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NOVAS ABORDAGENS NO ESTUDO DO POTENCIAL ALELOPÁTICO DE TRÊS ESPÉCIES DE *Schinus* L. (ANACARDIACEAE)

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Tese apresentada ao Programa de Pós-Graduação em Botânica do Instituto de Biociências da Universidade Federal do Rio Grande do Sul (UFRGS), como parte dos requisitos para obtenção do grau de Doutor em Botânica.

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RESUMO

Os óleos essenciais de três espécies de *Schinus* foram avaliados quanto à sua atividade fitotóxica na planta alvo *Arabidopsis thaliana*. Para esse fim, utilizou-se a abordagem convencional, que avalia o efeito dos aleloquímicos sobre a germinação e o crescimento de plântulas, mas também buscaram-se novas técnicas para compreender o modo de ação dos voláteis, a saber o efeito sobre o enraizamento adventício e a análise da expressão de genes por PCR em tempo real. Adicionalmente, foi realizada a caracterização química dos óleos essenciais por cromatografia gasosa bidimensional, demonstrando que o óleo de *S. lentiscifolius* é principalmente constituído por sesquiterpenos, enquanto que os de *S. molle* e *S. terebinthifolius* apresentam maior quantidade de monoterpenos. Os três óleos essenciais afetaram a porcentagem de germinação, o índice de velocidade de germinação, o crescimento da raiz primária e o crescimento da parte aérea de *A. thaliana*, mas não apresentaram efeito sobre a expressão dos genes ANP1 e CDKB1;1. Os voláteis também afetaram o processo de enraizamento adventício da planta alvo de uma maneira dose-dependente, afetando o tempo médio de enraizamento e o crescimento da maior raiz adventícia, mas não afetou a porcentagem de enraizamento nem o número de raízes por microestaca. A adição de uma auxina não reverteu os efeitos fitotóxicos induzidos pelos voláteis, mas a adição de uma citocina contribuiu na recuperação do atraso do processo de enraizamento quando os óleos essenciais de *S. lentiscifolius* e *S. molle* foram utilizados. Além disso, o Trolox®, um potente antioxidante, contribuiu na recuperação parcial dos efeitos inibitórios causados pelos óleos essenciais, indicando que eles induzem estresse oxidativo. Adicionalmente, foi realizada a detecção histoquímica de peróxido de hidrogênio nas microestacas expostas aos óleos essenciais. Os resultados demonstram que os efeitos fitotóxicos dos voláteis ocorrem por diversos mecanismos, incluindo estresse oxidativo e efeito sobre fitormônios. Esse estudo demonstrou que, devido às diversas técnicas que podem ser aplicadas, *A. thaliana* é uma espécie útil para ser utilizada em experimentos que avaliem o potencial alelopático de produtos naturais.

Palavras-chave: atividade fitotóxica; comprimento da raiz; estresse oxidativo.

ABSTRACT

The essential oils of three *Schinus* species were evaluated for phytotoxic activity using *Arabidopsis thaliana* as target species. It was used the conventional approach, evaluating allelochemical effects on seed germination and seedling growth. Additionally, it was also employed new techniques in allelopathic potential area to understand the modes of action of volatiles, as the phytotoxic effects on adventitious rooting and the analysis of expression of genes by RT-qPCR. Furthermore, chemical characterization of essential oils using two-dimensional gas chromatography was carried out. *S. lentiscifolius* essential oil is mainly composed by sesquiterpenes, whereas *S. molle* and *S. terebinthifolius* presented high amounts of monoterpenes. Essential oils affected the germination rate (%), speed of accumulated germination, primary root length and shoot length of *A. thaliana*. However, the expression of the genes ANP1 and CDKB1; 1 was not affected. Volatiles also interfere on *A. thaliana* adventitious rooting in a dose-dependent manner. Inhibitory effects were observed on mean rooting time and root length. Rooting percentage and number of roots per microcuttings were not affected by volatiles. Addition of an auxin not reversed phytotoxic effects induced by volatiles, but the addition of a cytokinin contributed to *A. thaiana* recover from the effects on mean rooting time caused by *S. lentiscifolius* and *S. molle* essential oils. Furthermore, Trolox®, a potent antioxidant, contributes to the partial recovery of the inhibitory effects caused by essential oils, indicating that volatiles induce oxidative stress. Histochemical detection of hydrogen peroxide after essential oil exposure was also carried out. Results demonstrated that the phytotoxic activity of volatiles may occur by several mechanisms, including oxidative stress and effects on phytohormones. This study also demonstrated *A. thaliana* is a useful species to be used in allelopathic potential evaluations due to the different techniques that can be applied.

