 77.3% in the seeds cultivated with coat, reaffirming the results for the control treatment, a factor which was eliminated with the removal of the coat at the moment of germination in vitro.

In relation to the height of the seedlings, the seeds exposed to liquid nitrogen with coat and germinated in vitro without coat presented vigorous seedlings, with an average height of 7.3 cm at the end of the 21 days of in vitro culture, and did not differ statistically in relation to the control (Table 3). However, in the seeds submitted to liquid nitrogen without coat, even though these presented high levels of germination, reduced vigor was observed, and in this case the plants presented an average height of 3.9cm (Table 3). Such plants produced also a lower number of roots (Table 4). As for the number of leaves, no significant differences were observed between the treatments, with around two leaves per plant after 21 days of cultivation. All the abnormal plants from zygotic embryo and from seeds exposed without coat, when acclimatized, were unable to withstand the acclimatization and died, while those with normal germination and development or those exposed to LN with tegument and germinated without coat survived in 100% in the greenhouse. Such plants demonstrated phenotypically normal growth after two months of acclimatization (Fig. 1), although freezing the seeds of *J. curcas* without the coat resulted in a significant increase in abnormal plants morphologically (31.7%)(Table 4).

In general, seeds exposed to liquid nitrogen present a lower accumulation of fresh and

TABLE 4  
Number of leaves, roots and abnormal plants (%) from cryopreserved (24h) *Jatropha curcas* seeds after 7, 14 and 21 days of culture

LN Treatment	In vitro culture	Number of leaves (per plant)			Number of roots (per plant)			Abnormal plants (%)		
		Days			Days			Days		
		7	14	21	7	14	21	7	14	21
(+) coat (+) LN	(+) coat	-	-	-	-	-	-	-	-	-
(+) coat (+) LN	(-) coat	2.0aB	2.1aB	2.0aB	4.7abA	4.8aA	5.0aA	1.7	11.7	13.3
(-) coat (+) LN	(-) coat	2.0aB	2.0aB	2.0aB	4.3bA	4.3bA	4.3bA	6.7	20.0	31.7
(+) coat (-) LN	(+) coat	-	-	-	-	-	-	-	-	-
(+) coat (-) LN	(-) coat	2.0aB	2.0aB	2.0aB	5.0aA	5.0aA	5.0aA	0.0	5.0	10.0

Means following by different letters, small letters within a column, and capital letters along rows, within each variable evaluated, differ for the Tukey's test ( $p \leq 0.05$ ).

(+) coat: seeds with coat; (-) teg: seeds without coat; (+) LN: exposure to liquid nitrogen; (-) LN: non exposure to liquid nitrogen.

dry mass, particularly those exposed without coat (Table 5). Thus, seeds with coat submitted to liquid nitrogen and cultivated without coat in culture medium resulted in seedlings with a fresh and dry weight which was lower than that of the control treatment, on average 1.3g and 0.12g, respectively. However, the fresh and dry weight of the root did not differ significantly from the control. The seedlings regenerated from the seeds exposed to the liquid nitrogen without coat presented lower vigor than the other treatments. However, the dry and fresh weight of the root did not differ significantly from the cryopreserved seeds with coat, presenting on average 0.1g and 0.01g respectively.

## DISCUSSION

Plant genetic resources or germplasm describes the total genetic diversity of cultivated species and their wild relatives (Ford-Lloyd & Jackson 1991), and conservation of germplasm is the most fundamental aspect of biological conservation. Plant germplasm can be derived from a variety of sources, which will in turn determine the method of its conservation. Seed storage is the most common method used to conserve plant germplasm because of the convenience and relatively low costs involved. This strategy, however, cannot be applied to all plant species as the post-harvest behaviour of their seeds, which determines the most suitable method of conservation, differs.

Cryopreservation of plant materials in the form of whole seeds, excised embryos, reproductive parts and vegetative tissues is gaining momentum as a method for the conservation of germplasm. Cryopreservation refers to preservation at  $-196^{\circ}\text{C}$ , the temperature of liquid nitrogen (LN). The basic concept of LN preservation is that, at this temperature, all metabolic processes in seeds are essentially reduced. Consequently, all sources of seed deterioration that are metabolically related are greatly reduced or stopped, thus providing 'indefinite' preservation. Storage in LN could prevent or significantly reduce such viability loss (Camillo *et al.* 2009). Thus, the result would be the improved maintenance of valuable genetic resources.

