

Endosperm and endocarp effects on the *Ilex paraguariensis* A. St.-Hil. (Aquifoliaceae) seed germination

N.R. DOLCE, L.A. MROGINSKI AND H.Y. REY

Instituto de Botánica del Nordeste-CONICET, Facultad de Ciencias Agrarias (UNNE), C.C. 209, Corrientes (3400), Argentina (E-mail: ndolce@agr.unne.edu.ar)

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Summary

To develop a procedure for regenerating plants directly from pyrenes, as an alternative to *in vitro* embryo culture, germination studies were done on maté tree, *Ilex paraguariensis*, seeds using aseptic conditions. The effect of the endosperm, the woody endocarp and cold treatment on embryo development and germination were examined by culturing isolated embryos, intact, acid-scarified and bisected pyrenes from mature fruits. The results show that maté tree plantlets may be obtained by *in vitro* culture of bisected pyrenes on solidified (0.65% agar) quarter-strength Murashige and Skoog medium containing 3% sucrose. It is recommended to pre-culture the pyrenes at $4 \pm 2^\circ\text{C}$ to obtain a higher germination rate and more vigorous seedlings. This is the first report of the *in vitro* culture of *Ilex* pyrenes. Compared to the culture of isolated embryos this procedure shortens the duration of the embryo culture technique and minimizes damage to the young embryos.

Introduction

The genus *Ilex* L. (family Aquifoliaceae) contains over 500 species of deciduous and evergreen trees and shrubs growing in the temperate and tropical regions (Hu, 1989; Giberti, 1995). *Ilex paraguariensis* A. St.-Hil., a perennial crop known locally as the “maté tree”, is used in Argentina, southern Brazil, Paraguay and Uruguay to make a very popular beverage highly appreciated for its flavour and stimulating properties due to its caffeine and theobromine contents (Filip *et al.*, 2001).

The dispersal unit of *Ilex* species is the pyrene, formed by the seed enclosed in a woody endocarp. Like other *Ilex* species (Ives, 1923; Hu, 1975) *I. paraguariensis* seeds contain rudimentary embryos, which remain at the immature heart or late-heart stage long after the fruits mature (Niklas, 1987). As such, a minimum of 5-9 months under optimal conditions is required for embryo maturation, and even then seed germination is very poor (Fontana *et al.*, 1990).

The dormancy of *I. paraguariensis* seeds has been attributed to: i) intrinsic causes related to embryo and/or endosperm (inhibitors, suspensor maintenance) and ii) extrinsic causes related to the seed coats (endocarp consistency, impermeability) (Almeida *et al.*, 2000).

Previous work (Hu, 1975; Hu *et al.*, 1979; Sansberro *et al.*, 1998; 2001) showed that embryonic growth resumed immediately after excision and placement of the embryos on a nutrient medium. As such, the *in situ* quiescent period of *Ilex* embryos is considered to be an imposed dormancy rather than a hereditary characteristic of the embryonic cells. That is, certain factor(s) must exist in the endosperm and/or endocarp to prevent further *in situ* development of the heart-shaped embryos.

The growth of isolated *I. aquifolium*, *I. cornuta* and *I. opaca* embryos *in vitro* is drastically reduced when the embryo is cultured adjacent to its endosperm (Hu *et al.*, 1979). This strongly suggests 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 supposed inhibitors. An opposing viewpoint reported by Cunha (1990) is that the development of *I. paraguariensis* embryos *in vitro* is not inhibited when the endosperm is in direct contact with the embryo.

Conversely, Mello (1980) and Jeske *et al.* (2000) concluded that the slow development of the embryos and the low germination rate are not directly related to the permeability of the pyrenes, which can absorb water although they have a woody endocarp. Alternatively, the endocarp may act as a mechanical barrier, interfering in the expansion of tissues rather than in water absorption.

