Dolce, N.R., Medina, R.D., Mroginski, L.A. and Rey, H.Y. (2015), *Seed Sci. & Technol.,* **43**, 2, 1-5.
http://doi.org/10.15258/sst.2015.43.2.04

# Research Note

# Sowing pyrenes under aseptic conditions enhances seed germination of *Ilex brasiliensis*, *I. pseudoboxus* and *I. theezans* (Aquifoliaceae)

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*(Accepted February 2015)*

## Abstract

Seeds of *Ilex* species are individually enclosed by a woody endocarp, forming a dispersal unit termed a pyrene. Embryos are undeveloped when fruits reach maturity. As a consequence, germination is low even after several months under proper conditions. In the present study, we present reliable and reproducible techniques that enhance seed germination of *Ilex brasiliensis*, *I. pseudoboxus* and *I. theezans* by aseptically sowing whole pyrenes. The highest germination percentages (53-92%) were reached by *in vitro* culturing whole pyrenes on solidified (0.65% agar) quarter-strength Murashige and Skoog medium with 3% sucrose and 0.1 mg $l^{-1}$ zeatin, and incubating in a growth room at 27 ± 2°C with a 14-hour photoperiod. Thus, the *in vitro* culture of whole pyrenes constitutes an easy and efficient technique to obtain quick germination of these *Ilex* species and may be an alternative to embryo rescue. Rapid progress in the application of this method to breeding and preservation programmes of *Ilex* species is expected.

## Experimental and discussion

The genus *Ilex* (Aquifoliaceae) contains several species of economic importance. The "maté tree" (*I. paraguariensis*) is a perennial crop whose leaves are used in Argentina, southern Brazil, Paraguay and Uruguay to make a very popular stimulatory beverage which has several health benefits (Schinella *et al.*, 2005). *Ilex brasiliensis* (Sprengel) Loes., *I. pseudoboxus* R. and *I. theezans* R. are almost entirely sympatric species with *I. paraguariensis* and lately they have been considered plant genetic resources of the maté crop.

The seeds of *Ilex* species are individually enclosed by a woody endocarp, forming a dispersal unit termed a pyrene (Giberti, 1994). They have undeveloped embryos when fruits reach maturity (Tsang and Corlett, 2005). The immature embryo requires several months under proper conditions to reach maturity and allow for seed germination.

However, although embryos reach maturity, germination is poor (Hu, 1975; Hu *et al*., 1979). For example, *I. opaca* germinates in nature after 1-3 years and the germination rate is about one in ten million (Ives, 1923). This extremely low germination rate constitutes a serious inconvenience for breeding and conservation programmes, since it leads to a loss of potentially valuable genotypes. When conventional sowing methods produce low or no germination, *in vitro* techniques may greatly enhance germination and growth rates by optimising the culture conditions and medium. Among *in vitro* techniques, embryo rescue has been successfully used to solve this problem for many *Ilex* species (Hu, 1975, 1989; Sansberro *et al*., 1998, 2001). However, since embryos are minute (0.16-0.35 mm long) and easily damaged during isolation, this technique is laborious and time-consuming. In this study, we examined the most suitable system for seed germination of *I. brasiliensis*, *I. pseudoboxus* and *I. theezans*, as a prerequisite for the establishment of seed preservation programmes of *Ilex* species.

Open-pollinated fruits of *Ilex brasiliensis*, *I. pseudoboxus* and *I. theezans* were collected from trees at the Estación Experimental Agropecuaria Cerro Azul (Misiones, Argentina) – Instituto Nacional de Tecnología Agropecuaria. Pyrenes were separated from the pulp and immediately distributed among different treatments. For cultures under aseptic conditions, pyrenes were surface-sterilised by soaking them in 70% ethanol for two minutes, followed by immersion in an aqueous solution of 2.5% sodium hypochlorite and 0.1% Triton X-100® for 60 minutes, and rinsed three times with sterile distilled water.

*In vitro germination of whole and cut pyrenes:* Two kinds of explants were cultured: i) whole pyrenes and ii) cut pyrenes. Cut pyrenes were prepared under a stereomicroscope by transversely cutting the pyrenes with a scalpel blade approximately one-third away from the micropylar end and then culturing this part which contains the immature embryo. Both explants were prepared in a laminar flow hood and cultured on 5 ml solidified (0.65% agar; Sigma A-1296) nutrient medium in 15 ml glass tubes (10 pyrenes/tube). Three culture media were tested consisting of ¼ MS (quarter-strength salts and vitamins of Murashige and Skoog (1962) medium with 3% sucrose) without growth-regulator or supplemented with 0.1 mg $l^{-1}$ zeatin (ZEA) or gibberellic acid ($GA_3$). ZEA was added to ¼ MS medium before autoclaving whereas $GA_3$ was filter-sterilised and then added to the cooled, autoclaved medium. The pH of the media was adjusted to 5.8 before adding agar. Tubes with culture media were sterilised by autoclaving at 142.2 kPa and 120°C for 20 minutes. Cultures were sealed with Resinite AF 50® and incubated in a growth room at 27 ± 2°C with a 14-hour photoperiod (116 µmol $m^{-2}$ $s^{-1}$ PPFD provided by cool white fluorescent lamps) or in darkness. Germination percentages were calculated 60 days after the culture, based on the number of whole and cut pyrenes that presented shoot or root emergence.

