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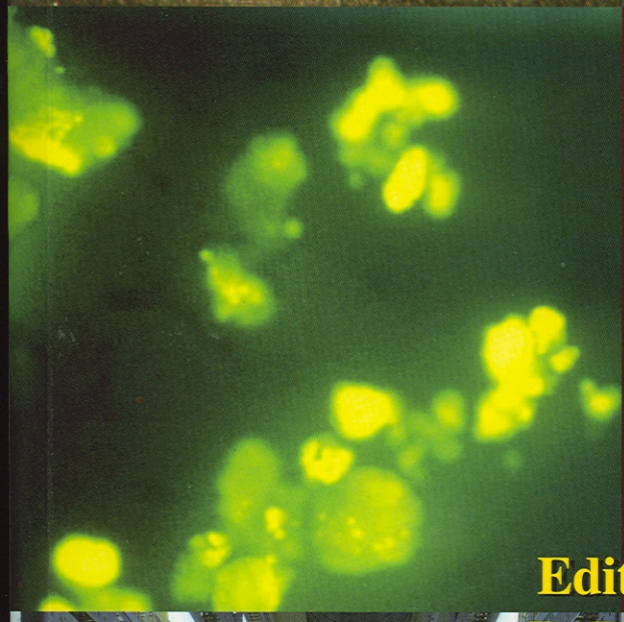

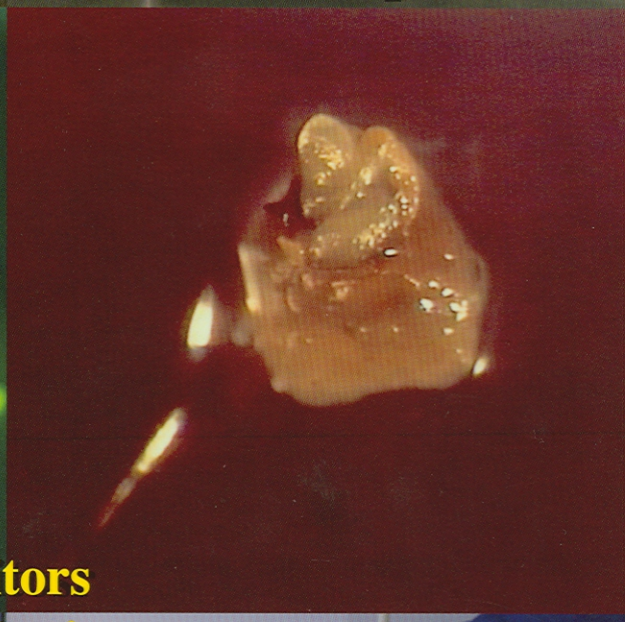

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1. Droplet freezing of potato meristems. Photograph by courtesy of Joachim Keller, IPK, Gatersben, Germany.
2. The NBPGR (National Bureau for Plant genetic resources, New Delhi, India) cryopreservation facilities. Photograph by courtesy of Bart Panis, Katholieke Universiteit Leuven, Leuven, Belgium.
3. FDA (fluorescein diacetate) test of cryopreserved banana cell suspensions, viable cells fluorescing yellow. Photograph by courtesy of Bart Panis, Katholieke Universiteit Leuven, Leuven, Belgium.
4. Recovering banana meristem, two weeks following droplet vitrification. Photograph by courtesy of Bart Panis, Katholieke Universiteit Leuven, Leuven, Belgium.
5. The Musa germplasm collection hosted by the Bioversity International Transit Centre. Photograph by courtesy of Bart Panis, Katholieke Universiteit Leuven, Leuven, Belgium.
6. Rack containing cryotubes removed from the liquid nitrogen tank. Photograph by courtesy of Bart Panis, Katholieke Universiteit Leuven, Leuven, Belgium.

