academicJournals

Vol. 12(21), pp. 3250-3254, 22 May, 2013
DOI: 10.5897/AJB2012.10391
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Seed cryopreservation of the native cacti *Discocactus zehntneri*, *Pilosocereus gounellei* and *Stephanocereus luetzelburgii* from Bahia, Brazil

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Accepted 20 May, 2013

The native cacti species from Bahia, *Discocactus zehntneri*, *Pilosocereus gounellei* and *Stephanocereus luetzelburgii*, as other members of the Family Cactaceae, have been dramatically affected by illegal traffic, and with the destruction and fragmentation of their habitats. Considering the potential extinction risks for endangered species, and the several applications of these species, some actions on *ex situ* conservation of cacti are required. Thus, this work aimed to evaluate the physiological quality of seeds from *D. zehntneri*, *P. gounellei* and *S. luetzelburgii* stored in liquid nitrogen (-196°C) for 0, 7 and 30 days. For this purpose, seeds of the three species were transferred to cryovials and then immersed directly in liquid nitrogen for 7 or 30 days. After the storage period, the seeds were removed from the nitrogen tank, and thawed for 1 h at ambient temperature and subsequently chemically sterilized. The seeds were inoculated onto Petri dishes lined with two layers of germitest paper. The germination rate of the three species was not reduced by exposure to liquid nitrogen, as well as *D. zehntneri* that had the best results for speed and germination rate observed in seeds stored for 30 days.

Key words: Cacti, cryoconservation, *ex situ* conservation, germination.

INTRODUCTION

The native cacti species from Bahia, *Discocactus zehntneri*, *Pilosocereus gounellei* and *Stephanocereus luetzelburgii*, as their Cactaceae congeners, have been dramatically affected by illegal traffic and by the destruction and fragmentation of habitat (Hubstenberger et al., 1992; Rojas-Aréchiga and Vázquez-Yanes, 2000; Rosas et al., 2001; Santos-Díaz et al., 2003; Dávila-Figueroa et al., 2005; Zappi et al., 2011). According to Zappi et al. (2011), because of the possible risk of extinction of endangered species and even the several applications of these species, some actions on *ex situ* conservation of cacti are required.

An alternative to long time conservation of genetic plant resources is cryopreservation, which ensures the storage of the genetic material and its integrity, requiring little space for the installation of the germplasm bank with a relatively low cost (Santos, 2001; Almeida et al., 2002; Carvalho and Vidal, 2003; Engelmann, 2004; Molina et al.,

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2006; Kaviani et al., 2009; Rocha et al., 2009; Engelmann, 2011; Zappi et al., 2011).

Several explants can be used in cryopreservation, such as cell suspensions, embryos, protoplasts, buds, callus and pollen (Carvalho and Vidal, 2003; Sarasan, 2006; Silva et al., 2011; Srinivasan, 2012; Zhou et al., 2012). However, seed conservation is the easiest, most common and efficient (Santos, 2001; Goldfarb et al., 2010; Pence, 2010) *ex situ* conservation.

Seeds preserve genetic variability, are easy to collect, do not take up a lot of space (Li and Pritchard, 2009) and, further, are the unit of natural propagation for most species of higher plants (Santos, 2001). Moreover, according to Silva et al. (2011), the seeds are the most appropriate structures for conservation because they are more organized systems.

Most of the *ex situ* conservation activities for plant biodiversity, such as cryopreservation, focus on crop species (Engelmann, 2011; Pilatti et al., 2011). Native species, rare and endangered, as many members of the family Cactaceae, remain poorly studied. So far, only a single cacti cryopreservation study is available in the literature (Veiga-Barbosa et al., 2010).

Therefore, this work aimed to evaluate the physiological quality of seeds of *D. zehntneri*, *P. gounellei* and *S. luetzelburgii* stored in liquid nitrogen (-196°C) for 0, 7 and 30 days.

MATERIALS AND METHODS

Mature fruits of *D. zehntneri*, *P. gounellei* and *S. luetzelburgii* were collected from different populations in May and August of 2010 in two localities: Morro do Chapéu and Mucugê, both in Chapada Diamantina, Bahia State. The seeds were extracted from the fruits, dried on filter paper, processed and afterwards stored in paper bags at ambient temperature until mounting of the experiment (October, 2011).