Keywords: phytotoxic activity; root length; oxidative stress.

SUMÁRIO

1 REFERENCIAL TEÓRICO

1.1 O gênero *Schinus* L.

O gênero *Schinus* L. (Anacardiaceae) é constituído por espécies arbóreas ou arbustivas lenhosas nativas do continente americano. Segundo Sobral e Jarenkow (2006), no estado do Rio Grande do Sul ocorrem cinco espécies: *S. lentiscifolius* March. (aroeira-cinzenta), na Serra do Sudeste e esporadicamente junto à floresta com araucária (Figura 1); *S. molle* L. (anacauíta, aroeira-salso), na Depressão Central e Serra do Sudeste (Figura 2); *S. pearcei* Engl., na região das Missões; *S. polygamus* (Cav.) Cabr. (assobiadeira), em todas as formações florestais; e *S. terebinthifolius* Raddi (aroeira-vermelha), em todas as formações do estado (Figura 3).

Figura 1 – *Schinus lentiscifolius* (aroeira-cinzenta)

Fonte: http://www.visoflora.com/photos-nature/photo-grand-schinus-lentiscifolius.html

Schinus molle e *S. terebinthifolius* são espécies importantes na recuperação e expansão de áreas florestais, pois crescem mesmo em solos muito degradados. Além disso, *S. terebinthifolius* é considerada uma espécie agressiva, pois ocupa rapidamente capoeiras ou outros espaços disponíveis pela ampla dispersão de seus frutos que são muito apreciados pelos pássaros e formigas (BACKES; IRGANG, 2002). Os frutos secos de *S. terebinthifolius* são também apreciados na culinária, sendo utilizados como condimento (BARBOSA *et al.*, 2007), a pimenta rosa. Tanto *S. molle* como *S. terebinthifolius* são amplamente cultivadas como ornamentais e

foram introduzidas em áreas subtropicais fora de sua área de ocorrência natural, por exemplo, América do Norte e sul da África, tornando-se espécies invasoras (IPONGA *et al.*, 2008; MORGAN; OVERHOLT, 2005; MUKHERJEE *et al.*, 2012; WILLIAMS *et al.*, 2005).

Figura 2 – *Schinus molle* (aroeira-salso)

Fonte: Autoria própria.

Figura 3 – *Schinus terebinthifolius* (aroeira-vermelha)

Fonte: Autoria própria.

Fitoquimicamente, o gênero *Schinus* é marcado pela produção de compostos fenólicos e terpenoides (CERUKS *et al.,* 2007; RICHTER *et al.*, 2010), sendo numerosos os estudos relacionados à composição química das espécies e as atividades biológicas de seus extratos e metabólitos. Nesse sentido, estudos

demonstraram atividade antipirética, anti-inflamatória e analgésica de *S. polygamus* (ERAZO *et al.,* 2006), antimicrobiana de *S. lentiscifolius* (GEHRKE *et al.,* 2012) e *S. terebinthifolius* (AMORIM; SANTOS, 2003), antidepressiva de *S. molle* (MACHADO *et al.*, 2007), e antitumoral (SANTANA *et al*., 2012) e cicatrizante (RIBAS *et al*., 2006) de *S. terebithifolius*. Sob o ponto de vista ecológico, estudos demonstram a atividade fitotóxica (ZAHED *et al.*, 2010), fungicida (DIKSHIT *et al.*, 1986; SANTOS *et al.*, 2010) e inseticida (FERRERO *et al.*, 2006; HUERTA *et al.*, 2010; LÓPEZ *et al*., 2014) de *S. molle* e propõem a aplicação dos produtos naturais dessa espécie no controle biológico de plantas daninhas, patógenos e pragas.

