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MICHELI SOSSAI SPADETO

PROPAGAÇÃO IN VITRO E DETERMINAÇÃO DO NÚMERO CROMOSSÔMICO DE *Myrsine coriacea* (Sw.) R.Br. ex Roem. & Schult.

ALEGRE, ES

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Dissertação apresentada à Universidade Federal do Espírito Santo, como parte das exigências do Programa de Pós-Graduação em Produção Vegetal, para obtenção do título de *Magister Scientiae*.

Orientador: Prof. Dr Wellington Ronildo Clarindo.

Coorientadoras: Profª. Drª. Tatiana Tavares Carrijo Profª. Drª. Maria Andréia Corrêa Mendonça

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Dedico com eterno amor e gratidão,

aos meus pais, Djame e Marlene, exemplos de determinação, humildade, simplicidade e fé, que muitas vezes renunciaram de seus sonhos, para que pudessem realizar os de seus filhos... Espero ter sido digna do esforço dedicado por vocês em todos os aspectos. Vocês são os verdadeiros responsáveis por esta realização e pelo meu desejo de ser uma pessoa melhor a cada dia. Amo imensamente vocês!

“Quando os ventos de mudança sopram, algumas pessoas levantam barreiras, enquanto outras constroem moinhos de vento”.

Érico Veríssimo

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BIOGRAFIA

Micheli Sossai Spadeto, filha de Djame Spadeto e Marlene Sossai Spadeto, nasceu em Venda Nova do Imigrante, Espírito Santo, no dia 03 de outubro de 1985. Em fevereiro de 2005, ingressou no Centro Universitário São Camilo – ES, graduando-se em Licenciatura Plena em Ciências Biológicas em Dezembro de 2009. Durante o período de graduação foi bolsista de iniciação científica da Embrapa Café, onde desenvolveu pesquisa em Biologia Molecular do cafeeiro. Em agosto de 2012 passou a cursar disciplinas como estudante especial no Programa de Pós-graduação em Produção Vegetal do Centro de Ciências agrárias da Universidade Federal do Espírito Santo. Em fevereiro de 2013 ingressou no curso de Mestrado, atuando na área de Biotecnologia e Ecofisiologia do Desenvolvimento de Plantas, sob orientação do Prof. Dr. Wellington Ronildo Clarindo, submetendo-se à defesa de dissertação em fevereiro de 2015.

SUMÁRIO

LISTA DE ABREVIATURAS

ABA	abscisic acid
APM	amiprophos-methyl
BAP	6-benzylaminopurine
CIM	calli induction medium
CV	coefficient of variation
2,4-D	2,4-dichlorophenoxyacetic acid
EIM	embryo induction medium
EMM	embryo maturation medium
FEC	embryogenic friable callus
FCM	flow cytometry
GA_3	gibberellic acid
GM	germination medium
HCl	hydrochloric acid
IAA	indoleacetic acid
IBA	indole butyiric acid
IVTC	in vitro tissue culture
IZE	immature zygotic embryos
MS	Murashige and Skoog
NAA	α- naphthaleneacetic acid
PI	propidium iodide
RM	regeneration medium
SE	somatic embryogenesis

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CAPÍTULO I

SPADETO, Micheli Sossai, M.Sc., Universidade Federal do Espírito Santo, fevereiro de 2015. Propagação in vitro e determinação do número cromossômico de *Myrsine coriacea* (Sw.) R.Br. ex Roem. & Schult. Orientador: Dr. Wellington Ronildo Clarindo. Coorientadoras: Drª. Tatiana Tavares Carrijo e Drª. Maria Andréia Corrêa Mendonça.

RESUMO

As técnicas de cultura de tecidos in vitro podem gerar subsídios à propagação e manutenção do germoplasma de espécies florestais de interesse, como *Myrsine coriacea* (Sw.) R.Br. ex Roem. & Schult. (Primulaceae). Essa espécie vem se destacando na indústria farmacêutica por produzir substâncias como o ácido mirsinóico B, de efeito antinociceptivo e a proteína lectina, com ação potencial na cura do câncer. Além disso, o estabelecimento in vitro de *M. coriacea*, é importante no fornecimento de material vegetal (folhas e raízes) para análises citogenéticas e de citometria de fluxo, que podem ser utilizadas para a caracterização cariotípica da espécie. Com base no exposto, o presente trabalho teve como objetivos: estabelecer um protocolo de propagação in vitro para *M. coriacea,* mensurar o conteúdo de DNA nuclear e determinar o número cromossômico dessa espécie. Desse modo, plântulas de *M. coriacea* foram obtidas por duas vias de propagação in vitro: germinação de sementes maduras e regeneração a partir de embriões zigóticos imaturos. A maior frequência de germinação (100%) foi observada para sementes previamente tratadas com HCl 10% e inoculadas em meio MS suplementado com 10.74 µM de ácido giberélico, no qual, as primeiras plântulas foram geradas após 41 dias de cultivo. Na embriogênese somática, utilizando embriões zigóticos imaturos, calos friáveis e embriões somáticos foram obtidos em meio MS suplementado com 4.44 µM de 6-benzilaminopurina, sendo que as plântulas foram regeneradas após 120 dias de cultivo. Folhas e raízes das plântulas obtidas in vitro foram utilizadas nas análises de citometria de fluxo, as quais revelaram uma variação intraespecífica no conteúdo de DNA nuclear de *M. coriacea*. Tal variação se confirmou nas análises citogenéticas, onde alguns indivíduos apresentaram 2n = 45 e outros 2n = 46 cromossomos. Tendo em vista a dioicia em *M. coriacea*, os dados sugerem que essa variação esteja relacionada à

presença de cromossomos sexuais. Além disso, grupos de pares de cromossomos morfologicamente idênticos também foram observados indicando uma possível origem poliploide da espécie. Os resultados obtidos nesse estudo contribuem com estudos sobre determinação do sistema sexual, evolução e cultivo in vitro do gênero *Myrsine.*

Palavras-chave: *Myrsine,* citogenética, citometria de fluxo, cultura de tecidos vegetais, embriogênese somática.