Cryopreservation of *Ilex dumosa* (*Aquifoliaceae*) Germplasm

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Keywords: seed, zygotic embryo, shoot-tip, desiccation, encapsulation, liquid nitrogen

Abstract

Most of the subtropical *Ilex* species have recalcitrant seeds and therefore, not suitable for long-term preservation using conventional seed storage methods. Thus, the germplasm of *Ilex* spp. is maintained in the field as ex situ genebanks. This work describes experiments demonstrating the feasibility of long-term conservation of *I. dumosa* through cryopreservation of both whole seeds and zygotic rudimentary embryos. Lately, this species has received greater attention from plant breeders because, besides the quality of its leaves for making the stimulatory beverage named 'maté' with less caffeine than the ones from *I. paraguariensis*, the plants are resistant to some pests. For cryopreservation of zygotic embryos and apical shoot-tips, the explants were aseptically removed, encapsulated in 3% calcium alginate beads and pregrown for 24 h intervals in liquid medium enriched with progressively increasing sucrose concentrations (0.5, 0.75 and 1 M). The beads were then dehydrated with silica gel to 25% water content and plunged rapidly in liquid nitrogen. The beads were rewarmed by immersion in a water bath at 30°C. Finally, the beads were transferred onto culture medium (1/4 MS, 3% sucrose, 0.1 mg L^{-1} zeatin, solidified with 0.8% agar) and incubated in a growth room at 27°C under a 14 h photoperiod (116 $\mu molm^{-2} s^{-1}$). By culturing cryopreserved embryos, as much as 42% of them produced plants. However, no plants were recovered when apical shoot-tips were cryopreserved. In addition, a procedure for cryopreservation of mature intact seeds of *I. dumosa* by desiccation with silica gel and rapid freezing was developed. Up to 40% of the cryopreserved seeds produced plants when they were cultivated in vitro in an appropriate culture medium.

INTRODUCTION

The genus *Ilex* (*Aquifoliaceae*) comprises about 600 species of deciduous or evergreen shrubs or trees that inhabit temperate and tropical regions of the world. Among them, 220 species are native to South America. *Ilex paraguariensis* ('yerba mate') is extensively cultivated in North-eastern Argentina, eastern Paraguay and South-eastern Brazil due to its economic importance (Loizeau, 1994; Giberti, 1995). Its leaves and shoots are used for making a stimulatory beverage named 'maté' which has several health benefits (Schinella et al., 2005). In the last years, *I. dumosa* ('yerba señorita') has generated interest from plant breeders because plants of this species are resistant to the attacks of certain pests. Also, 'maté' made from its leaves contains less caffeine than the ones from *I. paraguariensis* (Filip et al., 1999).

Like other species of *Ilex* (Hu, 1989), seeds of *I. dumosa* usually contain rudimentary embryos that remain at the immature heart-shaped stage for a long time after fruits reach maturity (Dolce et al., 2007) and they are also highly sensitive to desiccation and cold. In other words, according to Roberts (1973) the seeds of this species are recalcitrant and therefore unsuitable for the dry and/or cold seed storage procedures traditionally employed for plant germplasm preservation (Engelmann, 1991). Consequently, its germplasm is conserved as whole plants in field collections. Although some work has been done for cryopreservation of zygotic embryos of *I. dumosa*, the procedure involves the culture of embryos dissected from seeds of immature fruits for obtaining complete plants (Mroginski et al., 2006, 2008).

In this study, we describe procedures for cryopreservation of *I. dumosa* germplasm

using both seeds and zygotic embryos dissected from seeds of mature fruits.

MATERIALS AND METHODS

Plant Material

Ten-years-old field grown plants of *Ilex dumosa* var. *dumosa* R. (herbarium specimen is deposited in CTES as 321289) growing at Estación Experimental Agropecuaria (INTA) Cerro Azul, Misiones, Argentina were used. Three types of tissues were employed in this study: a) zygotic embryos, b) apical shoot-tips and c) mature seeds.

Experiment 1: Cryopreservation of Zygotic Embryos and Shoot-Tips

Mature fruits (drupes) were collected and stored at 4°C for 30 d. Subsequently the pulp was removed and the seeds were surface-sterilized by a 2 min immersion in 70% ethanol, followed by a 45 min treatment in 2% sodium hypochlorite solution containing 0.1% of Triton X-100® (Merk, Darmstadt, Germany). The seeds were then washed three times with sterile distilled water and left in the final rinse until use. Finally, rudimentary embryos (0.15-0.25 mm in length) at the heart developmental stage were aseptically removed.