For over 50 years, the embryo rescue technique has been used 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. paraguariensis* (Ferreira *et al.*, 1991; Sansberro *et al.*, 1998) plus 19 *Ilex* species (Hu, 1975; 1989; Sansberro *et al.*, 2001). Nevertheless, there are no reports of *in vitro* culture of *Ilex* species pyrenes. The aim of the present research was to develop a procedure to regenerate plants directly from *I. paraguariensis* pyrenes as an alternative to *in vitro* culture of embryos and to study the effect of various factors on seed germination under aseptic conditions. The results should prove useful for evaluating the survival in seed conservation programs.

Materials and methods

Plant material

Open pollinated ripe drupes of *Ilex paraguariensis* were collected in February, 2008, from trees at the EEA INTA Cerro Azul (Misiones, Argentina). The pyrenes were separated from the pulp and 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® (Merk, Darmstadt, Germany) for 60 min and then rinsed three times in sterile distilled water.

Culture media and conditions

A first assay was made to determine the effect of the endosperm on embryo development and germination. Embryos were cultured along with their own endosperm (bisected pyrenes) or without endosperm (isolated embryos). Sterilized bisected pyrenes were

prepared by transversely cutting with a scalpel blade and then culturing the micropylar one-third end with the rudimentary embryo (figure 1a-c). Isolated embryos at the heart or late-heart stage (0.24-0.26 mm long) were separated from the endosperm following the procedure describes by Mroginski *et al.* (2010) (figure 1d). In both of cases, the explants were prepared using a stereomicroscope in a laminar flow hood and cultured on 4 ml of solidified nutrient medium in 11 cm³ glass tubes (one explant/tube). The tubes were sealed with Resinite AF 50[®] (Casco S.A.I.C. Company, Bs. As.) and incubated in the darkness at 27 ± 2°C for 60 days, and then they were transferred to a growth room at 27 ± 2°C with a 14 h photoperiod (116 µmol.m⁻².s⁻¹). The culture medium of Sansberro *et al.* (1998) was used for *I. paraguariensis* embryo culture and contained quarter-strength salts and vitamins of Murashige and Skoog (1962) medium with 3% sucrose (¼ MS), 0.1 mg/L zeatin (ZEA) and 0.65% agar (A-1296; Sigma Chem. Co.). The pH of the medium was adjusted to 5.8 with KOH or HCl before adding of agar. The culture tubes were covered with aluminium foil and autoclaved at 1.45 kg.cm⁻² and 120°C for 20 min.

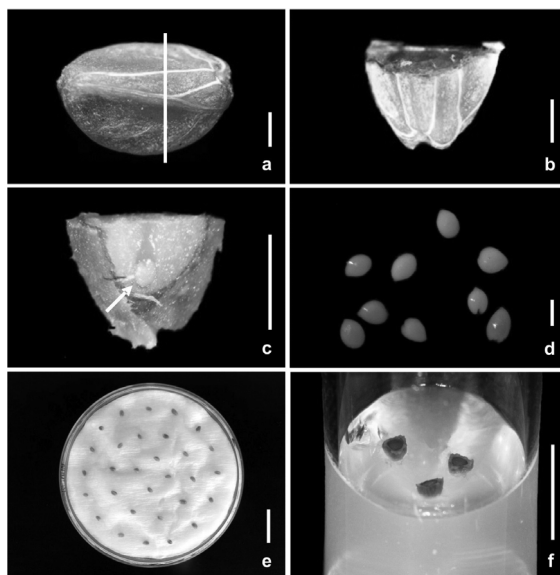


Figure 1. *Ilex paraguariensis* pyrenes and isolated embryos culture. (a) Intact pyrene, the line indicates the cross section for culture of bisected pyrenes (bar = 1mm); (b) pyrene cut transversely (bar = 1 mm) and (c) longitudinally (bar = 1 mm), showing the embryo position and small size; (d) isolated embryos at the heart or late-heart stage (bar = 250 µm). (e) Whole pyrenes sowed in a conventional germinator (bar = 2 cm); (f) bisected pyrenes cultured on solidified nutrient medium (bar = 5 mm).

Germination percentages were determined 60 days after inoculation. In the control treatment (isolated embryos) the gradual change in shape of the immature, cultured embryos was observed until germination. This change was characterized by radicle elongation followed by immediate hypocotyl elongation and cotyledon expansion. When the embryo cultures included the endosperm (bisected pyrenes), the emergence of a shoot and/or a root from the endosperm was used as the criterion to calculate germination rate.

