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Enhanced Seed Germination of *Ilex dumosa* R. (Aquifoliaceae) through In Vitro Culture of Cut Pyrenes

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Abstract. **An in vitro culture protocol was developed that increased the germination percentage and decreased the lag time to germination for *Ilex dumosa* R. pyrenes as a tool for replacing the laborious task of embryo rescue technique. This method involves transversely cutting surface-sterilized pyrenes with a scalpel blade, then placing the micropylar one-third end with the rudimentary embryo (≈0.25 mm long) on solidified (agar 0.65%) quarter-strength salts and vitamins of Murashige and Skoog, 1962 medium with 3% sucrose, and incubating in a growth room at 27 ± 2 °C with a 14-h photoperiod (116 μmol·m^{-2}·s^{-1}). Most of the cut pyrenes (greater than 50%) germinated within the first month after inoculation and achieved maximum germination (≈70%) in 2 months compared with whole pyrenes, which began to germinate 3 months after sowing and required more than 8 months for maximum germination (37%). Moreover, the germination percentage of cut pyrenes was significantly higher than the germination of isolated embryos (34%). Thus, the cut pyrenes culture is a simpler and more effective technique than embryo rescue. Easily, on average, a trained operator is able to culture ≈1000 cut pyrenes per day instead of ≈100 isolated embryos.**

The genus *Ilex* L. (Aquifoliaceae) is the largest genus of woody dioecious plants with at least 600 species (Galle, 1997; Loizeau and Spichiger, 2004). They are generally deciduous and evergreen trees or shrubs occurring in temperate and tropical regions (Giberti, 1995; Hu, 1989). Various plant species are economically important. The English holly (*I. aquifolium*), the American holly (*I. opaca*), and the Chinese holly (*I. cornuta*) are cultivated for landscape architecture (Hu, 1989). The maté tree (*I. paraguariensis*) is a perennial crop whose leaves are used in Argentina, southern Brazil, Paraguay, and Uruguay for making a very popular stimulatory beverage, which has several health benefits (Schinella et al., 2005). Additionally, other species such as *I. guayusa*, *I. tarapotina*, and *I. vomitoria* are used in infusions (Loizeau, 1994). Lately, *I. dumosa* has awakened an interest from plant breeders as a result of the quality of its leaves for making "maté" with less caffeine that from *I. paraguariensis* (Filip et al., 1999, 2001) and the resistance of the plants to the main pests of the maté tree (Prat Kricun and Belingheri, 1995).

The dispersal unit of *Ilex* species is the pyrene, formed by the seed enclosed in a woody endocarp. The seeds of *Ilex* species normally contain rudimentary embryos, which remain at the immature heart or late-heart stage long after the fruits mature (Hu, 1975; Ives, 1923; Niklas, 1987). As such, a minimum of 5 to 9 months under optimal conditions is required for embryo maturation, and even then seed germination by using conventional methods is very poor (Hu, 1975; Hu et al., 1979; Ives, 1923). This fact constitutes a serious inconvenience for breeding programs because it results in the loss of potentially valuable genotypes. Furthermore, for conservation purposes, seed as a source of plant material as compared with vegetative material is preferable because it has a wider genetic base. When conventional methods produce low or no germination, in vitro techniques may greatly enhance germination and growth rates by optimizing culture conditions and media (Fay, 1992).

The embryo rescue technique has been used for over 50 years to accelerate the maturation of rudimentary embryos in many crops (Sharma et al., 1996). Several attempts have been made to develop protocols for culturing isolated immature embryos of *I. dumosa* (Sansberro et al., 2001) plus 19 other *Ilex* species (Ferreira et al., 1991; Hu, 1975, 1989; Mroginski et al., 2010; Sansberro et al., 1998, 2001). However, because the embryos are minute (0.16 to 0.35 mm in length) and they are easily damaged during the isolation manipulation, it is necessary to know exactly the place of the seed in which they are located (Mroginski et al., 2010). Also it is a very laborious task (on average, a trained operator is able to isolate ≈100 embryos per day). Therefore, the development of a protocol for easy and efficient propagation of this species is necessary. In this study, we describe a reliable and reproducible method to propagate *I. dumosa* through in vitro culture of cut pyrenes.