Moisture content of seeds

The moisture content of the seeds was calculated using the oven method at 105 ± 3°C (Brasil, 2009). About 100 seeds of each species were weighed in analytical balance to obtain the fresh weight. Then, the samples were kept in an oven for 24 h and weighed again to obtain the dry weight.

Cryopreservation

Seeds of three species were transferred to cryovials (110 seeds per vial) and then directly plunged in liquid nitrogen (-196°C) for 7 or 30 days, except the control group, which were inoculated immediately. After the conclusion of each storage period, the seeds were removed from the nitrogen tank, thawed for 1 h at room temperature (24 to 25°C) and subsequently sterilized chemically for 1 min in absolute alcohol for 15 min in sodium hypochlorite solution (2.5% active chlorine), and finally washed three times in sterile water. They were seeded in Petri dishes lined with two sheets of germitest paper and wetted with distilled water at a ratio of 2.5 times the paper's mass.

Culture conditions

After inoculation, the experimental units were kept in germinator at 25 ± 3°C under fluorescent light (60 μmol^{-2} s^{-1}) and a 12 h photoperiod.

Variables, experimental design and statistical analysis

Daily, the evaluations were done for 26 days successively and seeds were considered to have germinated when the radicle measured in average had more than two millimeters. The variables analyzed follow' Santana and Ranal (2000) and consisted of: germination (%G), mean germination time (MGT), germination speed index (GSI) and germination uniformity coefficient (GUC). Aiming to break the dependence between the mean and variance of the germination data, expressed as percentages, they were transformed into arccosine.

The experimental design randomization was made by selection of four replications, each of them with 25 seeds. The data were subjected to analysis of variance and means were compared by Tukey test at 5% probability using the statistical program Sisvar 5.1 (Ferreira, 2008).

RESULTS AND DISCUSSION

The increase in time of exposure of seeds to liquid nitrogen did not reduce the germination in the species analyzed (Table 1). Similarly, no significant differences for seed germination were found for *D. zehntneri* and *P. gounellei* undergoing cryopreservation for different periods, as reported for other cacti species as *Cereus jamacaru*, *Melocactus concinnus*, *Melocactus paucispinus* and *Micranthocereus flaviflorus* (Veiga-Barbosa et al., 2010). Ours results corroborate those of others obtained for *Jatropha curcas* (Euphorbiacea) (Goldfarb et al., 2010; Silva et al., 2011) and *Prunus avium* (Rosaceae) (Chmielarz, 2009). However, for *Ricinus communis* (Almeida et al., 2002) and *Melocactus albicephalus* (Veiga-Barbosa et al., 2010), the storage period was inversely proportional to the germination of frozen seeds.

The reduction in germination after cryopreservation may be attributed to contamination with fungi (Salomão, 2002) and/or the high initial moisture content of the seeds (González-Benito et al., 1998; Salomão, 2002; Molina et al., 2006; Rocha et al., 2009; Silva et al., 2011). High levels of moisture facilitate the formation of intracellular ice crystals which cause rupture of the endomembrane system and results in loss of semi-permeability and cellular compartmentalization (Santos, 2001; Kaviani et al., 2009; Goldfarb et al., 2010).

D. zehntneri showed the best germination (20%) and germination speed index (0.205) in seeds stored for 30 days (Table 1). Similar results were obtained for other 15 species from different families: *Astragalus membranaceus, Hedysarum austro-kurilense, Oxytropis chankaensis, O. kamtschatica, Oxytropis ochotensis, Oxytropis revoluta, Oxytropis retusa, Sophora flavescens, Trifolium lupinaster* (Kholina and Voronkova, 2012), *Xyris*

Table 1. Effect of the period of storage in liquid nitrogen on germination (%G), mean germination time (MGT), germination speed index (GSI) and germination uniformity coefficient (GUC) in seeds of *D. zehntneri*, *P. gounellei* and *S. luetzelburgii*.