Given the variety and specific nature of factors affecting recovery after cryopreservation, specific *in vitro* conditions need to be optimized before cryopreservation can be attempted. Thus, the optimum culture medium for *in vitro* regeneration must also be ascertained. In this work, the initial moisture content of cryopreserved seeds was 9.8% with germination values of 82.4% in the treatment where the seeds were stored and germinated without coat. Seeds germinated without coat into MS and WPM media did not presented significant differences on the germination percentage and just for zygotic embryo the germination was significantly higher in the  $\frac{1}{2}$  MS medium than in the WPM medium, despite abnormal germination reached between 97% to 99% of zygotic embryo in culture, independently of media. It is interesting to note that MS medium has

TABLE 5  
Fresh (FW) and dry matter (DW) weight of aerial parts, roots and whole plant from cryopreserved (24h) *Jatropha curcas* seeds after 7, 14 and 21 days of culture

LN Treatment	<i>In vitro</i> culture	Aerial part FW (g)	Root FW (g)	Total FW (g)	Aerial part DW (g)	Root DW (g)	Total DW (g)
(+) coat (-) LN	(-) coat	1.6a	0.5a	2.1a	0.2a	0.04a	0.24a
(+) coat (+) LN	(-) coat	1.0b	0.3ab	1.3b	0.1b	0.02ab	0.12ab
(-) coat (+) LN	(-) coat	0.4c	0.1b	0.5c	0.05b	0.01b	0.06b

Means followed by different letter within a column differ for the Tukey's test ( $p \leq 0.05$ ).

(+) coat: seeds with coat; (-) coat: seeds without coat; (+) LN: exposure to liquid nitrogen; (-) LN: non exposure to liquid nitrogen.

been successfully used in the *in vitro* culture of various tropical woody plant species, such as *Caryocar brasiliense* Camb. (Landa *et al.* 2000), *Lychnophora pinaster* Mart. (Souza *et al.* 2003) and *Byrsonima intermedia* A. Juss. (Nogueira *et al.* 2004). Experiments with mature, immature and dry seeds of *J. curcas* also revealed that MS medium was efficient to sustain germination and continuous growth of plants under *in vitro* conditions (Nunes *et al.* 2008). On the other hand, seeds cultured *in vitro* with coat did not germinate, and there were no significant differences between these and the control (-LN). These results can probably be attributed to the presence of dormancy in the seeds, as observed by Añez *et al.* (2005) who studied the germination behavior of seeds of *J. elliptica*, and observed that these seeds present physical dormancy caused by the impermeability of the coat, requiring a scarification treatment. It is particularly important to observe also that the occurrence of contamination was 77% in the seeds cultivated with coat, reaffirming the results for the control treatment, a factor which was eliminated with the removal of the coat at the moment of germination *in vitro*.

However, for successful cryopreservation, it is necessary to avoid lethal intracellular freezing, which occur during rapid cooling in LN. In the present study, it was interesting to note that as well as the seeds cryopreserved with coat when seeds were cryopreserved without coat there was germination, but seedling presented reduced vigor, and in this case the plants presented an average height and number of roots per plant lower than those cryopreserved with coat. According to Normah *et al.* (1994) this decline in plant growth and number of roots was probably due to dormancy induced during dry storage of the seeds on embryo axis, as observed with hazelnut (*Corylus avellana*), besides a reduction in metabolism caused by exposure of the plant materials to subzero temperatures (Ellis & Roberts 1981, Hor *et al.* 2005). Furthermore, seeds without coat directly frozen in liquid nitrogen resulted in a significant increase in abnormal plants. This fact

can be attributed to injury in the seed tissues, caused by exposure to extreme temperatures.

These results confirm the importance of cryopreserving seeds of *J. curcas* with the coat intact. Almeida *et al.* (2002) reports that seeds with high oil content, due to their chemical composition, are more susceptible to injury and physical damage caused by the exposure to low temperatures. According to Crane *et al.* (2003) and Volk *et al.* (2006), low temperatures crystallize lipids during seed storage and water interactions with crystallized storage lipids can be lethal to seeds. Thus, the seed coat maintenance could prevent this physic-chemical reaction preserving the viability of the seeds, although the reason why water contacting crystallized triacylglycerols is lethal to seeds is not known.

