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Review

Cryoconservation of plant germplasm native to Brazil

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The preservation of biological material at -196°C, that is, at liquid nitrogen temperature, or its vapor (between -150 and -178°C), is a long-term storage procedure called cryopreservation or cryoconservation. This article reports studies made in Brazil for cryoconservation of native plant species and highlights what might be the gap in this area of germplasm conservation. Most studies use seeds as plant material, and the great majority of those is orthodox, which means that countless species are being left out of this conservation effort, such as those with recalcitrant seeds and plants that do not produce seeds. Many articles addressed in this present work studied species threatened by anthropogenic activities and chose cryoconservation as a way to safeguard their germplasm. The Brazilian cryobanks, on the other hand, are still developing, with many studies yet to be made and many accessions yet to be incorporated to collections in order for them to achieve an ideal stage of operation.

Key words: Biodiversity, biotechnology, conservation, cryopreservation, preservation, seed.

INTRODUCTION

Plant genetic resources consist in a natural reserve of genes with potential use for sustainable produce such as food, fibers and medications, is essential to mankind. Genetic erosion is characterized by gradual loss of this genetic reserve, emerging from disorganized growth of human population and uncontrolled exploitation of the ecosystems and their natural resources, and also from the evolutionary process species undergo in nature through natural selection and genetic drift. Such loss can be mitigated through long-term conservation techniques with as much as possible genetic and biological integrity (Santos, 2001; Raven and Havens, 2014).

Thus, *in situ* and *ex situ* conservation of germplasm from domestic crops and wild relatives of agronomic species, as well as native species with still unexplored potentials, is proposed in sight of the destruction of biodiversity which has been observed nowadays and the climate changes planet Earth is going through. Con-

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servation of plant genetic diversity is imperative in face of such threats and will enable mankind to reestablish species in the wild when conditions are favorable (Raven and Havens, 2014). Furthermore, the conservation of biodiversity is imperative for both long known species as well as recently discovered ones, since the genetic resources of a species may not be of interest to mankind at present but become fundamental for solving problems that already exist and the ones that may arise in the future (Gonzaga et al., 2003).

Ex situ conservation, that is, the conservation that takes place outside of the natural environment in which the species occur, allows for medium to long term preservation of the stored species genetic variability and their anatomic, physiological and biochemical study to support strategies of reintroduction, genetic engineering and education (Heywood and Iriondo, 2003) that is accomplished by preserving a representative sample of the natural populations in germplasm banks, like, for instance, *in vitro* banks, seed banks, cryobanks, or field collections. Seeds, pollen and spores of plants are adequate for medium to long term conservation because of their natural adaptation to sustaining viability for extended periods of time (Heywood and Iriondo, 2003).

In situ conservation, however, being the conservation of species in the environment where they naturally occur, preserves physical and biological processes which *ex situ* conservation is incapable of safeguarding, such as evolution and ecological relationships, which, in turn, makes them complementary and essential strategies (Maxted et al., 1997; Raven and Havens, 2014). Therefore, *in situ* conservation depends on the maintenance of natural populations and their careful monitoring, because just creating conservation areas does not guarantee the survival of species, particularly threatened species (Heywood and Iriondo, 2003).

Since *in situ* conservation of endemic species is based in theoretical actions that are not strictly followed, applied or respected, complementary strategies like the creation of *ex situ* germplasm banks are recommended (Tarré et al*.,* 2007). The preservation of biological material at -196°C, that is, at liquid nitrogen temperature, or its vapor (between -150 and -178°C), is a long-term storage procedure called cryopreservation or cryoconservation. It is based on the principle that under ultralow temperatures of -196°C all metabolical processes are essentially paralyzed and kept in a latent state, consequently providing indefinite preservation (Medeiros and Cavallari, 1992).

Cryoconservation can be applied to a series of plant materials such as suspension cultures, calli, shoots, somatic embryos or embryonic axes, fern gametophytes, axillary buds and seeds (Sarasan et al., 2006). Among these, the storage of seeds is the most used method of *ex situ* conservation, because seeds are the natural structures of plant reproduction and each one can represent a genetically distinct organism (Tarré et al*.,* 2007). Seeds may be stored for indefinite amounts of time in conditions which reduce their metabolism and deterioration (Tarré et al*.,* 2007; Hartmann et al., 2011) as well as prolong their long-term viability (Salomão, 2002; Wetzel et al. 2003; Lima et al., 2008), besides, depending on the species, being small in size and occupying little space.

The evaluation of seed behavior after storage is the first step for the establishment of ideal storage conditions, as well as desiccation tolerance and freezing (Tarré et al*.,* 2007). There may be abnormal germination or death from internal injuries, which may be related to certain seed traits such as size, water content and chemical com-position (Cromarty et al., 1982; Santos, 2001; Goldfarb et al., 2010; Silva et al., 2011). Thus, the effect of freezing in liquid nitrogen should be analyzed for each and every species targeted for a cryoconservation study.

Seed cryoconservation is advantageous for long-term conservation of tropical and subtropical forest biodiversity because the stages of embryo isolation and *in vitro* manipulation are not necessary. Even for orthodox and intermediary seeds, whose conservation is less problematic then for recalcitrant seeds, cryoconservation preserves seed longevity as long as liquid nitrogen levels are maintained, whereas dry storage of seeds at −20°C in banks for long periods may lead to physiological and genetic damage (Pilatti et al., 2010). The DNA damage, the risk of disease and plagues striking, besides the need to perform viability control, all typical of traditional storage, are reduced or eliminated all together with the use of seed cryoconservation (Tarré et al*.,* 2007).

Cryoconservation techniques have been much applied to long-term storage of seeds in germplasm banks (Lima et al., 2008). Cryoconservation is specially indicated for species of vegetative propagation, species with recalcitrant seeds, rare germplasm or even threatened species (Engelmann, 2011). However, few studies have been made with seeds of tropical climate (Lima et al., 2008), and the development of techniques for seed storage is fundamental in order to conserve genetic resources in germplasm banks and to promote the longevity of threatened species seeds (Vásques-Yanes and Orozco-Segovia, 1993; Raven and Havens, 2014).

