ANYELA MARCELA RÍOS RÍOS

PRODUÇÃO E EXTRAÇÃO DE METABÓLITOS SECUNDÁRIOS DE DUAS PLANTAS MEDICINAIS PROPAGADAS *IN VITRO* E PRODUÇÃO DE LEVOGLUCOSANA POR PIRÓLISE RÁPIDA DE BAGAÇO DE CANA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Agroquímica, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 27 de fevereiro de 2019.

Edgard Augusto de Toledo Picoli

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(Coorientador)

Sergio Antonio Fernandes
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BIOAGRAFIA

Anyela Marcela Rios Rios, filha de Luz Nelly Rios Valencia e José Marino Rios Nieto, nasceu em Pereira, Risaralda - Colômbia, em 12 de março de 1985. Graduou-se em Química Industrial em setembro do 2010 pela Universidade Tecnológica de Pereira. Durante os anos 2010 - 2012, trabalhou como assistente de pesquisa no Laboratório de Biotecnología - Productos Naturales (GB-PN) da Universidade Tecnológica de Pereira, desenvolvendo pesquisas nas áreas de cultura de tecidos vegetais *in vitro* e produtos naturais.

Iniciou o mestrado em Agroquímica em março de 2012 no Departamento de Química da Universidade Federal de Viçosa (UFV), tendo defendido a dissertação em fevereiro de 2014. Iniciou o doutorado em Agroquímica em março de 2014 na Universidade Federal de Viçosa (UFV), na linha de pesquisa de produtos naturais, tendo defendido tese em fevereiro de 2019 sob a orientação do Dr. Sérgio Antônio Fernandes e a coorientação do Dr. Wagner Campos Otoni.

SUMÁRIO

RESUMO

RÍOS-RÍOS, Anyela Marcela, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Produção e extração de metabólitos secundários de duas plantas medicinais propagadas *in vitro* e produção de levoglucosana por pirólise rápida de bagaço de cana.** Orientador: Sergio Antonio Fernandes. Coorientador: Wagner Campos Otoni.

Diversas espécies vegetais e combustíveis fósseis têm sido utilizados como fonte de compostos químicos de interesse comercial. A preocupação pela conservação dos recursos naturais tem levado ao desenvolvimento de estratégias bioeconômicas que permitam diminuir sua sobre-exploração. Plantas medicinais propagadas em condições *in vitro* têm sido utilizadas para a produção biotecnológica de substâncias bioativas como compostos orgânicos voláteis (VOCs) e fenóis; enquanto a biomassa de cana de açúcar tem sido empregada como fonte renovável de biocombustíveis e compostos químicos, como a levoglucosana (LGA). No entanto, protocolos de cultivo *in vitro* ainda têm que ser otimizados para incrementar o desenvolvimento das plantas e a produção de compostos de interesse em espécies como *Ruta graveolens* e *Piper crassinervium*. Assim mesmo, técnicas como a pirólise de biomassa para a produção de LGA ainda precisam ser aprimoradas. Em este trabalho foi estudado o efeito: (i) da qualidade espectral da luz sobre a produção de VOCs em plantas propagadas *in vitro* de *R. graveolens*; (ii) de diferentes condições de cultivo sobre a propagação *in vitro* de *P. crassinervium* e o acúmulo de compostos fenólicos (iii) do pré-tratamento ácido com cálix[*n*]areno de bagaço de cana para a obtenção de LGA via pirólise rápida. Para tal, plantas de *R. graveolens* foram cultivadas sob lâmpadas LEDs branca, azul, azul-vermelha, vermelha (V) e vermelha distante (VD); os VOCs foram extraídos por microextração em fase sólida através do headspace (HS-SPME) e as condições de extração foram otimizadas previamente. Fatores abióticos foram testados no desenvolvimento *in vitro* de plantas de *P. crassinervium* a partir de segmentos nodais (SN) cultivados sob condições controladas. O bagaço de cana foi tratado com ácido *p*-sulfônico calix[4]areno para a remoção de interferentes; posteriormente, a biomassa tratada foi pirolizada para a produção de açúcares pirolíticos (LGA). Como resultado, o tipo de recobrimento da fibra de SPME, o tempo de extração e a quantidade de biomassa utilizada para a extração de VOCs por HS-SPME de *R. graveolens* foram otimizadas; LEDs V e VD influenciaram na

produção de sesquiterpenos e alguns álcoois alifáticos nas plantas propagadas *in vitro* de *R. graveolens*. O protocolo de propagação *in vitro* de *P. crassinervium* estabelecido neste trabalho, resultou no incremento da biomassa e de compostos fenólicos. Foi demonstrado pela primeira vez que o pré-tatamento da biomassa com organocatalisadores como os cálix[*n*]arenos incrementa o rendimento da LGA em 12 vezes comparado com a biomassa não tratada. Este trabalho pode contribuir à elucidação de outros fatores que participam nas rotas de biossíntese de VOCs de interesse comercial; um protocolo de propagação *in vitro* eficiente, tanto para a obtenção de compostos fenólicos como de biomassa, foi estabelecido para *P. crassinervium*. O pré-tratamento ácido de bagaço de cana com calix[*n*]arenos forneceu uma metodologia eficiente para incrementar a produção de LGA.

ABSTRACT

RÍOS-RÍOS, Anyela Marcela, D.Sc., Universidade Federal de Viçosa, February, 2019. **Production and extraction of secondary metabolites of two medicinal plants propagated *in vitro* and production of levoglucosan by rapid pyrolysis of sugarcane bagasse.** Adviser: Sergio Antonio Fernandes. Co-Adviser: Wagner Campos Otoni.

Various plant species and fossil fuels have been used as a source of commercially available chemical compounds. Concern over the conservation of natural resources has led to the development of bioeconomic strategies to reduce their overexploitation. Medicinal plants propagated under *in vitro* conditions have been used for the biotechnological production of bioactive substances such as volatile organic compounds (VOCs) and phenols; while sugarcane biomass has been used as a renewable source of biofuels and chemical compounds, such as levoglucosan (LGA). However, *in vitro* culture protocols still have to be optimized to increase plant development and the production of compounds of interest in species such as *Ruta graveolens* and *Piper crassinervium*. Also, techniques such as biomass pyrolysis for LGA production still need to be improved. In this work the effect of: (i) spectral light quality on the production of VOCs in *in vitro* plants propagated of *R. graveolens*; (ii) different culture conditions on the *in vitro* propagation of *P. crassinervium* and the accumulation of phenolic compounds (iii) acid pretreatment of sugarcane bagasse with calix[*n*]arene to obtain LGA via rapid pyrolysis was studied. For this, *R. graveolens* plants were cultivated under white, blue, blue-red, red (R) and far-red (FR) LEDs; the VOCs were extracted by solid phase microextraction through the headspace (HS-SPME) and the extraction conditions were optimized previously. Abiotic factors were tested in the *in vitro* development of *P. crassinervium* plants from nodal segments (NS) grown under controlled conditions. The sugarcane bagasse was treated with *p*-sulfonic acid calix[4]arene for the removal of interferents; the treated biomass was pyrolyzed for the production of pyrolytic sugars (LGA). As a result, the SPME fiber coating type, the extraction time and the amount of biomass used for the extraction of VOCs by HS-SPME of *R. graveolens* were optimized; R and FR LEDs influenced the production of sesquiterpenes and some aliphatic alcohols in the *in vitro* propagated plants of *R. graveolens*. The *in vitro* propagation protocol of *P. crassinervium* established in this work resulted in the increase of

biomass and phenolic compounds. It has been demonstrated for the first time that biomass pretreatment with organocatalysts such as calix[*n*]arenes increases the yield of LGA by 12-fold compared to untreated biomass. This work can contribute to the elucidation of other factors that participate in the commercial interest VOCs biosynthesis routes; an efficient *in vitro* propagation protocol for both phenolic compounds and biomass was established for *P. crassinervium*. The pre-treatment of sugarcane bagasse with calix[n]arenes provided an efficient methodology to increase the production of LGA.

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INTRODUÇÃO

A biossíntese dos metabólitos nas plantas é afetada pela variação genética, mudanças climáticas, nutrientes do solo e invasão de patógenos ou herbívoros. Isso limita a obtenção de grandes quantidades de compostos bioativos a partir de plantas cultivadas em campo (Verpoorte et al. 1999; Isah et al. 2018). Diversos compostos têm sido sintetizados em laboratório; porém, a síntese química de alguns compostos de interesse comercial, como o taxol, é difícil ou economicamente inviável. O taxol é um diterpeno isolado da casca de *Taxus* spp., utilizado no tratamento contra o câncer e de doenças neurodegenerativas. A alta complexidade da estrutura do taxol e os requerimentos estereoquímicos têm limitado sua obtenção a partir da síntese química (Yue et al. 2014; Kundu et al. 2017; Matsuura et al. 2018). Em consequência, a demanda por plantas medicinais para o isolamento e síntese de compostos de interesse farmacológico incrementou, levando à superexploração de determinadas culturas. Com isso, metodologias alternativas, como a cultura *in vitro* de tecidos vegetais, são necessárias para conservar o germoplasma e induzir simultaneamente a produção de metabólitos secundários de interesse farmacológico e agroindustrial (Verpoorte et al. 2002; Kundu et al. 2017; Matsuura et al. 2018).

Por outro lado, a exploração de materiais fósseis para a produção de polpa celulósica e papel, etanol, combustíveis, dentre outros, tem incrementado. Isso leva à necessidade de desenvolver tecnologias que permitam o reaproveitamento de resíduos lignocelulósicos (Li et al. 2015; Kim et al. 2016). A biomassa tem atraído a atenção mundial como fonte de energia renovável; permite a produção de calor e energia de forma sustentável e neutra em CO_2 e pode ser convertida em combustíveis líquidos e produtos de valor agregado (Meier e Faix 1999; Anca-Couce 2016). No entanto, a conversão da biomassa em biocombustíveis ou produtos químicos requer tratamento por processos químicos, termoquímicos ou bioquímicos. A eficiência desses tratamentos depende do tipo de biomassa, da sua composição química e da estrutura do material lignocelulósico. Estabelecer novos métodos ou aprimorar os já existentes é um desafio para o aproveitamento dos resíduos agroindústrias para produção de compostos de alto valor agregado como levoglucosana e 5-hidroximetilfurfural (Sarkar et al. 2012; Zhang et al. 2013; David et al. 2017).

1. Obtenção de metabólitos secundários a partir de plantas medicinais cultivadas em condições *in vitro*

Plantas medicinais têm sido utilizadas como fonte natural de compostos ativos com aplicações nas indústrias farmacêutica, agroquímica e alimentícia, aumentando sua importância econômica (Lopes et al. 2008; Orlanda e Nascimento 2015; Peixoto et al. 2015). Alguns dos metabólitos de interesse comercial são compostos orgânicos voláteis (VOCs) e compostos fenólicos.

Os VOCs são principalmente da classe dos terpenos, derivados de ácidos graxos, fenilpropanoides/benzenoides e derivados de aminoácidos (Fig 1) (Dudareva et al. 2013; Fu et al. 2015). Os VOCs são biosintetizados ou armazenados em tricomas, osmóforos, ductos e cavidades secretoras presentes em folhas, caules, raízes e flores (Glas et al. 2012; Rehman et al. 2016).

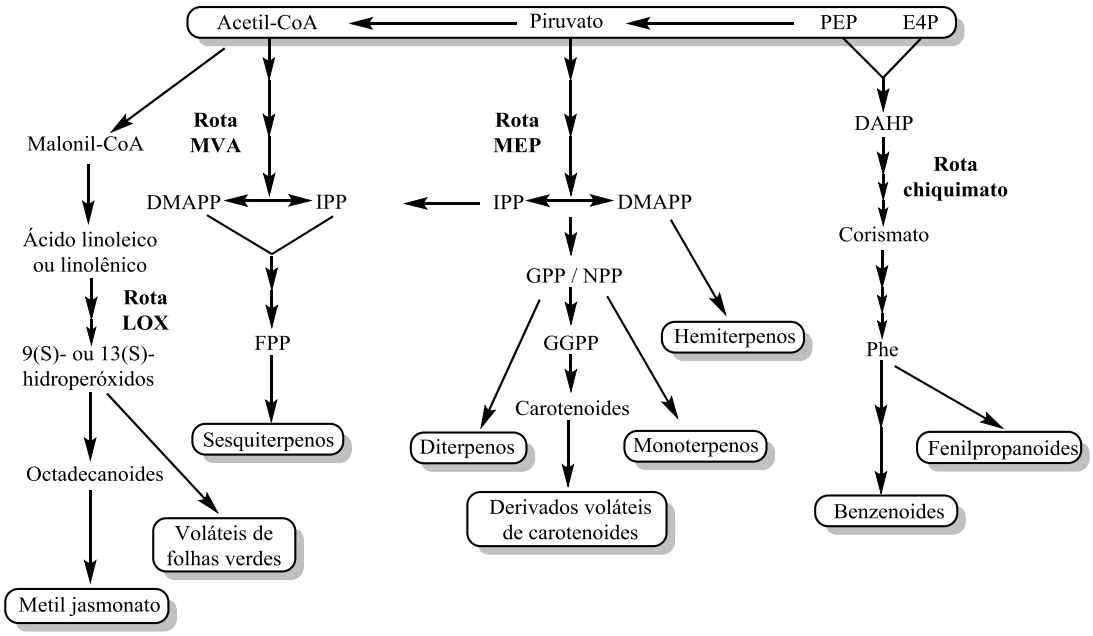

Figura 1. Principais rotas de biossíntese de compostos orgânicos voláteis nas plantas. **PEP**: fosfo*enol*piruvato; **E4P**: eritrose 4-fosfato; **MVA**: ácido mevalónico; **MEP**: metileritritol fosfato; **DAHP**: 3-desoxi-*D*-arabinoheptulosonato-7-fosfato; **DMAPP**: dimetilalil difosfato; **IPP**: isopentenil pirofosfato; **LOX**: lipoxigenase; **FPP**: farnesil pirofosfato; **GGPP**: geranilgeranil pirofosfato; **GPP**: geranil pirofosfato; **NPP**: neril pirofosfato; **Phe:** fenilalanina. Setas sobrepostas indicam o envolvimento de vàrias reações enzimáticas (Dudareva et al. 2013).

Terpenoides são formados a partir de unidades de isopreno (C_5): isopentenil difosfato (IPP) e o seu isômero dimetilalil difosfato (DMAPP). O IPP e o DMAPP são biossintetizados pela via do mevalonato (MVA), a partir da acetil-CoA no

citoplasma e, pela via do metileritritol fosfato (MEP), a partir do piruvato e do gliceraldehído-3-fosfato nos plastídeos. Em seguida, geranil difosfato (GPP), farnesil difosfato (FPP) e geranilgeranil difosfato (GGPP) são produzidos mediante reações de trans-prenilação. A trans-prenilação envolve a condensação de uma ou mais moléculas de IPP com o DMAPP, catalisada por enzimas terpeno sintase. O GPP, FPP e GGPP geram monoterpenos (C_{10}), sesquiterpenos (C_{15}) e diterpenos (C_{20}), respetivamente, envolvendo a formação de carbocations intramoleculares (Fig 2). Em alguns casos, mono-oxigenases, redutases, desidrogenases e transferase do citocromo P450, dentre outras, contribuem para a diversidade estrutural dos terpenos a través de reações de oxidação, deshidrogenação, metilação e acilação (Dudareva et al. 2004; Arimura et al. 2017; Ro et al. 2017).

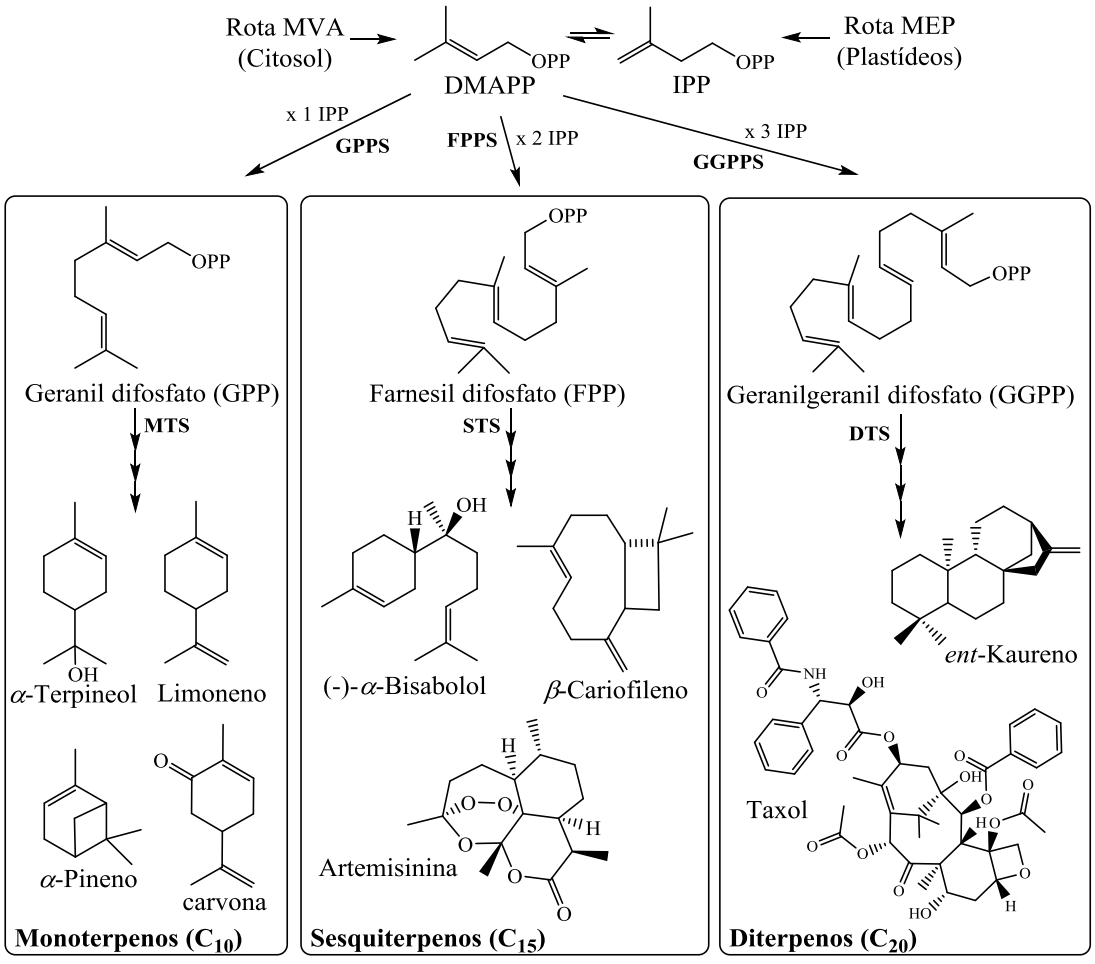

Figura 2. Esquema geral da rota de biossíntese de terpenoides. **MVA**: ácido mevalónico; **MEP**: metileritritol fosfato; **DMAPP**: dimetilalil difosfato; **IPP**: isopentenil pirofosfato; **GPPS**: geranil pirofosfato sintase; **FPPS**: farnesil pirofosfato sintase; **GGPPS**: geranilgeranil pirofosfato sintase; **MTS**: monoterpeno sintase; **STS**: sesquiterpeno sintase; **DTS**: diterpeno sintase. Setas sobrepostas indicam o

envolvimento de vàrias reações enzimáticas (Dudareva et al. 2004; Arimura et al. 2017; Ro et al. 2017).

VOCs da classe dos benzenoides, fenilpropanoides, fenilbutanoides e fenilpropenos são biossintetizados a partir dos precursores fosfo*enol*piruvato (PEP) e *D*-eritrose 4-fosfato (E4P) pela rota do chiquimato. Inicialmente o ácido cinâmico é formado a partir da desaminação do aminoácido fenilalanina (Phe) pela fenilalanina amônia liase (PAL). Nas seguintes etapas as enzimas benzaldeído desidrogenase (BALDH), ácido benzoico carboxil metiltransferase (BAMT), benzalacetona sintase (BAS), dentre outras, atuam produzindo álcool benzílico, benzoato de metila e fenilbutanoides. A vanilina sintase (VAN) catalisa a conversão direta do ácido ferúlico em vanilina. Fenilpropanoides C_6-C_2 (fenilacetaldeído, 2-feniletanol, acetato de 2-feniletila) podem ser formados diretamente a partir da Phe pela ação da fenilacetaldeído sintase (PAAS) ou em duas etapas, envolvendo reações de transaminação e descarboxilação, dependendo da espécie vegetal (Fig 3) (Dudareva et al. 2013; Arimura et al. 2017).

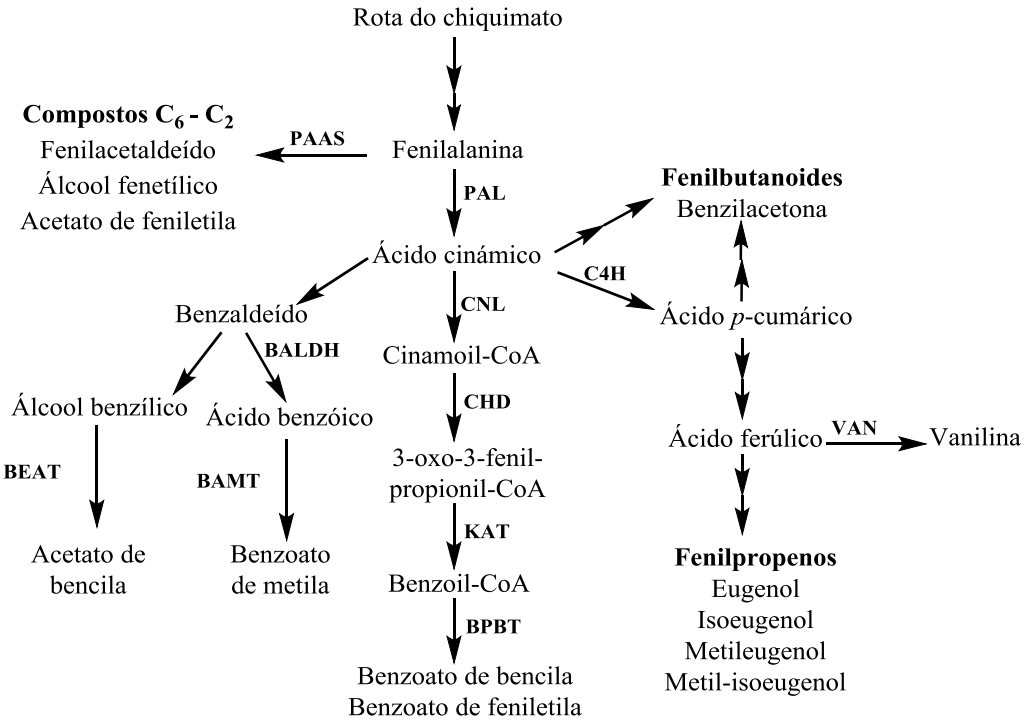

Figura 3. Principais etapas da biossíntese de fenilpropanoides/benzenoides pela via do chiquimato. **PAAS**: fenilacetaldeído sintase; **PAL**: fenilalanina amônia liase; **CNL**: cinamato:CoA ligase; **C4H**: cinamato-4-hidroxilase; **BALDH**: benzaldeído desidrogenase; **BEAT**: benzil álcool acetiltransferase; **BAMT**: ácido benzoico carboxi metiltransferase; **CHD**: cinamoil-CoA hidratase-desidrogenase; **KAT**: 3-cetoacil-CoA tiolase; **BPBT**: benzoil-CoA:benzil álcool/feniletanol benzoiltransferase; **VAN**: vanilina sintase (Arimura et al. 2017).

VOCs derivados de ácidos graxos como metil jasmonato, ésteres, álcoois e aldeídos (C_6 - C_9, conhecidos como voláteis de folhas verdes) são biossintetizados pela rota da lipoxigenase (LOX) ou oxilipina (Fig 1). Ácidos graxos insaturados C_{18} (ácidos linoleico ou linolênico) sofrem oxigenação estereoespecífica formando intermediários 9 e 13-hidroperoxi. Esses intermediários levam á formação de aldeídos C_6 e C_9 que posteriormente geram os álcoois e ésteres correspondentes, mediante reações enzimáticas com hidroperòxido liase e álcool desidrogenase (Dudareva et al. 2013; Ul Hassan et al. 2015). Metil cetonas voláteis (C_7 - C_{15}) são biossintetizadas a partir da descarboxilação de 3-cetoácidos catalisada por metil-cetona sintetase 1 (MKS1). Os 3-cetoácidos são obtidos mediante a hidrólise de intermediários da proteína transportadora 3-cetoacil-acil, produzidos durante a biossíntese de ácidos graxos e, catalisada pela MKS2 (Glas et al. 2012).

Os VOCs são sintetizados e emitidos pelas plantas em resposta a estresses bióticos (ataque de herbívoros ou patógenos) e abióticos (danos mecânicos, luz, temperatura, nutrientes) (Baldwin et al. 2006; Carvalho et al. 2016; Farzadfar et al. 2017). Os VOCs estão envolvidos nos mecanismos de defesa da planta induzindo ou ativando a resistência em tecidos distantes e/ou próximos ao local danificado e/ou em plantas vizinhas à planta afetada. Os VOCs podem ser emitidos pela planta para atrair predadores ou parasitas que atacam seus inimigos naturais ou para atrair polinizadores e agentes dispersores de sementes (Quintana-Rodriguez et al. 2015; Aljbory et al. 2018; Fontes et al. 2018). Alguns VOCs apresentam atividade alelopática inibindo a germinação ou o desenvolvimento de plantas vizinhas que competem pelos nutrientes do solo, fonte de luz ou polinizadores (de Feo et al. 2002).

Os compostos fenólicos apresentam um ou mais grupos hidroxila diretamente ligados a um anel benzênico. Entre os compostos fenólicos se encontram os taninos, flavonoides (flavonois, flavonas, isoflavonas, flavanonas, antocianidinas e flavanois), cumarinas, estilbenos, lignanas e ácidos fenólicos (ácido caféico, clorogênico, cinâmico, ferúlico, gálico, *p*-hidroxibenzoico, *p*-cumárico, protocatecúico, rosmarínico, siríngico e vanílico) (Quideau et al. 2011; Sevgi et al. 2015). Fenilpropanoides são biossintetizados pelas plantas através da via do chiquimato. Inicialmente, a Phe sofre desaminação produzindo o ácido cinâmico, que por sua vez, leva à biossíntese de cumarinas e de ácido *p*-cumárico. Posteriormente, o ácido *p*-

cumárico leva à formação de ácidos hidroxicinâmicos (ácidos caféico, ferúlio e sinápico), lignina ou flavonoides (Fig 4). A diversidade estrutural dos flavonoides é resultado de reações de glicosilação, metilação, hidroxilação, acilação e prenilação, dentre outras (Vog 2010; Deng e Lu 2017; Dias et al. 2016; Lee et al. 2018)

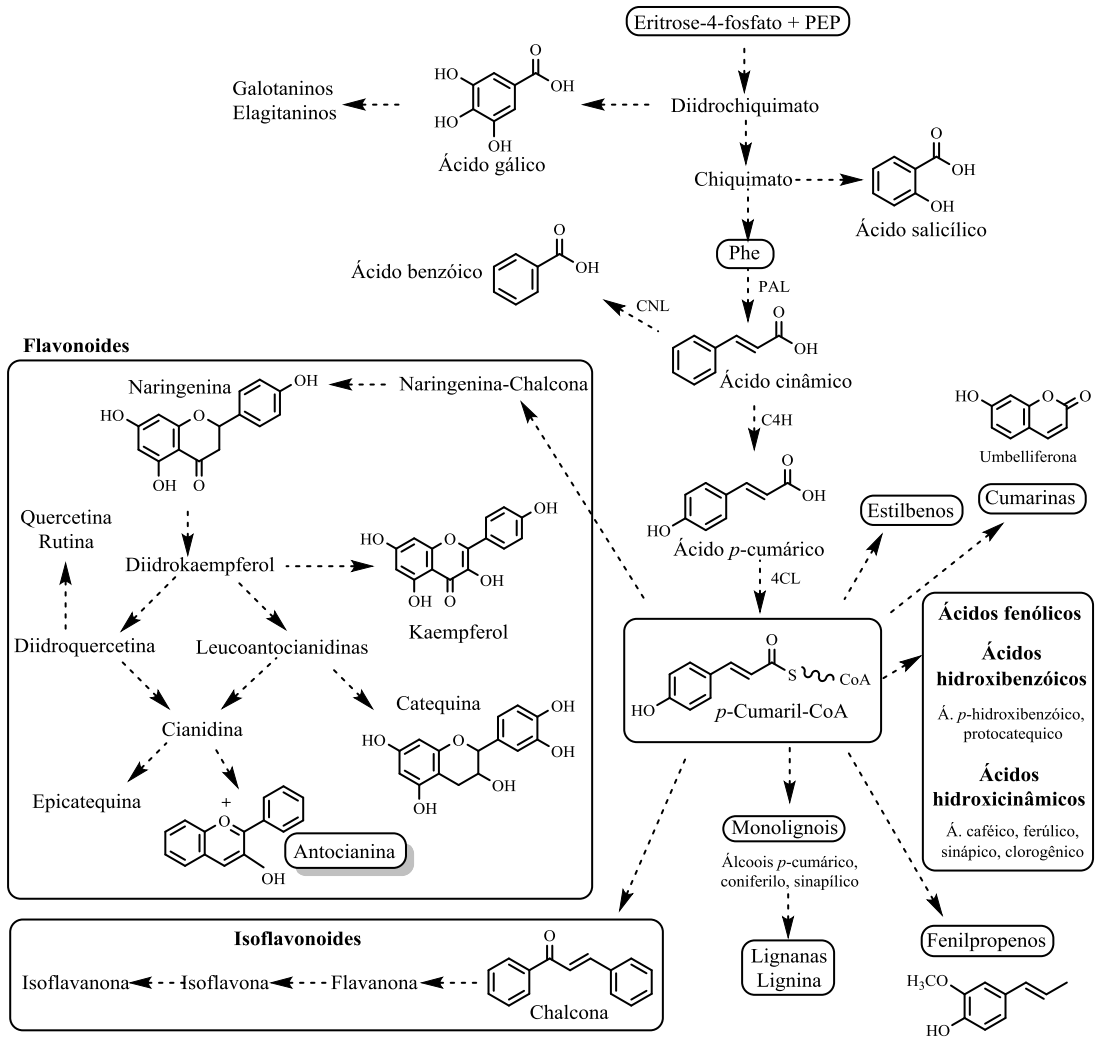

Figura 4. Rota de biossíntese de compostos fenólicos. **PEP**: fosfo*enol*piruvato; **Phe:** fenilalanina; **PAL**: fenilalanina amônia liase; **CNL**: cinamato:CoA ligase; **C4H**: cinamato-4-hidroxilase; **4CL**: hidroxicinamato-CoA ligase (Vog 2010; Deng e Lu 2017; Dias et al. 2016; Lee et al. 2018).

Os compostos fenólicos interferem na pigmentação da planta e conferem resistência contra patógenos, pragas, herbívoros e radiação solar (principalmente contra o dano do DNA causado pela luz UV-B) às plantas, dentre outros (Quideau et al. 2011; Dillon et al. 2018). Os mesmos possuem a capacidade de sequestrar espécies reativas de oxigênio (ROS) tais como $^{\bullet}OH$, $NO^{\bullet}$, $O_2^{-\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$, H_2O_2, O_2 e HOCl, atuando como antioxidantes e diminuindo o estresse oxidativo dos tecidos (Quideau et al. 2011; Szopa et al. 2018). Considerando o amplo número de funções

que os compostos fenólicos cumprem nas plantas e às propriedades físico-químicas do grupo funcional fenol, tem sido crescente o interesse sobre sua biossíntese em tecidos cultivados *in vitro* (Dias et al. 2016; Szopa et al. 2018). Apesar dos grandes avanços alcançados nos últimos anos, ainda é um desafio iminente estabelecer protocolos eficientes de propagação de plantas que permitam elucidar os fatores que interferem na rota de biossíntese dos fenóis, dentre outras classes de metabólitos secundários (Yue et al. 2014; Dias et al. 2016; Szopa et al. 2018).

Tanto órgãos especializados como células com diferentes graus de diferenciação (calos e suspensões celulares) cultivados *in vitro* de várias espécies vegetais, sintetizam metabólitos secundários de interesse farmacológico. Isso tem permitido que a produção biotecnológica em culturas de tecidos de plantas seja uma alternativa para a obtenção de compostos como artemisinina, capsaicina, morfina, resveratrol, tanshinona, taxol, vincristina e vimblastina, dentre outros (Atanasov et al. 2015; Yue et al. 2014; Cioć et al. 2018). A produção de metabólitos secundários em tecidos vegetais cultivados *in vitro* tem sido estimulada mediante o uso de elicitores bióticos (quitosana, micélio de fungos, bactérias e leveduras, dentre outros) e abióticos (composição do meio de cultura, qualidade e intensidade de luz, CO_2, dentre outros) (Yue et al. 2014; Batista et al. 2018; Cioć et al. 2018).

A qualidade da luz é um dos fatores abióticos mais estudados nos últimos anos visando incrementar a produção de biomassa e de compostos de interesse comercial. Variações na qualidade da luz afetam vários processos fisiológicos e bioquímicos das plantas, influenciando no crescimento e desenvolvimento dos tecidos vegetais. Diferentes qualidades espectrais da luz (comprimentos de onda) têm estimulado de forma distinta a produção de compostos fenólicos, enzimas antioxidantes e voláteis em tecidos vegetais cultivados *in vitro* de várias plantas medicinais. A qualidade da luz interfere na regulação fotoquímica da planta mediante a ativação seletiva dos fotorreceptores; isso leva a mudanças na expressão de genes envolvidos na biossíntese de diversos metabólitos primários e secundários, incluindo lignina e celulose (Alvarenga et al. 2015; Ahmad et al. 2016; Batista et al. 2018; Mamedes-Rodrigues et al. 2018).

Dentre as plantas medicinais utilizadas para a indução da produção de metabólitos de interesse em tecidos cultivados em condições *in vitro* se encontram

Piper crassinervium e *Ruta graveolens* (Kuzovkina et al. 2009; Delgado-Paredes et al. 2012; Faisal et al. 2018).

1.1 Plantas medicinais objeto de estudo

1.1.1 *Ruta graveolens*

Ruta graveolens L. (Rutaceae), mais conhecida como arruda é nativa do sul da Europa e distribuída no norte da África, Ásia, regiões do Mediterrâneo e zonas temperadas em todo o mundo. É usada na medicina tradicional para o tratamento de doenças gastrointestinais e ópticas, dores severas, reumatismo, rigidez do pescoço, tontura, dor de cabeça e artrite (Nazish et al. 2009; Khan et al. 2011). Na parte aérea da planta são encontrados esterois, esteroides, aminas, alcaloides, cumarinas (chalepeusina e graveliferona), flavonoides (rutina e hesperidina), taninos, proteínas, aminoácidos, carboidratos, resinas, lipídeos, mucilagem, metilcetonas, glicosídeos e óleos essenciais (Nazish et al. 2009; Hamad 2012; Giresha et al. 2012). Compostos esses responsáveis por atividades biológicas como alelopática, inseticida e antioxidante, dentre outras (de Feo et al. 2002; da Silva et al. 2014; Giresha et al. 2012).

