In Vitro Culture of Zygotic Embryos of *Ilex* Species

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Additional index words. plant regeneration, germination, embryo culture, holly, propagation

Abstract. Plants of *Ilex argentina* L., *I. brasiliensis* (S.) L., *I. brevicuspis* R., *I. dumosa* R., *I. integerrima* (V.C.) L., *I. microdonta* R., *I. pseudoboxus* R., and *I. theezans* C.M. were obtained by immature embryo culture. Heart-stage zygotic embryos were removed from immature fruits and cultured aseptically on quarter-strength Murashige and Skoog medium with 3% sucrose, 0.65% agar, and 0.1 mg·L⁻¹ zeatin. Cultures were incubated at 27 ± 2 °C for 4 weeks, in darkness and subsequently transferred to a culture room with a 14-hour photoperiod (116 μmol·m⁻²·s⁻¹) for another 4 weeks. Seedlings with two leaves, derived from germinated embryos, were successfully transplanted to pots containing 1 peat : 1 perlite : 1 sand (v/v) and were maintained in greenhouse conditions. From 95% to 100% of transplanted seedlings survived. Chemical name used: 6-(4-hydroxy-3-methylbut-2-enylamino) purine (zeatin).

The genus *Ilex* (Aquifoliaceae) is widely distributed with over 500 species that inhabit temperate and tropical regions of both hemispheres (Giberti, 1995; Hu, 1989). Some species of the genus are economically important. The “maté tree” (*Ilex paraguariensis* St. Hil.), a perennial crop, is an important source of income in some regions of northeastern Argentina, Paraguay, and southern Brazil. Its leaves are used for making a stimulatory beverage named “maté” (Giberti, 1995). *Ilex vomitoria* Aiton, *I. guayusa* Loes., and *I. tarapotina* Loes. are used in infusions (Loizeau, 1994). Chinese holly (*I. cornuta* Lindl. Hitchcock), American holly (*I. opaca* Ait. Farage), and English holly (*I. aquifolium* L.) are cultivated as landscape plants (Hu, 1989). One of the main barriers in breeding *Ilex* species is delayed germination of seeds, because of the presence of rudimentary embryos that remain in the immature, heart-shaped stage (Fig. 1A) long after fruits reach maturity (Hu, 1989).

For more than a half century, the technique of embryo rescue has been used successfully in many crops to accelerate the maturation of rudimentary embryos (Sharma et al., 1996). An in vitro culture medium capable of supporting the development of immature embryos is a prerequisite to the use of this technique for rapid seed germination. Several successful attempts have been made to develop protocols for culture of immature embryos

ensuring plant recovery in *Ilex paraguariensis* (Sansberro et al., 1998) as well as in 11 other species of *Ilex* (Hu, 1975, 1989). The objective of this investigation was to establish an efficient system recovering plants from rudimentary embryos of eight South American species of *Ilex*. In the breeding program of the Instituto Nacional de Tecnología Agropecuaria (INTA), these species serve as sources of resistance to disease and pests in interspecific hybridization with *I. paraguariensis*. However, breeding is severely limited by seed dormancy.

Materials and Methods

Estación Experimental Agropecuaria (INTA) Cerro Azul, Misiones, Argentina, kindly provided the plant material. Immature fruits (drupes) from greenhouse-grown plants (7–8 years old) of *Ilex* sp. (Table 1) were collected in Feb. 1999 (2–3 months after hand pollination). Fruits of single plants of each species were surface-disinfected by soaking in 70% ethanol for 5 min, followed by immersion in 1.8% sodium hypochlorite and two drops of Triton X-100® (Merck, Darmstadt, Germany) for 30 min, rinsed three times with sterile distilled water, and left in the final rinse until embryo excision. Seeds were separated from pulp under aseptic conditions and embryos were excised with a surgical blade under a

stereomicroscope before transfer to culture medium. The culture medium was that reported by Sansberro et al. (1998) for embryo culture of *I. paraguariensis* and consisted of 1/4-strength Murashige and Skoog (1962) medium with 3% sucrose, 0.65% agar (A-1296; Sigma Chemical Co., St. Louis), and 0.1 mg·L⁻¹ zeatin. Medium pH was adjusted to 5.8 with KOH or HCl before the addition of agar. Tubes were covered with aluminum foil and autoclaved at 1.46 kg·cm⁻² for 20 min.

Embryos at the heart-shaped stage (0.18–0.26 mm in length) were cultured on 3 mL of medium in 11-mL glass tubes (one embryo per tube) and sealed with Resinite AF 50 (Casco S.A.I.C. Co., Buenos Aires, Argentina); 10–14 embryos were cultured per species per experiment in a completely random design. Each experiment was repeated three times. Embryos were cultured for 28 d in darkness at 27 ± 2 °C. Tubes containing germinated embryos were placed at 27 ± 2 °C under a 14-h photoperiod with 36-W cool-white fluorescent lamps (116 μmol·m⁻²·s⁻¹). After another 28 d of culture, seedlings with at least two true leaves were transplanted to 5-cm-diameter pots containing 1 peat : 1 perlite : 1 sand (by volume) and kept in a greenhouse. Relative humidity was maintained at 95% to 100% during the first 7 d by a misting device and then decreased gradually.

