The Cornerstone for *in Vitro* Propagation and Ploidy Stability of *Myrsine* (Primulaceae) Species

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Summary *Myrsine coriacea* is a Primulaceae species considered ecologically important for colonizing degraded areas and providing fruits for birds. This species has been gaining attention at present due to possessing pharmacological compounds explored in cancer treatment. This study aimed to establish the first procedure for *in vitro* propagation of *M. coriacea* through seed germination and somatic embryogenesis. A high frequency of germination (100%) was observed from mature seeds pre-treated with hydrochloric acid and inoculated into medium supplemented with gibberellic acid. Plantlets were generated after 41 days from these seeds, a time relatively short in comparison to *ex vitro* methods used for propagation of this species. Besides the direct system starting with seed germination, the recovery of *M. coriacea* was established from indirect somatic embryogenesis using immature zygotic embryos. From these explants, friable calli were formed on medium supplemented with 6-benzylaminopurine, and somatic embryos were regenerated and plantlets recovered after 115 days. This result evidenced that the immature zygotic embryos of *M. coriacea* possess levels of endogenous phytohormones, mainly auxins, which are sufficient to trigger the embryogenic system. All *M. coriacea* plantlets showed the same nuclear 2C value and chromosome number in relation to explant donors. Thus, no karyotype change occurred during propagation in the *in vitro* system standardized. The first reliable and relatively rapid procedures provided morphologically normal seedlings of *M. coriacea*, providing the basis for other tissue culture applications. Furthermore, the plantlets regenerated can be used in reforestation and conservation programs, and for production of pharmacological compounds.

Key words Woody species, Seed germination, Somatic embryogenesis, Zygotic embryos, Plantlet recovery.

Myrsine coriacea (Sw.) R. Br. ex Roem. & Schult., previously *Rapanea ferruginea* Ruiz & Pav., is a woody species circumscribed to the family Primulaceae, subfamily Myrsinoideae (The Angiosperm Phylogeny Group 2009). This species is Neotropical, showing wide distribution in Colombia, Bolivia, Argentina, Paraguay, Uruguay and Brazil. *Myrsine coriacea* is an important forest species, widely used in reforestation programs in Brazil. This is due to individuals having fast growing having fruits that attract birds and be able to easily occupy unsheltered areas (Freitas and Carrijo 2008). The species is also used in civil engineering and for charcoal production and its bark is rich in tannic substances employed in the leather industry. In the pharmaceutical industry, *M. coriacea* has been highlighted for anti-inflammatory production from its mirsinoic acid B, which has anti-nociceptive effect. Further, the protein lectin

prospected from this species is considered a potential compound for cancer treatment (Medeiros *et al.* 2013). *Myrsine coriacea* also possesses ecological importance as fruit source for several bird species, and represents an initial tree species for inhabiting depredated areas (Freitas and Carrijo 2008).

Woody plants have been the focus of *in vitro* tissue culture (IVTC) approaches, which seek to optimize and increase the production of secondary metabolites and/or to mass propagate selected species with high wood production. IVTC has also been applied to propagate and preserve plantlets from other woody species of ecological importance, such as *Anthocephalus cadamba* (Roxb.) Miq. and *A. chinensis* (Lamk) A. Rich (Apurva and Thakur 2009), and *Caesalpinia echinata* Lam. (Werner *et al.* 2009), promoting the germplasm conservation. Although the propagation of some species is limited by challenges in the seed obtaining, advances through *in vitro* tissue culture systems is promoting a relatively rapid propagation of some woody species (Varshney and

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Anis 2014).

Considering that *M. coriacea* is a wood species of wide interest, the establishment of procedures for its *in vitro* propagation represents the first step for applications such as induction and increase of secondary metabolite production (Karuppusamy 2009) and large-scale regeneration from genotypes chosen for conservation in reforestation programs (Engelmann 2011). Among the IVTC techniques, seed germination can be a fast alternative method for seedling generation in relation to the germination of *M. coriacea* in *ex vitro* environment, which occurs only after the passage of seeds through the digestive tract of birds or asexually through cuttings. Somatic embryogenesis (SE), another IVTC application, is also considered suitable for mass propagation (Rout *et al.* 2006), representing the basis for other *in vitro* techniques. Independently of the IVTC method, the explant cells are stimulated by chemical and physical conditions. In this regard, auxins and cytokinins, which are involved in dedifferentiation and acquisition of cell division, are compounds that determine the morphogenic responses (Fehér *et al.* 2003).