In the three species, both whole and cut pyrenes germinated in all the media and light conditions assayed. However, the germination percentages of whole pyrenes (40.0 to 92.2% when they were incubated in light) were much higher than cut pyrenes in the same conditions (table 1). It is interesting to note that in the three *Ilex* species evaluated in this study, the presence of the intact endosperm (whole pyrenes) improved the embryo germination compared with broken endosperm (cut pyrenes), thus suggesting that the endosperm does not inhibit the development and germination of the embryo. This result is

Table 1. Germination (%) of *Ilex brasiliensis, I. pseudoboxus* and *I. theezans* whole and cut pyrenes cultured on quarter-strength MS medium without growth-regulator or supplemented with 0.1 mg $l^{-1}$ zeatin (ZEA) or gibberellic acid ($GA_3$) and incubated in a growth room at 27 ± 2°C with a 14 hour- photoperiod or in darkness.

| Culture conditions | *Ilex brasiliensis* | | *Ilex pseudoboxus* | | *Ilex theezans* | |
|---|---|---|---|---|---|---|
| | Light | Darkness | Light | Darkness | Light | Darkness |
| Quarter-strength MS | | | | | | |
| Whole | 43.3$^{b}$ | 23.3$^{d}$ | 55.5$^{b}$ | 27.2$^{d}$ | 55.6$^{b}$ | 28.9$^{d}$ |
| Cut | 20.6$^{d}$ | 17.8$^{d}$ | 4.4$^{e}$ | 3.9$^{e}$ | 16.7$^{ef}$ | 11.1$^{fg}$ |
| Quarter-strength MS + 0.1 mg $l^{-1}$ ZEA | | | | | | |
| Whole | 55.0$^{a}$ | 35.6$^{bc}$ | 92.2$^{a}$ | 60.0$^{b}$ | 64.4$^{a}$ | 38.3$^{c}$ |
| Cut | 21.7$^{d}$ | 18.9$^{d}$ | 7.2$^{e}$ | 6.7$^{e}$ | 21.1$^{e}$ | 17.8$^{ef}$ |
| Quarter-strength MS + 0.1 mg $l^{-1}$ $GA_3$ | | | | | | |
| Whole | 40.0$^{bc}$ | 32.8$^{c}$ | 60.0$^{b}$ | 45.6$^{c}$ | 55.7$^{b}$ | 37.2$^{c}$ |
| Cut | 18.3$^{d}$ | 16.1$^{d}$ | 6.7$^{e}$ | 4.4$^{e}$ | 17.8$^{ef}$ | 8.9$^{g}$ |

Data shown are the means of six replicates. Percentage data were transformed using the equation log(%germination). In both columns of each species, mean values followed by the same letters are not significantly different (Tukey's Multiple Comparison Test; $P < 0.05$).

in disagreement with those reported by Dolce *et al*. (2010, 2011) for *I. paraguariensis* and *I. dumosa*, whose embryo germination is promoted by cutting pyrenes. Meanwhile, Hu *et al*. (1979) reported that the *in vitro* growth of isolated embryos of *I. aquifolium*, *I. cornuta* and *I. opaca* was drastically reduced when the embryos were cultured adjacent to their own endosperm. Based on this result, they suggested the presence of growth-inhibitors in *Ilex* endosperm and/or in the membrane-like testa attached to the endosperm, which are responsible for maintaining embryo dormancy. Moreover, since the highest germination percentages were achieved by culturing whole pyrenes, in *I. brasiliensis*, *I. pseudoboxus* and *I. theezans* the woody endocarp would not act as a mechanical barrier interfering with the expansion of the tissues, as occur in *I. dumosa* and *I. paraguariensis* pyrenes (Jeske *et al*., 2000; Dolce *et al*., 2010; 2011). Therefore, different mechanisms may be involved in seed dormancy among *Ilex* species.

