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Tolerance to Desiccation and Cryopreservation of Seeds of Seven South American *Ilex* Species

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Abstract. **This research aimed at evaluating the desiccation tolerance and ability to withstand cryostorage of intact seeds of seven South American *Ilex* species, and comparing different methodologies for in vitro germination of fresh and cryostored seeds. Intact seeds were silica gel–desiccated from 2 to 14 hours, placed in cryovials, and immersed in liquid nitrogen (LN). Survival was assessed through in vitro germination of intact seeds, bisected seeds, or isolated embryos. Seeds of the seven *Ilex* species (*Ilex brasiliensis* (Sprengel) Loes., *Ilex brevicuspis* R., *Ilex dumosa* var. *dumosa* R., *Ilex integerrima* (Vell. Conc.) R., *Ilex paraguariensis* A. St. Hil., *Ilex pseudoboxus* R., and *Ilex theezans* R.) tolerated desiccation to ≈6% moisture content (MC) and could be successfully cryopreserved when MC decreased between 6.4% and 8.4% depending on the species, before immersion in LN. In addition, it was established as the optimal condition for in vitro seed germination of the seven *Ilex* species. A simple and cost-effective cryogenic procedure (which did not require the use of cryoprotectants or sophisticated facilities) was defined for seeds of seven *Ilex* species, which provides a new alternative for safe long-term preservation of *Ilex* germplasm.**

Ilex L. (Aquifoliaceae) is the largest genus of woody dioecious plants, with at least 600 species (Galle, 1997; Loizeau and Spichiger, 2004). Although the genus is almost cosmopolitan, most species occur in mesic subtropical or tropical midmontane habitats in East Asia and the Americas. They are mainly deciduous or evergreen shrubs or small trees (Tsang and Corlett, 2005). The genus *Ilex* comprises several species of economic importance. Some of them, commonly named "hollies," such as "English holly" (*Ilex aquifolium*), "Japanese holly" (*Ilex crenata*), and "American holly" (*Ilex opaca*), have long been symbolic of Christmas and have also been cultivated by nurserymen in Europe, Asia, and the United States for landscaping (Hu, 1989; Walden and Wright, 1995). The "maté tree" (*I. paraguariensis*) is an economically and culturally important South American crop used in Argentina, southern Brazil, Paraguay, and Uruguay for making drinks that stimulate the central nervous system because of the presence of xanthine alkaloids, such as caffeine and theobromine (Filip et al., 2001). In addition, *I. paraguariensis* contains antioxidant compounds such as phenolic and tannic acids and has hepatoprotective, diuretic, hypocholesterolemic, anti-inflammatory, and anti-obesity effects (Bracesco et al., 2011). *Ilex brasiliensis*, *I. brevicuspis*, *I. dumosa*, *I. integerrima*, *I. pseudoboxus*, and *I. theezans* are almost entirely sympatric species with *I. paraguariensis*, and lately, they have become in plant genetic resources of the maté crop (Giberti, 1999).

Ex situ conservation of *Ilex* germplasm is necessary to safeguard the threatened diversity of this genus, mainly because of negative anthropogenic impact. These species are usually conserved in field collections, where they remain exposed to diseases, pests, fire, drought, and human damage, leading to genetic erosion (Giberti, 1999; Zhang et al., 2014). Thus, research for alternative methods to field conservation for *Ilex* genetic resources has become a priority. Seed storage (with 3% to 7% MC at –20 °C) is the most effective and efficient method for ex situ conservation of genetic resources of plants which produce orthodox seeds, combining low storage costs (100 times cheaper than in situ conservation of individual trees) with ease of seed distribution and regeneration of whole plants from genetically diverse materials (Li and Pritchard, 2009; Linington and Pritchard, 2001).

