INSTITUTO FEDERAL DE EDUCAÇÃO, CIÊNCIA E TECNOLOGIA GOIANO – IF GOIANO - CAMPUS RIO VERDE
DIRETORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS AGRÁRIAS

CULTURA *in vitro* DE *Mouriri elliptica* (Mart.) SOB CONDIÇÕES FOTOMIXOTRÓFICAS: ESTUDOS ANATÔMICOS, FISIOLÓGICOS E DE CRESCIMENTO

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Orientador: Prof. Dr. Fabiano Guimarães Silva

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BIOGRAFIA DA AUTORA

ELISVANE SILVA DE ASSIS, filha de Valdeci de Assis e Noeme Batista da Silva, nasceu na cidade de Itarumã - GO em 11 de dezembro de 1983.

Em 2002 ingressou no Curso de Ciências Biológicas (Licenciatura) na Universidade Federal de Goiás (UFG) – Campus Jataí, concluiu em 2005. Neste mesmo ano, teve um filho "Pedro Lucas Silva Severino", hoje com 11 anos de idade.

Em março de 2009, iniciou no curso de mestrado no Programa de Pós-Graduação em Agronomia (Produção Vegetal) na Universidade Federal de Goiás, Campus Jataí UFG/Jataí, sob orientação do Professor Dr. Edésio Fialho dos Reis. Foi por 24 meses bolsista do CNPq, e, em junho de 2011, defendeu a dissertação intitulada por "Diversidade genética de gabirobeiras (*Campomanesia* spp) por meio de caracteres morfológicos e marcadores moleculares RAPD".

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Ab Ep T	Espessura da epiderme abaxial	µm
Ad Ep T	Espessura da epiderme adaxial	µm
CP T	Espessura do parênquima clorofiliano	µm
CV	Coeficiente de variação	-
CCC	Coeficiente de correlação cofenético	-
cm^2	Centímetro ao quadrado	-
ºC	Graus Celsius	-
CO_2	Dióxido de Carbono	-
DIC	Delineamento inteiramente ao acaso	-
ETR	Taxa relativa de transporte de elétrons	-
FV	Fonte de variação	-
GENES	Sofware de análise estatística	-
NaO Cl	Hipoclorito de sódio	-
PAS	Reação com ácido periódico e Reagente de Schiff	-
r_p	Coeficiente de correlação fenotípico	%
SISVAR	Sofware de análise estatística	-
St Cr Dn	Densidade de cripta stomática	Criptas mm^{-2}
St Cr Dp	Profundidade da cripta estomática	µm
St Cr O	Área de abertura da cripta estomática	µm
S.j	Importância relativa	%
S	Sul	-
SP	Parênquima esponjoso	-
pH	Potencial de hidrogênio	-
PP	Parênquima paliçádico	-
PVC	Polivinilcloreto	-
WPM	Wood Plant Medium	-
W	Oeste	-
UPGMA	Unweighted pair groups mean arithmetic	-
Fo	Fluorescência inicial	-
Fv/Fm	Rendimento quântico máximo do fotossistema II	-
Y(II)	Rendimento quântico efetivo do fotossistema II	-
µm	Micrômetro	-
µmol	Micromol	-
µmol $m^{-2}s^{-1}$	Micromol por metro quadrado por segundo	-

RESUMO

ASSIS, ELISVANE SILVA. Instituto Federal de Educação, Ciência e Tecnologia Goiano – IF Goiano - Campus Rio Verde. Dezembro de 2016. **Cultura *in vitro* de *Mouriri elliptica* (Mart.) sob condições fotomixotróficas: estudos anatômicos, fisiológicos e de crescimento**. Orientador: Dr. Fabiano Guimarães Silva, Coorientador: Dr. Aurélio Rubio Neto.

A planta croada (*Mouriri elliptica* Mart.), é frutífera nativa no domínio do cerrado com potencialidade para uso alimentício e medicinal. Há carência de estudos para espécie na área de propagação, visto ser esta etapa, importante no processo de domesticação da espécie. Assim, objetivou-se com este trabalho avaliar o crescimento e as características anatômicas e fisiológicas de *M. elliptica* (Mart.) sob condições de cultivo *in vitro* fotomixotróficas. O meio de cultivo utilizado em todos os ensaios foi o Wood Plant Medium. No primeiro capítulo, avaliou-se o crescimento e características anatômicas foliares de plântulas de croada cultivadas em diferentes irradiâncias (0, 50, 75, 100 e 150 µmol $m^{-2}s^{-1}$) e meio de cultivo com e sem sacarose. No segundo capítulo, estudou-se a dissimilaridade de plântulas de croada obtidas via cultivo *in vitro* fotomixotrófico e fotoautotrófico com plantas *in situ* a partir de características anatômicas. No terceiro capítulo, analisou-se o crescimento e desenvolvimento de croada *in vitro* utilizando materiais de suporte (vermiculita, fibra de jerivá e bagaço de cana) em comparação com ágar, avaliou-se também a interação destes suportes com o regulador de crescimento ácido naftaleno acético no enraizamento das plântulas. Por último, estudou-se a influência do cultivo *in vitro* fotomixotrófico sob atmosfera enriquecida com CO_2 e diferentes vedações na aclimatização de plântulas de croada. Em todos os ensaios experimentais utilizou-se delineamento inteiramente ao acaso, com esquema fatorial quando necessário. Na ausência de sacarose, notou-se capacidade de regeneração das plântulas de croada apenas com irradiância acima de 50 µmol $m^{-2}s^{-1}$, sendo observado

comportamento linear para número de brotos e folhas. Ponto máximo para acúmulo de matéria seca foi observado com intensidade luminosa de 100 µmol $m^{-2}s^{-1}$. Independente da presença de sacarose no meio, notou-se variações anatômicas nas folhas de croada em reposta as diferentes intensidades luminosas. Considerou-se que condições fotoautotróficas podem ser utilizadas para micropropagação da espécie. Contudo, notou-se a partir do estudo de dissimilaridade, que plântulas cultivadas na presença de sacarose e irradiâncias de 50 e 75 µmol m^{-2} s^{-1} desenvolveram características anatômicas foliares menos dissimilares as plantas *in situ*. Identificou-se as características de área de abertura da cripta estomática e densidade de criptas de maior importância relativa no estudo de dissimilaridade. Quanto ao cultivo utilizando diferentes materiais de suporte para os explante de croada, notou-se que vermiculita, seguido do bagaço de cana-de-açúcar são promissores para utilização *in vitro*. Não se observou diferença entre os suportes avaliados para as características de crescimento número de segmentos nodais, número de folhas e massa seca total. Maior número de raízes adventícias e raízes secundárias, foram obtidas em plântulas cultivadas em vermiculita. A presença do regulador ácido naftaleno acético no meio de cultivo não influenciou no enraizamento das plântulas. Plântulas de *M. elliptica* (Mart.) tiveram melhor performance na aclimatização quando propagadas em frascos vedados com tampa com 2 orifício de área de 2,24 10^{-4} m^2 com membrana microporosa e atmosfera ambiente de CO_2.

PALAVRAS-CHAVE: Croada, Fotoautotrofismo, Melastomataceae, intensidade luminosa, suportes.

ABSTRACT

ASSIS, ELISVANE SILVA. Federal Institute of Education, Science, and Technology of Goiás (IF Goiano) Rio Verde Campus. December 2016. ***In vitro* culture of *Mouriri elliptica* (Mart.) under photomixotrophic conditions: anatomical, physiological, and growth studies**. Advisor: Dr. Silva, Fabiano Guimarães; co-advisor: Dr. Rubio Neto, Aurélio.

Croada plant (*Mouriri elliptica* Mart.) is a native fruit in Brazilian cerrado (savannah) domain with potential for food and medicinal use. There is a lack of studies on species in the propagation area, and this stage is important in the species domestication process. Thus, this paper aimed to evaluate the growth and anatomical and physiological characteristics of *M. elliptica* (Mart.) under photomixotrophic *in vitro* culture conditions. Wood Plant Medium was the culture medium used in all experiments. In the first chapter, the growth and foliar anatomical characteristics of croada seedlings grown in different irradiances (0, 50, 75, 100, and 150 µmol $m^{-2}s^{-1}$) and culture medium with and without sucrose were evaluated. In the second chapter, the dissimilarity of croada seedlings obtained by photomixotrophic and photoautotrophic *in vitro* cultivation with plants *in situ* was studied based on anatomical characteristics. In the third chapter, the growth and development *in vitro* of croada using support materials [vermiculite, jerivá (Syagrus romanzoffiana) fiber, and sugarcane bagasse] in comparison with agar were analyzed. Interaction of these substrates with the naphthalene acetic acid growth regulator in seedling rooting was also evaluated. Finally, the influence of photomixotrophic on *in vitro* cultivation under CO_2 enriched atmosphere and different seals in the acclimatization of croada seedlings were studied. In all experimental trials, a completely randomized design was used with a factorial scheme when necessary. In the

absence of sucrose, the regeneration capacity of the croada seedlings was observed only with irradiance above 50 µmol $m^{-2}s^{-1}$, and a linear behavior was observed for number of shoots and leaves. Maximum point for dry matter accumulation was observed with luminous intensity of 100 µmol $m^{-2}s^{-1}$. Independently of sucrose presence in the medium, anatomical variations in the croada leaves were noted in response to the different light intensities. It was considered that photoautotrophic conditions can be used for species micropropagation. However, on the basis of the dissimilarity study, it was noted that seedlings grown in the presence of sucrose and irradiances of 50 and 75 µmol $m^{-2}s^{-1}$ developed less dissimilar foliar anatomical characteristics *in situ*. Characteristics of opening area of the stomatal crypt and crypt density of greater relative importance in dissimilarity study were identified. Regarding the cultivation using different support materials for the croaker explants, it was noted that vermiculite followed by sugarcane bagasse are promising for using *in vitro*. There was no difference among evaluated supports for growth characteristics, number of nodal segments, number of leaves and total dry mass. Greater number of adventitious roots and secondary roots were obtained in seedlings cultivated in vermiculite. The presence of the naphthalene acetic acid regulator in the culture medium did not influence the seedling rooting. Seedlings of *M. elliptica* (Mart.) had better acclimatization performance and higher survival rate when propagated in bottles sealed with hole lid area of 2.24 10^{-4} m^2 with microporous membrane and CO_2 ambient atmosphere.

KEYWORDS: Croada. Photoautotrophism. Melastomataceae. Luminous intensity. Support material.

INTRODUÇÃO GERAL

O Cerrado brasileiro é considerado um dos hotspot para a conservação da biodiversidade mundial, e, representa importante fonte de recursos vegetais (Batalha et al., 2011). Possui variedade de espécies frutíferas detentoras de características sensoriais peculiares pouco exploradas científica e comercialmente. Estudos que buscam conhecer os frutos nativos do cerrado, são imprescindíveis, agregam valor, desperta o interesse dos consumidores e contribui com a busca das indústrias por inovações (Siqueira et al., 2013; Morzelle et al., 2015).

Dentre as frutíferas, cita-se a *Mouriri elliptica* (Mart.), com potencialidades para ser utilizada pela população. Esta espécie possui hábito arbóreo e tem sido classificada como frutífera tropical não tradicional (Rufino et al., 2010). Os frutos de *M. elliptica* (Mart.) quando maduros são apreciados pela população, podendo ser consumidos *in natura* ou processados na forma de geleias (Silva et al., 2001). As folhas são ricas em compostos fenólicos, em especial flavonoides, que tem sido relacionado ao eficaz tratamento de doenças gastrointestinais, como úlceras gástricas ou doenças provocadas pelo micro-organismo *Helicobacter pylori* (Moleiro et al., 2009; Vasconcelos et al., 2010b).

As sementes de *M. elliptica* (Mart.) possuem tegumento muito rígido, dificultando sua reprodução sexual. Há a necessidade de aplicação de práticas que promovam à superação de dormência das sementes, no entanto, nenhuma das metodologias propostas conseguiram subsidiar 100% de germinação (Vasconcelos et al., 2010a). Assim, a cultura de tecidos representa ferramenta biotecnológica importante para produção massal de mudas da espécie, as quais poderão ser utilizadas em cultivos ou para reflorestamento.

Na cultura de tecidos, estudos são desenvolvidos com finalidade de melhorar as qualidades morfofisiológicas das plantas, focados principalmente em fatores físicos e químicos do ambiente (Chandra et al., 2010). Torna-se primordial a adequação da luminosidade, temperatura, umidade e fotoperíodo do ambiente de crescimento das plantas (Torres et al., 1998).

Cada espécie responde de forma dissimilar a uma condição de cultivo imposta, assim, além dos fatores físicos citados, é importante a otimização do meio de cultivo, ajustando as concentrações de sais, sacarose (Assis et al., 2012; Cabral et al., 2013; Assis et al., 2015). É destacado também a suplementação do meio de cultivo com regulador de crescimento na indução de brotos e raízes (Brondani et al., 2012; Hossain e Urbi, 2016; Aina et al., 2015).

Destaca-se que o sucesso da propagação *in vitro* depende da capacidade de transferência das plantas das condições *in vitro* para as *ex vitro* com alta taxa de sobrevivência e com qualidade (Chandra et al., 2010; Correia et al., 2012). Nesta perspectiva, tem sido investigado técnicas de propagação *in vitro* que estimulam o desenvolvimento autotrófico das plantas (Xiao et al., 2011), beneficiando assim a aclimatização das mesmas (Xiao e Kozai, 2006; Zhang et al., 2009; Cha-um et al., 2011; Iarema et al., 2012; Saldanha et al., 2014).

2. REVISÃO DE LITERATURA

2.1 Características gerais da espécie *Mouriri elliptica* (Mart.)

A espécie em estudo é a *Mouriri elliptica* (Mart.), uma das representantes da família Melastomataceae. É chamada neste trabalho de croada, no entanto é conhecida também como “coroa de frade, croadinha, puçá, puçazeiro e manipuçá”. É uma frutífera de hábito arbóreo (Figura 1A), podendo atingir quando adulta até 6 m de altura (Silva et al., 2001).

Figure 1. Planta adulta de *Mouriri elliptica* (Mart.) *in situ* (A), frutos em maturação (B) e sementes. Frutos maduros coletados em novembro de 2014, no Município de Montividiu – GO, Latitude "17º 19.201"S, Longitude "51 33.500"W, Altitude 982 m.

As flores de *M. elliptica* (Mart.) possuem pétalas brancas e cremes, estames amarelados e cálice verde. O fruto tem mesocarpo alaranjado e doce, são arredondados (Figura 1B), chegando a 35,22 mm de diâmetro equatorial, 28,68 mm de diâmetro longitudinal e pesa em média 21,69 g (Lima et al., 2016). A frutificação pode ocorrer de agosto a dezembro. Quando maduros, os frutos podem ser colhidos no chão ou na própria planta. Não são climatéricos, portanto, se colhidos verdes, os frutos não amadurecem. Animais silvestres dependem destes como base para sua alimentação, entre estes animais cita-se a raposa do campo (*L. vetulus)*, que tem sido considerada um potente dispersor de sementes (Dalponte e Lima, 1999).

Análise química dos frutos de croada identificou cerca de 40 mg de vitamina C, 3,4 mg de antocianinas, 17,7 mg de flavonoides e 3,4 mg de carotenoides para cada 100 g de material fresco (Rufino et al., 2010). Além do potencial nutricional dos frutos de croada, Rufino et al. (2011) indicam os frutos de croada juntamente com frutos de *Platonia insignis*, *Spondias mombin*, *Myrciaria dubia*, *Myrciaria cauliflora*, *Copernicia prunifera*, *Mouriri guianensis*, *Mouriri pusa*, *Syzygium cumini*, *Euterpe edulis*, *Blepharocalyx salicifoliu* como potentes antioxidantes, justificando seu uso na alimentação humana.

A espécie *M. elliptica* (Mart.) possui potencial medicinal, podendo ser um recurso para indústria farmacológica. Estudos de extratos das folhas de croada, indicam fitoquímicos derivados de ácidos fenólicos e taninos (Moleiro et al., 2009). Estes compostos podem agir neutralizando oxidantes reativos, conferindo desta forma,

atividade terapêutica contra doenças gástricas e duodenais (Moreira et al., 2004; Zayachkivska et al., 2005).

As sementes de croada (Figura 1 C) possuem rígido tegumento, que dificulta a absorção de água e difusão de gases durante a germinação. Vasconcelos et al. (2010) e Lima et al. (2016) relataram a dificuldade de obtenção de mudas de *M. ellipitca* (Mart.) via sementes, além de desuniformidade na emergência das plântulas. Assim, o aprimoramento de métodos alternativos para propagação massal da mesma torna-se importante e necessário.

Trabalhos com propagação *in vitro* da espécie são escassos (Lima et al., 2016). Estudos em nível de gênero vêm sendo desenvolvidos principalmente na quantificação nutricional dos frutos, estudos fitoquímicos das folhas, quebra de dormência das sementes, e, a partir deste trabalho, estudos com propagação *in vitro* da espécie (Tabela 1).

Tabela 1 - Principais estudos do gênero *Mouriri*, publicados no período de 1999 a 2016 (dados obtidos na Web of Science e Sciencedirect).

Espécie	Título	Parte da planta	Referências
M. elliptica	Disponibilidade de frutos e a dieta de *Lycalopexvetulus* (Carnivora – Canidae) em um cerrado de Mato Grosso, Brasil	Frutos	Dalponte, (1999)
M. elliptica	*Mouriri elliptica*: Validation of gastroprotective, healing and anti-*Helicobacter pylori* effects	Folhas	Moleiro et al. (2009)
M. elliptica	Métodos de superação de dormência em sementes de croada (*Mouriri elliptica* Mart)	Sementes	Vasconcelos et al. (2010a)
M. pusa	Effect of *Mouriri pusa* tannins and flavonoids on prevention and treatment against experimental gastric ulcer	Folhas	Vasconcelos et al. (2010b)
M. guianensis e *M. pusa*	Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil	Frutos	Rufino et al. (2010)
M. guianensis e *M. pusa*	Free radical scavenging behavior of	Frutos	Rufino et al.

	tem exotic tropical fruits extracts		(2011)
M. pusa	Absence of mutagenicity of plants used to treat gastrointestinal disorders	Folhas	Santos et al. (2013)
M. pusa	Comparison of Brazilian Plants Used to Treat Gastritis on the Oxidative Burst of *Helicobacter pylori*-Stimulated Neutrophil	Folhas	Bonacorsi et al. (2013)
M. elliptica	Germination and emergence of *Mouriri elliptica* Mart., a rare medicinal fruit tree native to the Brazilian Cerrado biom	Sementes	Lima et al. (2016)
M. elliptica (Mart.)	*In vitro* culture of *Mouriri elliptica* (Mart.) under conditions that stimulate photoautotrophic behavior	Sacarose e intensidade luminosa	Assis et al. (2016)
M. elliptica (Mart.)	Dissimilarity between *Mouriri elliptica* (Mart.) plants cultivated *in vitro* and *in situ* through anatomic parameters	Plantas *in situ* e *in vitro*	Assis et al. (2016)

Na literatura, cita-se ocorrência natural de plantas do gênero *Mouriri* no domínio do Cerrado, nos estados de Mato Grosso, Mato Grosso do Sul e Goiás (Silva et al., 2001), no entanto, vem perdendo seu habitat. De acordo com Pereira e Pasquaeto, (2011) o Cerrado sofre pressão antrópica, principalmente pela atividade pecuária, exploração extrativista e expansão da agricultura. As frutíferas nativas são fundamentais neste ecossistema, porém, mesmo com a crescente valorização e o emprego dos produtos regionais, os estudos científicos com essas espécies são limitados, carecendo de investimentos (Damiani et al., 2011).

2.2 Cultura *in vitro*: Propagação heterotrófica, fotoautotrófica e fotomixotrófica

Na cultura *in vitro*, objetiva-se produção de plantas, crescimento e multiplicação de células, tecidos e órgãos em meio de cultura específico, semissólido ou líquido sob condições ambientais controladas e, na ausência de patógenos (Thorpe, 2007; Chandra et al., 2010). Fontes de carbono, nutrientes (Macro e Micro) e energia encontram-se disponíveis no meio de cultivo, e estes, subsidiam o crescimento das plantas *in vitro* (Brondani et al., 2012).

Em comparação com outras técnicas de propagação, a cultura *in vitr*o contribui significativamente para produção de mudas de espécies silvestres ou cultivadas que possuem dificuldades de propagação pelos métodos convencionais, ou ainda, busca-se rapidez na obtenção de plântulas (Martendal et al., 2014; Mali e Chavan, 2016). Além disso, favorece a produção de mudas em escala comercial e conservação de muitas espécies vegetais (Mosaleeyanon et al., 2004).

Tradicionalmente, a cultura *in vitro* tem a sacarose como maior fonte de energia metabólica do meio de cultivo (Arigita et al., 2002). Os frascos utilizados, restringem trocas gasosas, mantendo alta umidade relativa do ar e baixa concentração de CO_2, e, a intensidade luminosa do ambiente de cultivo normalmente é baixa. Estas características de cultivo *in vitro* tornam as plantas dependentes da sacarose presente no meio, expondo as plantas a um comportamento heterotrófico (Kozai e Kubota, 2001).

Plantas cultivados sob regime heterotrófico desenvolvem tecidos com maior teor de água, brotos pouco desenvolvidos, folhas pequenas e finas, com menos tricomas e com desordens anatômicas e fisiológicas que não possibilitam que o aparato fotossintético opere normalmente (Cha-um et al., 2011; Xiao et al., 2011). Estas características causam grande risco de desidratação das mudas e morte durante a sua aclimatização (Kitaya et al., 2005) resultando na perda de mudas e de mão de obra, aumentando consideravelmente os custos de produção.

Com perspectivas de aprimorar a cultura *in vitro* e beneficiar a produção de mudas, tem sido estudado a propagação que estimula o comportamento autotrófico das plantas, conhecida como sistema fotoautotrófico. Este conceito foi estabelecido a mais de duas décadas, e, é caracterizado pela ausência de sacarose no meio de cultivo (Kozai, 1991; Xiao et al., 2011), estimulando as plantas a desenvolverem com eficiência seu aparato fotossintético. O comportamento autotrófico das plantas também pode ser desenvolvido em sistema fotomixotrófico, ajustando as condições da cultura *in vitro*, conforme pode ser observado nos estudos de Saldanha et al. (2012) e Iarema et al. (2012).

Os fatores que têm contribuído com o desenvolvimento autotrófico das plantas *in vitro*, beneficiando o crescimento e aclimatização são: aumento da intensidade luminosa, uso de materiais de suporte fibrosos ou porosos em substituição ao ágar, vedações que permitem maiores trocas gasosas e enriquecimento da atmosfera de cultivo com CO_2.

2.3 Intensidade luminosa, suportes alternativos, vedações e CO_2 na cultura *in vitro*

A intensidade e a qualidade da luz são fatores ambientais fundamentais que interferem diretamente na morfologia, fisiologia e metabolismo das plantas (Fukuda et al., 2008; Li e Kubota, 2009, Shin et al., 2013). A dependência das plantas à luz é um processo complexo que envolve a ação combinada de fotorreceptores que controlam estádios variados no desenvolvimento (Braga et al., 2009).

Em sala de crescimento, a intensidade luminosa fornecida para as culturas *in vitro* normalmente são baixas (< 50 µmol $m^{-2}s^{-1}$). Entretanto, quando objetiva-se induzir o autotrofismo das plantas *in vitro,* pode ser necessário aumentar a intensidade luminosa, especialmente quando se pretende utilizar meio de cultivo desprovido de sacarose (Kozai e Nguyen, 2003). Assim, sob cultivo fotoautotrófico a intensidade luminosa de 100 µmol $m^{-2}s^{-1}$ foi ideal para *Momordica grosvenori* (Zangh et al., 2009). Já, para o híbrido *Doritaenopsis* os autores obtiveram melhor crescimento com intensidade luminosa de 120 µmol $m^{-2}s^{-1}$ e para *M. elliptica* (Mart.) maior crescimento foi com 150 µmol $m^{-2}s^{-1}$ de luz (Assis et al., 2016).