The collecting of germplasm to ensure seed conservation is, on the other hand, of extreme importance on account of preserving the physiological quality of those seeds, maintaining genetic variability of threatened species and subsidizing reintroduction programs for extinct populations (Cabral et al., 2003; Lima et al., 2008; Guerrant et al., 2014). Technological studies of seeds are the actual starting point for mindful use and exploration of native species (Tresena et al*.,* 2010).

This article reports studies made in Brazil for cryoconservation of native plant species and highlights which might be the gaps in this area of germplasm conservation.

CRYOPRESERVATION OF NATIVE PLANTS

Brazil is among the most diverse countries in the world regarding plants and their genetic resources, and the species that occur there are used for food and beverages, medicinal and ornamental purposes, or have valuable wood. Many species are not cultivated, and therefore are threatened by illegal collection, and with the development and economic growth Brazil has been going through in the last few decades, urban and agricultural expansion is also a very serious threat to Brazilian genetic resources and environment preservation (Santos et al., 2013).

The development of protocols for the cryoconservation of native plants in Brazil remains limited, with most of the efforts directed to economically important species. Due to recalcitrance, limited supply of germplasm and lack of knowledge regarding the behavior of their seeds when stored, their adaptive physiology and their response to culture, groups of native and threatened species are not targeted for conservation studies often. *In vitro* manipulation and cryoconservation worksed with intermediary and orthodox seeds are the ones achieving the greatest advances in this area (Pilatti et al*.,* 2010).

There have been made approximately 24 cryoconservation studies with Brazilian plant species, which are listed in Table 1. Only five of these works applied some kind of cryoprotector in order to achieve conservation of the plant material, which may be related to the fact that these studies used calli, apexes and buds instead of only seeds (Charoensuba et al., 2003; Nogueira, 2010; Rodrigues et al., 2012; Porto, 2013); the one that did use seeds was a study with an Orchidaceae species (Galdiano et al., 2013). The use of vitrification procedures prevents most of the ice crystal formation typical of traditional procedures (Engelmann, 2011), which is associated to cellular rupture, damage of organelles and bubble formation (Fuller, 2004). All the other studies found in literature used only seeds as conserved plant material and did not apply cryoprotectors to them, which in turn may be due to the toxicity of this kind of treatment (Fuller, 2004) or to the raise in protocol costs. It could also be an unnecessary step to add in a protocol if the results of cryoconservation of seeds without cryoprotectors are already effective.

Most of the species cited in Table 1 had their seeds classified as orthodox or intermediary, with only a few species of the Passifloraceae family being labeled as recalcitrant. The desiccation of the seeds was performed prior to freezing at the authors' criteria in each study, varying according to the targeted species. Since desiccation decreases water content within the seed, it tends to lower the probability of damage taking place, normally associated to the presence of water in the tissues during the process of cryoconservation. According to Engelmann (2004), the survival of the frozen samples is optimized when seeds are frozen with water content between 10 and 20%, which is in accordance with the majority of species listed in Table 1. Some species, however, were stored with a water content above this limit range, like, for instance, *Blepharocalyx salicifolius*, *Eugenia jambolana*, *Parapiptadenia rigida* (Venzke et al., 2006), *Encholirium scrutor* and *Dyckia ursina* (Tarré et al., 2007). This reiterates that the water content limit range for cryoconservation of seeds varies depending on the target species. On the other hand, according to Pritchard and Nadaranjan (2007), moisture contents ≤ 8% do not affect the survival of orthodox seeds after cryoconservation. This is also in accordance with many species listed in Table 1, such as *Astronium urundeuva* (Medeiros and Cavallari, 1992), *Spondays mombin*, *Byrsonima basiloba* (Salomão, 2002), *Styrax camporum* (Lima et al., 2008), and *Tabebuia chrysotrica* (Tresena et al., 2010).

Regarding recalcitrant seeds, it is estimated that 70% of tropical forest species have seeds that belong to this category (Barbedo et al., 2002), and is still necessary to establish specific protocols related to water content and desiccation tolerance. According to Pilatti et al. (2010), during the last few decades efforts have been made to expand knowledge of plants with recalcitrant seeds, but in the present study this was not found in literature regarding native species targeted for cryoconservation. The conservation of such seeds tends to be more problematic than the conservation of orthodox and intermediary seeds, which is due, sometimes, not only to their intolerance to desiccation, but due to difficult access to these plants for sample collection, the devastation of their habitat, and the irregularity of seed production throughout the year (Pilatti et al., 2010; Walters et al*.,* 2013). All of these factors lead to lack of plant material available for conducting experiments, to lack of knowledge regarding the physiology and reproduction of these plants, and, finally, it is reflected in the shortage of studies about cryoconservation of species with recalcitrant seeds native to Brazil, as presented in this work.

Nonetheless, cryoconservation of recalcitrant seeds can be achieved using plant tissue culture technologies to isolate the embryo and germinate it *in vitro*, and with the use of cryoprotectors and the reduction of stress caused by free radicals, thus conserving the plant germplasm in liquid nitrogen (Walters et al*.,* 2013; Pammenter and Berjak, 2014). Moreover, cryoconservation studies with orthodox seeds should be used to improve the development of protocols for the germplasm of intermediary and recalcitrant seeds of native Brazilian species (Pilatti et al., 2010).

Concerning plants that only reproduce vegetatively, there were no studies of cryoconservation found for native species of Brazil that fall into that category. Nevertheless, protocols have been established successfully for many ornamental species and species of agronomic importance, be it fruit-bearing trees, tubers or

Table 1. Family, water content (%), plant material used desiccation prior to storage, use of cryoprotection and time of storage of cryoconserved plant species native to Brazil.