Materials and Methods

Plant material. Open-pollinated ripe drupes of *I. dumosa* (Fig. 1A) were collected in February 2008 from trees at the EEA INTA Cerro Azul (Misiones, Argentina). The fruits were separated into three lots: 1) used immediately or 2) stored in a refrigerator at 4 °C for 30 d or 3) for 60 d. Pyrenes from each lot were separated from the pulp and used immediately after isolation (Fig. 1B–C).

The plant material was prepared in a laminar flow hood. Pyrenes were surface-sterilized by soaking them in 70% ethanol for 2 min followed by immersion in an aqueous solution of 2.5% sodium hypochlorite and 0.1% Triton X-100® (Merck, Darmstadt, Germany) for 60 min and then rinsed three times in sterile distilled water. The sterilized pyrenes were separated into three lots: 1) used immediately or 2) stored in a refrigerator at 4 °C for 30 d or 3) for 60 d.

In vitro germination of whole and cut pyrenes or isolated embryos from fresh fruits. Fruits immediately after their collection were used as the source of explants in this experiment. Three kinds of explants were inoculated: 1) whole pyrenes (Fig. 1C); 2) cut pyrenes (Fig. 1D) that were prepared under a stereomicroscope by transversely cutting the pyrenes with a scalpel blade and then culturing the micropylar one-third end with the rudimentary embryo; and 3) isolated embryos at the heart stage (≈0.25 mm long) that were separated from the endosperm following the procedure described by Mroginski et al. (2010). The explants were in vitro-cultured on 4 mL of solidified (0.65% Sigma agar A-1296) MS/4 [quarter-strength salts and vitamins of Murashige and Skoog (1962) medium with 3% sucrose] in 11-cm^3 glass tubes (six explants/tube). The pH of the medium was adjusted to 5.8 with KOH or HCl before adding agar. The tubes with media were sterilized by autoclaving at 1.45 kg·cm^{-2} and 120 °C for 20 min. Cultures were sealed with Resinite AF 50® (Casco S.A.I.C. Company, Bs.As., Argentina) and incubated in a growth room at 27 ± 2 °C with a 14-h photoperiod (116 μmol·m^{-2}·s^{-1}).

In vitro germination of whole and cut pyrenes from cold stored fruits and pyrenes. Fruits and surface-sterilized pyrenes stored at 4 °C for 30 or 60 d were used as the source of explants in this experiment. Moreover, pyrenes from fresh fruits were used as a control treatment. In all cases, whole and cut pyrenes were in vitro-cultured on MS/4 as described previously and incubated in a growth room at 27 ± 2 °C with a 14-h photoperiod or in darkness.

Germination of whole or cut pyrenes on different substrates under aseptic or nonaseptic conditions. Whole or cut pyrenes were sowed under aseptic conditions in germinators consisting of 90 × 15-mm glass petri dishes (30 explants/dish) containing 40 mL of solidified MS/4 or with a 5-mm layer of cotton and filter paper or Sphagnum peat soaked with sterile

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distilled water. Pyrenes were surface-sterilized and petri dishes were sterilized as described previously. Additionally, whole or cut pyrenes were sowed under non-aseptic conditions in petri dishes containing 40 cm^3 of sand or with a 5-mm layer of cotton and filter paper or Sphagnum peat soaked with distilled water. In all cases, germinators with whole or cut pyrenes were sealed with Resinite AF 50® and incubated in a growth room at 27 ± 2 °C with a 14-h photoperiod.