Apical shoot-tips (2-3 mm in length) were dissected from 60-d-old in vitro regenerated plants obtained following the procedure describes by Luna et al. (2003).

Both zygotic embryos and apical shoot-tips were cryopreserved according to the protocol of Mroginski et al. (2008). The explants were encapsulated in 3% calcium alginate beads and pregrown for 24 h intervals in liquid medium enriched with progressively increasing sucrose concentration (0.5, 0.75 and 1 M). The beads were then dehydrated for 5 h with silica gel to 25% water content (fresh weight basis). Dried beads were placed in sterile 5 ml cryovials (10 beads/vial) and plunged rapidly in liquid nitrogen (LN) where they were kept for at least 24 h. The beads were rewarmed by immersing the cryovials for 1 min in a water bath at 30°C. In order to get whole plants, the beads containing zygotic embryos were transferred onto ¼ MS (quarter-strength salts and vitamins of Murashige and Skoog, 1962 medium) with 3% sucrose, 0.1 mg L^{-1} ZE (zeatin) and solidified with 0.8% agar, according to Sansberro et al. (2001). The beads containing shoot-tips were recultured on solidified ¼ MS, 3% sucrose and 1 mg L^{-1} BAP (6-benzylaminopurine) in order to achieve shoot regeneration. In all cases, the beads were incubated in a growth room at 27±2°C under a 14 h photoperiod (116 µmol $m^{-2}s^{-1}$).

Experiment 2: Cryopreservation of Seeds

Intact seeds obtained as described in Experiment 1 were dehydrated by using 30 g silica gel for 0-15 h in 100 ml air-tight containers. After dehydration to different water contents, the seeds were placed in sterile 5 ml cryovials (30 seeds/vial) and plunged directly in LN for at least 48 h. Subsequently, the seeds were rewarmed by immersing the cryovials in a water bath at 35°C for 2 min.

For germination test, the seeds were: a) cultured on solidified (0.8% agar) ¼ MS medium with 3% sucrose and 0.1 mg L^{-1} ZE; b) placed in a germinator with filter paper and cotton as substrate; or c) placed in a germinator with sand as substrate. Finally, the seeds were incubated at 27±2°C under a 14 h photoperiod (116 µmol m^{-2} s^{-1}).

The water content of beads and seeds was expressed on a fresh weight basis. For this purpose, the fresh weight of the samples was measured after different desiccation periods tested and subsequently they were placed in the oven at 103°C for 17 h to determinate their dry weight.

Survival Assessment and Statistical Analysis

The experiments were replicated three times with 30 samples per treatment. Survival of plant material was evaluated by counting the number of plants obtained after 60 days incubation (in the case of zygotic embryos and shoot-tips) or after 30, 60, 90, 120 and 150 days culture (in the case of intact seeds). Means are presented with their standard

errors. Data were subjected to analysis of variance (ANOVA) and comparison of mean was realized with Tukey's Multiple Comparison Test ($P<0.05$).

The water content of beads and seeds was measured using three replicates of 30 or 100 samples per treatment, respectively.

RESULTS AND DISCUSSION

Experiment 1

Both zygotic embryos and shoot-tips of *Ilex dumosa* showed relatively high percentages of plant regeneration (62.5 and 93.3% respectively). Likewise, there were no major differences in plant regeneration when the explants were dissected and encapsulated or pretreated with sucrose (Table 1). This fact suggests that these operations used in this work did not affect per se the viability of embryos and shoot-tips. Similar results were reported when zygotic embryos from immature fruits were used (Mroginski et al., 2008). However, when the explants were subsequently dehydrated, their behavior was completely different. While 50% of the zygotic embryos regenerated into plants, it did not happen with any shoot-tip (Table 1).

The immersion of the explants in LN resulted in the death of all shoot-tips, while when the zygotic embryos were appropriately encapsulated, pretreated with sucrose and dehydrated, 42% of them germinated and produced whole plants (Table 1).