Family	Species	Plant material	Water content (%)	Desiccation	Cryoprotection	Storage time	Reference
Anacardiaceae	*Anacardium othonianum*	Seed	12.0 - 14.0	Yes	No	20 days	Silva et al., 2013
Anacardiaceae	*Astronium fraxinifolium*	Seed	6.3	No	No	3 days	Salomão, 2002
Anacardiaceae	*Astronium urundeuva*	Seed	6	Yes	No	15 days	Medeiros et al., 1992
Anacardiaceae	*Astronium urundeuva*	Seed	9	No	No	5, 15 and 25 days	Gonzaga et al., 2003
Anacardiaceae	*Lithraea brasiliensis*	Seed	31	No	No	1 h	Venzke et al., 2006
Anacardiaceae	*Schinopsis brasiliensis*	Seed	6.7	No	No	3 days	Salomão, 2002
Anacardiaceae	*Schinopsis brasiliensis*	Seed	7.4	No	No	5, 15 and 25 days	Gonzaga et al., 2003
Anacardiaceae	*Spondays mombin*	Seed	4.1	No	No	3 days	Salomão, 2002
Anarcadiaceae	*Astronium fraxinifolium*	Seed	5.9	No	No	3 days	Lima et al., 2008
Anarcadiaceae	*Myracrodruon urundeuva*	Seed	12	No	No	3 days	Lima et al., 2008
Anarcadiaceae	*Schinopsis brasiliensis*	Seed	7.3	No	No	3 days	Lima et al., 2008
Apocynaceae	*Aspidorperma pyrifolium*	Seed	5.4	No	No	3 days	Salomão, 2002
Apocynaceae	*Aspidosperma discolor*	Seed	5.7	No	No	3 days	Salomão, 2002
Apocynaceae	*Aspidosperma parvifolium*	Seed	7.2	No	No	3 days	Salomão, 2002
Apocynaceae	*Aspidosperma pyrifolium*	Seed	6.8	No	No	3 days	Lima et al., 2008
Apocynaceae	*Hancornia speciosa*	Apical buds/ calli	-	-	Yes	60 mins/24 h	Nogueira, 2010
Bignonaceae	*Tabebuia chrysotrica*	Seed	4	Yes	No	5 days	Tresena et al., 2010
Bignonaceae	*Tabebuia heptaphylla*	Seed	8	Yes	No	30, 60 and 90 days	Tresena et al., 2009
Bignonaceae	*Tabebuia umbellata*	Seed	6	Yes	No	7 days	Wetzel et al., 2003
Bignoniaceae	*Anemopaegma arvense*	Seed	6.3	No	No	3 days	Salomão, 2002
Bignoniaceae	*Jacaranda brasiliana*	Seed	5.7	No	No	3 days	Lima et al., 2008
Bignoniaceae	*Jacaranda cuspidifolium*	Seed	8.5	No	No	3 days	Salomão, 2002
Bignoniaceae	*Jacaranda decurrens*	Seed	5.2	No	No	3 days	Salomão, 2002
Bignoniaceae	*Pithecoctenium squalus*	Seed	10.7	No	No	1 h	Venzke et al., 2006
Bignoniaceae	*Tabebuia aure*	Seed	6.7	No	No	3 days	Lima et al., 2008
Bignoniaceae	*Tabebuia aurea*	Seed	7	No	No	3 days	Salomão, 2002
Bignoniaceae	*Tabebuia impetiginosa*	Seed	5.8	No	No	3 days	Salomão, 2002
Bignoniaceae	*Tabebuia impetiginosa*	Seed	7.5	No	No	3 days	Lima et al., 2008
Bignoniaceae	*Tabebuia roseo-alba*	Seed	5.8	No	No	3 days	Salomão, 2002
Bignoniaceae	*Tabebuia serratifolia*	Seed	5.4	No	No	3 days	Salomão, 2002
Bignoniaceae	*Zeyheria montana*	Seed	5.5	No	No	3 days	Salomão, 2002
Bombacaceae	*Cavanillesia arborea*	Seed	8.5	No	No	3 days	Lima et al., 2008
Bombacaceae	*Chorisia pubiflora*	Seed	8.5	No	No	3 days	Salomão, 2002
Bombacaceae	*Chorisia speciosa*	Seed	5.8	Yes	No	7 days	Wetzel et al., 2003
Bombacaceae	*Eriotheca gracilipis*	Seed	6.5	No	No	3 days	Salomão, 2002

Table 1. Contd.

Bombacaceae	*Pseudobombax cf. tomentosum*	Seed	7	No	No	3 days	Salomão, 2002
Boraginaceae	*Cordia trichotoma*	Seed	7.3	No	No	3 days	Lima et al., 2008
Bromeliaceae	*Dyckia sordida*	Seed	13.4	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Dyckia ursina*	Seed	28.2	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium heloisae*	Seed	19.1	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium magalhaesii*	Seed	14.6	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium pedicellatum*	Seed	2.5	Yes	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium reflexum*	Seed	11.2	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium scrutor*	Seed	24.2	No	No	1 day	Tarré et al., 2007
Bromeliaceae	*Encholirium subsecundum*	Seed	12.7	No	No	1 day	Tarré et al., 2007
Cactaceae	*Cereus jamacaru*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Discocactus zehntneri*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Discocactus zehntneri*	Seed	9 - 12	No	No	7 and 30 days	Marchi et al., 2013
Cactaceae	*Melocactus albicephalus*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Melocactus concinnus*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Melocactus ernestii*	Seed	6.8	No	No	7, 34 and 120 days	Assis et al., 2011
Cactaceae	*Melocactus paucispinus*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Melocactus zehntneri*	Seed	8.3	No	No	7, 34 and 120 days	Assis et al., 2011
Cactaceae	*Micranthocereus flaviflorus*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Pilosocereus gounellei*	Seed	-	No	No	7, 30 and 120 days	Veiga-Barbosa et al., 2010
Cactaceae	*Pilosocereus gounellei*	Seed	9 - 12	No	No	7 and 30 days	Marchi et al., 2013
Cactaceae	*Stephanocereus luetzelburgii*	Seed	9 - 12	No	No	7 and 30 days	Marchi et al., 2013
Caesalpiniaceae	*Apuleia leiocarpa*	Seed	3.5	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Bauhinia acuruana*	Seed	5.7	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Bauhinia* sp.	Seed	6.9	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Bauhinia ungulata*	Seed	5.2	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Chamaecrista desvauxii*	Seed	7.7	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Copaifera langsdorffii*	Seed	5.9	No	No	3 days	Lima et al., 2008
Caesalpiniaceae	*Dialium divaricatum*	Seed	8.4	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Dimorphandra mollis*	Seed	8.4	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Hymenaea courbaril var.stilbocarpa*	Seed	5.8	No	No	3 days	Lima et al., 2008
Caesalpiniaceae	*Melanoxylum brauna*	Seed	9.9	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Peltogyne confertiflora*	Seed	10.1	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Sclerolobium paniculatum*	Seed	6.9	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Senna alata*	Seed	7.2	No	No	3 days	Salomão, 2002
Caesalpiniaceae	*Senna* sp.	Seed	9.3	No	No	3 days	Salomão, 2002