SPADETO, Micheli Sossai, M. Sc., Federal University of Espírito Santo, February 2015. In vitro propagation and determination of the chromosome number of *Myrsine coriacea* (Sw.) R. Br. Ex Roem. & Schult. Adviser: Dr. Wellington Ronildo Clarindo. Co-advisers: Drª. Tatiana Tavares Carrijo and Drª. Maria Andréia Corrêa Mendonça.

ABSTRACT

In vitro tissue culture techniques can generate subsidies for the propagation and germplasm maintenance of forest species of interest such as *Myrsine coriacea* (Sw.) R. Br. Ex Roem. & Schult. (Primulaceae). This species has been highlighted in the pharmaceutical industry for producing substances like mirsinoic acid B, showing analgesic effects, and the lectin protein, with potential câncer-healing action. Thus, the establishment and in vitro culture of *M. coriacea* is a potential alternative for the production of these substances and the generation of seedlings for reforestation programs. Besides the economic relevance, *M. coriacea* is a dioecious species with male and female representatives clearly defined in the reproductive period. Based on the above, this study aimed to establish a protocol for in vitro propagation of *M. coriacea*, measure the nuclear DNA content and determine the chromosome number of the species. *M. coriacea* seedlings were obtained by in vitro propagation through two routes: mature seed germination and regeneration from immature zygotic embryos. The highest frequency of germination (100%) was observed for seeds previously treated with 10% HCl and inoculated in medium MS (Murashige and Skoog) supplemented with 10.74 mM of gibberellic acid, in which the first seedlings were generated after 41 days of culture. In somatic embryogenesis using immature zygotic embryos, friable calli and somatic embryos were obtained in medium MS supplemented with 4.44 µM of 6-benzylaminopurine, and the plantlets were regenerated after 120 days of cultivation. Leaves and roots of the seedlings obtained in vitro were used in flow cytometric analysis, which revealed an intraspecific variation in nuclear DNA content in *M. coriacea*. This variation was confirmed by cytogenetic analysis,

where some individuals showed 2n = 45 and others 2n = 46 chromosomes. Given the dioecy of *M. coriacea*, the data suggest that this variation is related to the presence of sex chromosomes. Furthermore, groups of morphologically identical chromosome pairs were also observed indicating a possible polyploidy origin of the specie. The establishment of the in vitro culture protocol for *M. coriacea* enabled the mass propagation of the species and is useful for the large-scale production of plants with reduced time and space. The flow cytometry and cytogenetic data showed for the first time that the male and female individuals of *M. coriacea* differ in DNA content and chromosome number.

Keywords: *Myrsine,* cytogenetics, flow cytometry, plant tissue culture, somatic embryogenesis.

1. INTRODUÇÃO

Myrsine coriacea (Sw.) R. Br. ex Roem. & Schult. (Primulaceae) é uma espécie arbórea, dioica e de ampla distribuição na América do Sul. No contexto ambiental, *M. coriacea* é considerada importante como secundária inicial em estágios sucessionais (Freitas e Carrijo 2008) e fornecedora de frutos para a avifauna, sendo assim, recomendada para a recuperação de ecossistemas alterados e na manutenção da fauna de diferentes ecossistemas (Carvalho 2003). Atualmente, a espécie vem ganhando destaque também na indústria farmacêutica, em virtude da produção de substâncias de interesse como o ácido mirsinóico B, com ação antinociceptiva (Hess *et al.* 2002; Baccarin *et al.* 2011) e a proteína lectina, que tem apresentado efeito potencial na cura do câncer (Medeiros *et al.* 2013).

Para espécies arbóreas e florestais de interesse, como *M. coriacea*, técnicas de propagação in vitro são utilizadas para elevar a taxa de produção de mudas em tempo e espaço reduzidos, potencializar o fornecimento de metabólitos secundários úteis na fabricação de fármacos e facilitar a conservação de germoplasma e a preservação da espécie (Engelmann 1991; Toribio *et al.* 2008).

A germinação in vitro de algumas espécies vem se destacando em relação aos métodos convencionais, por utilizar metodologias e substâncias que aceleram a quebra de dormência das sementes (Ribas *et al.* 1996) e consequentemente, diminuindo o tempo de obtenção das mudas. Além da germinação direta de plântulas in vitro, a embriogênese somática tem se destacado por ser uma técnica eficaz no aumento das taxas de multiplicação

dos explantes, possibilitando a produção de mudas em larga escala (Rout *et al.* 2006).

Dada a relevância de *M. coriacea*, a regeneração e o estabelecimento in vitro dessa espécie representam o passo inicial para a aplicação de outras técnicas da cultura de tecidos in vitro e pode ser uma fonte de mudas para programas de reflorestamento e estudos farmacológicos. Além disso, as plântulas de *M. coriacea* obtidas e mantidas in vitro podem ser utilizadas em estudos de citometria de fluxo e citogenética da espécie.