Entre os tipos de suportes utilizados *in vitro*, o ágar é o agente geleificante do meio de cultura tradicional (Thorpe et al., 2008). Entretanto, devido o uso em abundância, torna-se o ingrediente mais caro do meio de cultivo, além disso, as plântulas têm desenvolvido raízes mal formadas e geralmente não possuindo pelos absorventes. Tais características podem dificultar a aclimatização e sobrevivência das plântulas às condições *ex vitro* (Braga et al., 2011). Diante dessa problemática, vêm sendo testado suportes alternativos, em especial porosos, acrescido de meio de cultura líquido (Mohan et al., 2005).

Suportes porosos aumentam a condutividade hidráulica, favorece a absorção de nutrientes do meio de cultura e proporciona melhor aeração de tecidos e raízes do que seria no cultivo com ágar, melhorando potencialmente o vigor da planta e, assim, a taxa de sobrevivência no processo de aclimatização (Kozai, 2010; Xiao et al., 2011; Saldanha et al., 2014). Entre os tipos de suportes que podem ser utilizados *in vitro*, cita-se vermiculita (Xiao e Kozai, 2006; Cha-um et al. 2011), combinação de vermiculita e fibra de celulose (Florialite®) (Xiao e Kozai, 2006), bagaço de cana-de-açúcar (Mohan et al., 2005), entre outros, como a fibra de Jerivá utilizada neste trabalho.

Para *Pfaffia glomerata* (Spreng) Pedersen, a retirada da sacarose do meio de cultura não afetou o crescimento das plântulas quando se utilizou frascos com vedações possuindo membranas permeáveis aos gases (Iarema et al., 2012). Neste trabalho o objetivo de promover o comportamento autotrófico de *P. glomerata* foi alcançado, pois plantas desenvolveram morfologia e características fisiológicas necessárias para o processo de aclimatização.

Comportamento fotoautotrófico também foi observado em *Annona glabra* L., II. (Santana et al., 2008), no qual compararam o crescimento das plantas em meio sem sacarose e com tampas permeáveis a gases ao invés de vedação fechadas. Neste, os autores observaram que as raízes foram maiores e as plantas desenvolveram maior número de raízes secundárias. Observou-se também que as espessuras dos tecidos foliares possuíam semelhanças com plantas cultivadas *ex vitro*, e confere maior sustentação e plasticidade. Essa capacidade de alterar a estrutura das folhas em resposta à aeração dos frascos, revela adaptação da planta.

Quando se aborda umidade relativa do ar dentro do frasco de cultivo, maiores trocas gasosas gasosas com o ambiente externo, pode aumentar significativamente a taxa de transpiração da planta, e consequentemente, a absorção de água e de nutrientes (Aitken-Christie et al., 1995). Ao mesmo tempo, a redução da umidade relativa reduz a incidência de hiperhidricidade nas plantas, favorece a formação de cutícula nas folhas e o funcionamento normal dos estômatos, aumentando a tolerância ao estresse hídrico na aclimatização (Zobayed et al., 2001).

Carboidratos exógenos são fornecidos à cultura *in vitro* devido à concentração de CO_2 no interior do recipiente ser baixa limitando a fotossíntese (Kozai, 2010; Xiao et al., 2011). Esta limitação fotossintética pode ser revertida quando permite maior aeração no interior dos recipientes de cultivo, conforme resultados de Iarema et al. (2012) ou ainda, proporciona enriquecimento da atmosfera de cultivo com Dióxido de Carbono (CO_2), fornecendo substrato para fotossíntese (Saldanha et al., 2014).

Trabalhos com propagação *in vitro* fotoautotrófica têm sido desenvolvidos para um variado número de espécies, potencializando a obtenção de mudas e beneficiando o setor produtivo. Estudos com espécies frutíferas do cerrado são excassos, assim, este trabalho de pesquisa teve como base os estudos citados na tabela 2. Nestes, os principais fatores em estudo são CO_2, intensidade luminosa, concentrações de sacarose e vedações.

Tabela 2 - Principais estudos com propagação fotoautotrófica, publicados no período de 2007 a 2016 (dados obtidos na Web of Science e Sciencedirect).

Espécie	Título	Fator em estudo	Referências
Dendrobium candidum	Growth and photosynthesis of *Dendrobium candidum* plantlets cultured photoautotrophically	CO_2	Xiao et al. (2007)
Uniola paniculata	Influence of *in vitro* growth conditions on *in vitro* and ex vitro photosynthetic rates of easy- and difficult-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes	CO_2	Valero-Aracama et al. (2007)
Annona glabra	Estímulo do comportamento fotoautotrófico durante o enraizamento *in vitro* de *Annona glabra* l., II. Aspectos da anatomia da folha antes da aclimatização	Intensidade luminosa e sacarose	Santana et al. (2008)
Momordica grosvenori	Growth and photosynthethetic capability of *Momordica grosvenori* plantlets grown photoautotrophically in response to light intensity	Intensidade luminosa	Zhang et al. (2009)
Macadamia tetraphylla	Promoting root induction and growth of *in vitro* macadamia (*Macadamia tetraphylla* L. ‘‘Keaau’’) plantlets using CO_2-enriched photoautotrophic conditions.	Sacarose, CO_2 e vedações	Cha-um et al. (2011)
Castanea sativa	Increased light intensity during *in vitro* culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels	Intensidade luminosa	Sáez et al. (2012)
Pfaffia glomerata	A low-cost alternative membrane system that promotes growth in nodal cultures of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen].	Vedações	Saldanha et al. (2012)
Pfaffia glomerata	Photoautotrophic propagation of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]	Sacarose e vedações	Iarema et al. (2012)

Pfaffia glomerata	A CO_2-enriched atmosphere improves *in vitro* growth of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]	Sacarose e CO_2	Saldanha et al. (2013)
Pfaffia glomerata	CO_2-enriched atmosphere and supporting material impact the growth, morphophysiology and ultrastructure of *in vitro* brazilian-ginseng [*pfaffia glomerata* (spreng.) pedersen] plantlets	CO_2	Saldanha et al. (2014)
Bilbergia zebrine	Impacts of photoautotrophic and photomixotrophic conditions on *in vitro* propagated *Bilbergia zebrine* (Bromeliaceae).	Sacarose e vedações	Martins et al. (2015)
Carica papaya	Effects of different culture conditions (photoautotrophic, hotomixotrophic) and the auxin indole-butyric acid on the *in vitro* acclimatization of papaya (*Carica papaya* L. var. Red Maradol) plants using zeolite as support	Sacarose e regulador de crescimento	Pérez et al. (2015)
Anacardium othonianum Rizz.	Effects of photomixotrophic conditions and type of culture vessel closure on *Anacardium othonianum* Rizz. grown *in vitro*	Sacarose e vedações	Assis et al. (2015)
Mouriri elliptica (Mart.)	*In vitro* culture of *Mouriri elliptica* (Mart.) under conditions that stimulate photoautotrophic behavior	Sacarose e intensidade luminosa	Assis et al. (2016)

2.4 Aclimatização

A aclimatização das plantas é a etapa mais crítica do processo de propagação *in vitro*, visto o estresse pelo qual as plantas são submetidas. Estas deixam as condições de cultivo *in vitro* totalmente controladas e passam para o meio *ex vitro* no qual geralmente são expostas à condição de alta luminosidade, baixa umidade relativa do ar, possível estresse hídrico, entre outras. Assim, para o sucesso da técnica, é de suma importância que as plantas possuam características morfológicas e fisiológicas adaptativas (Tanno e Biasi, 2013).

É justamente na etapa da aclimatização que se viabiliza a metodologia de produção *in vitro*, pois é nela que se obtém o número de plântulas aptas ao plantio, ou seja, maior sobrevivência de mudas com qualidade (Correia et al., 2012). A condição de cultivo *in vitro* que estimula o comportamento autotrófico das plantas favorece sua aclimatização ao ambiente *ex vitro*. Entre as características cita-se, maior biomassa,

maior número de raízes adventícias, aumento de raízes secundárias, presença de pelos radiculares, estômatos funcionais, maior teor de clorofila, altas taxas fotossintéticas, incremento na espessura dos tecidos foliares, tecidos lignificados e maior depósito cutícula (Santana et al., 2008; Zhang et al., 2009; Xiao et al., 2011; Shin et al., 2013; Saldanha et al., 2014).

Nota-se os esforços dos pesquisadores em aprimorar a cultura de tecidos, em especial com o desenvolvimento de técnicas fotoautotróficas beneficiando o sistema de produção de mudas e contribuindo com informações úteis para o desenvolvimento de novos trabalhos. É um desafio o estabelecimento de protocolos de propagação de mudas em larga escala, e com sucesso na etapa final, que é aclimatização das plantas as condições *ex vit*ro. Assim, os trabalhos desenvolvidos nesta pesquisa serão de suma importância para área de cultura de tecidos, além de valorização e início de um processo de domesticação da espécie *M. elliptica* (Mart.).

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OBJETIVOS

Geral

Avaliar as características anatômicas, fisiológicas e de crescimento em plântulas de *Mouriri elliptica* (Mart.) sob condições fotomixotróficas de cultivo *in vitro*.

Específicos

- Estimular o comportamento autotrófico de plântulas de croada utilizando diferentes irradiâncias em combinação com meio de cultivo com e sem sacarose;
- Avaliar a dissimilaridade entre plantas de *M. elliptica* (Mart.) cultivadas *in vitro* e *in situ* a partir de parâmetros anatômicos com auxílio de técnicas de estatística multivariada.
- Verificar se o tipo de suporte mais poroso ou fibroso influencia no crescimento inicial de plântulas de croada, em especial na formação de raíz;
- Aclimatizar mudas de croada obtidas no processo de micropropagação fotoautotrófica ou fotomixotrófica.

CAPÍTULO I

(Normas de acordo com a revista Australian Journal of Croop Science. Artigo publicado em fevereiro de 2016, v. 10, n. 2, p. 229-236.

In vitro culture of *Mouriri elliptica* (Mart.) under conditions that stimulate photoautotrophic behavior

Abstract

Micropropagation has been efficiently used to mass-produce seedlings of species that are difficult to multiply via conventional methods. Thus, the present study aimed to analyze the *in vitro* culture of *Mouriri elliptica* (Mart.) seedlings under conditions that stimulate photoautotrophic behavior. Nodal segments were grown in 50% salt Wood Plant Medium in the absence and presence of sucrose and subjected to differents lights intensities (0, 50, 75, 100, and 150 µmol $m^{-2}s^{-1}$). Evaluations were performed after 60 days of culture, considering growth and morphoanatomic characteristics. There was an exponential increase in the number of shoots and leaves in seedlings cultured in the absence of sucrose with increasing light intensity. Additionally, greater total and leaf dry weights were recorded in seedlings cultured in sucrose-supplemented medium at an light intensity close to 100 µmol $m^{-2}s^{-1}$. Morphoanatomic changes were observed in leaves at differents lights intensities, both in the presence and absence of sucrose. As the light intensity increased, the supplementation of the medium with sucrose became unnecessary. Thus, photoautotrophic conditions can be used for micropropagation of the species.

Keywords: autotrophic micropropagation; "croada"; light intensity; morphoanatomy; stomatal crypt; sucrose.

1.1 Introduction

Mouriri elliptica (Mart.) belongs to the family Melastomataceae. It is a fruit tree that occurs naturally in several brazilian states, being very common in the Goiás Cerrado (savannah), and it has been classified as a non-traditional tropical fruit (Rufino et al., 2010). It is popularly known as "croada", "croadinha", "coroa de frade", "puçá", "puçazeiro", or "manipuçá". When ripe, its fruit are sweet and rich in antioxidant compounds such as vitamin C, anthocyanins, carotenoids and flavonoids and can be eaten raw by humans or processed into jellies (Silva et al., 2001; Rufino et al., 2010; Rufino et al., 2011).

The plant also has medicinal potential, the application of *M. pusa* and *M. elliptica* leaf extracts in rodents is an alternative treatment for acute ulcers, with these extracts exhibiting a gastroprotective effect and promoting healing. The extracts also have an anti-*Helicobacter pylori* effect, which is a microorganism that causes serious gastrointestinal diseases. These effects have been attributed to phenolic constituents, in the form of flavonoids and tannins identified in the plants' leaves (Moleiro et al., 2009; Vasconcelos et al., 2010b). Leaf extracts from this species show no toxicity in treated animals, which is an important factor in its pharmacological applicability (Moleiro et al., 2009).

There are currently no studies on "croada" micropropagation, despite the plant's various uses. It is known that its seeds have a rigid coat, hindering its sexual reproduction, as reported by Vasconcelos et al. (2010a), requiring the application of practices that promote overcoming dormancy. Therefore, propagation of this important species by seeds may not meet seedling demands. *In vitro* propagation provides a greater chance of producing seedlings that could be used for crops or reforestation.

Traditionally, explants are cultured in flasks that restrict gas exchange, with a high relative humidity, high ethylene concentration, low CO_2 concentration, low-density flow of photosynthetically active photons, and the use of sucrose as the main metabolic energy source. This system may cause anatomical and physiological disorders in the seedlings, hindering the normal function of the photosynthetic apparatus (Xiao et al., 2011), as observed in the *in vitro* culture of *Billbergia zebrina* (Herbert), in which the supply of sucrose reduces the quantity of photosynthetic pigments (Martins et al., 2015).

This is one of the features that may cause seedling losses during the acclimatization process, increasing production costs.

Thus, photoautotrophic micropropagation has been investigated using a number of different practices, such as total or partial elimination of sucrose from the culture medium (Xiao and Kozai, 2006), enrichment of atmospheric CO_2 (Saldanha et al., 2013; Saldanha et al., 2014), reduction of the relative humidity and ethylene concentration in the culture flask using seals that allow greater gas exchange (Saldanha et al., 2012), replacement of agar with alternative support materials such as Florialite® (Saldanha et al., 2014) or leaf litter and coconut fiber (Deb and Pongener, 2013), and increases in light intensity (Zhang et al., 2009; Sáez et al., 2012). These conditions can increase plant growth, improve physiological characteristics, and facilitate seedling acclimatization to *ex vitro* conditions by promoting the development of the photosynthetic apparatus (Walters, 2005; Santana et al., 2008; Iarema et al., 2012).

Anatomical and physiological evaluations and growth analysis can be performed to investigate autotrophic development, as observed in studies by Iarema et al. (2012), who evaluated the photoautotrophic propagation of *Pfaffia glomerata* (Spreng.) Sáez et al. (2012), in the culture of *Castanea sativa* Mill; Fan et al. (2013), in *Solanum lycopersicum* L.; and Dong et al. (2014), during *in vitro* culture of *Triticum aestivum* L.

Thus, the present study aimed to analyze the behavior of *Mouriri elliptica* (Mat.) seedlings subjected to an absence of sucrose in the culture medium and an increased light intensity in the environment by evaluating growth and morphoanatomic characteristics.

1.2 Results and discussion

The increase in light intensity eliminated the requirement for *M. elliptica* (Mart.) seedlings for sucrose in the culture medium

There was an interaction between lights intensities (0, 50, 75, 100, and 150 µmol $m^{-2}s^{-1}$) and sucrose levels regarding seedling length, the number of shoots and leaves, total dry weight and leaf dry weight. An isolated effect of the factors on the leaf area and specific leaf area was observed ($p \leq 0.05$).

Traditionally, in *in vitro* culturing, seedlings are kept in a growth room under low light intensity, and sucrose is used as the metabolic energy source for explants

(Zhang et al., 2009; Arigita et al., 2002). Fig 1 shows *Mouriri elliptica* (Mart.) seedling growth in culture medium supplemented with sucrose (A-E) and without sucrose (F-J).

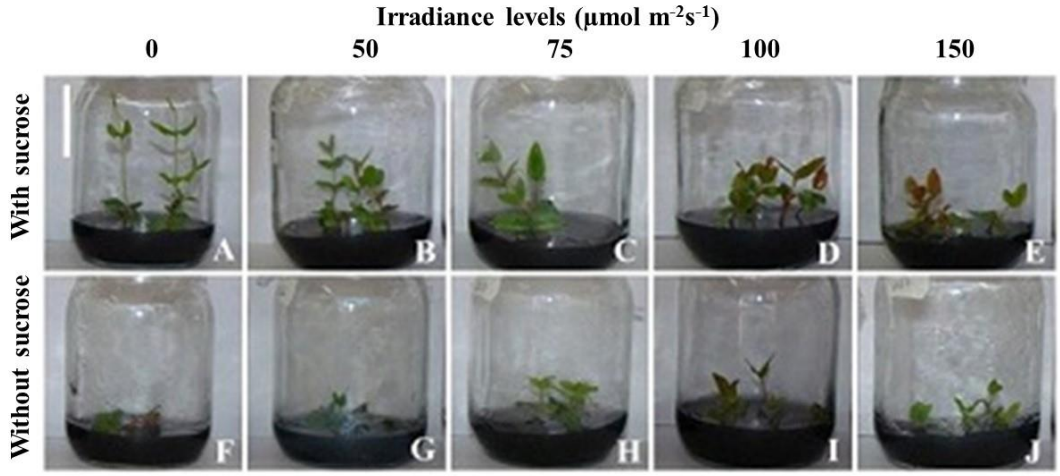

Figure 1. Growth of *Mouriri elliptica* (Mart.) seedlings in culture medium supplemented with sucrose and without sucrose at lights intensities diferentes. *In vitro* culture for 45 days. Scale bar = 2 cm.

A greater seedling length was observed in the absence of light and in the presence of sucrose (Figs 1 and 2), demonstrating etiolation characteristics, most likely due to the seedlings' sensitivity to endogenous auxin (George, 1993), considering that sucrose availability in the culture medium induced the *M. elliptica* (Mart.) seedlings to metabolize auxin, a result previously observed in *Arabidopsis* (Sairanen et al., 2012).

Etiolation of these seedlings *in vitro* can be advantageous to the multiplication process, allowing their nodal segments to be used as explants, as observed in a study by Suzuki et al. (2004), and to obtain new shoots of crop species, as in pineapple plants (Moreira et al., 2003). However, etiolation is a characteristic related to inefficiency of the photosynthetic apparatus (Solymosi and Schoefs, 2010) and susceptibility to photoinhibition (Long, 1994), which may compromise the acclimatization process.

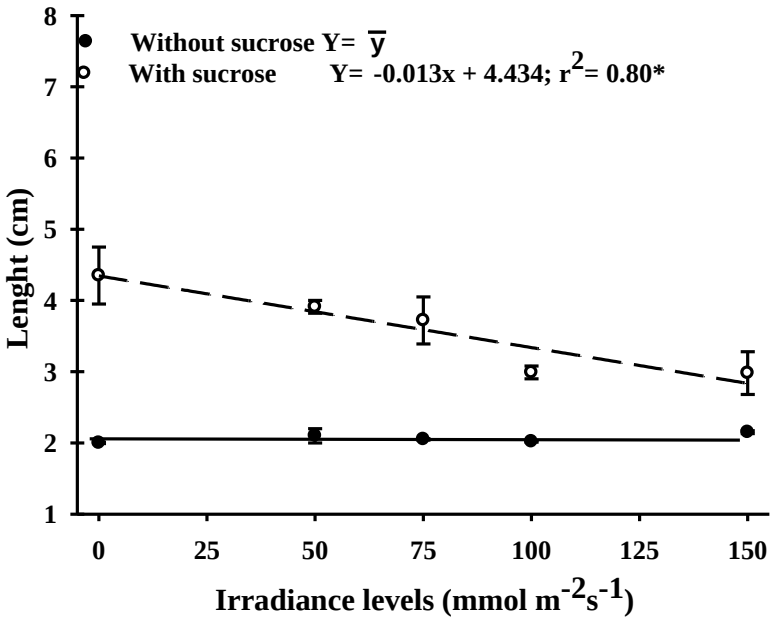

Figure 2. Length of *Mouriri elliptica* (Mart.) seedlings in culture medium with and without sucrose at lights intensities of 0, 50, 75, 100, and 150 µmol $m^{-2}s^{-1}$ for 45 days of *in vitro* culture. *$p < 0.05$.

The maximum number of leaves (3.8 leaves per plant) was obtained in seedlings cultured in medium with sucrose at a 67 µmol $m^{-2}s^{-1}$ light intensity (Fig 3a). The *M. elliptica* (Mart.) seedlings remained less dependent on high lights intensities when sucrose was supplemented in the culture medium, forming tissue even in the absence of light (Fig 1a). However, the highest accumulated total dry weight (40.36 mg) and leaf dry weight (26.67 mg) occurred when the seedlings were cultured at an approximately 100 µmol $m^{-2}s^{-1}$ light intensity (Fig 3c and 3d). These results corroborate those of Zhang et al. (2009), who observed higher fresh and dry weights of *Momordica grosvenori* plants under increased environment light intensity.

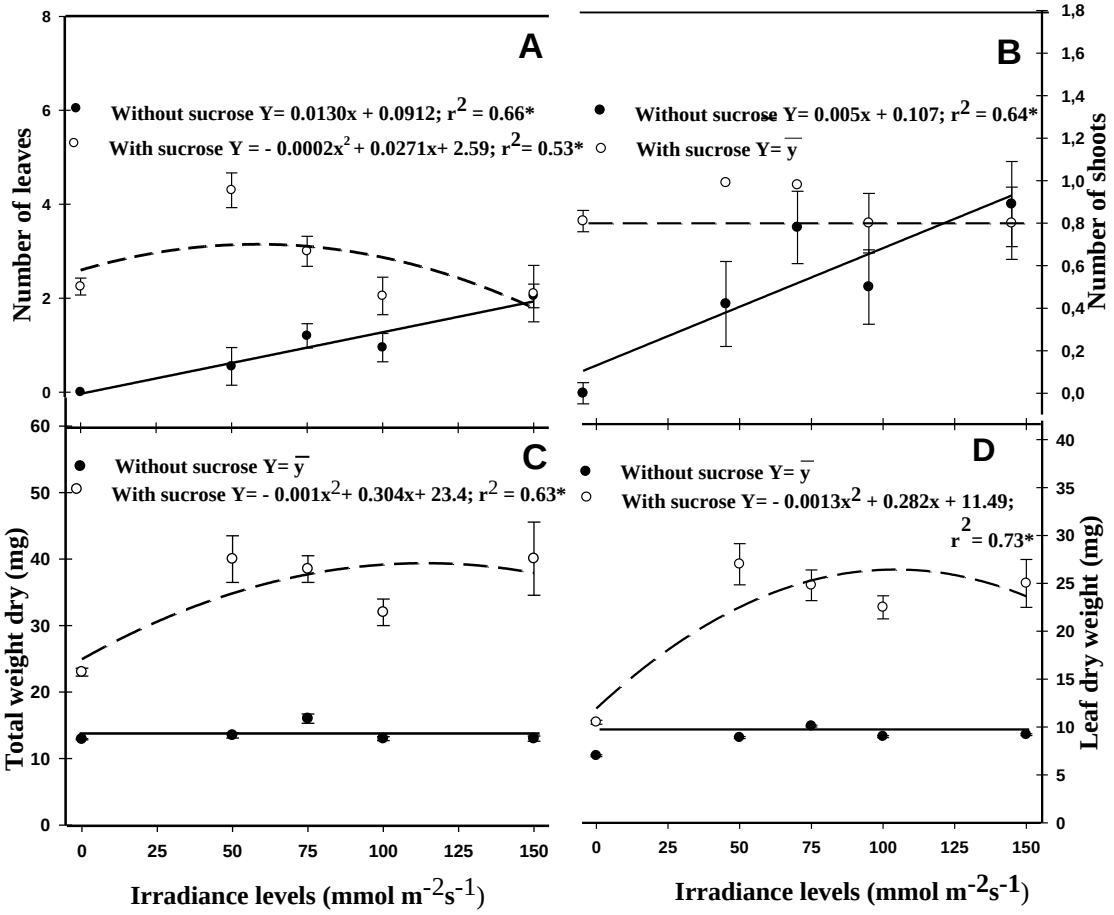

Figure 3. Number of leaves (A), number of shoots (B), total dry weight (C), and leaf dry weight (D) of *M. elliptica* (Mart.) seedlings cultured in medium with and without sucrose at lights intensities of 0, 50, 75, 100, and 150 µmol $m^{-2}s^{-1}$. *$p < 0.05$.

