Tropical seed species' responses to liquid nitrogen exposure

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Received: 01/02/2002, Accepted: 18/07/2002

The ability to tolerate ultra-low temperature (-196 ºC) exposure was evaluated in 66 tropical orthodox seed species of 21 botanical families from the Cerrado (Brazilian Savannah) and Atlantic Forest Brazilian biomes. Liquid nitrogen had no effect on the germinability of 51 seed species. The stimulatory effect of cryogenic temperature on germinability, with or without subsequent chemical scarification, was observed in nine seed species with deep physical dormancy, or heterogeneous levels of seed hardness, or with no dormancy. Significant reduction in germinability occurred in six seed species, presumably because of factors acting individually or in combination on these seeds, such as inappropriate moisture content, or the potentially detrimental effect of rapid cooling (263 ºC.min^{-1}) or fungal contamination. The results obtained suggest that cryopreservation may be a promising alternative for storing most of the seed species tested.

Key words: cryopreservation, *ex situ* conservation, seed dormancy.

Respostas de sementes de espécies tropicais à exposição ao nitrogênio líquido: A habilidade de tolerar a exposição à temperatura subzero (-196 ºC) foi avaliada em sementes ortodoxas de 66 espécies tropicais, pertencentes a 21 famílias botânicas e procedentes dos biomas Cerrado e Mata Atlântica. Nitrogênio líquido não teve efeito sobre a germinabilidade de sementes de 51 espécies. O efeito favorável da temperatura criogênica, seguida ou não de escarificação química, sobre a germinação foi observado em sementes de nove espécies apresentando dormência física ou heterogeneidade de dureza da testa ou não dormentes. Redução significativa de germinabilidade foi observada em sementes de seis espécies, possivelmente devido a fatores atuando individualmente ou não nas sementes, tais como, conteúdo de umidade inadequado, efeito prejudicial do rápido congelamento (263 ºC.min^{-1}) ou contaminação fúngica. Os resultados obtidos sugerem que a criopreservação pode ser uma alternativa promissora para o armazenamento da maioria das sementes das espécies testadas.

Palavras-chave: Conservação *ex situ*, criopreservação, dormência de sementes.

Neotropical ecosystems have long been submitted to constant pressure by human activity (Brown and Brown, 1992). In these ecosystems the number of plants at risk of genetic depletion or extinction and the loss of important genetic resources is continually on the increase. *Ex situ* conservation practices seem to be one of the priority procedures in safeguarding genetic resources especially through germplasm repositories, e.g., in field gene banks or in conventional germplasm gene banks at –20 ºC. But such methods may not represent the ideal conditions for maintaining germplasm securely in the long term (Stanwood, 1985). In field gene banks, germplasm integrity is subjected to the vagaries of environmental biotic and abiotic components (Al-Madeni and Tisserat, 1986; Stushnoff, 1991). Seeds having intermediate or recalcitrant storage behaviour are cold-sensitive; consequently, they cannot be stored in standard seed gene banks at –20 ºC (Roberts, 1973; Ellis et al., 1990).

Although living organisms may suffer severe stresses due to the freezing process, it has been postulated that biological activities are greatly minimised under cryogenic conditions (Stanwood and Bass, 1981). Thus cryopreservation is proposed as the most favourable and safest technique for preserving germplasm and preventing its deterioration (Stanwood, 1985). Protocols for cryopreserving agricultural, horticultural and economically important clonal or heterozygotic crops and fruit species have been developed and can be routinely

adopted, according to previous knowledge about physiological and biochemical species properties, ideal moisture content and cooling/thawing rates (Stanwood and Roos, 1979; Engelmann, 2000). Recently cryopreservation methods have been applied to endangered, threatened and wild species germplasm from a number of diverse, mainly temperate, florae (Pence, 1991; Iriondo et al., 1992; Touchell and Dixon, 1993, 1994; Gonzalez-Benito et al., 1998). Nevertheless, further studies are required to determine the feasibility of using cryogenic storage conditions, especially for tropical species.