Os óleos essenciais (OEs) da arruda possuem atividade nematicida, acaricida, inseticida e alelopática, dentre outras (de Feo et al. 2002; Faria et al. 2015; da Silva et al. 2014). Entre os componentes principais dos OEs encontram-se: undecan-2-ona, nonan-2-ona, undecan-2-ol e nonan-2-ol; terpenoides como limoneno, α-pineno, linalol e 1,8-cineol; sesquiterpenos, furanocumarinas, ostol, halepensina, rutacultina, pregeijereno, geijereno, ácido hexanóico, ácido octanóico e xantotoxina (Fig. 5) (de Feo et al. 2002; Kuzovkina et al. 2009; Orlanda e Nascimento 2015). Compostos como a nonan-2-ona e undecan-2-ona têm atividade inseticida contra mosquitos vetores da febre amarela e da malária, o que tem incrementado a importância econômica desta espécie (da Silva et al. 2014). No entanto, os fatores que interferem na produção *in vitro* de VOCs em *R. graveolens* têm sido pouco explorados.

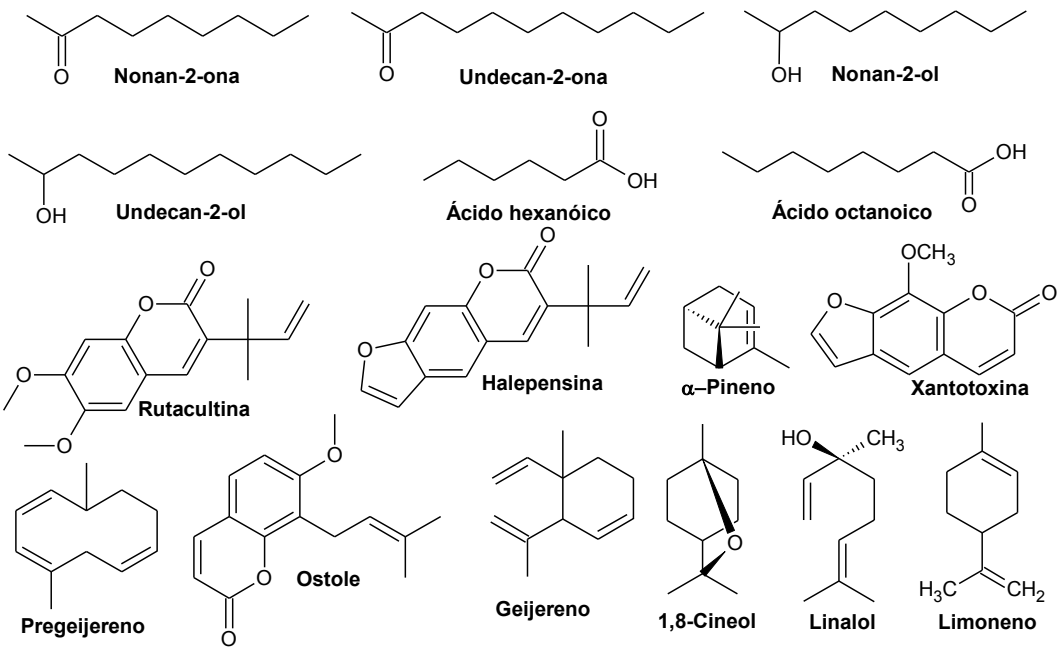

Figura 5. Componentes principais do óleo essencial de *Ruta graveolens* (de Feo et al. 2002; Kuzovkina et al. 2009; Orlanda e Nascimento 2015).

1.1.2 *Piper crassinervium*

Piper crassinervium (Piperaceae) é uma planta medicinal do tipo arbustiva, distribuída na Colômbia, Equador, Peru e na Floresta Atlântica do Brasil. Floresce nos meses de abril e outubro; as inflorescências são do tipo espiga, de coloração amarela quando maduras (Albieiro et al. 2005). As atividades biológicas mais relatadas para essa espécie são antifúngica, antioxidante e anti-chagásica (Yamaguchi et al. 2006; Lopes et al. 2008; Lago e Kato 2007).

Extratos de *P. crassinervium* contêm derivados de piperidona (3α, 4α-epoxi-2-piperidona), flavanonas (naringenina e sakuranetina), ácidos 4-hidroxibenzóicos prenilados [ácido 4-hidroxi-(3',7'-dimetil-1'-oxo-2'*E*,6'-dienil)benzóico (**1**); ácido 4-hidroxi-3-(3',7'-dimetil-3'-hidroxi-1'-oxo-6'-octenil)benzóico (**2**)] e hidroquinonas preniladas [1,4-dihidroxi-2-(3',7'-dimetil-1'-oxo-2'*E*,6-octadienil)benzeno (**3**)] (Fig. 6) (Lago et al. 2004; Lago e Kato, 2007; Lopes et al. 2008). Os compostos prenilados possuem grupos prenilo, geranilo ou nerolidol formados pela prenilação com dimetilalil pirofosfato (DMAPP), geranil pirofosfato (GPP) ou farnesil pirofosfato (FPP) (Kato e Furlan 2007).

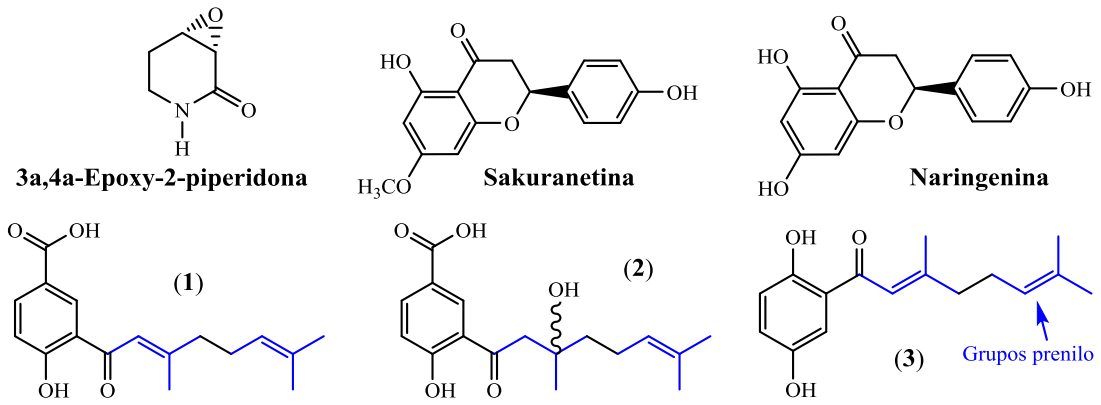

Figura 6. Alguns dos compostos biossintetizados por *Piper crassinervium* (Lopes et al. 2008; Lago et al. 2004).

A produção de biomassa de *Piper crassinervium* e outras espécies do gênero *Piper* em condições de cultivo *in vitro* ainda é ineficiente. Isso limita o estudo dos fatores bióticos e abióticos que estimulam a biossíntese de metabólitos de interesse, como os compostos fenólicos (Delgado-Paredes et al. 2012; Ahmad et al. 2013; Silva et al. 2014)

1.2 Produção e extração de compostos orgânicos voláteis (VOCs) a partir de plantas propagadas em condições *in vitro*

A produção de VOCs em células e tecidos vegetais de diversas plantas medicinais mantidos *in vitro* tem sido otimizada através da manipulação de fatores bióticos como: exposição a herbívoros, nematóides, bactérias, fungos, óleo essencial de plantas medicinais e plantas vizinhas infectadas ou não por patógenos (Maes e Debergh 2003; Faria et al. 2015; Zhou et al. 2016; Cellini et al. 2018). Fatores abióticos como: intensidade e qualidade da luz, fotoperíodo, concentração de nutrientes, sacarose, reguladores de crescimento, agentes gelificantes e osmóticos, CO_2 e etileno também interferem na biossíntese *in vitro* de VOCs (Fig. 7) (Musarurwa et al. 2012; Alvarenga et al. 2015; Batista et al. 2017a; Batista et al. 2017b; Batista et al. 2018; Jesionek et al. 2017; Bekircan et al. 2018). A produção de VOCs pode ser maximizada através do uso de biorreatores ou estratégias para coletar dinamicamente os VOCs durante a propagação *in vitro* das plantas (Durenne et al. 2017; Jesionek et al. 2017).

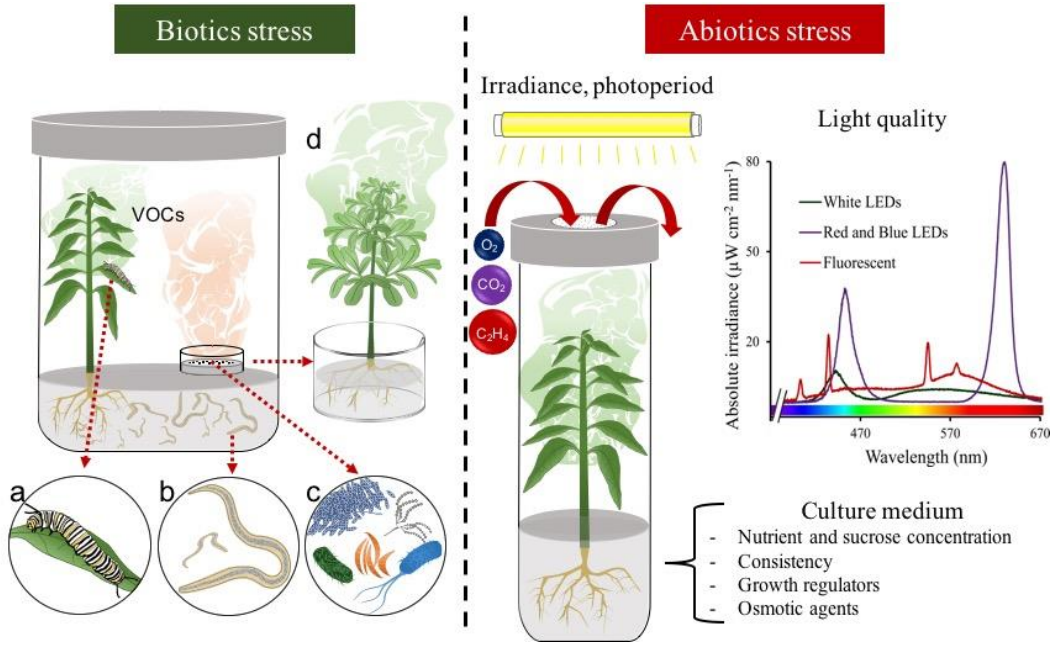

Figura 7. Fatores bióticos e abióticos que interferem na biossíntese de compostos orgânicos voláteis (**VOCs**) em células vegetais e tecidos cultivados em condições *in vitro*. (Plantas micropropagadas expostas a herbívoros (**a**), nematóides (**b**), bactérias, fungos (**c**) e outras plantas aromáticas (**d**); **O_2**: Oxigênio; **CO_2**: dióxido de carbono; **C_2H_4**: etileno. Setas curvas vermelhas indicam a troca gasosa entre os ambientes *in vitro* e *ex vitro*.

No entanto, a biomassa disponível para a extração de VOCs e OEs de material cultivado *in vitro* é limitada. A microextração em fase sólida através do headspace (HS-SPME) tem sido um dos métodos mais adequados para a análise de VOCs a partir de baixas quantidades de biomassa (Bassolino et al. 2015; Jesionek et al. 2017). A SPME é uma técnica livre de solventes, simples e rápida que permite a extração direta de VOCs, evitando sua degradação ou perda em comparação com outros métodos de extração de OEs (Stashenko e Martínez 2007; Mesquita et al. 2017).

Na extração HS-SPME os VOCs presentes no headspace acima da amostra são absorvidos ou adsorvidos em uma pequena quantidade de revestimento líquido ou sólido (polímero polar ou não polar ou adsorvente bipolar) disperso em um suporte (fibra óptica de sílica fundida ou fibra de aço inoxidável). Os VOCs são concentrados na fibra após um certo tempo de exposição e são subseqüentemente dessorvidos diretamente no injetor do cromatógrafo a gás para ser análisados (Pawliszyn 2012; Liu e Ouyang 2017; Mesquita et al. 2017).

A extração de VOCs por HS-SPME a partir de material vegetal cultivado *in vitro* tem sido realizada diretamente no frasco de cultura (Maes et al. 2001; Bassolino et al. 2015). No entanto, os recipientes de cultivo contêm alguns VOCs contaminantes (hidrocarbonetos, ftalatos e aldeídos): I) emitidos pela tampa de plástico usada para selar o frasco (Bassolino et al. 2015); II) remanescente de outras culturas (como terpenos) que não foram removidas durante a limpeza do material e III) adsorvido durante a esterilização por autoclavagem dos vasos contendo ou não o meio de cultura (Maes et al. 2001), o que pode interferir nas análises. Além disso, o meio de cultura pode absorver alguns VOCs polares emitidos pelo material vegetal reduzindo sua concentração no headspace e limitando a sua extração (Maes et al. 2001). Com isso, o mais recomendado é realizar a extração de VOCs por HS-SPME transferindo uma quantidade determinada do material vegetal para um outro frasco livre de contaminantes (Passinho-Soares et al. 2017).

As fibras de SPME revestidas com polidimetilsiloxano (PDMS, 100 μm), carboxen/polidimetilsiloxano (CAR/PDMS, 75 μm), polidimetilsiloxano/divinilbenzeno (PDMS/DVB, 65 μm), divinilbenzeno/Carboxen sobre PDMS (DVB/CAR/PDMS, 50/30 μm) e poliacrilato (PA, 85 μm) têm sido utilizadas para a extração de VOCs de plantas propagadas *in vitro*. O tempo de equilíbrio e o tempo de exposição da fibra variaram de 15-60 min e a temperatura de extração de 25-80 ° C (Maes et al. 2001; Maes e Debergh 2003; Musarurwa et al. 2012; Passinho-Soares et al. 2013; Bekircan et al. 2018; Cellini et al. 2018).

A eficiência da extração de VOCs por HS-SPME é afetada pelo revestimento da fibra, temperatura, tempo de equilíbrio dos VOCs no headspace e tempo de exposição das fibras (Mesquita et al. 2017). Portanto, as condições de extração do HS-SPME devem ser otimizadas dependendo da espécie e do material vegetal analisado.

2. Obtenção de açúcares a partir de biomassa residual de processos industriais

A cana-de-açúcar é utilizada como matéria prima para a produção industrial de etanol de segunda geração, empregado como um combustível alternativo à gasolina que reduz a emissão de gases responsáveis pelo efeito estufa (CO_2, CH_4 e NO_2). Grandes quantidades de biomassa lignocelulósica são geradas como

subproduto após esse tipo de processo agroindustrial. Essa biomassa pode ser convertida em biocombustíveis ou produtos químicos após ser submetida a processos químicos, termoquímicos ou bioquímicos (Ortiz et al. 2013; Kan et al. 2016).

A composição média do bagaço de cana varia de 40 a 50% de celulose, 20 a 30% de hemicelulose, 20 a 25% de lignina e 1,5 a 3% de cinzas (Garcia-Perez et al. 2002; Ortiz et al. 2013). O bagaço de cana é utilizado pelas indústrias sucroalcooleiras para a geração de energia através da sua combustão ou para a produção de etanol de segunda geração; no entanto, grande quantidade desse bagaço residual é descartado (Garcia-Perez et al. 2002; Hoi et al. 2013). Açúcares constituintes da biomassa podem ser utilizados para a produção de compostos com importantes aplicações industriais (Sarkar et al. 2012).

A conversão de biomassa em açúcares tem sido realizada através da tecnologia de pirólise (convencional ou rápida) (Zhang et al. 2013; Dewangan et al. 2016). A pirólise convencional maximiza o rendimento de gás combustível utilizando alta temperatura (500 °C), baixa taxa de aquecimento (10 °C min^{-1}) e longo tempo de residência dos vapores (60 min), produzindo principalmente bio-óleo, biocarvão e gases não condensáveis. A pirólise rápida é realizada com elevada taxa de aquecimento (>100 °C min^{-1}) e de transferência de calor na ausência de oxigênio. Pequenas partículas de biomassa (<2 mm) são alimentadas no reator a altas temperaturas (450 - 650 °C) seguido do rápido esfriamento dos vapores resultantes da pirólise para suprimir reações secundárias, gerando o bio-óleo bruto (Sarkar et al. 2012; Jiang et al. 2015; Kan et al. 2016).

A maioria dos processos de pirólise é realizada com o objetivo de obter bio-óleo a partir da degradação térmica de celulose, hemicelulose, lignina e outras biomoléculas originalmente presentes na biomassa vegetal. Celulose e hemicelulose contribuem para a produção de bio-óleo, enquanto a lignina incrementa o rendimento de biocarvão. O bio-óleo é constituído por 15 – 35 wt% de água e uma mistura complexa de compostos orgânicos (ácidos, álcoois, fenóis, estéres, sacarídeos), sendo a 1,6-anidro-*β*-*D*-glucopiranose (levoglucosana, LGA) um dos componentes de maior interesse (Akhtar e Saidina Amin 2012; Yang et al. 2014; Kan et al. 2016).

A degradação de celulose durante a pirolise acontece em duas etapas: uma leva à formação direta de produtos moleculares pequenos [LGA, furano e

glicoaldeído]; enquanto oligômeros com baixo grau de polimerização são formados na outra etapa (Guo et al. 2015). Inicialmente a celulose é despolimerizada (R1) produzindo um composto líquido intermediário (celulose ativa); subsequentemente, compostos de baixo peso molecular e LGA são obtidos a partir de reações de fragmentação (R2) e transglicosilação (R3), respetivamente. Reações de carbonização (R4 e R5) e de craqueamento dos voláteis (R6) acontecem posteriormente (Fig 8). No entanto, o mecanismo da pirólise da biomassa ainda não foi bem estabelecido; várias propostas mecanísticas têm sido reportadas na literatura, incluindo os mecanismos heterolítico e homolítico e um mecanismo concertado (Anca-Couce 2016).

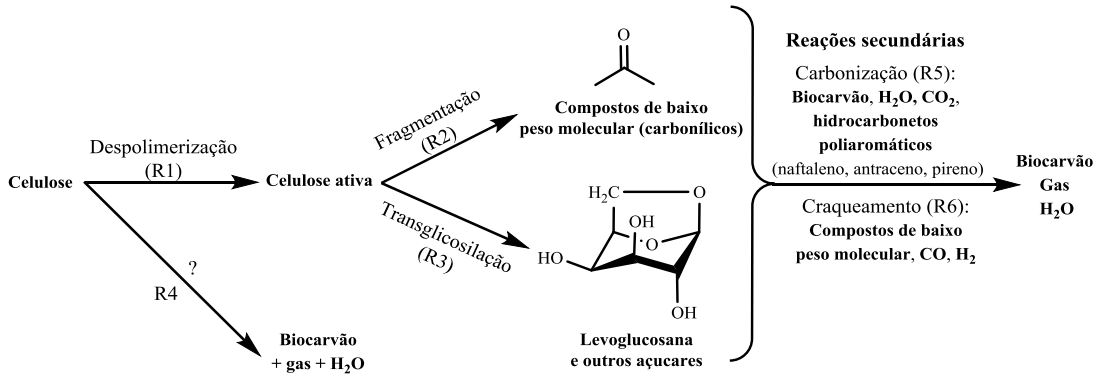

Figura 8. Esquema geral da pirólise de celulose (Anca-Couce 2016).

Ponder et al. (1999) propuseram que a formação de LGA a partir da pirólise de polissacarídeos ocorre através da clivagem heterolítica da ligação glicosídica. A clivagem heterolítica produz um cátion glucosilo que posteriormente é estabilizado pela formação de um intermediário de cadeia 1,6-anhidro glucopiranose terminal. Subsequentemente, a cisão glicosídica da unidade terminal libera uma molécula de LGA e outro cátion glucosilo que continua reagindo (Fig 9). Patwardhan et al. (2009) indicaram que a LGA e os compostos de baixo peso molecular são formados através de reações de pirólise competitivas e não por reações sequenciais. Estes autores concluíram que a formação de LGA foi menor a partir de polissacarídeos com ligações 1,6, devido que a orientação ou a posição das ligações glicosídicas influenciaram na distribuição do produto.

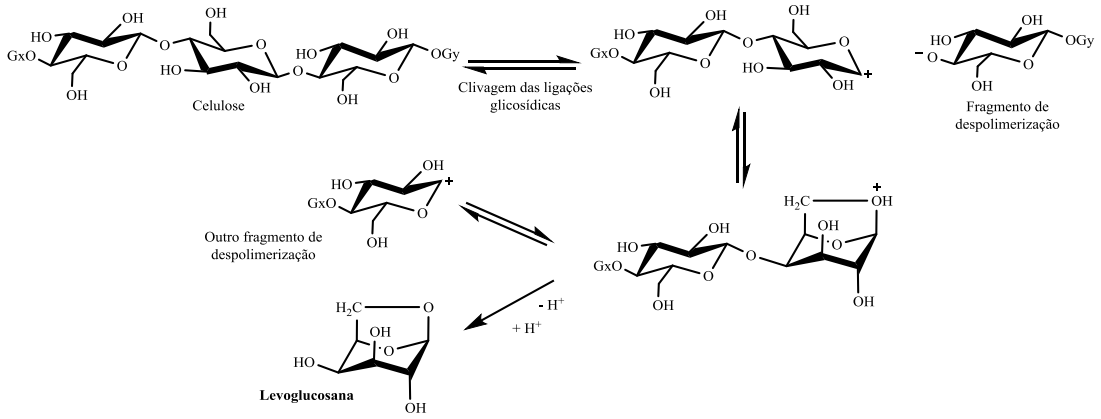

Figura 9. Mecanismo da formação de levoglucosana através da clivagem heterolítica da ligação glicosídica de polissacarídeos durante a pirólise (Ponder et al. 1999).

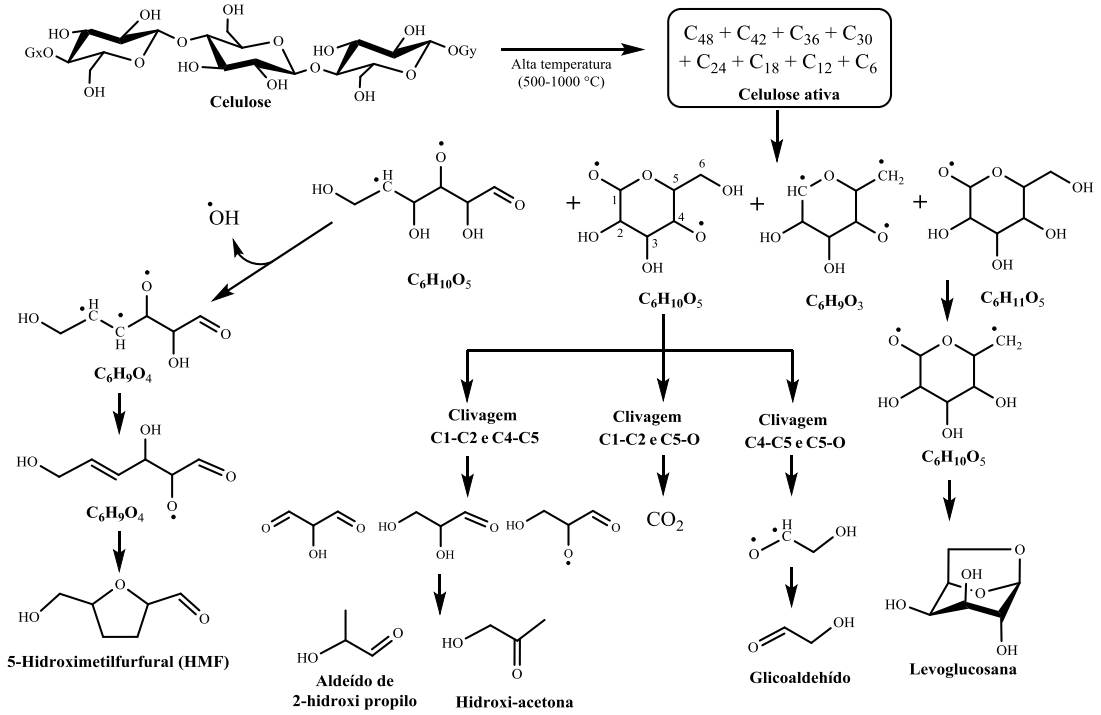

Figura 10. Mecanismo da pirólise da celulose via radicais livres proposto por Zheng et al. (2016a).

No mecanismo proposto por Zheng et al. (2016a) a celulose é despolimerizada pela clivagem homolítica das ligações *β*-1,4-glicosídicas (C-O), originando fragmentos ativos de celobiose, celutriose e outros compostos intermediários com diferentes graus de polimerização (celulose ativa). Em seguida, os intermediários formados se descompõem em monômeros com diferentes sítios ativos. Alguns desses monômeros abstraem radicais hidroxila gerando os precursores da LGA após a modificação da configuração da estrutura. Subsequentemente, a LGA

é formada pela transglicosilação de monómeros do tipo $C_6H_{10}O_5$. A clivagem das ligações C-O do anel de 6 membros também pode acontecer, em menor grau, produzindo espécies radicalares de cadeia aberta; esses intermediários levam à formação de compostos como o 5-hidroximetilfurfural (HMF). A clivagem de ligações C-C nos monômeros ativos também acontece durante a pirólise produzindo moléculas menores como o glicoaldehído, hidroxi-acetona e CO_2 (Fig 10).

O mecanismo com reações concertadas envolve a clivagem da celulose para formar LGA e RN sem a formação de glicose como produto intermediário, embora a LGA também possa ser produzida a partir da glicose. A LGA é formada por reações combinadas e não apenas de extremidades LGA, mas também de extremidades não redutoras.

O bio-óleo obtido da pirólise pode conter mais de 10% em peso de LGA, dependendo do tipo de material lignocelulósico, tamanho das partículas de biomassa e das condições de pirólise (temperatura e tempo de residência dos vapores e da biomassa) (Zhuang et al. 2001; Layton et al. 2011; Lian et al. 2013). Cada grama de LGA custa ao redor de R$ 800 g^{-1} (Sigma-Aldrich Co. LLC) e é utilizada como fonte de carbono para a produção de compostos de interesse industrial tais como: lipídios, ácido cítrico, etanol, aldeído pirúvico, furfural e HMF, dentre outros produtos de fermentação (Zhuang et al. 2001; Layton et al. 2011; Lian et al. 2013).

A LGA é obtida geralmente a partir de produtos petroquímicos via ciclização, seguida por várias reações de oxidação catalítica, passo limitante do processo que incrementa o custo de produção da LGA. Assim, a pirólise de biomassa surge como uma alternativa para contornar os problemas de produção da LGA (Zhang et al. 2017). No entanto, a pirólise rápida da biomassa produz LGA com baixos rendimentos, em comparação com o rendimento teórico baseado na fração de celulose do material lignocelulósico. O baixo rendimento de compostos químicos de alto valor agregado faz com que o processo da pirólise seja economicamente inviável para a obtenção desses compostos em grande escala. Assim, o material lignocelulósico deve passar por um pré-tratamento antes da pirólise com o intuito de eliminar interferentes e disponibilizar maior quantidade de celulose que levem a incrementar a produção de LGA (Jiang et al. 2015; Zhang et al. 2017).

Materiais inorgânicos aceleram as reações de desidratação e carbonização e favorecem as reações heterocíclicas da formação de compostos de baixo peso molecular durante a pirólise (Akhtar e Saidina Amin 2012; Anca-Couce 2016). A biomassa lignocelulósica contém ~ 10% de metais alcalinos (Na e K), alcalino-terrosos (Ca, Mg e Ba) e outros metais tais como Mn, Al, Co, Ni, Cu e Zn (AAEMs) (David et al. 2017). AAEMs podem interaturar com a cadeia terminal da celulose e catalisar reações de fragmentação e da formação do biocarvão durante a pirólise, diminuindo o rendimento do bio-óleo (Fahmi et al. 2008). A presença de AAEMs na biomassa é considerada uma das principais causas da redução do teor de LGA no bio-óleo (Hou et al. 2013; Guo et al. 2015). Processos de pré-tratamento da biomassa antes da pirólise diminuem ou eliminam o conteúdo de AAEMs incrementando o rendimento de LGA em até 80% (Kuzhiyil et al. 2012).

Estratégias de pré-tratamento têm sido estudadas visando remover ou atenuar os efeitos AAEMs na biomassa para incrementar a formação de levoglucosana durante a pirólise. Essas estratégias incluem: pré-tratamento da biomassa em micro-ondas utilizando glicerol como solvente (Zheng et al. 2016b), pré-tratamento hidrotérmico sob alta pressão e temperatura (Chang et al. 2013), troca iônica usando ácido diluído (Scott et al. 2000), processo de desmineralização (Hosoya et al. 2007), lavagem da biomassa lignocelulósica com diferentes tipos de ácidos (Hong et al. 2009; Dong et al. 2015). Catalisadores ácidos têm sido utilizados no pré-tratamento da biomassa incrementando a formação de LGA (Zhou et al. 2013; Wang et al. 2016; David et al. 2017; Zhang et al. 2017). A lignina também reduz a formação de LGA durante a pirólise, sendo assim, métodos de pré-tratamento para deslignificação total da biomassa devem ser aprimorados (Sarkar et al. 2012; Tran et al. 2013).

2.1 Calix[*n*]arenos para a conversão de biomassa

Calix[*n*]arenos são compostos macrocíclicos em forma de cone, formados por unidades fenólicas ligados por grupos metileno e caracterizados por apresentarem a cavidade central e anéis superior e inferior bem definidos (Fig. 11). Os calix[*n*]arenos podem formar complexos de inclusão atuando como hospedeiros para formar arranjos supramoleculares com diversos íons e moléculas, devido às suas variáveis conformações e tamanho de cavidade (Varejão et al. 2013). Calix[*n*]arenos hidrossolúveis e anfifílicos (possuem um extremo hidrofílico e outro hidrófobo)

podem ser sintetizados devido à relativa facilidade com que os anéis superior e inferior podem ser derivatizados. Esses calix[*n*]arenos possuem diversas aplicações, incluindo a atuação como organocatalisadores em transformações orgânicas (Simões et al. 2012; Sathicq et al. 2015).

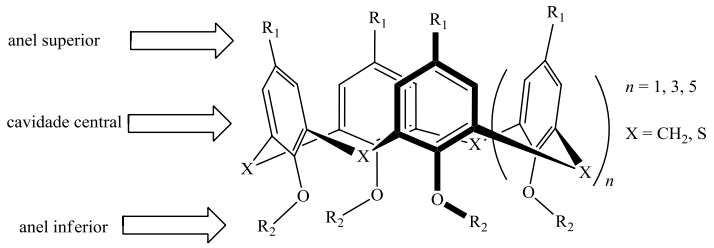

Figura 11. Estrutura geral dos calix[*n*]arenos.

Calix[*n*]arenos como o ácido *p*-sulfônico calix[4]areno ($CX4SO_3H$) e ácido *p*-sulfônico calix[6]areno ($CX6SO_3H$) são utilizados para a esterificação de ácidos carboxílicos e transesterificação (Fernandes et al. 2012; Almeida et al. 2015). Nos últimos anos os calix[*n*]arenos vem sendo empregados em diversas transformações químicas e sendo neste trabalho avaliado no pré-tratamento de biomassa e posterior pirólise para a produção de LGA.

Com base no citado anteriormente, este trabalho teve como objetivos:

1. Adaptar o método de HS-SPME para a extração de compostos orgânicos voláteis a partir do cultivo *in vitro* de *Ruta graveolens.*
2. Determinar o efeito da qualidade de luz sobre a produção de compostos orgânicos voláteis em plantas propagadas em condições *in vitro* de *Ruta graveolens.*
3. Otimizar o protocolo de propagação *in vitro* de *Piper crassinervium*, visando a máxima produção de biomassa e de compostos fenólicos.
4. Estudar a eficiência do ácido *p*-sulfônico calix[4]areno ($CX4SO_3H$) como organocatalisador sobre o pré-tratamento ácido de bagaço de cana e posterior pirólise para a produção de açúcares pirolíticos (levoglucosana).

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CHAPTER 1

HS-SPME: optimization of the extraction conditions and volatile profile analysis from *in vitro* propagated plants of *Ruta graveolens* under light spectral qualities

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Abstract

In vitro propagation protocols have been established aiming to increase the production of volatile organic compounds (VOCs) in medicinal plants such as *Ruta graveolens*. Low biomass production is a limitation to determine the volatile profile of the *in vitro* plants. The headspace solid-phase microextraction (HS-SPME) is considered a fast, inexpensive and useful technique for capturing and characterizing VOCs. However, the extraction conditions of HS-SPME should be optimized. The fiber coating type, sample amount and extraction time were studied in this work. Subsequently, the HS-SPME method was applied to determine the effect of light spectral quality on the production of VOCs in *in vitro* plants. DVB/CAR/PDMS (50/30 μm) fiber was the most efficient for the extraction of VOCs and was the most selective for the extraction of ethers and some esters, ketones and monoterpenes. The highest extraction efficiency was achieved with 200 mg of fresh shoot mass, DVB/CAR/PDMS fiber and 30 min of extraction. Plants grown under control (white LEDs) showed ketones (66.4%), esters (11.7%), and sesquiterpenes (10.5%), mainly.

The major constituents in all samples was nonan-2-one (~53%). Red and far red LEDs have interfered with the production of geyrene, geijerene and nonan-1-ol. This work is the first evidence of the influence of light quality on VOCs production in *R. graveolens* plants. The HS-SPME methodology established in this work can be used to determine other biotic or abiotic factors that alter the production of VOCs in *in vitro* propagated plants of *R. graveolens*. The volatile fraction of plant species composed of VOCs similar to those of rue can be analyzed by HS-SPME using the DVB/CAR /PDMS or PDMS/DVB fibers.

Key words: LED, medicinal plant, rue, SPME fibers, spectrum of light, volatile metabolites.

Introduction

The plants emit volatile organic compounds (VOCs) in response to herbivore or pathogen attack, mechanical damage, environmental or nutritional changes and to attract pollinators, among others (Maes and Debergh, 2003; Zhou et al. 2016; Cellini et al. 2018; Fontes et al. 2018). VOCs biosynthesis varies in each plant species depending on the genotype, chemotype, age, organ and growing period (Olbricht et al. 2011; Ulrich et al. 2015; Krayni et al. 2015; Mesquita et al. 2017). Complex mixtures of VOCs, such as terpenes, fatty acid derivatives, and phenylpropanoids/benzenoids, constitute the essential oils (EOs) of plants (Fu et al. 2015).

The medicinal plant *Ruta graveolens* L. (Rutaceae), known as rue, produces EOs predominantly of the fatty acid derivatives classes. The EOs have multiple biological activities: antifungal (Reddy and Al-Rajab 2016), allelopathic (de Feo et al. 2002), antibacterial (Haddouchi et al. 2013; Orlanda and Nascimento 2015), nematicide (Faria et al. 2015), acaricide (Castagnino and de Oliveira Orsi 2012), insecticide (Aivazi and Vijayan 2010; Perera et al. 2017), among others. Nonan-2-one, undecan-2-one and decan-2-one are one of the major components of OEs and the vapor phase of rue plants. These ketones have larvicidal and nematicidal activity against *Aedes aegypti*, *Anopheles quadrimaculatus* (mosquitoes vectors of yellow fever and malaria) and *Meloidogyne incognita* (pest of coffee and other crops) (Ali et al. 2013; da Silva et al. 2014). These biological activities increas the economic importance of *R. graveolens.*

The extraction of the volatile fraction or the EOs, in *R. graveolens* and several medicinal plants, has been made from fresh, dry and lyophilized biomass. Inadequate temperatures and lyophilization during drying of plant material can degrade VOCs, changing the composition of EO and inducing errors in chemical characterization (Antal et al. 2011). The hydrodistillation, conventional extraction method, can cause rearrangements in the chemical structure of thermally unstable VOCs modifying the natural composition of EO (Jordan et al. 1986; Asfaw et al. 2005). Modern extraction methods such as assisted extraction by microwave, ultrasound and supercritical CO_2 allow the production of higher quality EOs; however, these extraction methods

require expensive equipment (Asfaw et al. 2005; Eblaghi et al. 2016; Baldino et al. 2018; Golmakani et al. 2017).