Results and Discussion

Most of the embryos that had been dissected at the heart-shaped stage (Fig. 1A) of all eight *Ilex* species started to grow shortly after incubation on the nutrient medium. During the first week of culture, 1% of the tubes became contaminated with bacteria, fungi, or both. Within 1 month of incubation the embryos passed through the following sequential stages: heart-shaped, torpedo, mature embryo, and germination. The frequency of embryos that became seedlings was high and, depending on the plant species, ranged from 45% to 97% (Table 1). These frequencies are in agreement with those obtained for other *Ilex* species (Hu, 1989; Sansberro et al., 1998).

In most species, mature embryos were observed after 14–21 d of culture, whereas embryos of all species tested germinated after 21–28 d of culture. These results are consistent with those reported for other *Ilex* species (Hu, 1989; Sansberro et al., 1998), where embryos required 10–30 d of culture to germinate. In contrast, embryos dissected from *Ilex*

Table 1. Plant regeneration by in vitro culture of rudimentary embryos of eight species of *Ilex* cultured on 1/4 MS with 3% sucrose and 0.1 mg·L⁻¹ zeatin.

Plant species	Accession no.	Place of collection	No. of observations	Embryos forming seedlings (% ± SE)
<i>I. argentina</i>	111	Achelar, Tucumán, Argentina	35	97 ± 6
<i>I. brasiliensis</i>	059	Rio Branco do Sul, Brazil	32	82 ± 1
<i>I. brevicuspis</i>	088	Palmas, Brazil	33	94 ± 3
<i>I. dumosa</i>	090	Palmas, Brazil	36	45 ± 7
<i>I. integerrima</i>	056	Tijucas do Sul, Brazil	33	61 ± 13
<i>I. microdonta</i>	064	Reserva Marumbi, Brazil	34	89 ± 3
<i>I. pseudoboxus</i>	132	Tramandaí, Brazil	42	93 ± 4
<i>I. theezans</i>	046	San Antonio, Mnes., Argentina	33	97 ± 3

Received for publication 20 Oct 1999. Accepted for publication 28 July 2000. This research was supported by the Secretaría General de Ciencia y Técnica (Universidad Nacional del Nordeste) and by Consejo Nacional de Investigaciones Científicas y Técnicas. We thank A. Goytía for preparing the illustrations. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

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Fig. 1. Plants of *Ilex* sp. obtained by in vitro culture of immature embryos. Vertical bars represents 1 mm (A), 1 cm (B, C, D, E, and F), and 10 cm (G). (A) Heart-stage embryo of *I. pseudoboxus* cultured in vitro. (B) Seedling of *I. dumosa*. (C) Seedling of *I. brasiliensis*. (D) Seedling of *I. theezans*. (E) Seedling of *I. integririma*. (F) Multiple shoot regeneration of *I. microdonta*. (G) Plants of *I. pseudoboxus* obtained by embryo culture.

aquifolium required an incubation period of 6–8 weeks (Hu, 1975).

When tubes containing germinated embryos were transferred from darkness to light, whole plants were obtained. Most showed a normal phenotype with several leaves and a well-developed root system (Fig. 1B–E). However, some embryos of *I. microdonta* produced multiple shoots without roots (Fig. 1F). In addition, some embryos (3% to 36%) of *I. brasiliensis*, *I. brevicuspis*, *I. dumosa*, and *I. integririma* failed to survive in vitro or remained without any visible response. Also, for 2% to 11% of embryos of *I. argentina*, *I. dumosa*, and *I. integririma*, callus growth was noticeable after 2–3 weeks of culture. In embryo culture of *Ilex* species, browning of the explants was not observed; such browning is common after culture of nodal segments (Mroginski et al., 1999).

Seedlings of all *Ilex* species in this study were successfully transplanted with a survival rate of 95% to 100% after 3 months (Fig. 1G). This high survival rate agrees with those for other species of *Ilex*, such as *I. aquifolium*, *I. crenata*, *I. glabra*, *I. opaca*, *I. verticillata* (Hu,

1989), and *I. paraguariensis* (Sansberro et al., 1998).

This work demonstrates for the first time that plantlets of *I. argentina*, *I. brasiliensis*, *I. brevicuspis*, *I. dumosa*, *I. integririma*, *I. microdonta*, *I. pseudoboxus*, and *I. theezans* can be achieved readily by in vitro culture of immature (heart stage) embryos on a semi-solid 1/4-strength Murashige and Skoog (1962) medium with 3% sucrose and 0.1 mg·L⁻¹ zeatin. Rapid progress in the application of this method to breeding programs for *I. paraguariensis* is expected. We are currently using embryo culture to develop a system for cryopreservation of *Ilex* germplasm (Scocchi et al., 1998).

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