Table 1. Contd

Caesalpinioideae	*Caesalpinia echinata*	Seed	-	-	No	30, 90 and 180 days	Zanotti et al., 2007
Caesalpinoideae	*Bauhinia* sp.	Seed	6.9	Yes	No	7 days	Wetzel et al., 2003
Caesalpinoideae	*Cassia ferruginea*	Seed	6.5	Yes	No	7 days	Wetzel et al., 2003
Caesalpinoideae	*Hymenae stignocarpa*	Seed	5	Yes	No	7 days	Wetzel et al., 2003
Caesalpinoideae	*Hymeneae courbaril*	Seed	-	No	No	7 days	Farias et al., 2006
Caesalpinoideae	*Sclerolobium aureum*	Seed	6.5	Yes	No	7 days	Wetzel et al., 2003
Combretaceae	*Buchenavia tomentosa*	Seed	8.1	No	No	3 days	Salomão, 2002
Cucurbitaceae	*Lagenaria vulgaris*	Seed	13	No	No	1 h	Venzke et al., 2006
Cucurbitaceae	*Luffa cylindrica*	Seed	12	No	No	1 h	Venzke et al., 2006
Dioscoreaceae	*Dioscorea* sp.	Seed	12.4	No	No	3 days	Salomão, 2002
Eriocaulaceae	*Syngonanhus arthrotrichus*	Seed	-	No	No	40, 80 and 120 days	Duarte, 2009
Eriocaulaceae	*Syngonanthus elegans*	Seed	-	No	No	40, 80 and 120 days	Duarte, 2009
Euphorbiaceae	*Jatropha curcas*	Seed	8	Yes	No	5 days	Goldfarb et al., 2008
Euphorbiaceae	*Jatropha curcas*	Seed	8.3	Yes	No	30, 60 and 90 days	Silva et al., 2011
Euphorbiaceae	*Manihot esculenta*	Axillary apices	-	No	Yes	2 h	Charoensuba et al., 2003
Euphorbiaceae	*Manihot esculenta*	Meristems	-	No	Yes	15 days	Rodrigues et al., 2012
Fabaceae	*Amburana cearensis*	Seed	5.3	No	No	3 days	Salomão, 2002
Fabaceae	*Amburana cearensis*	Seed	8.9	No	No	3 days	Lima et al., 2008
Fabaceae	*Anadenanthera colubrina*	Seed	7.1	No	No	3 days	Salomão, 2002
Fabaceae	*Bowdichia virgilioides*	Seed	5.9	No	No	3 days	Salomão, 2002
Fabaceae	*Crotalaria cf. spectabilis*	Seed	15	No	No	3 days	Salomão, 2002
Fabaceae	*Cyclolobium cf. blanchetianum*	Seed	6.8	No	No	3 days	Salomão, 2002
Fabaceae	*Dalbergia miscolobium*	Seed	8.6	No	No	3 days	Salomão, 2002
Fabaceae	*Lonchocarpus montanus*	Seed	5.3	No	No	3 days	Lima et al., 2008
Fabaceae	*Machaerium aculeatum*	Seed	4.2	No	No	3 days	Salomão, 2002
Fabaceae	*Machaerium brasiliensis*	Seed	4.9	No	No	3 days	Salomão, 2002
Fabaceae	*Machaerium cf. acutifolium*	Seed	6.4	No	No	3 days	Salomão, 2002
Fabaceae	*Machaerium scleroxylon*	Seed	8.5	No	No	3 days	Lima et al., 2008
Fabaceae	*Ormosia fastigiata*	Seed	3	No	No	3 days	Salomão, 2002
Fabaceae	*Platypodium elegans*	Seed	8.4	No	No	3 days	Salomão, 2002
Fabaceae	*Pterodon emarginatus*	Seed	5.3	No	No	3 days	Salomão, 2002
Fabaceae	*Stryphnodendron adstrigens*	Calli/shoot tips/seeds	6 - 9	Yes	Yes	1 h/1 h/24 h	Porto, 2013
Gramineae	*Euchlaena mexicana*	Seed	16	No	No	1 h	Venzke et al., 2006
Guttiferae	*Kielmeyera coriacea*	Seed	8.7	No	No	3 days	Salomão, 2002
Lecythidaceae	*Cariniana estrellensis*	Seed	5.9	No	No	3 days	Salomão, 2002
Lecythidaceae	*Cariniana legalis*	Seed	7.2	No	No	3 days	Salomão, 2002

Table 1. Contd.