High amounts of biomass are required to extract, isolate, identify and determine biological activities of OEs (Dob et al. 2008; Aivazi and Vijayan 2010; da Silva et al. 2014). Furthermore, using high amounts of biomass to satisfy the demand of VOCs of commercial interest can reduce native populations of medicinal and aromatic plants affecting biodiversity (Verpoorte et al. 2002; Jesionek et al. 2017). Thus, the *in vitro* propagation constitutes an alternative technique for the production of plants of commercial interest, avoiding the overexploitation of native plants used in the production of essential oils (Musarurwa et al. 2012; Pistelli et al. 2013; Kirimer et al. 2017; Espinosa Leal et al. 2018; Isah et al. 2018).

The establishment of adequate *in vitro* propagation protocols improves the propagation of *R. graveolens* and the production of secondary metabolites, including essential oils (Corduan and Reinhard 1972; Kuzovkina et al. 2009; Kengar and Parakar 2015; Faisal et al. 2018). The production of VOCs in plant cells and tissues maintained *in vitro* can be optimized by manipulating of biotic and abiotic factors (Mulder-Krieger et al. 1988; Maes and Debergh 2003; Zhou et al. 2016; Passinho-Soares et al. 2017; Bekircan et al. 2018; Cellini et al. 2018). The production of VOCs can be maximized through the use of bioreactors (Jesionek et al. 2017) or strategies for collecting dynamically the VOCs during the *in vitro* propagation of plants under optimized culture conditions (Durenne et al. 2017).

The propagation of *in vitro* plants favors the biosynthesis of essential oils but limits the production of biomass to extract VOCs using conventional extraction methods. The headspace - solid phase micro-extraction (HS-SPME) is a simple and fast technique to analyze VOCs (Castellar et al. 2013; Bassolino et al. 2015; Jesionek et al. 2017). The SPME allows to extract VOCs directly without using solvents, limiting the generation of artifacts that modify the original composition of VOCs, compared to the extraction methods mentioned above (Stashenko et al. 2004; Xiao et al. 2014; Zhu and Chen 2017; Mesquita et al. 2017).

In HS-SPME extraction, the VOCs present in the headspace above the sample are absorbed or adsorbed onto a small amount of liquid or solid coatings (polar or nonpolar polymer or bi-polar adsorbent) dispersed on a support (fused silica optical

fiber or stainless steel fiber). The VOCs are concentrated in the fiber after a certain time of exposure and are subsequently desorbed directly in the gas chromatograph injector (Pawliszyn 2012; Liu and Ouyang 2017; Mesquita et al. 2017).

HS-SPME extraction of VOCs from *in vitro* cultured plant material has been performed directly on the culture flask as a form of non-destructive headspace sampling. However, contaminant VOCs emitted by the flask, lids or culture medium, among others, may interfere with the analyzes (Maes et al. 2001; Maes and Debergh 2003; Deng et al. 2004; Bassolino et al. 2015). The culture medium can adsorb some polar VOCs emitted by the plant material reducing their concentration (Maes et al. 2001). Transfer of a determined amount of plant material to a sealed vial allow the extraction of VOCs by HS-SPME eliminating most of the cited interferers (Musarurwa et al. 2012; Castellar et al 2013; Passinho-Soares et al. 2017).

The efficiency of VOCs extraction by HS-SPME is affected by the fiber coating (polarity, thickness and length), temperature, time of equilibrium of the VOCs in the headspace and the time of fiber exposure (Stashenko et al. 2004; Xiao et al. 2014; Liu and Ouyang 2017; Mesquita et al. 2017). HS-SPME extraction conditions should be optimized depending on the species and the plant material analyzed.

In order to determine the effect of different light spectral quality on VOCs biosynthesis in *Ruta graveolens in vitro* propagated plants, the extraction conditions of HS-SPME were optimized in this work. The SPME fiber coating type, the amount of plant material and the time of exposure of the fiber were tested. We also determined the location of essential oils in the leaves of *in vitro* plants of rue using histochemical analyzes. Blue, red, blue/red, far red and white LEDs were tested on the production of VOCs.

Materials and Methods

Establishment of the plants *in vitro*

Ruta graveolens L. seeds (ISLA PAK, Brazil) were disinfected following the methodology of Ríos-Ríos et al. (2019) with modifications in the immersion times of the seeds to the different disinfectant solutions used. The immersion times used were:

(I) 20 min in the neutral detergent; (II) 6 min in commercial sodium hypochlorite solution (NaOCl) with plus Tween 20®; (III) 3 min in ethyl alcohol (70%, v/v) and (IV) 10 min in NaOCl solution (25%, v/v) with Tween 20®.

The seeds were inoculated in glass flasks (350 mL) containing 60 mL of MS salts and vitamins (Murashige and Skoog 1962), 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose (Sigma-Aldrich® Co, St. Louis, MO) and 6.5 g L^{-1} of AgarGel® (São Paulo, SP, Brazil). The pH was adjusted to 5.8 ± 0.1 prior to autoclaving at 121°C, 108 kPa for 20 min. The seeds were germinated in a growth-room at 25 ± 2 ºC, under a photoperiod of 16:8 h (light:dark) and 80 μmol m^{-2} s^{-1} of irradiance quantified by radiometer (LI-COR®, LI-250A Light Meter) and provided by fluorescent lamps (HO Sylvania T12, 110W, São Paulo, Brazil). The flasks were closed with polypropylene lids with one orifice of 10 mm covered with a porous membrane (Saldanha et al. 2012).

The *in vitro* plants were subcultured from stem segments (~ 1.5 cm, with at least one node) on modified JADS medium [JADSm, JADS without the antioxidant polyvinylpyrrolidone (PVP); Correia et al. 1995; stock solution] under the same culture conditions used for seed germination. The MS medium was replaced by JADSm to decrease the hyperhydricity and calogenesis induced at the explant base during long periods of *in vitro* culture of *R. graveolens* (effect observed in previous experiments, un-published data). Explants for the first cycle of subcultures were excised from germinated seedlings *in vitro*.

In vitro propagation of *Ruta graveolens* plants and light treatment

Leafless nodal segments (~1.5 cm) with 1-2 axillary buds were excised from plants maintained *in vitro* and inoculated into 500-mL glass flasks containing 100 mL of MS medium semisolid with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose and 6.5 g L of AgarGel®. Three explants were inoculated into each culture flask, constituting a biological replicate.

The cultures were maintained for 45 days in a growth-room under LED (light-emitting diode) lamps with light spectra: white (SMD 100, 18 W, Vilux®, Vitória, ES, Brazil), blue/red (whit a balanced rate of blue and red; Tec-Lamp-660/450-120 cm, 36 W, Tecnal®, Piracicaba, Brazil), blue, red (LabPAR LL-HR/DB-

480, 11,6 W; LabLumens®, Carapicuíba, SP, Brazil) and far-red (LED lighting system Tec-Lux, Tecnal®), totaling five treatments. The irradiance was standardized at 80 µmol m^{-2} s^{-1}. The absorption spectra at wavelengths of 350 to 800 nm were obtained by spectroradiometer using the Ocean Optics Spectra-Suite (OceanOptics®, Dunedin, FL) data acquisition software system (Fig. 1).

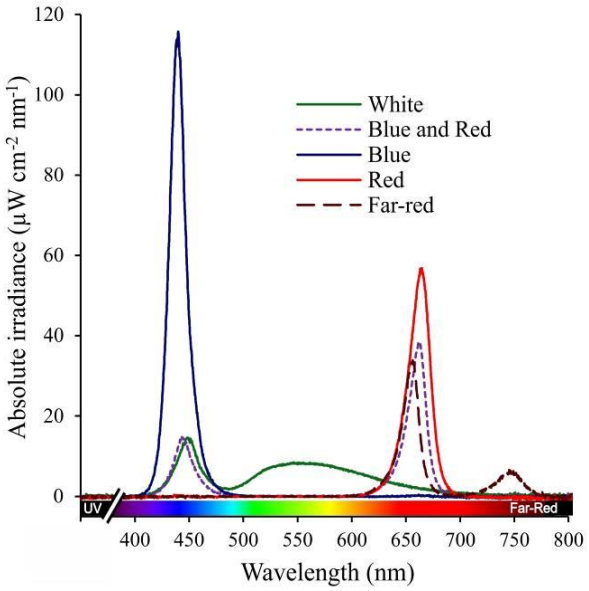

Figure 1 Spectral distribution of light-emitting diodes (LED) lamps used in the *in vitro* propagation of *Ruta graveolens* plants. The absorption spectra were obtained by spectroradiometer using an Ocean Optics Spectra-Suite data acquisition software system.

Histochemical analysis for essential oil detection

In vitro plants grown under white LED lamps was used for the histochemical analysis after 45 days of cultivation. Completely expanded fresh leaves were removed from the third and fourth nodes (counted from the shoot tip), sectioned transversely on table microtome (LPC, Rolemberg & Bohering, Retail and Import Ltda., Belo Horizonte, Brazil) and treated with Nadi reagent (David and Carde 1964) for the detection of essential oils. Images were captured with an Olympus AX70TRF microscope (Olympus Optical, Tokyo, Japan) with a U-Photo Camera System (Spot Insight Color 3.2.0, Diagnostic Instruments Inc., USA).

Headspace-Solid phase microextraction (HS-SPME): fiber selection and optimization of extraction conditions

The HS-SPME methodology was based on Xiao et al. (2014), Ulrich et al. (2015) and Mesquita et al. (2017). *In vitro* plants grown under white LED lamps were used in order to optimize the extraction method by HS-SPME. The shoots without roots were collected after 45 days of culture, immediately frozen with liquid nitrogen and stored at - 80 °C. Subsequently, the frozen plant material from various culture flasks was pulverized, homogenized and separated into 200 and 500 mg aliquots, placed in 2 mL microtubes and stored at - 80 °C until the VOCs extraction by HS-SPME.

The fresh mass frozen (500 mg) placed in 2 mL microtubes was rapidly transferred to a 20 mL glass vials, which were immediately sealed with butyl rubber stoppers and maintained at -20 °C for 8 - 18 h before analyses. The sealed vials were left at room temperature (R_t = 24 ± 1 °C) for 15 min to reach equilibrium before extraction by HS-SPME. After this, the SPME fiber was inserted into the vial and was exposed over the headspace to 1 cm above the plant material for 30 min at R_t. Then the fiber was removed and inserted directly into the GC injector, remained in the GC inlet for 5 min at 250 ºC for desorption of the volatile constituents.

The SPME fibers: carboxen/polydimethylsiloxane (CAR/PDMS, 75 µm), polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 µm), divinylbenzene/carboxen on polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm), polydimethylsiloxane (PDMS, 100 µm) and polyacrylate (PA, 85 µm) (Supelco®, Bellefonte, PA, USA) were tested as adsorbent for extraction by HS-SPME. The extractions were performed in triplicate, using one sample at a time, for the type of SPME fiber tested.

Different plant fresh weight (200 and 500 mg) and fiber exposure times (15 and 30 min) were tested in a 2x2 factorial scheme (totaling 4 treatment), after the fiber selection for the optimization of the extraction conditions by HS-SPME. The extractions were performed in triplicate.

The residual VOCs remaining in the glass vials and butyl rubber stoppers, used in previous extractions, were eliminated based on the methodology proposed by Maes et al. (2001). For this, the glass vials were washed with neutral soap, wrapped

in foil and left for at least 4 h in an oven at 100-120 °C before use. The butyl rubber stoppers were left in neutral soap overnight, wiped with water and submerged in dichloromethane (DCM), leaving them on ultrasound for 5 min; subsequently, the lids were washed again with neutral soap and oven dried at 100 °C for 20-30 min.

VOCs extraction by HS-SPME of *in vitro* plants of *Ruta graveolens* grown on different light spectral qualities

The shoots grown under the white, blue, blue/red, red and far red LEDs were collected after 45 days of culture, immediately frozen with liquid nitrogen and stored at -80 °C. The sample preparation was performed as mentioned in the previous section; however, the plant material from each culture flask was stored separately, forming a biological repetition. The HS-SPME was performed using 200 mg of the frozen material pulverized, DVB/CAR/PDMS (50/30 µm) fiber and 30 min of fiber exposure times (Fig. 2). Three biological repeats were used for each light spectral quality tested and each of these repeats was analyzed in triplicate in the GC-FID.

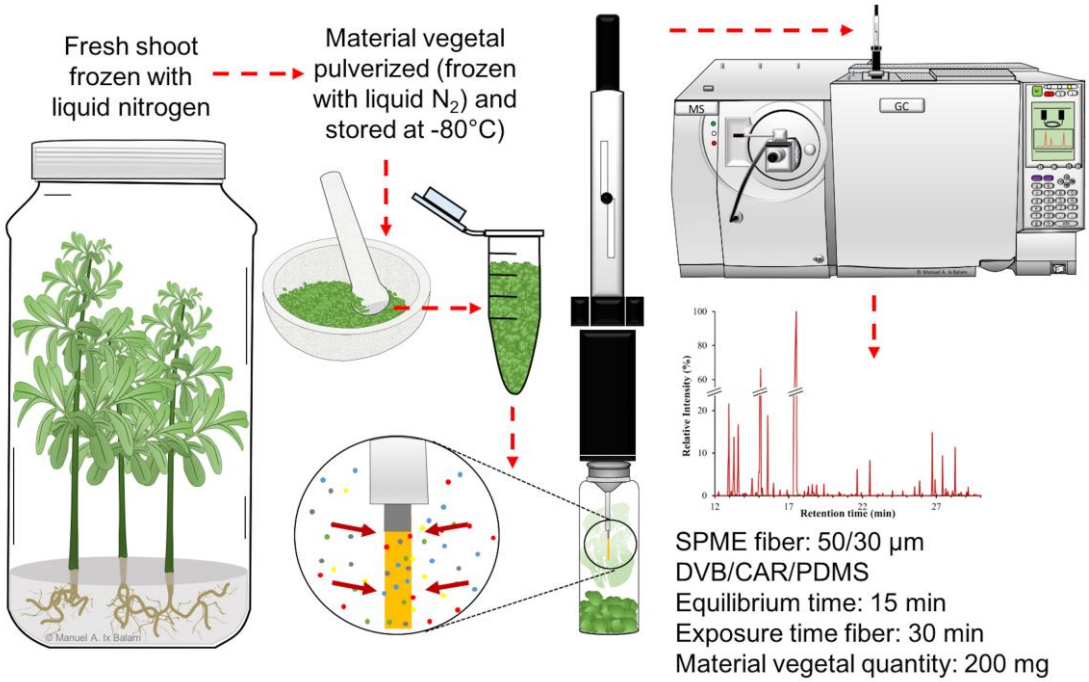

Figure 2. Extraction methodology of volatile organic compounds by HS-SPME from *Ruta graveolens* plants propagated under *in vitro* conditions.

GC-MS and GC-FID analyses

The VOCs extracted by HS-SPME were analyzed on a gas chromatography coupled to a mass spectrometer (GCMS-QP2010C Ultra mass spectrometer, Shimadzu®, Kyoto, Japan) with a data system (CGMS solution 4.2) with NIST (2005; 2008; 2011 and 2014) and Wiley 7v100 mass spectral data libraries. The GC was equipped whit a capillary SLB®-5MS column [coated with silphenylene polymer virtually equivalent in polarity to poly (5% diphenyl/95% dimethyl siloxane); 30 m length x 0.25 mm internal diameter x 0.25 μm film thickness, Supelco®]. The injection mode was split (1:8 ratio) with volumetric flow rate of the carrier gas (Helium) of 1.6 mL min^{-1}. The injector and detector (interface and ion source) temperatures were maintained at 250 °C. GC oven temperature was programmed from 40 °C (hold 5 min) to 200 °C (5 °C min^{-1}), hold in 200 °C for 5 min, then heated to 250 °C at 10 °C min^{-1} (hold 10 min), with a total time of 57 min (Stashenko et al. 2004). Mass spectra were obtained by electron impact ionization at 70 eV in scan mode with range of *m/z* 35 to 400 (0.5 s intervals) and solvent curt time of 4 min (Golmakani et al. 2017). The VOCs of all samples used to select SPME fiber type and the extraction conditions (fresh mass vs. fiber exposure time) were analyzed only in GC-MS.

The VOCs of the plants grown under the different light spectral qualities were identified in GC-MS and quantified on a GC-17A (Shimadzu®) equipped with flame ionization detection (FID). A SPB5 capillary column (30 m length x 0.25 mm internal diameter x 0.25 μm film thickness, Supelco®) and the injection in mode split (1:3 ratio) were used. The method and the injector and detector temperatures were the same ones used in GC-MS.

Compound identification and relative composition (%) of VOCs

The volatiles constituents were identified by comparison of (I) their mass spectra with stored spectra in the data system library NIST (2005; 2008; 2011 and 2014) and Wiley 7v100 (match ≥ 95% or ≥ 90% for compounds previously reported in the literature for the analyzed plant or for other plants of the same family); (II) the linear retention indexes (LRI), relative to a series of *n*-Alkanes (C_7-C_{30}, Supelco®) with those reported in the literature for similar chromatographic columns (Adams

2007; NIST Chemistry WebBook). The LRI were calculated using the Van Den Dool and Kratz (1963) equation.

The integration of the peaks was performed with the same parameters in all the chromatograms (peaks with area ≥ 45000 in the GC-MS). The total area of detected peaks, total peak area mg^{-1} weight fresh and the number of detected peaks, obtained in the GC-MS analysis, were used to select the type of SPME fiber and the best extraction conditions (plant fresh weight and fiber exposure time). The relative concentration of the VOCs was expressed as percentage of relative area of the chromatographic peaks detected measured without correction factors.

Statistical analyses

For the type of SPME fiber selection and optimization of the HS-SPME conditions were analyzed the variable: total peak area (sum of area of detected peaks), total peak area mg^{-1} weight fresh and the number of detected peaks. In the quality spectral light experiment (white, blue, red and far-red LEDs lamps) the analyzed variable was the relative composition (%) of individual VOCs and the VOCs classified by chemical class. The data were submitted to ANOVA and the means compared by the Tukey's test at the 5% level of significance, using the Genes program, version Windows/2011.9.0 (Cruz 2013).

A data matrix containing the relative concentration (%) of 24 VOCs and 15 samples of five SPME fibers [CAR/PDMS (75 μm), PDMS/DVB (65 μm), DVB/CAR/PDMS (50/30 μm), PDMS (100 μm) and PA (85 μm)] was submitted to the principal component analysis (PCA) with mean center and normalize pretreatments, using the Chemoface software (Nunes et al. 2012). The PCA was employed to intensify the clustering trends of different SPME fibers, based on the VOCs extracted. The heatmap analysis was done for fiber type and VOCs using online tool Heatmapper (average linkage, Euclidian distance) (Babicki et al. 2016).

Results and discussion

Histological localization of essential oils of the *in vitro* plants of *Ruta graveolens* leaves

The EOs were evidenced by their blue coloration after the reaction with the Nadi reagent in the leaves of *in vitro* plants of *R. graveolens*. EOs were localized in the epidermal cells of both leaf faces, vascular bundles and into the secretory cavities and glandular trichomes and/or in epidermal cells associated with these structures (Fig. 3). EOs have been detected in the cuticle, epidermal cells and glandular trichomes of several medicinal plants propagated *in vitro*; these plants have the same secretory structures and secretions found in nature plants (Santos et al. 2013; Zuzarte et al. 2010; Batista et al. 2017; Passinho-Soares et al. 2017). Secretory structures and glandular trichomes contain VOCs associated with the chemical defense of the plant or the attraction of pollinators and seed dispersing agents; these VOCs are released rapidly when the leaf surface secretory structures are damaged (Dudareva et al. 2004; Maffei et al. 2010).

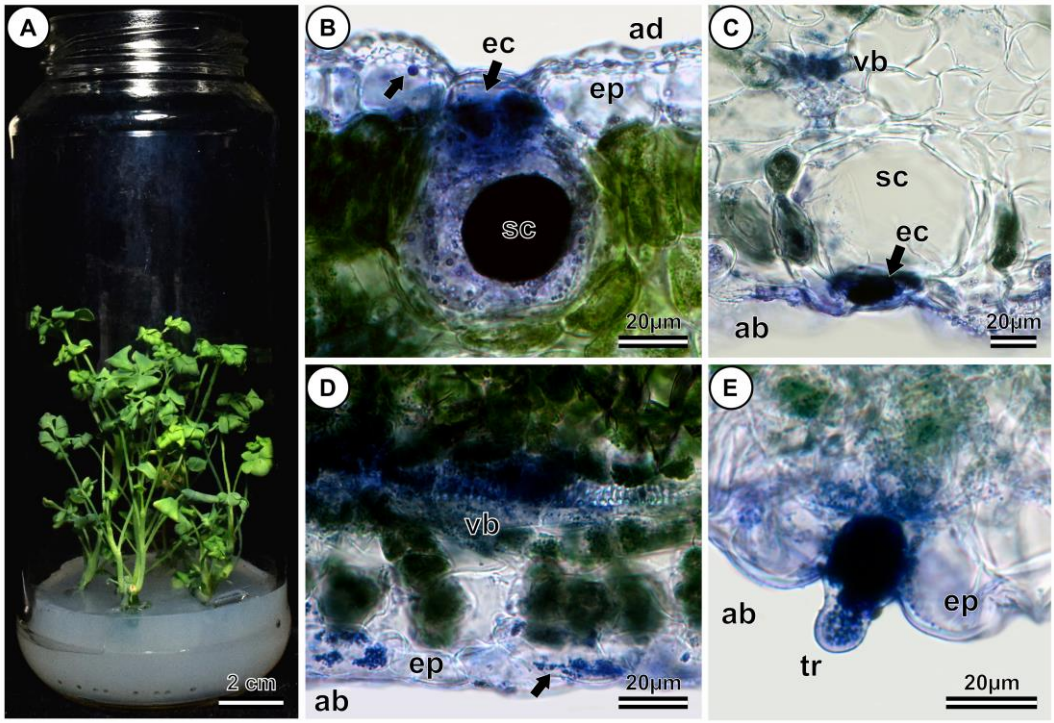

Figure 3. *In vitro* propagated plants of *Ruta graveolens* after 45 days growth (**A**) and cross sections of leaf stained with Nadi reagent (**B-E**). Plants grown in MS medium supplemented with 30 g L^{-1} sucrose under irradiance of 80 µmol m^{-2} s^{-1} provided by white LEDs lamps. **ad**: adaxial surface of the epidermis; **ep**: epiderme; **sc**: secretory

cavity; **ec**: epidermal cells associated with secretory cavities; **vb**: vascular bundle; **ab**: abaxial surface of the epidermis; **tr**: trichome.

The DVB/CAR/PDMS and PDMS/DVB fibers were the most efficient for the extraction of VOCs from the *in vitro* plants of *R. graveolens*

The highest number of detected peaks was obtained with the DVB/CAR/PDMS and PDMS/DVB fibers, being 108 and 101, respectively. The PA fiber extracted the smallest number of compounds, being detected 43 peaks (Fig. 4A). The total area of the peaks detected was higher with the DVB/CAR/PDMS and PDMS/DVB fibers, followed by PDMS = CAR/PDMS > PA fibers (Fig. 4B).

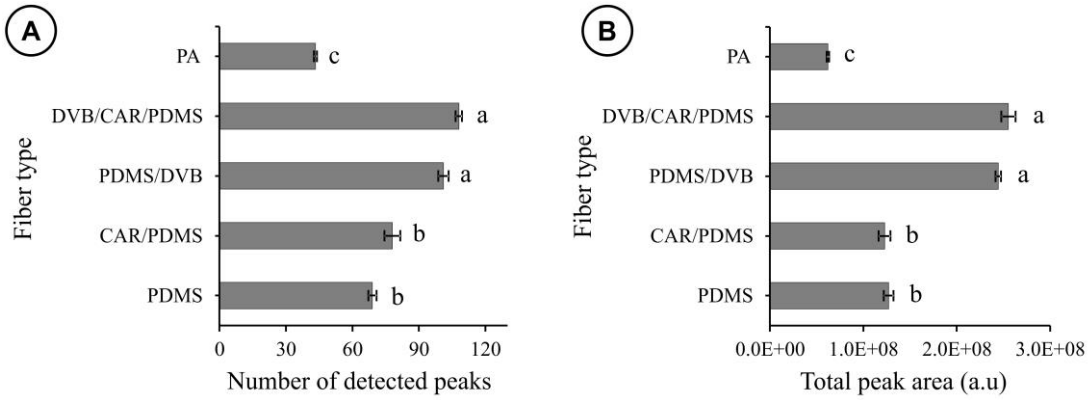

Figure 4. Efficiency of different types of fiber coating for the extraction of volatile organic compounds from *in vitro* plants of *Ruta graveolens* by HS-SPME**. A**: number of detected peaks; **B**: total area of the detected peaks. **PDMS**: polydimethylsiloxane fiber, 100 µm; **PA**: polyacrylate fiber, 85 µm; **CAR/PDMS**: carboxen/polydimethylsiloxane fiber, 75 µm; **PDMS/DVB**: polydimethylsiloxane/divinylbenzene fiber, 65 µm; **DVB/CAR/PDMS**: divinylbenzene/Carboxen on polydimethylsiloxane fiber, 50/30 µm. Means followed by the same lowercase letter did not differ significantly among the treatments (Tukey's test at 5%). Error bars indicate the standard error among replicates (n = 3).

The fiber coating efficiency depends on the complexity of the sample, the molecular mass, size, volatility, polarity and concentration of the analytes (Shirey 2012; Mesquita et al. 2017; Silva et al. 2018). The PDMS and PA fibers have high capacity to absorb polar volatile and apolar semi-volatile compounds, respectively (Risticevic et al. 2010; Shirey 2012). While in mixed coating fibers such as CAR/PDMS, PDMS/DVB and DVB/CAR/PDMS the extraction is done by adsorption and depends on the size of the analyte and the pore diameter of the fiber.

The surface of the fiber and the analyte can interact by π-π bonding, dehydrogen bonding or van der Waals interactions. Thus, mixed coatings adsorb a wide variety of polar and nonpolar analytes with different physico-chemical properties (Risticevic et al. 2010; Pawliszyn 2012; Shirey 2012).

The PDMS/DVB (65 μm) fiber absorbs volatiles of molecular weight of 50-300 g mol^{-1}; while the DVB/CAR/PDMS fiber (50/30 μm) adsorb C_3-C_{20} volatile and semi-volatile compounds with a molecular weight of 40-275 g mol^{-1} (Risticevic et al. 2010; Shirey 2012). The VOCs biosynthesized in the *in vitro* cultivated shoots of *R. graveolens* were C_7-C_{21} volatile and semi-volatile compounds, mainly fatty acid derivatives and terpenes, with molecular weight 128 – 214 g mol^{-1} and polarities different; these characteristics led to greater affinity between rue VOCs and the mixed adsorbents PDMS/DVB and DVB/CAR/PDMS.

The Fig. 5 shows the chromatograms of the volatile fraction extracted with the SPME fibers used, highlighting 44 peaks with relative abundance ≥ 0.09% and whose area was larger with the DVB/CAR/PDMS and PDMS/DVB fibers. Twenty - nine VOCs were identified, corresponding to 87.55 - 90.31% of the total area of the extracted peaks. The volatile fraction of *in vitro* plants of *R. graveolens* consisted mainly of ketones (48.28 - 59.85%), esters (15.84 - 22.71%), sesquiterpenes (8.65 - 16.10%), aldehydes (0.30 - 1.50%), alcohols (0.71 - 1.75%), ethers (0.16 - 0.95%) and monoterpenes (0.38 - 1.34%) (Table 1). Perera et al. (2017) identified this same compounds class in the volatile fraction of leaves of native plants of rue extracted by HS-SPME with the fibers PA (85 μm) and PDMS (30 μm).

PDMS/DVB and DVB/CAR/PDMS fibers have proven efficiency for the extraction of aldehydes, alcohols, esters, monoterpenes, sesquiterpenes and ketones (Xiao et al. 2014; Elizalde-González and Segura-Rivera 2018; Camelo et al. 2019). Thus, the higher affinity of these two types of SPME coatings by VOCs derivate of fatty acid influenced the results obtained in this work; since the *in vitro* plants of *R. graveolens* produced high content of ketones and esters.

The relative percentage (R%) of VOCs varied with the SPME fiber used. The major constituents in the shoots of *R. graveolens* were nonan-2-one (32.34 - 48.04%), undecan-2-one (6.86 – 15.46%), undecan-2-yl-acetate (4.46 - 11.97%), nonan-2-yl acetate (4.51 - 7.68%), decan-2-one (3.72-4.44%) and *n*-nonyl acetate

(1.09 - 2.21%). The R% of nonan-2-one was about three times higher than the R% of undecan-2-one and this proportion varied with the SPME fiber used in this work (Table 1).

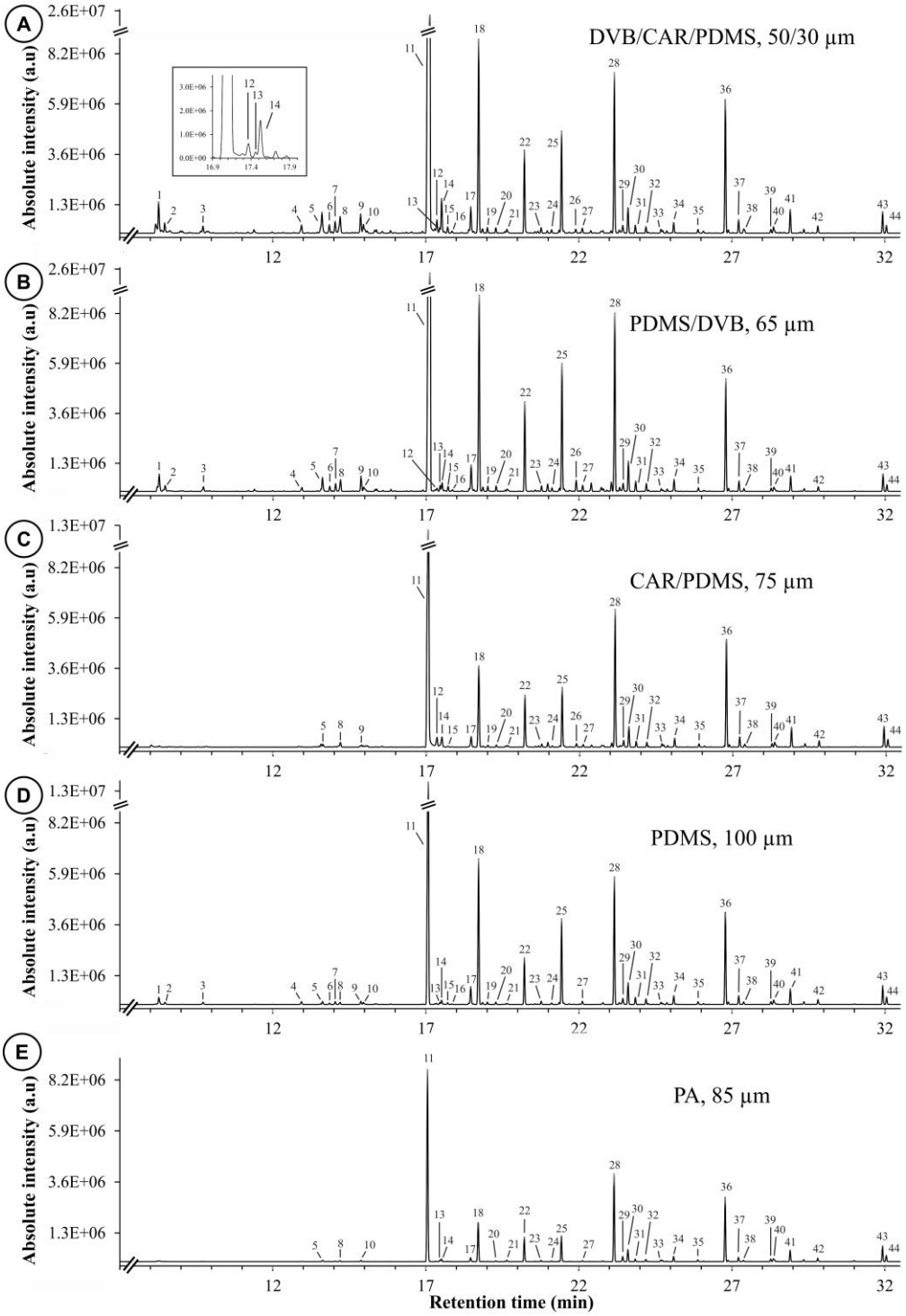

Figure 5. Volatile fraction chromatogram (GC-MS) of *in vitro* plants of *Ruta graveolens* extracted by HS-SPME using different SPME fibers. **PDMS**: polydimethylsiloxane fiber, 100 µm; **CAR/PDMS**: carboxen/polydimethylsiloxane fiber, 75 µm; **PDMS/DVB**: polydimethylsiloxane/divinylbenzene fiber, 65 µm; **DVB/CAR/PDMS**: divinylbenzene/Carboxen/polydimethylsiloxane fiber, 50/30 µm; **PA**: polyacrylate fiber, 85 µm. The peaks identified are shown in the table 1.

Table 1. Volatile organic compounds extracted by HS-SPME from *in vitro* plants of *Ruta graveolens* with different SPME fibers.