These results show, on one hand, the necessity of finding some system that allows the cryopreservation of shoot-tips and, on the other hand, demonstrate that it is possible to use zygotic embryos extracted from mature fruits as explants, with similar results to those obtained when embryos of immature fruits are used (Mroginski et al., 2008).

Experiment 2

In general, independent of the treatment and the substrate used, the germination of seeds was very slow and the maximum values were reached after 120-150 days incubation (Fig. 1).

Results of seed cryopreservation of *Ilex dumosa* are given in Table 2. An analysis of these results shows that:

1. The seeds which have not been dehydrated (43.3% water content) lose their viability totally after immersion in LN. Thus, these results indicate clearly that this procedure was always lethal. Meanwhile, the control seeds (without desiccation or cryopreservation) have shown germination percentages between 1.7 and 20.0%. These values are lower than the ones reported earlier from the culture of isolated embryos (Mroginski et al., 2006; 2008). There are at least two possible explanations for this response. First, the experiments were done in different years and second, in these experiments we used mature seeds instead immature ones.
2. A period of 9 to 13 h dehydration (seeds with a water content of 7.6 to 5.9%) gave the biggest germination values when seeds were cryopreserved (25.6 to 40.0%).
3. The substrate for seed germination proved to be very important, as highest percentages of seed germination were obtained when cultured in vitro in an appropriate culture medium. It is probable that this fact is related with the immature embryos requirement of substances which are not present in a germinator with cotton or sand. Moreover, seedlings emerged from seed sowed on cotton become chlorotic and had slight grown (Fig. 2).

In conclusion, these results might be considered as the basis for a new concept of germplasm preservation of *Ilex dumosa* involving direct cryogenic storage of whole seeds, previously desiccated with silica gel to water content around 7%. For plant recovery the best procedure was the in vitro culture of the seeds on ¼ MS with 3% sucrose and 0.1 mg L^{-1} ZE.

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Literature Cited

Dolce, N.R., Gonzalo, A.G. and Rey, H.Y. 2007. Embriología de *Ilex dumosa* e *I. brevicuspis* (*Aquifoliaceae*). Bol. Soc. Arg. Bot. 42(Suppl.):40-41.

Engelmann, F. 1991. In vitro conservation of tropical plant germplasm – a review. Euphytica 57:227-243.

Filip, R., López, P.J. and Ferraro, G.E. 1999. Phytochemical study of *Ilex dumosa*. Acta Hort. 501:333-336.

Giberti, G.C. 1995. Florística, sistemática y potencialidades con relación a un banco de germoplasma para la yerba mate. p.303-312. In: H. Winge, A.G. Ferreira, J.E. de Araujo Mariath and L. Tarasconi (eds.), Erva-Mate: Biologia e Cultura no Cono Sul, A. UFRGS, Brasil.

Hu, C.Y. 1989. Holly (*Ilex* spp.). p.412-427. In: Y.P.S. Bajaj (ed.), Biotechnology in Agriculture and Forestry, Vol. 5. Springer Verlag, Berlin.

Loizeau, P.A. 1994. Les Aquifoliaceas péruviennes. Boissiera 48:1-306.

Luna, C., Sansberro, P., Mroginski, L. and Tarragó, J. 2003. Micropropagation of *Ilex dumosa* (Aquifoliaceae) from nodal segments in a tissue culture system. Biocell. 27:205-212.

Mroginski, L.A., Sansberro, P.A., Scocchi, A.M., Luna, C.V. and Rey, H.Y. 2006. Effect of fruit cryopreservation on in vitro germination of zygotic embryos of several species of *Ilex*. Acta Hort. 725:417-420.

Mroginski, L.A., Sansberro, P.A., Scocchi, A.M., Luna, C. and Rey, H.Y. 2008. A cryopreservation protocol for immature zygotic embryos of species of *Ilex* (Aquifoliaceae). Biocell. 32:33-39.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant.15:473-497.

Roberts, E.H. 1973. Predicting the storage life of seeds. Seed Sci. Technol. 1: 499-514.