A citometria de fluxo é considerada importante para detectar pequenas diferenças no conteúdo de DNA nuclear e mensurar níveis de ploidia, identificando possíveis variações intraespecíficas relacionadas ao cariótipo (Yanpaisan *et al.* 1999; Doležel e Bartos 2005; Doležel *et al.* 2007), contribuindo para a caracterização genômica e estudos evolutivos. Já a citogenética compreende o estudo dos cromossomos nos aspectos numéricos, estruturais e funcionais. Assim, a partir da caracterização do cariótipo dos diferentes indivíduos de *M. coriacea*, as informações obtidas podem ser correlacionadas com o sistema de determinação sexual da espécie.

A ampla importância e aplicabilidade de *M. coriacea* e o fato da espécie ter sua dioicia evidente no período reprodutivo, fundamentaram os três objetivos deste trabalho, divididos em dois capítulos: Capítulo 1 - estabelecer e propagar *M.* coriacea in vitro; capítulo 2 - mensurar o conteúdo de DNA nuclear e determinar seu número cromossômico dessa espécie.

2. OBJETIVOS

- Estabelecer e propagar *M. coriacea* in vitro.

- Mensurar o conteúdo de DNA nuclear de *M. coriacea*.

- Determinar o número cromossômico e caracterizar o cariótipo de *M. coriacea.*

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Chapter 1

The comerstone for in vitro propagation of *Myrsine coriacea* (Sw.) R.Br. ex Roem. & Schult. (Primulaceae)

1. Abstract

Myrsine coriacea (Sw.) R. Br. ex Roem. & Schult 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 10% HCl and inoculated into medium supplemented with 10.74 µM of gibberellic acid. From these seeds plantlets were generated after 41 days, a time relatively short in relation 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 (IZE). From these explants, friable calli were formed on medium supplemented with 6-benzylaminopurine, and somatic embryos were regenerated and plantlets recovered after 125 days. This result evidenced that the IZE of *M. coriacea* possess levels of endogenous phytohormones, mainly auxins, that are sufficient to trigger the embryogenic system. Therefore, the first reliable and relatively rapid procedures allowed obtaining morphologically normal seedlings of *M. coriacea*, providing the basis for other in

vitro tissue culture applications. Furthermore, the plantlets obtained here can be used in reforestation and conservation programs, and for production of pharmacological compounds of interest.

Keywords: Woody species; seed germination; somatic embryogenesis; zygotic embryos; plantlet recovery.

2. Introduction

Myrsine coriacea (Sw.) R. Br. ex Roem. & Schult*., previously Rapanea ferruginea* Ruiz & Pav., is a woody species circumscribed to the family Primulaceae, subfamily Myrsinoidea (APG III 2009). This species occurs in the Neotropical region, showing wide distribution in Colombia, Bolivia, Argentina, Paraguay, Uruguay and Brazil (Backes and Irgang 2004). *M. coriacea* is used in civil engineering and for charcoal production (Freitas 2003), and its bark is rich in tannic substances employed in the leather industry (Monteiro *et al.* 2005). In the pharmaceutical industry, *M. coriacea* has been highlighted for anti-inflammatory production from its mirsinoic acid B, which has anti-nociceptive effect (Hess *et al.* 2010; Baccarin *et al.* 2011). Further, the protein lectin has been prospected from this species and considered a potential compound for cancer treatment (Medeiros *et al.* 2013). *M. coriacea* also possesses ecological importance as fruit source for several bird species (Pascotto 2007), 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 (*Cinnamomum camphora* Sieb, Du *et al.* 2007), and/or to mass propagate selected species with high wood production (*Swietenia macrophylla* R. A. King, Marzalina 2002). IVTC has also been applied to propagate and preserve plantlets from other woody species of ecological importance, such as *Anthocephalus cadamba* (Roxb.) Miq. *Syn A. chinensis* (Lamk) A. Rich (Apurva and Thakur 2009) and *Caesalpinia echinata* Lam. (Werner *et al.* 2009),

promoting the germplasm conservation. Because *M. coriacea* is a species of wide interest, the establishment of procedures for its in vitro propagation represents the first step for other 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 1991; 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 (Nascimento 2013) or asexually through cuttings (Elias 2010). Somatic embryogenesis (SE), another IVTC application, is also considered suitable for mass propagation (Cevallos *et al.* 2002; 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 (Peres and Kerbauy 1999). 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. 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 (Chand and Singh 2005; 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.

3. Material and methods

3.1. 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.

3.2. Seed germination and plantlet propagation

Mature seeds of *M. coriacea* were submersed in hydrochloric acid (HCl) solution at 0%, 5% or 10% (Ali *et al.* 2007). After 24 hours 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, M1–M7), which were constituted by ½MS 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, the media M2–M7 were supplemented with 10.74 or 21.48 µM of gibberellic acid (GA_3), NAA or IBA (Table 1a).

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

3.3. 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, M8–M10) composed by ½MS basal medium (MS salts), 10 mL L^{-1} B5 vitamins (Murashige and Skoog 1962), 0.08 g L^{-1} L-cysteine, 0.4 g L^{-1} L-glutamine, 0.1 g L^{-1} myo-inositol, 30 g L^{-1} sucrose, 2.8 g L^{-1} Phytagel, 4.44 µM BAP, with and without different concentrations of 2,4-D (Table 1b). For each concentration of 2,4-D were prepared seven Petri dishes, each containing five IZE. The dishes were kept in the dark at 25°C.