N°	Compound	MW (g mol^{-1})	t_R[a] (min)	RI[b]	RI[c]	Relative composition[e] (%) ± SE				
						PDMS	CAR/PDMS	PDMS/DVB	DVB/CAR/ PDMS	PA
1	Ethyl 2-methylbutanoate	130	8.175	848	846	0.85 ± 0.11	0.44 ± 0.01	1.32 ± 0.11	2.20 ± 0.08	0.39 ± 0.03
2	Ethyl 3-methylbutanoate	130	8.408	854	856	0.21 ± 0.02	0.06 ± 0.04	0.39 ± 0.01	0.62 ± 0.11	0.02 ± 0.02
3	Propan-2-yl 2-methylbutanoate	144	9.696	887	880	0.20 ± 0.03	tr	0.30 ± 0.01	0.45 ± 0.04	tr
4	Sabinene	136	12.916	972	972[d]	0.26 ± 0.02	0.07 ± 0.04	0.19 ± 0.03	0.39 ± 0.02	tr
5	Octan-2-one	128	13.592	990	994	0.26 ± 0.02	0.50 ± 0.04	0.75 ± 0.04	1.24 ± 0.02	0.29 ± 0.01
6	N.I.	-	13.838	997	-	0.10 ± 0.01	0.05 ± 0.03	0.25 ± 0.01	0.38 ± 0.04	tr
7	2-Methylpropyl 2-methylbutanoate	158	14.043	1003	1002	0.24 ± 0.02	0.10 ± 0.01	0.40 ± 0.02	0.50 ± 0.06	0.06 ± 0.03
8	1-Methoxy-2-methylbenzene	122	14.207	1008	1009	0.19 ± 0.03	0.59 ± 0.06	0.70 ± 0.05	0.72 ± 0.04	0.16 ± 0.01
9	Methyl 4-methylhex-2-enoate	142	14.876	1027	-	0.27 ± 0.02	0.24 ± 0.02	0.79 ± 0.04	0.87 ± 0.06	0.21 ± 0.02
10	Limonene	136	14.963	1030	1030[d]	0.17 ± 0.02	0.16 ± 0.02	0.21 ± 0.03	0.44 ± 0.02	tr
11	Nonan-2-one	142	17.140	1094	1093[d]	32.34 ± 1.33	39.27 ± 0.69	46.77 ± 0.68	48.04 ± 0.57	36.64 ± 0.82
12	Linalool	154	17.365	1100	1101[d]	0.19 ± 0.09	0.76 ± 0.28	0.32 ± 0.15	0.52 ± 0.07	0.38 ± 0.38
13	Nonan-2-ol	144	17.465	1103	1101[d]	0.18 ± 0.01	0.60 ± 0.39	0.27 ± 0.11	0.17 ± 0.08	0.30 ± 0.02
14	Nonanal	142	17.522	1105	1106[d]	0.32 ± 0.13	0.63 ± 0.41	1.02 ± 0.33	1.50 ± 0.26	0.30 ± 0.11
15	3-Methylbut-3-enyl 2-methylbutanoate	170	17.719	1112	-	0.15 ± 0.01	0.15 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	tr
16	3-Methylbut-3-enyl 3-methylbutanoate	170	17.860	1116	1116	0.05 ± 0.00	0.05 ± 0.00	0.09 ± 0.01	0.10 ± 0.00	tr
17	Geijerene or geyrene[f]	162	18.473	1136	1132	1.71 ± 0.01	1.14 ± 0.11	1.10 ± 0.14	1.23 ± 0.03	0.71 ± 0.04
18	Geijerene or geyrene[f]	162	18.734	1144	1142	13.91 ± 0.26	8.24 ± 0.22	7.94 ± 1.09	8.69 ± 0.18	7.48 ± 0.44
19	2-Methoxy-3-isopropyl-5-methyl pyrazine	166	19.015	1153	1149[g]	0.17 ± 0.01	0.15 ± 0.00	0.25 ± 0.02	0.25 ± 0.01	tr
20	N.I	-	19.290	1162	-	0.19 ± 0.01	0.18 ± 0.00	0.24 ± 0.00	0.24 ± 0.01	0.14 ± 0.00
21	Nonan-1-ol	144	19.657	1174	1174[d]	0.14 ± 0.01	0.19 ± 0.01	0.18 ± 0.02	0.17 ± 0.02	0.32 ± 0.03
22	Decan-2-one	156	20.226	1192	1192	3.94 ± 0.10	4.44 ± 0.14	3.93 ± 0.04	3.72 ± 0.16	4.22 ± 0.02
23	*n*-Octyl acetate	172	20.771	1210	1211	0.26 ± 0.02	0.27 ± 0.01	0.25 ± 0.00	0.24 ± 0.01	0.21 ± 0.01
24	N.I	-	21.113	1222	-	0.13 ± 0.00	0.10 ± 0.00	0.17 ± 0.01	0.16 ± 0.01	0.08 ± 0.01
25	Nonan-2-yl acetate	186	21.440	1233	1229	7.68 ± 0.35	5.42 ± 0.49	5.10 ± 0.34	4.51 ± 0.24	4.74 ± 0.14
26	N.I.	-	21.901	1249	-	0.01 ± 0.01	0.35 ± 0.17	0.63 ± 0.12	0.19 ± 0.03	tr
27	N.I.	-	22.118	1257	-	0.30 ± 0.01	0.23 ± 0.02	0.27 ± 0.01	0.25 ± 0.01	0.18 ± 0.01
28	Undecan-2-one	170	23.168	1293	1292[d]	11.74 ± 0.21	11.67 ± 0.97	8.08 ± 0.23	6.86 ± 0.18	15.46 ± 0.19
29	Undecan-2-ol	172	23.441	1303	1307	0.68 ± 0.04	0.61 ± 0.05	0.44 ± 0.02	0.38 ± 0.04	1.14 ± 0.09
30	*n*-Nonyl acetate	186	23.611	1309	1308	2.00 ± 0.11	1.76 ± 0.17	1.28 ± 0.03	1.09 ± 0.03	2.21 ± 0.06

31	N.I	-	23.850	1318	-	0.68 ± 0.09	0.49 ± 0.05	0.40 ± 0.02	0.33 ± 0.01	0.47 ± 0.04
32	Decan-2-yl-acetate	200	24.197	1331	-	0.53 ± 0.06	0.39 ± 0.04	0.31 ± 0.02	0.26 ± 0.01	0.42 ± 0.04
33	Ethyl 3-phenylpropanoate	178	24.688	1349	1352[d]	0.19 ± 0.02	0.29 ± 0.02	0.19 ± 0.04	0.14 ± 0.01	0.32 ± 0.05
34	N.I	184	25.103	1365	-	0.84 ± 0.07	0.71 ± 0.06	0.51 ± 0.02	0.42 ± 0.02	1.03 ± 0.08
35	N.I	184	25.899	1394	-	0.26 ± 0.02	0.26 ± 0.01	0.18 ± 0.02	0.14 ± 0.01	0.39 ± 0.03
36	Undecan-2-yl acetate	214	26.793	1429	-	9.79 ± 0.72	8.68 ± 0.31	5.50 ± 0.49	4.46 ± 0.55	11.97 ± 0.46
37	N.I.	-	27.224	1446	-	0.89 ± 0.06	0.80 ± 0.04	0.55 ± 0.07	0.44 ± 0.05	0.80 ± 0.04
38	N.I.	-	27.392	1453	-	0.25 ± 0.02	0.31 ± 0.05	0.16 ± 0.03	0.21 ± 0.03	0.22 ± 0.01
39	2-phenylethyl 2-methylbutanoate	206	28.278	1487	1488	0.28 ± 0.02	0.29 ± 0.01	0.18 ± 0.02	0.13 ± 0.01	0.49 ± 0.03
40	(*Z*,*E*)-alpha-farnesene	204	28.364	1491	1492	0.49 ± 0.06	0.39 ± 0.06	0.34 ± 0.08	0.17 ± 0.05	0.47 ± 0.12
41	N.I	-	28.912	1513	-	1.53 ± 0.10	1.45 ± 0.08	0.90 ± 0.17	0.69 ± 0.13	2.02 ± 0.06
42	N.I	-	29.815	1551	-	0.45 ± 0.03	0.45 ± 0.07	0.29 ± 0.06	0.23 ± 0.04	0.58 ± 0.02
43	N.I	-	31.930	1642	-	1.70 ± 0.07	1.56 ± 0.25	0.94 ± 0.16	0.60 ± 0.13	2.50 ± 0.16
44	N.I	-	32.057	1647	-	0.66 ± 0.02	0.57 ± 0.09	0.35 ± 0.06	0.21 ± 0.05	0.97 ± 0.07
	Alcohols					0.99 ± 0.06	1.40 ± 0.40	0.88 ± 0.02	0.71 ± 0.09	1.75 ± 0.14
	Aldehydes					0.32 ± 0.13	0.63 ± 0.41	1.02 ± 0.33	1.50 ± 0.26	0.30 ± 0.11
	Esters					22.71 ± 0.88	18.14 ± 0.45	16.34 ± 0.43	15.84 ± 0.30	21.05 ± 0.66
	Ethers					0.37 ± 0.03	0.74 ± 0.06	0.95 ± 0.06	0.97 ± 0.04	0.16 ± 0.01
	Ketones					48.28 ± 1.27	55.88 ± 0.51	59.53 ± 0.90	59.85 ± 0.74	56.61 ± 0.82
	Monoterpenes					0.62 ± 0.10	0.99 ± 0.29	0.72 ± 0.17	1.34 ± 0.09	0.38 ± 0.38
	Sesquiterpenes					16.10 ± 0.22	9.78 ± 0.36	9.38 ± 1.15	10.10 ± 0.20	8.65 ± 0.47
	Unidentified compounds					8.00 ± 0.31	7.51 ± 0.37	5.83 ± 0.69	4.48 ± 0.38	9.38 ± 0.12
	Total					97.40 ± 0.15	95.07 ± 0.33	94.65 ± 3.75	94.78 ± 0.03	98.29 ± 0.12

[a] Average of retention time on the SLB-5MS column (30 m x 0.25 mm d_i x 0.25 µm film thickness); [b] Retention index (**RI**) relative to a series of *n*-Alkanes (C_7-C_{30}), calculated using the Van Den Dool and Kratz (1963) equation; [c] RI published in the literature for similar chromatographic columns to SLB-5MS (Adams 2007; NIST Chemistry WebBook); [d] RI published in the literature using the SLB-5MS column; [e] Averages of percentage of relative area of the chromatographic peaks detected (n = 3); [f] Diastereoisomers reported in the essential oil of *Ruta graveolens* by Soleimani et al. (2009); the assignment of the corresponding peak to the geigerene or geyrene in this work was not possible. [g] **MW**: molecular weight; **SE**: standard error (n = 3); **PDMS**: polydimethylsiloxane fiber, 100 µm; **CAR/PDMS**: carboxen/polydimethylsiloxane fiber, 75 µm; **PDMS/DVB**: polydimethylsiloxane/divinylbenzene fiber, 65 µm; **DVB/CAR/PDMS**: divinylbenzene/carboxen on PDMS fiber, 50/30 µm; **PA**: polyacrylate fiber, 85 µm; **tr**: trace.

EOs of native rue plants obtained by hydrodistillation have been characterized by the higher concentration of undeca-2-one (34-55%) in comparison with nona-2-one (8-39%) (Soleimani et al. 2009; Haddouchi et al. 2013; Orlanda et al. 2015). The concentration of these two ketones varies with the extraction method used. EOs of *R. chalepensis* extracted by hydrodistillation were constituted by 15% of nonan-2-one and 23% of undeca-2-one; while the volatile fraction extracted by static headspace showed 33% of nonan-2-one and 14% of undeca-2-one (Fakhfakh et al. 2012).

The VOCs with higher volatility and lower molecular weight are easily released by the plant to the vapor phase, whereat the profile of the EOs varies with respect to the volatile fraction of the plants. Compounds with low volatility and higher molecular weight, such as sesquiterpenes, are less frequent in the volatile fraction. The emission of these compounds to the atmosphere is restricted, although they are concentrated in specific storage structures in the leaves in considerable amounts. Thus, extraction processes more drastic than the HS-SPME are required for its release. In contrast, some VOCs are often lost during these extraction processes (Ormeño et al. 2011). In the case of *R. graveolens*, the greater number of carbon atoms in the undecan-2-one chain, compared to nonan-2-one, decreases their volatility reducing their concentration in the headspace.

The diastereoisomers geyrene and geijerene were also detected in the vapor phase of *in vitro* plants of *R. graveolens*. These compounds were reported previously in this species with IR of 1132 and 1142, respectively (Soleimani et al. 2009). However, the assignment of the corresponding peak to the geigerene or geyrene was not possible in this work; due to the similarity of the mass spectra and the variation of IRs reported in the literature (1132 to 1146) (Adams 2007; NIST Chemistry WebBook; Fakhfakh et al. 2012; da Silva et al. 2014). These two VOCs constituted 0.71 - 1.71% and 7.48 - 13.91% of the vapour phase of the shoots of *R. graveolens* (Table 1). In addition, most of the VOCs identified in this work (Table 1) have been reported previously in the EOs of this species and other Rutaceaes (Berger et al. 1990; Soleimani et al. 2009; da Silva et al. 2014; Bennaoum et al. 2017). The compound 2-methoxy-3-isopropyl-5-methylpyrazine was reported by Dob et al. (2008) in the EO of *R. chalepensis*.

The relative percentage (R%) of 30 VOCs extracted with different types of fiber coating were grouped according to the profile of VOCs obtained by the hierarchical cluster analysis (HCA). The variation of the R% of VOCs between the fibers was represented by the color change in the heatmap; the color ranged from dark red to light green with the increase of the relative percentage (Fig. 6). The dendrogram associated with the heatmap showed that the DVB/CAR/PDMS (50/30 μm) and PDMS/DVB (65 μm) fibers were similar and formed a separated group in relation to the other fibers tested (top of Fig. 6).

These fibers were more selective for 14 of the 30 VOCs identified in the samples, in comparison with the other fibers. These VOCs were: 2 ketones [nonan-2-one (a) and octan-2-one (b)], 2 ether [2-methoxy-3-isopropyl-5-methyl pyrazine (c) and 1-methoxy-2-methylbenzene (d)], 7 esters [methyl-4-methylhex-2-enoate (e), 3-methylbut-3-enyl 3-methylbutanoate (f), ethyl 3-methylbutanoate (g), 2-methylpropyl 2-methylbutanoate (h), 3-methylbut-3-enyl 2-methylbutanoate (i), propan-2-yl-methylbutanoate (j) and ethyl 2-methylbutanoate (k)], one aldehyde [nonanal (l)] and 2 monoterpenes [limonene (m) and sabinene (n)]. The VOCs b, g, h, j, k, l, m represented about 7.34% of the total area of detected peaks and their R% was highest in the DVB/CAR/PDMS fiber, in comparison with PDMS/DVB fiber. The efficiency of the extraction for the remaining VOCs, including nonan-2-one, was similar between these two fibers and repeats and the R% was about 50.75% (Fig. 6).

DVB/CAR/PDMS fiber improves the desorption of high molecular weight analytes compared to CAR/PDMS fiber and extracts low molecular weight analytes to compensate for the limitations of PDMS/DVB fiber (mainly extracts VOCs C_{18}-C_{24}). Thus, the extraction of compounds over a wide molecular weight range its possible, increasing the effectiveness of analyte extraction in a complex mixture (Risticevic et al. 2010; Shirey 2012). The DVB/CAR/PDMS fiber has been more efficient than PDMS/DVB and PDMS fibers for the extraction of low molecular weight compounds such as aldehydes, alcohols, esters and ketones (Xiao et al. 2014; Elizalde-González and Segura-Rivera 2018).

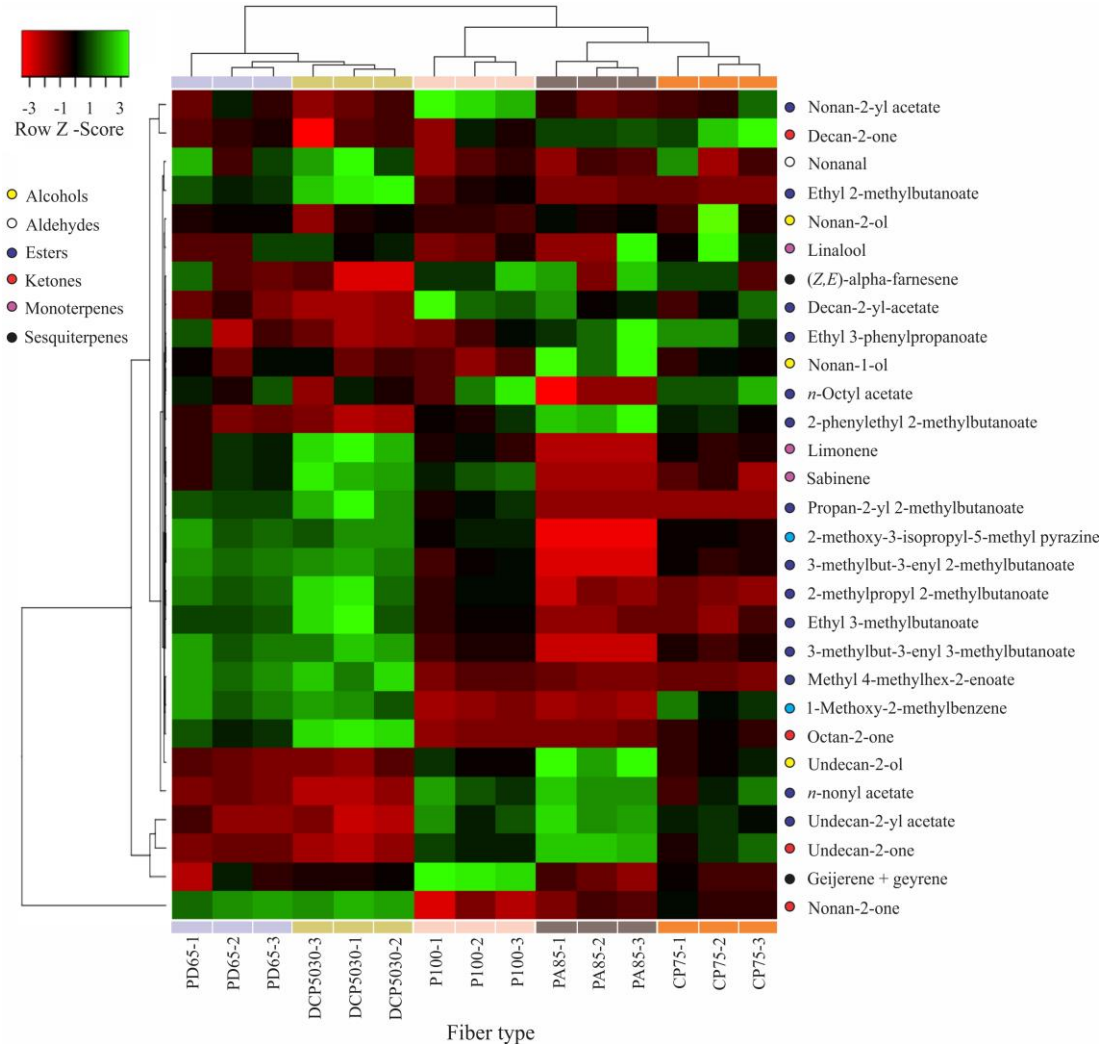

Figure 6. Hierarchical clustering analysis dendrogram associated with heatmap of the volatiles organic compounds extracted by HS-SPME from *in vitro* plants of *Ruta graveolens* using different SPME fibers. **PD65**: polydimethylsiloxane/divinylbenzene fiber (65 µm); **DCP5030**: divinylbenzene/carboxen on PDMS fiber (50/30 µm); **P100**: polydimethylsiloxane fiber (100 µm); **PA85**: polyacrylate fiber (85 µm); **CP75**: carboxen/polydimethylsiloxane fiber (75 µm). The numbers 1-3 indicate the repetitions for each fiber.

The PDMS fiber was more selective for the esters nonan-2-yl acetate (7.68%) and decan-2-yl acetate (0.53%) and the sesquiterpenes geyrene and geijerene (15.62%); which are equivalent to about 23.83% of the total area of detected peaks. The relative percentage of undecan-2-one (15.46%), undecan-2-yl-acetate (11.97%), *n*-nonyl acetate (2.21%), ethyl 3-phenylpropanoate (0.32%), 2-phenylethyl 2-methylbutanoate (0.49%), undecan-2-ol (1.14%) and nonal-1-ol (0.32%) was highest in PA fiber; these VOCs presented ~ 31.91% of the total area of detected peaks (Fig 6). The PDMS and PA fibers can be used to study the effect of different biotic and

abiotic factors on these particular compounds. This greater selectivity eliminates the interference of compounds of lower interest; although the amount of VOCs extracted in terms of detected area peak was smaller with these two fibers (Fig. 4).

Similar to that obtained in this study, Perera et al. (2017) reported that PDMS fiber (30 μm) was more efficient for the extraction of most VOCs from native plants of *R. graveolens*, in comparisson to PA fiber (85 μm). These authors also obtained greater selectivity for undecan-2-one with PA fiber. In addition, the high selectivite of PDMS fiber to absorb sesquiterpenes had already been reported for *Salvia dolomitica* shoots and callus cultivated under *in vitro* conditions and in greenhouse plants (Bassolino et al. 2015).

The principal component analysis (PCA) of the VOCs extracted from the *in vitro* plants of *R. graveolens* with different type of fiber coating indicated that the compounds nonan-2-one (10), undecan-2-one (22), undecanyl acetate (27), geyrene + geijerene (16) and nonanal (13) contributed the most to discriminate the fibers, which were classified into three groups: (i) DVB/CAR/PDMS and PDMS/DVB, (ii) CAR/PDMS and PA and (iii) PDMS. The compound 22 was associated with the PA fiber, the 10 with the DVB/CAR/PDMS and PDMS/DVB fibers and the 16 with the PDMS fiber (Fig. 7A). The analysis indicated that ketones, esters, ethers and monoterpenes contributed the most to the classification of the fibers (Fig. 7B). These results were correlated with that obtained in the HCA (Fig. 6)

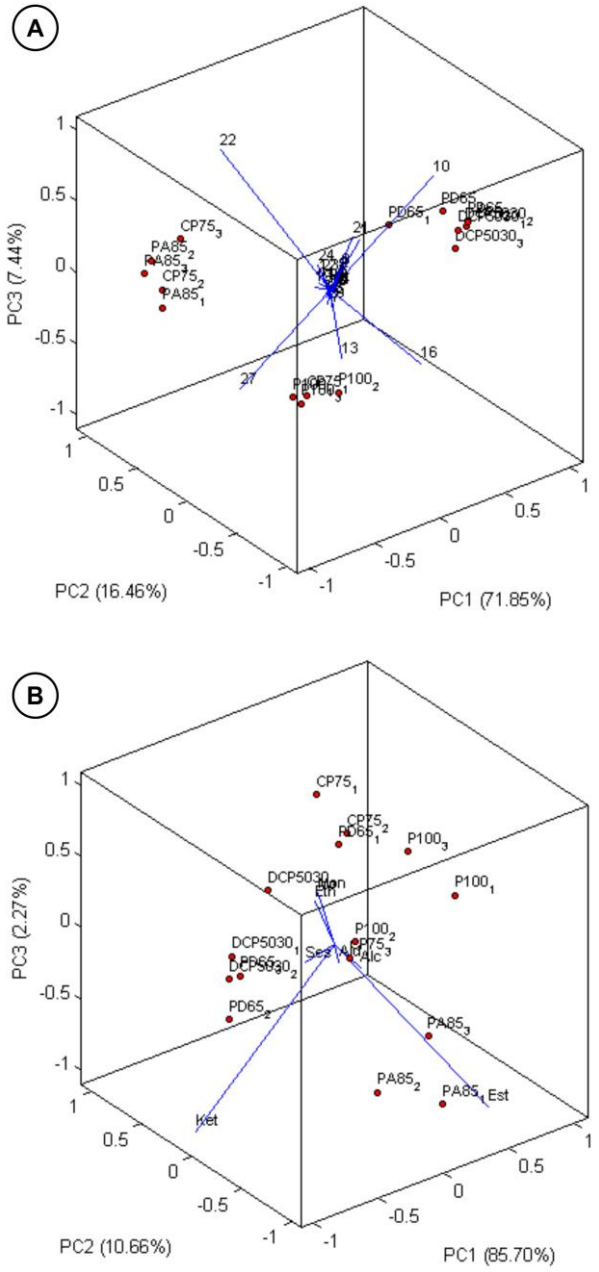

Figure 7. Principal component analysis of the volatiles extracted by HS-SPME from *in vitro* plants of *Ruta graveolens* using different SPME fibers. **A**: Loading plot; **B**: Score plot. **PD65**: polydimethylsiloxane/divinylbenzene fiber (65 µm); **DCP5030**: divinylbenzene/carboxen on PDMS fiber (50/30 µm); **P100**: polydimethylsiloxane fiber (100 µm); **PA85**: polyacrylate fiber (85 µm); **CP75**: carboxen/polydimethylsiloxane fiber (75 µm). Nonan-2-one (10), undecan-2-one (22), undecanyl acetate (27), geyrene + geijerene (16) and nonanal (13)

The extraction time affected the efficiency of VOCs extraction

The chromatogram of different fresh plant weight (200 and 500 mg) and fiber exposure times (15 and 30 min) tested were shown from Supplementary Material (Fig. S1). The exposure time of the fiber to the vapor phase affected the efficiency of VOCs extraction by HS-SPME from *in vitro* plants of *R. graveolens*. The number of peaks detected and the total peak area increased with the longer exposure time of the fiber (30 min), regardless of the amount of biomass used (Fig. 8A and B).

The SPME depends on the diffusion rate and the coefficient of distribution of the analytes between the fiber coating, the headspace and the sample matrix. Thus, the extraction efficiency of some analytes varies with the time of exposure of the fiber, depending on the volatility and chemical nature of VOCs (Stashenko et al. 2004; Pawliszyn 2012). Extraction times of 15 - 45 min have been used to extract the analytes with greater relative abundance in the headspace of several plant species using DVB/CAR/PDMS fiber (Musarurwa et al. 2012; Silva et al. 2018). Extraction times longer than 30 min were not considered to avoid extending the total analysis time for each sample in this work. As in *R. graveolens*, exposure of the fiber for 30 min to the headspace has allowed greater adsorption of VOCs from *Agastache rugosa* and ground almond (*Prunus dulcis*) samples (Zielińska et al. 2011; Xiao et al. 2014).

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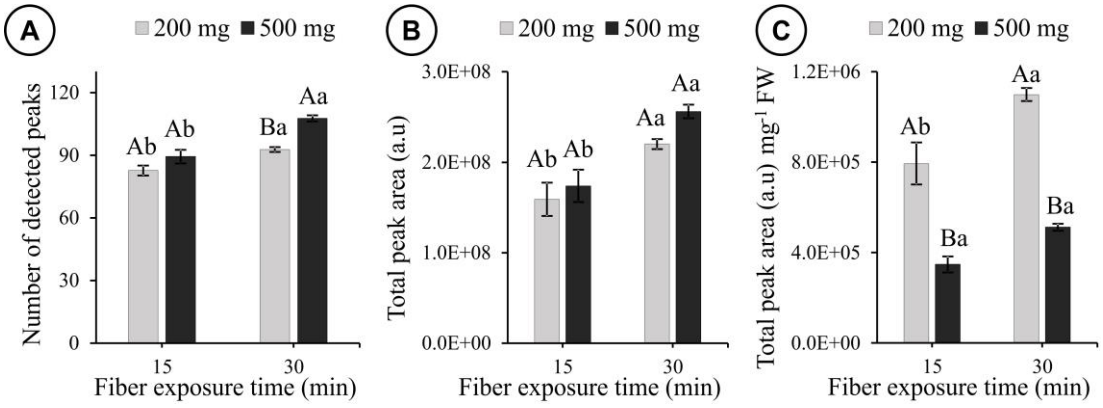

Figure 8. Effect of the plant fresh weight and the time of exposure of the fiber on the extraction of volatile organic compounds from *in vitro* plants of *Ruta graveolens* by HS-SPME**. A**: number of detected peaks; **B**: total area of the detected peaks. **C**: total area of the detected peaks mg^{-1} fresh weight (**FW**). The SPME fiber used was divinylbenzene/Carboxen on polydimethylsiloxane fiber, 50/30 µm. Means followed by the same lowercase letter do not differ statistically for time of exposure; means followed by the same capital letter did not differ significantly among the plant fresh

weight (Tukey's test at 5%). Error bars indicate the standard error among replicates (n = 3).

The relative abundance of ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, propan-2-yl-2-methylbutanoate and sabinene was ~ 1.5 - 2.5 times higher when the fiber was exposed for 15 min at headspace of both 200 mg and 500 mg fresh weight, in comparison with 30 min extraction. The relative percentage of linalool, nonanal, (*Z*,*E*)-α-farnesene increased ~ 1.2 - 5 times with the longer fiber exposure time (Table 2). The concentration of one of the geyjerene/geyrene isomers varied with the extraction time only when 500 mg of biomass were used; the relative percentage of one of these isomers decreased from 10.24 to 8.69% by increasing the fiber exposure time from 15 to 30 min. However, the relative concentration of the major compounds: nonan-2-one, undecan-2-one, undecan-2-yl-acetate, nonan-2-yl acetate, decan-2-one and *n*-nonyl acetate was not altered by changes in biomass and time of exposure of the fiber (Table 2).

Table 2. Volatile organic compounds extracted from *in vitro* plants of *Ruta graveolens* by HS-SPME varying the amount of plant fresh weight and fiber exposure times.

N°	Compound	Relative composition[a] (%) ± SE			
		200 mg x 15 min	200 mg x 30 min	500 mg x 15 min	500 mg x 30 min
1	Ethyl 2-methylbutanoate	3.47 ± 0.79	1.29 ± 0.01	3.36 ± 0.38	2.20 ± 0.08
2	Ethyl 3-methylbutanoate	0.83 ± 0.18	0.45 ± 0.03	1.18 ± 0.18	0.62 ± 0.11
3	Propan-2-yl 2-methylbutanoate	0.62 ± 0.08	0.23 ± 0.01	0.72 ± 0.15	0.45 ± 0.04
4	Sabinene	0.52 ± 0.06	0.26 ± 0.02	0.69 ± 0.17	0.39 ± 0.02
5	Octan-2-one	1.08 ± 0.05	0.83 ± 0.11	1.21 ± 0.20	1.24 ± 0.02
7	2-methylpropyl 2-methylbutanoate	0.50 ± 0.03	0.29 ± 0.03	0.56 ± 0.13	0.50 ± 0.06
8	1-Methoxy-2-methylbenzene	0.71 ± 0.03	0.60 ± 0.07	0.75 ± 0.15	0.72 ± 0.04
9	Methyl 4-methylhex-2-enoate	0.78 ± 0.02	0.62 ± 0.06	0.78 ± 0.10	0.87 ± 0.06
10	Limonene	0.38 ± 0.07	0.22 ± 0.01	0.52 ± 0.17	0.44 ± 0.02
11	Nonan-2-one	48.76 ± 0.47	46.81 ± 1.87	46.47 ± 0.89	48.04 ± 0.57
12	Linalool	0.28 ± 0.02	0.36 ± 0.09	0.27 ± 0.00	0.52 ± 0.07
13	Nonan-2-ol	0.29 ± 0.03	0.35 ± 0.03	0.29 ± 0.04	0.17 ± 0.08
14	Nonanal	0.54 ± 0.05	0.98 ± 0.09	0.30 ± 0.03	1.50 ± 0.26
15	3-Methylbut-3-enyl 2-methylbutanoate	0.21 ± 0.01	0.21 ± 0.01	0.21 ± 0.02	0.27 ± 0.01
16	3-Methylbut-3-enyl 3-methylbutanoate	0.07 ± 0.00	0.08 ± 0.01	0.07 ± 0.01	0.10 ± 0.00
17	Geijerene or geyrene[b]	1.22 ± 0.02	1.26 ± 0.11	1.37 ± 0.02	1.23 ± 0.03
18	Geijerene or geyrene[b]	9.45 ± 0.13	9.16 ± 0.84	10.24 ± 0.24	8.69 ± 0.18
19	2-Methoxy-3-isopropyl-5-methyl pyrazine	0.18 ±0.01	0.23 ± 0.01	0.17 ± 0.01	0.25 ± 0.01
21	Nonan-1-ol	0.15 ± 0.01	0.31 ± 0.06	0.15 ± 0.02	0.17 ± 0.02
22	Decan-2-one	3.64 ± 0.10	4.22 ± 0.08	3.50 ± 0.16	3.72 ± 0.16
23	*n*-Octyl acetate	0.21 ± 0.01	0.26 ± 0.01	0.21 ± 0.01	0.24 ± 0.01
25	Nonan-2-yl acetate	5.13 ± 0.12	5.13 ± 0.28	5.13 ± 0.32	4.51 ± 0.24
28	Undecan-2-one	7.55 ± 0.25	8.83 ± 0.45	7.42 ± 0.74	6.86 ± 0.18

29	Undecan-2-ol	0.35 ± 0.02	0.53 ± 0.04	0.38 ± 0.06	0.38 ± 0.04
30	*n*-Nonyl acetate	1.14 ± 0.06	1.32 ± 0.08	1.15 ± 0.12	1.09 ± 0.03
32	Decan-2-yl-acetate	0.26 ± 0.02	0.30 ± 0.02	0.28 ± 0.03	0.26 ± 0.01
33	Ethyl 3-phenylpropanoate	0.11 ± 0.01	0.20 ± 0.02	0.10 ± 0.01	0.14 ± 0.01
36	Undecan-2-yl acetate	4.12 ± 0.19	5.27 ± 0.22	4.34 ± 0.62	4.46 ± 0.55
39	2-Phenylethyl 2-methylbutanoate	0.12 ± 0.01	0.19 ± 0.02	0.12 ± 0.02	0.13 ± 0.01
40	(*Z*,*E*)-alpha-farnesene	0.06 ± 0.03	0.21 ± 0.02	0.09 ± 0.02	0.17 ± 0.05
	Alcohols	0.79 ± 0.06	1.19 ± 0.11	0.81 ± 0.12	0.71 ± 0.09
	Aldehydes	0.54 ± 0.05	1.50 ± 0.26	0.30 ± 0.03	1.50 ± 0.26
	Esters	17.57 ±0.73	15.84 ± 0.49	18.21 ± 0.45	15.84 ± 0.30
	Ethers	0.89 ± 0.03	0.83 ± 0.07	0.92 ± 0.16	0.97 ± 0.04
	Ketones	61.04 ± 0.41	60.69 ± 1.47	58.60 ± 0.34	59.85 ± 0.74
	Monoterpenes	1.18 ± 0.13	0.83 ± 0.09	1.49 ± 0.34	1.34 ± 0.09
	Sesquiterpenes	10.73 ± 0.16	10.62 ± 0.95	11.70 ± 0.27	10.10 ± 0.20
	Unidentified compounds	3.74 ± 0.18	5.11 ± 0.18	3.87 ± 0.34	4.48 ± 0.38
	Total	96.48 ± 0.06	96.08 ± 0.13	95.90 ± 0.36	94.78 ± 0.03

[a] Averages of percentage of relative area of the chromatographic peaks detected (n = 3); [b] Diastereoisomers reported in the essential oil of *Ruta graveolens* by Soleimani et al. (2009); however, the assignment of the corresponding peak to the geigerene or geyrene in this work was not possible. **SE**: standard error (n = 3); fresh weight (**200** and **500 mg**) and fiber exposure times (**15** and **30 min**). The divinylbenzene/carboxen on PDMS fiber (50/30 μm) was used. The compounds are listed in the same sequence shown in Table 1; the unidentified peaks were excluded.

The coating of solid sorbents has a well-defined crystalline structure which can reduce the diffusion coefficients of VOCs. The adsorption occurs only on the porous surface of the solid coating and the analytes compete for the active sites of the adsorbent depending on their affinity. VOCs with low affinity for the adsorbent form weaker bonds and are displaced by higher affinity or more abundant analytes in the sample, when longer extraction times are used (Stashenko and Martínez 2007; Pawliszyn 2012; Shirey 2012). Some of the VOCs emitted by the *R. graveolens* plants, such as the geyrene isomer, may be displaced from the DVB/CAR/PDMS fiber by other compounds with higher affinity or higher concentration in the headspace, especially when using 500 mg of fresh mass (Table 2). These displacement problems between the analytes of interest can be decreased by using less concentrated samples and shorter extraction times. Therefore, the parameters such as amount of sample and time of extraction should be optimized for HS-SPME depending on the complexity of the sample (Risticevic et al. 2010).

The number of detected peaks increased (93 to 108) when the fresh weight of the plant was increased from 200 to 500 mg, with 30 min of fiber exposure (Fig. 7A and B). The total peak area mg^{-1} fresh weihgt decreased with the increase of the biomass used, regardless of the fiber exposure time; however, this variable was not altered by the time of fiber exposure when used 500 mg of biomass (Fig. 8C).

Biomass of 0.1 - 1 g has been used to extract VOCs from plants propagated *in vitro* and *ex vitro* using the PDMS/DVB or DVB/CAR/PDMS fibers (Musarurwa et al. 2012; Zielińska et al. 2011; Castellar et al. 2013; Passinho-Soares et al. 2017). The efficiency of VOCs extraction by HS-SPME is strongly affected by the amount of sample as compared to temperature and time of extraction (Camelo et al. 2019).

Increasing the amount of sample enhances the concentration of VOCs in the headspace; however, this increase is not always linear for some of the VOCs extracted (Zhang et al. 2007; George et al. 2018). The increase of the biomass decreases the repeatability of the extraction and possibly affect the absorption rate of the analytes in the fiber. The excess sample may also lead to fiber saturation; with this, the area of detected peaks must remain almost constant from a certain amount of sample used (George et al. 2018). In this work, the VOCs detected in the *in vitro* plants of *R. graveolens* increased when using 500 mg of fresh mass; however, the lowest total peak area mg^{-1} fresh weihgt obtained indicates that this biomass led to saturation of the fiber, so that 200 mg of fresh mass is sufficient for the extraction of VOCs.