Ilex seeds are regularly shed at high water contents (greater than 30%), a feature that is often associated with recalcitrant behavior (Berjak et al., 1992). However, the tolerance of *Ilex* seeds to desiccation and low temperature storage has not been properly investigated. Moreover, seeds of *Ilex* species are individually enclosed by a woody endocarp and have undeveloped embryos (mostly at the heart stage) when fruits reach maturity (Dolce et al., 2007; Martin, 1946; Niklas, 1987; Tsang and Corlett, 2005), resulting in a deep dormancy and low germination rate (Hu, 1975; Hu et al., 1979). For example, *I. opaca* seeds germinate in nature after 1–3 years and the germination rate is about one in 10 million (Ives, 1923). This extremely low seed germination constitutes a serious inconvenience for breeding and conservation programs because it leads to the loss of potentially valuable genotypes. Besides, the small size of the embryos (160–350 μm in length) and the high level of dormancy of *Ilex* seeds have hampered efforts to gain knowledge about their storage characteristics.

Seed cryopreservation, i.e., storage at the ultralow temperature of LN (–196 °C), may be of particular importance for the long-term storage (10–100s years) of problem species (Pritchard, 2007; Walters et al., 2004). This is an easy and reliable storage technology because only storage in LN leads to a virtually complete arrest of metabolism and ensures long-term conservation of seeds (Bonner, 1990). Cryopreservation prevents the depletion of reserve substances, accumulation of toxins, decomposition and inactivation of enzyme complexes, and autoxidation of lipids (Stanwood, 1985), thereby avoiding the risk of genetic and epigenetic changes (Bonner, 1990). Thus, seed cryobanks are promising for conserving the biological diversity of plant species and for maintaining the stability of their genotypes (Kholina and Voronkova, 2008).

This work aimed at evaluating the desiccation tolerance and ability to withstand cryostorage of intact seeds of seven South American *Ilex* species and comparing different methodologies for in vitro germination of fresh and cryostored seeds, including *I. paraguariensis* (the source of raw material for the production of maté, the most popular beverage in the south cone of South America) and wild relatives with different valuable characteristics for the genetic improvement of maté crop.

Materials and Methods

Plant material. Fresh seeds (pyrenes) of seven South American *Ilex* species, *I. brasiliensis* Loes., *I. brevicuspis* R., *I. dumosa* R. var. *dumosa*, *I. integerrima* (Vell.) R., *I. paraguariensis* A. St. Hil., *I. pseudoboxus* R., and *I. theezans* Mart. ex R., were used in

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this study. Open-pollinated ripened fruits (≈3 months after anthesis) were hand-harvested from trees growing at the Estación Experimental Agropecuaria Cerro Azul (Misiones, Argentina)—Instituto Nacional de Tecnología Agropecuaria. Seeds were removed from the fruits, cleaned of the pulp, and immediately used for experiments.

Seed desiccation and cryopreservation. Seeds were desiccated by placing them in tightly sealed containers (100 cm^3) over 30 g silica gel (Honeywell Riedel-de Haën AG, 30926, Seelze, Germany) for 0–14 h. Samples were taken at 2-h intervals to determine MC. After every desiccation period tested, three replications of 50 seeds were used as desiccation controls (–LN) and three other samples of 50 seeds were placed in 2-mL polypropylene vials (Nalgene®; Nalge Company, Rochester, NY) and immersed in LN (+LN). After cryostorage for 1 week, the cryovials were retrieved from LN and rewarmed in a water bath at 40 °C for 2 min.

Seed MC determination. After the different desiccation periods tested, the MC of intact seeds was determined gravimetrically after oven-drying at 103 ± 2 °C for 17 h and expressed on a percentage fresh weight basis. Five replicates of 300 seeds were used for each of these determinations.