Light regime had an important effect on germination of whole pyrenes (table 1) and subsequent seedling growth. In the three species evaluated, incubation with a 14-hour photoperiod resulted in the highest germination percentages and seedling growth. After 60 days of incubation, the light-germinated seedlings were green and vigorous, with fully developed cotyledons and 3-5 true leaves on most seedlings. However, the dark-germinated seedlings were etiolated, with underdeveloped cotyledons and no true leaves. This response differs from that reported by Hu (1976) and Ferreira and Hu (1989) for isolated embryos of *I. opaca*, *I. aquifolium* and *I. paraguariensis*, whose *in vitro* late embryogeny was light-inhibited.

Although 43.3-58.9% of whole pyrenes incubated in light germinated and grew into seedlings when sown on quarter-strength MS medium lacking growth-regulator, the addition of 0.1 mg $l^{-1}$ ZEA to the medium significantly improved germination, resulting

in the highest germination percentages (55.0 to 92.2%) in the three *Ilex* species evaluated (table 1). This result is consistent with those reported by Sansberro *et al*. (1998, 2001) for isolated zygotic embryos of nine *Ilex* species.

*Seed germination on aseptic vs. non-aseptic conditions:* Whole and cut pyrenes were sown under aseptic conditions in 90 mm-diameter × 15 mm-deep glass Petri dishes (30 explants/dish) with a 5 mm layer of cotton and filter paper or containing 40 ml sand or *Sphagnum* peat moss soaked with sterile distilled water. Pyrenes were surface-sterilised and Petri dishes were autoclaved as described above. Additionally, pyrenes were sown under non-aseptic conditions in Petri dishes prepared in the same way as above but without autoclaving. In all cases, Petri dishes were sealed with Resinite AF 50® and incubated in a growth room at 27 ± 2°C with a 14-hour photoperiod. Germination percentages were calculated 60 days after culturing as described above.

Although pyrenes germinated in all the substrates assayed, germination percentages were significantly higher (50.6 to 63.3%) when whole pyrenes were sown under aseptic conditions on cotton-paper (table 2). Thus, the germination percentage was similar to that reached by *in vitro* culturing whole pyrenes on quarter-strength MS medium lacking growth-regulator. However, when pyrenes were cultured under non-aseptic conditions, germination was very poor regardless of the substrate. The reduced germination may be a result of pre-germination mortality caused by pathogens during the extended period of time required for embryo maturation. Similar results have been reported for cut pyrenes of *I. dumosa* (Dolce *et al*., 2011). Consequently, sowing pyrenes under aseptic conditions may be considered to enhance seed germination of *Ilex* species.

Table 2. Effect of different substrates on germination (%) of *Ilex brasiliensis*, *I. pseudoboxus* and *I. theezans* whole and cut pyrenes sowed under aseptic and non-aseptic conditions. Cultures were incubated in a growth room at 27 ± 2°C with a 14-hour photoperiod.

| Culture conditions | *Ilex brasiliensis* | | *Ilex pseudoboxus* | | *Ilex theezans* | |
|---|---|---|---|---|---|---|
| | Whole | Cut | Whole | Cut | Whole | Cut |
| Aseptic | | | | | | |
| Cotton-paper | 50.6[a] | 25.0[cd] | 63.3[a] | 3.9[e] | 61.1[a] | 17.2[c] |
| Sand | 30.0[c] | 17.2[de] | 27.8[c] | 3.3[e] | 25.6[b] | 15.0[c] |
| Sphagnum moss | 38.9[b] | 16.7[e] | 36.1[b] | 4.4[e] | 31.1[b] | 17.8[c] |
| Non-aseptic | | | | | | |
| Cotton-paper | 2.2[f] | 1.1[f] | 2.8[e] | 1.7[e] | 2.8[d] | 1.7[d] |
| Sand | 2.8[f] | 1.7[f] | 5.6[de] | 2.8[e] | 3.3[d] | 2.2[d] |
| Sphagnum moss | 7.8[f] | 2.8[f] | 10.6[d] | 3.9[e] | 5.6[d] | 3.3[d] |

Data shown are the means of six replicates. Percentage data were transformed using the equation log(%germination). In both columns of each species, mean values followed by the same letters are not significantly different (Tukey's Multiple Comparison Test; $P < 0.05$).

In conclusion, we have demonstrated for the first time that seed germination of *Ilex brasiliensis*, *I. pseudoboxus* and *I. theezans* may be achieved readily by sowing whole pyrenes under aseptic conditions and incubating in light. Since seed germination of *Ilex* species is very poor when conventional methods are used, the procedures presented here become particularly relevant. Rapid progress in the application of this method to breeding and preservation programmes of *Ilex* species is expected.

## Acknowledgements

Special thanks are extended to CONICET, ANPCyT and SGCyT (UNNE) for the financial support.

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