The higher volume occupied by the sample decreases the headspace and diffusion of the VOCs between the sample, the headspace and the fiber; even solid particles of the sample can adhere to the fiber because of their proximity, which reduces the contact surface between the fiber and the adsorbed VOCs (Zhang et al. 2007; George et al. 2018). However, the distance between the fiber and the sample used in this study was sufficient to avoid direct contact between them.

Thus, the conditions 200 mg of fresh plant weight x 30 min of fiber exposure were selected as the optimal conditions for the extraction of VOCs by HS-SPME using the CAR/PDMS/DVB fiber.

The composition of the volatile fraction of *R. graveolens* plants *in vitro* varied with the light spectral quality

Chromatographic profiles of the samples obtained in GC-FID differed for peaks 12-16 (linalool, nonan-2-ol, nonanal, 3-methylbut-3-enyl 2-methylbutanoate and 3-methylbut-3-enyl 3-methylbutanoate) compared to GC-MS (Fig. 9). The separation of these peaks in GC-FID was insufficient for their quantification (Fig.

9B). The VOCs identified in plants grown under blue, B/R, red, and white LEDs corresponded to 90.25, 91.11, 89.33, 90.4 and 90.35% of the total area of the detected peaks in GC-FID, respectively (Table 3).

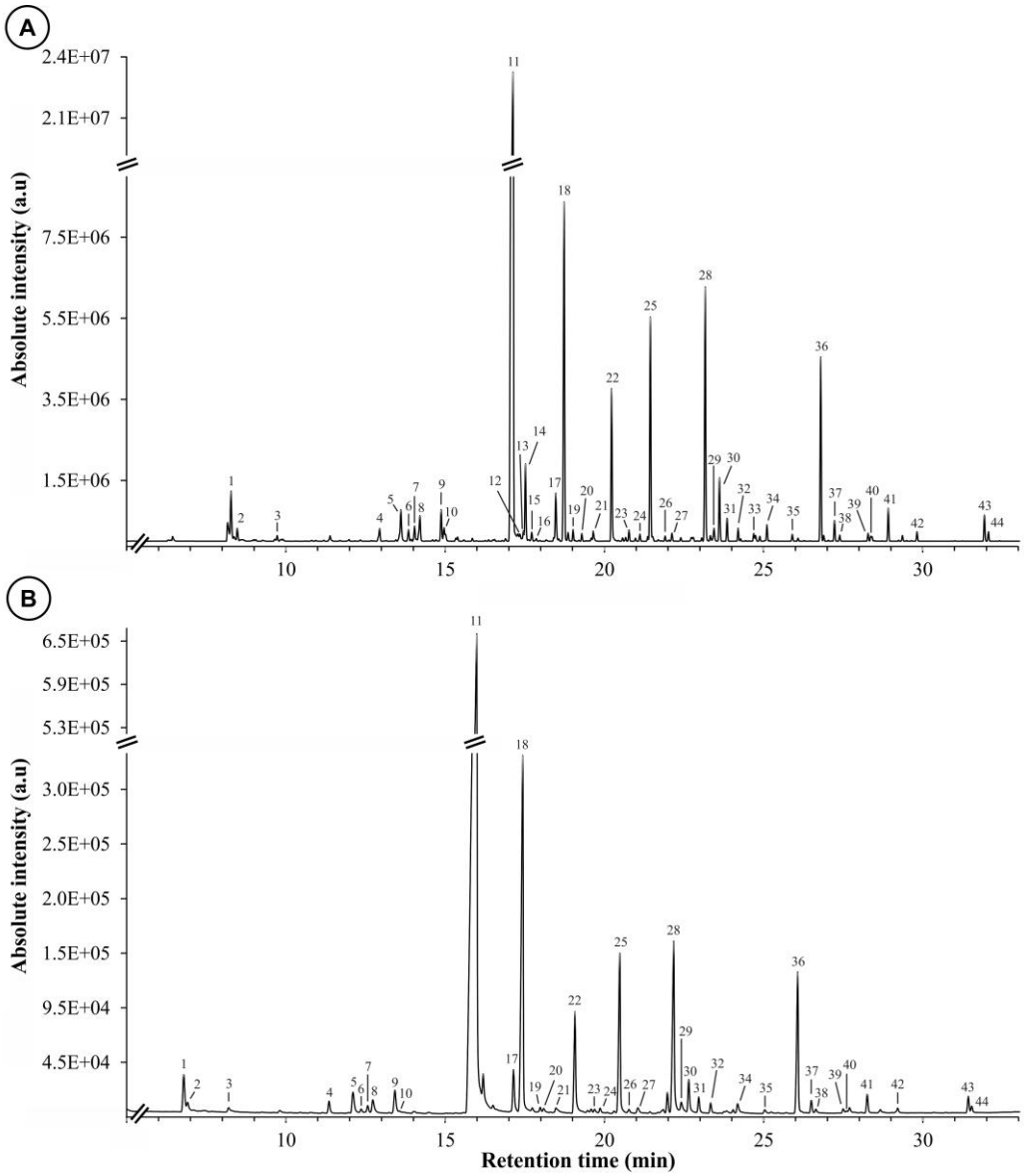

Figure 9. Chromatographic profile of the volatile fraction of *in vitro* plants of *Ruta graveolens* grown under white LEDs lamp. **A**: GC-MS, SLB®-5MS column (30 m x 0.25 mm x 0.25 µm film thickness); **B**: GC-FID, SPB5 column (30 m x 0.25 mm x 0.25 µm film thickness). Extraction performed by HS-SPME with the divinylbenzene/Carboxen/polydimethylsiloxane fiber (50/30 µm) exposed for 30 min to the vapor phase of 200 mg fresh mass. The peaks identified are shown in the table 3.

The relative concentration of nonan-1-ol and geyrene and geijerene isomers varied depending LED lamp used. *In vitro* plants of *R. graveolens* cultivated under red LEDs had a higher relative percentage of geyrene + geijerene (17.88 ± 2.47%).

Thus, the content of sesquiterpenes was that most differed among the treatments, obtaining a greater percentage under red LEDs. The relative percentage of geyrene + geijerene decreases with light quality in the following order: Red (R) > Far red (FR) > White (W) > Blue/Red (B/R) > Blue (B) LEDs. The relative percentage of nonan-1-ol increased under white LED (0.28 ± 0.02) and the effect of the R and FR lamps was inverse to that obtained for the geyrene isomers. Nonan-1-ol varied with the lamps tested in the following order: W > B = B/R > R = FR LEDs. The other compounds identified in the vapor phase of the plants did not differ significantly between the light qualities tested (Table 3). The geyrene isomers are volatile derived from isoprenoid pathways; while nonan-1-ol is biosynthesized by lipoxygenase pathway. According to the results obtained, both biosynthesis pathways were altered mainly by the red and far red LEDs.

Table 3. Volatile organic compounds from *in vitro* plants of *Ruta graveolens* grown under different light qualities.

N°	Compound	t_R[a] (min)	Relative composition[b] (%) ± SE				
			Blue	Blue/Red	Red	Far red	White
1	Ethyl 2-methylbutanoate	6.805	1.25 ± 0.60 a	1.31 ± 0.14 a	1.37 ± 0.60 a	0.59 ± 0.25 a	1.13 ± 0.26 a
2	Ethyl 3-methylbutanoate	6.929	0.53 ± 0.13 a	0.41 ± 0.08 a	0.32 ± 0.10 a	0.17 ± 0.02 a	0.35 ± 0.08 a
3	Propan-2-yl 2-methylbutanoate	8.215	0.42 ± 0.10 a	0.26 ± 0.06 a	0.34 ± 0.08 a	0.22 ± 0.08 a	0.25 ± 0.02 a
4	Sabinene	11.367	0.22 ± 0.06 a	0.28 ± 0.05 a	0.59 ± 0.22 a	0.40 ± 0.14 a	0.32 ± 0.06 a
5	Octan-2-one	12.117	0.81 ± 0.14 a	0.86 ± 0.04 a	0.64 ± 0.06 a	0.64 ± 0.07 a	0.78 ± 0.07 a
7	2-methylpropyl 2-methylbutanoate	12.576	0.32 ± 0.07 a	0.23 ± 0.02 a	0.42 ± 0.08 a	0.22 ± 0.03 a	0.24 ± 0.08 a
8	1-Methoxy-2-methylbenzene	12.740	0.65 ± 0.17 a	0.39 ± 0.03 a	0.39 ± 0.04 a	0.59 ± 0.27 a	0.48 ± 0.13 a
9	Methyl 4-methylhex-2-enoate	13.428	0.55 ± 0.10 a	0.68 ± 0.05 a	0.94 ± 0.30 a	0.61 ± 0.21 a	0.61 ± 0.13 a
10	Limonene	13.579	0.12 ± 0.03 a	0.04 ± 0.01 a	0.18 ± 0.04 a	0.09 ± 0.02 a	0.10 ± 0.04 a
11	Nonan-2-one	15.992	57.51 ± 1.65 a	56.41 ± 1.93 a	46.31 ± 4.84 a	50.94 ± 3.62 a	56.11 ± 2.66 a
17	Geijerene + geyrene[c]	17.281	6.58 ± 0.16 c	9.04 ± 1.64 bc	17.88 ± 2.47 a	15.44 ± 2.24 ab	10.41 ± 0.70 abc
19	2-methoxy-3-isopropyl-5-methyl pyrazine	17.976	0.09 ± 0.05 a	0.05 ± 0.02 a	0.17 ± 0.02 a	0.11 ± 0.03 a	0.11 ± 0.02 a
21	Nonan-1-ol	18.501	0.19 ± 0.05 ab	0.21 ± 0.01 ab	0.08 ± 0.04 b	0.10 ± 0.02 b	0.28 ± 0.02 a
22	Decan-2-one	19.044	4.27 ± 0.52 a	3.56 ± 0.10 a	4.02 ± 0.12 a	4.14 ± 0.35 a	2.98 ± 0.31 a
23	*n*-Octyl acetate	19.677	0.21 ± 0.07 a	0.11 ± 0.01 a	0.11 ± 0.02 a	0.10 ± 0.00 a	0.11 ± 0.01 a
25	Nonan-2-yl acetate	20.575	3.86 ± 1.50 a	3.43 ± 0.30 a	3.00 ± 0.57 a	3.42 ± 1.13 a	3.73 ± 0.58 a

28	Undecan-2-one	22.192	7.20 ± 2.80 a	7.94 ± 0.43 a	7.26 ± 0.57 a	7.85 ± 1.48 a	6.48 ± 1.74 a
29	Undecan-2-ol	22.420	0.41 ± 0.11 a	0.48 ± 0.00 a	0.38 ± 0.04 a	0.49 ± 0.07 a	0.51 ± 0.12 a
30	*n*-nonyl acetate	22.651	1.13 ± 0.21 a	0.88 ± 0.05 a	0.83 ± 0.05 a	0.72 ± 0.04 a	0.86 ± 0.10 a
32	Decan-2-yl acetate	23.326	0.25 ± 0.08 a	0.22 ± 0.03 a	0.26 ± 0.06 a	0.23 ± 0.05 a	0.23 ± 0.01 a
33	Ethyl 3-phenylpropanoate	23.774	0.08 ± 0.03 a	0.08 ± 0.01 a	0.06 ± 0.02 a	0.05 ± 0.01 a	0.06 ± 0.01 a
36	Undecan-2-yl acetate	26.068	3.37 ± 0.41 a	4.03 ± 0.09 a	3.61 ± 0.42 a	3.10 ± 0.41 a	3.96 ± 0.50 a
39	2-phenylethyl 2-methylbutanoate	27.371	0.14 ± 0.05 a	0.15 ± 0.03 a	0.11 ± 0.04 a	0.14 ± 0.05 a	0.21 ± 0.11 a
40	(*Z*,*E*)-alpha-farnesene	27.450	0.07 ± 0.02 a	0.08 ± 0.02 a	0.05 ± 0.01 a	0.04 ± 0.01 a	0.07 ± 0.02 a
	Alcohols		0.60 ± 0.13 a	0.70 ± 0.01 a	0.46 ± 0.07 a	0.58 ± 0.06 a	0.78 ± 0.14 a
	Esters		12.12 ± 2.23 a	11.77 ± 0.72 a	11.37 ± 2.20 a	9.58 ± 2.08 a	11.72 ± 0.44 a
	Ethers		0.75 ± 0.20 a	0.44 ± 0.03 a	0.56 ± 0.05 a	0.69 ± 0.29 a	0.59 ± 0.15 a
	Ketones		69.79 ± 1.69 a	68.77 ± 2.35 a	58.23 ± 5.39 a	63.57 ± 5.30 a	66.35 ± 1.12 a
	Monoterpenes		0.34 ± 0.08 a	0.32 ± 0.06 a	0.78 ± 0.26 a	0.49 ± 0.15 a	0.42 ± 0.04 a
	Sesquiterpenes		6.65 ± 0.14 c	9.11 ± 1.65 bc	17.93 ± 2.49 a	15.48 ± 2.25 ab	10.48 ± 0.70 abc
	Total		90.25 ± 0.25	91.11 ± 0.12	89.33 ± 0.72	90.40 ± 0.90	90.35 ± 0.61

[a] Average of retention time on SPB5 column (30 m x 0.25 mm d_i x 0.25 μm film thickness) in the GC-FID; [b] Averages of percentage of relative area of the chromatographic peaks detected (n = 3); [c] Diastereoisomers reported in the essential oil of *Ruta graveolens* by Soleimani et al. (2009); however, the assignment of the corresponding peak to the geigerene or geyrene in this work was not possible. **SE**: standard error (n = 3). Extraction performed by HS-SPME with the divinylbenzene/Carboxen/polydimethylsiloxane fiber (50/30 μm) exposed for 30 min to the vapor phase of 200 mg fresh mass. The compounds are listed in the same sequence shown in Table 1; the unidentified peaks were excluded. Means followed by the same lowercase letter did not differ significantly among the treatments (Tukey's test at 5%).

The light spectral quality regulates the production of some VOCs involved in the chemical defense of the plant against different types of stress (Ballaré et al. 2014; Legris et al. 2017; Batista et al. 2018). Changes in light quality are perceived by photoreceptors such as phytochrome B (phyB) and phototropins (Ballaré et al. 2014; Li et al. 2017; Batista et al. 2018). R: FR high ratio increases the phyB activity and the concentration of sesquiterpenes and some ketones, improving the plant response to herbivores, pathogens and other plants (Kegge et al. 2013; Ballaré et al. 2014; Kegge et al. 2015). Red light alters the production of monoterpenes and sesquiterpenes depending of the plant species. The red light increases the abundance of oxygenated sesquiterpenes in *Petroselinum crispum* and monoterpenes in the *in vitro* plants of *Achillea millefolium* (Alvarenga et al. 2015; Ascrizzi et al. 2018). As

the content of monoterpenes in the *in vitro* plants of *Hyptis suaveolens* decreased with the red LED (Andrade et al. 2015).

Fu et al. (2015) have shown that blue and red light regulates the enzymatic activity of terpene synthase (TPS), 9/13-lipoxygenase (LOX) and phenylalanine ammonialyase (PAL) in preharvest tea leaves. These enzymes are involved in the biosynthesis of three classes of VOCs: (i) terpenes, by isoprenoid pathways (methylerythritol phosphate and mevalonate pathways); (ii) fatty acid derivatives, by lipoxygenase pathway from C_{18} unsaturated fatty acids (linoleic or linolenic acids) and (iii) phenylpropanoids/benzenoids from shikimate *via* L-phenylalanine. These authors also determined that the content of nonan-1-ol was slightly altered by exposure to monochromatic light blue, red and near-infrared light. Thus, red LEDs stimulated the production of sesquiterpenes in the *in vitro* plants of *R. graveolens*, possibly by the increase in the TPS activity, among others. Meanwhile, the biosynthesis of nonan-1-ol was downregulated by the lamps R and FR.

The initial step of the isoprenoid biosynthetic pathway depends on 3-hydroxy-3-methylglutaryl coenzyme A (HMC-CoA) reductase and their activity is suppressed more effectively by blue light than red in *Arabidopsis seedlings* (Learned 1996). The lower production of sesquiterpenes was obtained in the plants of *R. graveolens* cultivated under blue LED; the activity of the HMC-CoA reductase must have been reduced by this monochromatic light.

The effect of light quality on VOCs fatty acid derivatives production is still poorly explored, so the mechanism by which monochromatic light regulated the production of compounds such as nonan-1-ol is unclear.

Conclusions

The HS-SPME method was adapted to characterize the volatile fraction of *in vitro* plants of *Ruta graveolens*. The DVB/CAR/PDMS fiber, followed by the PDMS/DVB fiber, was the most efficient for the extraction of the VOCs emitted by plants of rue, obtaining the largest area of the peaks. Although the area of the identified peaks was smaller with PA and PDMS firing, these fibers were more

selective for some major compounds such as geyrene isomers and undecan-2-one, reducing the interference of other peaks of lower interest.

HS-SPME was used to determine the effect of light spectral quality on the production of VOCs in *in vitro* propagated *Ruta graveolens* plants. Red and far red monochromatic light altered the biosynthesis of the geyrene and geyjerene isomers and nonan-1-ol; while the major compounds nonan-2-one and undecan-2-one did not differ with light quality. This work provides new information on the regulation of the biosynthesis of VOCs fatty acid derivatives by light quality; this work is the first evidence of the influence of light spectral quality on VOCs production in *Ruta graveolens* plants.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Supplementary material

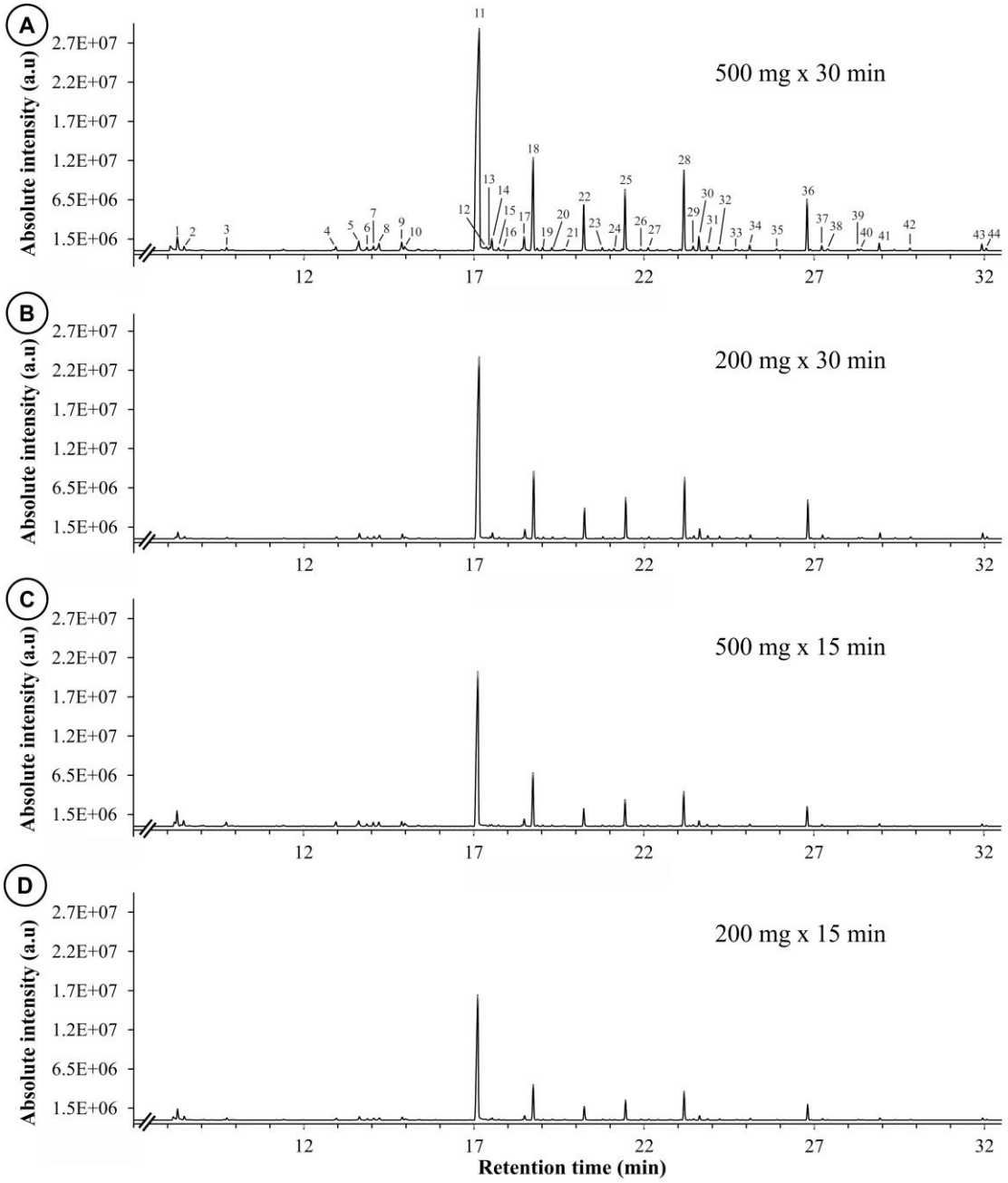

Figure S1. Volatile fraction chromatogram (GC-MS) extracted from *in vitro* plants of *Ruta graveolens* by HS-SPME varying the amount sample and fiber exposure times. The peaks identified are shown in the table 1.

CHAPTER 2

Micropropagation of *Piper crassinervium*: an improved protocol for faster growth and augmented production of phenolic compounds

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Highlights

1. Adjustments in levels of sucrose, irradiance and gas exchange reduced the time between micropropagation cycles in *Piper crassinervium*.
2. DKW/Junglans and MS are the most effective media for *in vitro* phenolic compounds production in *P. crassinervium.*
3. Explants with preexisting leaf are the most responsive for the micropropagation of *P. crassinervium.*

Abstract

The medicinal plant *Piper crassinervium* is a source of bioactive compounds with potential use in agrochemical and pharmaceutical industries. However, its propagation is slow and influenced by the composition of the culture medium and growth conditions. In the genus *Piper*, tissue oxidation limits the biomass and recovery of bioactive compounds such as phenols. We evaluated the effect of medium formulations, sucrose, growth regulators, medium consistency, explant type, gas exchange and irradiance levels on growth and phenols and total flavonoids content in *P. crassinervium in vitro* plants. Murashige and Skoog (MS) and DKW/Juglans (DKW/J) media induced the highest production of chlorophyll and phenolic compounds; however, the regeneration rate of the explants in the DKW/J medium was the lowest (~75%) in comparison with the other media (90%). The maximum biomass production was achieved when half-strength MS (½MS) liquid medium was used; while the optimum sucrose level depended on the medium salt concentration used. Notably, the irradiance altered the accumulation of biomass and phenolic compounds. The presence of one remaining leaf in the nodal segments, used as explants, produced taller plants in ½MS liquid medium, combined or not with 2-iP. The optimal growth conditions for biomass and phenolic compound production in *P. crassinervium in vitro* plants established were: nodal explant with a preexisting leaf cultured on ½MS stationary liquid medium, 15 g L^{-1} sucrose, using lids with one PTFE 0.45-µm-pore size membrane and an irradiance of 100 µmol m^{-2} s^{-1}.

Key words: Gas exchange, irradiance, medium composition, secondary metabolism, sucrose.

Introduction

The genus *Piper* (Piperaceae) includes ~ 2000 species, divided among herbs, small trees, climbers and shrubs, distributed in Neotropics, Asian Tropics and the South Pacific regions (Quijano-Abril et al. 2006; Jaramillo et al. 2008). *Piper* plants are recognized as producers of bioactive secondary metabolites (Kato and Furlan 2007). *Piper crassinervium* Kunth essential oil contains monoterpenes, sesquiterpenes, phenylpropanoids and phenols (Tognolini et al. 2006; Morandim-Giannetti et al. 2010), which are known for their broad spectrum of biological activity and important antifungal (Lago and Kato 2007; Morandim-Giannetti et al. 2010), antioxidant (Yamaguchi et al. 2006), antibacterial (Perigo et al. 2016) and anti-chagasic (Lopes et al. 2008) pharmacological activities.

These biological activities of the *P. crassinervium* extracts have increased their potential as a source of new bioactive compounds and biocatalysts of interest for the pharmaceutical and agrochemical industries (Lopes et al. 2008; López et al. 2010; Cáceres and Kato 2014). However, *P. crassinervium* has a low propagation rate since it is a light-demanding species, does not propagate vegetatively in nature and reproduces mainly by seeds (Figueiredo and Sazima 2004). *Piper* spp. seeds are recalcitrant, with slow germination and strongly influenced by light; they are produced once or twice a year depending on temperature, rainfall, and day length (Dousseau et al. 2011; Delgado-Paredes et al. 2012; Valentin-Silva et al. 2018). Propagation of *P. crassinervium* and other *Piper* species has been performed through stem cuttings, which is inefficient for large-scale production of plant material (Ahmad et al. 2014; Gomes and Krinski 2018), and *in vitro* propagation is an alternative method to increase biomass production without affecting wild populations of *Piper* species (Delgado-Paredes et al. 2012; Ahmad et al. 2014).

In vitro culture of plant cells, tissues and organs has been widely used to evaluate and/or manipulate factors affecting the biosynthesis of secondary metabolites aiming at the maximum production to meet growing demands of the industry (Savio et al. 2012; Dias et al. 2016; Szopa et al. 2017; Isah et al. 2018; Matsuura et al. 2018; Pérez-López et al. 2018). Production of phenolic compounds has been stimulated in tissues cultured *in vitro* of *P. crassinervium* and *P. nigrum* (Danelutte et al. 2005; Ahmad et al. 2014), but optimization of the process in *Piper* spp. remains largely unexplored.

Explant oxidation is one of the main problems for *in vitro* propagation of *Piper* spp., since it reduces regeneration capacity (Balbuena et al. 2009; Rani and Dantu 2012; Nirmal Babu et al. 2016), and antioxidants (ascorbic acid, polyvinylpyrrolidone and activated carbon) have been tested to decrease or neutralize plant oxidation in most *in vitro* propagation protocols of *Piper* (Rani and Dantu 2012; Nirmal Babu et al. 2016). However, the use of antioxidants may reduce the production of the phenolic compounds of interest and *in vitro* propagation protocols of *Piper* spp. must therefore be optimized.

In this study, we tested different culture conditions in order to optimize the production of biomass and phenolic compounds in *P. crassinervium* cultured *in vitro*.

Materials and Methods

Establishment of *in vitro* plants

Mature inflorescences of *P. crassinervium* were collected in April of 2015 from Mata do Paraíso Forest Reserve (20º47'-48'S, 42º50'-52'W), Viçosa, Minas Gerais, Brazil. The voucher specimens (numbers 32,175 and 32,177) were deposited in the BOTU Herbarium, São Paulo State University - UNESP (Valentin-Silva et al. 2018).

The seeds were removed according to Cavalcante et al. (2002), and were disinfected in a laminar flow chamber, as in Delgado-Paredes et al. (2012) with modifications. They were immersed in: (I) neutral detergent for 20 min; (II) commercial sodium hypochlorite solution (NaOCl; 2.5% (w/v) active chlorine; Super Globo®, Rio de Janeiro, Brazil) plus Tween 20® (4 drops 100 mL^{-1}) for 1 min; (III) 70% (v/v) ethyl alcohol for 1 min and (IV) NaOCl with Tween 20® (4 drops 100 mL^{-1}) for 15 min. Seeds were then rinsed five times with sterile deionized water. The recipient containing the seeds was shaken for 1 min at 5 min intervals during the different disinfestation steps.

The seeds were inoculated in glass flasks (350 mL) containing 60 mL of Murashige and Skoog (1962; MS) medium at half-strength of macro and micronutrients (½MS), with MS vitamins, 100 mg L^{-1} of myo-inositol, 20 g L^{-1} of sucrose (Sigma-Aldrich® Co, St. Louis, MO), and 7 g L^{-1} of granulated agar (Merck™, Darmstadt, Germany). The pH was adjusted to 5.8 ± 0.1 prior to

autoclaving at 121°C, 108 kPa for 20 min. The seeds were germinated at 25 ± 2ºC, under a photoperiod of 16 h light and irradiance of 70 μmol m^{-2} s^{-1} quantified by radiometer (LI-COR®, LI-250A Light Meter). Blue/red LED lamps (LabPAR LL-HR/DB-480, 11.6 W; LabLumens®, Carapicuíba, SP, Brazil) were used with a balanced rate of blue and red (that most stimulated germination of *P. crassinervium* seeds in previous studies; un-published data).

Plants were propagated by monthly subcultures of stem segments (~1.5 cm) with 1-2 nodes and at least one leaf. Initial explants were excised from *in vitro* germinated seedlings. MS liquid media (with a Germitest® paper bridge to support the explant) with 30 g L^{-1} of sucrose, and fluorescent lamps (HO Sylvania T12, 110W, São Paulo, Brazil) with irradiance of 45 μmol m^{-2} s^{-1} were used.

Effect of *in vitro* culture conditions on the biomass and phenolic compound production in *Piper crassinervium*

Culture conditions were tested in the following order: (I) salt medium composition; (II) culture medium consistency and gas exchange rates; (III) MS medium salt concentration; (IV) light intensity; (V) photoperiod; (VI) growth regulators; (VII) explant type; (VIII) 2-iP concentrations; (IX) medium strength (½MS and ½OM [Rugini Olive medium (Rugini 1984)]) in combination with sucrose (Fig. 1).

Effect of salt medium composition on shoot growth and phenolic compound production

Stem segments of ~1.5 cm with 1-2 nodes without leaves, from plants maintained *in vitro*, were inoculated into glass tubes (25 x 150 mm) containing 10 mL of liquid media. The liquid media used were: (I) MS (Phytotechnology® Lab, EUA), (II) DKW (DKW/Junglans; Driver and Kuniyuki 1984; McGranahan et al. 1987; Sigma-Aldrich®), (III) QL (Quoirin and Lepoivre; Quoirin et al. 1977; Sigma-Aldrich®), (IV) WPM (McCown's Woody Plant; Lloyd and McCown 1981; Sigma-Aldrich®) and (V) modified JADS [JADS without the antioxidant polyvinylpyrrolidone (PVP); Correia et al. 1995; stock solution] (Online Resource 1).

Media were supplemented with the DKW vitamins nicotinic acid (1.0 mg L^{-1}) and thiamine (2.0 mg L^{-1}), glycine (2.0 mg L^{-1}), myo-inositol (100 mg L^{-1}) and sucrose (30 g L^{-1}). The liquid medium was used based on studies with other *Piper*

species (Sencie-Tarazona et al. 2015). The pH of media was adjusted to 5.8 ± 0.01. Tubes were sealed with rigid polypropylene lids and cultures were maintained for 35 days under a photoperiod of 16 h light, irradiance of 45 µmol m^{-2} s^{-1} (provided by fluorescent lamps), and temperature of 25 ± 2°C. Similar explants, concentrations of myo-inositol and sucrose, and growth conditions (including fluorescent lamps) were employed in the subsequent experiments.

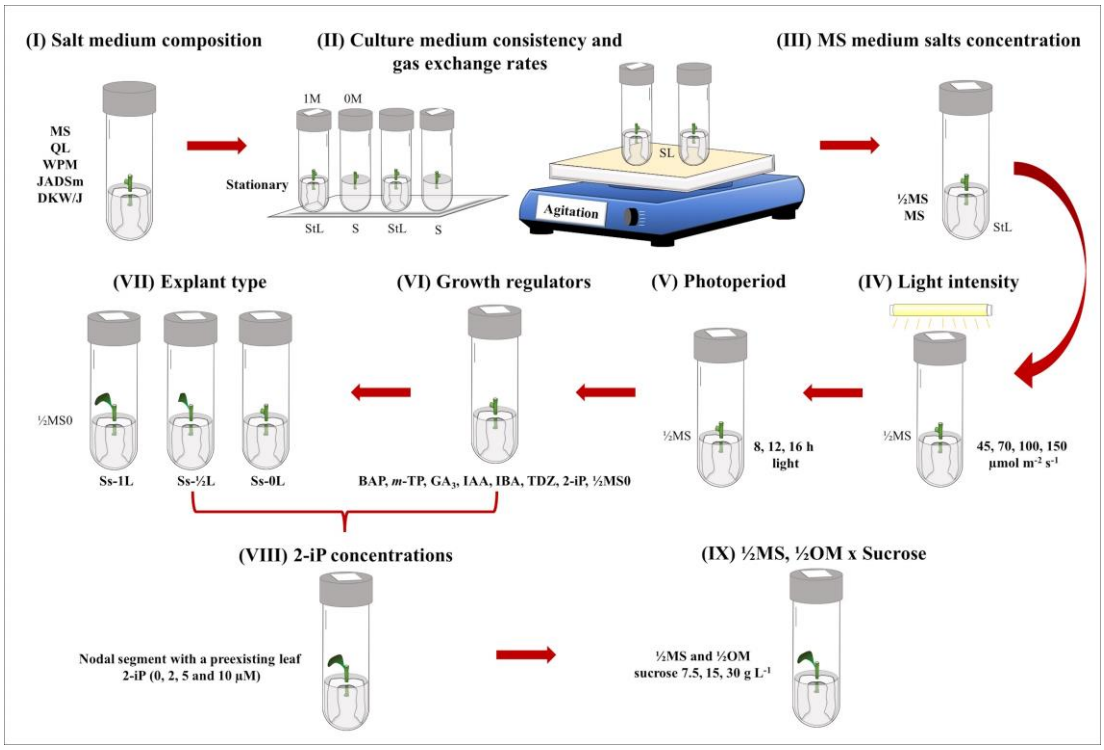

Fig. 1 – Steps in optimizing *in vitro* propagation of *Piper crassinervium*. (**MS**: Murashige and Skoog; **QL**: Quoirin and Lepoivre; **WPM**: McCown's Woody Plant; **JADSm**: JADS without the antioxidant polyvinylpyrrolidone (PVP); **DKW/J**: DKW/Junglans; **0M** and **1M**: rigid polypropylene lid without membrane and cover with 1 membrane; **StL**: Stationary liquid medium; **S**: Semi-solid medium; **SL**: Stirring liquid medium; **½MS** and **½OM**: MS medium and Rugini Olive medium (OM) with half the concentration of macro and micronutrients; **BAP**: 6-benzylaminopurine; ***m*-TP**: *meta*-topoline; **GA_3**: gibberellic acid; **IAA**: indole-3-acetic acid; **IBA**: indole-3-butyric acid; **TDZ**: thidiazuron; **2-iP**: N^6-(2-isopentenyl)adenine; **½MS0**: ½MS medium without growth regulators; **Ss-0L**, **Ss-½L** and **Ss-1L**: Stem segments without, with half-leaf blade and one entire preexisting leaf, respectively).

Effect of culture medium consistency and gas exchange rates on shoot growth

The explants were cultured in semi-solid (6.5 g L^{-1} of AgarGel®, São Paulo, SP, Brazil) and liquid [stationary and in constant agitation (50 rpm, Kline NT-150 agitator, Nova Técnica, Brazil)] MS medium salts and vitamins. Tested sealing

systems were: (I) rigid polypropylene lids (RL) without orifice [CO_2 exchange rate ($TTCO_2$): 1.33 μL L^{-1} s^{-1} (Pinheiro et al. 2013)] and (II) RL with a hole covered by porous membrane and $TTCO_2$ of 1.84 μL L^{-1} s^{-1} (Saldanha et al. 2012). The irradiance was 70 μmol m^{-2} s^{-1}.