The number of leaves and shoots increased linearly in the seedlings cultured in medium without sucrose with an increasing light intensity (Figs 3a and 3b). There was no difference in these characteristics between culture medium with and without sucrose at light intensity of 100 and 150 µmol $m^{-2}s^{-1}$. This is an important observation for photoautotrophic culture, in which an increased light intensityin the culture environment suppressed the need to sucrose on *M. elliptica* (Mart.) seedling regeneration. According to Kozai and Nguyen (2003), light intensity must be increased to stimulate autotrophic behavior in seedlings *in vitro* using media sucrose-free. Light, as the primary energy source, is one of the most important environmental factors for growth, directly influencing the development of morphophysiological mechanisms for adaptation to light

variation (Li and Kubota, 2009), such as through altering leaf structure (Zhang et al., 2003).

Although the *M. elliptica* (Mart.) seedlings regenerated in the absence of sucrose, increasing light intensity did not affect the accumulated total and leaf dry weights (Figs 3c and 3d). Additionally, these characteristics presented lower values at all lights intensities compared with the seedlings cultured in medium with sucrose (Figs 3c and d). The seedlings cultured in medium without sucrose reached a mean total dry weight of 13.6 mg, which was 2.5 times lower than the mean for the seedlings cultured in the presence of sucrose. These results can be explained by the fact that these plants only photosynthetic system as a way to accumulate carbon, however, types of alternative seals that result in greater gas exchange were not evaluated in the present study. According to Iarema et al. (2012), seals with membranes that allow greater ventilation within a flask must be used when performing culture without sucrose supplementation. Greater ventilation within the *in vitro* culture flask allows a sufficient CO_2 concentration to ensure photosynthesis and seedling growth (Kitaya et al., 2005).

The plant leaf area is another characteristic related to the accumulated dry weight for this variable, greater investment (2.408 cm^2) was observed when the *M. elliptica* (Mart.) seedlings were cultured in the presence of sucrose, while a lower leaf area (1.67 cm^2) was observed in the absence of sucrose, regardless of the light intensity. This parameter is very important, as the leaf is responsible for the largest portion of carbohydrate production essential for plant growth and development (Marafon, 2012). This information corroborates the observed lower specific leaf area values associated with sucrose availability in the medium, representing a greater accumulated dry weight by area. A mean specific leaf area of 112.706 cm^2g^{-1} was obtained during culture in the presence of sucrose, while a value of 199.266 cm^2g^{-1} was recorded in the absence of sucrose.

Regarding the effect of lights intensities on the specific leaf area, the seedlings showed the highest value (182.1 cm^2g^{-1}) at absence light, and increasing light intensity induced a decrease in this parameter ($Y = -0.349x + 182.171$; $r^2 = 0.635$, $p < 5\%$). Low light levels can generally lead to an increased specific leaf area of the plant to intercept more radiation, reducing leaf thickness and, thus, the net assimilation rate (NAR), corresponding to an increased accumulated dry matter weight in the plant per available leaf area unit (Marafon, 2012). Steinger et al. (2003) considered this an adaptation to meet photosynthetic demands.

To test the maximum production of shoots and leaves and biomass accumulation in *M. elliptica* (Mart.) seedlings cultured in the absence of sucrose, the development of other studies involving a light supply above 150 µmol $m^{-2}s^{-1}$ will be necessary, or even the development of cultures that allow greater gas exchange between the culture environment and the external atmosphere (Iarema et al., 2012), combined with high lights intensities and alternative support for the culture medium (Saldanha et al., 2014). These conditions characterize the photoautotrophic system (Xiao et al., 2011).

Anatomical characteristics: *M. elliptica* (Mart.) exhibits leaf plasticity

Studies that demonstrate the effect of different lights intensities on the morphoanatomic characteristics of native Cerrado plants cultured *in vitro* are still scarce, especially when correlated with presence or absence of sucrose in the culture medium. There are no available studies that demonstrate such characteristics for *M. elliptica* (Mart.).

Morphoanatomic and physiological changes in the leaves are common plant adaptive responses to different environmental conditions (Pereira et al., 2013). The *M. elliptica* (Mart.) explants cultured in the absence of sucrose and light did not possess the ability to form tissues and did not regenerate new seedlings. Thus, the comparisons conducted in micromorphometric analyses of the leaves of seedlings cultured *in vitro* only correspond to lights intensities of 50, 75, 100, and 150 µmol $m^{-2}s^{-1}$, independently presence of sucrose in the medium.

There was a significant interaction between the light intensity and culture medium for the chlorophyll parenchyma thickness (CP T), stomatal crypt density (St Cr Dn), stomatal crypt depth (St Cr Dp), and stomatal crypt opening area (St Cr O). An isolated effect of these factors was observed for the adaxial epidermis thickness (Ad Ep T) and abaxial epidermis thickness (Ab Ep T) ($p \leq 0.05$).

It was noted the stomata presence in adaptive structures known as stomatal crypts (Figs 4a and 5 a-h), and they were only identified on the abaxial surface; therefore, the plants can be classified as hypostomatic. The adaptive significance of the stomatal crypts is still under discussion, and they probably evolved in response to several environmental factors, most likely as a resource for xerophilic plants to reduce water loss via reduced leaf transpiration (Hassiotou et al., 2009). This concept is reinforced by the frequency of trichomes that are generally identified in the crypts

(Rotondi et al., 2003; Jordan et al., 2008), which was not observed in the plants under study. Stomata positioned in crypts may be more protected environmental stressors than stomata located at the leaf surface (Haworth and McElwain, 2008). However, Roth-Nebelsick et al. (2009), who studied the functions of the stomatal crypts, concluded that future studies should focus on the effects on water vapor and CO_2 diffusion.

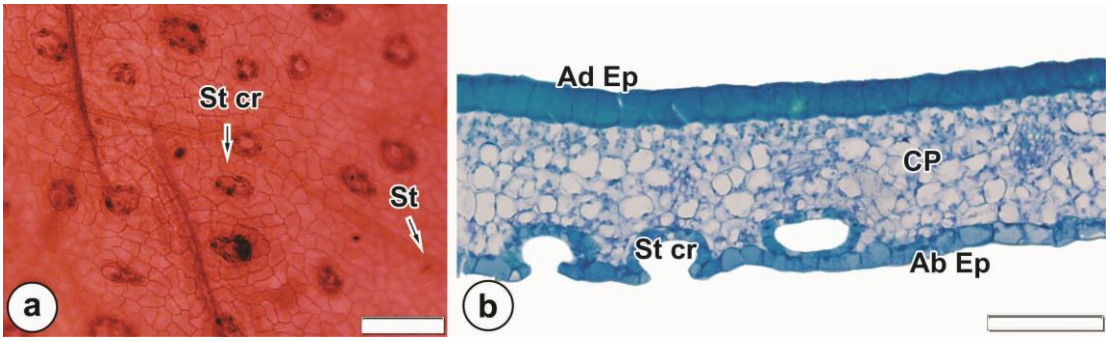

Figure 4. Photomicrographs of *Mouriri elliptica* (Mart.) leaves *in vitro* in the absence of light and the presence of sucrose. (a) A portion of the abaxial epidermis with stomatal crypts (St Cr) and outside the stomatal crypt, (b) cross-section of the blade's median region showing the cell arrangement in the adaxial epidermis (Ad Ep), chlorophyll parenchyma (CP), abaxial epidermis (Ab Ep), and stomatal crypt (St Cr). Scale bar = 100 µm

Stomatal crypts were also identified in leaves from seedlings cultured in the dark with sucrose as the metabolic energy source (Fig 4a). However, the density of 75.56 crypts/mm^2 observed under these conditions was lower than in seedlings cultured in light. A higher St Cr Dn may benefit the "croada" seedlings during the acclimatization process by providing greater control over gas exchange, enabling a reduction of water loss (Hassiotou et al., 2009).

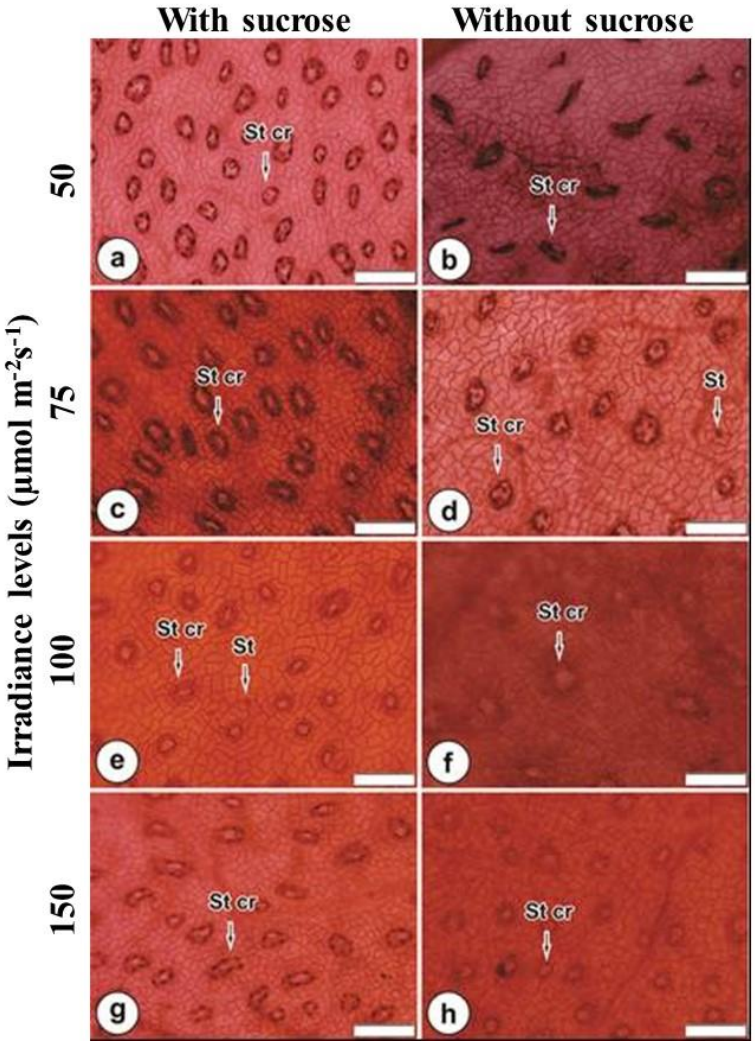

Figure 5. Photomicrographs of *Mouriri elliptica* (Mart.) leaves *in vitro*, showing the abaxial epidermis with stomatal crypts (St Cr). Scale bar = 100 µm.

The chlorophyll parenchyma modified its structural organization according to the environment, ranging from homogenous, as observed in the leaves of seedlings cultured in the dark (Fig 4b), to dorsiventrally heterogeneous, with palisade parenchyma (columnar cells) located under the adaxial epidermis and spongy parenchyma (irregular shaped cells) under the abaxial epidermis (Fig 6a-h), demonstrating great leaf plasticity for adaptation to different environmental conditions. When the palisade parenchyma is more developed, it facilitates the absorption of carbon dioxide (CO_2) into the mesophyll cells when they are directly exposed to light (Terashima et al., 2005). In addition, the palisade parenchyma can be responsible for reduced leaf heating, maintaining optimal temperatures for physiological processes (Taiz and Zeiger, 2009).

The chlorophyll parenchyma thickness was greater in the absence of sucrose at all tested lights intensities. However, a greater total dry weight and leaf dry weight was observed in seedlings cultured in the presence of sucrose; such results can be explained by the accumulation of polysaccharides as starch grains within the cells (Figs 6a, 6c, 6e and 6g), which was not observed in the leaf tissues of seedlings without sucrose (Fig 6b, 6d, 6f and 6h). Thus, supplying sucrose to the culture medium expanded the starch reserves of the micropropagated plants.

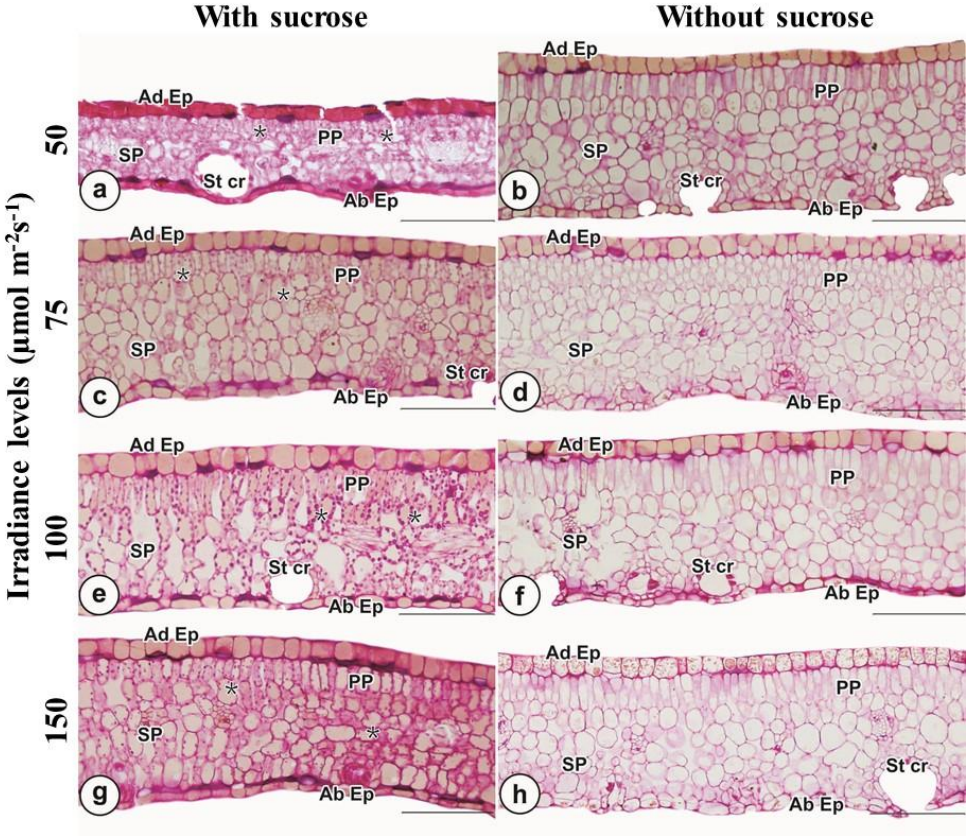

Figure 6. Photomicrographs of cross-sections of the median region of *M. elliptica* (Mart.) leaves *in vitro*, showing the cellular arrangement of the adaxial epidermis (Ad Ep), palisade parenchyma (PP), spongy parenchyma (SP), abaxial epidermis (Ab Ep), and stomatal crypts (St Cr). *Polysaccharides accumulated within the cells, tissue stained via the PAS method. Scale bar = 100 µm.

In cross-sections of the *M. elliptica* (Mat.) leaves, a square-to-rectangular uniseriate adaxial and abaxial epidermis was observed (Fig 6a-h). The adaxial epidermis thickness (Ad Ep T) remained unaffected by the differents lights intensities (Fig 7a);

these results corroborate those obtained by Espindola-Júnior et al. (2009) in a study on *Mikania glomerata* Spreng. plants subjected to different light conditions.

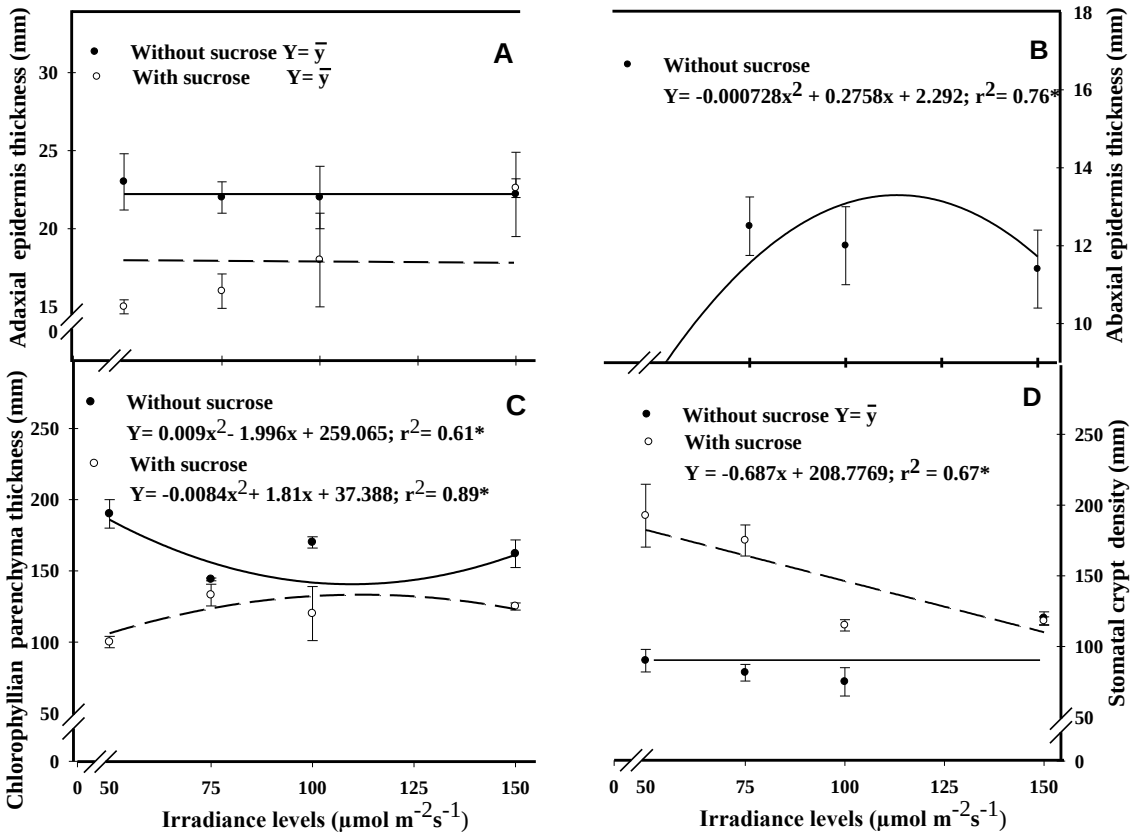

Figure 7. Adaxial epidermis thickness (A), abaxial epidermis thickness (B), chlorophyllian parenchyma thickness (C), and stomatal crypt density (D) of *M. elliptica* (Mart.) seedlings cultured in medium with and without sucrose at lights intensities 0, 50, 75, 100, and 150 µmol $m^{-2}s^{-1}$. *$p < 0.05$.

A difference in the Ad Ep thickness was only observed for the type of culture medium, with a value of 22.03 µm in cultures without sucrose and a mean thickness of 18.61 µm in supplemented medium. In a study by Santana et al. (2008) using a photoautotrophic stimulus culture system for *Annona glabra* L., a thicker epidermis formed on the adaxial surface compared with a heterotrophic culture system. These authors identified characteristics in the plants that developed in the photoautotrophic environment similar to the characteristics of plants grown *ex vitro*, which is considered an important factor in the acclimatization process.

The abaxial epidermis thickness (Ab Ep T) varied according to the environmental energy supply (Fig 7b). An light intensity of 120 µmol $m^{-2}s^{-1}$ induced a

greater Ab Ep thickness in the "croada" leaves, regardless of the presence or absence of sucrose in the medium. Epidermis thickness is related to greater lignin synthesis in this tissue and is directly conditioned to environmental light, as light interferes with enzymatic activities, promoting the formation of phenylalanine and tyrosine. The presence of enzymes in different tissues catalyzes the deamination of these substances for the synthesis of aromatic monomer units, which are precursors of lignin (Abreu, 1994).

At lights intensities of 75 and 150 µmol $m^{-2}s^{-1}$ in the absence of sucrose, the obtained St Cr O values were 560.38 and 340.25 µm, respectively, which were higher than the values observed in the presence of sucrose (244.276 and 175.095 µm, respectively). At lights intensities of 50 and 100 µmol $m^{-2}s^{-1}$, there was no difference in the St Cr O values recorded in the absence of sucrose (298.97 and 261.55 µm, respectively) and presence (296.31 and 213.92 µm, respectively) . The Stomatal crypt openings are can be observed in Fig 5.

Linear behavior ($Y = 29.364 + 0.1372x$; $r^2 = 0.908$, $p < 5\%$) was observed for St Cr Dp as a function of light levels in the absence of sucrose. Deeper stomatal crypts can facilitate CO_2 diffusion to assimilation sites (Roth-Nebelsick et al., 2009). None of the tested mathematical models fit the St Cr Dp data in the presence of sucrose.

1.3 Materials and methods

Obtaining plant material and *in vitro* establishment

Nodal segments (2 cm-long) with two axillary buds were removed from *Mouriri elliptica* (Mat.) seedlings that were obtained from seeds and emerged in trays with sand. After obtaining the segments, they were disinfected under running water with three drops of neutral detergent for 15 minutes and 30 seconds in 70% alcohol and 15 minutes in a 0.5% commercial sodium hypochlorite.

Following disinfection, the explants were inoculated in test tubes containing 20 mL of culture medium with only water and agar and were maintained in a growth room for 15 days at 25±2°C, under a photoperiod of 16/8 hours (light/dark), with light being provided by 40-Watt fluorescent lights. After this period, these explants were transferred to flasks containing 50 mL of Wood Plant Medium (WPM) (Lloyd and Mccown , 1981) with 50% salt and 2 g of activated charcoal and solidified with 3.5 gL^{-1}

of agar. The pH of the culture medium was adjusted to 5.7±0.03 prior to autoclaving at 121°C for 20 minutes. PVC film was used to seal the flasks after inoculation.

***In vitro* culture of nodal segments of *M. elliptica* (Mart.)**

Two types of medium were used, without and with 30 gL^{-1} of sucrose. To test the effect of lights intensities of 0, 50, 75, 100, and 150 µmol m^2s^{-1} in the *in vitro* culture of *Mouriri elliptica* (Mat.), the flasks were placed in a climatic chamber (Fitotron®) at 25°±2°C with 60% relative humidity. Light levels were adjusted using a QSO-S photosynthetically active radiation sensor (Decagon Devices, Pullman, WA, USA).

Growth evaluation

Evaluations were performed after 60 days of *in vitro* culture. The following parameters were evaluated: seedling length (cm), the number of shoots and leaves, total and leaf dry weights (mg), leaf area (cm^2), and the specific leaf area (cm^2g^{-1}). Leaf area was obtained through image integration using image analysis software (ImageJ®). Length measurements were obtained with a millimeter ruler. Total dry weight and leaf dry weight were determined on a digital analytical balance after drying the material in a forced air oven at 65ºC for 72 hours. The specific leaf area was obtained from the ratio between the leaf area (cm^2) and leaf dry weight (grams).

Anatomical characterization

For the anatomical analyses, leaf samples were fixed in Karnovsky solution (Karnovsky, 1965) for 48 hours, then dehydrated in an ascending ethanol series, pre-infiltrated, and infiltrated with historesin (Historesin Leica, Erviegas Ltda: São Paulo - SP, Brazil), according to the manufacturer's recommendations. After drying the blocks, the material was transversely sectioned into 5 µm-thick samples in a rotary microtome (RM 2155 model, Leica). The sectioned material was stained with 0.05% toluidine blue, pH 4.0 (O'Brien et al., 1965), to evaluate the epidermis thickness of both surfaces, chlorophyll parenchyma thickness and the depth of the stomatal crypts (St Cr Dp).