Germinability assessment. Before culturing, all seeds coming from both the desiccation (–LN) and desiccation + cryostorage (+LN) treatments were pre-humidified for 24 h at 27 ± 2 °C to prevent imbibition damage. This was accomplished by placing the seeds over distilled water in hermetically sealed glass bottles. The seeds were then surface-sterilized by soaking them in 70% ethanol for 1 min, followed by immersion in an aqueous solution of 2.5% sodium hypochlorite and 0.1% Triton X-100® (Merck & Co., Darmstadt, Germany) for 60 min with continuous shaking on a rotatory shaker (100 rpm). The seeds were rinsed three times with sterile distilled water before being processed for inoculation.

The initial germinability (fresh seeds), survival after desiccation (–LN), and desiccation + cryostorage (+LN) were assessed by in vitro culturing of different explants (i.e., intact seeds, bisected seeds, and isolated embryos), as recommended by Dolce et al. (2011, 2015) and Sansberro et al. (1998) for different *Ilex* species. Explants were cultured on 4 mL solidified (A1296 SIGMA Agar; Merck KGaA, Darmstadt, Germany) quarter-strength Murashige and Skoog (1962) basal medium supplemented with 0.1 $mg \cdot L^{-1}$ zeatin, in 11-cm^3 glass tubes. The pH of the media was adjusted to 5.8 before adding agar. Tubes with culture medium were sterilized by autoclaving at 1.45 $kg \cdot cm^{-2}$ and 120 °C for 20 min. The culture tubes were sealed with Resinite AF 50® (Casco. S.A.I.C. Co., Buenos Aires, Argentina) and incubated in a growth room at 27 ± 2 °C with a 14-h light/10-h dark photoperiod, under an irradiance of 116 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density provided by cool-white fluorescent lamps (intact and bisected seeds), or in permanent darkness for the first 30 d and then transferred to light condition (isolated embryos). Germinability was scored after 60 d of culture by counting the seeds and isolated embryos, which formed normal seedlings.

Seedlings derived from desiccation (–LN) and desiccation + cryostorage (+LN) treatments were carefully washed with tap water and transferred to pots containing lateritic acid soil from the natural maté-growing region. During the first weeks, plantlets were kept in a growth room at 27 ± 2 °C with a 14-h light/10-h dark photoperiod and covered with translucent plastic bags to maintain a high relative humidity and prevent dehydration. The relative humidity was gradually decreased by removing the bags for increasingly longer periods of time. Finally, the plantlets were deprived of the bags and kept in greenhouse conditions for acclimatization.

Experimental design and data analysis. Treatments were arranged in a completely randomized design with three replications of 50 seeds per treatment. Data were expressed as mean ± SE and subjected to analysis of variance. The significance of mean differences was determined using Duncan's multiple comparison test ($P < 0.05$). The Shapiro–Wilks test was used to verify the normality of the data.

Results

Seed MC determination. Seed MC decreased from an initial average of 40.9% ± 0.2% to an average of 6.2% ± 0.1% after 14 h desiccation. Seeds of *I. brevicuspis*, *I. dumosa*, and *I. paraguariensis* showed faster dehydration rates compared with the other *Ilex* species. They reached ≈8% of MC after 6 h desiccation, whereas seeds of *I. brasiliensis*, *I. integerrima*, *I. pseudoboxus*, and *I. theezans* reached the same MC only after 10–12 h exposure to silica gel (Fig. 1).

Germinability assessment. The germination percentage of fresh seeds (nondesiccated and noncryostored) differed depending on the species and type of explant cultured (Table 1). For *I. dumosa*, *I. brevicuspis*, and *I. paraguariensis*, the highest germination percentages were achieved by in vitro culturing bisected seeds or isolated embryos. On the other hand, *I. pseudoboxus*, *I. brasiliensis*, and *I. theezans* germinated best when intact seeds or isolated embryos were cultured, whereas for *I. integerrima*, high germination percentages were only possible by culturing isolated embryos.