Owing to this role, the majority of the IVTC strategies for woody species involve the supplementation of the tissue culture medium with growth regulators, since it is believed that the embryogenic tissue formation and somatic embryo recovering are triggered by the exogenous application of growth regulators (Fehér *et al.* 2003, Fehér 2008). The most used ones are auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D) (Werner *et al.* 2009), α -naphthalene acetic acid (NAA) (Shi *et al.* 2009), indoleacetic acid (IAA) (Chaturvedi *et al.* 2004) and indolebutyric acid (IBA) (Thengane *et al.* 2006). In addition to auxins, the cytokinin 6-benzylaminopurine (BAP) is widely used (Fotso *et al.* 2008), followed by kinetin (Shekhawat *et al.* 2009), zeatin (Naval *et al.* 2009) and thidiazuron (Chaturvedi *et al.* 2004).

Given the relevance of *M. coriacea*, the establishment of this species *in vitro* represents the first step for application of other IVTC techniques, and provides a substantial source of plantlets for reforestation programs and pharmacological studies. Thus, the purpose of this study was to accomplish an initial procedure for *M. coriacea* propagation and regeneration *in vitro*.

Materials and methods

Plant material

Mature and immature fruits of *M. coriacea* were collected at the city of Venda Nova do Imigrante – ES, Brazil (20°20'6"S 41°7'49"W). The pericarp was manually removed and the seeds were dried on filter paper.

Seed germination and plantlet propagation

Mature seeds of *M. coriacea* were submersed in hydrochloric acid (HCl) solution at 10% (Ali *et al.* 2007).

After 24h the seeds were disinfected under laminar flow using 70% ethanol (Merck®) for 1 min and sodium hypochlorite (Merck®) solution supplemented with 0.1% (v/v) Tween 20 (Merck®). The seeds were then rinsed three times in sterile distilled water (Oliveira *et al.* 2013).

The seeds were inoculated in germination medium (GM), which were constituted by 1/2 MS basal medium (MS salts), 10 mL L⁻¹ MS vitamins (Murashige and Skoog 1962), 30.0 g L⁻¹ sucrose and 7.0 g L⁻¹ agar type A. Additionally, some media were supplemented with 10.74 or 21.48 μ M of gibberellic acid (GA₃), NAA or IBA.

The experiment followed a completely randomized design with seven treatments. Each treatment had thirty repetitions, consisting of one seed per tube. The tubes were kept under a 16/8h light/dark regimen, with 36 μ mol m⁻² s⁻¹ light radiation provided by two fluorescent lamps (20 W, Osram®), at 25°C.

Somatic embryogenesis

Immature fruits of *M. coriacea* were collected in the field at 15, 30, and 45 days after anthesis, totaling three samples. After pericarp removal, the seeds were disinfected as described in the previous section. Immature zygotic embryos (IZE) were excised and inoculated into Petri dishes containing calli induction medium (CIM) composed by 1/2 MS basal medium (MS salts), 10 mL L⁻¹ MS vitamins (Murashige and Skoog 1962), 0.08 g L⁻¹ L-cysteine, 0.4 g L⁻¹ L-glutamine, 30 g L⁻¹ sucrose, 2.8 g L⁻¹ Phytigel, 4.44 μ M BAP, without and with two concentrations of 2,4-D: 9.06 or 18.12 μ M. For each medium were prepared ten Petri dishes, each containing five IZE. The dishes were kept in the dark at 25°C.

Friable embryogenic calli (FEC) were transferred to embryogenic induction medium (EIM) until the emergence of somatic embryos. This medium was similar to CIM, without BAP and 2,4-D, and with the addition of 2 g L⁻¹ activated charcoal. Petri dishes were kept under a 16/8h light/dark regimen, with 36 μ mol m⁻² s⁻¹ light radiation provided by two fluorescent lamps (20 W, Osram®), at 25°C. Subsequently, the somatic embryos were isolated and transferred to embryo maturation medium (EMM). This medium was similar to EIM, with addition and 0.5 μ M abscisic acid (ABA). The cotyledonary somatic embryos were converted into plantlets after inoculation into flasks containing the regeneration medium (RM), which was identical to EIM.

Nuclear 2C value and karyotype evaluation

Flow cytometry (FCM) was performed using leaves of *M. coriacea* plantlets obtained from seed germination (controls) and SE (samples) and from *Pisum sativum* (2C=9.16 pg, Praça-Fontes *et al.* 2011). Leaf fragments of *M. coriacea* and *P. sativum* were simultaneously chopped, for 30s, with 0.5 mL⁻¹ OTTO-I buffer (0.1 M citric acid-Merck KGaA® and 0.5% Tween 20-Merck KGaA®, Otto 1990), containing 2.0 mM dithiothrei-