In this work the responses to liquid nitrogen exposure were evaluated for 66 tropical orthodox seed species from the Cerrado (Brazilian Savannah) and the Atlantic Forest biomes with a view to adopting cryopreservation as an alternative to conventional seed bank storage for plant germplasm conservation. Fruits of the 66 species of 21 botanical families shown in tables 1 and 2 were collected in the states of Goiás (Cerrado) and Minas Gerais (Atlantic Forest), Brazil. The seeds were extracted from the fruits by pounding, depulping, threshing or removing parts of fruits, according to the fruits' characteristics at the Seed Processing Laboratory. Cleaned seeds without any dehydration treatment were placed in paper or cotton bags and remained at the Seed Processing Laboratory (25 ± 2 ºC) for no longer than three days, after which they were sent to the Seed Quality Control Laboratory. The moisture content (mc) was the determined with three replicates of a variable number of whole seeds, according to seed size and availability, by oven drying at 105 ºC for 24 h. The results were expressed as an average of percentages based on the fresh weight.

No desiccation procedures were carried out because of the wide variation in seed mc. Thus the seeds with their original mc without cryoprotectant treatments were placed in aluminium foil laminate bags and immersed directly in liquid nitrogen (LN). After 3 days' exposure the samples were removed and thawed at room temperature (25 ± 2 ºC) for 3 h. Cooling and thawing rates were measured with Thermocouple Delta OHM TP 956. Before and after cryogenic treatment, germination tests were performed at 25 ºC incubation temperature, photoperiod 16 h light (74.98 $\mu M.m^{-2}.s^{-1}$), with four replicates of a variable number of seeds according to seed availability, rolled in moistened paper towel. The duration of the germination tests was from seven to ca. 60 d. Germination was expressed as a percentage of normal seedlings.

The water-impermeable non-imbibed legume seeds (species in Caesalpiniaceae, Fabaceae and Mimosaceae - table1) that had not germinated at least 30 d after being sown (control and frozen seeds) were scarified with concentrated sulphuric acid for 60 min. Then the seeds were washed in distilled water and sown as described previously.

Data of the final germination percentages were transformed to arc-sine and analysis of variance (ANOVA) was performed.

The results showed that no seed species tested seemed to be completely sensitive to ultra-low temperature exposure (tables 1 and 2). Little loss of viability was recorded after LN treatment when seeds had mc ranging from 3.0 % to 15.0 %, and were cooled at 263 $^{o}C.min^{-1}$ and thawed at 5 $ºC.min^{-1}$. However, different effects of LN on seed germination were observed. No significant difference in germination was observed for control and LN exposed seeds (table 1 and 2) in 51 species.

Dormancy in most legume seeds was not broken by LN exposure. However, this treatment when combined with chemical scarification favoured these seeds' germination, as observed in table 1. These combined treatments improved germination significantly in *Mimosa somnians* var. *viscida* and *Stryphnodendron polyphyllum* . Stimulatory and significant effect of LN in germination occurred in *Bowdichia virgilioides, Pterodon emarginatus, Apeiba tiboubour* species that had hard seeds and no hard seeds in their samples (table 1), or in *Cariniana estrellensis* and *Crotalaria* cf. *spectabilis* with apparently no hard seeds in the samples (table 2).

The action of LN varied among the species whose seeds were surrounded by an endocarp. In *Buchenavia tomentosa* and *Guettarda pohliana,* LN had no effect on germination. Although *Byrsonima basiloba* exhibited low germination values in treated and untreated seeds, the germination percentage increased after LN exposure. Germination percentages increased significantly in *Schinopsis brasiliensis* and *Spondias mombin* in comparison to the control (table 1).

The germination values of *Dimorphandra mollis* (table 1), *Bauhinia acuruana*, *Bauhinia ungulata*, *Dalbergia miscolobium*, *Sterculia striata*, *Styrax camporum* (table 2) seeds declined significantly after LN exposure. After being set to germinate, *D. mollis*, *D. miscolobium*, *B. acuruana* and *B. ungulata* seeds that had

been frozen showed high levels of fungal contamination, mainly by *Aspergillus* spp., *Penicillium* sp., *Rhizopus* sp.. After freezing, the same fungus species proliferation was also apparent for *Ormosia fastigiata* (table 1), *Pseudobombax* cf. *tomentosum* and *Machaerium aculeatum* seeds (table 2). It is possible that the interaction between inappropriate moisture content and rapid cooling rate (263 ºC.min^{-1}) had negatively influenced *S. striata* and *S. camporum* seed response to LN. These combined factors, in addition to fungal infection, presumably affected similarly *B. acuruana* and *B. ungulata* seed germinability after freezing.