Species	Cryopreservation period (days)		
	0	7	30
G (%)			
D. zehntneri	0[b]	3[ab]	20[a]
P. gounellei	61[a]	51[a]	63[a]
S. luetzelburgii	0[a]	3[a]	6[a]
MGT (days)			
D. zehntneri	0.00[a]	5.75[a]	14.16[a]
P. gounellei	15.55[a]	16.24[a]	15.18[a]
S. luetzelburgii	0.00[a]	3.25a	13.00[a]
GSI			
D. zehntneri	0.000[b]	0.010[b]	0.205[a]
P. gounellei	1.182[a]	1.137[a]	1.467[a]
S. luetzelburgii	0.000[a]	0.020[a]	0.020[a]
GUC			
D. zehntneri	0.000[a]	0.000[a]	0.065[a]
P. gounellei	0.382[a]	0.115[a]	0.087[a]
S. luetzelburgii	0.000[a]	0.000[a]	0.000[a]

G (%), Germination; MGT, mean germination time; GSI, germination speed index; and GUC, germination uniformity coefficient. Means followed by the same letter in lines do not differ significantly by Tukey test (p>0.05).

tennesseensis (Johnson et al., 2012), *Melocactus albicephalus*, *M. concinnus*, *M.s paucispinus* (Veiga-Barbosa et al., 2010), *Ricinus communis* (Almeida et al., 2002) and *Gossypium hirsutum* (Gonzalez-Benito et al., 1998; Rocha et al., 2009). This increase in germination percentage can be attributed to changes in temperature (Almeida et al., 2002; Rocha et al., 2009) and the breaking or formation of a crack network in the structures surrounding the embryo (Salomão, 2002), which may have caused the breaking of physical dormancy in seeds subjected to ultra-low temperatures. However, *Rubus* spp. treatments with liquid nitrogen did not surpass the efficiency of sulfuric acid in breaking physical dormancy in the seeds (Peacock and Hummer, 1996).

According to Veiga-Barbosa et al. (2010), *D. zehntneri* appears to have some deep dormancy. Thus, the low percentage of germination obtained in this study (Table 1) also corroborates these observations. However, the similar low germination obtained for *S. luetzelburgii* (Table 1) requires more investigation.

Although, protocols for seed cryopreservation of some species emphasized the need of drying the plant material (Kaviani et al., 2009; Graiver et al., 2011) for *D. zehntneri*, *P. gounellei* and *S. luetzelburgii*, the absence of a cryoprotection step did not result in loss of seed viability (Table 1). The same results were registered in literature (Salomão, 2002; Veiga-Barbosa et al., 2010; Johnson et al., 2012; Kholina and Voronkova, 2012), in addition to the simplification of cryopreservation protocols, significantly reducing its costs. According to Engelmann (2004, 2011), some materials such as orthodox seeds and dormant buds exhibit a natural dehydration process and can be cryopreserved without any pretreatment. The seeds showed moisture content between 9 and 12%; standard type of species that have orthodox seeds. The physical intracellular characteristics of seeds (minimization of vacuolation, protection of DNA integrity and orderly dismantling of cytoskeletal elements, balance between reactive oxygen species and abundant antioxidant protein LEA, accumulation of carbohydrate, lipid composition, endogenous amphiphilic substances, oleosinas, amongst others), maintains the physical and physiological quality of desiccation tolerance (Berjak and Pammenter, 2008).

No physical damage was observed after freezing, such as cracks or broken structures in the seed, or unusual development of the plants (Figure 1). Similar results were obtained for *A. membranaceus, H. austro-kurilense, Hedysarum sachalinense O. chankaensis, O. kamtschatica, O. ochotensis, O. revoluta, O. retusa, S. flavescens, T. lupinaster, Vicia amurensis* (Kholina and Voronkova, 2012), *D. zehntneri*, *P. gounellei*, *Melocactus concinnus*, *M. paucispinus, M. albicephalus*, *M. flaviflorus* (Veiga-Barbosa et al., 2010), *Rubus ursinus, R. leucodermis, R. eustephanos, R. multibracteatus, R. chamaemorus, R. parviflorus* (Peacock and Hummer, 1996) and other 66 species of tropical orthodox seeds (Salomão, 2002). Nevertheless, these results disagree with those obtained by Silva et al. (2011), in which prolonged exposure to liquid nitrogen resulted in the development of attached, atrophied and not chlorophyllous cotyledons, in callus formations on the adaxial side of the cotyledons and on the base of the hypocotyl and in plants consisting only of hypocotyl.

Conclusion

Cryopreservation did not alter the physiological quality of the seeds and therefore may be a suitable method for *ex situ* conservation of seeds of *P. gounellei*. However, the evaluation of a longer storage time is suggested.