Friable embryogenic calli (FEC, Fig.1a) were transferred to embryo induction medium (EIM) until the emergence of somatic embryos. This medium was identical to M8, with the addition of 2 g L^{-1} activated charcoal. Petri dishes

were kept under a 16/8 h light/dark regimen, with 36 µmol m^{-2} s^{-1} light radiation provided by two fluorescent lamps (20 W, Osram®), at 25°C. Subsequently, the somatic embryos (Fig. 1b) were isolated and transferred to embryo maturation medium (EMM). This medium was identical to M8, with addition of 2 g L^{-1} activated charcoal and 0.132 g L^{-1} abscisic acid (ABA). The cotyledonary somatic embryos were converted into plantlets (Fig. 1c) after inoculation into flasks containing the regeneration medium (RM), which was identical to M8 without BAP.

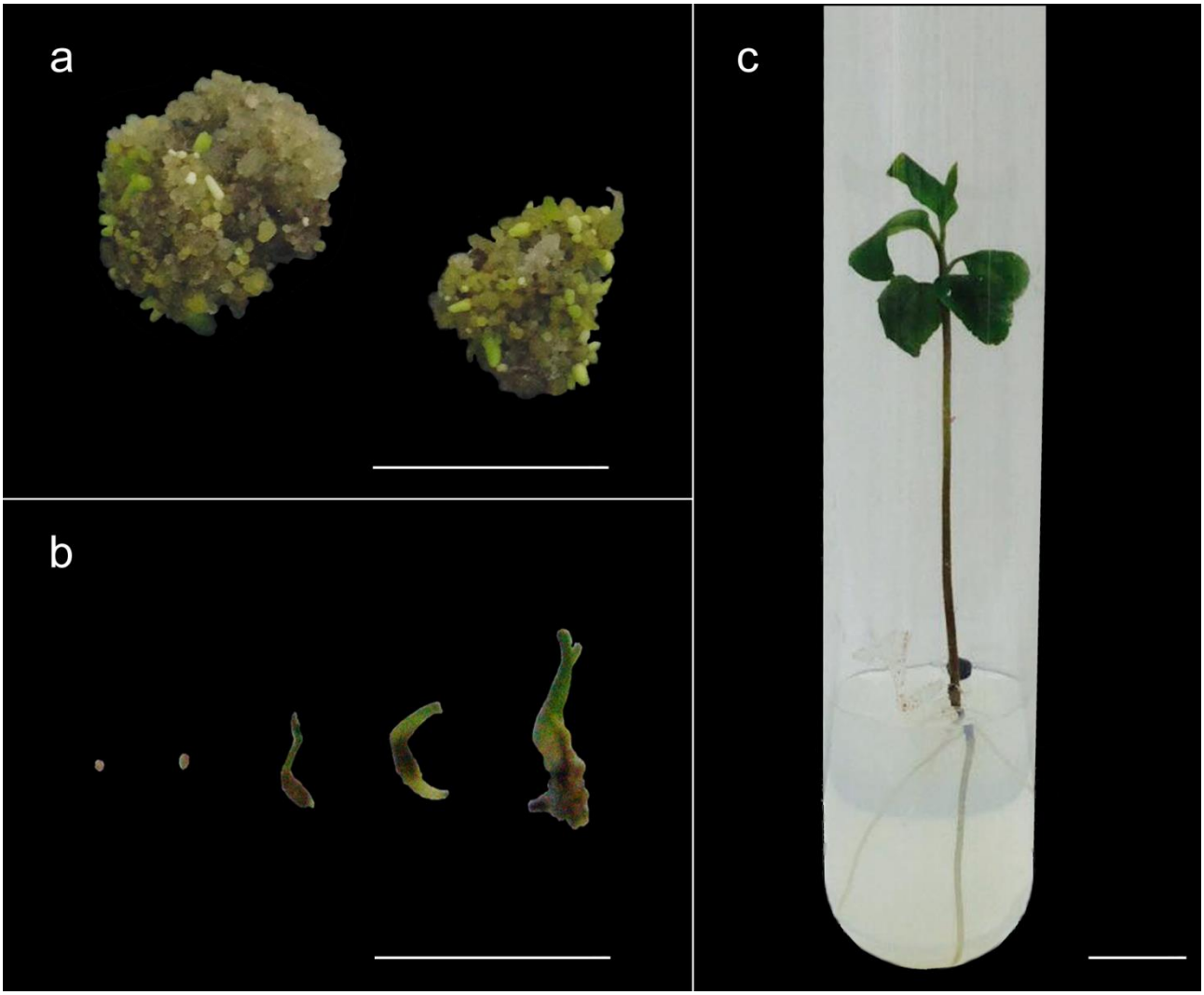

Fig. 1: Indirect somatic embryogenesis of *M. coriacea*. a) FEC obtained in medium CIM supplemented with growth regulator BAP. Note the presence of somatic embryos at distinct stages. b) Somatic embryos at distinct development stages, from globular stage to the mature cotyledonary. c) Regenerated plantlet of *M. coriacea*. Bars = 1 cm.