Regardless of the seed response to LN or chemical scarification, no physical seed damage, such as cracking or detachment of seed structures, nor abnormal seedling development, was observed.

It is expected that desiccation-tolerant seeds should be well adapted to withstand LN exposure without compromising their physiological and physical integrity. However, these seeds differ in their tolerance or response to ultra-low temperature exposure (Dussert et al., 1998). Moisture content is the prime factor in restricting seed tolerance of cryostorage conditions. Associated with the proper mc, the rates of cooling and thawing are decisive to avoid any damaging effect on seed tissues and cells. The range of acceptable moisture limits to cryogenic temperature tolerance, as well the cooling/thawing rates, is species-specific and requires individual determination (Dickie and Smith, 1995; Pritchard, 1995; Potts and Lumpkin, 1997). Seeds of 60 species with mc varying from 3.0 % to 15.0 % preserved or increased their germination values after LN exposure, using rapid cooling (263 ºC.min^{-1}) and slow thawing (5 ºC.min^{-1}). Loss of germinability after LN exposure, probably due to the interaction between inappropriate moisture content and rapid cooling rate (263 ºC.min^{-1}), was verified in *Styrax camporum* (3.0 % mc) and *Sterculia striata* (11.1 % mc). It is possible that the low or high moisture limits of these seeds was surpassed, demanding manipulation of the cooling rate. Reduction in the germinability or in survival level, as a result of inappropriate mc and cooling/warming rates, was reported for soybean and sunflower (Verttuci, 1989), *Populus deltoides* (Pence, 1996), *Coffea costatifructa*, *Coffea sessiliflora* and *Coffea arabica* (Dussert et al., 1998, 2000). LN treatment did not harm germination in seeds with mc higher than *S. striata*, such as *Mimosa somnians* var. *viscida* (12.2 %), *Dioscorea* sp. (12.4 %), *Stryphnodendron pulcherrimum*. (13.0 %), *Crotalaria* cf. *spectabilis* (15.0 %) or almost equal to *S. camporum*, such as *Apuleia leiocarpa* (3.5 %) and *Byrsonima basiloba* (3.0 %). The freezing behaviour of these seeds makes it evident that the interaction between mc and cooling rate is complex and species-specific. An analogous response to LN is not found even for related species (Pence, 1991). This was evident for the three species of *Bauhinia. Bauhinia* sp. (6.9 % mc) retained seed germinability after being frozen, while *Bauhinia acuruana* (5.7 % mc) and *Bauhinia ungulata* (5.2 % mc) frozen seed germination decreased significantly and exhibited a great predisposition to fungal proliferation.

It has been reported that LN removes physical dormancy, inducing disruption or a network of cracks in the structures that surround the seed, such as the endocarp (Normah and Vengadasalam, 1992; Salomão, 1995) or immediately surround the embryo, such as the seed coat (Busse,1930; Brant et. al., 1971). These findings are reinforced by the results obtained in this study for the species that have seeds surrounded by an endocarp. The final germination percentages after LN treatment increased in *Buchenavia tomentosa, Byrsonima basiloba and Guettarda pohliana* and, more remarkably, in *Schinopsis brasiliensis* and *Spondias mombin*. In this case, it is supposed that the level of mechanical restraint imposed by the endocarp varied among these species. In *B. tomentosa* and *G. pohliana* the endocarp seemed not to restrain the germination process severely, and LN had no significant effect on germination. Liquid nitrogen had a partially beneficial effect on *B. basiloba*, which seemed to have a harder endocarp or an poor original seed quality, due to the low germination values observed before and after LN treatment. *S. brasiliensis* and *S. mombin* probably have an intermediate level of endocarp hardness and LN acted more efficaciously.