A second experiment was done to study the effect of the woody endocarp and cold treatment on germination. Intact, acid-scarified and bisected pyrenes were assayed to evaluate whether or not the woody endocarp acted as a mechanical barrier for *I. paraguariensis* embryo germination. Bisected pyrenes were prepared as described above. Dry, whole pyrenes were acid-scarified in concentrated H₂SO₄ for 1, 5, 10, 15, 30 and 45 min. The pyrenes were immediately copiously rinsed with tap water and the excedent H₂SO₄ was neutralized with Ca(OH)₂. Pyrenes subjected to scarification were surface-sterilized after treatment by soaking them in 70% ethanol for 1 min, followed by immersion in 1.6% sodium hypochlorite for 45 min and then rinsed three times with sterile, distilled water.

Pyrenes were placed in conventional germinators (30 pyrenes/germinator), consisting of 90 × 15 mm glass Petri dishes with a 5 mm layer of cotton and filter paper moistened with sterile, distilled water (figure 1e) or cultured on 4 ml of solidified (0.65% agar) nutrient medium in 11 cm³ glass tubes (three pyrenes/tube; figure 1f). Three culture media were tested, i.e. ¼ MS without growth-regulators or supplemented with 0.1 mg/L ZEA or gibberellic acid (GA₃). ZEA was added to ¼ MS medium before autoclaving, whereas GA₃ was filter-sterilized and then added to the cooled, autoclaved medium. The germinators and tubes with solidified nutrient medium were sterilized by autoclaving (1.45 kg.cm⁻² and 120°C) for 20 min. After inoculation, whole and bisected pyrenes were incubated at 4 ± 2 or 27 ± 2°C in dark for 60 days, and then they were transferred to a growth room at 27 ± 2°C with a 14 h photoperiod (116 µmol.m⁻².s⁻¹).

All treatments were assayed weekly for seedling germination until 12 months after inoculation. Germination percentages were determined 120 days after culture (60 days after they were transferred to a warm and illuminated growth room) when seed germination ceased in all treatments. Emergence of the shoot and/or root from intact and bisected pyrenes was used to calculate the germination rates.

Statistical analysis

The treatments were arranged in a completely randomized design with three replicates of 30 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 ($P < 0.05$).

Results and discussion

Effect of the endosperm on embryo development and germination

The analysis of variance showed no significant differences between the germination percentages of *I. paraguariensis* isolated embryos (40.0 ± 5.8%) and bisected pyrenes (43.3 ± 3.3%) suggesting that the endosperm does not inhibit the development or germination of the embryos. As such, the excision and culture of isolated embryos would be unnecessary, which disagrees with the results of Hu *et al.* (1979) for embryo germination of *I. aquifolium*, *I. cornuta* and *I. opaca*.

To minimize damage to the fragile embryos and to improve the rapidity of the embryo culture technique, we will use the culture of bisected pyrenes for future studies.

Effect of the woody endocarp and cold treatment on germination

The mean percentages of germination are summarized in table 1.

Table 1. Germination of *Ilex paraguariensis* intact, acid-scarified and bisected pyrenes with or without a cold treatment on different media.

Culture medium	4°C treatment	Germination (%)		
		intact pyrenes	acid-scarified pyrenes ^a	bisected pyrenes
¼ MS + ZEA 0,1 mg/L	No	0.0	0.0	46.7 b ^c
¼ MS + ZEA 0,1 mg/L	Yes	0.0	0.0	57.8 bc
¼ MS + GA ₃ 0,1 mg/L	No	0.0	0.0	38.9 b
¼ MS + GA ₃ 0,1 mg/L	Yes	0.0	0.0	55.6 bc
¼ MS	No	0.0	0.0	43.3 b
¼ MS	Yes	0.0	0.0	74.4 c
Paper-cotton	No	0.0	0.0	0.0 a
Paper-cotton	Yes	0.0	--- ^b	0.0 a

^aAll acid-scarification treatments tested; ^bNot determined; ^cWithin the columns means followed by different letters are significantly different (Tukey's Multiple Comparison Test; P < 0.05).