Effect of MS medium salt concentration on shoot growth

Stationary MS and ½MS liquid media, in combination with 30 g L^{-1} of sucrose, were tested. The vitamins of MS medium, RL with membrane (used in subsequent experiments) and irradiance of 70 μmol m^{-2} s^{-1} were used.

Effect of light intensity on shoot growth and phenolic compounds production

Explants were grown in the ½MS stationary liquid medium with 30 g L^{-1} of sucrose (½MS-LE-30S) and kept under irradiance of 45, 70, 100 and 150 μmol m^{-2} s^{-1}, provided by fluorescent lamps.

Influence of explant type (preexisting leaves on nodal explants) on shoot development

Stem nodal segments without leaves (Ss-0L); with a half leaf (Ss-½L) and with one leaf (Ss-1L) were evaluated. The explants were inoculated into ½MS-LE-30S medium under 100 μmol m^{-2} s^{-1} irradiance (used in subsequent experiments).

Effect of 2-iP on growth of nodal segments with a preexisting leaf and production of phenolic compounds

Stem segments with one leaf (Ss-1L) were inoculated into ½MS-LE-30S medium supplemented with 2-iP (0, 2, 5 and 10 μM).

Effect of medium strength (½MS and ½OM), in combination with sucrose, on shoot growth and phenolic compounds accumulation

The salt composition of ½MS and ½OM media in combination with sucrose (7.5, 15 and 30 g L^{-1}) was tested for development and phenol content of *P. crassinervium* plants. Ss-1L explants were inoculated onto ½MS and ½OM liquid media (Stock

solutions) with MS medium vitamins under the optimized culture conditions identified in previous steps.

Growth analysis and quantification of pigments

Plant and root length (mm), number of roots and leaves, leaf area (cm^2 $plant^{-1}$), total fresh and dry weight (mg $plant^{-1}$) and the photosynthetic pigments content were determined after 35 days of culture in all experiments. The leaf area was calculated using leaf images (excised and fixed individually on millimeter white paper) captured with digital camera and analyzed in the Image J program (Schneider et al. 2012).

The photosynthetic pigments (chlorophylls *a* and *b* and carotenoids) were quantified according to Saldanha et al. (2012), with the extraction following Santos et al. (2008) and calculated according to Wellburn (1994) equations. Five leaf discs (5 mm diameter) from the second and third expanded leaf counted from the apex were used.

Quantification of total phenols and flavonoids

Phytochemical analysis was performed with plants obtained from experiments (I) salt medium composition, (II) light intensity, (III) 2-iP concentrations and (IV) medium strength (½ MS and ½ OM) in combination with sucrose.

Shoots and roots of 2-3 plants (constituting a biological replicate) were collected after 35 days of culture, immediately frozen with liquid nitrogen and stored at -80°C until use. The extraction methodology was adapted from Giri et al. (2012) and Araujo et al. (2015). Samples were lyophilized (Lyophilizer L101 LIOTOP, Liobras, Brazil), pulverized in porcelain mortar with pestle, weighed and placed in 2 mL microtubes. Aliquots of 1 mL MeOH, 80% (v/v) analytical grade (Vetec®), for each 50 mg of plant material, were added and the contents homogenized and left in an ultrasonic bath (Branson 1210, Marshall Scientific©, USA) for 5 min at room temperature (25 ± 2°C). Samples were shaken at 60 rpm for 25 min (total extraction time 30 min), and centrifuged at 12.000 rpm for 5 min at 25 ± 2°C. The supernatant was recovered and the residue was extracted again twice. The three supernatants

were combined in 5 mL vials and stored at 8°C. Finally, extracts were diluted with 80% (v/v) MeOH with dilution factors of 10 to 40 for subsequent chemical analyzes.

The total phenols content was determined by the colorimetric method of Folin-Ciocalteu (Singleton and Rossi 1965) and the methodology was adapted from Saidani et al. (2017). In 96-well microplates, 100 µL of water, 25 µL of the diluted extract and 25 µL of Folin-Ciocalteu reagent (1:10, v/v) were homogenized and allowed to react for 1 min in the dark. Then, 50 µl aliquots of 10% (w/v) sodium carbonate were added and incubated at 25 ± 3°C for 2 h in the dark. The absorbance was measured at 760 nm in UV/visible spectrophotometer with a microplate reader (Multiskan™ GO, Thermo Scientific™, Germany). The calibration curve ($R^2 = 0.999$) was performed with gallic acid (1-60 µg mL^{-1}). Results were expressed in µg of gallic acid equivalents (GAE) per mg dry weight (DW).

Quantification of total flavonoids was performed using the colorimetric method of Zhishen et al. (1999), following the methodology of Mosquera et al. (2015). Diluted extract (20 µL) and the standard catechin (1-100 µg mL^{-1}) were used. The flavonoid content was calculated (calibration curve, $R^2 = 0.995$) and the results were expressed in µg of catechin equivalents (CE) per mg DW.

The productivity, in terms of mg of GAE and CE $plant^{-1}$, was calculated from the mean dry weight (shoot + root) and average of the phenol and flavonoid content in the whole plant.

Statistical analyzes

All *in vitro* propagation experiments were performed in randomized block design (RBD), two blocks with 10-40 repetitions each (varied depending on the amount of analysis performed by experiment), the experimental unit being a tube with one explant. For each experiment, 10-12 biological replicates were used for growth analyzes (plant and root length, number of roots and leaves, leaf area, total fresh and dry weight), 6-8 for the quantification of photosynthetic pigments (chlorophyll *a* and *b* and carotenoids) and 5 for the determination of total phenolics and flavonoids. The total phenolics and flavonoids quantification was made with 3 technical replicates (at least two readings with error percentage less than 2%) for each biological repetition (consisting of 1-3 plants selected at random). The experiment of ½MS and ½OM media in combination with sucrose was performed in a 2x3 factorial scheme, totaling

6 treatments. The data were submitted to ANOVA and the means compared by the Tukey's test at the 5% level of significance, using the Genes program, version Windows/2011.9.0 (Cruz 2013).

Results

The medium composition influenced the accumulation of photosynthetic pigments and phenolic compounds

The accumulation of dry mass and the other growth parameters evaluated were similar for *P. crassinervium* plants in the different media tested (Fig. 2a, b) [Online Resource 2 (Fig. a-f)]. The explant response percentage was lower with the DKW/J medium (~ 75%) due to tissue oxidation; the regeneration rate of the explants in the other culture media tested was greater than 90%. The content of chlorophylls *a* and *b* was higher in MS medium, while the carotenoid content was similar in all media (Fig. 2c, d). Total phenolic and flavonoid production in the whole plant (shoot and root) was higher in DKW/J medium (68.9 μg GAE mg^{-1} DW and 47.0 CE mg^{-1} DW), followed by MS medium (64.9 μg GAE mg^{-1} DW and 38.3 μg CE mg^{-1} DW) (Fig. 2e). However, the productivity of these metabolites, considering the dry mass of each plant, was similar among media tested; each plant produced ~ 0.6 mg GAE and 0.3 - 0.4 mg CE (Table 1). MS medium was selected for subsequent experiments as it induced a large production of phenolic compounds coupled less oxidation of explants.

A liquid medium combined with higher gas exchange favored the growth of *Piper crassinervium*

Plants grown in semi-solid medium presented lower development, and poorly expanded, deformed and chlorotic leaves, regardless of the type of seal used [(Fig. 2f and Online Resource 2 (Fig. g-l)]. The dry weight was higher in the shaken liquid medium (SL), followed by stationary (StL), in combination with lids with membrane (SL1M and St1M) (Fig. 2g). The St1M medium was used in subsequent experiments based on the dry weight of *P. crassinervium* and easiness to maintain the cultures.

Lower concentration of MS medium salts favored the development of *Piper crassinervium*

Explants inoculated in MS medium showed higher oxidation (~ 35%), thicker and deformed leaves, compared to ½MS medium (Fig. 2h). However, the dry weight and content of chlorophyll *a* and *b* and carotenoids did not vary between MS and ½MS media (Fig. 2 i-k). Reducing mineral salts concentration (macro and micronutrients) led to the formation of plants with greater root length, leaf number, and root and leaf area [Online Resource 2 (Fig. m-r)]. Thus, the ½MS medium was used in subsequent *in vitro* propagation experiments.

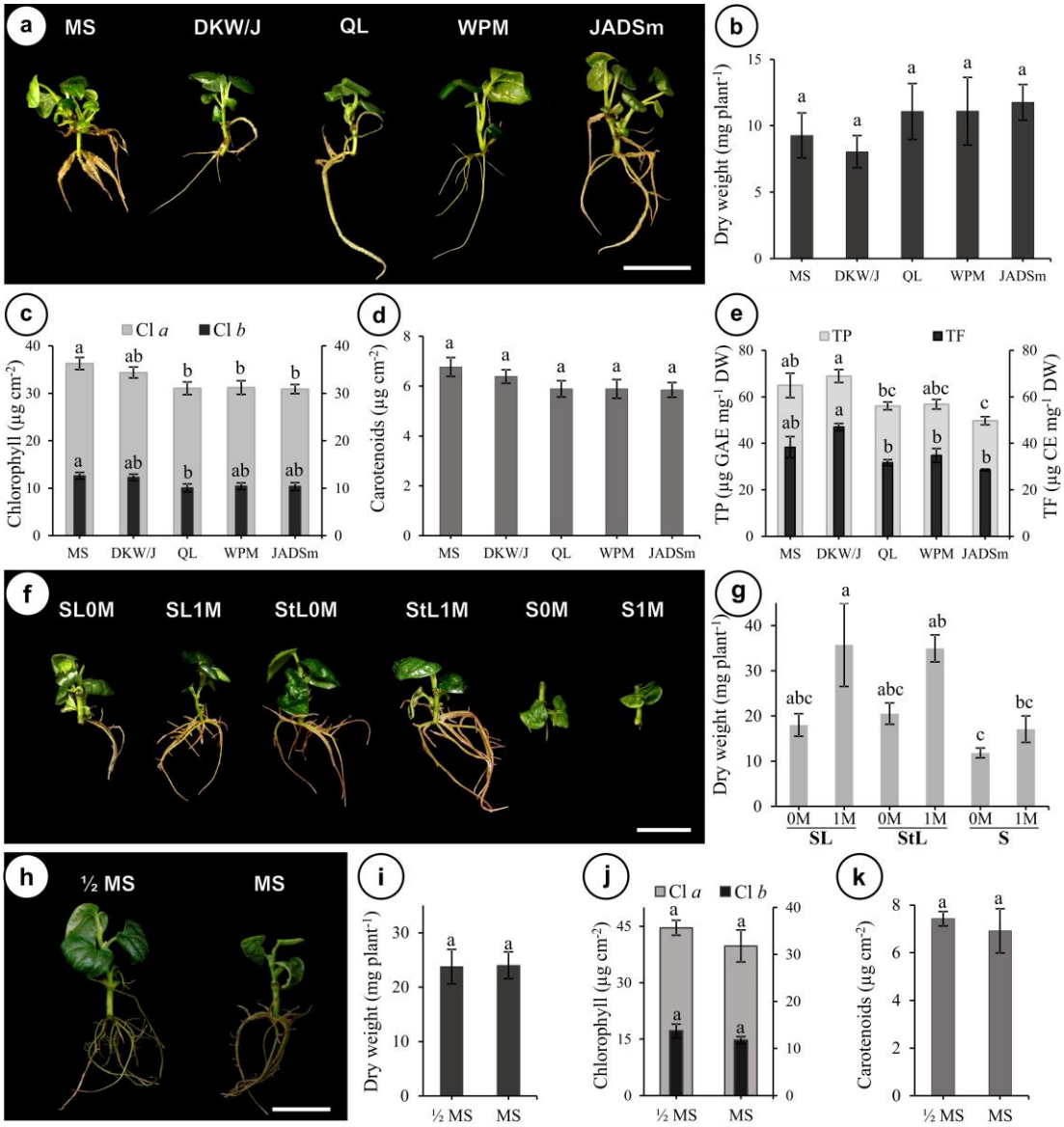

Fig. 2 - *Piper crassinervium* plants and growth variables after 35 days of culture under different salt media composition (**a-e**), culture medium consistency and gas exchange levels (**f**, **g**) and salt concentration of MS medium (**h**–**k**). (**MS**: Murashige

and Skoog; **DKW/J**: DKW/Junglans; **QL**: Quoirin and Lepoivre; **WPM**: McCown's Woody Plant; **JADSm**: JADS without the antioxidant polyvinylpyrrolidone (PVP); **Cl *a*** and **Cl *b***: Chlorophyll *a* and *b*; **TP**: Total phenols; **GAE**: Gallic acid equivalent; **TF**: Total flavonoids; **CE**: Catechin equivalent; **DW**: Dry weight; **SL**: Stirring liquid medium; **StL**: Stationary liquid medium; **S**: Semi-solid medium; **0M** and **1M**: rigid polypropylene lid without membrane and cover with 1 membrane; **½MS**: MS medium with half macro and micronutrients concentration. Means followed by the same lowercase letter did not differ significantly among treatments (Tukey's test at 5%). Error bars indicate the standard error among replicates. Bar = 2 cm in Figures **a**, **f** and **h**)

Table 1 - Productivity of total phenols and flavonoids in *Piper crassinervium* plants grown in different culture media, irradiance and concentrations of N^6-(2-isopentenyl)adenine (2-iP)

Treatment	Total phenols (mg GAE plant^{-1})	Total flavonoids (mg CE plant^{-1})
Basal composition of culture medium salts		
MS	0.6	0.4
DKW/J	0.6	0.4
QL	0.6	0.4
WPM	0.6	0.4
JADSm	0.6	0.3
Irradiance (μmol m^{-2} s^{-1})		
45	1.2	0.7
70	1.8	1.2
100	2.0	1.1
150	1.8	1.1
2-iP (μM)		
0	5.0	2.7
2	5.0	3.0
5	4.4	2.3
10	4.9	2.9

Productivity calculated from the mean of the total dry mass (shoot+ root) and that of the phenol and flavonoids content in the whole plant. **MS**: Murashige and Skoog; **DKW/J**: DKW/Junglans; **QL**: Quorin and Lepoivre; **WPM**: McCown's Woody Plant; **JADSm**: JADS without the antioxidant polyvinylpyrrolidone (PVP); **GAE**: Gallic acid equivalent; **CE**: Catechin equivalent.

Light intensity affected growth and production of photosynthetic pigments in *Piper crassinervium*

Irradiance of 100 μmol m^{-2} s^{-1} induced the highest development of shoots and roots, increasing dry weight accumulation (Fig. 3a, b); while 70 μmol m^{-2} s^{-1} restricted root formation (Online Resource 3, Fig. a-f). Chlorophyll *a* and *b*, carotenoids, phenols and total flavonoids were all higher at 70 μmol m^{-2} s^{-1} (Fig. 3 c-f). However, phenols and flavonoids productivity was similar to that of 100 μmol m^{-2} s^{-1}. Each plant produced on average 1.9 mg GAE and 1.13 mg CE (Table 1).

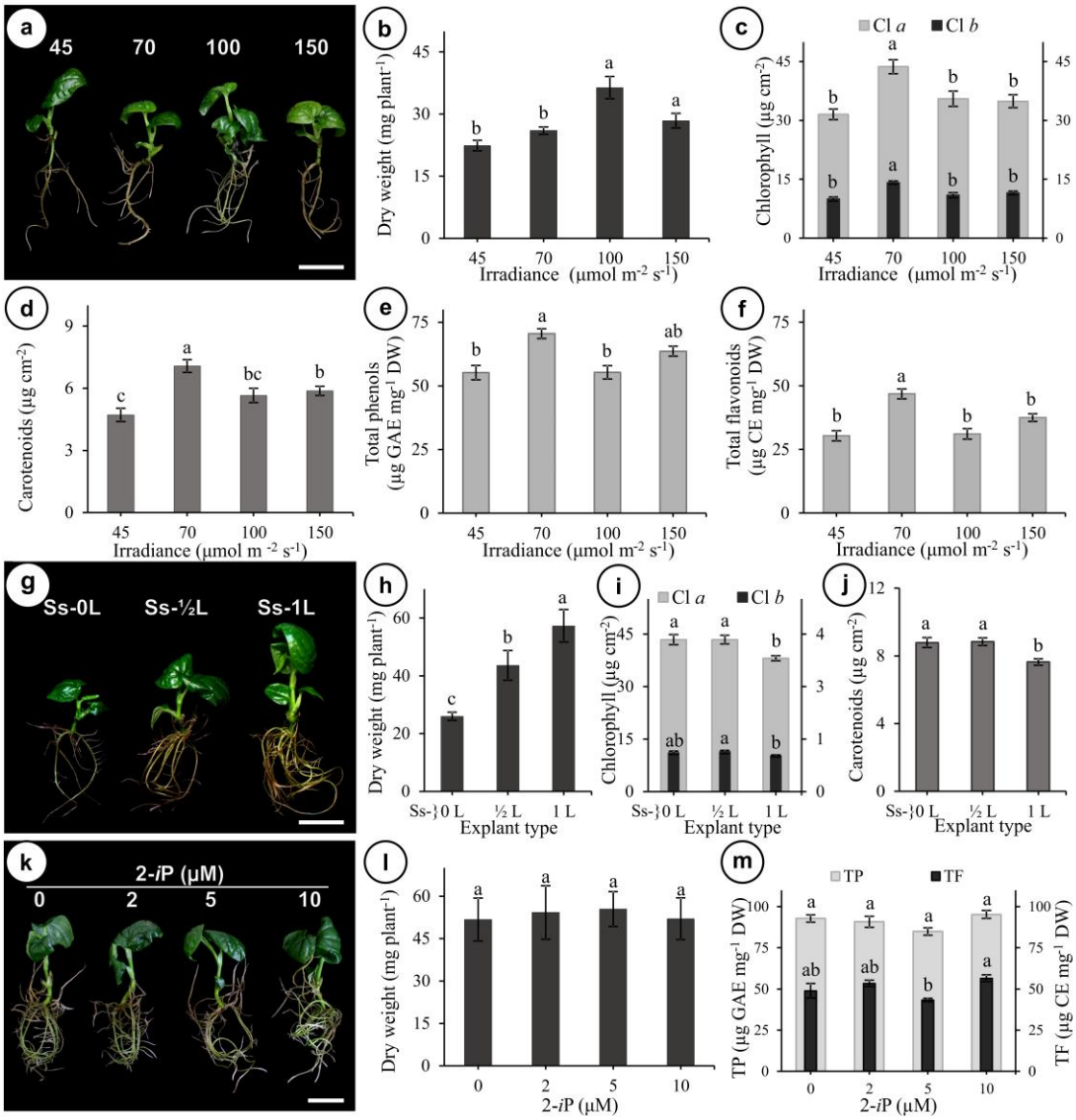

Fig. 3 - *Piper crassinervium* plants and growth variables after 35 days of *in vitro* culture in liquid medium (½MS) under different irradiances (**a-f**), explant (**g-j**) and 2-iP (2, 5 and 10 µM) (**h-m**). (**Cl *a*** and **Cl *b***: Chlorophyll *a* and *b*; **GAE**: Gallic acid equivalent; **CE**: Catechin equivalent; **DW**: Dry weight; **Ss-0L**, **Ss-½L** and **Ss-1L**: Stem segments without, with half-leaf blade and one entire preexisting leaf, respectively; **TP**: Total phenols; **TF**: Total flavonoids; **2-iP**:N^6-(2-isopentenyl)adenine. Means followed by the same lowercase letter did not differ significantly among the treatments (Tukey's test at 5%). Error bars indicate the standard error among replicates. Bar = 2 cm in Figures a, g and k).

Besides the irradiance, photoperiods of 8, 12 and 16 h light were also tested in preliminary assays, but only altered the carotenoid content, which was higher with a 16 h light photoperiod (Online Resource 4). Based on this, an irradiance of 100 µmol m^{-2} s^{-1} and a photoperiod of 16/8 h were selected for the *in vitro* propagation of *P. crassinervium*.

Use of nodal explants with a remaining leaf increased rhizogenesis and *in vitro* development of *Piper crassinervium* without use of 2-iP

Stem segments with one preexisting leaf (Ss-1L) generated plants with higher growth and dry weight (Fig. 3g, h), followed by explants Ss-½F [Online Resource 3 (Fig. g-l)]. The chlorophyll *a* and carotenoid content increased in plants from the Ss-0L and Ss-½L explants; while plants grown from Ss-½L explants had higher chlorophyll *b* content (Fig. 3i, j). Root formation in *P. crassinervium* Ss-½L and Ss-1L explants was faster, compared to Ss-0L explants (Fig. 4). The Ss-1L explant was selected for subsequent experiments based on the highest biomass produced.

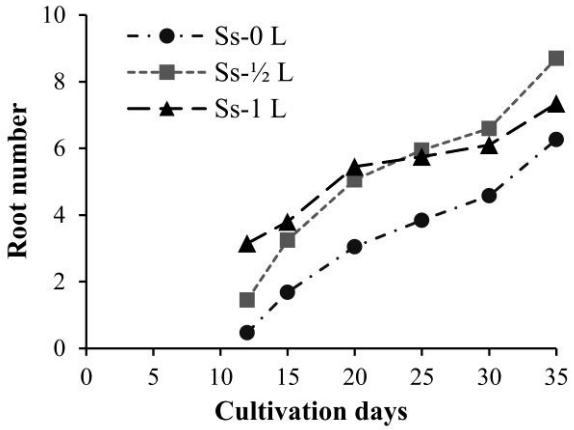

Fig. 4 - Root formation in stem segments of *Piper crassinervium* without, with half-leaf blade and one entire preexisting leaf (Ss-0, Ss-½ and Ss-1L) during 35 days of culture in the half-strength MS (½MS) stationary liquid medium, under irradiance of 100 μmol m^{-2} s^{-1}.

Among the growth regulators IBA (7 μM), TDZ and 2-iP (2, 5 and 10 μM) and the combination of BAP or *m*-TP (0.2 μM) with IAA (0.1 μM) and GA_3 (0.05 μM) tested, 2-iP (10 μM) best favored the growth of plants from Ss-0L explants; although dry weight of plants did not differ between treatments (Online Resource 5).

2-iP (10 μM) increased the total flavonoid content in plants when Ss-1L explants were used, but did not alter any of the other growth variables evaluated [Fig. 3k-m, Online Resource 6 (Fig. a-f)]. Phenols and flavonoids productivity was similar between 0, 2 and 10 μM 2-iP concentrations, which varied from 4.9 to 5.0 mg GAE $plant^{-1}$ and 2.7 to 3.0 mg CE $plant^{-1}$ (Table 1).

Half-strength OM (½OM) or MS (½MS) media influenced growth and accumulation of phenolic compounds

The ½OM and ½MS media favored growth of P. crassinervium [Fig. 5a and Online Resource 6 (Fig. j–o)]. The accumulation of dry weight did not differ between sucrose doses used in ½MS and ½OM media (Fig. 5b). The production of chlorophylls a and b and carotenoids was higher in ½MS medium in the three concentrations of sucrose tested (Fig. 5c–e).

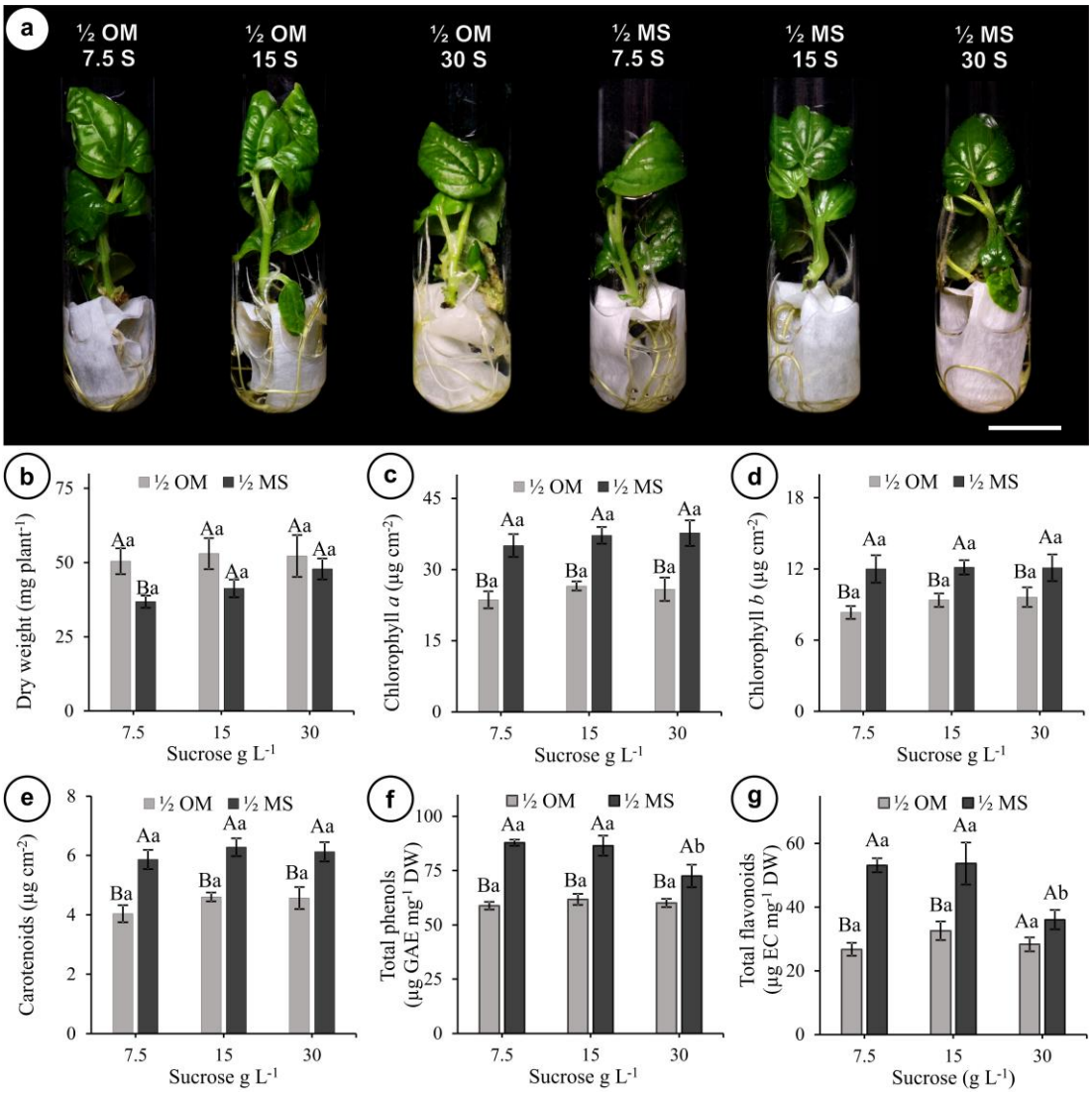

Fig. 5 - *Piper crassinervium* plants (**a**) and growth variables (**b**–**g**) after 35 days of *in vitro* culture in the liquid medium ½OM and ½MS with 7.5, 15 and 30 g L^{-1} of sucrose. (**½OM**: Rugini Olive Medium (OM) and **½MS**: Murashige and Skoog (MS) medium with half the concentration of macro and micronutrients; **Cl *a*** and **Cl *b***: Chlorophyll *a* and *b*; **GAE**: Gallic acid equivalent; **CE**: Catechin equivalent; **DW**: Dry weight. Means followed by the same lowercase letter do not differ statistically for sucrose concentration; means followed by the same capital letter did not differ

significantly among the ½OM and ½MS media (Tukey's test at 5%). Error bars indicate the standard error among replicates. Bar = 2 cm, in **a**).

Total phenol content was higher in ½MS medium, regardless of sucrose dose, being on average 82.3 µg GAE mg^{-1} DW; whereas the accumulation of flavonoids was higher in ½MS medium containing 7.5 and 15 g L^{-1} of sucrose (~ 53.4 µg CE mg^{-1} DW) (Fig. 5f, g). Phenols and flavonoids productivity was higher in ½MS medium with 15 g L^{-1} of sucrose, at ~ 3.6 mg GAE and 2.2 mg CE per plant (Table 2). Total phenolic and flavonoid contents were three and five times higher, respectively, in roots than shoots in all treatments; plant roots contained on average 53.3 µg GAE mg^{-1} DW and 31.8 µg CE mg-1 DW, while the shoot had 18.0 µg GAE mg-1 DW and 6.6 µg CE mg-1 DW.

Table 2 - Productivity of total phenols and flavonoids in *Piper crassinervium* plants grown in liquid culture medium ½OM and ½MS with7.5, 15 and 30 g L^{-1} sucrose.

Variable	Culture medium	Sucrose (g L^{-1})		
		7.5	15	30
Total phenols (mg GAE $plant^{-1}$)	½OM	3.0	3.3	3.1
	½MS	3.2	3.6	3.5
Total flavonoids (mg CE $plant^{-1}$)	½OM	1.4	1.7	1.5
	½MS	2.0	2.2	1.7

GAE: Gallic acid equivalent; **CE**: Catechin equivalent. Productivity calculated from the mean of the total dry mass (aerial part + root) and that of the phenol and flavonoids content in the whole plant.

Discussion

The salts composition of the medium mainly altered the synthesis of chlorophylls and phenolic compounds in *Piper crassinervium* plants. The higher concentration of NO_3^- and NH_4^+, as well as the low NO_3^-:NH_4^+ ratio (2:1 mM), in MS medium compared to media DKW/J, QL, WPM and JADS increased chlorophyll production in *P. crassinervium* plants. High N concentration stimulated photosynthetic capacity and chlorophyll biosynthesis in *in vitro* plants of *Salvia stenophylla* (Burch. Ex Benth) (Musarurwa et al. 2012); whereas a decrease of the NO_3^-:NH_4^+ (10:4) ratio increased the chlorophyll content in tomato plants grown under greenhouse conditions (Flores et al. 2001).

The higher concentration of Ca^{2+} and Mn^{2+}, in DKW/J medium, and the N, NO_3^- and NH_4^+ in the DKW/J and MS media influenced the increased total phenol and flavonoid content in *P. crassinervium* plants. Ca^{2+}, NO_3^-, Mg^{2+} and Mn^{2+} are involved in the regulation of enzymes and genes associated with phenolic compound biosynthesis and the production of reactive oxygen species (ROS) (Farzadfar et al. 2017; Martins et al. 2018; Zhou et al. 2018). The interaction of Cu^{2+}, Zn^{2+}, Fe^{2+}, Mo and B in DKW/J and MS media influenced the accumulation of phenols in *P. crassinervium*. Cu, Zn, Mo, Fe and B are required as enzymatic cofactors of enzymes involved in oxidative stress and/or participate in the regulation of genes involved in the biosynthetic pathway of phenolic compounds; the deficiency of any of these ions may decrease phenols production (Panhwar et al. 2015; Schulten and Kramer 2017; Clemente et al. 2018).

KNO_3, $CaCl_2$ and KH_2PO_4 in the media may have also altered flavonoid production in *P. crassinervium*, as reported by Parmar and Jasrai (2017) for leaves and roots of *Oroxylum indicum*. Moreover phenolic acid biosynthesis was shown to depend on the amount of PO_4^{3-} available in the medium (Magangana et al. 2018).

MS liquid medium (agitated and stationary) induced greater accumulation of biomass in *P. crassinervium* plants compared to the semi-solid medium. The liquid medium increases the availability, absorption and substitution of nutrients and growth regulators on the cell surface and allows larger contact area of the explant with the culture medium (Szopa et al. 2017; Stevens and Pijut 2018). The higher diffusion rate of the liquid medium decreases the negative effect of growth inhibitors exuded by explants (mainly phenolic compounds) on shoot development. These inhibitors are concentrated around the explant in the semisolid environment, inducing greater stress in tissues (Gupta and Timmis 2005; Savio et al. 2012). With other *Piper* species, liquid has been more efficient than semisolid medium for induction, elongation and/or rooting of shoots (Rani and Dantu 2012; Sencie-Tarazona et al. 2015).

The Germitest® paperbridge, used as support for *P. crassinervium* explants, guaranteeing their partial immersion in the liquid medium, allowed for better aeration of the plant material favoring biomass gain. The stirring or stationary liquid media with the use of inert substrates increase tissue aeration and facilitate nutrient uptake (Savio et al. 2012; Cuenca et al. 2017; Stevens and Pijut 2018).

In this work, the increased gas exchange rates inside the culture vessel, through use of membranes (Saldanha et al. 2012; Pinheiro et al. 2013; Batista et al. 2017), stimulated growth of *P. crassinervium* plants, especially when using liquid medium (shaking and stationary). This higher level of gaseous exchange increases nutrient absorption, transpiration and photosynthetic capacity of tissues and reduces ethylene accumulation inside the container, favoring the plant material development (Kozai 2010; Hoang et al. 2017). This increase of gas exchange rate, along with the use of liquid medium, further favors the aeration of tissues and nutrient absorption (Cuenca et al. 2017).

Reducing the MS medium salts concentration by half (½MS) decreased explant oxidation and favored the development of *P. crassinervium*. At higher concentrations than those required naturally by plants, salts may cause ion toxicity *in vitro*, diminish the water potential and induce osmotic stress (Baque et al. 2010; Cui et al. 2013). As a result, absorption of water and minerals from the medium decreases and cell elongation and division, biomass accumulation and metabolic biosynthesis are reduced causing eventual explant necrosis (Baque et al. 2010; Cui et al. 2013; Silva et al. 2014). Lower concentrations of macro and micronutrients decrease the osmotic pressure of media, stimulating growth and rooting of shoots in *Piper* spp. (Bhat et al. 1992; Anand and Rao 2000) as in several medicinal plants (Cuenca et al. 2017; Ghimire et al. 2018). The greater availability of nutrients in liquid medium influenced the higher efficiency of ½MS medium when compared to MS. Explants of *P. crassinervium* did not require large amounts of nutrients, the use of liquid ½MS medium being sufficient for their development.

Irradiance of 100 µmol m^{-2} s^{-1} provided the best development of *P. crassinervium* and favored phenolic biosynthesis. High irradiance increases photosynthesis rate, water and nutrient absorption due to greater transpiration in the leaves, and CO_2 fixation; this accelerates the assimilation of nutrients such as nitrate and stimulates biomass accumulation (Poorter and Nagel 2000; Fu et al. 2017).

Although an irradiance of 70 µmol m^{-2} s^{-1} less stimulated plant growth in *P. crassinervium*, it induced higher chlorophyll, carotenoids and phenolic compounds accumulation. Such inverse relationship between the content of photosynthetic pigments and biomass at irradiances of 60-70 µmol m^{-2} s^{-1} has been reported in other plants grown *in vitro* or not (Alvarenga et al. 2015; Fu et al. 2017). Low or excess

light intensity reduces the photosynthetic rate, CO_2 assimilation, increases leaf transpiration and damages the photosynthetic system. Oxidative stress can be induced in light conditions decreasing the growth and altering photosynthetic pigments and secondary metabolites production (Alvarenga et al. 2015; Zhu et al. 2017). Some enzymes associated with Rubisco and phytochrome-mediated transcriptional regulators, located in plastids and chloroplasts, participate in the response to oxidative stress by altering the production of photosynthetic pigments and phenols (Wang et al. 2012). Carotenoid production increases in response to oxidative stress, in order to avoid or minimize photoinhibition, inactivating ROS (Sáez et al. 2013).

The production of phenolic compounds, as well as antioxidant enzyme activity, increases or decreases as a defense mechanism under light-induced oxidative stress (Zhu et al. 2017; Pérez-López et al. 2018). The response to light changes is controlled by photoreceptors that activate signal transduction in the regulation of genes involved in the biosynthesis of phenolic compounds such as flavonoids and anthocyanins, associated with photoprotection (Zoratti et al. 2014; Dias et al. 2016).