All treatments were assayed every 30 d for germination percentages until 300 d after inoculation. When isolated embryos were cultured, the gradual change in shape of the immature embryos was observed until germination. This change was characterized by radicle elongation followed by immediate hypocotyl elongation and cotyledon expansion. When whole and bisected pyrenes were cultured, the emergence of the shoot and/or root was used as the criterion to calculate germination rate (Fig. 1E–F).

Statistical analysis. The treatments were arranged in a completely randomized design with three replicates of 30 or 60 samples per treatment. The data were subjected to analysis of variance (ANOVA) and the significance of mean differences was determined using Tukey's multiple comparison test ($P < 0.05$).

Percentage data were transformed using the square root for the cold storage experiment and the transformation log(2+%germination) for the different substrates experiment. The normality of the transformations was verified before applying the ANOVA by the Shapiro-Wilks test ($P < 0.05$).

Results and Discussion

After 30 d inoculation, the results of in vitro culture of whole and cut pyrenes or isolated embryos from fresh fruits (Fig. 2) showed that although the germination of whole pyrenes was nil, as much as 51% of the cut pyrenes germinated. This percentage was significantly higher than that of isolated embryos (34.4%), which is similar to the one obtained previously (Sansberro et al., 2001). After 60 d of culture, this difference increased, achieving germination percentages of 67.2% and 34.4% from cut pyrenes and isolated embryos, respectively. Thus, the in vitro culture of cut pyrenes, besides being a simpler technique, is more efficient than the culture of isolated embryos to obtain quick germination of *I. dumosa* embryos.

It is interesting to note that the rest of its own endosperm (cut pyrenes) improved the germination of *I. dumosa* embryos compared with inoculation of isolated embryos (Fig. 2). This result is in disagreement with that reported by Hu et al. (1979) who have reported that the growth of isolated embryos of *I. aquifolium*, *I. cornuta*, and *I. opaca* in vitro was drastically reduced when the embryos were cultured adjacent to their own endosperm and they have strongly suggested the presence of growth inhibitors in *Ilex* endosperm and/or in the membrane-like testa attached to the endosperm. However, no attempt has been made to isolate and identify these putative inhibitors.

On the other hand, germination of whole pyrenes was not observed until after at least 3 months and it still took 9 months to achieve maximum germination (37.8%). Therefore, cutting pyrenes increased germination and decreased the time to maximum germination compared with whole pyrenes. Similar results have been reported by Miller et al. (1992) and Ke et al. (1985) for *Fragaria* achenes and *Rubus* seeds, respectively. Thus, this technique may be applicable to many crop seeds that have a hard coat surrounding the embryo.

It is not very well known how cutting affects pyrenes, achenes, and other seeds with