The periodic acid-Schiff (PAS) reaction was used to observe neutral polysaccharides. The PAS reaction was controlled through the acetylation of the material or via the omission of oxidation by periodic acid (McManus, 1948).

The diaphanization technique was used to determine the density of the stomatal crypts (St Cr Dn) of the leaf surface and the crypt opening area. For this purpose, leaf samples were immersed in 5% sodium hydroxide for 24 hours, then clarified with chloral hydrate (1.6:1, p/v) for 24 additional hours and stained with 1% safranin in 50% ethanol (Arnott, 1959).

Images were obtained under an optical microscope (BX61 model, Olympus) with the U-photo system in the Laboratory of Plant Anatomy (Laboratório de Anatomia Vegetal) of the Goiás Federal Institute of Education, Science, and Technology – Rio Verde Campus, Brazil.

Statistical analysis

The experiment was arranged in a completely randomized design (CRD) under a 2x5 factorial scheme, with two types of culture medium, with and without 30 gL^{-1} of sucrose, and five different lights intensities (0, 50, 75, 100, and 150 µmol $m^{-2}s^{-1}$), with four replicates and four explants per flask.

The data were subjected to analysis of variance (ANOVA) using the F test, with regression analysis at the 5% probability level for light intensity factors (5% probability).

1.4 Conclusion

It was possible to regenerate *Mouriri elliptica* (Mart.) seedlings in the absence of sucrose by providing a higher light intensity (at least 50 µmol $m^{-2}s^{1}$) to the culture environment. However, better seedling performance was obtained when sucrose was used as the metabolic energy source.

The species under study exhibits great leaf plasticity when cultured under photoautotrophic conditions. Thus, the plants show a great ability to adapt to environmental variation, especially regarding light.

1.5 Acknowledgements

The authors would like to thank the Laboratory of Plant Tissue Culture (Laboratório de cultura de tecidos vegetais) of the Goiás Federal Institute of Science Education and Technology Rio Verde Campus – Goiás, Brazil, for the infrastructure and the experimental material. The authors would also like to thank the Goiás Research Foundation (Fundação de Amparo à Pesquisa do Estado de Goiás – FAPEG) and the

Brazilian Federal Agency for the Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES) for financial support.

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CAPÍTULO II

(Normas de acordo com a revista Genetics and Molecular Research. Artigo publicado em outubro de 2016, v. 15, n. 4, p. 1-11)

Dissimilarity between plants of *Mouriri elliptica* (Mart.) cultivated *in vitro* and *in situ* through anatomic parameters

Abstract

The species *Mouriri elliptica* (Mart.) is a genetic resource of the Cerrado domain, as it has potential food and medicinal uses. There have been few studies on its *in vitro* propagation, and there are no studies examining the dissimilarities between plants of this species when cultivated *in situ* or *in vitro*. Therefore, the objective of this study was to identify *in vitro* cultivation conditions that allow the formation of plantlets with leaf anatomical features that are less dissimilar to plants *in situ*. Thus, an anatomical study of the leaves was conducted, in which, it is considered the adaxial epidermis thickness, the abaxial epidermis thickness, the chlorenchyma thickness, the stomatal crypt depth, the stomatal crypt density and the leaf surface stomatal crypt aperture area. The distance between phenotypes was determined based on micromorphometric data, and the UPGMA cluster was then determined. Four different groups were tested, and cultivation conditions in the presence of sucrose and irradiance of 50 and 75 μmol $m^{-2}s^{-1}$ were identified as promoters of plantlet development that maximized the similarity to *in situ* plant. The most important anatomical parameters in this identification were the stomatal crypt aperture area and crypt density. This study

holds great importance for the anatomical characterization of the leaves of *M. elliptica* (Mart.), as it identifies plasticity as a function of *in vitro* culture conditions.

Keywords: Leaf micromorphometrics; micropropagation; phenotype; plantlets; UPGMA clustering

2.1 Introduction

Mouriri elliptica (Mart.) (Melastomataceae family) is a native plant of the Cerrado domain and is a potential resource for the human population. It produces nutritious fruits that contain antioxidant compounds and that can be consumed *in natura* or processed into jam (Rufino et al., 2010; Rufino et al., 2011). Its leaves and/or bark have been used to treat gastric ulcers and gastritis, and they may have an antimicrobial effect. These medicinal effects stem from immune system stimulation against pathogens (Moleiro et al., 2009; Vasconcelos et al., 2010) and may also be due to inhibition of the oxidative capacity of *Helicobacter pylori* (Bonacorsi et al., 2013).

The sexed reproduction of the species *M. elliptica* (Mart.) is very complex, due in part to the presence in the seeds of a hard tegument that renders germination difficult, as reported by Vasconcelos et al. (2010) and Lima et al. (2016). Thus, it is necessary to utilize practices that allow dormancy to be overcome or that obtain seedlings through vegetative propagation. Plant tissue culture has been an indispensable tool for obtaining seedlings of various native and cultivated species, such as *Anacardium othonianum* Rizz (Assis et al., 2015), *Byrsonima cydoniifolia* A. Juss. (Martendal et al., 2013 and 2014) and *Pfaffia glomerata* (Spreng.) Pedersen (Saldanha et al., 2014) and banana (*Musa spp* AAA) plantlets (Kaçar et al., 2010).

When cultivating *in vitro*, it is important to obtain plantlets that have characteristics akin to plants grown *in situ*. I*n vitro* cultivation conditions normally permit rapid plant growth and multiplication but may induce structural and physiological changes that render plants unfit to survive adverse environmental conditions (Rout et al., 2006). Thus, photoautotrophic micropropagation has been investigated, in which the absence of sucrose in the growth medium and increased irradiance from the environment are used to promote the formation of plantlets with characteristics that benefit their survival when they undergo the acclimatization process (Xiao and Kozai, 2006; Sáez et al., 2012; Iarema et al., 2012; Assis et al., 2016).

To determine whether plantlets that are micropropagated in a photoautotrophic system develop morphophysiological characteristics that are similar to those of *in situ* plants, the use of multivariate techniques is appropriate. According to Cruz (2011), multivariate analysis has diverse applications, meeting the needs of investigators with wide ranges of interests and knowledge. The use of the technique is common in plant breeding programs (Silva et al., 2015) as a way to evaluate genetic diversity (Assis et al., 2013) or superior genotypes (Oda et al., 2015) and to conduct environmental studies (Leo et al., 2015; Ma et al., 2016) in which the technique has been successful and has clarified data.

Knows of no studies to date that used multivariate analysis for the comparative evaluation of plants *M. elliptica* (Mart.) cultivated *in vitro* and *in situ* based on anatomical features. Anatomical parameters can support future studies with morphogenetic characterization of populations of this species, and genetic diversity studies, and, based on plant breeding programs. Thus, this study aimed to use multivariate statistical techniques to evaluate the dissimilarity between plants cultivated *in vitro* and *in situ* based on anatomical parameters.

2.2 Material and methods

Plant material and *in vitro* cultivation conditions

The study material consisted of leaves from mature plants of *M. elliptica* (Mart.) under natural conditions (*in situ*) and leaves of plantlets cultivated *in vitro*. Both the leaves of the mature plants and the seeds for the plantlets were collected on a private property located in the Planalto Verde District, municipality of Montividiu, Goiás, Brazil (17° 19.201’ S, 51° 33.500’ W and 982 m altitude).

Before implementing the experiment, 2-cm-long nodal segments with 2 axillary buds each were inoculated in test tubes containing 20 mL of growth medium composed of water and agar. The tubes were then kept in a growth room for 15 days at a temperature of 25° ± 2°C and with a photoperiod of 16/8 h (light/dark) under irradiance from 40-W fluorescent lamps. After that period, the explants were transferred to vials containing 50 mL of WPM (wood plant medium) (Lloyd and McCown, 1981) with 50% salts and 2.5 g of added activated carbon that was solidified with 3.5 g L^{-1} agar and either contained or lacked 30 g L^{-1} sucrose. The pH of the growth medium was adjusted to 5.7 ± 0.03 before autoclaving at 121°C for 20 minutes. The vials were sealed after inoculation with PVC plastic film.

The experiment was organized with a completely randomized design (CRD) in a growth chamber (Fitotron®) at 25° ± 2°C and with a relative humidity of 60%. Irradiances of 0, 50, 75, 100 and 150 µmol $m^{-2}s^{-1}$ were evaluated and were adjusted based on periodic tests with the aid of a QSO-S photosynthetically active radiation sensor (Decagon Devices, Pullman, WA, USA).

As no plantlets were formed in the absence of both sucrose and light, 9 *in vitro* cultivation conditions were used for the study, as follows: C1, presence of sucrose and zero irradiance; C2, presence of sucrose and 50 µmol $m^{-2}s^{-1}$; C3, presence of sucrose and 75 µmol $m^{-2}s^{-1}$; C4, presence of sucrose and 100 µmol $m^{-2}s^{-1}$; C5, presence of sucrose and 150 µmol $m^{-2}s^{-1}$; C6, absence of sucrose and 50 µmol $m^{-2}s^{-1}$; C7, absence of sucrose and 75 µmol $m^{-2}s^{-1}$; C8, absence of sucrose and 100 µmol $m^{-2}s^{-1}$; and C9, absence of sucrose and 150 µmol $m^{-2}s^{-1}$. These conditions were compared and correlated with each other and with *in situ* plants (CO).

Anatomical study of *M. elliptica* (Mart.) leaves

The leaves of *M. elliptica* (Mart.) were subjected to 2 analytical processes, namely fixation and diaphonization. For fixation, leaves were submerged in Karnovsky's solution (Karnovsky, 1965) for 48 hours, dehydrated in a graded ethanol series, and pre-infiltrated and infiltrated with Historesin (Leica) according to the manufacturer's instructions. The material was sectioned into 5-µm sections with a rotary microtome (RM 2155, Leica). The sections were stained with 0.05% toluidine blue at pH 4.0 (O'Brien et al., 1965).

For diaphonization, leaf samples were immersed in a 5% sodium hydroxide solution for 24 hours, clearified with chloral hydrate (1.6:1, p/v) for 24 additional hours and stained with 1% safranin in 50% ethanol (Arnott, 1959). After these procedures, the slides with the material were covered with a cover slip using Canada balsam.

Images were obtained under an optical microscope (BX61, Olympus) with a U-photo system at the Plant Anatomy Laboratory of the Federal Institute of Education, Science and Technology of Goias – Rio Verde Campus. The adaxial epidermis thickness (Ad Ep T), the abaxial epidermis thickness (Ab Ep T), the chlorenchyma thickness (Ch T), the stomatal crypt depth (St Cr Dp), the stomatal crypt density (St Cr D) and the leaf surface stomatal crypt aperture area (St Cr A) were evaluated.

Four fully formed leaves were extracted from three randomly chosen plants grown *in situ* or for each cultivation *in vitro* condition. For each feature in the study, was evaluated 10 images per repetition (leaf tissue), totaling 40 measurements by plant.

Statistical analysis

To access dissimilarity between plants grown *in situ* and *in vitro*, the data were subjected to a analysis of variance (ANOVA) by F testing at a 5% probability. Based on the ANOVA, the variance matrix and residual covariance were obtained. The dissimilarity matrix between the plant growth conditions was then determined by the generalized Mahalanobis distance (D^2), while the relative contribution of the micromorphometric features (S.j) was obtained according to Singh (1981), by software GENES (Cruz, 2013). Subsequent clustering was conducted by the average linkage between groups (Unweighted pair groups mean arithmetic, UPGMA) using the cluster package in R (Maechler, 2010).

To assess the clustering accuracy, the cophenetic correlation coefficient (CCC) was calculated, which was obtained with 1000 simulations with the help of the GENES software (Cruz, 2013). A descriptive analysis of the anatomical features was also conducted.

2.3 Results

Analysis of dissimilarity between *M. elliptica* (Mart.) plants *in situ* and *in vitro*

ANOVA results are presented in Table 1. Difference were observed between *M. elliptica* (Mart.) plants cultivated *in vitro* and *in situ* condition for all traits investigated in this study. The coefficient of variation (CV) was between 7.39 and 24.26%, demonstrating good experimental consistency.

Table 1 - Summary of the analysis of variance informing the mean square, mean and coefficient of variation (CV) of the anatomical features.

FV	d.f.	Mean square					
		Ad Ep	Ch T	Ab Ep T	St Cr Dp	St Cr A	St Cr Dp
Tr	9	23,39*	5862,03**	10,55**	185,59**	37055,13**	6041,56**
Re	20	7,86	206,84	1,83	9,42	4738, 05	407,31
Mean		19,65	141,82	11,22	41,49	283,63	118,79
CV (%)		14,27	10,14	12,05	7,39	24,26	16,98

Adaxial epidermis thickness (Ad Ep T - µm), chlorenchyma thickness (Ch T - µm), abaxial epidermis thickness (Ab Ep T - µm), stomatal crypt depth (St Cr Dp - µm), stomatal crypt aperture area (St Cr A - µm) and stomatal crypt density (St Cr D crypts/mm^2), were evaluated in leaves of *Mouriri elliptica* (Mart.).**And* significance of 0.01 and 0.05, respectively, by test F.

Estimates of the phenotypic correlations (r_p) among the 6 micromorphometric features leaves are shown in Table 2. A stronger but negative correlation (-0.73) was identified between the characteristics Ad Ep T and St Cr D; therefore, cultivation conditions that provide higher St Cr D tend to form plantlets with thinner Ad Ep. A positive correlation (0.52) was observed between the parameters stomatal crypt depth and stomatal crypt density. Based on the positive correlation of 0.47, plantlets with higher chlorenchyma thickness tended to develop deeper stomatal crypts, representing the acclimatization of the plantlet to the cultivation condition.

Table 2 - Phenotypic correlation coefficients (rp) between micromorphometric features.

Features	Ad Ep T	Ch T	Ab Ep T	St Cr Dp	St Cr A	St Cr D
Ad Ep T	1					
Ch T	0.267	1				
Ab Ep T	0.052	-0.340	1			
St Cr Dp	0.072	0.478	0.354	1		
St Cr A	0.330	-0.046	-0.233	-0.053	1	
St Cr D	-0.737	0.209	0.102	0.521	-0.415	1

Adaxial epidermis thickness (Ad Ep T), abaxial epidermis thickness (Ab Ep T), chlorenchyma thickness (Ch T), stomatal crypt density (St Cr D), stomatal crypt depth (St Cr Dp) and stomatal crypt aperture area (St Cr A) were evaluated in leaves of *Mouriri elliptica* (Mart.).

The dissimilarities between *M. elliptica* (Mart.) plants in each of the 10 cultivation conditions ranged from 0.2 to 2.7 (Table 3). Plantlets micropropagated under

conditions C2 (presence of sucrose and irradiance of 50 µmol $m^{-2}s^{-1}$) and C3 (presence of sucrose and irradiance of 75 µmol $m^{-2}s^{-1}$) were less dissimilar to *in situ* plants, as the observed dissimilarities were 0.7 and 0.6, respectively. A higher dissimilarity (2.7) was observed between *in situ* plants and C1 conditions (presence of sucrose and absence of light) or C7 conditions (absence of sucrose and irradiance of 75 µmol $m^{-2}s^{-1}$). A lower dissimilarity (0.2) was observed between the *in vitro* cultivation conditions C4 (presence of sucrose and irradiance of 100 µmol $m^{-2}s^{-1}$) and C5 (presence of sucrose and irradiance of 150 µmol $m^{-2}s^{-1}$).

Table 3 - Dissimilarity matrix obtained by the generalized Mahalanobis distance (D2) between *M. elliptica* (Mart.) plantlets under different cultivation conditions *in vitro* and *in situ*.

Environment	CO	C1	C2	C3	C4	C5	C6	C7	C8	C9
CO	0									
C1	2.7	0								
C2	0.7	1.3	0							
C3	0.6	2.1	0.6	0						
C4	1.3	0.8	1.1	0.7	0					
C5	1.8	1.7	1.9	0.9	0.2	0				
C6	1.9	1.5	1.9	2.4	0.9	1.6	0			
C7	2.7	1.7	2.2	2.1	1.03	1.3	0.9	0		
C8	1.7	1.2	1.7	1.6	0.4	0.6	0.3	0.6	0	
C9	1.2	1.8	1.3	1.1	0.5	0.7	0.6	0.5	0.3	0

CO, *in situ*; C1, presence of sucrose and zero irradiance; C2, presence of sucrose and 50 µmol $m^{-2}s^{-1}$; C3, presence of sucrose and 75 µmol $m^{-2}s^{-1}$; C4, presence of sucrose and 100 µmol $m^{-2}s^{-1}$; C5, presence of sucrose and 150 µmol $m^{-2}s^{-1}$; C6, absence of sucrose and 50 µmol $m^{-2}s^{-1}$; C7, absence of sucrose and 75 µmol $m^{-2}s^{-1}$; C8, absence of sucrose and 100 µmol $m^{-2}s^{-1}$; and C9, absence of sucrose and 150 µmol $m^{-2}s^{-1}$ of irradiance.

Based on the dissimilarity matrix among the 10 *M. elliptica* (Mart.) cultivation conditions, it was possible to identify UPGM clustering. The CCC was 0.75, demonstrating agreement between the original dissimilarity values and those represented by the dendrogram. The cultivation conditions caused leaf anatomical changes that were responsible for the discrepancies among the test plants. Thus, 4

distinct groups of plants were identified, with a dendrogram cut of approximately 50% (Figure 1).

The plantlets that were micropropagated under photoautotrophic growth conditions grouped into the same cluster (1), which is consistent with the plantlet responses, as they were grown in the absence of sucrose (Figure 1). Phenotypic characteristics more similar to *in situ* plants developed in plantlets grown under the photo-mixotrophic conditions C2 (presence of sucrose and irradiance of 50 µmol $m^{-2}s^{-1}$) and C3 (presence of sucrose and irradiance of 75 µmol $m^{-2}s^{-1}$) (Figure 1), with the latter being the most similar to *in situ* plants.

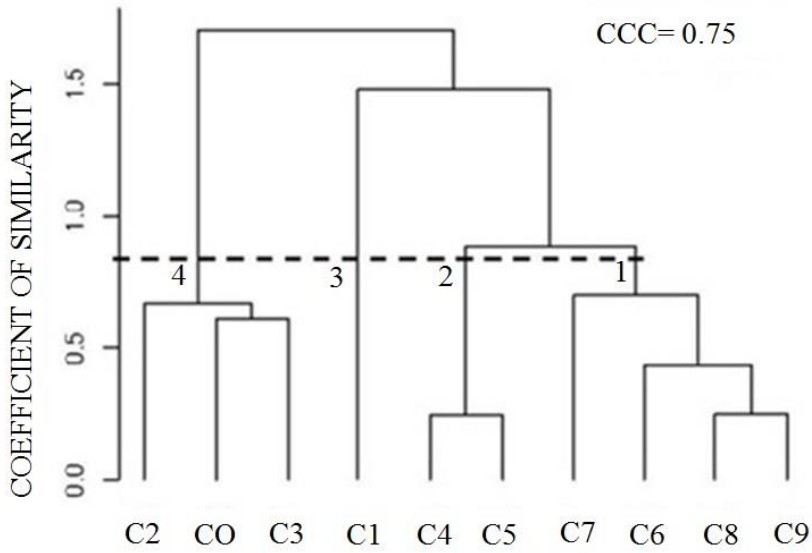

Figure 1: UPGMA clustering of the 10 phenotypes of *Mouriri elliptica* (Mart.). Dashed line: dendrogram cut indicating approximately 50% dissimilarity. CCC, cophenetic correlation coefficient; CO, *in situ* plantlets; C1 to C9, plantlets grown *in vitro*, as follows: C1, presence of sucrose and zero irradiance; C2, presence of sucrose and 50 µmol $m^{-2}s^{-1}$; C3, presence of sucrose and 75 µmol $m^{-2}s^{-1}$; C4, presence of sucrose and 100 µmol $m^{-2}s^{-1}$; C5, presence of sucrose and 150 µmol $m^{-2}s^{-1}$; C6, absence of sucrose and 50 µmol $m^{-2}s^{-1}$; C7, absence of sucrose and 75 µmol $m^{-2}s^{-1}$; C8, absence of sucrose and 100 µmol $m^{-2}s^{-1}$; and C9, absence of sucrose and 150 µmol $m^{-2}s^{-1}$ of irradiance.

Plantlets grown in the presence of sucrose but without light (C1) showed greater differences from the others in their anatomical features, and they thus formed their own group (3). Irradiance at 100 and 150 µmol $m^{-2}s^{-1}$ resulted in the development

of plantlets with very similar anatomical features, both in the presence (C4 and C5) and absence of sucrose (C8 and C9) (Figure 1).

The relative importance of each assessed anatomical feature is shown in Table 4. The feature stomatal crypt aperture area was identified as being most important in the cluster study of *M. elliptica* (Mart.) plants *in situ* and under different *in vitro* cultivation conditions, with a contribution of 75.34%. The features stomatal crypt density and chlorenchyma thickness also contributed 12.28% and 11.92% of the clustering, respectively. The features adaxial epidermis thickness, abaxial epidermis thickness and stomatal crypt depth were less important, with clustering contributions of 0.05%, 0.02% and 0.37%, respectively.

Table 4 - Relative importance (S.j) of micromorphometric features in the divergence study of *M. elliptica* (Mart.) plants grown in situ and plantlets subjected to different *in vitro* cultivation conditions.

Parameters	S.j	S.j (%)
St Cr A (µm)	1,111,653.92	75.35
St Cr D (mm^2)	181,247.03	12.28
Ch T (µm)	175,860.93	11.92
St Cr Dp (µm)	5,567.96	0.38
Ad Ep T (µm)	701.93	0.05
Ab Ep T (µm)	316.70	0.02

Ad Ep T, adaxial epidermis thickness; Ab Ep T, abaxial epidermis thickness; Ch T, chlorenchyma thickness; St Cr D, stomatal crypt density; St Cr Dp, stomatal crypt depth; and St Cr A, crypt aperture area.

Anatomic descriptions of *M. elliptica* (Mart.) leaves *in situ* and *in vitro*

The leaves of *M. elliptica* (Mart.), both *in situ* and *in vitro*, have their stomata allocated into stomatal chambers called stomatal crypts (Figure 2a). Stomatal crypts were observed only on the abaxial surface of the leaves, classifying them as hypostomatic. The adaxial epidermal cells of the leaves developed an overlapping tetrahedral shape, regardless of the plant growth condition (Figure 2b).

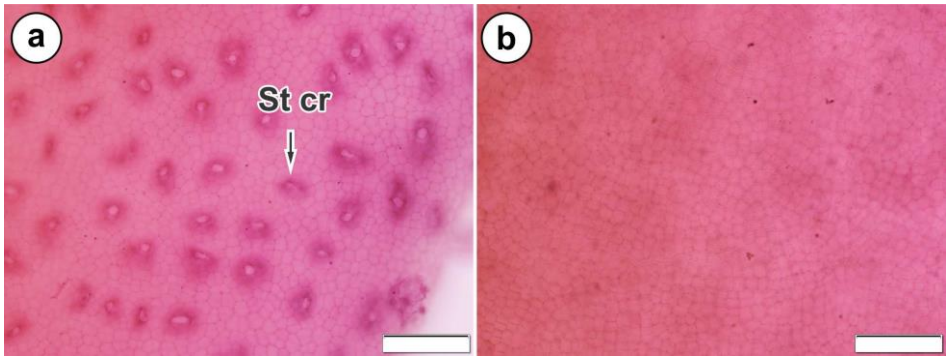

Figure 2. Photomicrographs of an *Mouriri elliptica* (Mart.) leaf from a plant grown *in situ*. Abaxial epidermis with stomatal crypts (St Cr) (a) and adaxial epidermis (b). Scale bar = 100 µm.