Sansberro, P.A., Rey, H.Y and Mroginski, L.A. 2001. In vitro culture of zygotic embryos of *Ilex* species. HortSci. 36:351-352.

Schinella, G., Fantinelli, J.C. and Mosca, S.M. 2005. Cardioprotective effect of *Ilex paraguariensis* extract: evidence for a nitric oxide-dependent mechanism. Clin. Nutr. 24:360-366.

Tables

Table 1. Explants forming plantlets (%) for control and cryopreserved shoot-tips and zygotic embryos of *Ilex dumosa*, employing the encapsulation-dehydration technique.

Treatments				% Explants forming plants	
Encapsulation	Pretreatment with sucrose	Dehydration (5 h)	LN	Shoot-tips	Zygotic embryos
-	-	-	-	93.3	62.5
+	-	-	-	90.0	56.3
+	+	-	-	83.3	68.8
+	+	+	-	0.0	50.0
+	+	+	+	0.0	42.0
+	-	-	+	0.0	0.0
+	+	-	+	0.0	0.0

Table 2. Effect of different dehydration periods with silica gel followed or not by direct plunging of seeds into LN, on seed germination (%) after 120 days culture. Means in each column followed by different letters are different according to Tukey's multiple comparison test with $p \leq 0.05$.

Treatments		Seed germination (%)		
Dehydration (h) [1]	LN	Semisolid medium [2]	Cotton [3]	Sand [4]
0 (43.3 ± 0.5)	-	20.0 b	14.4 b	1.7 bc
9 (7.6 ± 0.2)	-	27.8 ab	13.3 bc	2.0 bc
11 (6.5 ± 0.2)	-	24.4 b	22.2 b	1.3 bc
13 (5.9 ± 0.2)	-	30.0 ab	19.9 b	1.7 bc
15 (5.7 ± 0.1)	-	18.9 b	13.3 bc	1.0 bc
0 (43.3 ± 0.5)	+	0.0 c	0.0 c	0.0 c
9 (7.6 ± 0.2)	+	25.6 ab	15.6 b	0.0 c
11 (6.5 ± 0.2)	+	27.8 ab	23.3 b	1.0 bc
13 (5.9 ± 0.2)	+	40.0 a	26.7 b	3.3 b
15 (5.7 ± 0.1)	+	21.1 b	14.4 b	1.7 bc

[1] Values in parenthesis correspond to the moisture content (% FW basis) for each dehydration time. Means are presented with their standard errors.

[2] Solidified ¼ MS, with 3% sucrose and 0.1 mg L^{-1} zeatin (0.8% Agar).

[3] Germinator with 5 mm layer of cotton and filter paper as substrate, soaked with distilled water.

[4] Germinator with sand as substrate, soaked with distilled water.