ACKNOWLEDGEMENTS

We would like to thank the Programa de Pesquisa em Biodiversidade (PPBio) for the funds provided for this work. M.N.G. Marchi was supported with scholarship by the 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior' (CAPES).

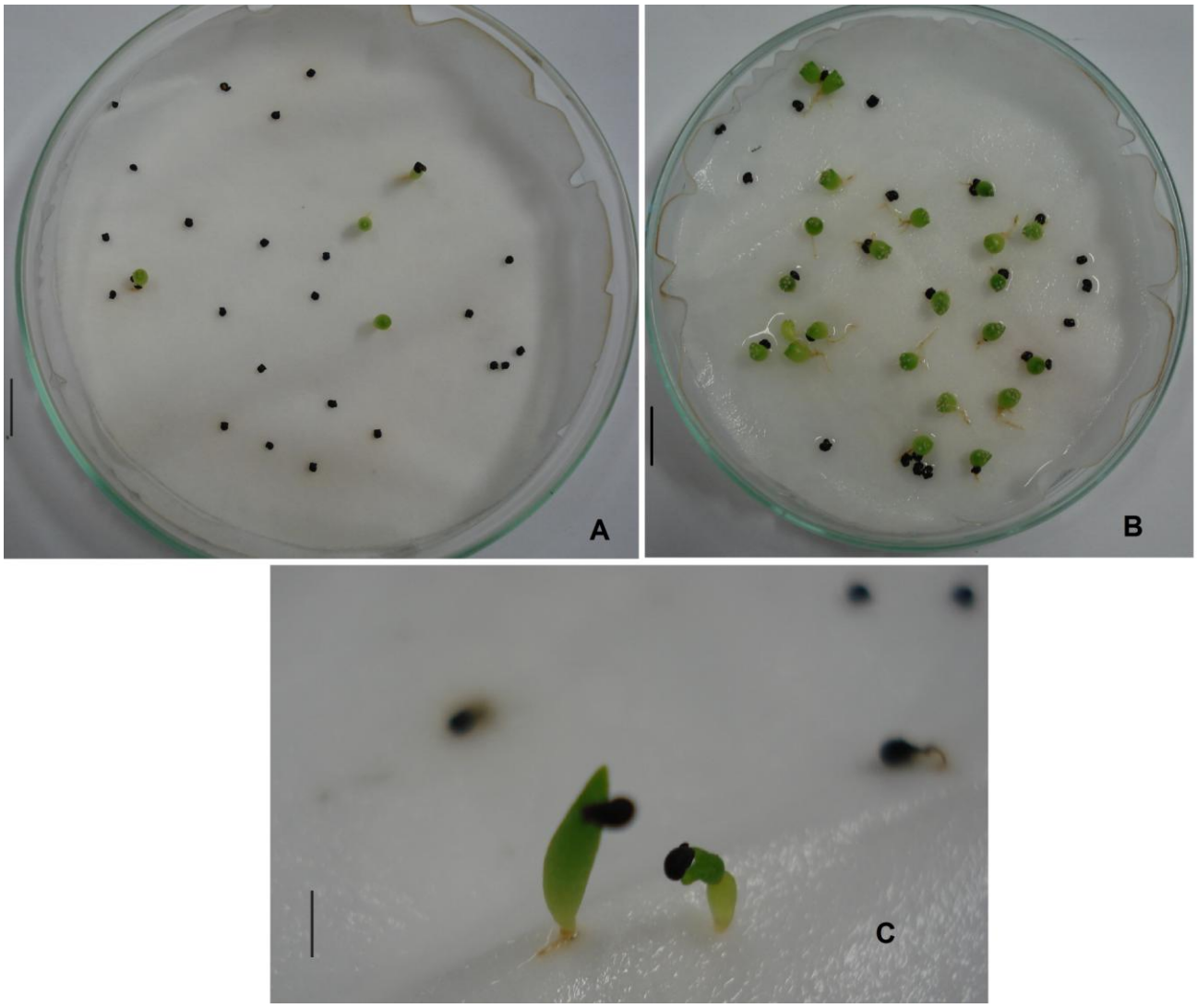

Figure 1. Germination of *D. zehntneri* (A), *P. gounellei* (B) and *S. luetzelburgii* (C) after cryopreservation. (Beam = 1 cm).

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