Table 1: Rate (%) of seed germination and plantlet conversion (a), calli formation and plantlets regeneration (b) of *Myrsine coriacea* propagated in distinct media.

a. Seed germination and plantlet conversion

Medium	Growth regulator	0% HCl	% Converted plantlets	5% HCl	Converted plantlets	10% HCl	Converted plantlets
M1	–	20	20	36.6	16	66.6	20
M2	10.74 µM NAA	20	-	20	-	60	-
M3	21.48 µM NAA	30	-	20	-	10	-
M4	10.74 µM GA_3	20	20	80	50	100	80
M5	21.48 µM GA_3	0	-	60	20	0	-
M6	10.74 µM IBA	10	10	0	-	20	30
M7	21.48 µM IBA	0	-	40	20	0	-
Total*		15,5%	10%	34,7%	15,5%	53,3%	18,8%

b. Somatic embriogenesys

Medium	Growth regulator	calli formation	Mean number of somatic embryos	Plantlets regenerated from somatic embryos
M8	–	25,7%	92	5,7%
M9	9.06 µM 2,4-D	0	0	0
M10	18.12 µM 2,4-D	0	0	0

*Mean rate (%) of seed germination and plantlet conversion for each HCl concentration

4. Results and Discussion

4.1. 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 (Table 1). The high rate of seed germination and, consequently, of plantlets (100%, Table 1) was obtained from the seeds treated with HCl 10% and inoculated into medium M4. In some treatments (HCL 0% and HCl 10%: M5 and M7; HCl 5%: M6), no seeds germinated (Table 1a).

Independently of the tissue culture medium, the average germination rate was higher in pre-treatment with 10% HCl (53.3%), and decreased from 5% HCl (34.7%) to 0% HCl (15.5%). Acid scarification with 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 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 *Tabebuia roseo-alba* Roble (Abbade *et al.* 2010) and *Albizia lebbeck* 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 (Backes and Irgang 2004) and 120 days (Lorenzi 2002; Carvalho *et al.* 2003), with rates varying from 33% (Lorenzi 2002) to 73% (Lorenzi 2002; Mori *et al.* 2012; Carvalho 2003). 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*.

4.2. 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 M8 (Table 1b). After 50 days, the FEC were transferred to medium EIM, and immature somatic embryos at distinct stages were thus recovered (Fig. 1b) 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 (Srivastava 2001; Jiménez 2005). Its high concentrations have been associated with best rates of embryogenic response in some species (Jimenez 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 (Delbarre *et al.* 1996; Berros *et al.* 2005; Chen *et al.* 2010). During maturation the IAA rate decreases (Jiménez 2005; Konieczny *et al.* 2010) in the cotyledonary embryo.

Besides the chronological, physiological and ontogenetic ages of the IZE, also the composition of the tissue culture media was fundamental for establishment of SE in *M. coriacea*. Plantlets were recovered only in medium M8, which was supplemented with the cytokinin BAP (Table 1b). This growth regulator stimulates the induction of cell division (Bronner *et al.* 1994), leading to FEC formation and regeneration of somatic embryos of *M. coriacea*. Plantlets of other woody species, such as *Dalbergia sissoo* Roxb. (Chand and Singh 2005) and *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 (Michalczuk *et al.* 1992; Féher *et al.* 2003). Differently, *M. coriacea*

plantlets were not obtained in the media containing 2,4-D (Table 1b). 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 at 45 days, 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 (Taras *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 indirect SE, differently from the result obtained by seed germination.

This research was pioneering in the establishment and propagation of *M. coriacea* in vitro using two different systems: seed germination and SE. This first step is relevant to the IVTC of *M. coriacea* and other species of the genus, since the propagation is fundamental for other biotechnological techniques and the sustainable exploration of this potential germplasm.

5. Conclusion

In conclusion, this research was pioneer in the establishment and propagation of *M. coriacea* in vitro using two different systems, seed germination and SE. This first step is relevant to IVTC of *M. coriacea* and other species of the genus, since the propagation is fundamental for other biotechnology techniques and sustainable exploration of this potential germplasm.

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Chapter 2

Variation in the nuclear 2C value and chromosome number of *Myrsine coriacea* (Sw.) R.Br. ex Roem. & Schult. (Primulaceae)

1. Abstract

Dioecy is a sexual system expressed in 6% of the angiosperms, including most of the *Myrsine* species (Primulaceae), which have male and female individuals clearly distinguishable during the reproductive period. Considering this biological aspect, the genus represents a suitable model for accomplishment of karyotype approaches, which provide data to understand the origin and evolution of the sexual chromosomes in plants. Using flow cytometry and cytogenetic procedures, this study aimed to characterize the karyotype of *Myrsine coriacea* (Sw.) R.Br. ex Roem & Schult. An intraspecific variation in the nuclear 2C value was found and initially attributed to the presence of secondary metabolites. However, karyotype analysis showed that this variation was rather associated to the presence of individuals with 2n = 45 or 2n = 46 chromosomes. Thus, the results indicate that the detected difference can be related to sex chromosomes in *M. coriacea*. Furthermore, groups of morphologically identical chromosomes were found, suggesting a polyploid origin for this species. Based on genome size and karyotype data, the dioecy of *M. coriacea* can be associated to genes that occur in specific sex chromosomes.

Keywords: Dioecy, *Myrsine*, cytogenetics, flow cytometry, sex chromosome, karyotype evolution.

2. Introduction

The family Primulaceae comprises 58 genera (Stevens 2008), including *Myrsine,* which presents dioecious species (Freitas 2003). Dioecy is controlled by chromosomal, genetic factors, hormonal and/or environmental. However, the determination system of most dioecious species involves sex chromosomes (Ming and Moore 2007).