Lytraceae	*Lafoensia pacari*	Seed	9.2	No	No	3 days	Salomão, 2002
Malpighiaceae	*Byrsonima basiloba*	Seed	3	No	No	3 days	Salomão, 2002
Meliaceae	*Cedrela fissili*	Seed	8	No	No	3 days	Lima et al., 2008
Meliaceae	*Cedrella fissilis*	Seed	9.8	No	No	3 days	Salomão, 2002
Meliaceae	*Cedrella fissilis*	Seed	-	No	No	1 h	Nunes et al., 2003
Mimosaceae	*Acacia farnesiana*	Seed	6.3	No	No	3 days	Salomão, 2002
Mimosaceae	*Acacia polyphylla*	Seed	9.4	No	No	3 days	Lima et al., 2008
Mimosaceae	*Albizia* sp.	Seed	6.3	No	No	3 days	Salomão, 2002
Mimosaceae	*Anadenanthera colubrina*	Seed	8.8	No	No	3 days	Lima et al., 2008
Mimosaceae	*Enterolobium contortisiliquum*	Seed	7.7	No	No	3 days	Salomão, 2002
Mimosaceae	*Enterolobium contortisiliquum*	Seed	7.3	No	No	3 days	Lima et al., 2008
Mimosaceae	*Enterolobium gummiferum*	Seed	7.3	No	No	3 days	Salomão, 2002
Mimosaceae	*Mimosa somnians var. viscida*	Seed	12.2	No	No	3 days	Salomão, 2002
Mimosaceae	*Mimosa* sp.	Seed	7.3	No	No	3 days	Salomão, 2002
Mimosaceae	*Parapiptadenia rigida*	Seed	35	No	No	1 h	Venzke et al., 2006
Mimosaceae	*Stryphnodendron polyphyllum*	Seed	5.1	No	No	3 days	Salomão, 2002
Mimosaceae	*Stryphnodendron pulcherrimum*	Seed	13	No	No	3 days	Salomão, 2002
Mimosoideae	*Albizia lebbeck*	Seed	6.1	Yes	No	7 days	Wetzel et al., 2003
Mimosoideae	*Anadenanthera macrocarpa*	Seed	5	Yes	No	7 days	Wetzel et al., 2003
Mimosoideae	*Mimosa setosa Benth*	Seed	3.5	Yes	No	7 days	Wetzel et al., 2003
Monimiaceae	*Siparuna guianensis*	Seed	6.4	No	No	3 days	Salomão, 2002
Myrsinaceae	*Myrsine laetevirens.*	Seed	16	No	No	1 h	Venzke et al., 2006
Myrtaceae	*Blepharocalyx salicifolius*	Seed	32	No	No	1 h	Venzke et al., 2006
Myrtaceae	*Eugenia jambolana*	Seed	52	No	No	1 h	Venzke et al., 2006
Myrtaceae	*Psidium guajava*	Seed	7	No	No	1 h	Venzke et al., 2006
Myrtaceae	*Psidium guajava L. v. pomifera*	Seed	11.7	No	No	1 h	Venzke et al., 2006
Orchidaceae	*Oncidium flexuosum*	Seed	11.3	No	Yes	30 min	Galdiano et al., 2013
Papilionoideae	*Platypodium elegans*	Seed	5.4	Yes	No	7 days	Wetzel et al., 2003
Passifloraceae	*Passiflora edulis*	Seed	22-34	Yes	No	10 days	Meletti et al., 2007
Passifloraceae	*Passiflora nitida*	Seed	30	Yes	No	10 days	Meletti et al., 2007
Passifloraceae	*Passiflora serrato-digitata*	Seed	21	Yes	No	10 days	Meletti et al., 2007
Polygonaceae	*Triplaris gardneriana*	Seed	5.6	No	No	3 days	Salomão, 2002
Proteaceae	*Roupala montana*	Seed	7.5	Yes	No	7 days	Wetzel et al., 2003
Rubiaceae	*Guettarda pohliana*	Seed	3.5	No	No	3 days	Salomão, 2002
Rubiaceae	*Tocoyena formosa*	Seed	5.9	No	No	3 days	Salomão, 2002
Sapindaceae	*Magonia pubescens*	Seed	5	No	No	3 days	Salomão, 2002
Sterculiaceae	*Sterculia striata*	Seed	11.1	No	No	3 days	Salomão, 2002

Table 1. Contd.

Sterculiaceae	*Sterculia striata*	Seed	10.9	No	No	3 days	Lima et al., 2008
Styracaceae	*Styrax camporum*	Seed	3	No	No	3 days	Salomão, 2002
Styracaceae	*Tiliaceae Luehea* sp.	Seed	7.5	No	No	3 days	Salomão, 2002
Tiliaceae	*Apeiba tibourbou*	Seed	6.9	No	No	3 days	Salomão, 2002
Verbenaceae	*Aegiphila lhotzkiana*	Seed	4.6	Yes	No	7 days	Wetzel et al., 2003
Vochysiaceae	*Qualea parviflora*	Seed	8.5	Yes	No	7 days	Wetzel et al., 2003

roots, of tropical or temperate origins, through the use of vitrification and other strategies (Engelmann, 2011). The same strategies of tissue cryoconservation available for plants with recalcitrant seeds could also be used for preserving the genetic diversity of plants that only reproduce vegetatively or that possess seeds or spores that traditional methods are unable to preserve *ex situ* (Pence, 2014).

In the next sections studies found in literature regarding cryoconservation of Brazilian plant species will be presented distributed in four categories, namely, Forest species, Medicinal and edible species, and Ornamental species. These categories are arbitrary, chosen for better exploration of contents, and many species might fit in more than one of them, despite being allocated here in the category representing their main usage by man. The complete list of works and species can be found in Table 1. A survey of the literature from 1992 to 2014 for studies regarding the cryoconservation of Brazilian native plant species was conducted. These works are available on the Internet and were obtained through searches in databases such as Scielo (Scientific Electronic Library Online), Google Scholar and "Portal de Periódicos CAPES/MEC" (http://www.periodicos.capes.gov.br/). To the best knowledge of the authors of this article, all of the papers regarding the cryoconservation of plant germplasm native to Brazil that were available for download are presented in the following sections.

Forest species

The capability to tolerate ultralow temperatures (-196°C) was tested for 66 tropical species, belonging to 21 botanical families of Cerrado and Atlantic Forest (Salomão, 2002). In 51 of these species germinability (germination percentage) was not affected by cryoconservation, and in nine of them there was an enhancement in germinability or a break of seed dormancy with freezing, while six showed significant reduction in germinability (Table 1).

In the Laboratory of Seed Physiology of Embrapa (Brazilian Agricultural Research Corporation) Genetic Resources and Biotechnology, the behavior of forest species seeds collected in the Brazilian Cerrado was evaluated after seven days of storage in liquid nitrogen (Wetzel et al., 2003). Seeds of thirteen species of seven different families were studied (Table 1). They were previously dehydrated in drying chamber (20±3°C and 12±3% of relative humidity). The initial average germination was 64.92%, and after the freezing period the average was 62.23%, which indicated that dry seeds of the studied species resisted the temperature of -196°C, suggesting that this technique can be used for the conservation of native forest species in germplasm banks (Wetzel et al., 2003).