Fig. 1. Indirect somatic embryogenesis of *M. coriacea*. (A) FEC obtained in medium CIM supplemented with growth regulator BAP. Note the presence of somatic embryos at distinct stages. (B) Somatic embryos at distinct development stages, from globular stage to the mature cotyledonary. (C) Regenerated plantlet of *M. coriacea*. Bars=1 cm. (D) Representative histogram showing G_0/G_1 of *in vitro* *M. coriacea* plantlet (channel 144, $2C=6.60$ pg) and *P. sativum* (channel 200, $2C=9.16$ pg). (E) Karyotype of the *M. coriacea* plantlets exhibiting $2n=46$ chromosomes. Bar=5 μ m.

tol (Sigma®) and 50 mg mL⁻¹ RNase (Sigma®). A volume of 0.5 mL of the same buffer was added to each suspension, which was filtered through 30 μ m nylon filter (Partec®) and centrifuged (ALC® microCentrifugette® 4214) at 100g for 5 min. The supernatant was scattered, and the pellet was resuspended and incubated for 5 min in 100 μ L OTTO-I (Praça-Fontes *et al.* 2011). The suspension was stained in the dark with 1.5 mL of buffer solution OTTOI:OTTO-II (2:1) supplemented with 75 mM propidium iodide (PI, Sigma®), 2.0 mM dithiothreitol (Sigma®) and 50 mg mL⁻¹ RNase. After 40 min, the suspensions were filtered through nylon filter with mesh diameter of 20 μ m (Partec®), and analyzed in Partec PAS® flow cytometer (Partec® GmbH, Munster, Germany). DNA content measurement was performed based on G_0/G_1 peaks of the internal standard, controls and samples using FlowMax® software (Partec®). The

nuclear 2C values were reported in picograms (pg).

Roots obtained from *in vitro* plantlets were excised and treated with 5 μ M of the microtubule inhibiting agent amiprophos-methyl (APM, Nihon Bayer Agrochem K. K®), for 15 h at 4°C. Subsequently, the roots were washed with distilled water for 20 min and fixed with methanol:acetic acid (Merck®) solution (3:1), with three changes of fixative solution. The roots were stored at -20°C for 24 h, washed for 20 min, and incubated for 2 h 45 min at 34°C in pectinase solution (Sigma®) at a concentration of 1:8 (enzyme:water). Next, the roots were washed for 10 min, fixed and stored at -20°C. Slides were prepared applying the dissociation and air-drying techniques, placed on a hot plate at 50°C for 10 min, stained with 5% Giemsa (Merck®) for 5 min, washed in distilled water and air dried (Carvalho *et al.* 2007). Chromosome images were captured using a Me-

dia Cybernetics® Camera Evolution™ charge-coupled device video camera, mounted on a Nikon 80i microscope (Nikon, Japan).

Results and discussion

Seed germination and plantlet propagation

After 22 days of inoculation, the first signs of germination were observed. From the first rooting, the germination rate was weekly evaluated until 60 days from inoculation. Distinct germination rates were found in relation to the previous treatment with type and concentration of growth regulators. The high rate of seed germination and, consequently, of plantlets (100%) was obtained from the seeds inoculated into medium supplemented with $10.74\mu\text{M}$ of GA_3 . In media with $21.48\mu\text{M}$ of GA_3 and $21.48\mu\text{M}$ of IBA no seeds germinated. 66.6% of the seeds germinated in the medium without growth regulators, 60% in the $10.74\mu\text{M}$ NAA, 10% in the $21.48\mu\text{M}$ NAA, and 20% in the $10.74\mu\text{M}$ IBA.

Acid scarification with 10% HCl was used to promote dormancy breaking, resembling the digestive tract of birds. This procedure promoted disorganization of the endocarp's anatomical structure, becoming more permeable to compounds of the culture medium and facilitating the radicle protrusion (Freitas *et al.* 1990).

The supplementation of the MS medium with GA_3 was suitable for germination of the *M. coriacea* seeds, as reported for other woody species of Angiosperms such as *Albizia lebbbeck* L. Benth (Perveen *et al.* 2013). In IVTC, GA_3 activates processes associated with seed germination, including the loss of the pericarp, expansion and development of the zygotic embryo and mobilization of stored nutrient reserves (Finkelstein *et al.* 2008).

Seed germination *ex vitro* has been evaluated for *M. coriacea* under different conditions of temperature and substrate. With this system, germination occurred between 30 and 120 days (Lorenzi 2002), with rates varying from 33 to 73% (Lorenzi 2002). Thus, germination *in vitro* of *M. coriacea* was more suitable than *ex vitro*, supporting that seed germination rate of some species may increase when tissue culture techniques are applied (Fay 1992).

In vitro germination was successfully conducted for *M. coriacea*, providing plantlets that can be used for distinct purposes. Thus, the *in vitro* method represents an alternative application for propagation of *M. coriacea*.

Somatic embryogenesis

Besides germination and direct plantlet conversion, this study also focused on the regeneration of *M. coriacea* through SE. FEC (Fig. 1a) were only obtained from IZE collected 45 days after anthesis and inoculated into medium CIM without 2,4-D. After 50 days, the FEC were transferred to medium EIM, and immature somatic embryos at distinct stages were thus recovered (Fig.