Cytogenetic approaches have reported the chromosome number for the genus *Myrsine*. However, these studies have focused only on the karyotype aspect of several species belonging to different families. For the *Myrsine* species *M. coxxi* Cockayne, *M. divaricata* A. Cunn, *M. kermadecensis* Cheeseman, *M. nummularia* Hook, *M. salicina* Hook. f. (Beuzenberg and Hair 1983), *M. chathamica* F. Muell., *M. oliveri* Allan, *M. aff. divaricata* A. Cunn, *M. argentea* Heenan et de Lange (Dawson 1995; 2000), *M. matensis* (Mez) Otegui *(*Molero *et al.* 2002) and *M. africana* L. (Hanson *et al.* 2003) the chromosome number was determined as 2n = 46, while *M. guianensis* (Aubl.) Kuntze showed 2n = 48 chromosomes. Furthermore, only one study (Hanson *et al.* 2003) reported the nuclear DNA content of *Myrsine* species. Using Feulgen densitometry technique, the nuclear 2C value of *M. africana* was identified as 2.46 picograms (pg).

Cytogenetic and nuclear genome size data have been applied for discrimination and karyotype characterization of male and female plants, as in *Silene latifolia* Poir. and *Silene dioica* (L.) Clairv. (Costich *et al.* 1991; Doležel and Gohde 1995), *Rumex acetosa* L. (Ainsworth *et al.* 1995) and *Humulus lupulus* L. (Beatson *et al.* 2003). Moreover, this information is very useful for

evolutionary research based on euploid origin in some species, such as *Glycine max* (L.) Merr. (Clarindo *et al.* 2007), *Paullinia cupana* 'Sorbilis' (Freitas *et al.* 2007), *Coffea arabica* L. (Clarindo and Carvalho 2009) and *Coffea* "Híbrido de Timor" (Clarindo *et al.* 2013).

In view of all the above, this study aimed to measure the nuclear genome size and to determine the chromosome number of *Myrsine coriacea* (Sw.) R. Br. ex Roem. & Schult. This species was chosen as model because its plants can be unmistakably identified as male and female during the reproductive period.

Material and Methods

3.1. In vitro establishment of *M. coriacea*

M. coriacea seeds were collected at the city Iúna – ES, Brazil (20°21'6"S 41°31'58"W). At first, the pericarp was removed manually and the seeds were washed under running water and placed to dry on filter paper. Under laminar flow hood, the seeds were disinfested using 70% ethanol (Merck®) for 1 min, and after in a sodium hypochlorite (Merck®) solution supplemented with 0.1% (v/v) Tween 20 (Merck®). Subsequently, the seeds were rinsed three times with sterile distilled water (Oliveira *et al.* 2013).

After disinfestation, the seeds were inoculated into glass tubes (150 mm x 25 mm) containing tissue culture medium composed of 2.15 g L^{-1} of ½MS basal medium (MS salts), 10 mL^{-1} MS vitamins (Murashige and Skoog 1962), 30 g L^{-1} sucrose and 7.0 g L^{-1} agar type A. The pH was adjusted to 5.7 before autoclaving. The tubes were kept under a 16/8 h light/dark regimen, with 36

µmol m^{-2} s^{-1} light radiation provided by two fluorescent lamps (20 W, Osram®), at 25–28°C.

3.2. Nuclear DNA content of *M. coriacea*

Initially, flow cytometry (FCM) was performed using leaves from male and female specimens of *M coriacea* collected at the city of Iúna – ES, Brazil (20°21'6''S 41°31'58''W), and at greenhouse for *Pisum sativum* (2C = 9.16 pg, Praça-Fontes *et al.* 2011). Leaf fragments from the male and female plants of *M. coriacea* and *P. sativum* were simultaneously chopped (Galbraith et al. 1983), for 30 s, 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 dithiothreitol (Sigma®) and 50 g mL^{-1} RNAse (Sigma®). A volume of 0.5 mL of the same buffer was added to each suspension, which was filtered in through 30 µm nylon filter (Partec®) and centrifuged in ALC®microCentrifugette®4214) at 100 g for 5 min. The supernatant was scattered, and the pellet was resuspended and incubated for 5 min in 100 µL OTTO-I (Clarindo and Carvalho 2009). 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 µm (Partec®).

The suspensions were analyzed in Partec PAS® flow cytometer (Partec® GmbH, Munster, Germany) after calibration of the equipment. DNA content measurement was performed based on G_0/G_1 peaks of the internal standard and sample using FlowMax® software (Partec®). The data were reported in

picograms (pg) and base pairs (bp). Based on the results, the genome size was also measured using leaf or root fragments obtained from standard and sample plantlets cultured in vitro.

3.3. Karyotype of *M. coriacea*

Roots obtained from in vitro plantlets were excised and treated with 5 µM 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 distillated 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 -20ºC for 24 h, washed for 20 min, and incubated for 2h 45 min at 34ºC in pectinase solution (Sigma®) at a concentration of 1:8 (enzyme:water). Next, the roots were washed for 10 min, fixed and stored at -20ºC.

Slides were prepared applying the dissociation and air-drying technique, placed on a hot plate at 50ºC for 10 min, stained with 5% Giemsa (Merck®) for five minutes, washed in distilled water and air dried (Carvalho *et al.* 2007). Chromosome images were captured using a Media Cybernetics® Camera Evolution™ charge-coupled device video camera, mounted on a Nikon 80i microscope (Nikon, Japan).

4. Results

After 45 days, morphologically normal seedlings of *M. coriacea* were obtained (Fig. 1a), providing sufficient roots and leaves for cytogenetic and FCM analyses.