Zanotti et al. (2007) evaluated the development of seedlings and saplings of pau-brasil (*Caesalpinia echinata*) originated from cryoconserved seeds. Regarding the initial development, the authors noted significant differences, although these differences diminished as the plants grew. According to the data obtained there were no significant differences between treatments, and plant growth was normal even for plants from up to 180 days of storage at -18 and -196°C (Zanotti et al., 2007).

Aiming to increase germination in the field for ecological restoration projects by direct seeding of dry forest tree species of the Paranã river basin (GO), Lima et al. (2008) investigated if seeds of such species alter their germinability after being stored in natural conditions for three to 15 months and in germplasm banks at -20 and -196°C. Storage at -20 and -196°C was efficient for preservation of seed physiological qualities for most of the arboreal species from deciduous forests of the Paranã river basin, being an alternative for *ex situ* conservation and for the increase of field germination in restoration projects by direct seeding (Lima et al., 2008).

The yellow ipê (*Tabebuia chrysotrica*) has seeds of short life spam, hindering its use in reforestation and the commercialization of its seedlings (Tresena et al., 2010). It was determined that the most appropriate water content limit for the cryoconservation of yellow "ipê" seeds

subjected to the temperature of -196°C, for five days, is 4%. No studies were made with longer periods of cryoconservation of this species, but the data obtained by Tresena et al. (2010) established that the yellow "ipê" resists freezing and thawing with a maximum of 4% of water content in its seeds.

Medicinal and edible species

Medeiros and Cavallari (1992) conducted an experiment to analyze the germination of "aroeira" seeds *(Astronium urundeuva*), Anacardiaceae, after they had been dried and immersed in liquid nitrogen, in order to conserve the germplasm of this species in the Genetic Resources Conservation Area of Embrapa Genetic Resources and Biotechnology. This species is at risk of extinction because of the extraction of its wood, widely used in construction industry, and also for having medicinal use. The bark can be used to treat diseases of the airways and urinary tract, and its resin is applied for its tonic effect (Medeiros and Cavallari, 1992; Gonzaga et al*.,* 2003). The seeds were subjected to drying in a drying chamber (25°C and 10-15% of relative humidity) and, afterwards, samples were immediately conditioned in hermetic packages and immersed directly in liquid nitrogen (-196°C) for 15 days. The results indicate that "aroeira" seeds can be classified as orthodox and previously dehydrated up to 6% of water content to be directly stored in liquid nitrogen, and thus conserved by the cryoconservation method (Medeiros and Cavallari, 1992).

Gonzaga et al. (2003) studied not only the cryoconservation of "aroeira", but also of "baraúna" (*Schinopsis brasiliensis*), Anacardiaceae, another threatened species of the Brazilian semi-arid environment. Its wood is used for manufacture of furniture and in construction, but also for its digestive and analgesic action in popular medicine. Seeds were conserved in liquid nitrogen (-96°C) and in nitrogen vapor (-170°C), for a period of 25 days. The authors concluded that the seeds of both "aroeira" and "baraúna" can be cryoconserved in liquid nitrogen as well as in its vapor, and that "baraúna" seeds have their germinability increased when conserved in these temperatures for 25 days compared to control, which indicates that, during this time, seed dormancy break happens.

The "jatobá" (*Hymeneae courbaril*) is found in the Amazon Forest and the Brazilian Atlantic Forest, where it occurs naturally from the state of Piauí to the north of Paraná state. Its wood is also used for manufactoring furniture and in construction, and its fruits are consumed as food, and its leaves and seeds are utilized in the cosmetic and pharmaceutical industries (Farias et al*.,* 2006). "Jatobá" seeds from the tip and the middle section of the pod were subjected to different temperatures and it was concluded that the cryoconservation at -170°C showed higher germination rates and vigor (91% and 72%, respectively), being, thus, recommended (Farias et al., 2006).

The passionfruit (Passifloraceae) is a native plant broadly used in Brazilian alimentation, and at least a third of the *Passiflora* species has its center of origin in Brazil (Meletti et al., 2007). Seeds of six accessions of passionfruit from the Active Germplasm Plant of the Agronomic Institute of Campinas (IAC) were subjected to cryoconservation with or without previous desiccation, and different responses to storage in liquid nitrogen were observed (Meletti et al., 2007). The commercial varieties of *P. edulis* can be cryoconserved after desiccation, but *P. nitida* and *P. serrato-digitata* behaved as recalcitrant and intermediary seeds, respectively, and did not have their physiological quality improved by cryoconservation.

The physic nut (*Jatropha curcas*) is an oleaginous species with potential for biofuel production, found in the semi-arid regions of Brazil (Goldfarb et al., 2008; Silva et al., 2011). Its seeds were dehydrated in drying chamber and then rehydrated until the water content ranged between 4 and 14% for different treatments and were cryoconserved for five days, being gradually thawed at the end of the period (Goldfarb et al., 2008). The water content limit suggested for the species was 8%, but with 4% to 8% of humidity the seeds did not have their quality altered significantly. Seeds with 8% of water content were cryoconserved for 30, 60 and 90 days immersed in the nitrogen vapor or liquid nitrogen (-170 and -196°C, respectively), maintaining their vigor and viability at both temperatures and during all of the storage periods (Goldfarb et al., 2010). In another more recent study, seeds were dried with silica gel for 24 or 48 h, and then stored in liquid nitrogen for 60 and 90 days (Silva et al., 2011). In this case, cryoconserved seeds also maintained their viability high with moisture content close to 8%, but the desiccation combined with cryoconservation damaged cells and tissues, causing abnormalities in plants.