Plasticity was observed in the development of the chlorenchyma. *In situ* plants (CO) developed isobilateral chlorenchyma, with layers of palisade cells facing both the adaxial surface and the abaxial surface and with spongy parenchyma between the 2 regions of palisade cells (Figure 3a). In plantlets grown in the presence of sucrose but without light (C1), chlorenchyma stratification was not observed; instead, the chlorenchyma was homogeneous (Figure 3b). In the leaves of *in situ* plants, there were epidermal cells on the adaxial and abaxial surfaces that contained mucilage, which was colored purple by toluidine blue staining of the leaf tissue; however, this feature was not observed in the leaves of plantlets grown under condition C1 (Figure 3a and b).

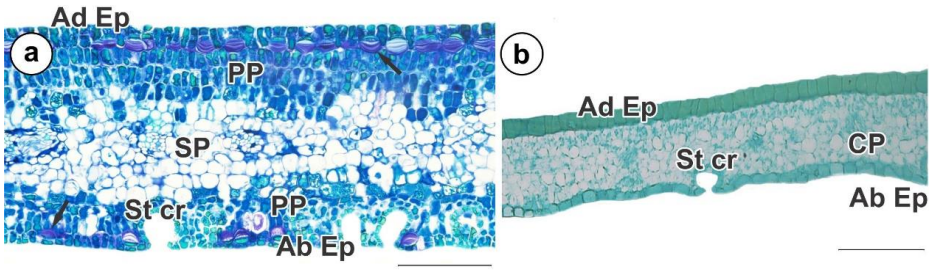

Figure 3. Cross sections of the middle region of the leaves *Mouriri elliptica* (Mart.) *in situ* (a) and *in vitro* in the presence of sucrose and the absence of light (b). Toluidine blue was used to stain the tissue. Ad Ep, adaxial epidermis; Ab Ep, abaxial epidermis; PP, palisade parenchyma; SP, spongy parenchyma; St Cr, stomatal crypt; and CP, chlorenchyma. The arrows indicate cells containing mucilage. Scale bars = 100 µm.

In plantlets grown under *in vitro* conditions (in the presence or absence of sucrose in the growth medium) with irradiance starting at 50 µmol $m^{-2}s^{-1}$, dorsiventral

chlorenchyma was observed, with 2-3 layers of palisade parenchyma cells facing the adaxial surface (Figure 4a - h). Spongy parenchyma, with more space between cells, was observed in the leaves of plants grown in the presence of sucrose, irrespective of the light intensity (Figure 4 a, c, e, g). Epidermal cells with mucilage content were also observed in *in vitro* cultivation conditions with irradiance above 50 µmol $m^{-2}s^{-1}$; however, toluidine blue dye only lightly stained these cells, possibly due to reduced mucilage accumulation (Figure 4a - h).

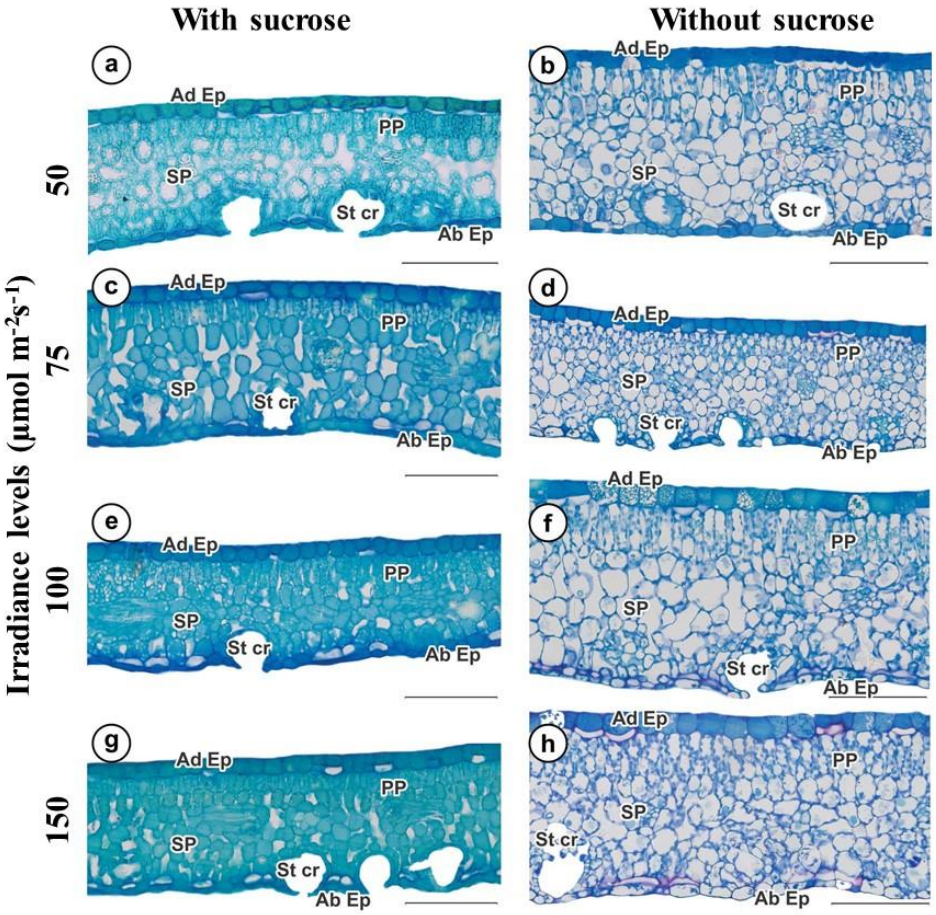

Figure 4: Cross sections of the middle region of the leaves *Mouriri elliptica* (Mart.). Ad Ep, adaxial epidermis; Ab Ep, abaxial epidermis; PP, palisade parenchyma; SP, spongy parenchyma; and St Cr, stomatal crypt. Scale bars = 100 µm.

2.4 Discussion

Anatomical plasticity between *M. elliptica* (Mart.) plantlets grown *in vitro* and *in situ* plants generates 4 distinct groups after UPGMA clustering

Based on the phenotypic variations in the micromorphometric data from *M. elliptica* (Mart.) leaves grown *in situ* and *in vitro*, it was possible to estimate the dissimilarity between them. According to Cruz et al. (2011), phenotypic characteristics typically show a continuous distribution and are determined by many genes with small individual contributions while being influenced by the environment. Thus, it was possible to determine which *in vitro* cultivation conditions resulted in leaf development with anatomical characteristics that were less dissimilar to the *in situ* plants.

The leaf anatomic characteristics that developed on the *in vitro* plantlets are important for adaptation to growth conditions, as they influence physiological processes, especially the ability to perform photosynthesis. In plantlets grown in the presence of sucrose but without light (C1), there was no stratification of the chlorenchyma; instead, it was homogeneous, thus demonstrating little tissue differentiation. Light intensities greater than 50 μmol $m^{-2}s^{-1}$ (C2 to C9) facilitated better leaf development, with stratification of the chlorenchyma into palisade and spongy zones of the dorsiventral type. The anatomical plasticity observed for leaves of *M. elliptica* (Mart.) due to cultivation conditions represents the acclimatization capacity of the species.

The presence of stomatal crypts is an important feature of *in situ* plants that was also observed for *in vitro* plants. Stomatal crypts are considered as features that characterize the species in their natural habitat; many species are found in arid environments (Jordan et al., 2008). Crypts favor the development of plants in such environments, as they restrict transpiration, reducing water loss and promoting gas exchange at appropriate times (Hassiotou et al., 2009).

The anatomical parameters St Cr A and St Cr D together accounted for 87.63% of the relative importance (S.j) in the UPGMA cluster. Therefore, these characteristics were considered as key factors for the dissimilarity study between *M. elliptica* (Mart.) plants cultivated *in situ* and *in vitro*. Four different groups were obtained with UPGMA clustering, based on a dendrogram cut indicating approximately 50% dissimilarity. The CCC was 0.75, allowing us to infer that the clustering was consistent. Silva and Dias (2013) consider the assessment of cluster consistency by the CCC very important so that the conclusions on similarities between individuals may be considered trustworthy.

According to Cruz and Carneiro (2006), a higher CCC value corresponds to a lower clustering distortion.

None of the *in vitro* conditions used in this study permitted the formation of plantlets with anatomical features that were similar to the *in situ* plants, but the C2 and C3 conditions, both of which were photomixotrophic, formed plantlets that were less dissimilar to *in situ* plants. This information suggests that these plants have the highest chances of survival when subjected to *ex vitro* conditions.

The first study on micropropagation of the species *M. elliptica* (Mart.) revealed the regeneration ability of plantlets under photoautotrophic conditions and with irradiance above 50 µmol $m^{-2}s^{-1}$ (Assis et al., 2016). In the present study, the clustering analysis between *in situ* and *in vitro* plantlets based on leaf anatomical features showed that plantlets cultivated under a photoautotrophic system were more dissimilar to *in situ* plantlets. However, more studies on micropropagation of the species should be conducted in which plants are taken to the acclimatization stage to ensure their survivability, as observed in studies of Corrêa et al. (2015) on the interactions between genotypes of *P. glomerata* (Spreng.) in the photoautotrophic culture and studies by Rodrigues et al. (2015) with *Etlingera elatior* (Jack) rm smith (torch ginger).

Several studies on plant micropropagation have been developed to obtain plantlets with anatomical and physiological characteristics that increase their ability to survive the acclimatization process, as it is a stressful stage for the plant. Among the successfully developed studies cited with native plants *Byrsonima cydoniifolia* A. Juss. (Martendal et al., 2014), *Billbergia zebrine* (Martins et al., 2015), and cultivated plants of commercial importance as *Carica papaya* L. var. Red and Maradol (Pérez et al., 2015).

2.4 Conclusion

The anatomical characteristics studied in the leaves of *M. elliptica* (Mart.) supported a dissimilarity study between plants grown *in situ* and those cultivated *in vitro* under photomixotrophic and photoautotrophic conditions. UPGMA clustering was used to determine that *in vitro* cultivation conditions in the presence of sucrose and irradiances of 50 and 75 µmol $m^{-2}s^{-1}$ supported the growth of plantlets with leaf anatomical features that were less dissimilar to *in situ* plants that were placed in the same group.

2.5 Conflicts of interest

The authors declare no conflict of interest.

2.6 Acknowledgments

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CAPÍTULO III

(Normas de acordo com a revista Cerne, em processo de revisão)

Alternative support materials to agar in the *in vit*ro cultivation of *Mouriri elliptica* (Mart.)

Abstract

Alternative supports can be successfully used in place of agar for *in vitro* culture to increase seedling quality and subsidize root formation. The objective of the present study was to evaluate the efficiency of alternative support materials compared to agar in the *in vitro* cultivation of *Mouriri elliptica* (Mart.) in the absence or presence of Naphthalene Acetic Acid (NAA). Nodal segments were grown in 50% salt Wood Plant Medium, with 30 gL^{-1} of sucrose and 2,5 gL^{-1} of activated charcoal. The alternative support materials used were medium-granulometry vermiculite, sugarcane (*Saccharum spp.* L.) bagasse and queen palm fiber [*Syagrus romanzoffiana* (Chamisso) Glassman] in compared to culture medium solidified with agar. No differences were observed between agar, vermiculite and sugarcane bagasse cultures for growth characteristics number of nodal segments, number of leaves and total dry mass. Greater numbers of adventitious and secondary roots and greater root length were observed in plantlets grown in the cultivation of vermiculite and the absence of NAA. In the agar culture, roots had weak points and poorly differentiated tissues, with parenchymal tissue predominating. The concentration of 2.0 mg L^{-1} NAA used this study did not stimulate rooting of *M. elliptica* (Mart.) plantlets. It was possible to regenerate plantlets in both support materials used, with vermiculite and sugarcane bagasse representing promising agar substitutes to obtain seedlings with roots.

Keywords: Anatomical characteristics, croada, melastomataceae, micropropagation, rooting.

3.1 Introduction

Mouriri elliptica (Mart.) (Melastomataceae) is a tree typical of the Cerrado domain (Brazil) and is popularly known as coroa de frade (friar's crown), croada or croadinha and puçá ou puçazeiro. In addition to its importance to the forest, its fruits rich in nutrient and antioxidant compounds have been recommended for human consumption (RUFINO et al., 2011). Its trunk and leaves can be used medicinally to treat gastric ulcers and gastritis (MOLEIRO et al., 2009). These characteristics demonstrate the economic potential of the species, however, it is little known and studied.

Pioneering studies, such as those by Vasconcelos et al. (2010) and De Lima et al. (2016), reported the difficulty of producing *M. elliptica* (Mart.) seedlings from seeds, justifying the use of alternative methods for mass propagation. The *in vitro* propagation technique is a viable tool to produce seedlings of wild or domesticated species that are difficult to propagate by conventional methods, accelerate plantlet production (ASSIS et al., 2012; MARTENDAL et al., 2014; MALI; CHAVAN, 2016) and conservation of endangered species (PATEL et al., 2014).

In the *in vitro* propagation, the agar is the most widely used support material for explants in culture medium. However, problems have been reported in plantlets grown in agar medium, such as poor root formation, resulting in losses (XIAO et al., 2011). These observations, coupled with the abundant use of agar, make this agent costly for *in vitro* multiplication (BRAGA et al., 2013). Thus, alternative support materials that can reduce production costs and possibly improve plantlet vigor, facilitating their acclimatization, have been tested (MOHAN et al., 2005; SALDANHA et al., 2014).

In addition to the need to use alternative support, the suitability of the culture medium and the use of growth regulators are fundamental. In the *in vitro* propagation, growth regulators are use to the induction of cellular division and root differentiation (Navarro-García et al., 2016; ABDULMALIK et al., 2012). IBA (indole-3-butyric acid), IAA (indole-3-acetic acid) and NAA (naphthalene acetic acid) are the auxins generally used for *in vitro* rooting of plants (BARPETE et al., 2014).

The endogenous auxin level greatly influences root induction, and the application of plant growth regulators can significantly increase either low or high concentrations of auxin (HOSSAIN; URBI, 2016). For some species *in vitro* rooting acclimatization aiming unnecessary (AINA et al., 2015; SHEKHAWAT; MANOKARI, 2016). Studies of the interaction between growth regulators and culture medium support materials have been rare and are of extreme importance.

The first study on vegetative propagation of *M. elliptica* (Mart.) by means of tissue culture was conducted by Assis et al. (2016). In that study, it was possible to produce plantlets in traditional and photoautotrophic culture conditions by increasing the irradiance (50 to 150 μmol $m^{-2}s^{-1}$) in the environment. However, the importance of developing new methods associated with the photoautotrophic system that enhance the *in vitro* production of seedlings of this species was discussed, citing the use of materials as alternative supports to agar.

Studies evaluating the interaction between different media materials with growth regulator are non - existent for propagation of the species. Thus, this study sought to evaluate the influence of support materials alternative to agar for the *in vitro* rooting of plantlets of this species in the presence or absence of NAA, making the plantlets more resistant.

3.2 Material and methods

Collection of fruits, plantlets and explants, disinfection and inoculation

Plantlets with at least two axillary buds were used as explant sources, as they had nodal segments 1.5 cm in length. The disinfection process followed, in which the explants were wrapped with gauze and placed under running water with three drops of neutral detergent for 15 minutes. In a laminar flow hood, the explants were immersed in 70% (v/v) ethanol for 30 seconds and then submerged in 20% sodium hypochlorite - NaOCl solution (commercial bleach 2.0 – 2.5% active chlorine) for 15 minutes. To complete the disinfection, the explants were washed three times in distilled and autoclaved water.

The explants were subsequently inoculated into Magenta® culture flasks containing the alternative support materials or agar for plant culture. The alternative support materials studied were sugarcane bagasse (sugarcane B.) (*Saccharum spp.* L.), queen palm fiber (queen palm F.) [*Syagrus romanzoffiana* (Chamisso) Glassman] and medium-grain vermiculite. The influence of the growth regulator naphthalene acetic

acid (NAA) (Sigma®) on plantlet rooting was also studied at concentrations of 0.0 and 2.0 mg L^{-1}. The nutrient medium WPM (Wood Plant Medium) developed by Lloyd and McCown 1981 with 50% salts, 30 g L^{-1} sucrose, 3.5 g L^{-1} agar (Dinâmica®) and 2 g L^{-1} activated carbon at (Synth®) was used, and a volume of 50 mL was used in each Magenta® culture flasks, which was sealed with a polypropylene closure. The pH of the growth medium was adjusted to 5.7 ± 0.03 before autoclaving at 121°C for 20 minutes.

Five grams of alternative support materials was used in each Magenta® culture containers. The volume of culture medium used was determined by calculating the amount of water to substrate (BRASIL, 2009), with some modifications. Thus, the support material (5 g) and the known volume of water (100 mL) were placed in a funnel with filter paper and left to rest for 45 minutes to drain the water. The volume retained in the support material was used. Therefore, the volumes of liquid culture media (WPM, with 50%) used in 5 g of alternative support material were 41.0; 30.0 and 21.3 mL for sugarcane B., queen palm F. and vermiculite, respectively.

Water loss through evaporation was measured every 3 days in flasks containing the alternative support materials without plant culture using an analytical balance. The observed water loss was 0.24 mL day^{-1} per flasks. The culture medium in each support material was replenished at 15 and 30 days of *in vitro* culture. The culture was maintained in growth room for 45 days under light intensity of 45.

Purification of sugarcane B. and queen palm F. support materials for *in vitro* cultivation

To obtain the queen palm F., ripe fruits were placed in a fruit and vegetable depulper (Ker Mod. 1.5, Tortugan®) for 40 minutes to separate the epicarp and mesocarp (total pulp) from the diaspore (endocarp with seed). The dry sugarcane B. was derived from the Nova Gale located in Acreúna, Goiás, Brazil.

The fibrous support materials were washed in running water, using the methodology of Mohan et al. (2005) as a reference. For further purification of queen palm F., 10 consecutive washes with running water were necessary. Lastly, the fibrous material was washed 2 times with distilled water (heated to 95 ± 5 ºC).

Both queen palm F. and sugarcane B. were dried in an oven at 40°C for 72 hours and were then ground using a Willye (TE-650) grinder with a 5-mm sieve. Before using the supporting materials, including the vermiculite, they were washed with

distilled water to remove ions. Afterwards, they were dried in an oven and autoclaved at 121 ºC for 20 min.

Physical characterization of the alternative support materials

The physical characteristics of the alternative substrates were evaluated according to the recommendations of Zorzeto et al. (2014) and following the standard instructions established by the Brazilian Ministry of Agriculture, Livestock and Supply (BRASIL, 2007), the official Brazilian government agency that regulates the use of substrates for plants destined for agriculture.

The wet bulk density (WD) was determined from the ratio of the mass occupied by the substrate to the volume at current moisture in a 250-cm^3 plastic beaker dropped under the action of its own weight from a height of 10 cm ten consecutive times (Brasil, 2008). After autocompaction, the samples were dried in an oven at 65°C to constant weight (BRASIL, 2007), and the values were used to determine the dry bulk density (DD).

Other substrate samples were packed in PVC cylinders measuring 4 cm in diameter and 5 cm in height and subjected to saturation with distilled water for 24 hours at 10 to 100 hPa to determine water retention curves (DE BOODT; VERDONCK, 1972; BRASIL, 2008). The following parameters were determined: total porosity (TP), which considers the volumetric water content present in the saturated samples (0 hPa); aeration space (AS), the difference between the total porosity and volumetric water content at 10 hPa; available water (AW), which corresponds to the volume of water between 10 and 100 hPa; and remaining water (RW), the amount of water remaining in the sample after it was subjected to 100 hPa matric potential and equivalent to micropore water.

Growth evaluations

Evaluations were performed after 45 days of *in vitro* cultivation using the characteristics of plantlet length (cm), number of nodal segments, number of leaves, total dry mass (mg), numbers of adventitious and secondary roots, length of largest root (cm) and water content of plantlets (%). Measurements of length were obtained using a millimeter ruler.

To obtain the dry mass, the plant material remained in a ventilation oven forced to a temperature of 65 ºC for 72 hours, and weighing was performed on a digital

analytical balance. The plantlet water content was determined from the difference between total fresh mass and total dry mass and was expressed as a percentage.

Anatomical characteristics

Four plantlets of *M. elliptica* (Mart.) submitted to the different types of culture were fixed in Karnovsky solution (KARNOVSKY, 1965) for 48 hours. The region of the stem with root formation was submitted to an embedding procedure, for which the samples were dehydrated in a graded ethylic series (30, 50, 70, 96 and 100%), pre-infiltrated, infiltrated and polymerized in historesin (Historesin Leica, Erviegas Ltda: São Paulo - SP, Brazil) according to the recommendations of the manufacturer. Embedding molds were used to obtain polymerized blocks.

After drying, the blocks with plant material were cut into 5-µm-thick cross-sections in a rotary microtome (model RM 2155, Leica). The sections were stained with toluidine blue dye 0.05%, pH 4.0 and were mounted on slides with Canada Balsam. Images were obtained with an Olympus model BX61 microscope with a DP-72 camera.

Experimental design and statistical analysis

The experiment was conducted in a completely randomized design (CRD) in factorial arrangement (4x2), with four types of support materials for the culture medium and the absence or presence of NAA. Four replications with three explants each per Magenta® containers were performed for each factor studied. The data were subjected to analysis of variance (ANOVA), applying the F test and the means were compared using the Tukey test (5% probability). SISVAR software (FERREIRA, 2011) was used for the data analysis.

3.3 Results

Physical attributes of the support materials

Significant differences between the types of supports were observed for all physical attributes evaluated ($p < 0.01$). The queen palm F. support had the highest WD (694.07 kg m^{-3}) and DD (507.56 kg m^{-3}) values, followed by vermiculite. Lower WD and DD values were observed for sugarcane B. of 466.55 and 364.67 kg m^{-3}, respectively (Table 1).

Greater total porosity (0.92 m^3 m^{-3}) and aeration space (0.35 m^3 m^{-3}) were observed in the support material queen palm F. Lower values of these characteristics

were observed in the support material vermiculite, with values of 0.57 and 0.15 $m^3 m^{-3}$, respectively. In sugarcane B., the TP was 0.73 $m^3 m^{-3}$, which was between the values detected for queen palm F. and vermiculite. The AS of sugarcane B. was 0.12 $m^3 m^{-3}$ and did not differ from the value observed for vermiculite (Table 1).

Table 1 - Physical characteristics of the alternative support materials used for *in vitro* cultivation of *M. elliptica* (Mart.) plantlets. Total porosity (TP), available water (AW), aeration space (AS), remaining water (RW), wet density (WD) and dry density (DD).

Characteristics	Support materials		
	Vermiculite	**Sugarcane B.**	**Queen palm F.**
TP ($m^3 m^{-3}$)	0.57 ± 0.01c[z1]	0.71 ± 0.00 b	0.93 ± 0.01 a
AW ($m^3 m^{-3}$)	0.08 ± 0.00 b	0.38 ± 0.02 a	0.35 ± 0.03 a
AS ($m^3 m^{-3}$)	0.13 ± 0.00 b	0.12 ± 0.01 b	0.35 ± 0.02 a
RW ($m^3 m^{-3}$)	0.34 ± 0.00 a	0.23 ± 0.01 b	0.22 ± 0.01 b
WD ($kg\ m^{-3}$)	654.13 ± 2.73 b	466.55 ± 2.86 c	694.07 ± 4.77 a
DD ($kg\ m^{-3}$)	493.83 ± 1.82 b	364.67 ± 2.44 c	507.56 ± 3.27 a

[z]Means followed by the same letter in rows do not differ among according to the Tukey, $p < 0.05$. [1]± Standard error from the mean.