Like the irradiance, the photoperiod affected biomass production and that of primary and secondary metabolites in *in vitro* tissues of *P. nigrum* (Ahmad et al. 2014). However, it only altered the carotenoid content in *P. crassinervium* plants.

For the first time we established that single-node stem segments with either a half (Ss-½L) or one intact leaf (Ss-1L), induced the highest growth and development of *in vitro P. crassinervium* plants. The presence of leaves in explant stem segments stimulated the propagation of several of forest and medicinal plants (Tchoundjeu et al. 2002; Quiala et al. 2012), by providing a greater amount of photo-assimilates and other nutrients necessary for development of shoots. Auxin, such as indole-3-acetic acid (IAA), and soluble carbohydrates are translocated from the leaf to the base of the stem segment during the first days of excision, inducing rooting and stimulating shooting and total dry weight gain (Newton et al. 1992; Da Costa et al. 2013). Stalks with foliar tissue remain photosynthetically active during propagation, so a higher photosynthetic rate is induced (Mesén et al. 1997). Hence, the leaf tissue remaining in the Ss-½L and Ss-1L explants was a source of auxin and other nutrients necessary for the increased *in vitro* rhizogenic responses of *P. crassinervium*, after their

translocation to the base of the stem segment in contact with the medium. Gomes and Krinski (2018) observed the same during propagation of *P. crassinervium* from stem cuttings under greenhouse conditions. Furthermore, here, the faster root formation in *P. crassinervium* Ss-1L explant increased uptake of nutrients in the first 10 days of culture, leading to the highest growth and development of plants.

The results showed that 2-iP was not required to enhance the growth of *P. crassinervium*, as also found in *P. solmsianum* (Balbuena et al. 2009) and *P. aduncum* (Silva et al. 2014). Similarly, 2-iP was shown to be less efficient for *in vitro* propagation of *P. lonqum* (Bhat et al. 1992).

OM medium stimulates *in vitro* plant biomass production of *P. crassinervium* compared to MS medium (data not shown). In addition, Silva et al. (2019) showed that OM medium, in combination or not with sucrose, induces the production of biomass and primary and secondary metabolites in *Pfaffia glomerata* (Silva et al. 2019). However, the effect of ½OM and ½MS media depended on sucrose under the optimized culture conditions (stationary liquid medium, lids with membrane, 100 µmol m^{-2} s^{-1} of irradiance, 16 h photoperiod and Ss-1L explant) in *P. crassinervium*.

The ½MS medium contains lower Mg^{2+} concentration than ½OM medium. This, in combination with the lower quantity of sucrose in the medium (7.5 g L^{-1}) reduced the biomass gain of *P. crassinervium*. Mg^{2+} participates in the regulation of enzymes involved in the fixation of carbon in the plant. Low Mg^{2+} decreases uptake and translocation of carbohydrates through the phloem, reducing the biomass gain in plants (Hermans and Verbruggen 2005; Guo et al. 2016).

The higher production of photosynthetic pigments in *P. crassinervium* plants cultivated in the ½MS medium, compared with ½OM medium, is related to the greater amount of N and lower NO_3^-:NH_4^+ ratio in ½ MS medium (Flores et al. 2001; Musarurwa et al. 2012).

The ½MS medium contains higher concentration of N, NO_3^-, NH_4^+, a higher Mg^{2+}:Mn^{2+} ratio and the lowest amount of PO_4^{3-}, Cu^{2+}, Zn^{2+} and B, compared to the ½OM medium. This favored the accumulation of phenols in *P. crassinervium* plants in the ½MS medium, regardless of sucrose concentration used. Several authors reported that changes in concentration of these ions in the medium altered the biosynthesis of phenolic compounds in different species (Farzadfar et al. 2017;

Schulten and Kramer 2017; Clemente et al. 2018; Magangana et al. 2018; Zhou et al. 2018). In addition, the presence of KCl in OM medium decreased the phenol biosynthesis in *P. crassinervium*, since KCl induces osmotic/oxidative stress altering the metabolic profile of flavonoids in a specific way (Lucini et al. 2016).

Concentrations of 30 g L^{-1} sucrose in ½MS liquid medium decreased the phenols and flavonoids content in *P. crassinervium* plants without affecting biomass production. The effect of sucrose on the production of phenylpropanoids varies depending on the species and plant tissue grown *in vitro* and the culture conditions (Cui et al. 2013; Ghimire et al. 2018; Modarres et al. 2018). In *P. crassinervium* the variation of the sucrose effect on the biosynthesis of phenolic compounds depended on the salt composition of the medium.

The enhanced gas exchange in the *P. crassinervium* cultures influenced the responses to the different concentrations of sucrose in ½OM and ½MS media. The addition of sucrose to the medium, in combination with the appropriate gas exchange rate inside vessels, forced the plants to develop under photomixotrophic conditions, modifying their metabolism and influencing phenolic compound production among others (Kozai 2010; Badr et al. 2011; Mohamed and Ibrahim 2012).

Conclusions

Here we found that the best propagation protocol to increase the production of biomass and phenolic compounds in *P. crassinervium in vitro* consisted of stem segments with a node and one preexisting leaf grown in ½MS stationary liquid medium plus 15 g L^{-1} sucrose, using lids with membrane as a sealing system, under irradiation of 100 μmol m^{-2} s^{-1} and 16 h photoperiod. Addition of 2-iP (10 μM) to the culture medium increased the total flavonoid content in shoots. This is the first *in vitro* propagation protocol that optimized the general conditions for a faster and more efficient growth of *Piper*.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' contributions

AMRR, JVSS and JVMF raised the *in vitro* plants for the experiments and performed the experiments. AMRR, JVSS, JVMF, TDS, KC and DVF performed growth and photosynthetic pigments analysis. AMRR, JVSS, JVMF and DVF performed extractions and phytochemical analysis. AMRR, DSB and MVMP performed statistical analyses. AMRR, DSB, MVMP, DVF, WCO and SAF designed the research, interpreted the data and wrote the paper.

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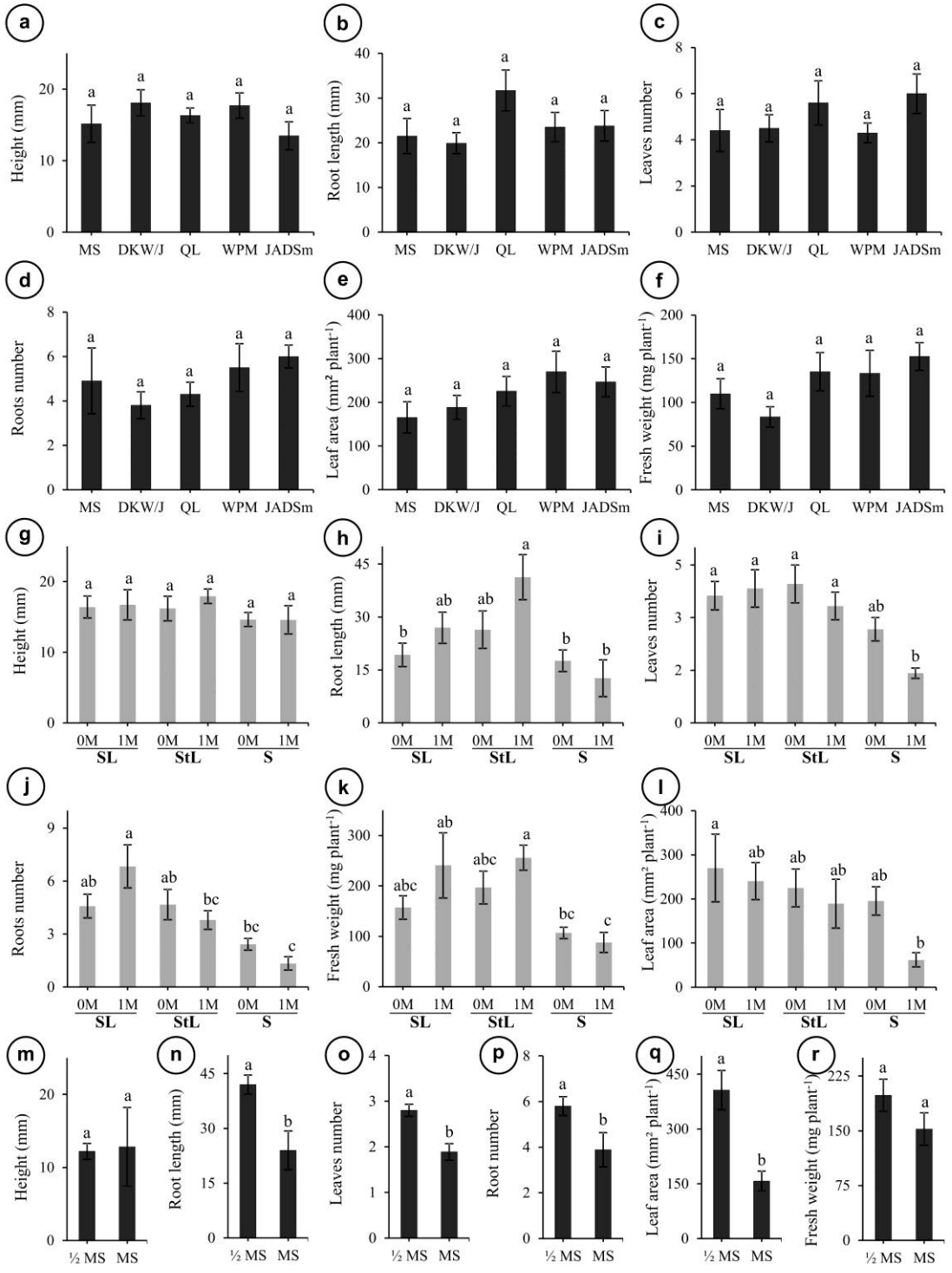
Electronic supplementary material

Online Resource 1 - Basal salt composition of the tested media.

COMPONENT	MOLECULAR FÓRMULA	MS	DKW/ J	QL	WPM	JADS	OM
MACRONUTRIENTS (mM)							
Ammonium nitrate	NH_4NO_3	20.6	17.7	5.0	5.0	4.0	5.2
Calcium chloride	$CaCl_2$	3.0	1.0	-	0.7	-	-
	$CaCl_2.2H_2O$	-	-	-	-	-	3.0
Calcium nitrate	$Ca(NO_3)_2$	-	8.3	5.1	2.4	-	-
	$Ca(NO_3)_2.4H_2O$	-	-	-	-	5.0	2.5
Magnesium Sulfate	$MgSO_4$	1.5	3.0	1.5	1.5	-	-
	$MgSO_4.7H_2O$	-	-	-	-	3.0	6.1
Potassium chloride	KCl	-	-	-	-	-	6.7
Potassium nitrate	KNO_3	18.8	-	17.8	-	8.0	10.9
Potassium Sulfate	K_2SO_4	-	9.0	-	5.7	-	-
Potassium phosphate	KH_2PO_4	1.3	2.0	2.0	1.3	3.0	2.5
MICRONUTRIENTS (µM)							
Boric acid	H_3BO_3	100.3	77.6	100.3	100.3	50.1	200.5
Cobalt chloride	$CoCl_2.6H_2O$	0.1	-	0.1	-	1.0	0.1
Copper Sulfate	$CuSO_4.5H_2O$	0.1	1.0	0.1	1.0	5.0	1.0
Manganese Sulfate	$MnSO_4.H_2O$	100.0	198.2	4.5	131.9	-	100.0
	$MnSO_4.4H_2O$	-	-	-	-	75.8	-
Nickel sulfate	$NiSO_4.6H_2O$	-	0.02	-	-	-	-
Potassium Iodide	KI	5.0	-	0.5	-	-	5.0
Sodium Molybdate	$Na_2MoO_4.2H_2O$	1.0	1.6	1.0	1.0	0.6	1.0
Nitrate of zinc	$Zn(NO_3)_2.6H_2O$	-	57.1	-	-	-	-
Zinc sulfate	$ZnSO_4,7H_2O$	30.0	-	30.0	30.0	15.0	50.0
Sodium EDTA	Na_2EDTA	-	134.2	-	110.3	200.0	-
	$Na_2EDTA.2H_2O$	100.0	-	100.2	-	-	100.7
Iron Sulphate	$FeSO_4.7H_2O$	100.0	121.6	100.0	100.0	200.0	100.0

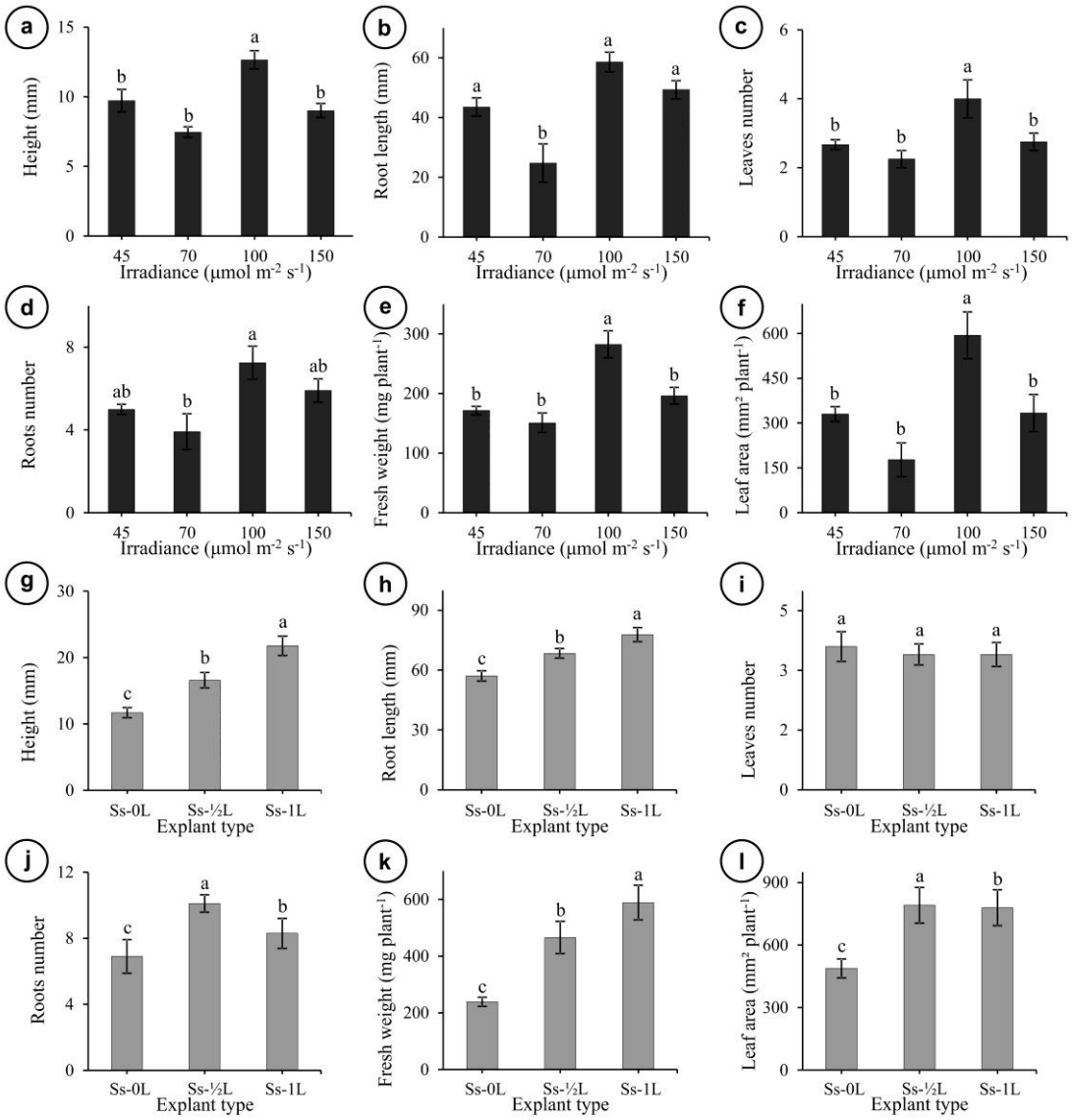
*Final concentration in the culture medium

MS: Murashige and Skoog; **DKW/J**: DKW/Junglans; **QL**: Quorin and Lepoivre; **WPM**: McCown's Woody Plant; **OM**: Rugini Olive medium



Online Resource 2 - *In vitro* plant growth variables of *Piper crassinervium* after 35 days of culture under different salt medium composition (**a** – **f**), culture medium consistency and sealing systems (**g** – **l**) and salt concentration of MS medium (**m** – **r**). (**MS**: Murashige and Skoog; **DKW/J**: DKW/Junglans; **QL**: Quoirin and Lepoivre; **WPM**: McCown's Woody Plant; **JADSm**: JADS without the antioxidant polyvinylpyrrolidone (PVP); **SL**: Stirring liquid medium; **StL**: Stationary liquid medium; **S**: Semi-solid medium; **0M** and **1M**: Autoclavable rigid polypropylene lid without membrane and cover with 1 membrane; **½MS**: MS medium with half the concentration of macro and micronutrients. Means followed by the same lowercase

letter did not differ significantly among treatments (Tukey's test at 5%). Error bars indicate the standard error among replicates).



Online Resource 3 - *In vitro* plant growth variables of *Piper crassinervium* after 35 days of culture under irradiance of 45, 70, 100 and 150 µmol m^{-2} s^{-1} (**a** – **f**) and from stem segments without, with half and one preexisting leaf, respectively (**0L**, **½L** and **1L**) (**g** – **l**). (Means followed by the same lowercase letter do not differ significantly among treatments (Tukey's test at 5%). Error bars indicate the standard error among the replicates).

Online Resource 4 - Growth variables of *Piper crassinervium* plantlet grown *in vitro* under different photoperiods.

Variable	Photoperiod - Light/Darkness (h)		
	8 / 16	12 / 12	16 / 8
Heigth (mm)	8.2 a	8.1 a	7.7 a
Length of largest root (mm)	45.1 a	51.2 a	45.3 a
Leaves number	3.9 a	2.6 a	2.9 a
Roots number	5.1 a	4.9 a	4.2 a
Fresh weight (mg)	135.6 a	131.0 a	137.2 a
Dry weight (mg)	14.6 a	15.1 a	17.6 a
Leaf area (mm^2)	265.2 a	302.8 a	289.1 a
Photosynthetic pigments per leaf area ($\mu g\ cm^{-2}$)			
Chlorophyll *a*	33.4 a	37.9 a	39.7 a
Chlorophyll *b*	8.9 a	10.5 a	10.9 a
Total carotenoids	6.9 b	7.7 ab	8.2 a

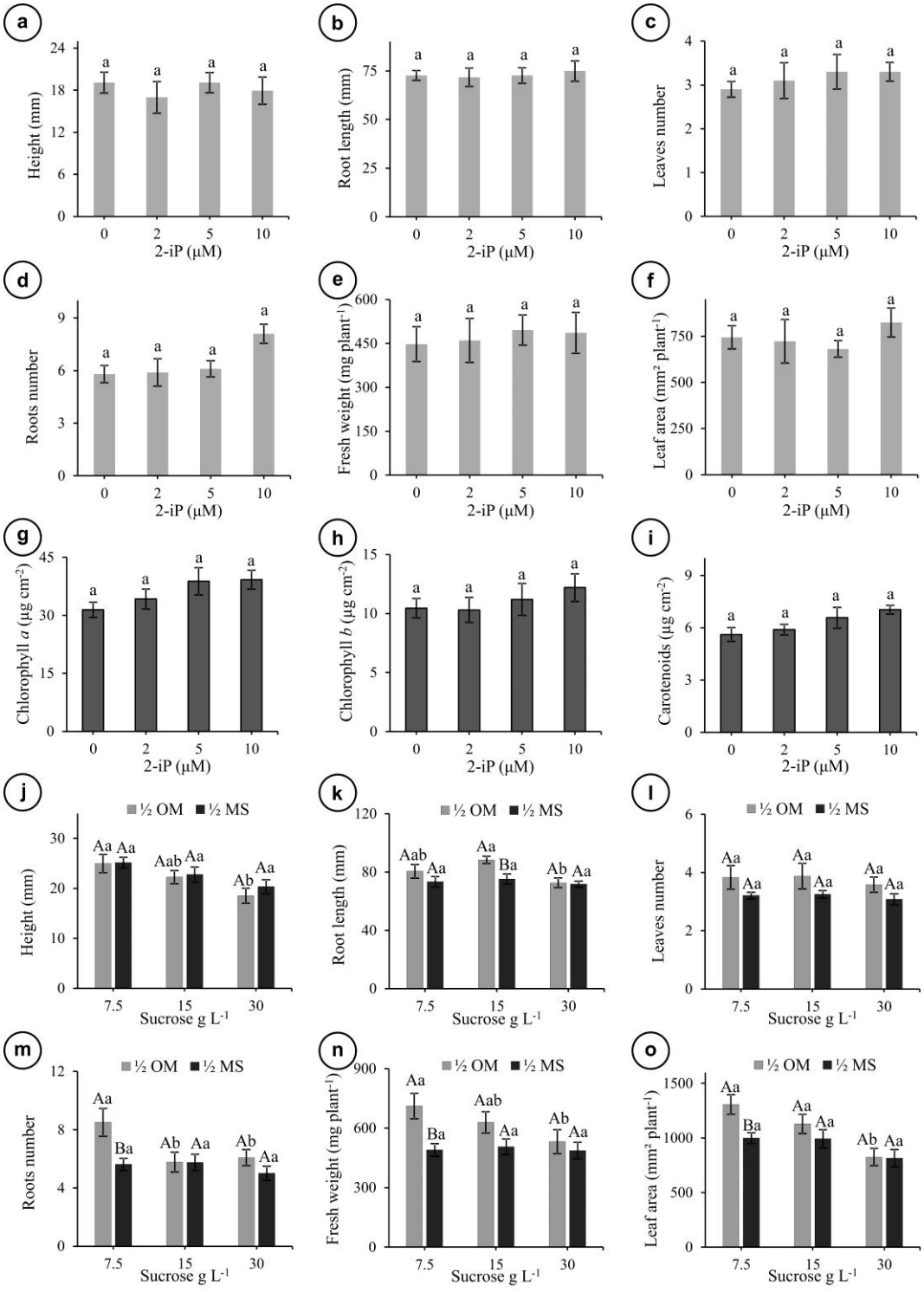
Segments of stem without leaves grown in tubes with ½ MS stationary liquid medium under 100 µmol $m^{-2}\ s^{-1}$ irradiance, autoclavable rigid polypropylene lids cover with 1 membrane. Means followed by the same lowercase letter did not differ significantly among the treatments (Tukey's test at 5%).

Online Resource 5 - Growth variables of *Piper crassinervium* plantlet grown *in vitro* with different growth regulators.

Variable	Treatment						
	BAP + GA_3 + IAA	*m*-TP + GA_3 + IAA	2-iP 2	2-iP 5	2-iP 10	IBA	½MS0
Heigth (mm)	8.2 c	10.4 bc	12.3 abc	12.5 abc	16.5 a	8.48 c	15.1 ab
Length of largest root (mm)	41.0bc	24.1 c	65.5 ab	62.9 ab	66.7 a	44.6 abc	65.9 ab
Leaves number	3.0 b	4.4 ab	4.0 ab	4.1 ab	3.7 ab	3.6 ab	5.1 a
Roots number	2.5 c	3.2 c	4.8 bc	5.0 bc	5.3 abc	8.2 a	7.1 ab
Fresh weight (mg)	167.1 b	197.8 b	241.2 ab	271.9 ab	328.19 a	330.0 a*	329.8 a
Dry weight (mg)	26.4 a	33.1 a	27.3 a	31.4 a	34.4 a	29.5 a*	37.2 a
Leaf area (mm^2)	239.5 d	335.3 cd	516.2 bc	642.67 ab	798.6 a	215.8 d	776.8 ab
Photosynthetic pigments per leaf area ($\mu g\ cm^{-2}$)							
Chlorophyll *a*	35.0 b	40.6 ab	40.3 ab	42.4 ab	44.7 ab	47.1 a	45.3 ab
Chlorophyll *b*	11.1 a	13.0 a	12.1 a	12.2 a	13.3 a	14.0 a	13.6 a
Total carotenoids	6.4 b	7.4 ab	7.4 ab	7.5 ab	8.1 a	8.6 a	8.1 a

* Fresh and dry weight are mainly attributed to root development.

Segments of stem without leaves grown in tubes with ½ MS stationary liquid medium under 100 µmol $m^{-2}\ s^{-1}$ irradiance, autoclavable rigid polypropylene lid cover with 1 membrane. Means followed by the same lowercase letter did not differ significantly among the treatments (Tukey's test at 5%). **BAP + GA_3 + IAA**: BAP (0.2 µM) + IAA (0.1 µM) + GA_3 (0.05 µM); ***m*-TP + GA_3 + IAA**: *m*-TP (0.2 µM) + IAA (0.1 µM) + GA_3 (0.05 µM); **2-iP 2 - 10**: 2-iP (2, 5 e 10 µM, respectively); **IBA**: IBA (7 µM); **½ MS0**: MS medium with half the concentration of macro and micronutrients without growth regulators.



Online Resource 6 - *In vitro* plant growth variables of *Piper crassinervium* after 35 days of *in vitro* culture in ½MS liquid medium supplemented with 2-iP (2, 5 and 10 μM) (**a - i**) and ½OM and ½MS medium in combination with sucrose (7.5, 15 and 30 g L^{-1}) (**j - o**). (**2-iP**: N^6-(2-isopentenyl) adenine; **½OM** and **½MS**: Rugini Olive medium (OM) and Murashige and Skoog (MS) medium with half the concentration of macro and micronutrients. Means followed by the same lowercase letter do not differ significantly between 2-iP and sucrose concentrations; Averages followed by the same capital letter do not differ when comparing means ½OM and ½MS (Tukey's test at 5%). Error bars indicate the standard error among the replicates).

CHAPTER 3

The use of *p*-sulfonic acid calix[4]arene as organocatalyst for pretreatment of sugarcane bagasse increased the production of levoglucosan

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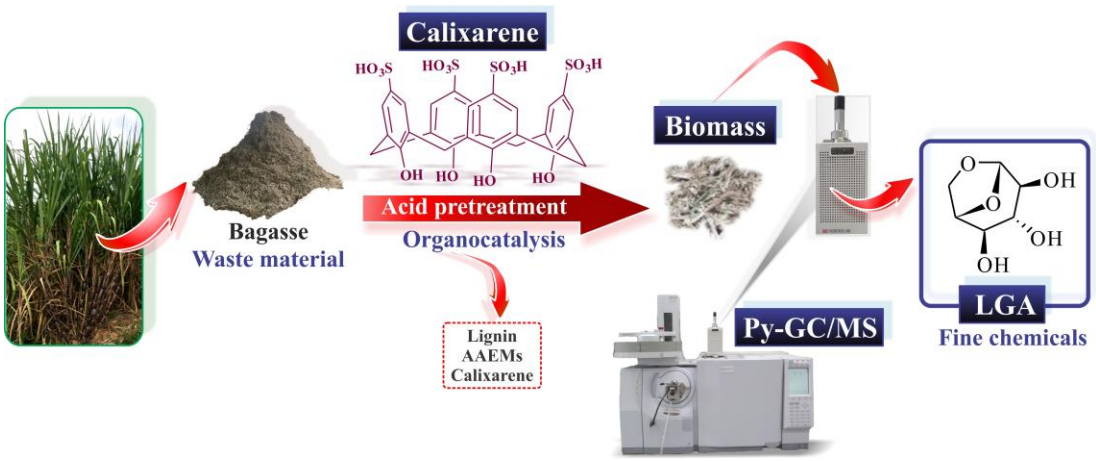
Highlights

- Levoglucosan is a source of carbon to produce several compounds of industrial interest.
- Acid catalyst-pretreated sugarcane bagasse improved the levoglucosan production.
- High levoglucosan yield was attained during pyrolysis process under acid condition.
- Calixarene CX4SO3H was the most efficient catalyst for producing levoglucosan

Graphical abstract



Abstract

This paper presents a novel method for the production of a biomass-derived chemical platform, levoglucosan (LGA), from sugarcane bagasse via pretreatment with an organocatalyst ($CX4SO_3H$) and fast pyrolysis. The effectiveness of this platform for producing LGA from sugarcane bagasse was screened using a microscale pyrolysis reactor coupled with gas chromatography-mass spectrometry (Py-GC/MS). The effects of $CX4SO_3H$ loading, temperature and pretreatment time were studied, and the best pretreatment conditions were 3 wt/wt%, 1 h and 23 °C. The yields of LGA from sugarcane bagasse in the presence and absence of $CX4SO_3H$ were 32.3 and 4.6%, respectively, compared to raw biomass. The presence of $CX4SO_3H$ for the treatment of biomass may make this process an interesting and very useful alternative for the production of a biomass-derived chemical platform, especially for levoglucosan production, as renewable feedstock for biofuels production and other bioproducts.

Keywords: Biorefinery; Biomass; Sugarcane bagasse; Levoglucosan; Organocatalysis; Calixarenes.

Introduction

One of the major challenges facing modern society is the use of unconventional and renewable resource-based green technologies for the production of fuels and commodity chemicals (Kim et al., 2016; Xu et al., 2015; Zang et al., 2018). Plant carbohydrates and abundant nonfood plant biomass as renewable energy sources for the production of fuels, chemicals, and materials have received considerable attention in recent years (Bridgwater, 2003; Caes et al., 2013; Dauenhauer et al., 2007; Huber et al., 2006; Iwamoto et al., 2018).

The advanced biorefinery methods must convert lignocellulosic biomass into a variety of chemical products through modern and green technologies including pyrolysis, liquefaction, hydrogenation, fermentation, gasification and catalytic conversion (Ye et al., 2017; Zhang et al., 2010). In the last decades, a fast pyrolysis-based biorefinery method has received considerable attention from both academia and industry (Agblevor et al., 2016; Black et al., 2016; Meier and Faix, 1999; Melero et al., 2012; Zheng et al., 2017).

Slow and fast pyrolysis, a thermochemical process, are promising technology for converting biomass into several compounds of industrial interest. The type of reactor to be used in the pyrolysis depends on the product desired, *i.e.* bio-oil or bio-char. In slow pyrolysis, the most common reactors are drum, rotatory kilns, and screw/auger. In fast pyrolysis systems, reactors can be fluidized bed, rotating cones, entrained flow, vacuum and ablative (Perkins et al., 2018; Roy and Dias, 2017). In the fast pyrolysis, large volumes of bio-oil is produced, approximately 80% on a dry biomass basis (Asadullah et al., 2007; Drummond and Drummond, 1996; Montoya et al., 2015; Zhou et al., 2013). The yield and quality of products obtained by pyrolysis depend of several factors, as the pyrolysis technology used, the configurations of equipment, operating conditions, type of sample, pre-treatment conditions, among other factors (Roy and Dias, 2017).

However, crude bio-oil obtained from traditional pyrolysis is a complex mixture, which makes it difficult to apply in chemical industries (Carpenter et al., 2014; Mortensen et al., 2011; Ranzi et al., 2017). Recently, interest in levoglucosan (LGA), has increased dramatically because it can be used as a source of carbon for the production of various compounds of industrial interest, such as lipids, citric acid,

ethanol, 5-hydroxymethylfurfural, furans, aromatic hydrocarbons among other fermentation products (Hu et al., 2012; Layton et al., 2011; Lian et al., 2013; Mullen and Boateng, 2015; Xiong et al., 2016; Zhuang et al., 2001). As a result, the cost of LGA is currently high, which prohibits a detailed evaluation of its full potential as a biorenewable platform chemical, and it is essential to perform a pretreatment before pyrolysis process (Carpenter et al., 2014; Chang et al., 2013; Jiang et al., 2015; Zheng et al., 2015).

The use of acid catalysts for the pretreatment of biomass before pyrolysis is an interesting route to produce the pyrolytic sugar. Various catalysts have been effective including H_2SO_4, HNO_3, H_3PO_4, solid superacid SO_4^{2-}/ZrO_2 and HZSM-5 (David et al., 2017; Kudo et al., 2017; Li et al., 2013; Wang et al., 2011; Ye et al., 2017; Zhou et al., 2013). The application of organocatalysts in biorefining for the pretreatment of biomass for the production of LGA remains largely unexplored (David et al., 2018; Feng et al., 2016). Among the available organocatalysts, calix[*n*]arenes have been gaining prominence in several organic transformations in recent years (Abranches et al., 2018; da Silva et al., 2015; de Assis et al., 2016; Homden and Redshaw, 2008; Natalino et al., 2014; Palermo et al., 2016; Simões et al., 2014, 2013, 2012). To the best of our knowledge, this is the first application of *p*-sulfonic acid calix[4]arene ($CX4SO_3H$) as an organocatalysts for the pretreatment of biomass for the production of LGA (Fig. 1).

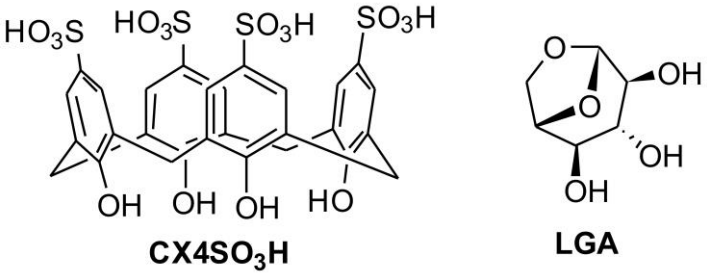


Fig. 1. Chemical structures of *p*-sulfonic acid calix[4]arene ($CX4SO_3H$) and levoglucosan (LGA).

Materials and methods

Biomass collection

Sugarcane bagasse from Brazilian Paraiso's Sucroalcohol Industry located north of Rio de Janeiro, Brazil, was collected. All reagents used were of analytical-reagent grade.

General procedure for the synthesis of *p*-sulfonic acid calix[4]arene ($CX4SO_3H$)

The *p*-sulfonic acid calix[4]arene were synthesized in our laboratory following the procedures published elsewhere (ESI). First, *p-tert*-butylcalix[4]arene was prepared by the Gutsche and Iqbal (1990) as presented in the supplementary information (Scheme 1). Then, the conversion of *p-tert*-butylcalix[4]arene to calix[4]arene was achieved using aluminium chloride in the presence of toluene and phenol according methodology described by Ungaro and co-works (Casnati et al., 2004) as presented in the supplementary information (Scheme 2). Finally, *p*-sulfonic acid calix[4]arene ($CX4SO_3H$) was obtain from calix[4]arene by adding concentrated sulfuric acid (98% wt) to the latter compound (Shinkai et al., 1987) as presented in the supplementary information (Scheme 3). The conversion of calix[4]arene to *p*-sulfonic acid calix[4]arene ($CX4SO_3H$) was monitored by removing small aliquots of the reaction mixture followed by the addition of a minimum quantity of water on this mixture. If no precipitation was observed, it means that all calix[4]arene was converted to *p*-sulfonic acid calix[4]arene ($CX4SO_3H$). The complete sulfonation is observed, in general, after 4h of the addition of the sulfuric acid (Casnati et al., 2004; Gutsche and Iqbal, 1990; Shinkai et al., 1987).

General procedure for biomass acid pretreatments

The sugarcane bagasse was milled up to 0.420 mm and then utilized to verify the effect of the different acid catalysts on the pretreatment of biomass. A vial containing a mixture of sugarcane bagasse (~1.000 g) and catalyst in deionized water (10 mL) was sealed and kept under constant stirring for 1 h using a magnetic stirrer at room temperature (23 °C). Then, the biomass was filtered, washed with 10 mL deionized water and dried at 105 °C for 24 h (constant weight) (Fig. 2). *p*-Sulfonic acid calix[4]arene ($CX4SO_3H$), phosphoric acid, nitric acid, sulfuric acid, *p*-toluenesulfonic acid (PTSA), *p*-hydroxybenzenesulfonic acid (PHA) and acetic acid

were tested. All catalysts were used with the same hydrogen ion concentration, considering the number of acid sites of each catalyst.