1a, b) and matured in medium EMM for 40 days. From the cotyledonary embryos, plantlets were regenerated (Fig. 1c) after 25 days in RM medium. Therefore, approximately 115 days were needed for regeneration of *M. coriacea*.

In periods of less than 45 days after anthesis, the explants did not respond to *in vitro* conditions. This result suggests that the 45-day IZE of *M. coriacea* possess auxin in adequate concentration to maintain cell totipotency and proliferation. The endogenous IAA content is important to induce competence and to acquire determination of an explant (Jiménez 2005). Its high concentrations have been associated with best rates of embryogenic response in some species (Jiménez 2001), such as *Pennisetum purpureum* Schum. (Filippini *et al.* 1992), *Triticum* spp. (Jimenez and Bangerth 2001a) and *Zea mays* L. (Jimenez and Bangerth 2001b). These authors have reported that the decrease in endogenous levels of IAA during the development of zygotic embryos resulted in reduction of embryogenic capacity.

The embryogenic capacity of the IZE is commonly related to their stage of development, which can be associated with fluctuations in the endogenous balance of IAA (Pescador *et al.* 2012). In the immature stages (globular, heart, torpedo and initial cotyledonary) the high level of IAA enables the initiation and maintenance of the bilateral symmetry of the embryo (Chen *et al.* 2010). During maturation the IAA rate decreases (Konieczny *et al.* 2010) in the cotyledonary embryo.

Besides the chronological, physiological and ontogenetic ages of the IZE, the composition of the tissue culture media also was fundamental for establishment of SE in *M. coriacea*. Plantlets were recovered only in medium CIM, which was supplemented with the cytokinin BAP. This growth regulator stimulates the induction of cell division, leading to FEC formation and regeneration of somatic embryos of *M. coriacea*. Plantlets of other woody species, such *Swietenia macrophylla* (Brunetta *et al.* 2006), were recovered in media containing only the growth regulator BAP.

Several protocols use media containing auxins, for instance 2,4-D, for SE induction (Michalczyk *et al.* 1992, Fehér *et al.* 2003). Differently, *M. coriacea* plantlets were not obtained in the media containing 2,4-D. The main effect of the auxins is to stimulate the biosynthesis of the phytohormone IAA (Fehér *et al.* 2001, 2002). As a suitable embryogenic response from IZE of *M. coriacea* was found, it can be concluded that the addition of 2,4-D led to the accumulation of endogenous IAA in the cells of the explant, causing failure to stimulate and preventing the proliferation (Pasternak *et al.* 2007) of FEC, and consequently somatic embryos and recovery of plantlets.

The data demonstrate that explant age and tissue culture composition were crucial for SE in *M. coriacea*. After approximately 115 days, several plantlets of this species were regenerated from one explant through in-

direct SE, differently from the result obtained by seed germination.

Nuclear 2C value and karyotype evaluation

FCM histograms showed G_0/G_1 peaks of the *M. coriacea* controls/samples and the *P. sativum* standard ($2C=9.16\text{pg}$) with coefficient of variation lower than 5%. Nuclear 2C values of *M. coriacea* controls/samples was $2C=6.60\text{pg}$ (Fig. 1d), demonstrating that there was no variation in DNA content between the control and sample plantlets of *M. coriacea*. Therefore, it was possible to verify the genome size stability of the regenerated *M. coriacea* plantlets. Besides this application, the DNA ploidy level determination using FCM was rapid, accurate and reproducible (Dhooghe *et al.* 2011). These advantages have turned the FCM into the main application to previous screening of somaclonal regenerants.

Cytogenetic protocol using APM ($5\mu\text{M}$ for 15h), enzymatic maceration (1:8 solution for 2h 45min), cell dissociation and air-drying provided prometaphases and metaphases with adequate chromosome morphology. These cells showed individualized chromosomes, flattened on the slide, without chromatin deformations and cytoplasmic background noise. Chromosome number of all *M. coriacea* plantlets was $2n=46$ (Fig. 1e). Chromosomal instability is a frequent somaclonal variation (SV) phenomenon associated with regenerated plantlets from long-term cultures or cultures that involve a callus phase or high rates of multiplication treatments (Roux *et al.* 2004, Bairu *et al.* 2011). However, in the present study, FCM and chromosome counts showed no occurrence of SV in seedlings, even those grown in medium with growth regulators.

Conclusion

The propagation is fundamental step for other biotechnological techniques and the sustainable exploration of potential germplasm. The establishment and propagation of *M. coriacea in vitro* using seed germination and SE is the first step to the IVTC of this forest species, and it can be applied to other species of the same genus.

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