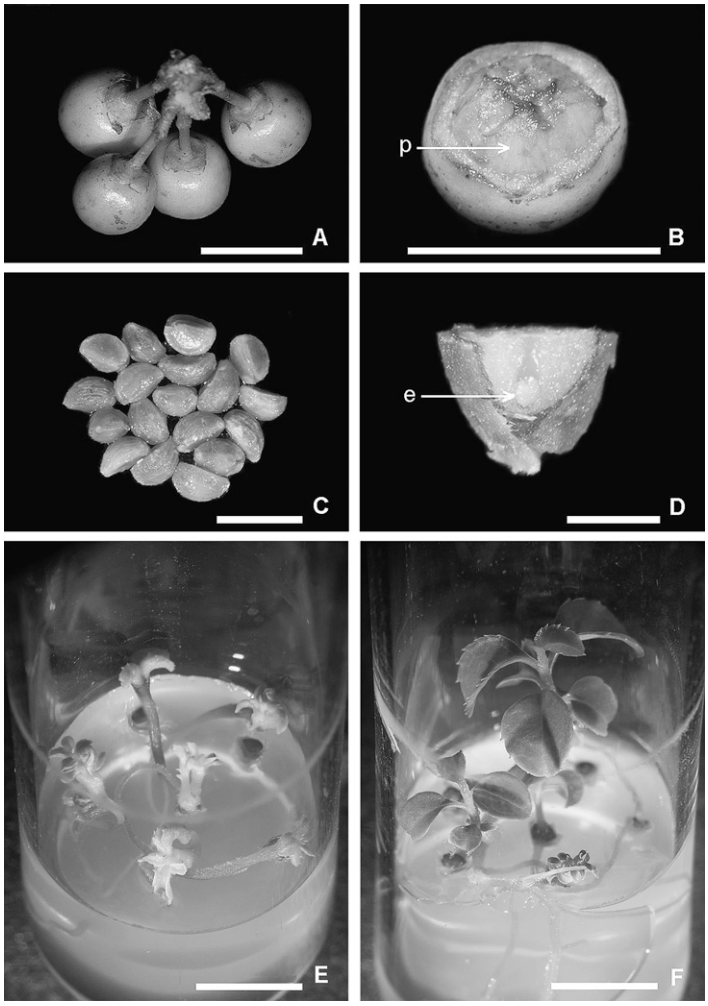

Fig. 1. *Ilex dumosa* (**A**) fruits. (**B**) Fruit showing four pyrenes. Arrows shows a pyrene. (**C**) Pyrenes. (**D**) Cut pyrene showing the embryo. (**E–F**) Cut pyrenes germinated after (**E**) 30 d and (**F**) 60 d cultured on MS/4 and incubated in a growth room at 27 ± 2 °C with a 14-h photoperiod. Bars represent 5 mm in **A, B, C, E, F**; in **D** represent 1 mm.

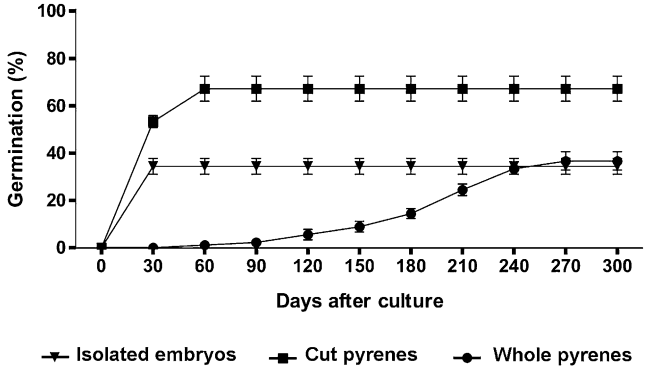

Fig. 2. Germination time courses of isolated embryos, whole and cut *Ilex dumosa* pyrenes cultured on MS/4 and incubated in a growth room at 27 ± 2 °C with a 14-h photoperiod. Bars indicate SE.

Table 1. Germination (%) of *Ilex dumosa* pyrenes with or without a cold treatment (4 °C) after 30 d cultured on MS/4 and incubated in a growth room at 27 ± 2 °C with a 14-h photoperiod or in darkness.[z]

	Culture condition			
	Light		Darkness	
Treatment	Whole pyrenes	Cut pyrenes	Whole pyrenes	Cut pyrenes
Fresh fruits, AT	0.0	56.7 a	0.0	27.8 a
Fruits, 30 d 4 °C	0.0	47.8 ab	0.0	16.7 a
Fruits, 60 d 4 °C	0.0	23.3 c	0.0	10.0 a
Pyrenes, 30 d 4 °C	0.0	50.0 ab	0.0	18.9 a
Pyrenes, 60 d 4 °C	0.0	31.1 bc	0.0	12.2 a

[z]Within the columns means followed by different letters are significantly different (Tukey's multiple comparison test; $P < 0.05$).
AT = ambient temperature.