Seeds of all species tolerated desiccation down to ≈6% MC without significant

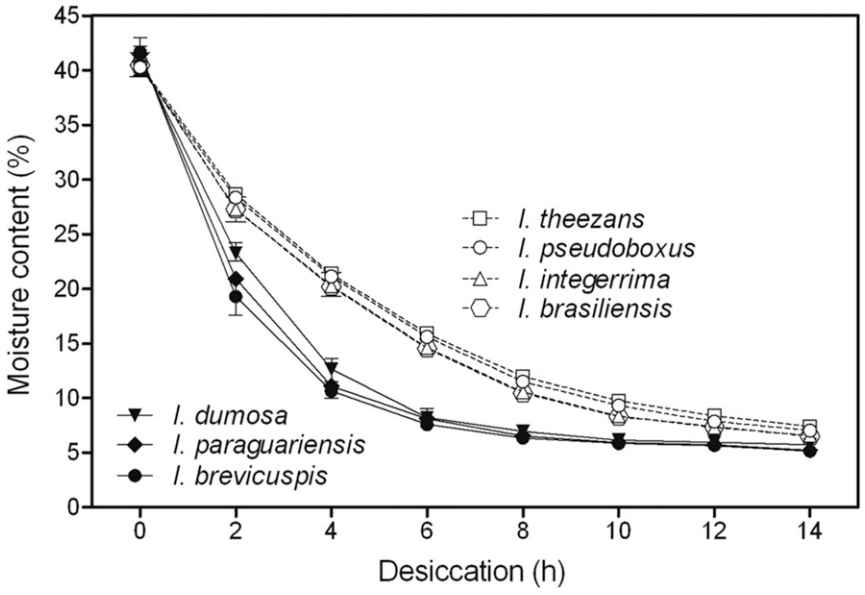

Fig. 1. Effect of desiccation duration (h) on seed moisture content (%MC, fresh weight basis) of seven South American *Ilex* species. Data are the mean of five replicates. Bars indicate SE.

Table 1. In vitro germination (%)[z] of intact seeds, bisected seeds, and isolated embryos of nontreated (nondesiccated and noncryostored) seeds from seven *Ilex* species.

	Explant		
Species	Intact seeds	Bisected seeds	Isolated embryos
Ilex brasiliensis	54.7 ± 3.5 b	20.7 ± 2.9 a	50.0 ± 4.2 b
Ilex brevicuspis	2.0 ± 3.5 a	42.7 ± 2.9 b	40.7 ± 2.4 b
Ilex dumosa	2.7 ± 3.5 a	59.3 ± 4.8 c	46.0 ± 4.2 b
Ilex integerrima	0.0 ± 0.0 a	7.3 ± 2.4 a	76.7 ± 4.4 b
Ilex paraguariensis	0.0 ± 0.0 a	46.0 ± 4.2 b	45.3 ± 2.4 b
Ilex pseudoboxus	81.3 ± 3.5 c	3.3 ± 1.3 a	65.3 ± 3.7 b
Ilex theezans	52.7 ± 2.9 b	20.7 ± 3.5 a	58.0 ± 4.2 b

[z]Data are the mean of three replicates ± SE. Within each row, means followed by different letters are significantly different (Duncan's multiple comparison test; $P < 0.05$).

differences on germination percentages (by in vitro culturing of intact seeds, bisected seeds, and isolated embryos) compared with that of their respective controls (nondesiccated and noncryostored seeds) (data not shown). The effect of desiccation duration on seed germination after cryostorage (+LN) of the seven *Ilex* species is presented in Fig. 2. Fresh seeds (MC ≈40%) of any species did not tolerate cryostorage, but the ability to withstand immersion in LN increased in line with the desiccation time. Once seed MC was optimized before immersion in LN, cryostorage did not significantly affect seed survival compared with their controls (nondesiccated and noncryostored seeds). Moreover, in the case of *I. brasiliensis* (54.7% vs. 66.7%), *I. brevicuspis* (42.7% vs. 51.3%), and *I. theezans* (52.7% vs. 76.7%), LN exposure significantly enhanced germination compared with the control seeds.