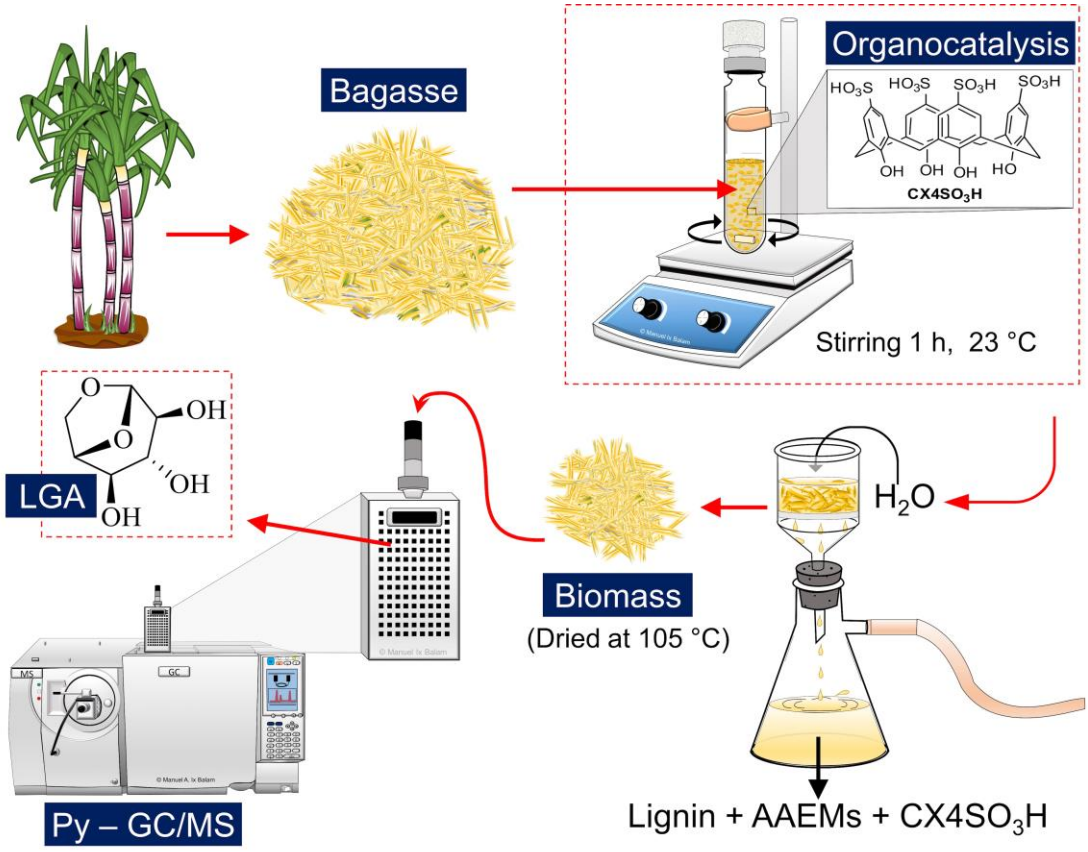


Fig. 2. Stages of pretreatment of sugarcane bagasse with the $CX4SO_3H$. **LGA**: levoglucosan; **AAEMs**: alkaline and alkaline earth metals; **Py-GC/MS**: pyrolysis reactor coupled with gas chromatography-mass spectrometry.

Analytical methods

Py-GC/MS of the biomass previously treated with acids and untreated

Biomass samples (0.5 mg) were subjected to pyrolysis in a Py-GC/MS reactor at 500 °C, after the acid pretreatment of sugarcane bagasse. Pyrolysis was carried out in a Single-shot Pyrolyzer PY-3030S (Frontier Lab Inc., Fukushima, Japan) connected to a GC-2010 Plus system with a GCMS-QP2010 Ultra spectrometer (Shimadzu, Kyoto, Japan). The temperature of the valve interface was set up at 290 °C, and the pyrolysis temperature was 500 °C. The compounds obtained after the pyrolysis were separated using the Rtx-5MS GC column (30 m x 0.25 mm d.i x 0.25 µm d_f) (Restek® corporation, Bellefonte, USA). The GC injector temperature was set up at 250 °C. The initial oven temperature was set at 40 °C and was maintained for 1

min, next, the oven was heated at a rate of 6 °C min^{-1} to 280 °C and then held for 15 min. Helium was used as carrier gas at a flow rate of 1 mL min^{-1} in a split mode, and the split ratio was 20:1. The peaks of the compounds obtained after the pyrolysis of sugarcane bagasse were identified based on the NIST (2005; 2008; 2011 and 2014) and Wiley 7v100 mass spectral data libraries. All analyzes were performed in duplicate.

Levoglucosan formed during the pyrolysis was quantified based on the external standard technique (levoglucosan from Carbosynth Ltd., Berkshire, UK). Standard solutions were prepared in methanol, at concentrations at 5-300 mg mL^{-1} and injected on GC/MS (injected volume 1 μL) under the same conditions of separation of the vapors formed in the biomass pyrolysis. Calibration curve (r^2 = 0.9991) was obtained with respect to the mass of the standard injected into GC/MS (0.005-0.300 mg of LGA) as presented in the supplementary information (Fig. S1). The percentage of levoglucosan yield (wt.%) was calculated based on the amount of pyrolysed biomass (0.5 mg) (Elias et al., 2001; Ghalibaf et al., 2017; Zhuang et al., 2001).

Results and Discussion

Herein, we report an efficient and simple method for the pretreatment of sugarcane bagasse with the $CX4SO_3H$ organocatalyst before pyrolysis to produce levoglucosan (LGA). After biomass treatment with the organocatalyst, the pyrolysis tests were carried out. For this study, were used as pyrolysis temperature 500 °C, this was selected due previous works published by our research group, which favored the LGA production (David et al., 2018). Besides LGA, other compounds were identified in the sample's chromatograms of sugarcane bagasse, the compounds names with their respective percentage of area and their corresponding chromatograms are presented in the supplementary information (Fig. S2 and Table S1).

The effect of the $CX4SO_3H$ concentration catalyst on the pretreatment of sugarcane bagasse (removal of lignin, alkali and alkaline earth metals) and

subsequent pyrolysis of the cellulose and hemicellulose to produce LGA was studied via tests using various concentrations of $CX4SO_3H$ (0.0 to 7.0 wt/wt%) (Fig. 3).

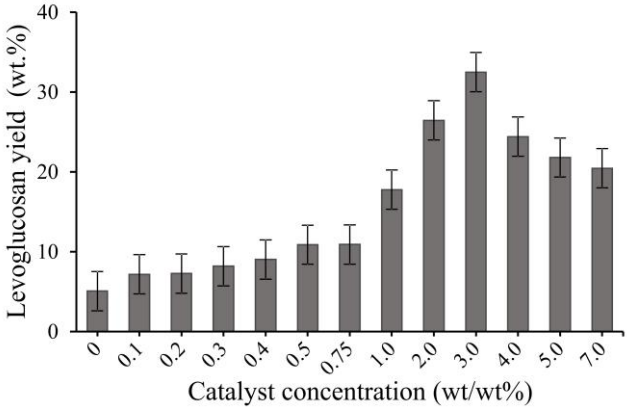


Fig. 3. Production of levoglucosan with different quantities of $CX4SO_3H$ catalyst used in the pretreatment of sugarcane bagasse. Error bars indicate the standard error among the replicates (n = 2).

Upon increasing the $CX4SO_3H$ concentration for the pretreatment of sugarcane bagasse to produce LGA, the yield increased gradually to 3 wt/wt%, which was the best catalyst concentration as a result of the sugarcane bagasse treatment. Concentrations greater than 3 wt/wt% catalyst decreased the LGA yield (Fig. 3). Additionally, it was observed that the biomass appeared dark during the drying process when treated with concentrations greater than 3 wt/wt%. Zhou et al. (2013) observed that the LGA yield decreased with an increase in the sulfuric acid concentration, the authors suggest that this behavior occurs due to an acceleration of the dehydration reactions when free acid was present. It is possible that this behavior is the same in this work because of the excess catalyst in the pretreatment step.

The effect of time on the pretreatment of sugarcane bagasse and subsequent pyrolysis to produce LGA was also investigated. Pretreatment reaction times of 0.5 h to 1 h increased the had low difference in the conversion of sugarcane bagasse to LGA. The yield did not change significantly after this period (Fig. 4). Based on the conversion and selectivity results, a reasonable pretreatment time is 1 h to produce LGA.

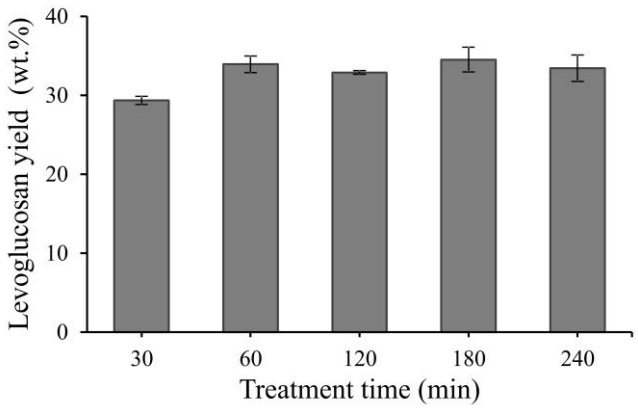


Fig. 4. Effect of the pretreatment time (min) of sugarcane bagasse with $CX4SO_3H$ prior to pyrolysis on levoglucosan production. Error bars indicate the standard error among the replicates (n = 2)

To investigate the effect of the temperature on the pretreatment of sugarcane bagasse, different temperatures (23-100 °C) were evaluated. An increase in the pretreatment temperature did not significantly alter the production of LGA (Fig. 5). The pretreatment of sugarcane bagasse with $CX4SO_3H$ (3 wt/wt%) for 1 h at room temperature (23 °C) prior to pyrolysis increased its conversion to LGA.

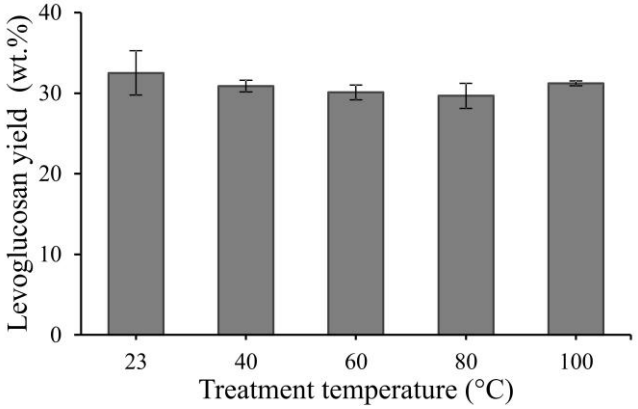


Fig. 5. Effect of the temperature on the pretreatment of sugarcane bagasse treated with $CX4SO_3H$ (3 wt/wt%) for levoglucosan production. Error bars indicate the standard error among the replicates (n = 2).

To prove the superior efficacy of the $CX4SO_3H$ catalyst for the pretreatment of sugarcane bagasse to produce LGA, other Brønsted acids were used under the best reaction conditions (Table 1). Nevertheless, to our satisfaction, none of the tested Brønsted acids were as efficient as $CX4SO_3H$ for the pretreatment of sugarcane bagasse to produce LGA (Table 1). Phosphoric acid, nitric acid and sulfuric acid (Table 1, inputs 2-4) showed good catalytic activity for pre-treatment of sugarcane

bagasse, getting LGA with yields of 17.3 - 22.1% (on average 7.2 times more than the raw biomass). However, $CX4SO_3H$ was even better at the acid pretreatment, producing an LGA yield of 32.3% (12.0-fold more than the raw biomass) (Table 1, entry 1). The use of *p*-toluenesulfonic acid (PTSA) and *p*-hydroxybenzenesulfonic acid (PHA) as catalysts afforded LGA after pyrolysis with yields of 10.5 and 8.8% (only 3.9 and 3.3-fold compared to raw biomass), respectively (Table 1, entries 5-6). The use of acetic acid as a catalyst for the pretreatment of sugarcane bagasse provided an LGA yield of 6.9% (2.6-folds), while the non-catalyzed reaction furnished an LGA yield of 4.6% (1.7-fold compared to raw biomass) (Table 1, entries 7-8).

The best performance exhibited by $CX4SO_3H$, for the pretreatment of sugarcane bagasse to produce LGA, compared with PTSA and PHA could be due to the presence of water in the process. We have demonstrated that water expressively decreased the catalytic activity of these later catalyst, while no significant effect was observed when $CX4SO_3H$ was used in esterification reactions (Natalino et al., 2014). The negative water-effect on palmitic acid esterification reaction catalyzed by H_2SO_4 was also observed by us, but this effect was not significant as for PTSA and PHA (Natalino et al., 2014). Indeed, the efficacy of H_2SO_4 was higher than PTSA and PHA (Table 1) (Natalino et al., 2014); this could be also the reason why H_3PO_4 and HNO_3 were more efficient than these two later catalysts.

Table 1. Effect of different Brønsted acid catalysts for the pretreatment of sugarcane bagasse to produce LGA.

Entry	Catalyst	Yield (%) ± SE[a]	Increase in LGA yield[b]
1	$CX4SO_3H$	32.3 ± 1.0	12.0
2	H_3PO_4	22.1 ± 0.6	8.2
3	HNO_3	19.1 ± 0.1	7.0
4	H_2SO_4	17.3 ± 3.5	6.4
5	PTSA	10.5 ± 1.0	3.9
6	PHA	8.8 ± 0.7	3.3
7	CH_3CO_2H	6.9 ± 0.4	2.6
8	-	4.6 ± 0.1	1.7
Raw biomass	-	2.7 ± 0.1	-

[a] Yield was determined by standard (levoglucosan) calibration curve GC/MS analysis. [b] Increase in levoglucosan yield compared to raw biomass; SE: standard error among the replicates (n = 2).

The acid pretreatment of the biomass separates part of the cell wall structure and removes lignin and other non-carbohydrate components (alkaline and alkaline earth metals), increasing the accessibility to cellulose and the hemicelluloses present in the lignocellulosic material (Gomes et al., 2014; de Carvalho et al., 2015). Removal of these lignocellulosic components increases the thermal conversion of cellulose to levoglucosan during rapid pyrolysis of biomass (Zheng et al., 2018).

Alkaline and alkaline earth compounds have been removed during the pretreatment of the biomass with different acid catalysts (acetic, sulfuric, nitric and phosphoric acid) (David et al., 2018; Pecha et al., 2015). Mourant et al. (2011) observed that the acid-washing, i.e. an ion-exchanging process to replace these AAEM species with H^+, would have resulted in the disintegration of these AAEM-related crosslinks in the biomass structure, i.e. some degree of structure relaxation in biomass (lower degree of cross-linking). Possibly the largest number of acid sites in the $CX4SO_3H$ increases the removal of lignocellulosic compounds when compared to the other catalysts evaluated.

The improvement of levoglucosan production during the fast pyrolysis of the pretreated biomass with acid catalysts may be due to the subtraction of lignin (Table S1, entries 18, 19, 23, 30, 32 and 33) and/or alkali and alkaline earth metals (AAEM) present in the sugarcane bagasse. Indeed, AAEMs such as K, Fe, Ca, S, Mg, P, Mn, Zn, Ba and Cu are very abundant in sugarcane bagasse and their negative effects on the chemical processing of lignocellulosic biomass are well known (David et al., 2018; Pecha et al., 2015; Zhou et al., 2013). Actually, the presence of inorganic metals can be responsible for secondary reactions (cracking and/or condensation) of pyrolytic compounds initially produced by primary pyrolysis reactions, which results in changes in chemical distributions in bio-oil and its yield (Eom et al., 2012; Mourant et al., 2011).

During the pyrolysis process, the AAEMs alter the structure of the carbohydrate by interacting with the oxygen atoms, affecting the stereochemistry of the molecules during the reactions, where rearrangement and dehydration reactions are intensified, followed by the fragmentation reaction present (Mayes et al., 2015; Pecha et al., 2015). Patwardhan et al. (2010) reported that mineral salts and higher temperatures accelerate the reactions, leading to the formation of low molecular weight compounds from cellulose inhibition of levoglucosan formation. Haddad et

al. (2017) studied pyrolysis mechanism of decomposition of lignocellulosic biomass with and without the AAEMs. These authors observed that potassium and sodium accelerated the thermal degradation of hemicellulose and modified the catalytic effects on cellulose degradation followed by condensation of low molecular weight compounds and consequently an increase in the yield of biochar. In this sense, the pretreatment with Brønsted acid, for instance, the acetic acid, is able to remove these negative interferes leading to a 9-fold improvement of levoglucosan production when compared with the non-treated biomass (David et al., 2018). In fact, the acid pretreatment of several types of biomasses aiming to remove the AAEM salts and consequently to improve levoglucosan production are described elsewhere in the literature (Dobele et al., 2001; Dong et al., 2015; Eom et al., 2011; Oudenhoven et al., 2013; Tan and Wang, 2009; Wang et al., 2014; Zhou et al., 2013). Based on the prementioned studies, the higher production of levoglucosan from $CX4SO_3H$-pretreated sugarcane bagasse under our reaction conditions could be, in part, explained by the ability of calix[*n*]arenes acts as both, a Brønsted acid (Abranches et al., 2018; da Silva et al., 2015; de Assis et al., 2016; Binder and Raines, 2010; Homden and Redshaw, 2008; Natalino et al., 2014; Palermo et al., 2016; Simões et al., 2014, 2013, 2012) and a metal scavenger (Martins et al., 2016; Sliwa and Girek, 2010). The $CX4SO_3H$ can be removed from the biomass with deionized water and easily recovered from the aqueous phase to be reused, in addition to promoting the formation of levoglucosan during pyrolysis; furthermore the $CX4SO_3H$ is non-toxic (Abranches et al., 2018; Simões et al., 2012).

Conclusions

In this study, an integration of technology from biomass pretreatment to pyrolysis was used to obtain levoglucosan (LGA). For the first time, we demonstrated that sugarcane bagasse treatment with $CX4SO_3H$ favored the formation of LGA in 32.2% yield (12.0-fold more than the raw biomass). It has been found that an important factor in the biomass pretreatment is the catalyst loading, and the temperature and time do not decisively influence the LGA yield. These results clearly indicate that this organocatalyst ($CX4SO_3H$) is superior to previously reported catalysts with respect to the sugarcane bagasse treatment to improve pyrolytic sugar LGA.

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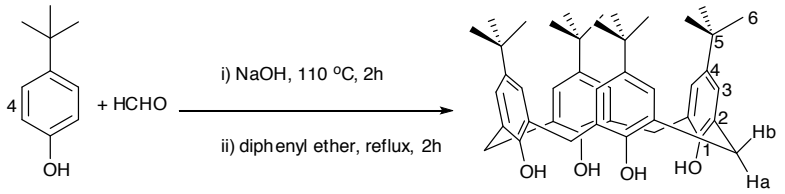
Supplementary material

Experimental procedures

Synthesis of calix[*n*]arenes

Synthesis of the *p-tert*-butylcalix[4]arene

The synthesis of the *p-tert*-butylcalix[4]arene involving the condensation of the *p-tert*-butylphenol, formaldehyde solution with a basic medium and under heating, as shown in Scheme 1, following the methodology described by Gutsche et al. [1]. The product was obtained as a white solid in 77% yield.



Scheme 1 - Reaction for obtaining the *p-tert*-butylcalix[4]arene

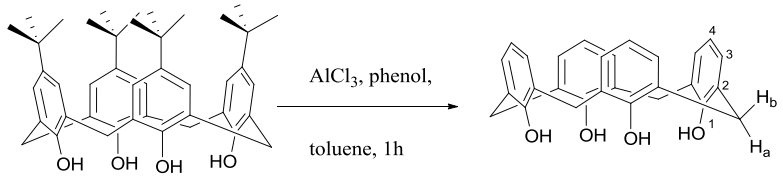
^{1}H NMR (300 MHz, $CDCl_3$): 1.21 (s, 36H, H-6), 3.48 (d, 4H, *J* 12.4, CH_2-Ha), 4.28 (d, 4H, *J* 12.4, CH_2-Hb), 7.05 (s, 8H, H-3), 10.34 (s, 4H, OH).

^{13}C NMR (75 MHz, $CDCl_3$): 31.4 (C-6), 32.7 (CH_2), 34.0 (C-5), 125.9 (C-3), 127.6 (C-2), 144.3 (C-4), 146.7 (C-1).

IR (ATR, cm^{-1}): 3161, 3058, 3025, 2953, 1737, 1604, 1480, 1456, 1391,1362, 1231, 1200, 871, 814, 781.

Synthesis of the calix[4]arene

The synthesis of calix[4]arene was carried out using *p-tert*-butilcalix[4]arene, phenol and aluminum chloride anhydrous in toluene following the methodology described by Gutsche et al. [2]. The system was kept under stirring and nitrogen atmosphere at room temperature for one hour (Scheme 2). The desired product, a white solid, was obtained in 81% yield after recrystallization in methanol-chloroform.



Scheme 2 - Reaction for obtaining the calix[4]arene.

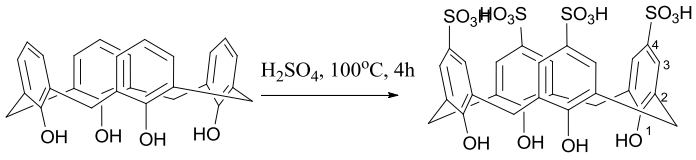
^{1}H NMR (300 MHz, $CDCl_3$): 3.56 (d, 4H, *J* 12.6, H-a), 4.27 (d, 4H, *J* 12.6, H-b), 6.79 (t, 4H, *J* 7.5, H-4), 7.08 (d, 8H, *J* 7.5 Hz, H-3), 10.23 (s, 4H, OH).

^{13}C NMR (75 MHz; $CDCl_3$): 31.9 (CH_2), 122.5 (C-4), 128.5 (C-2), 129.2 (C-3), 149.0 (C-1).

IR (ATR, cm^{-1}): 3145, 3091, 2933, 1465, 1448, 1376, 1361, 1212, 773, 752.

Synthesis of the *p*-sulfonic acid calix[4]arene ($CX4SO_3H$)

Catalyst *p*-sulfonic acid calix[4]arene was conducted from calix[4]arene in the presence of concentrated sulfuric acid and heated for four hours as described by Gutsche et al. [3] (scheme 3). The product was obtained in 75% yield as a solid white.



Scheme 3 - Reaction for obtaining of the *p*-sulfonic acid calix[4]arene ($CX4SO_3H$).

^{1}H NMR (300 MHz, D_2O): 3.84 (s, 8H, CH_2), 7.39 (s, 8H, H-3).

^{13}C NMR (75 MHz, D_2O): 30.7 (CH_2), 126.6 (C-3), 128.2 (C-2), 135.8 (C-4), 151.9 (C-1).

IR (ATR, cm^{-1}): 3234, 1705, 1636, 1599, 1455, 1162, 1117, 630.

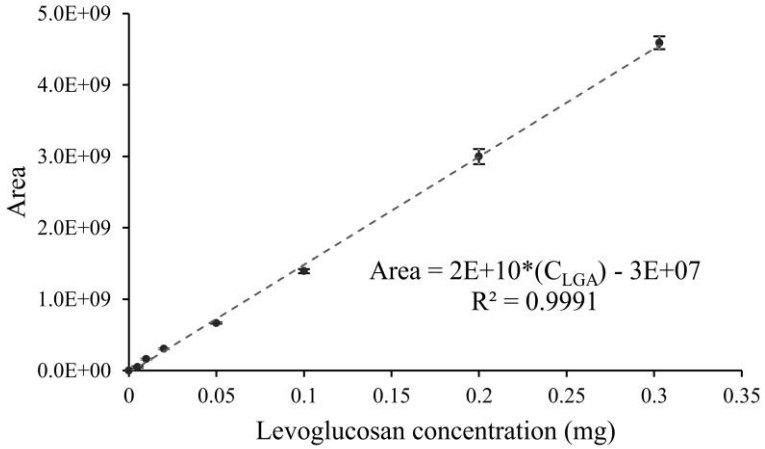


Fig. S1. Levoglucosan calibration curve. C_{LGA}= Levoglucosan concentration.

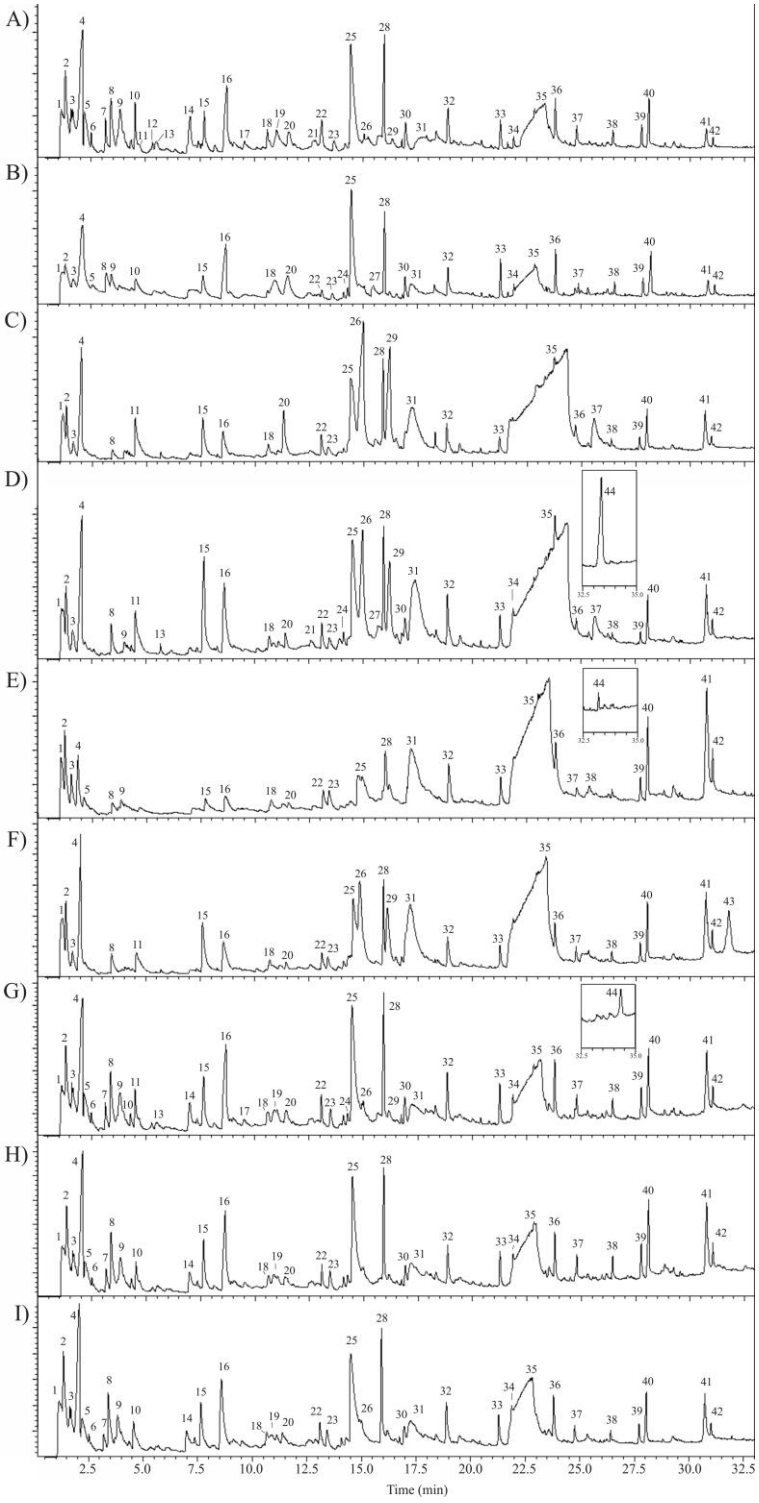


Fig. S2. Pyrolysis-GC/MS chromatogram of condensable compounds at 500 °C for treated sugarcane bagasse: a) Raw biomass, b) Control experiment, c) $CX4SO_3H$, d) H_3PO_4, e) HNO_3, f) H_2SO_4, g) PTSA, h) PHA and i) CH_3CO_2H.

Table S1. Percentages of areas of compounds formed from sugarcane bagasse pyrolysis.

Peak	Compounds	% Areas of compounds formed from sugarcane bagasse pyrolysis								
		Catalysts								
		Raw	Control	Calix	H_3PO_4	HNO_3	H_2SO_4	$C_7H_8O_3S$	$C_6H_6O_4S$	CH_3COOH
1	Carbon dioxide	4.39	4.68	2.18	2.75	3.31	4.32	2.22	4.59	5.58
2	Acetaldehyde	6.45	5.34	1.23	2.05	3.51	2.90	5.55	5.76	7.71
3	2-Oxopropanoic acid	1.17	2.58	0.43	0.81	1.84	1.04	1.19	0.91	1.53
4	Acetic acid	11.96	12.25	2.47	4.55	2.19	4.60	9.46	8.36	10.47
5	2-Oxopropanal	3.55	-	-	-	0.39	-	3.00	1.94	1.18
6	NI	0.35	2.01	-	-	-	-	0.16	0.16	0.19
7	Prop-2-enoic acid, methyl ester	1.48	-	-	-	-	-	1.11	0.96	0.80
8	1-Hydroxypropan-2-one	4.03	1.64	0.2	0.82	0.60	0.72	3.27	2.85	3.13
9	Methyl pyruvate	4.70	1.68	0.31	0.34	0.33	-	2.42	1.43	1.31
10	2-methypentano	2.14	-	-	-	-	-	0.38	0.91	0.99
11	Furfural	0.54	1.78	1.59	0.80	-	0.77	1.60	-	-
12	2-methylbutyl acetate	0.33	-	-	-	-	-	-	-	-
13	2-Furanmethanol	0.66	-	0.10	0.15	-	-	0.21	-	-
14	Cyclohexanone	2.53	-	-	-	-	-	1.53	1.06	2.09
15	Propanoic acid, 3-hydroxy-, hydrazide	1.74	5.64	0.97	3.15	0.55	2.70	2.42	2.46	2.02
16	NI	5.27	4.26	0.78	3.20	1.10	2.27	5.01	-	6.15
17	2-Hydroxy-3-methyl-2-cyclopentene-1-one	0.72	-	-	-	-	-	0.36	5.26	-
18	2-Methoxyphenol	0.64	4.26	0.27	0.35	0.72	0.50	0.70	0.50	0.82
19	4-methylphenol	1.64	-	-	-	-	-	1.07	1.34	0.20
20	Pentanal	1.30	0.42	1.69	0.39	0.29	0.28	0.91	0.61	0.88
21	Levoglucosenone	0.85	-	0.18	0.22	-	-	-	-	-
22	4-Hydroxycyclohexanone	1.38	0.48	0.34	0.61	0.83	0.62	0.82	0.68	1.06

23	2-Methoxy-4-methylphenol	0.78	0.29	0.28	0.41	0.92	0.39	1.04	0.90	0.97
24	4-Ethoxycyclohexanone	0.33	-	0.19	0.51	-	-	0.30	-	-
25	3-Hydroxycyclohexanone	10.62	11.37	1.55	5.62	1.90	3.76	9.05	9.16	9.42
26	2,3-Dihydrobenzofuran	0.27	3.8	2.94	5.68	-	6.49	0.98	-	4.39
27	beta-d-Ribopyranoside, methyl, 3-acetate	0.74	1.4	-	0.65	-	-	-	-	-
28	2-Methoxy-4-vinylphenol	4.06	1.24	1.26	2.08	2.00	2.21	3.55	3.51	0.66
29	3,4-Anhydro-d-galactosan	0.43	1.18	3.74	2.92	-	3.97	-	-	-
30	2,6-Dimethoxyphenol	1.44	0.54	0.28	0.66	-	-	0.82	0.72	3.29
31	D-Glucose, 4-O-alpha-D-glucopyranosyl	0.43	2.28	3.36	8.49	7.06	8.67	2.84	3.61	2.19
32	1,2,4-Trimethoxybenzene	1.77	1.83	0.44	1.48	1.63	1.26	1.71	1.59	1.23
33	3',5'-Dimethoxyacetophenone	1.25	1.75	0.26	0.64	0.89	0.61	1.10	1.18	0.68
34	2,6-dimethoxy-4-(2-propenyl)phenol	0.40	-	0.41	0.38	-	-	0.88	1.49	1.27
35	Levoglucosan	12.89	19.46	57.21	42.15	42.95	42.20	28.29	27.66	22.17
36	Benzaldehyde, 4-hydroxy-3,5-dimethoxy	1.71	1.92	1.17	0.46	1.59	1.04	1.51	1.62	1.84
37	1,5-Anhydro-d-mannitol	0.85	0.66	0.08	1.48	0.27	0.43	0.45	0.67	0.60
38	Pentadecanoic acid	0.38	0.48	2.3	0.10	0.24	0.22	0.20	0.62	0.24
39	2-hydroxycyclopentadecanone	0.77	0.86	8.98	0.18	0.54	0.47	0.20	1.22	0.61
40	Hexadecanoic acid	1.92	2.63	3.62	0.85	2.04	1.47	0.31	2.65	1.83
41	NI	0.89	1.07	0.32	1.28	5.75	2.48	1.38	3.14	2.04
42	Desaspidinol	0.25	0.30	0.34	0.26	1.28	0.58	0.79	-	0.38
43	NI	-	-	-	-	-	3.03	-	-	-
44	Squalene	-	-	1.58	3.53	0.34	-	2.65	-	0.15

NI – not identified.

CONCLUSÕES GERAIS

Um protocolo eficiente para a caraterização da fração volátil de tecidos vegetais cultivados *in vitro* por HS-SPME foi estabelecido. A metodologia otimizada neste trabalho pode ser aplicada para futuras pesquisas envolvendo a elicitação da biossíntese de compostos orgânicos voláteis em material vegetal cultivado *in vitro* de *Ruta graveolens* e de outras plantas medicinais com VOCs quimicamente semelhantes aos encontrados na arruda.

O efeito da qualidade de luz sobre a produção de VOCs derivados de ácidos graxos foi estudado pela primeira vez em plantas propagadas *in vitro* de *Ruta graveolens*. Este trabalho pode contribuir à elucidação de outros fatores que participam nas rotas de biossíntese de diversos VOCs de interesse comercial.

Neste trabalho constatou-se que raízes de plantas de *P. crassinervium* apresentaram maior conteúdo de fenóis e flavonoides totais, em comparação com a parte aérea. O incremento da produção de compostos fenólicos correlacionou com o maior estresse induzido nas plantas, evidenciado pelo menor acúmulo de biomassa, em algumas das condições de cultivo testadas. No entanto, um protocolo de propagação *in vitro* eficiente, tanto para a obtenção de compostos fenólicos como de biomassa, foi estabelecido para *P. crassinervium*. Este é o primeiro estudo para a espécie em que sistematizou-se e buscou-se a otimização de alguns fatores (formulações de meios, consistências do meio, concentrações de sacarose, irradiâncias, trocas gasosas, dentre outros) com reflexos positivos sobre o desempenho *in vitro* de plantas de *Piper*.

As condições reacionais para produção de levoglucosana via pirólise rápida de biomassa foram otimizadas utilizando pela primeira vez o ácido *p*-sulfônico calix[4]areno como organocatalisador. O pré-tratamento ácido de bagaço de cana com calix[*n*]arenos forneceu uma metodologia eficiente para incrementar a produção de levoglucosana.