Figures

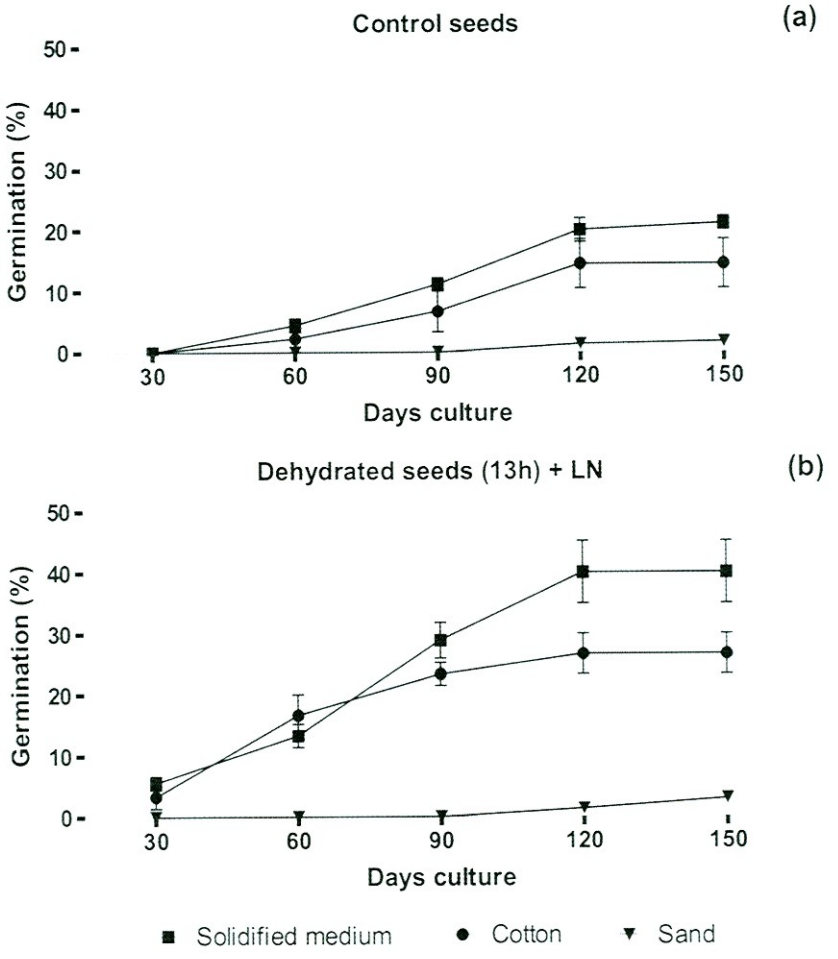

Fig. 1. Germination (%) of control **(a)** and cryopreserved **(b)** seeds of *Ilex dumosa* along 150 days culture on solidified medium (¼ MS 3% sucrose, 0.1 mg L^{-1} zeatin and 0.8% agar) and germinators with cotton or sand as substrate. Means are presented with their standard errors.

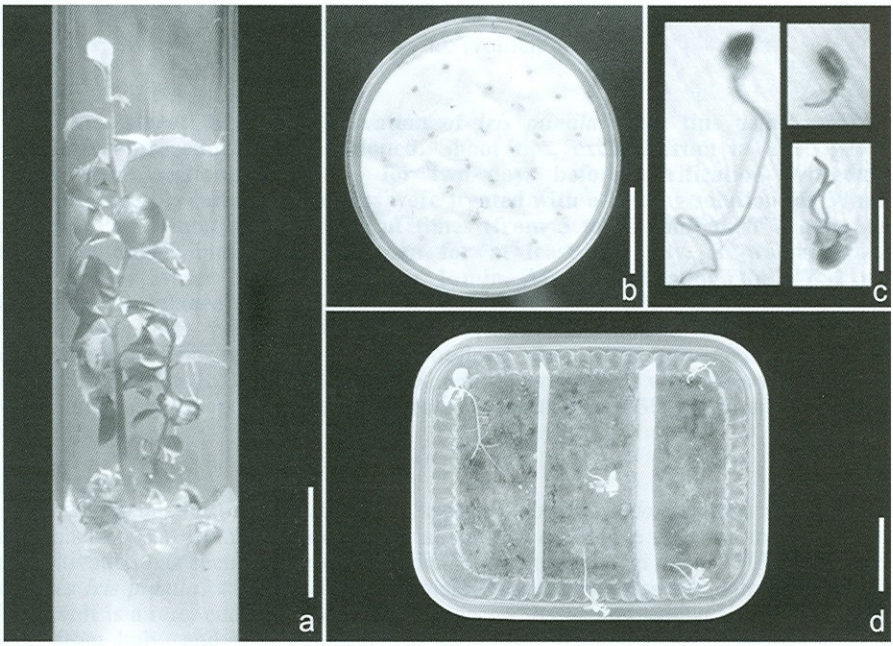

Fig. 2. Germination of cryopreserved seeds of *Ilex dumosa* after 120 days culture. **(a)** Plantlets originated from seeds cultured in vitro on solidified ¼ MS medium, with 3% sucrose and 0.1 mg L^{-1} zeatin (bar = 1 cm); **(b)** germination of seeds sowed in germinators with cotton as substrate (bar = 2 cm); **(c)** detail of germinated seeds showing that there was not ulterior growth of the seedlings when seeds were cultured on cotton (bar = 1 cm), and **(d)** plantlets originated from seeds placed in sand (bar = 5 cm).