Higher AW values were obtained for sugarcane B. (0.38 $m^3 m^{-3}$) and queen palm F. (0.35 $m^3 m^{-3}$), and lower RW values were observed in these two support materials: 0.23 $m^3 m^{-3}$ in sugarcane B. and 0.22 $m^3 m^{-3}$ in queen palm F. A lower AW value (0.08 $m^3 m^{-3}$) and higher RW value (0.34 $m^3 m^{-3}$) were detected in vermiculite (Table 1).

In vitro regeneration of *M. elliptica* (Mart.) plantlets in different culture medium support materials in the presence or absence of NAA

The growth patterns of the plantlets after 45 days of *in vitro* cultivation in culture medium support materials agar, sugarcane B., queen palm F. and vermiculite can be seen in Figure 1 (A – F). Greater plantlet length (2.85 cm) occurred in the agar culture, and shorter length (1.69 cm) occurred in the queen palm F. culture (Figure 2A). An increase of 86.66% in plantlet shoot length was seen when plantlets were grown in agar medium, with an initial explant length of 1.50 cm as the base. Increases in growth

of 37.70, 34.10 and 13.00% were observed in the sugarcane B., vermiculite and queen palm F. cultures, respectively.

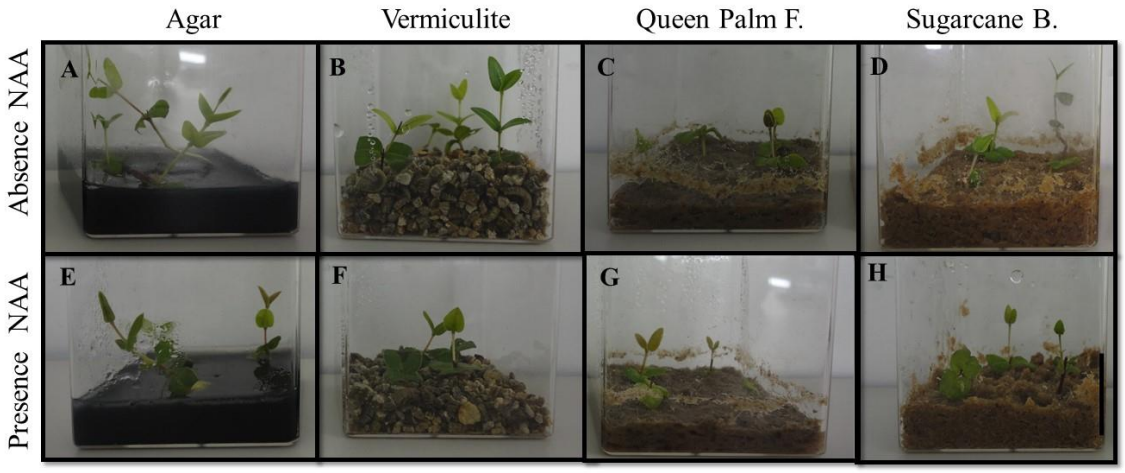

Figure 1. *In vitro* cultivation of *Mouriri elliptica* (Mart.) plantlets in different culture medium support materials for 45 days. Plantlet formed in different support materials in the absence or presence of Naphthalene Acetic Acid - NAA. Scale bar: 2 cm.

Differences between the support materials agar, vermiculite and sugarcane B. were not observed for the characteristics of number of nodal segments, number of leaves and total dry mass of plantlets (Figure 2B, C and D). In these supports, NAA did not influence these characteristics. An average of 2.0 nodal segments per plantlet was obtained in the agar culture, vermiculite and sugarcane B. (Figure 2B). In sugarcane B. a higher number of nodal segments (2.0) in the absence of the regulator (Figure 2B).

The averages observed for number of leaves in agar, vermiculite, and sugarcane B. were 4.5, 3.7 and 3.3, respectively. As observed for the characteristic number of nodal segments, a difference between the presence and absence of NAA for leaf regeneration was observed only in sugarcane B., where it was higher (4.0 leaves per plantlet on average) in the absence of the growth regulator (Figure 2C). Regarding total dry mass, plantlets grown in agar, vermiculite and sugarcane B. had averages of 35.73, 31.66 and 26.59 mg, respectively. Smaller numbers of nodal segments (1.0) and numbers of leaves (2.41) and lower total dry mass (23.00 mg) of plantlets were observed in the queen palm F. culture (Figure 2B, C and D).

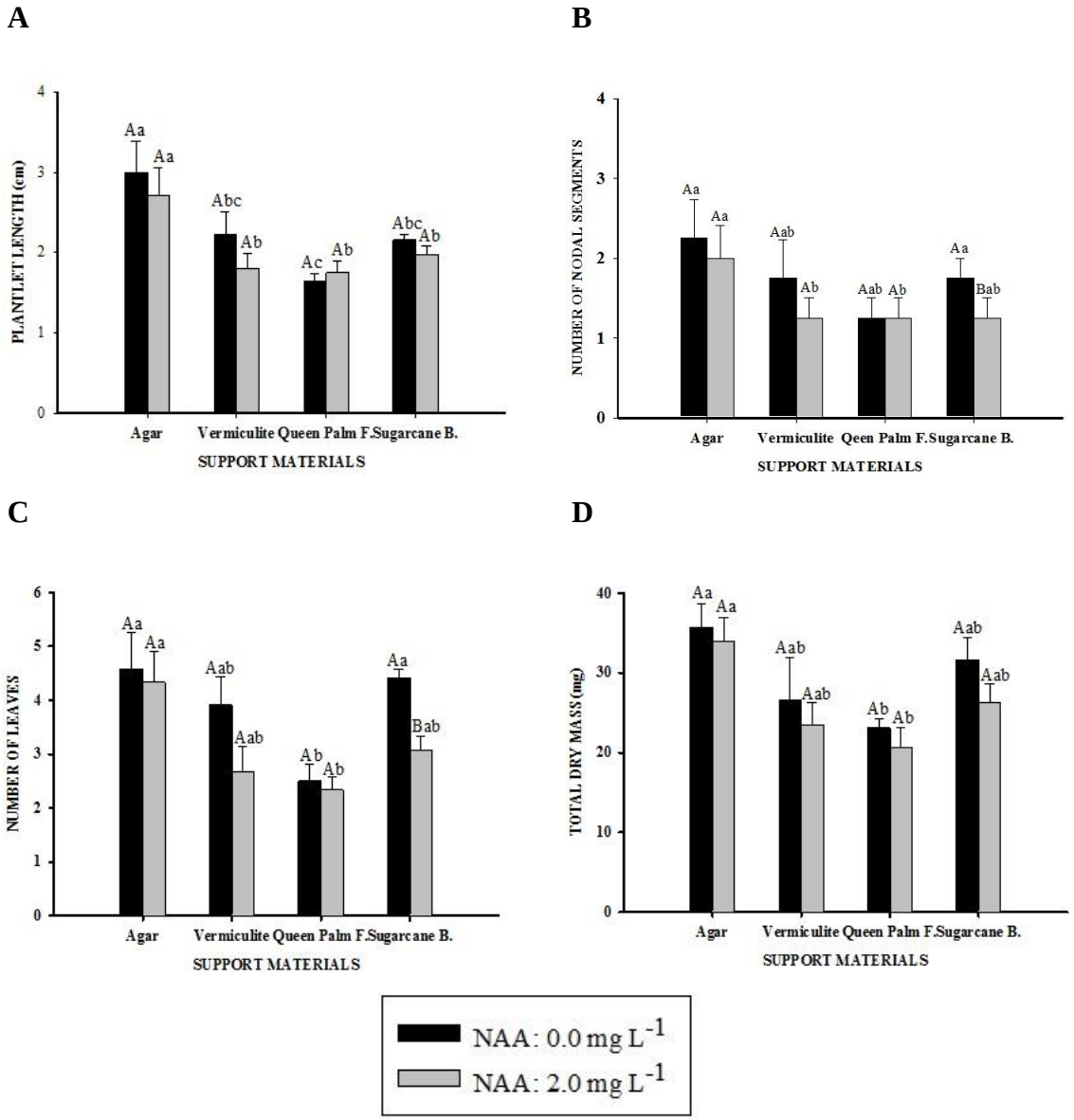

Figure 2. Length of plantlets (A), number of segments (B), number of leaves (C), and total dry mass (D) of *M. elliptica* (Mart.) plantlets with 45 days of *in vitro* cultivation. Means followed by the same uppercase letter do not differ between the presence and absence of NAA, and means followed by the same lowercase letter do not differ among support materials, according to the Tukey test, $p < 0.05$.

A higher number of adventitious roots (1.33), a longer main root (5.66 cm) and longer secondary roots (3.13) were observed in the vermiculite culture when the medium was absence of NAA. Root formation was insignificant in the queen palm F. culture even in the presence of NAA. Among vermiculite, sugarcane B. and agar, there were no differences in these characteristics (Figure 3A, B, C).

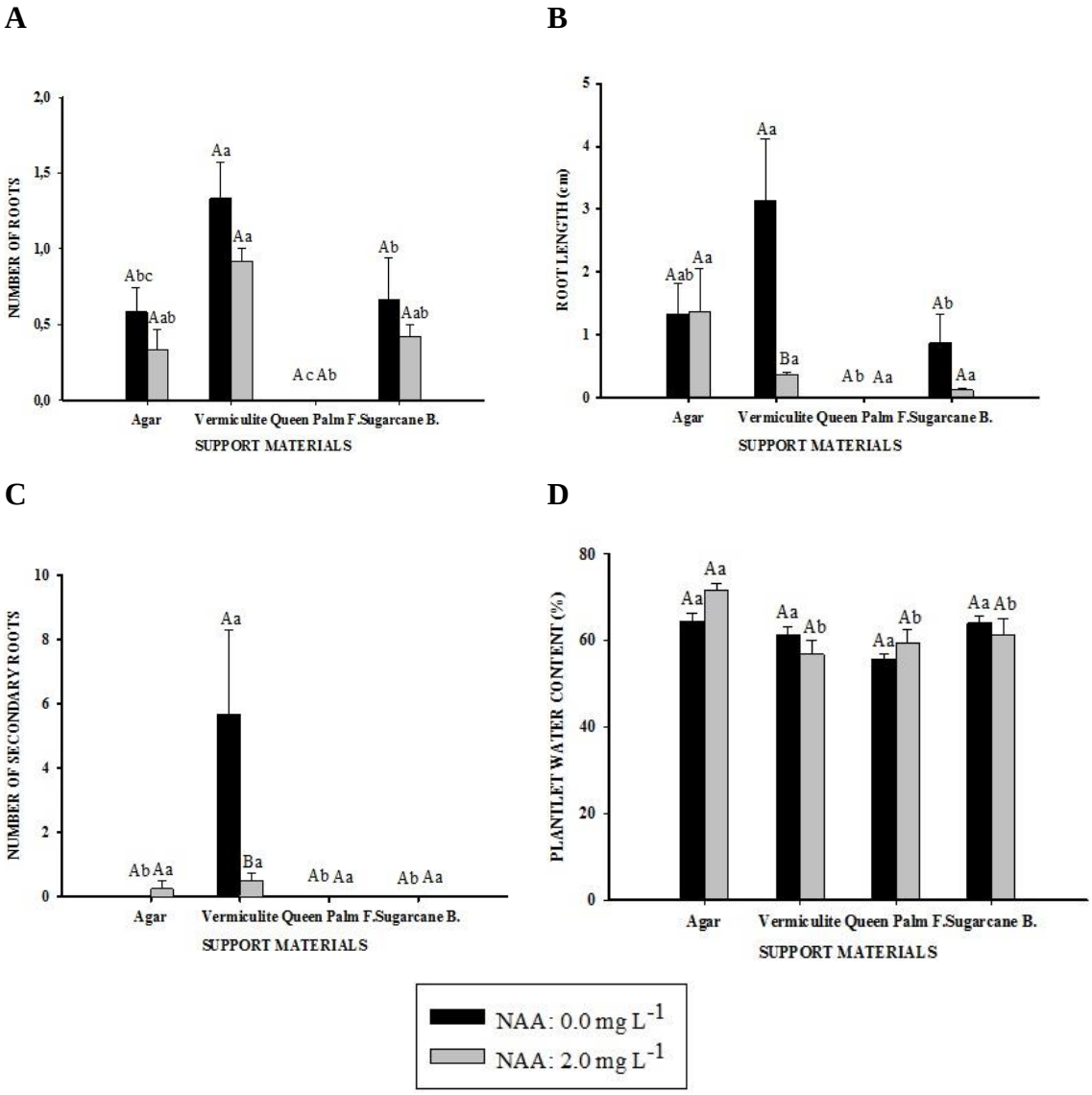

Figure 3. Number of roots (A), root length (B), number of secondary roots (C) and total water content (D) of *M. elliptica* (Mart.) plantlets with 45 days of *in vitro* cultivation. Averages followed by the same uppercase letter do not differ between the presence and absence of NAA, and averages followed by the same lowercase letter do not differ among support materials, according to the Tukey test, $p < 0.05$.

Higher water content (71.64%) was observed in plantlets produced in agar culture under increased NAA. There was no difference between the alternative support materials sugarcane B., queen palm F. and vermiculite, with averages of 61.41, 59.58 and 56.85%, respectively (Figure 3D). Plantlet hyperhydricity was not observed in any support materials used.

Anatomical characteristics of roots formed in different culture medium support materials in the presence and absence of NAA

At the time of assessment (45 days of *in vitro* cultivation), many roots of the plantlets obtained in the agar culture without NAA broke during the measurement of length, thus, these roots were considered fragile (Figure 4A and E). Visually, more resistant roots were formed in the vermiculite (Figure 4B) and sugarcane B. cultures (Figure 4D) in the absence NAA. No root formation occurred in the queen palm F. (Figure 4C and G). Callus formation was obtained in vermiculite cultivation with addition of NAA (Figure 4F).

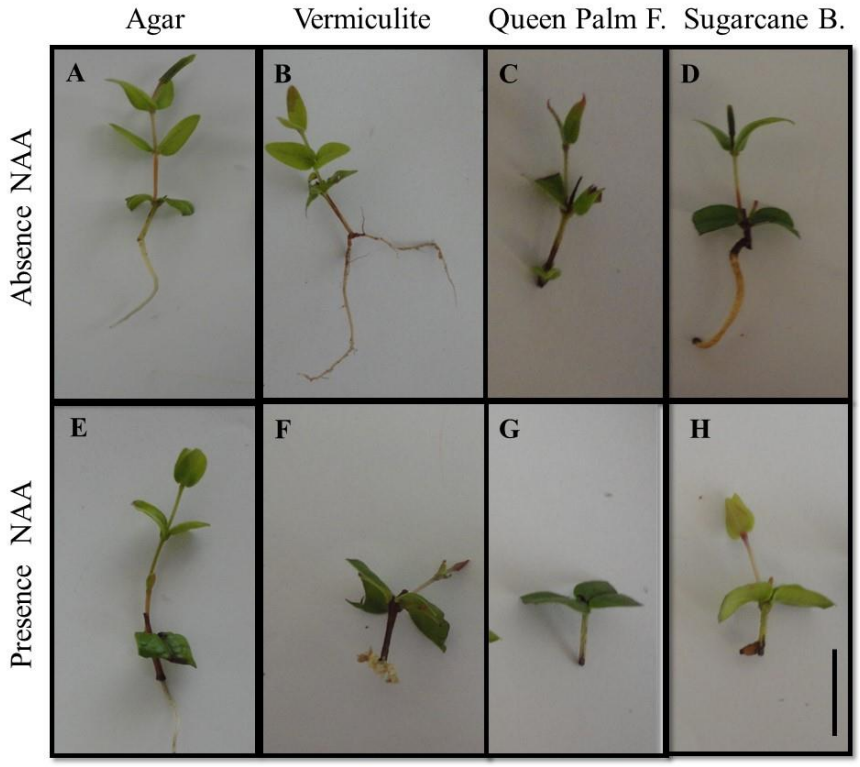

Figure 4. *Mouriri elliptica* (Mart.) plantlets with 45 days of *in vitro* cultivation. Plantlet formed in different support materials in the absence or presence of Naphthalene Acetic Acid - NAA. Scale bar = 2 cm.

Roots with disorganized vascular cambium and no vascular cylinder and with predominant parenchymal tissue formed in plantlets grown in agar without NAA and in sugarcane B. in the presence of the regulator (Figure 5A, B, D). Adventitious roots with differentiated tissues were obtained in the *in vitro* culture without growth regulator and using sugarcane B. (Figure 5C) and vermiculite (Figure 5G) as support material.

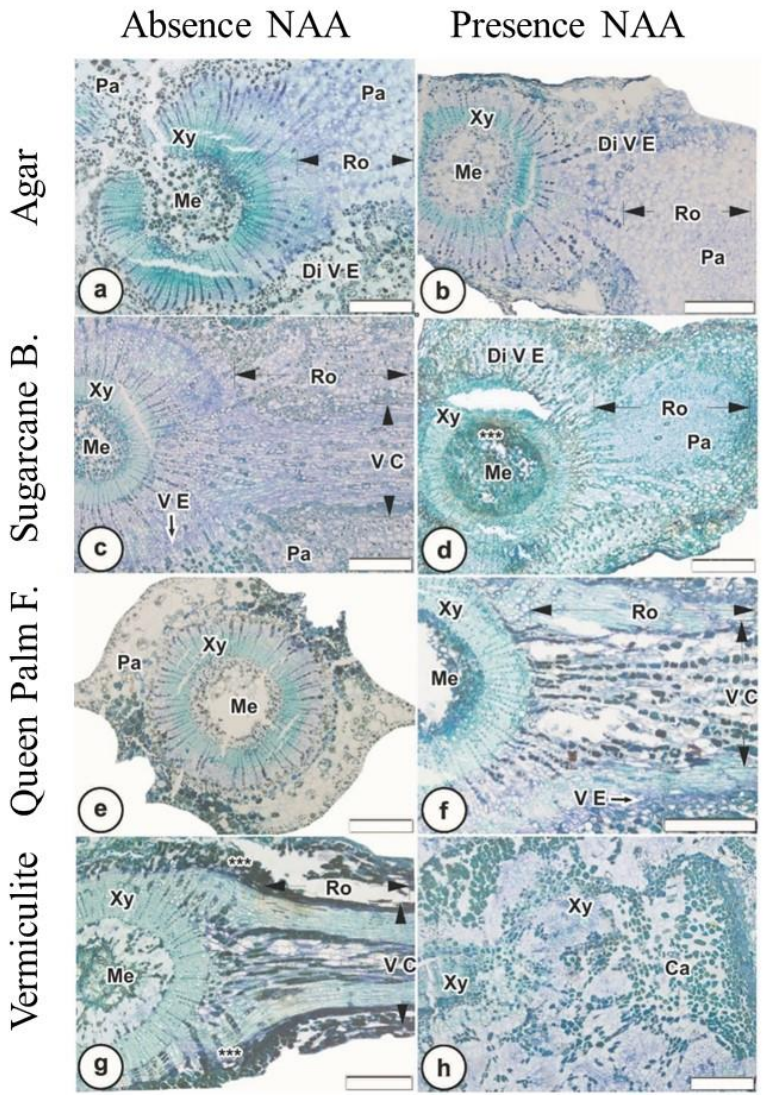

Figure 5. Anatomy roots *Mouriri elliptica* (Mart.) formad under *in vitro* culture for 45 days and, different support materials. Culture in the absence or presence of Naphthalene Acetic Acid - NAA. Parenchyma – Pa; xylem – Xy; root – Ro; medulla – Me; vascular cambium – V E; disorganized vascular cambium – Di V E; vascular cylinder – V C; callus – Ca and necrotic tissue – ***. Scale bar = 100 µm.

Roots formed in the support sugarcane B. and vermiculite had the vascular cylinder connected to the vascular cambium of the stem was identified in these roots (Figure 5c and g). This characteristic was also observed in roots of plantlets produced in the queen palm F. culture when the medium was supplemented with NAA (Figure 5F).

The presence of NAA in the culture medim stimulated the formation of callus at the base of segments of plantlets grown in vermiculite at a rate of 41.66%. In Figure 4F, the morphological pattern of the callus at the base of the plantlet stems can be

observed, and its internal organization can be observed in Figure 5H. Disorganized tissues can be seen, but with a certain level of differentiation and the presence of xylem (Xy) (Figure 5H).

3.4 Discussion

The results of this study demonstrate that vermiculite, followed by sugarcane B., can be used as agar substitutes in *in vitro* culture of *M. elliptica* (Mart.). This finding is based on the regeneration ability of seedling shoots and, in particular, root development. Woody plants are usually difficult to root, and thus, a material that facilitates *in vitro* rooting is beneficial in micropropagation systems by enabling the regulation of the growth environment based on the physical properties of the support material (XIAO et al., 2011).

Despite recommendations for the use of support materials, few studies have physically characterized these materials for use *in vitro*. The distribution of water, air and solids in alternative supports depends on several factors such as pore space, density, particle size and spatial distribution of pores (OH et al., 2012). Such evaluations are common for use in nurseries, as the physical quality of the substrate is an important factor for seedling growth and development, providing nutrients, retaining water and moisture and being financially viable (PAGLIARINI et al., 2012; DORNELLES et al., 2014).

Ideal substrates are those that promote better aeration and water infiltration and drainage. Vermiculite, followed by sugarcane B., provided good development of the seedlings under study for all evaluated characteristics. These supports had smaller pore spaces than queen palm F. These results suggest that queen palm F. exerts a negative effect on seedling growth, particularly on root formation, even in the presence of NAA in the culture medium. Although queen palm F. did not promote good growth in the plantlets under study, it still has potential for *in vitro* use, as the response of plantlets is in part dependent on genotype. Different genotypes may respond differently to the same cultivation condition (CORRÊA et al., 2015; 2016).

For growth of plants in containers, the best values for AW are between 0.24 and 0.40 $m^3\ m^{-3}$ (DE BOODT; VERDONCK, 1972). For RW, the ideal range is between 0.25 and 0.30 $m^3\ m^{-3}$ (VERDONCK; GABRIËLS, 1988). Although TP, AW and RW values outside of the ideal range for *in vitro* cultivation of plants in containers were observed in vermiculite, this support material allowed for better formation of

adventitious and secondary roots and did not differ from agar and sugarcane B. in terms of shoot formation. The observed positive results reflect the ability of the seedlings to use the nutrient solution added to the vermiculite.

Our results demonstrate that the support material does not need to be excessively porous for proper root development of *M. elliptica* (Mart.) seedlings. In the case of vermiculite, a lower TP associated with a higher RW (water retained at matric potentials higher than 100 hPa and present in the form of water films around the particles) resulting from the volumetric expansion of the mineral particles during their production promoted adequate gas exchange through the roots and provided greater substrate/root contact surface area, contributing to nutrient absorption processes and thus to plant growth and development.

Sugarcane B. had intermediate values for TP and AW and RW values within the ideal range (DE BOODT; VERDONCK, 1972; VERDONCK; GABRIËLS, 1988). For plantlet regeneration, it was less effective than vermiculite for root formation, although roots formed on plantlets grown in sugarcane B. formed differentiated tissues with a vascular cylinder connected to the vascular cambium of the stem, as also observed in roots formed in vermiculite. Roots formed in the agar culture, regardless of the presence or absence of NAA, were fragile and had poorly differentiated tissues and no secondary roots. Similar problems in root formation using agar have been reported and can cause problems in the acclimatization process (BRAGA et al., 2011).

The presence of NAA in the culture environment with vermiculite as the support material was a limiting factor in root formation, as the root length was shorter and the number of secondary roots was lower. In addition, a relatively high percentage of calluses was observed at the base of the stem. This characteristic was considered undesirable in consideration of the principal objective of the study, the multiplication and rooting of *M. elliptica* (Mart.). However, this observation offers an opportunity to study callus formation from the stem of this species.