This study confirmed that *J. Curcas* is an orthodox seed and whole seeds can be cryopreserved under certain conditions. To our knowledge this is the first report of cryopreservation of seeds of *J. curcas*, which can contribute to the development of an efficient method for long-term conservation of genetic resources of this species. The procedure described for seedling recovery of cryopreserved seeds of *Jatropha curcas* is relatively simple and reliable whereby cryopreserved seeds germinated and seedlings developed into normal and healthy plants. Although this investigation has been a screening test to establish if *J. curcas* seeds are tolerant to liquid nitrogen when exposure is for a short time, the results obtained indicate that these seeds can withstand LN exposure without detrimental effects and cryopreservation would be another option to their germplasm conservation. Although under *in vitro* condition the germination percentages are high when seeds are deprived of coat, the presence of coat prior to LN exposure was essential to allow proper germination of cryopreserved seeds. In the present study, with the exception of seedlings originating from zygotic embryos which did not revert the characteristics of abnormality, all the seedlings of the other treatments returned to normal development after acclimatization. The application of this protocol for seedling

recovery to other *Jatropha* species and accessions is currently under investigation.

## ACKNOWLEDGMENTS

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasília, DF, Brazil, for financial support and fellowships, and Embrapa Agroenergia, Brasília, DF, Brazil, for providing the seeds for the experiments.

## RESUMEN

Actualmente, el germoplasma de las especies de *Jatropha* ssp. se conserva como plantas enteras en las colecciones de campo. Bajo este método de almacenamiento, los recursos genéticos están expuestos a enfermedades, plagas y desastres naturales tales como el error humano, la sequía y las inclemencias del tiempo. Además, los bancos de germoplasma de campo son costosos de mantener y requieren bastante personal capacitado. Por lo tanto, el desarrollo de técnicas eficientes para asegurar su conservación segura así como su regeneración, es de suma importancia. En este trabajo se describe un método de recuperación para semillas y plántulas criopuestas de *Jatropha curcas* después de descongeladas. En un primer experimento, se llevó a cabo un protocolo eficiente para la recuperación de plantas *in vitro* mediante el uso de embriones cigóticos o semillas con o sin testa. En un segundo experimento, las semillas disecadas, con o sin testa fueron expuestas a nitrógeno líquido y se evaluaron después de la crioposición. Los porcentajes de germinación fueron variables entre los tratamientos, y las semillas demostraron tolerancia a la exposición del nitrógeno líquido bajo ciertas condiciones. Las semillas de *J. curcas* presentaron hasta un 99.6% de germinación después de la eliminación de la testa. Las semillas con la testa cultivadas *in vitro* no germinaron, y el 60% se contaminaron. La germinación de los embriones cigóticos fue significativamente alta en el medio ½ MS (93.1%) en comparación con el medio WPM (76.2%), pero desde los embriones zigóticos, las plántulas anormales alcanzaron más del 99%. Semillas con la testa inmersa en nitrógeno líquido mostraron un 60% de germinación en cultivos después de la remoción de la testa con un buen crecimiento de la planta, y las semillas criopreservadas sin testa presentaron un 82% de germinación, pero las plántulas mostraron un reducido vigor y un incremento significativo de plantas anormales. Semillas con testa cultivadas *in vitro* no germinaron, independientemente de la criopreservación o no. Este estudio reporta el primer éxito *in vitro* de una metodología de recuperación de plántulas para semillas de *Jatropha curcas*, después de un tratamiento de criopreservación, que se recomienda como un procedimiento

eficaz para la recuperación de plantas *in vitro*, cuando las semillas se conservan en bancos de germoplasma a bajas o crio-temperaturas.

**Palabras clave:** *Jatropha* sp., conservación *ex situ*, criopreservación, recursos fitogenéticos, cultivo de tejidos vegetales, agroenergía, biodiesel.