Table 2. Effect of different substrates on germination percentage of *Ilex dumosa* pyrenes after 30 d sowed and incubated in a growth room at 27 ± 2 °C with a 14-h photoperiod.[z]

		Germination (%)	
Culture condition		Whole pyrenes	Cut pyrenes
Aseptic	MS/4	0.0	52.2 a
	Paper	0.0	14.4 ab
	Sphagnum	0.0	5.6 bc
Non-aseptic	Sand	0.0	2.2 c
	Paper	0.0	3.3 bc
	Sphagnum	0.0	2.2 c

[z]Within the columns means followed by different letters are significantly different (Tukey's multiple comparison test; $P < 0.05$).

hard coats and promotes faster and higher germination percentages than for control seeds. Possible explanations for the cut seed effect are: 1) cutting may mimic mechanical scarification, which makes the tissue surrounding the embryo permeable to water and gases (Hartmann and Kester, 1959); 2) the hard endocarp acts as a physical barrier to the expansion and germination of the embryo and cutting seems to be an effective means to break the barrier; and 3) the endosperm may contain one or more endogenous germination inhibitors. After cutting the seeds, the inhibitor(s) may diffuse away from the embryo and germination might occur (Miller et al., 1992).

The cold storage (4 °C) of either fruits or isolated pyrenes did not improve the germination percentage of *I. dumosa* pyrenes (Table 1). Although cut pyrenes from fruits and whole pyrenes stored for 30 d at 4 °C exhibited similar germination percentages as the cut unstored control pyrenes, the cold storage for a longer period (60 d) decreased germination compared with the control treatment when cut pyrenes were incubated in light. These results are not in agreement with Dolce et al. (2010) and Sansberro et al. (2000) who have found that low-temperature treatment increased the germination percentage of *I. paraguariensis* isolated embryos and cut pyrenes cultured in vitro. In many species, exposition of seeds to low temperatures decreases the endogenous content of abscisic acid and increases the gibberellin and cytokinin levels, which interact in a sequential way to break dormancy (Bewley and Black, 1994). On the other hand, the light regime had a significant effect on germination of *I. dumosa* cut pyrenes (Table 1) and subsequent seedling growth. Incubation in continuous dark resulted in significantly lower germination and severe inhibition of the seedling growth. Cultures maintained in darkness were etiolated with cotyledonary leaves scarcely developed and lacked true leaves. Instead, seedlings germinated under a 14-h photoperiod were green and vigorous with four true leaves in most of cases after 2 months of cutting and sowing the pyrenes. This response differs from that reported by Ferreira and Hu (1989) and Hu (1976) for isolated embryos of *I. opaca*, *I. aquifolium*, and *I. paraguariensis*, whose in vitro late embryogeny was light-inhibited.

The substrate had a relevant effect on *I. dumosa* seed germination (Table 2). Although cut pyrenes germinated after 30 d culture in all of the substrates assayed, the germination percentage was significantly higher when they were inoculated in aseptic conditions on solidified MS/4. Moreover, when cut pyrenes were cultured under non-aseptic conditions, the germination was nearly void. On the other hand, the germination of whole pyrenes was nil in all the conditions assayed after 30 d culture. Although 8 months after sowing, they germinated, the percentages were very poor (between 1% and 5%) when whole pyrenes were cultured under non-aseptic conditions. For comparison, when whole pyrenes were sowed under aseptic conditions, they achieved higher germination percentages (36%, 23%, and 12% from pyrenes placed on MS/4, paper–cotton, and Sphagnum peat, respectively) after 8 months of culture. The cause of the poor germination for pyrenes inoculated under non-aseptic conditions may be largely pathogen responsibility. During the extended time required for the embryo maturation, the seeds are prone to suffering attack by pathogens and heavy pre-germination mortality.

In conclusion, these studies demonstrate for the first time that the in vitro germination of *I. dumosa* cut pyrenes may be achieved readily and may be an alternative to embryo rescue. The culture of cut pyrenes is a simpler and more effective technique than the culture of isolated embryos. Easily, on average, a trained operator is able to culture ≈1000 cut pyrenes per day instead of ≈100 isolated embryos.

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