Thus, the characteristics of plantlets evaluated in this study informed the determination of physical attributes of alternative support materials to agar that were ideal for the *in vitro* cultivation of *M. elliptica* (Mart.) and that favored plantlet growth. However, studies on the micropropagation of this species using alternative support materials could be developed to evaluate the influence of other factors that typically promote development of more resistant plantlets, such as the elimination of sucrose in

the culture medium, the use of gaskets that allow for increased gas exchange, the increase of light intensity and/or atmospheric enrichment with carbon dioxide (CO_2).

3.5 Conclusions

The alternative support materials vermiculite followed by sugarcane B. can be used as substitutes for agar for micropropagation of *M. elliptica* (Mart.). These support materials promoted shoot growth equal to that of agar and greater root formation and tissue differentiation, thus increasing the resistance of the plantlets and survival of the acclimatization process.

The use of the growth regulator NAA did not stimulate increased rooting of *M. elliptica* (Mart.) plantlets in the types of support materials used in this study.

3.6 Acknowledgments

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CAPÍTULO IV

(Normas de acordo com a revista Acta Scientiarum – Agronomy)

Aclimatização de *Mouriri elliptica* (Mart.) propagadas *in vitro* sob atmosfera enriquecida com CO_2 e diferentes vedações

Resumo

A croada (*Mouriri elliptica* Mart.), é uma frutífera nativa do cerrado com potencialidade para ser utilizada pela população, no entanto, carece de estudos sobre sua propagação vegetativa, em especial sobre a influência das condições de cultivo *in vitro* na aclimatização. Assim, objetivou-se com este estudo, avaliar a performance na aclimatização de plântulas de *M. elliptica* Mart. cultivadas *in vitro* sob enriquecimento da atmosfera com CO_2 e uso de diferentes vedações do frasco de cultivo. No cultivo, utilizou-se 6 g de substrato vermiculita por frasco e solução nutritiva do meio Wood Plant Medium. As vedações utilizadas, foram i) tampa convencional de polipropileno (T. conv), ii) tampa com dois orifícios de área de 2,24 10^{-4} m^2 vedados com membrana microporosa (T. orif) e iii) vedação com vedafilme (PVC). Os frascos de cultivo foram mantidos em câmaras climáticas (Fitotron®) com atmosfera enriquecida com CO_2 (800 ± 35 µmol mol L^{-1}) e atmosfera ambiente de CO_2 (400 ± 59 µmol mol L^{-1}) sob irradiância de 150 ± 10 µmol $m^{-2}s^{-1}$, temperatura média de 25 ± 0,04 ºC e umidade relativa de 60 ± 0,18%. Sobrevivência de 100% foi obtida para plântulas micropropagadas em frascos vedados com T. orif sob atmosfera ambiente de CO_2. Na propagação *in vitr*o de *M. elliptica* Mart. o enriquecimento da atmosfera de cultivo com CO_2 (800 ± 35 µmol mol L^{-1}) não proporcionou incremento no crescimento das plântulas, exceto com a utilização da vedação do tipo PVC.

Palavras-chave: Croada, Melastomataceae, fotoautotrófico e fluorescência.

4.1 Introdução

Dentro da rica biodiversidade do Cerrado, destacam-se as espécies frutíferas, citando a *Mouriri elliptica* Mart. (Família Melastomataceae), que possui potencialidades para ser utilizada pela população. Seus frutos por serem ricos em nutrientes e em compostos antioxidantes, como a vitamina C são indicados para consumo, sendo considerados promotores da saúde humana (Rufino, Alves, Fernandes, & Brito, 2011). Em estudos fitoquímicos das folhas, constatou-se a presença de compostos fenólicos, como os flavonoides e taninos, sendo estes relacionados ao tratamento de doenças gastrointestinais como úlceras gástricas e gastrite (Moleiro et al, 2009; Vasconcelos, Andreo, Vilegas, Hiruma-Lima, Pellizzona & Vasconcelos, 2010b).

As sementes de *M. elliptica* Mart. possuem rígido tegumento, que tem sido relacionado à dormência física, problema que ocasiona baixa germinação e emergência desuniforme das plântulas (Vasconcelos et al., 2010a). Além disso, com a expansão da agropecuária e também de fatores limitantes a produção de frutos pelas plantas em condições naturais, a perpetuação da espécie está comprometida. Assim, para produção de mudas em grande escala, a técnica de propagação *in vitro,* torna-se importante, além de subsidiar na domesticação e conservação da espécie.

Trabalhos pioneiros com propagação *in vitro* dessa planta destacaram o cultivo fotoautotrófico (cultivo sem sacarose) como promissor para obtenção de mudas da espécie (Assis et al., 2016a e 2016b). De acordo com Xiao, Niu and Kozai, (2011) o cultivo fotoautotrófico interfere nas características morfoanatômica e fisiológicas das plântulas, tornando o aparato fotossintético funcional (Iarema et al., 2012). Assis et al. (2016b) consideraram de suma importância o aprimoramento do cultivo *in vitro* para a espécie, sendo necessário também, aclimatização às condições *ex vitro*.

Entre os trabalhos desenvolvidos com sucesso na literatura, cita-se o enriquecimento da atmosfera de cultivo com CO_2, com expressivo aumento da biomassa das plantas e influência na morfologia dos estômatos e cloroplastos (Saldanha et al., 2013; Saldanha et al., 2014), redução da umidade relativa e da concentração de etileno do frasco de cultivo, utilizando vedações que permitem maiores trocas gasosas (Iarema et al., 2012; Saldanha et al., 2012) e, substituição do ágar por materiais de suporte fibrosos ou porosos (Mohan, Chui, Biasi & Soccol, 2005; Saldanha et al., 2014).

Nesta pesquisa, objetivou-se aprimorar o cultivo *in vitro* fotoautotrófico de *M. elliptica* Mart. com enriquecimento ou não da atmosfera com CO_2 e utilização de diferentes vedações do frasco de cultivo. Tem-se como perspectiva a obtenção de plântulas mais resistentes, favorecendo assim o processo de aclimatização, visto ser esta etapa estressante para as plantas micropropagadas.

4.2 Material e métodos

Condições de cultivo *in vitro*

Os explantes foram constituídos de segmentos nodais (2 cm) com duas gemas axilares. Estes foram retirados de plântulas com 90 dias, mantidas em bandeja (Assis et al., 2016). Os explantes foram revestidos por gaze e colocados em água corrente (15 minutos), adicionou-se três gotas de detergente neutro. Posteriormente, os segmentos nodais foram imersos em álcool 70% (v/v) por 30 segundos, e em seguida submersos em solução de hipoclorito de sódio (20%) durante 15 minutos. Para finalizar a desinfestação, os explantes foram lavados por três vezes em água destilada e autoclavada.

Os explantes foram inoculados em frascos contendo 6 g de substrato vermiculita umedecido com 26 mL de solução nutritiva do meio Wood Plant Medium - WPM (Lloyd & Mccown, 1980) com 50% de sais e apenas 5 gL^{-1} de sacarose. O pH do meio foi ajustado para 5,73 ± 0,03, e, autoclavado a 120 ºC por 20 min. Em seguida, os frascos foram introduzidos em duas câmaras climática Fitotron® sob irradiância de 150 ± 10 µmol $m^{-2}s^{-1}$, temperatura média de 25 ± 0,04 ºC e umidade relativa de 60 ± 0,18% (Figura 1A e B) (Assis et al., 2016). Realizou-se reposição do meio de cultivo a cada 7 dias, conforme perda de água dos frascos.

A B

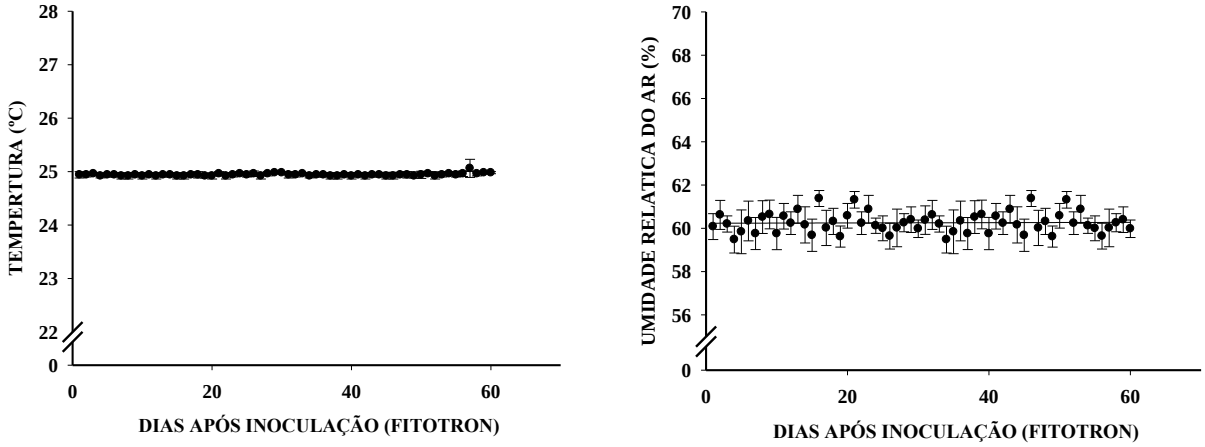

Figura 1. Dados de temperatura (A) e umidade relativa do ar (B) dentro das câmaras climáticas (Fitotron®) utilizadas por 60 dias para cultivo *in vitro* de *Mouriri elliptica* (Mart.).

Avaliou-se por meio das câmaras climáticas a atmosfera enriquecida com 800 ± 35 µmol Mol^{-1} de CO_2 e atmosfera ambiente de 400 ± 59 µmol Mol^{-1} de CO_2. Verificou-se também a influência de três tipos de vedações do frasco de cultivo, sendo estas: i) tampa convencional de polipropileno (T. conv.), ii) tampa com 2 orifício de área de 2,24 10^{-4} m^2 vedados com membrana microporosa (T. orif.), conforme descritos por Saldanha et al. (2012) e iii) frascos vedados com vedafilme (PVC).

O experimento foi inteiramente ao acaso, esquema fatorial 2 x 3, com 15 repetições, de duas plântulas. Ao final de 60 dias de cultivo *in vitro* realizou-se o transplantio das mudas para casa de vegetação.

Aclimatização

As plântulas de *M. elliptica* (Mart.) propagadas *in vitro* sob influência dos fatores CO_2 e vedações (Figura 2A - F) foram transplantadas, no mês de abril de 2016 para casa de vegetação e foram mantidas sob sombrite. A irradiância variou no decorrer do dia, sendo a mínima de 29 µmol $m^{-2}s^{-1}$ e máxima de 322 µmol $m^{-2}s^{-1}$, temperatura média de 23,24 ± 1,42ºC e umidade relativa do ar de 74 ± 2,58 %. Utilizou-se vasos plásticos (10,2 x 7,8 x 7,8 cm; volume de 415 mL) com substrato Bioplant® e realizou-se irrigação diária. A cada 15 dias, aplicou-se em cada vaso, 20 mL de solução nutritiva WPM com 50% de sais (Macro e micronutrientes).

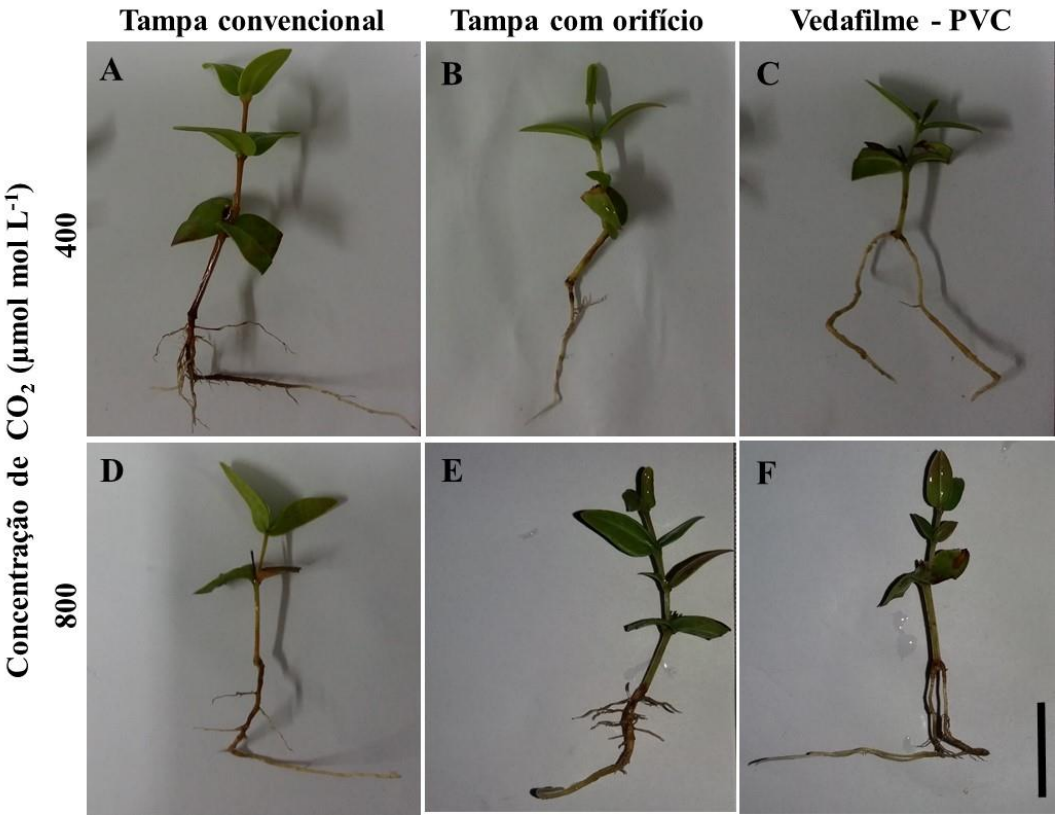

Figura 2. Plântulas de *Mouriri elliptica* (Mart.) micropropagadas em sistema fotoautotrófico sob duas concentrações atmosférica de CO_2 e três vedações do frasco de cultivo. Barra = 2 cm.

Após 60 dias de aclimatização, realizou-se a avaliação das plântulas. As características de crescimento avaliadas foram: comprimento da parte aérea, número de folhas, número de segmentos nodais, área foliar (cm^2), número de raízes adventícias e secundárias, comprimento da raiz principal (cm), massa seca parte aérea (mg) e massa seca de raiz (mg). Para medir o comprimento da parte aérea das plântulas e das raízes, utilizou-se régua milimétrica e a massa seca da parte aérea e de raiz foi obtida pesando o material vegetal em balança analítica após secagem por 72 h a 65ºC em estufa de ventilação forçada.

Características fisiológicas

Por meio da fluorescência por imagem da clorofila *a*, analisou-se: fluorescência inicial - Fo, rendimento quântico máximo do fotossistema II - Fv/Fm, rendimento quântico efetivo – Y(II), coeficiente de extinção não fotoquímica Y(NPQ) e taxa

transporte elétrons – ETR). Para obtenção das imagens da fluorescência da clorofila *a* foi utilizado o fluorômetro modulado Imaging-PAM (Heinz Walz, Effeltrich, Germany). As imagens de fluorescência foram capturadas por uma câmera CCD acoplada ao aparelho (Oxborough, 2004).

Características anatômicas

Utilizou-se para o estudo anatômico 6 folhas de plântulas de *M. elliptica* cultivadas nas diferentes condições *in vitro*. Para o estudo de superfície realizou-se a diafanização das folhas. Para tanto, as folhas foram imersas em hidróxido de sódio 5% por 24 horas, clarificadas com Clorol hidratado, 1,6:1 (p/v) por mais 24 horas e coradas com safranina 1% em etanol 50% (Arnott, 1959).

Após o procedimento citado, as lâminas com material foram cobertas com lamínula utilizando Bálsamo do Canadá. As imagens foram obtidas em microscópio óptico (modelo BX61, Olympus) com sistema U-photo, do Laboratório de Anatomia Vegetal do Instituto Federal de Educação, Ciência e Tecnologia Goiano – Campus Rio Verde. As imagens foram processadas com auxílio do *software* ImageJ®. Considerou-se as características densidade de cripta estomática (Cripta estomática/mm^2) e área de abertura da cripta.

Análise estatística

Os dados observados foram submetidos à análise de variância aplicando-se o teste F ($p \leq 0,05$). Os dados referentes aos tipos de vedações foram comparados pelo teste Tukey ($p \leq 0,05$). Realizou-se também análise descritiva das variações morfoanatômica.

4.3 Resultados

Notou-se que frascos vedados com T. conv restringiram mais a perda de água na forma de vapor, determinando maior umidade no ambiente de cultivo. Já os frascos vedados com T. orif e PVC chegaram a perder em média 25% de água em 15 dias de cultivo na câmara clomática Fitotron® (Figura 3). Estes resultados inferem sobre a capacidade de maior troca gasosas entre o ambiente interno e externo do cultivo *in vitro* ao utilizar vedações do tipo T. orif e PVC. Foi de suma importância avaliar a perda de

água em cada frasco de cultivo, repondo a cada 7 dias a solução nutritiva, evitando assim, déficit hídrico para as plantas.

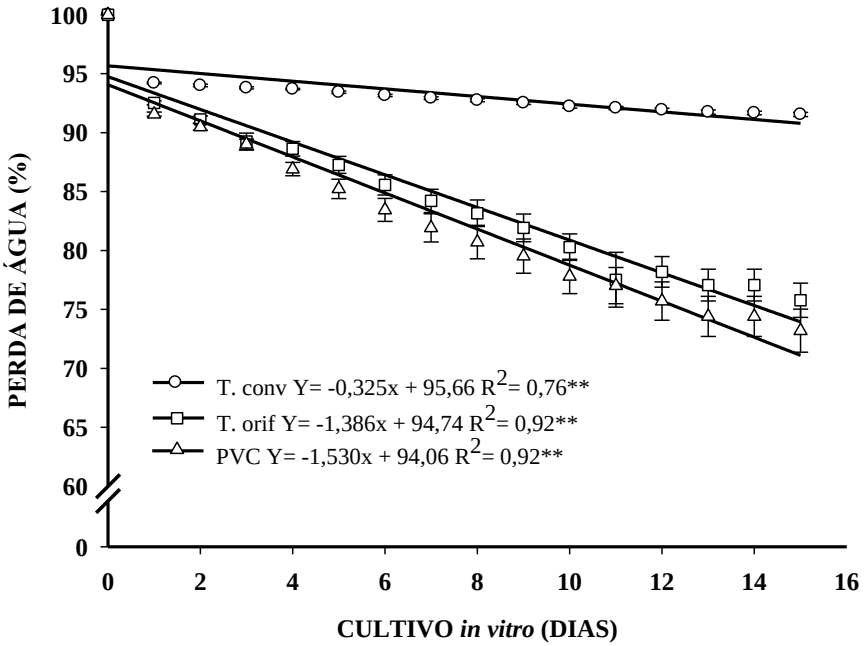

Figura 3. Porcentagem de perda de água em cada frasco de cultivo com as vedações: tampa convencional (T. conv), tampa com orifício e membrana microporosa (T. orif) e vedafilme (PVC) em função dos dias de cultivo *in vitro*. **p < 0,01.

Performance das plântulas de *M. elliptica* (Mart.) após 60 dias de aclimatização

O perfil morfológico das plantas após 60 dias de aclimatização e conforme a procedência de cultivo *in vitro* pode ser observado na Figura 4 (A – F). Sobrevivência de 100% foi obtida para plântulas micropropagadas em frascos vedados com T. orif sob atmosfera ambiente de CO_2. Maior porcentagem de mortalidade (13%) foi observado em plântulas cultivadas em frascos vedados com T. conv e atmosfera ambiente de CO_2 (Figura 5). Para as demais procedências de cultivo *in vitro*, notou-se 6,6% de mortalidade.

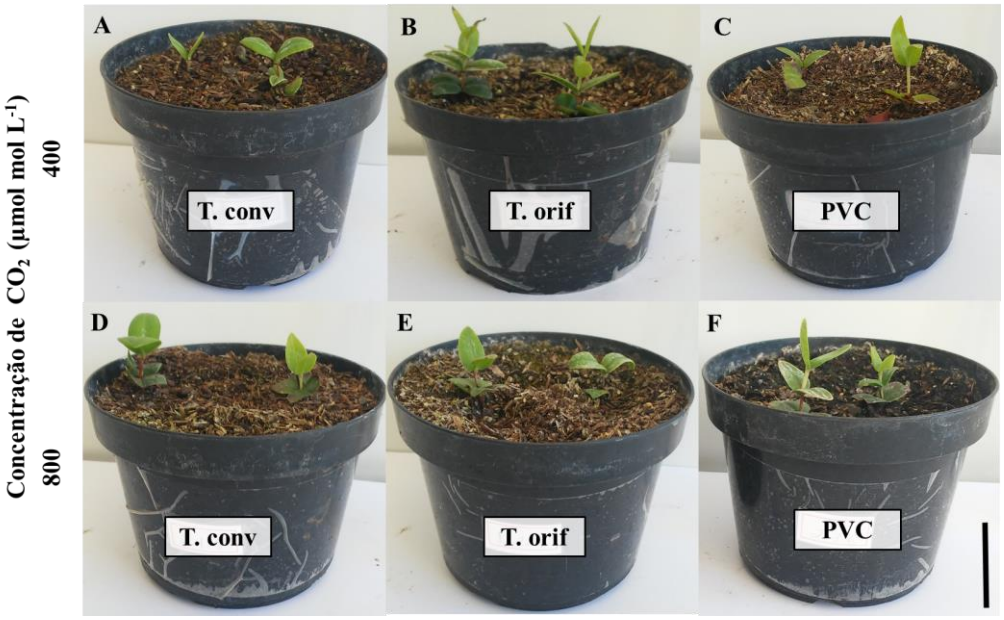

Figura 4. Plântulas de *Mouriri elliptica* (Mart.) aclimatizadas por 60 dias. Plantas estas oriundas do cultivo fotoautotrófico sob duas concentrações atmosférica de CO_2 e três vedações do frasco. Barra de 2 cm.

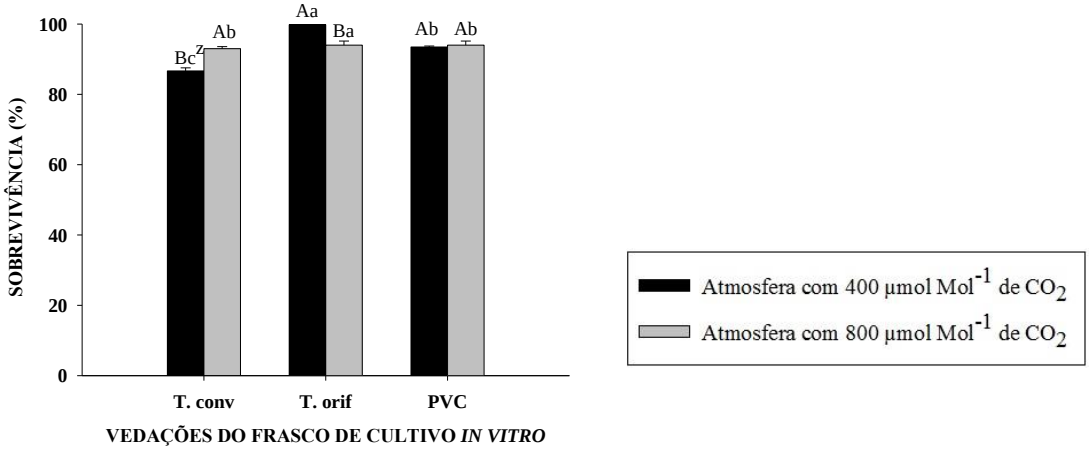

Figura 5. Porcentagem de sobrevivência das plântulas de *Mouriri elliptica* (Mart.) após 60 dias de aclimatização. [z]Médias seguidas pela mesma letra maiúsculas não diferem entre si quanto a concentração ambiente de CO_2, e, minúsculas iguais não diferem entre si, em relação aos tipos de vedações do frasco pelo teste Tukey, $p < 0,05$.