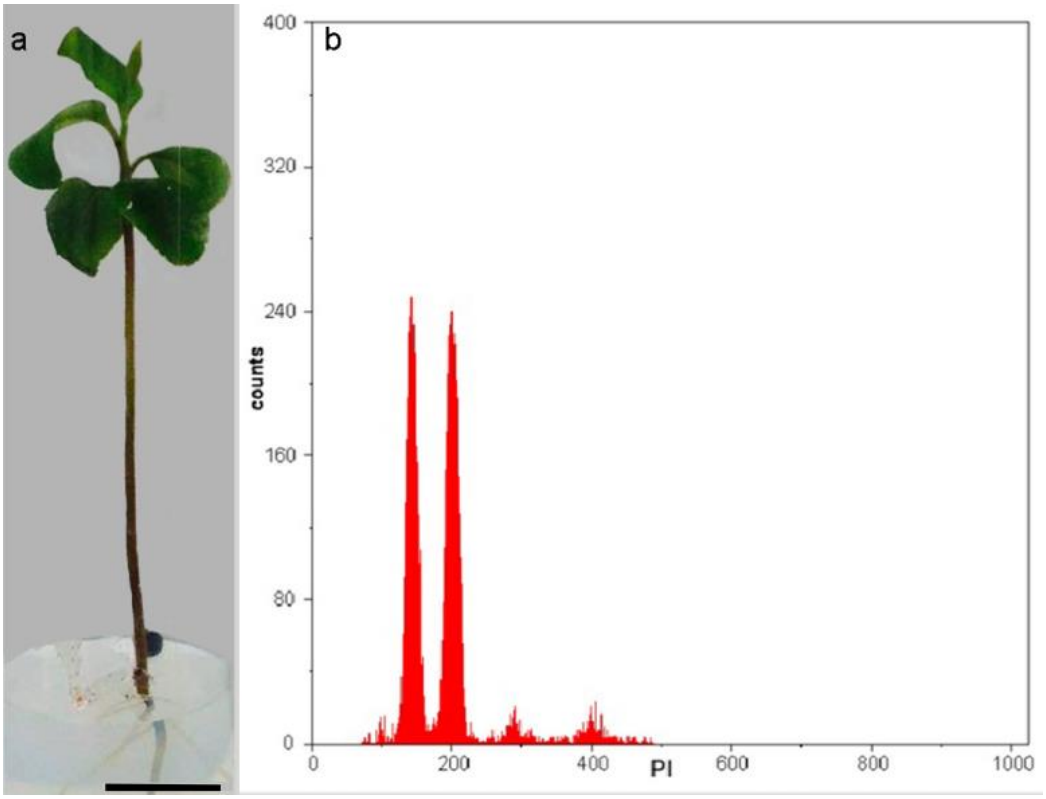

Fig. 1 a) Plantlet of *M. coriacea* after 45 days in the propagation medium b) Representative histogram exhibiting G_0/G_1 peaks corresponding to nuclei suspensions of *P. sativum* in the channel 200 (2C = 9.16 pg) and of one *M. coriacea* plantlet in the channel 144 (2C = 6.60 pg).

The initial FCM analysis of leaves collected from the plant in the field did not generate timeline peaks in the histograms. Because of this result, the measurement of the DNA content was performed alternatively using leaves and roots, separately from the sample and standard plantlets cultivated in vitro. The FCM histograms, for leaves and roots generated G_0/G_1 peaks with coefficient of

variation (CV) below 5%, indicating that the nuclear suspensions were suitable, composed by intact, isolated nuclei stained stoichiometrically.

From comparison between the G_0/G_1 peaks of the sample and the standard *P. sativum* (2C = 9.16 pg), the 2C value was measured for each *M. coriacea* plantlet. The values varied from 2C = 6.47 pg to 6.93 pg for roots (mean value 2C = 6.74 ± 0.18 pg) and from 2C = 6.36 pg to 6.86 pg (mean value 2C = 6.60 ± 0.14 pg, Fig. 1b) for leaves. Independently of the tissue, an intraspecific variation in the genome size was found *for M. coriacea*.

In accordance with FCM data, the chromosome number also varied between male and female specimens. Some slides showed metaphases with 2n = 45 (Fig. 1a) chromosomes and others with 2n = 46 (Fig. 1b, c). The protocol using APM (5 µM for 15 h), enzymatic maceration (1:8 solution for 2 h 45 min), cell dissociation and air-drying provided metaphase chromosomes with adequate morphology. The metaphases showed individualized chromosomes, flattened on the slide, without chromatin deformations and cytoplasmic background noise.