Seeds of "mangabeira" (*Hancornia speciosa*) have low germination rate and recalcitrance, which makes this Cerrado species difficult to conserve and multiply (Nogueira, 2010). Synthetic seeds made of apical buds from the "mangabeira" tree in a sodium alginate matrix were subjected to 60 mins in liquid nitrogen, but no regeneration was observed from such propagules after storage (Nogueira, 2010). The cryoconservation of mangabeira calli was achieved with previous dehydration followed by pre-treatment with cryoprotectors and immersion in liquid nitrogen for 24 h (Nogueira, 2010). The cryoconserved calli showed decline of cellular viability after 30 days of culture, and analysis identified plasmolyzed cells and residual cryoprotectors after cryoconservation.

The cassava (*Manihot esculenta*), a plant native to the Amazonas region, is estimated to have two thirds of its genetic diversity in *in situ* collections or natural habitat, with only one third being maintained *ex situ* (Santos et al., 2013). Recently, plant tissue culture methods have

been used to conserve and multiply accessions of cassava, as well as to assist in the cryoconservation of this species (Charoensuba et al., 2003; Rodrigues et al., 2012). Axillary apices of *M. esculenta* were cultured on medium supplemented with 0.3 M sucrose for 16 hours, followed by a treatment with 2 M glycerol and 0.4 M sucrose for 20 min for cryoprotection (Charoensuba et al., 2003). After 45 min in a solution of PVS2 and then two hs in liquid nitrogen, the apices were cultured and resumed growth, with a regeneration rate of 70% (Charoensuba et al., 2003). Other tests showed that meristems exposed to a vitrification solution containing 0.5% of Tween-20 were able to achieve an *in vitro* regeneration rate of 50% after cryoconservation (Rodrigues et al., 2012).

The "barbatimão" (*Styphnodendron adstringens*), also found in the Brazilian Cerrado, is rich in tannins and has medicinal applications (Porto, 2013). Calli obtained from cotyledon explants induced with 1 mg L^{-1} of 2,4-D (dichlorophenoxyacetic acid) in MS medium (Murashigue and Skoog, 1962) were cryoconserved, but after storage growth was not resumed in the cultures (Porto, 2013). Shoot tips cultured in medium with 0.25 mg L^{-1} of 6-benzylaminopurine (BAP) were exposed to PVS2 (30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide and MS medium with 0.4 M of sucrose) or modified PVS2 (40% glycerol, 10% ethylene glycol, 10% dimethyl sulfoxide and MS medium with 0.4 M of sucrose) for different periods of time and then cryoconserved. The exposure for 15 and 30 min followed by culture in medium with gibberelic acid (GA_3) and 2.0 mg L^{-1} of BAP obtained the highest survival rate and the longest shoot length, respectively (Porto, 2013). "Barbatimão" seeds were also cryoconserved by Porto (2013), and, with previous desiccation, seeds that originated seedlings with higher dry weight and length were those with moisture content between 6 and 9%.

The "caju-de-árvore-do-Cerrado" (*Anacardium othonianum*), another species from the Brazilian Cerrado, is appreciated for its fruits eaten *in natura* or as juice, liqueur and sweets, as well as for its roasted nut (Silva et al., 2013). Its seeds were cryoconserved by Silva et al. (2013) with the aim of testing the influence of water content and different thawing methods over its germination. The water content limit found was the range of 12 to 14%, being that lower contents caused decrease in germination, and most of the thawing methods tested did not interfere with seed viability, except the microwave, which caused an inferior germinative response.

Ornamental species

Tarré et al. (2007) studied the effect of desiccation, storage at low temperature and cryoconservation in eight species of Bromeliaceae, six of these from the genus *Encholirium* and two of the genus *Dyckia*, selected according to vulnerability and endemism criteria. The germinability after freezing in liquid nitrogen was higher or similar to the one obtained in the control for all species studied, but for *E. pedicellatum* tolerance to freezing was only achieved when seeds were dried until they contained 2,5% of water. Considering tolerance to low temperature storage and desiccation, seeds of these genera can be classified as orthodox and, according to the results obtained by Tarré et al. (2007), cryoconserved.

Similarly to bromeliads, "sempre-vivas" are exploited for their ornamental value and usage in decorations, which is why two of these commercialized species were cryoconserved by Duarte (2009): *Syngonanthus arthrotrichus* and *S. elegans*. They can be successfully cryoconserved for 40, 80 and 120 days and thawed at room temperature or in a microwave oven.

The wood of the pink "ipê" (*Tabebuia heptahylla*) is used for various purposes and the ornamental potential of this species is explored in landscaping and the afforestation of streets and avenues (Tresena et al., 2009). Its seeds can be cryoconserved, because the mean values of germinability and vigor were maintained after exposure for 90 days to the temperatures of -170 and -196°C (Tresena et al., 2009).

Cryoconservation by direct immersion of seeds in liquid nitrogen for 7, 30 and 120 days did not affect the germination of cacti native to Brazil with different degrees of threat and endemism (*Cereus jamacaru, Discocactus zehntneri, Melocactus xalbicephalus, M. concinnus, M. paucispinus, M. zehntneri, M. ernestii, Micranthocereus flaviflorus, Pilosocereus gounellei and Stephanocereus luetzelburgii*) (Veiga-Barbosa et al., 2010; Assis et al., 2011, Marchi et al., 2013). The cacti species investigated in these conservation studies are threatened by illegal trade that supplies the ornamental plant market, but also by the destruction and fragmentation of their habitat (Marchi et al., 2013). In the work reported by Assis et al. (2011), the seeds of *M*. *zehntneri* and *M. ernestii* showed higher germinability after cryoconservation when compared to control, which also happened to seeds of *D. zehntneri* after 30 days of storage in liquid nitrogen (Marchi et al., 2013). An advantage for cryoconservation of seeds of these cacti species is the characteristically low moisture content of orthodox seeds. Besides, another favorable feature for cactus conservation is the small dimension of the seeds of such species, including the ones of the threatened genera *Melocactus* and *Discocactus*.

The orchid *Oncidium flexuosum* is native to Brazil, Paraguay and Argentina, and it is used for its ornamental and medicinal qualities (Galdiano et al., 2013). In order to cryoconserve this species, cryoprotectors were added to the vitrification solution, and it was observed that vitrification using PVS2 for 120 mins with 1% of phloroglucinol is the best alternative to cryoconserve mature seeds of *O. flexuosum* (Galdiano et al., 2013).