Maximal survival after cryostorage was achieved when seed MC was between 6.4% and 8.4%, depending on the species. For *I. pseudoboxus*, *I. brasiliensis*, and *I. theezans*, germination after LN exposure was significantly higher by desiccating seeds for 10–14 h and in vitro culturing intact seeds or isolated embryos (Fig. 2A–C). On the other hand, the highest survival of *I. brevicuspis*, *I. dumosa*, and *I. paraguariensis* was achieved by desiccating seeds for 8–10 h and in vitro culturing bisected seeds or isolated embryos (Fig. 2E–G). For *I. integerrima*, the germination percentages after cryostorage were significantly higher when seeds were desiccated for 10–12 h and then its isolated embryos were cultured (Fig. 2D). Thus, it was clearly established the different requirements for in vitro germination of cryostored and noncryostored seeds, depending on the *Ilex* species. In both cases (with and without cryostorage), the optimal condition coincides for in vitro seed germination of each species.

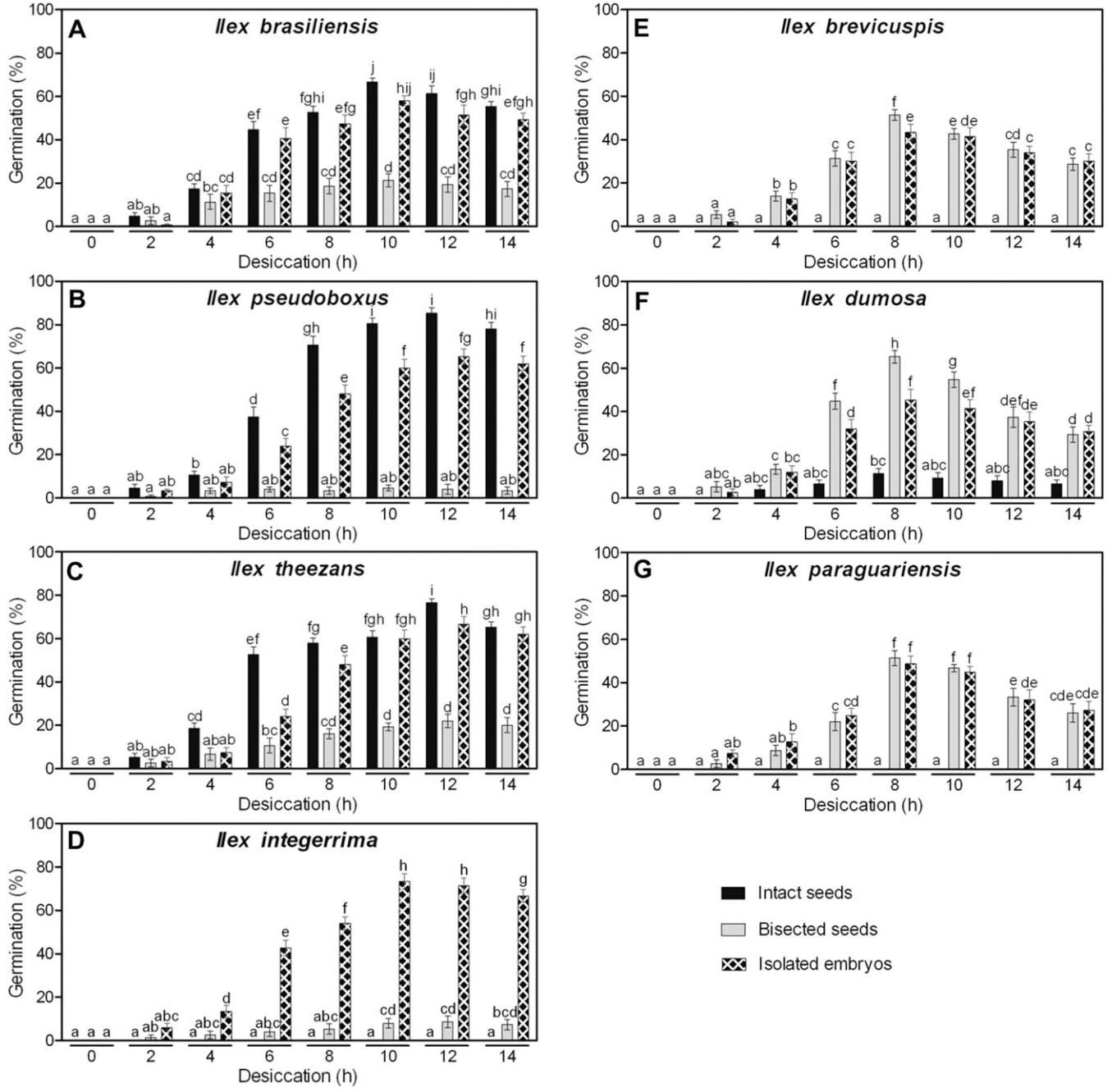

Fig. 2. Effect of desiccation duration (h) on survival after seed cryostorage (+liquid nitrogen) of seven South American *Ilex* species. Survival was assessed by in vitro culturing of intact seeds, bisected seeds, and isolated embryos. Data are the mean of three replications. Bars indicate SE. Different letters denote significant differences according to Duncan's multiple comparison test ($P < 0.05$).

Seedlings of the seven *Ilex* species tested developed into healthy plants and were successfully established in pots and kept under greenhouse conditions with a high survival (90% to 95%) of 12 months after their acclimatization (Fig. 3).

Discussion