Whole pyrenes (intact or acid-scarified) did not germinate after 120 days culture on either paper - cotton or solidified nutrient media. However, bisected pyrenes of *I. paraguariensis* germinated. This response suggests that the woody endocarp may act as a mechanical barrier interfering with the expansion of the tissues, as mentioned by Mello (1980) and Jeske *et al.* (2000). Similar results have been reported by Torresán *et al.* (1996) who cultured whole and cut achenes of *Helianthus annuus* on a non-aseptic medium to germinate, as an alternative to *in vitro* culture of embryos. Likewise, Miller *et al.* (1992) and Ke *et al.* (1985) increased *Fragaria* and *Rubus* germination through *in vitro* culture of cut achenes and seeds respectively, enhancing germination and decreasing the time to maximum germination relative to whole seeds. Thus, this technique may be used for many crop seeds that have a hard endocarp surrounding the embryo.

It is not known how cutting affects pyrenes, achenes and other seeds with hard seed coats and promotes faster and higher germination percentages than for control seeds. Possible explanations for the cut seed effect are: (i) 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. (ii) seeds may contain one or more endogenous germination inhibitors in the endosperm and/or pericarpium. After cutting the seeds, the inhibitor(s) may diffuse away from the embryo, allowing germination to occur (Miller *et al.*, 1992).

Acid-scarification did not enhance the germination of whole pyrenes, either after short periods of exposition (1, 5 or 10 min) or after longer H₂SO₄ exposition (15, 30 or 45 min). The periods of scarification that we used may have been insufficient for modifying the hard seed coat; however, longer exposure to the acid might affect embryo viability.

The substrate had a relevant effect on seeds germination. Germination only occurred when the bisected pyrenes were cultured on a solidified nutrient medium (figure 2a, b) but not in conventional germinators (figure 2c) moistened with sterile, distilled water. This might be related to the immature embryos requiring a substance not present in a germinator with moistened cotton - paper substrate. Such substances may normally be present in the endosperm, which was mostly removed by bisecting the pyrenes. Miller *et al.* (1992) reported that when cut achenes were placed on moistened filter paper, the germinating seedlings died within a week after germination. Apparently, the germinating embryos required sucrose and/or other nutrients in the culture medium to replace the energy source normally provided by the cotyledons, which had been removed.

Conversely, the germination rate of the bisected pyrenes on nutrient medium did not vary significantly with or without growth-regulators. Gibberellins may promote germination in absence of inhibitors, but when inhibitors are present, cytokinins are required to counteract their negative effects (Leadem, 1987). In our experiment, *I. paraguariensis* embryos were converted into seedlings when cultured on a nutrient medium lacking growth-regulators. This suggests that exogenous cytokinins or gibberellins are not required for the embryo maturation by culturing bisected pyrenes. As well, the emergence of abnormal shoots or multiple embryos from the endosperm was observed when pyrenes were cultured with ZEA (figure 2d, e).

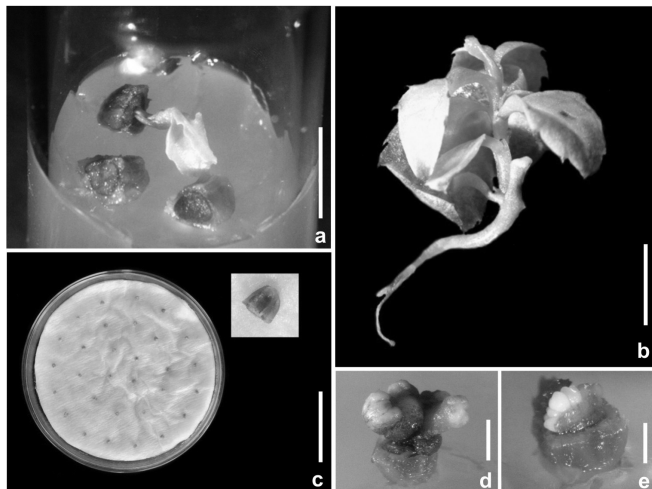


Figure 2. Culture of bisected *Ilex paraguariensis* pyrenes (a, b) on $\frac{1}{4}$ MS medium (bar = 5 mm), after 60 (a) and 120 (b) days culture; (c) in a conventional germinator after 120 days culture (detail shows that pyrenes remain without changes since they were sowed) (bar = 3 cm); and (d, e) on $\frac{1}{4}$ MS with 0.1 mg/L ZEA, showing emergence of abnormal shoot (d) and multiple embryos (e) from the endosperm (bar = 1 mm).