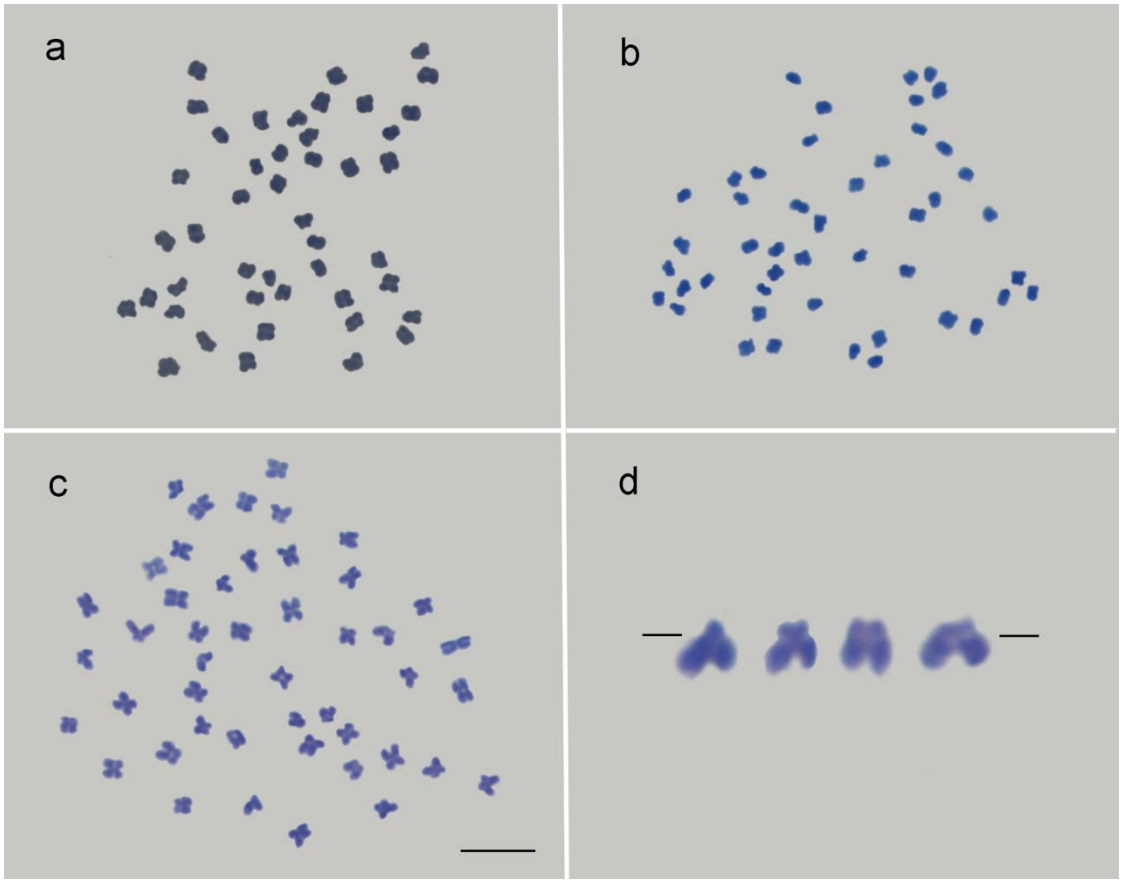

Fig. 2. (a - c) Karyotypes of *M. coriacea* showing chromosomes with different chromatin compaction levels. Metaphase exhibiting (a) 2n = 45 and (b, c) 2n = 46 chromosomes. d) Family portrait with four morphologically identical acrocentric chromosomes.

5. Discussion

The FCM analysis using *M. coriacea* leaves collected in the field (city of Iúna – ES, Brazil) did not generate timeline peaks in the histograms, possibly due to influence of secondary metabolites. Phenolic compounds present in the leaves of *M. coriacea* (Luna 2013), probably reduced or prevented the binding of the fluorochrome propidium iodide to the DNA molecule.

In order to solve this problem, leaves and roots of in vitro plantlets were used, seeing that variation in the amount of secondary metabolites occurs in different plant tissues as well as under in vitro and ex vitro propagation conditions. This effective strategy provided histograms with G_0/G_1 peaks showing CV below 5%, thus allowing to measure the nuclear DNA content of *M. coriacea*. Based on the obtained values, the leaves were more adequate for FCM due to lower standard deviation (mean value 2C = 6.60 ±0.14 pg). Therefore, use of leaves from in vitro plantlets as source of nuclei is suggested for FCM in *Myrsine*.

Besides secondary metabolites, the intraspecific variation in the measured 2C values is also associated to karyotype differences. As each slide exhibited metaphases with constant chromosome number, it can be concluded that *M. coriacea* is represented by individuals with 2n = 45 (Fig. 1a) or 2n = 46 chromosomes (Fig. 1b,c). Considering the fixed dioecy of this species, the occurrence of individuals with odd chromosome number can be related to a chromosomal system of sex determination of the type XY_1Y_2 as in *Silene latifolia* (females 2n = 14 and males 2n = 15, Ainsworth *et al.* 1998). Another possibility is the system X0, although the existence of an XX-XO sex determination system has not been demonstrated convincingly in plants. Additional cytogenetic approaches should be carried out to elucidate the evolutionary mechanisms of the sexual dimorphism in this species.

The karyotype analysis also evidenced the presence of morphologically identical chromosome groups (Fig. 1d), suggesting a polyploid origin for *M. coriacea* and showing the relevance of the polyploidization events during evolution of this taxon. This phenomenon can be triggered by chromosome

duplication in somatic cells (endopolyploidization or endomitosis) or fusion of non-reduced reproductive cells. Another mechanism that may result in polyploidy is hybridization (Stebbins 1950; Otto and Whitton 2000; Mallet 2007). These processes play a significant role in the speciation of plants (Soltis *et al*. 2003) and their detection is useful to elucidate the mechanisms of diversification and the spatial distribution of species.

6. Conclusions

For the first time, cytogenetic and FCM data showed that individuals of the same *Myrsine* species exhibit intraspecific variation associated to the karyotype. This result can be a consequence of the presence of sex chromosomes in *M. coriacea*. Besides, a polyploid origin of this species was suggested by the occurrence of groups of morphologically identical chromosomes.

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