The physical dormancy caused by testa impermeability to water is a characteristic shared by a great number of Caesalpinoideae, Mimosoideae and Fabaceae species. The intensity of seed hardness varies among the legume species and within the seeds of the same sample, possibly due to the differences in their seed coat structures, physical and chemical properties (Kelly et. al., 1992; Morrison et. al., 1998). These differences might be reflected in seed response to LN

treatment. As verified in species with seeds covered by endocarp, LN significantly enhanced germination in *Bowdichia virgilioides* and *Pterodon emarginatus* (Fabaceae) and *Apeiba tibourbou* (Tiliaceae) with variable numbers of hard seeds in the samples. In contrast, LN did not revoke the deep seed physical dormancy in *Bauhinia* sp., *Chamaecrista desvauxii*, *Dimorphandra mollis*, *Senna* sp., *Albizia* sp., *Enterolobium contortisiliquum*, *Enterolobium gummiferum, Mimosa somnians* var. *viscida*, *Mimosa* sp., *Stryphnodendron polyphyllum, Stryphnodendron pulcherrimum* and *Ormosia fastigiata.* For these species, the hardseededness was removed by sulphuric acid treatment. The stimulatory effect of the combined treatments, LN exposure followed by chemical scarification, was more evident in *Mimosa somnians* var. *viscida*. and *S. polyphyllum* seed germination. For the other species, these combined treatments did not lead to reduction in germination percentages when compared to the control, with the exception of *D. mollis* and *O. fastigiata*.

It has previously been observed that LN can render seed tissues more susceptible to fungal proliferation (Berjak and Dumet, 1996). A high level of fungal infection by saprophytic species of *Aspegillus, Penicillium* and *Rhizopus* occurred after LN exposure in *Pseudobombax* cf. *tomentosum*, *Machaerium aculeatum*, *Dalbergia miscolobium*, *Dimorphandra mollis, Ormosia fastigiata*, *Bauhinia acuruana* and *Bauhinia ungulata*. This contamination interfered with the germination assessment and consequently with the evaluation of the response of those seeds to LN.

It appeared that rapid cooling (263 ºC.min^{-1}) and subsequent slow warming (5 ºC.min^{-1}) or LN exposure followed by chemical scarification did not induce seed physical damage or abnormal seedling development for most species tested. In most cases, the seed moisture contents and cooling/warming rates seemed to be adequate for ultra-low temperature exposure, except for *Bauhinia acuruana*, *Bauhinia ungulata*, *Sterculia striata* and *Styrax camporum* seeds.

Table 1. Seed moisture content and germination percentages before and after liquid nitrogen exposure of seed species exhibiting physical restraint

Family	Species	Physical restraint to germination	mc (%)	Germination (%)	
				Control	LN
Anacardiaceae	*Schinopsis brasiliensis*	Endocarp	6.7	45	86*
	Spondias mombin	Endocarp	4.1	11	53*
Caesalpiniaceae	*Apuleia leiocarpa*	Seed coat[a]	3.5	31	50
	Bauhinia sp.	Seed coab[b]	6.9	72	88
	Chamaecrista desvauxii	Seed coat[b]	7.7	80	84
	Dimorphandra mollis	Seed coat[b]	8.4	95	55*
	Sclerolobium paniculatum	Seed coat[b]	6.9	37	55
	Senna alata	Seed coat[b]	7.2	90	98
Combretaceae	*Buchenavia tomentosa*	Endocarp	8.1	50	59
Fabaceae	*Bowdichia virgilioides*	Seed coat[a]	5.9	20	85*
	Ormosia fastigiata	Seed coat[b]	3.0	82	77
	Pterodon emarginatus	Seed coat[a]	5.3	50	80*
Malpighiaceae	*Byrsonima basiloba*	Endocarp	3.0	13	30
Mimosaceae	*Albizia* sp.	Seed coat[b]	6,3	88	98
	Enterolobium contortisiliquum	Seed coat[b]	7.7	90	91
	Enterolobium gummiferum	Seed coat[b]	7.3	88	98
	Mimosa somnians var. *viscida*	Seed coat[b]	12.2	65	90*
	Mimosa sp.	Seed coat[b]	7.3	89	86
	Stryphnodendron polyphyllum	Seed coat[b]	5.1	50	90*
	Stryphnodendron pulcherrimum	Seed coat[b]	13.0	93	88
Rubiaceae	*Guettarda pohliana*	Endocarp	3.5	72	78
Tiliaceae	*Apeiba tibourbou*	Seed coat[a]	6.9	22	54*