The cold treatment significantly increased the germination percentages of bisected pyrenes inoculated on solidified nutrient medium. Similar results were obtained by Sansberro *et al.* (2000) who exposed the fruits to low temperature before culturing isolated embryos. In many species exposition of seeds to low temperatures decreases the endogen content of abscisic acid (ABA) and increases the gibberellin and cytokinin levels, which interact in a sequential way to break dormancy (Bewley and Black, 1994).

In conclusion, our results show that *I. paraguariensis* plantlets can be obtained by *in vitro* culture of bisected pyrenes on solidified ¼ MS medium lacking growth-regulators. It is recommended to preculture the pyrenes at $4 \pm 2^\circ\text{C}$ to obtain higher germination rate and more vigorous seedlings (data not shown).

This is the first report of the *in vitro* culture of *Ilex* pyrenes. Compared to the culture of isolated embryos this procedure shortens the duration of the embryo culture technique and minimizes damage to the tender and fragile embryos. This method also decreases the cost of *in vitro* regeneration of *I. paraguariensis* plants since no growth-regulators are required.

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References

- Almeida, J.P., Zanatta, B. and Winge, H. (2000). Avaliação do efeito do endocarpo na germinação da semente de erva-mate (*Ilex paraguariensis* A. St. Hil., Aquifoliaceae). [Effect of the endocarp on the germination of maté tree (*Ilex paraguariensis* A. St. Hil., Aquifoliaceae) seeds]. In *Annals II Congresso Sul-Americano da Erva-Mate e III Reunião Técnica da Erva-Mate*, (ed. H. Winge), pp. 427-431, UFRGS and FEPAGRO, Porto Alegre, Brazil.
- Bewley, J.D. and Black, M. (1994). *Seeds: Physiology of development and germination* (Second edition). Plenum Press, New York, NY, 445 pp.
- Cunha, G.G. (1990). Cultura de embriões de erva-mate (*Ilex paraguariensis* St. Hil.) *in vitro*. [In vitro embryo culture of maté tree (*Ilex paraguariensis* St. Hil.)]. Thesis for a Master's degree in Biological Sciences. UFRGS, Porto Alegre, Brazil.
- Ferreira, A.G., Cunha, G.G., da Silva, T.S. and Hu, C.Y. (1991). In vitro germination of immature embryos of *Ilex paraguariensis* St. Hil. *Phyton*, **52**, 27-32.
- Filip, R., López, P., Giberti, G.C., Coussio, J. and Ferraro, G. (2001). Phenolic compounds in seven South American *Ilex* species. *Fitoterapia*, **72**, 774-778.
- Fontana, H.P., Prat Krikum, S.D. and Belingheri, L.D. (1990). Estudios sobre la germinación y conservación de semillas de yerba mate (*Ilex paraguariensis* St. Hil.). [Studies on the germination and preservation of maté tree (*Ilex paraguariensis* St. Hil.) seeds]. Informe Técnico 52 EEA INTA Cerro Azul, Misiones, Argentina.
- Giberti, G.C. (1995). Florística, sistemática y potencialidades con relación a un banco de germoplasma para la yerba mate. [Floristic, systematic and potentialities with relation to a germplasm bank of maté tree]. In *Erva-Mate: Biología e Cultura no Cono Sul*, (ed. H. Winge, A.G. Ferreira, J.E. de Araujo Mariíta and L. Tarasconi), pp. 303-312, A. UFRGS, Brazil.
- Hu, C.Y. (1975). In vitro culture of rudimentary embryos of eleven *Ilex* species. *Journal of the American Society for Horticultural Science*, **100**, 221-225.