Avoidance of intracellular ice formation during exposure to cryogenic temperatures is a prerequisite for successful cryopreservation of living organs, tissues, and cells (Endoh et al., 2018). The most critical factor affecting cryopreservation of seeds is MC (Michalak et al., 2015a; Verdier et al., 2013), so it should be determined for each species the accurate MC which allows seeds to tolerate cryostorage. Dehydration must be sufficient to avoid lethal intracellular freezing during cooling in LN but not so intense to induce extended desiccation injury. In optimal cases, no significant differences are observed in the survival rates of desiccated control and cryopreserved material (Da Silva et al., 2017; Endoh et al., 2018; Michalak et al., 2015b; Pammenter and Berjak, 1999).

Our data indicate that intact seeds of the seven *Ilex* species tested withstand cryostorage with no reduction in germinability when they are properly desiccated (6.4% to 8.4% MC) before immersion in LN. Even more, as was observed in three species, cryostorage enhanced germination. Similar response was reported for several orchid seeds (Nikishina et al., 2007; Popov et al., 2004); for *Pyrus communis* (Reed et al., 2001), *Halimium atriplicifolium*, *Helianthemum apenninum*, *Helianthemum squamatum* (Pérez-García and González-Benito, 2008), *Angelica ursina*, *Oxytropis chankaenis*, *Oxytropis retusa* (Kholina and Voronkova, 2008), *Elaeis guineensis* (Camillo et al., 2009), *Passiflora suberosa*, and *Passiflora edulis* (Araújo et al., 2016); and for wild medicinal legume species (Kholina and Voronkova, 2012) after seed cryostorage. It has been suggested that LN exposure may enhance the germination of treated seeds by breaking seed dormancy or softening of the seed coat (Kholina and Voronkova, 2008; Reed et al., 2001). In many species, exposition of seeds to low temperatures decreases the endogen content of abscisic acid and increases the gibberellin and cytokinin levels, which interact in a sequential way to break dormancy (Bewley and Black, 1994).