[a] Species that had different levels of seed coat hardness in the same sample.
[b] Seed species scarified with sulphuric acid.
* Means significantly different at $p < 0.01$.

Table 2. Seed moisture content and germination percentages before and after exposure to liquid nitrogen.

Family	Species	mc (%)	Germination (%)	
			Control	LN
Anacardiaceae	*Astronium fraxinifolium*	6.3	98	95
Apocynaceae	*Aspidosperma discolor*	5.7	99	95
	Aspidosperma parvifolium	7.2	94	93
	Aspidorperma pyrifolium	5.4	94	91
Bignoniaceae	*Anemopaegma arvense*	6.3	68	84
	Jacaranda cuspidifolium	8.5	78	89
	Jacaranda decurrens	5.2	89	89
	Tabebuia aurea	7.0	66	71
	Tabebuia impetiginosa	5.8	88	89
	Tabebuia roseo-alba	5.8	98	94
	Tabebuia serratifolia	5.4	88	93
	Zeyheria montana	5.5	95	88
Bombacaceae	*Chorisia pubiflora*	8.5	55	53
	Eriotheca gracilipis	6.5	100	100
	Pseudobombax cf. *tomentosum*	7.0	85	63
Caesalpiniaceae	*Bauhinia acuruana*	5.7	95	65*
	Bauhinia ungulata	5.2	98	82*
	Dialium divaricatum	8.4	95	92
	Melanoxylum brauna	9.9	99	96
	Peltogyne confertiflora	10.1	95	98
	Senna sp.	9.3	95	99
Dioscoreaceae	*Dioscorea* sp.	12.4	43	61
Fabaceae	*Amburana cearensis*	5.3	92	94
	Anadenanthera colubrina	7.1	99	93
	Crotalaria cf.*spectabilis*	15.0	73	94*
	Cyclolobium cf. *blanchetianum*	6.8	95	97
	Dalbergia miscolobium	8.6	100	53*
	Machaerium aculeatum	4.2	43	28
	Machaerium cf. *acutifolium*	6.4	45	68
	Machaerium brasiliensis	4.9	94	100
	Platypodium elegans	8.4	85	83
Guttiferae	*Kielmeyera coriacea*	8.7	65	58
Lecythidaceae	*Cariniana estrellensis*	5.9	4	53*
	Cariniana legalis	7.2	50	74
Lytraceae	*Lafoensia pacari*	9.2	86	90
Meliaceae	*Cedrella fissilis*	9.8	80	70
Mimosaceae	*Acacia farnesiana*	6.3	98	100
Monimiaceae	*Siparuna guianensis*	6.4	50	58
Polygonaceae	*Triplaris gardneriana*	5.6	60	60
Rubiaceae	*Tocoyena formosa*	5.9	87	83
Sapindaceae	*Magonia pubescens*	5.0	98	100
Sterculiaceae	*Sterculia striata*	11.1	100	85*
Styracaceae	*Styrax camporum*	3.0	90	36*
Tiliaceae	*Luehea* sp.	7.5	40	46

* Means significantly different at $p < 0.01$

The results obtained suggested that seed storability at cryogenic temperature is feasible for all the seed species tested. The ideal mc has to be determined to optimise cryostorage of *Sterculia striata*, *Styrax camporum, Bauhinia acuruana* and *Bauhinia ungulata*.

Acknowledgements: The author is indebted to B.M.T. Walter. J.A. da Silva, A.A. Santos, S.P.C. Silva, G.P. Santos, T.B. Cavalcanti, and A. C. Sevilha for seed supply.

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