- Hu, C.Y., Rogalski, F. and Ward, C. (1979) Factors maintaining *Ilex* rudimentary embryos in the quiescent state and the ultrastructural changes during in vitro activation. *Botanical Gazette*, **140**, 272-279.
- Hu, C.Y. (1989). Holly (*Ilex* spp.). In *Biotechnology in Agriculture and Forestry. 5, Trees II*, (ed. Y.P.S. Bajaj), pp. 412-427, Springer Verlag, Berlin.
- Ives, S.A. (1923). Maturation and germination of seeds of *Ilex opaca*. *Botanical Gazette*, **76**, 60-77.
- Jeske, V.L., da Silva, P.F. and Heuser, E.D. (2000). Embebição em pirenos de *Ilex paraguariensis* A. St. Hil. [Imbibition in pyrenes of *Ilex paraguariensis* A. St. Hil.]. In *Annals II Congresso Sul-Americano da Erva-Mate e III Reunião Técnica da Erva-Mate*, (ed. H. Winge), pp. 435-437, UFRGS and FEPAGRO, Porto Alegre, Brazil.
- Ke, S., Skirvin, R.M., McPheeters, K.D., Otterbacher, A.G. and Galleta, G. (1985). In vitro germination and growth of *Rubus* seeds and embryos. *HortScience*, **20**, 1047-1049.
- Leadem, C.L. (1987). The role of plant growth regulators in the germination of forest tree seeds. *Plant Growth Regulation*, **6**, 61-93.
- Mello, V.D.C. (1980). Morfologia e germinação de sementes de erva-mate (*Ilex paraguariensis* A. St. Hil.). [Morphology and germination of maté tree (*Ilex paraguariensis* A. St. Hil.) seeds]. Thesis for a Master's degree in Agricultural Sciences. UFPel, Pelotas, Brazil.
- Miller, A.R., Scheerens, J.C. and Erb, P.S. (1992). Enhanced strawberry seed germination through in vitro culture of cut achenes. *Journal of the American Society for Horticultural Science*, **117**, 313-316.
- Mroginski, L., Dolce, N., Sansberro, P., Luna, C., Gonzalez, A. and Rey, H. (2010). Techniques and protocol on the cryopreservation of *Ilex* immature zygotic embryo. In *Plant Embryo Culture: Methods and Protocols*, (ed. T. Thorpe and C. Yeung), Human Press, New York, NY (in press).
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15**, 473-497.
- Niklas, C.O. (1987). Estudios embriológicos y citológicos en la yerba mate *Ilex paraguariensis* (Aquifoliaceae). [Embryological and cytological studies in the maté tree *Ilex paraguariensis* (Aquifoliaceae)]. *Bonplandia*, **6**, 45-56.
- Sansberro, P.A., Rey, H.Y., Mroginski, L.A. and Collavino, M.M. (1998). In vitro culture of rudimentary embryos of *Ilex paraguariensis*: Responses to exogenous cytokinins. *Journal of Plant Growth Regulation*, **17**, 101-105.
- Sansberro, P.A., Rey, H.Y. and Mroginski, L.A. (2000). Efectos de bajas temperaturas en la germinación in vitro de embriones inmaduros de yerba mate. [Effect of low temperatures on in vitro germination of immature embryos of maté tree]. In *Annals II Congresso Sul-Americano da Erva-Mate e III Reunião Técnica da Erva-Mate*, (ed. H. Winge), pp. 443-446, UFRGS and FEPAGRO, Porto Alegre, Brazil.
- Sansberro, P.A., Rey, H.Y. and Mroginski, L.A. (2001). In vitro culture of zygotic embryos of *Ilex* species. *HortScience*, **36**, 351-352.
- Sharma, D.R., Kaur, R. and Kumar, K. (1996). Embryo rescue in plants—A review. *Euphytica*, **89**, 325-337.
- Torresán, A., Kesteloot, J., Castaño, F., Rodríguez, R. and Colabelli, M. (1996). Use of immature seed germination technique as an alternative to in vitro culture of sunflower (*Helianthus annuus* L.) embryos. *Euphytica*, **91**, 1-3.