The present study showed that cryopreservation of seeds of the seven *Ilex* species tested could be successfully achieved using the desiccation technique. This is a very simple and cost-effective cryopreservation method because neither cryoprotectants nor any sophisticated facilities are necessary. The previous recommendations for cryopreserving *Ilex* germplasm included isolating embryos and using encapsulation–dehydration (Mroginski et al., 2008, 2011). Compared with intact seed cryopreservation, that protocol has some disadvantages such as time and labor consumption (encapsulation of excised embryos and exogenous application of cryoprotectants). In this work, we propose a simplified protocol for *Ilex* germplasm cryostorage. The best procedure involves desiccation of intact seeds to 6.4% to 8.4% MC, immersion in LN, followed by rapid rewarming and in vitro germination by culturing intact/bisected seeds or isolated embryos, depending on the species (average survival for the seven species = 67% ± 2%). This protocol, besides being very simple, allows obtaining higher recovery rates than cryopreservation of isolated embryos using encapsulation–dehydration (average survival for the same seven species = 37% ± 9%) (Mroginski et al., 2011). Taking this into account that conventional seed germination of *Ilex* species is very poor, it is interesting to note the relevant role of the in vitro tissue culture techniques for assessing seed germinability in conservation programs. In previous works, it was studied the requirements for germination of several *Ilex* seeds (Dolce et al., 2010, 2011, 2015; Hu, 1975; Hu et al., 1979; Sansberro et al., 1998). These investigations allowed determining that the better culture condition differs with different *Ilex* species (e.g., intact seeds for *I. brasiliensis*, *I. pseudoboxus*, and *I. theezans*; bisected seeds for *I. brevicuspis* and *I. dumosa*; and isolated embryos for *I. integerrima*, *I. aquifolium*, *Ilex cornuta*, and *I. opaca*). Such differences were confirmed in this study.

This article presents, for the first time, information on the tolerance of desiccation and cryostorage of intact seeds of seven *Ilex* species. A simple and cost-effective cryogenic procedure (which did not require the use of cryoprotectants or sophisticated equipment) was defined for seeds of seven *Ilex* species. This approach will contribute to the development of new ex situ conservation programs for safeguarding the genetic diversity of *Ilex* species, as a complementary option to field collections.

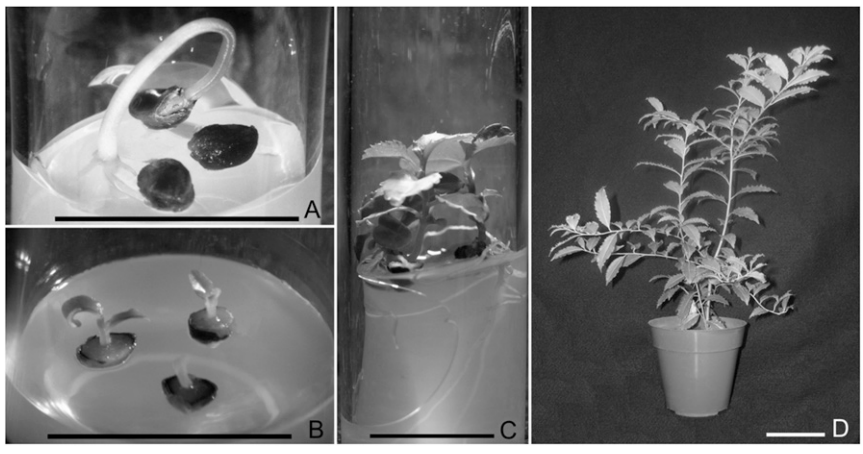

Fig. 3. Plant regeneration from cryostored seeds of *Ilex* spp. (**A** and **B**) In vitro germination of *Ilex theezans* intact seeds (**A**) and of *Ilex brevicuspis* bisected seeds (**B**) after 30 d of culture, bar = 1 cm. (**C**) In vitro plantlets of *I. theezans* after 60 d of culture, bar = 1 cm. (**D**) *Ilex brevicuspis* plant after 12 months of pot culture in a greenhouse, bar = 10 cm.

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