Não se observou influência das concentrações de CO_2 ou tipos de vedações para as características comprimento da parte aérea, número de folhas, número de segmentos nodais, número de raízes adventícias e secundárias. O comprimento médio das plântulas foi de 2,78 cm, com 4 folhas e 2 segmentos nodais. Observou-se média de uma raiz

adventícia por planta, sendo cada raiz com média de três raízes secundárias. Influência das condições de cultivo *in vitro* foi observado para as características área foliar, massa seca da parte aérea, massa seca de raiz e comprimento da raiz (Figura 6).

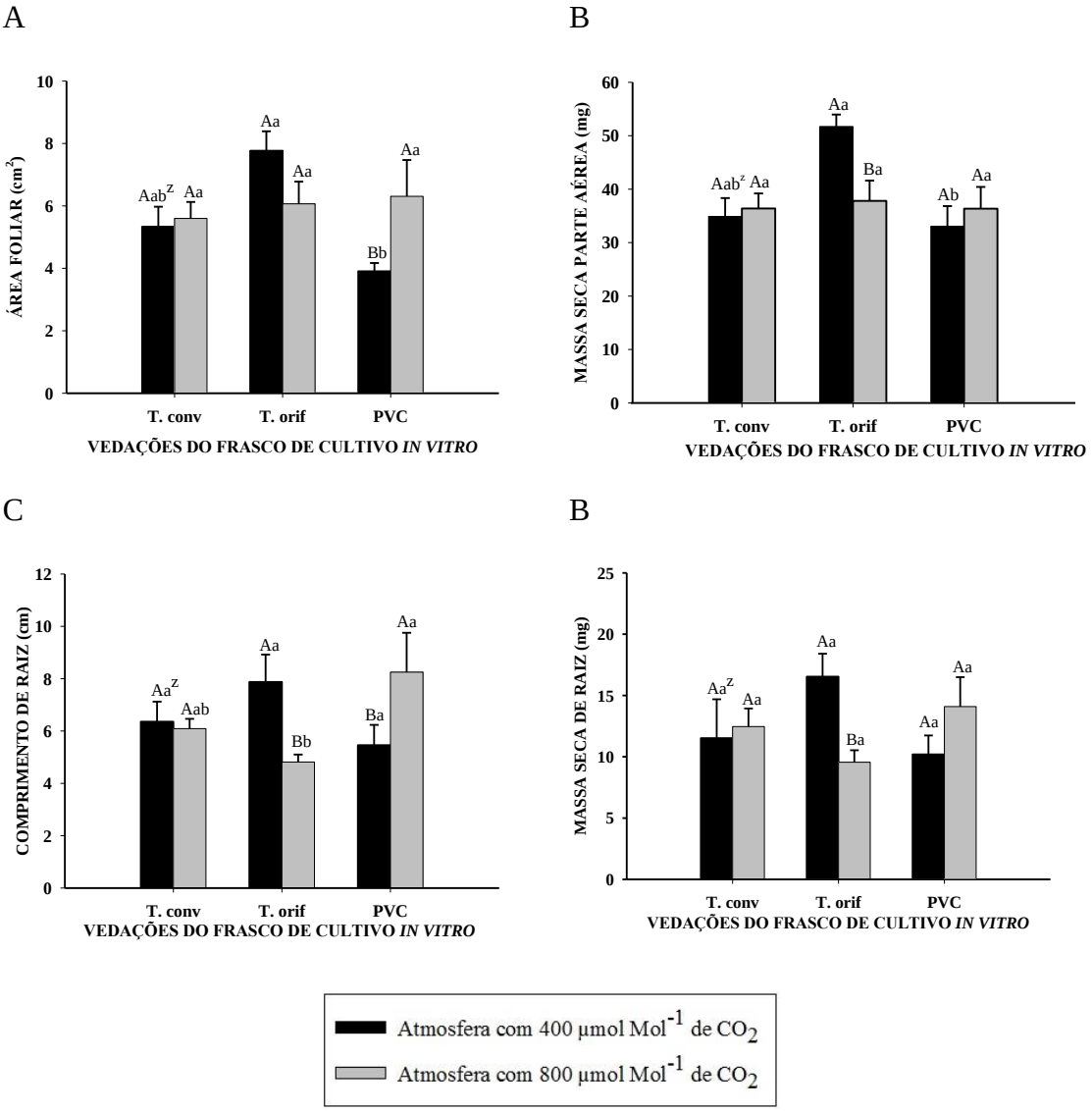

Figura 6. Influência das condições de cultivo *in vitro* nas **c**aracterísticas de crescimento de plântulas de *Mouriri elliptica* (Mart.) após 60 dias de aclimatização. Área foliar (A), massa seca parte aérea (B), comprimento de raiz (C) e massa seca de raiz (D). ᶻMédias seguidas pela mesma letra maiúsculas não diferem entre si quanto a concentração ambiente de CO_2, e, minúsculas iguais não diferem entre si, em relação aos tipos de vedações do frasco pelo teste Tukey, $p < 0,05$.

Plântulas procedentes do cultivo *in vitro* sob atmosfera ambiente de CO_2 tiveram maior investimento na formação da parte aérea quando cultivadas em frascos vedados

com T. orif, sendo observado média de 7,76 cm^2 de área foliar e 51,74 mg de massa seca da parte aérea (Figura 6A e B). Utilizando-se frascos vedados com PVC, obteve-se maior área foliar (6,8 cm^2) com atmosfera enriquecida com CO_2 (Figura 6A).

Quanto a formação radicular, observou-se diferença entre os tipos de vedação apenas para comprimento de raiz, quando as plântulas foram cultivadas sob atmosfera enriquecida com CO_2. Nesta condição de cultivo, frascos vedados com PVC tiveram maior influência no comprimento de raiz, com média de 8,25 cm (Figura 6 C). Notou-se que ao utilizar a vedação do tipo T. orif maior comprimento de raiz e massa seca de raiz foram obtidos em atmosfera ambiente de CO_2 (Figura 6C e D). Já, ao utilizar a vedação com PVC o enriquecimento da atmosfera com CO_2 proporcionou incremento de 33% no comprimento das raízes das plântulas após aclimatização. Desta forma, para as características de crescimento em estudo, o enriquecimento da atmosfera de cultivo *in vitro* com CO_2 representa ganhos apenas se o frasco for vedado com PVC.

Características fisiológicas e anatômicas das plântulas de *M. elliptica* (Mart.) após 60 dias de aclimatização

Não se observou diferença ou interação entre as concentrações de CO_2 e tipos de vedações para índices fisiológicos fluorescência inicial – Fo, rendimento quântico máximo do fotossistema II - Fv/Fm, rendimento quântico efetivo do fotossistema II – Y(II) e taxa de transporte de elétrons (ETR). Para estas características as médias observadas foram 0,084; 0,65; 0,240 e 0,982 respectivamente. A Figura 7A – F apresenta as imagens obtidas para Fo e Fv/Fm de plântulas de *M. elliptica* Mart. após 60 dias de aclimatização.

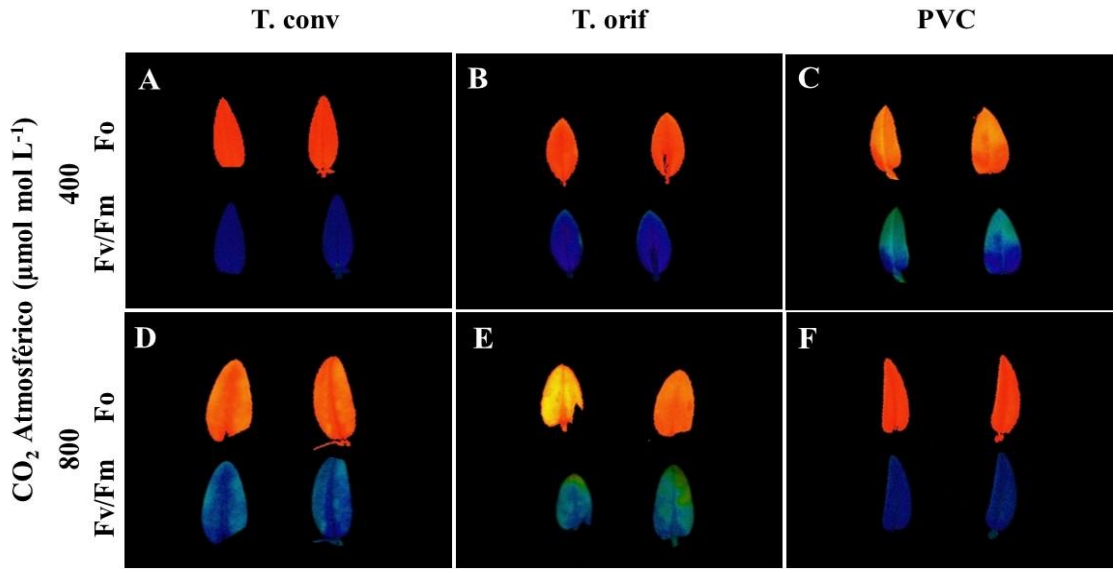

Figura 7. Imagens de fluorescência inicial (Fo) e rendimento quântico máximo do fotossistema II (Fv/Fm) de folhas de *Mouriri elliptica* (Mart) aclimatizadas por 60 dias. Plantas estas oriundas do cultivo fotoautotrófico sob duas concentrações atmosférica de CO_2 e três vedações do frasco.

Maior dissipação não fotoquímica Y(NPQ) das folhas foi obtida em plântulas oriundas do cultivo *in vitro* com frascos vedados com T. orif independente da concentração de CO_2. Para esta variável não se observou diferença entre as vedações T. conv e PVC (Figura 8A).

Na avaliação da superfície das folhas de *M. elliptica* Mart., maior densidade de criptas (157,30 cripta/mm^2) foi obtido em plântulas cultivadas em frascos vedados com T. orif (Figura 8B) independente da concentração de CO_2 do ambiente. Interação entre as concentrações de CO_2 e tipo de vedação foi observada para área de abertura da cripta (Figura 8C). Plântulas sob atmosfera ambiente de CO_2 tiveram maior área de abertura da cripta ao serem cultivadas em frascos vedados com T. orif, já sob atmosfera enriquecida com CO_2, não houve diferença entre os tipos de vedação, e a média observada foi de 136,66 cripta/mm^2.

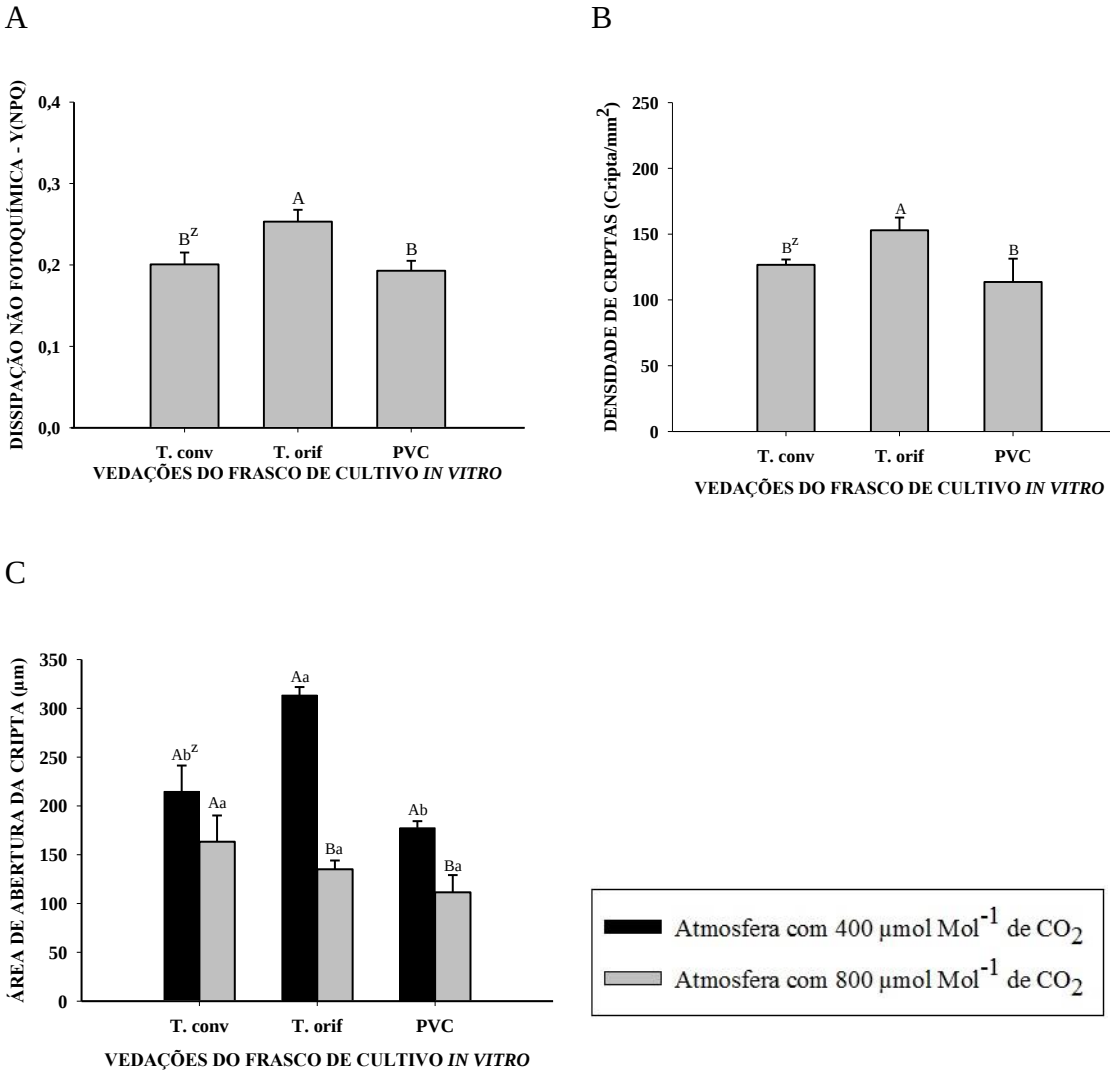

Figura 8. Índice de dissipação não fotoquímica – Y (NPQ) (A), densidade de cripta estomática (B) e área de abertura da cripta estomática (C) de plântulas de *Mouriri elliptica* (Mart.) após 60 dias de aclimatização em resposta as diferentes condições de cultivo *in vitro*. [z]Médias seguidas pela mesma letra não diferem entre si pelo teste Tukey, $p < 0,05$.

Notou-se que o enriquecimento da atmosfera com CO_2, representou fator limitante na característica de abertura da cripta estomática. Quando as plântulas foram cultivadas em frascos vedados com T. orif e PVC, as médias para área de abertura foram 135,13 e 111,51 µm respectivamente, valores estes bem abaixo do observado sob atmosfera ambiente de CO_2, no qual os valores obtidos foram 313,47 µm em T. orif e 177,56 µm em PVC (Figura 8C).

Na Figura 9 (a – f) observa-se a superfície abaxial das folhas de *M. elliptica* (Mart.) Para todas as condições de cultivo *in vitro*, não se notou presença de estômatos distribuídos na face adaxial ou fora das criptas estomáticas da face abaxial das folhas, mantendo a característica da espécie, sendo estas hipoestomáticas. Alteração na estrutura morfológica da cripta estomática só foi observada em folhas de plântulas cultivadas em frascos vedados com PVC e sob atmosfera enriquecida com CO_2 (Figura 9f). Nesta, observou-se maior ocorrência de criptas com área fechada ou com menor área de abertura.

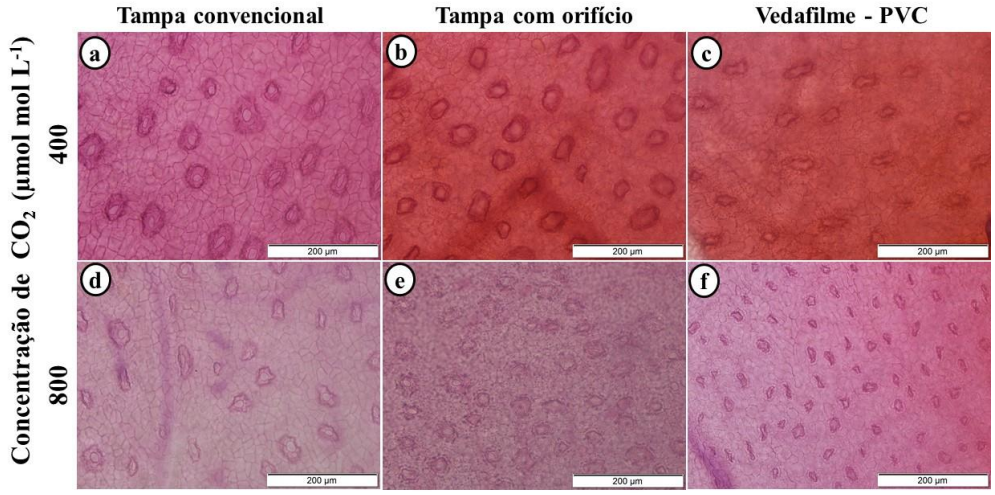

Figura 9. Superfície abaxial das folhas de *Mouriri elliptica* (Mart.) após 60 dias de aclimatização. Plantas oriundas do cultivo fotoautotrófico sob duas concentrações atmosférica de CO_2 e três vedações do frasco.

4.4 Discussão

Nas condições de cultivo estabelecidas neste trabalho, as plantas de *M. elliptica* (Mart.) cresceram e desenvolveram características que proporcionaram sua sobrevivência na casa de vegetação, em especial quando as mesmas foram cultivadas em frascos vedados com T. orif sob atmosfera ambiente de CO_2. Nesta condição de cultivo, obteve-se 100% de sobrevivência.

Utilizando-se frascos vedados com T. conv observou-se maior taxa de mortalidade. Neste sistema de vedação, a perda de água na forma de vapor para o ambiente foi menor em comparação com os demais tipos de vedação, assim, a umidade dentro dos frascos tornou-se maior. Alta umidade dentro dos frascos compromete a deposição de ceras epicuticulares e formação de estômatos funcionais, características

que comprometem a sobrevivência das plantas na aclimatização (Chandra, Bandopadhyay, Kumar & Chandra 2009; Saldanha et al., 2012).

Frascos vedados com T. orif, conforme apresentado por Saldanha et al. (2012), propicia trocas gasosas adequadas e incremento de CO_2 beneficiando os processos fotossintéticos das plantas. Estas vedações favorecem a diferenciação de tecidos parenquimáticos e vasculares das folhas, e com isso melhor é o crescimento *in vitro* (Ribeiro, Picoli, Lani, Vendrame & Otoni, 2009).

Para as espécies *Pfaffia glomerata* (Saldanha et al., 2013 e 2014) e *M. tetraphylla* (Cha-um, Chanseetis, Chitakovid, Pichakum & Supaibulwatana, 2011) o enriquecimento da atmosfera de cultivo *in vitro* com CO_2 proporcionou maior acúmulo de biomassa e alterações anatômicas e fisiológicas que indicam maior capacidade de sobreviver à aclimatização. Em *M. elliptica* (Mart.) a concentração de CO_2 (800 ± 35 µmol mol L^{-1}) utilizada neste trabalho não proporcionou maior vantagem para obtenção de mudas, demonstrando que os genótipos respondem de forma diferenciada às condições de cultivo *in vitro*. Entretanto, nas folhas observou-se influência da maior concentração de CO_2 na área de abertura das criptas estomáticas, diminuindo a área, em especial ao utilizar vedações que proporcionaram maior trocas gasosas com o ambiente.

Característica importante observada nas plântulas de *M. elliptica* (Mart.) foi a presença de sistema radicular, sendo este fator que beneficia a aclimatização (Saldanha et al., 2014). Neste estudo, o uso do suporte vermiculita associado à condições fotoautotróficas proporcionou enraizamento das plântulas *M. elliptica* (Mart.) *in vitro*, não sendo necessário a utilização de regulador de crescimento.

Plântulas de *M. elliptica* (Mart.) oriundas do cultivo *in vitro* com frascos vedados com PVC, tiveram maior incremento no comprimento das raízes na aclimatização, no entanto, apenas sob enriquecimento da atmosfera com CO_2. Este resultado não representou maior sucesso de aclimatização dessas plantas, pois, não significou maior incremento em biomassa da parte aérea e nem maior taxa de sobrevivência.

As plântulas cultivadas sob atmosfera ambiente de CO_2 e frascos vedados com T. orif tiveram melhor performance na aclimatização, no entanto, apresentou maior índice de dissipação não fotoquímica – Y (NPQ) e juntamente com as demais plantas, tiveram valores para rendimento quântico máximo do fotossistema II - Fv/Fm relativamente baixos (0,65). Valor este, observados em plantas sob condições de estresse, em *Hymenaea stigonocarpa* Mart. sob estresse luminoso e hídrico (Costa et al.,

2015) e em *Solanum lycopersicum* L. após inoculadas com *Xanthomonas gardneri* (Silveira et al., 2015).

Notou-se, portanto, que apesar das plântulas de *M. elliptica* (Mart.) obtidas *in vitro*, terem adquirido características que favoreceram a sobrevivência durante a aclimatização, este processo foi estressante para as mesmas. O que nos propõe hipóteses a serem testadas e respondidas em futuros trabalhos com a espécie, como por exemplo tipo de substrato, a água disponibilizada ou ainda aplicação de solução nutritiva. Inferindo se estes interferem na qualidade morfofisiológica das plantas no decorrer da aclimatização e proporcionam maior crescimento.

A aclimatização é a etapa mais crítica do processo de micropropagação, visto o estresse pelo qual as plantas são submetidas. As plantas deixam as condições de cultivo *in vitro* totalmente controladas e passam para o meio *ex vitro* no qual geralmente são expostas à condições adversas. Assim, para o sucesso da técnica, é de suma importância que as plantas possuam características morfológicas e fisiológicas adaptativas, conseguindo sobreviver nas condições *ex vitro* (Tanno & Biasi, 2013; Chandra et al., 2009; Bozena & Gabryszewska, 2016).

4.5 Conclusão

Melhor performance na aclimatização foi obtida em plântulas de *M. elliptica* (Mart.) cultivadas em frascos vedados com T. orif e atmosfera ambiente de CO_2.

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CONCLUSÃO GERAL

As plântulas de *M. elliptica* (Mart.) responderam positivamente as condições fotoautotrócas de cultivo *in vitro*, no qual o aumento da intensidade luminosa suprimiu a necessidade das plantas por sacarose no meio de cultivo. Verificou-se que as diferentes intensidades luminosas utilizadas neste estudo foram suficientes para compreender o comportamento desta espécie *in vitro*, subsidiar futuros trabalhos, visando assim maior produção de mudas.

Com o auxílio da técnica de estatística multivariada identificou que plântulas cultivadas sob condições fotoautotróficas desenvolveram características anatômicas foliares mais dissimilares as plantas *in situ*. Tal conclusão, indicou a necessidade de aclimatização das plântulas de *M. elliptica* (Mart.) micropropagadas sob condições fotoautotróficas.

Os suportes alternativos vermiculita, seguido do bagaço de cana-de-açúcar são promissores para utilização no cultivo *in vitro* da espécie *M. elliptica* (Mart.), visto a formação de sistema radicular nas plântulas com alto nível de diferenciação dos tecidos.

Foi possível obter 100% de sobrevivência de *M. elliptica* (Mart.) propagadas *in vitro* de forma fotoautotrófica e em cultivo com frascos vedados com tampa e orifício com membrana microporosa (T. orif) em atmosfera ambiente de CO_2.

Futuros trabalhos devem ser desenvolvidos com aclimatização da espécie, testando por exemplo tipos de substratos e presença ou não de câmara úmida, visando aumentar a qualidade fisiológica das mudas e diminuir o estresse ocasionado pelo processo.