This treatment enhanced the *in vitro* germination of the species by 68% and the plants originated showed normal development and no ploidy level changes (Galdiano et al., 2013).

CRYOBANKS IN BRAZIL

For cryoconservation to be effective, germplasm banks need to work with hundreds of thousands of accessions, which can only be achieved with high efficiency approaches and cryoconservation technical knowledge. In order to do so, the development of equipment and installations, control of the sampling process and of the manipulation of accessions, creation of inventory and database, quality control, standardization of industry practices, presence of biosecurity systems and the valorization of germplasm are all necessary (Varga and Tiersch, 2012).

When installing cryobanks, differences between cryoconservation methodologies must be avoided among different institutions so that a general germplasm bank may be formed to improve conserved material exchange. Thus, comparative validations of methods between different laboratories or cryoconserved species must be made, as well as economic analysis. The duplication of cryoconserved resources is advised, to limit the risk of loss (Keller et al., 2008).

According to Santos and Salomão (2010), the Embrapa Genetic Resources and Biotechnology has a cryobank for storage of a great number of samples in the facility where collections are stored, where there is a cryotank of great capacity, besides installations in which to perform experiments and establish protocols for various materials. Efforts have been made in the last two decades in order to cryoconserve native plant species, cultivated or not, that were not present in the traditional seed banks and collections (Santos et al., 2013), but most efforts have been targeting species of economic interest.

The Embrapa Genetic Resources and Biotechnology (previously known as National Centre of Genetic Resources - CENARGEN) is the main institution in Brazil that deals with the conservation of genetic resources (Embrapa, 2014), and cryoconservation is one of the goals for long-term conservation in this corporation. The company plans on building cryobanks to be used as base collections, which should include samples of species that are already stored in active collections, as well as species or products that are not yet stored in any bank or collection (Pádua, personal communication, 2014). Species with non-orthodox seeds, which will have their vegetative structures stored, and also species with orthodox seeds, will be cryoconserved in these cryobanks.

Periodic evaluations of genetic stability and biological integrity of the conserved material are recommended, although there are no protocols regarding which would be the ideal time interval for monitoring (Santos and Salomão, 2010). Information is necessary about the minimum acceptable levels of viability, the adequate sample size for each accession, the periodicity of monitoring and the effect of cryogenic protocols and regeneration over genetic stability of the material kept in liquid nitrogen (Santos, 2000).

Aiming to cryogenically store seeds of importance to biodiversity and of economic relevance in semi-arid region of Brazil, Cavalcanti et al. (2011) proposed a modeling for a cryogenic germplasm bank which included traceability of stored seeds. The software BCSeed (applied to management and control of seeds) was created with satisfactory performance in all topics in which it was structured after tests with various products (Cavalcanti et al., 2011).

A GLOBAL VIEW

Plant rarity is increasing worldwide and many species that need protection are not given proper attention not only because they are underappreciated but also conservation projects are often underfunded or neglected (Havens et al., 2014). Nevertheless, throughout the world, countries are concerned with environmental preservation and conservation, and many have been making efforts to safeguard the genetic resources of their native plant species for decades, even though, very similarly to Brazil, many are still establishing cryobanks and cryoconservation protocols.

Theres is lack of research on seed storage biology and germination, propagation and acclimatization protocols for Eastern Australian rainforest species, which have yet to be cryoconserved since few studies have applied *in vitro* and cryopreservation techniques to the conservation of these species (Ashmore et al., 2011). Research and projects are underway to improve the *ex situ* conservation of Eastern Australian rainforest species (Ashmore et al., 2011), as much as for most of the Brazilian flora. South Africa is also progressing in cryostorage research and its application through major efforts made by the authorities to achieve cryoconservation of native plant species, endangered or of economic importance (Berjak et al., 2011). The exchange of knowledge between Brazil and these countries could benefit germplasm conservation efforts in all parties involved.

In the United States of America, native and threatened plant species are being studied, documented and safely stored in seed banks, and the country has the largest somatic tissue culture collection of rare species in the world stored in liquid nitrogen through the cryogenic conservation program at Kings Park and Botanic Garden (Havens et al., 2014). In the Russian Academy of Sciences (Moscow), 15 species of rare medicinal plants and crops are conserved in liquid nitrogen through cell strains and seeds of 230 endangered plant species collected

collected in the Russian territory are cryoconserved, as well as seeds from 22 rare tropical and Russian orchids (Popov et al., 2006). Both of these countries are more advanced in cryoconservation of native plant germplasm than Brazil, and Brazilian institutions could also benefit from collaborations with their research groups and institutions.

In Latin America and the Caribbean, Brazil is one amongst 34 countries that are considered megadiverse regarding the biodiversity that exists in their territory (González-Arnao and Engelmann, 2013). The cryoconservation progress and development of this region of the world has been documented since studies started in the period of 1988-1990, with the professional training of researchers (González-Arnao and Engelmann, 2013).

Since then Latin American national institutions have developed projects with advanced technologies to increase knowledge regarding establishment and optimization of cryoconservation protocols, but also to verify stability of the conserved material (González-Arnao and Engelmann, 2013). The collaboration among national institutions and universities where cryoconservation research is performed in Latin America and the Caribbean is encouraged in order to establish cryobanks in the member countries and further research and genetic preservation of resources (González-Arnao and Engelmann, 2013).

CONCLUSIONS

More studies are necessary to better comprise the Brazilian biodiversity in regards to the cryoconservation of its genetic resources. Most studies use seeds as plant material, which means that species that do not propagate through seeds, or whose seeds are difficult to obtain, are not being prioritized because they are more difficult to study or have protocols established for them, and recalcitrant seeds can be highlighted in this aspect. Many of the works here addressed studied species threatened by anthropogenic activities and chose cryoconservation as a way to safeguard their germplasm. This calls attention to the threats which species native to Brazil are suffering and how much genetic resources may be lost if there is no intervention in favor of their conservation. The Brazilian cryobanks, however, seem to be in a state of shaping and development, with many studies yet to be made and many accessions yet to be incorporated to collections in order for them to achieve an ideal stage of operation and collaboration with other institutions worldwide.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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