## REFERENCES

- Almeida, F.A.C., A.M. Morais, J.M.F.C. Carvalho & J.P.G. Gouveia. 2002. Criopreservação de sementes de mamona das variedades nordestina e pernambucana. Rev. Bras. Eng. Agr. Amb. 6: 295-302.
- Añez, L.M.M., M.F.B. Coelho, M.C.F. Albuquerque & J.L.D. Dombroski. 2005. Caracterização e morfologia dos frutos, das sementes e do desenvolvimento das plântulas de *Jatropha elliptica* Mull. Arg. (Euphorbiaceae). Rev. Bras. Bot. 28: 563-568.
- Camillo, J., Z.G. Luis & J.E. Scherwinski-Pereira. 2009. Tolerância de sementes de dendezeiro à criopreservação. Pesq. Agropec. Bras. 44: 211-215.
- Carvalho, C.R., W.R. Clarindo, M.M. Praca, F.S. Araujo & N. Carels. 2008. Genome size, base composition and karyotype of *Jatropha curcas* L., an important biofuel plant. Plant Sci. 174: 613-617.
- Chmielarz, P. 2009a. Cryopreservation of dormant European ash (*Fraxinus excelsior*) orthodox seeds. Tree Phys. 29: 1279-1285.
- Chmielarz, P. 2009b. Cryopreservation of dormant orthodox seed of forest trees: mazzard cherry (*Prunus avium* L.). Ann. For. Sci. 66: 405.
- Crane, J., A.L. Miller, J.W. Van Roekel & C. Walters. 2003. Triacylglycerols determine the unusual storage physiology of *Cuphea* seed. Planta 217: 699-708.
- Deore, A.C. & T.S. Johnson. 2008. High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. Plant Biotechnol. Rep. 2: 7-11.
- Ellis, R.H. & E.H. Roberts. 1981. The quantification of ageing and survival in orthodox seeds. Seed Sci. Technol. 9: 373-409.
- Engelmann, F. 1991. *In vitro* conservation of tropical plant germplasm—a review. Euphytica 57: 227-243.
- Engelmann, F. 2004. Plant cryopreservation: Progress and prospects. In vitro Cell. Dev. B. 40: 427-433.

- Ford-Lloyd, B.V. & M.T. Jackson. 1991. Biotechnology and methods of conservation of plant genetic resources. *J. Biotechnol.* 17: 247-256.
- Gubitz, G.M., M. Mittelbach & M. Trabi. 1999. Exploitation of the tropical oil seed plant *Jatropha curcas* L. *Bio. Technol.* 67: 73-82.
- Hor, Y.L., Y.J. Kim, A. Ugap, N. Chabrilange, U.R. Sinniah, F. Engelmann & S. Dussert. 2005. Optimal hydration status for cryopreservation of intermediate oily seeds: citrus as a case study. *Ann. Bot.* 95: 1153-1161.
- Landa, F.S.L., R. Paiva, P.D.O. Paiva & J.S.S. Bueno-Filho. 2000. Indução *in vitro* de calos em explantes foliares de pequi ( *Caryocar brasiliense* Camb.). *Ciênc. Agro.* 24: 56-63.
- Lloyd, G. & B. McCown. 1980. Commercially-feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot-tip culture. *Proc. Int. Plant Prop. Soc.* 30: 421-427.
- Murashige, T. & F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15: 473-497.
- Nogueira, R.C., R. Paiva, A.H. Castro, C.V. Vieira, L.C. Abbade & A.A. Alvarenga. 2004. Germinação *in vitro* de murici-pequeno (*Byrsonima intermedia* A. Juss.). *Ciênc. Agro.* 28: 1053-1059.
- Normah, M.N., B.M. Reed & X.L. Yu. 1994. Seed storage and cryoexposure behaviour in hazelnut (*Corylus avellana* L. cv. Barcelona). *Cryo-Letters* 15: 315-322.
- Nunes, C.F., M. Pasqual, D.N. Santos, T.N. Custódio & A.G. Araújo. 2008. Diferentes suplementos no cultivo *in vitro* de embriões de pinhão-mansão. *Pesq. Agropec. Bras.* 43: 9-14.
- Openshaw, K. 2000. A review of *Jatropha curcas*: an oil palm plant of unfulfilled promise. *Biomass Bioenerg.* 19: 1-15.
- Souza, A.V., J.E.B.P. Pinto, S.K.V. Bertolucci, R.M. Correa & E.M. Castro. 2003. Germinação de embriões e multiplicação *in vitro* de *Lychnophora pinaster* Mart. *Ciênc. Agro.* 26: 1532-1538.
- Swarup, R. 2004. Biotechnological interventions to improve *Jatropha* seeds and oil quality. *SAARC Oil & Fats Today* 8: 39-41.
- Volk, G.M., J. Crane, A.M. Caspersen, L.M. Hill, C. Gardner & C. Walters. 2006. Massive cellular disruption occurs during early imbibitions of *Cuphea* seeds containing crystallized triacylglycerols. *Planta* 224: 1415-1426.
- Walters, C., L. Wheeler & P.C. Stanwood. 2004. Longevity of cryogenically stored seeds. *Cryobiology* 48: 229-244.