1.2 Óleos essenciais

Os óleos essenciais são denominados óleos por serem líquidos de aparência oleosa à temperatura ambiente, geralmente amarelados e solúveis em solventes orgânicos de baixa polaridade. São também chamados de óleos voláteis, ou apenas voláteis, devido ao fato de volatilizarem à temperatura ambiente. Ainda, podem ser denominados como essências, devido ao aroma agradável e intenso da maioria deles (DAMASCENO, 2007; SIMÕES; SPITZER, 2007).

Comercialmente, os óleos essenciais são amplamente utilizados como flavorizantes de alimentos e bebidas e como ingredientes de perfumes, cosméticos, produtos de limpeza e produtos de higiene pessoal, sendo também utilizados para fins medicinais (ADORJAN; BUCHBAUER, 2010). Ecologicamente, os óleos essenciais exercem importantes funções na defesa vegetal, em interações ecológicas como alelopatia, atuando como potentes inibidores da germinação de sementes e do crescimento de plantas, na atração de polinizadores, na comunicação entre plantas e na proteção contra predadores (CROTEAU *et al.*, 2000; DUDAREVA *et al.*, 2006).

A produção de óleos essenciais não está restrita a nenhum grupo taxonômico específico, ocorrendo amplamente em todo o reino vegetal. Entretanto, em relação à quantidade de óleo produzido, destacam-se as coníferas e as angiospermas eudicotiledôneas, em especial as famílias Asteraceae, Apiaceae, Lamiaceae, Lauraceae, Myrtaceae, Piperaceae e Rutaceae (SIMÕES; SPITZER, 2007). Os óleos essenciais podem ser estocados em certos órgãos, tais como flores, folhas, caule, raízes, rizomas, frutos ou sementes. Embora todos os órgãos de uma planta

possam acumular óleos voláteis, sua composição pode variar segundo a localização (SANGWAN *et al.*, 2001; SIMÕES; SPITZER, 2007).

De acordo com a família da planta, os óleos essenciais podem ocorrer em estruturas secretoras especializadas, tais como pêlos glandulares (Lamiaceae), células parenquimáticas diferenciadas (Lauraceae, Piperaceae, Poaceae), canais oleíferos (Apiaceae), bolsas lisígenas ou esquizógenas (Rutaceae), entre outras. Em Anacardiaceae, os óleos essenciais são encontrados em canais secretores nos órgãos vegetativos, no floema primário, secundário e medula, (LACCHIA; CARMELLO-GUERREIRO, 2009) e em cavidades secretoras nos órgãos reprodutivos, associadas ao floema (MACHADO; CARMELLO-GUERREIRO, 2001).

Diferentemente dos óleos fixos, que são substâncias lipídicas, os óleos essenciais são misturas complexas constituídas principalmente por mono- e sesquiternoides, além de fenilpropanoides (ADORJAN; BUCHBAUER, 2010; SIMÕES; SPITZER, 2007). Tais substâncias podem existir na forma de álcoois, ácidos, ésteres, epóxidos, aldeídos, cetonas, aminas, entre outros (BAŞER; DEMIRCI, 2007). Os terpenoides são derivados de duas rotas metabólicas secundárias: a rota do mevalonato, que é ativada no citosol, e a rota do 2-C-metil-D-eritritol-4-fosfato (MEP, do inglês "*2-C-methyl-D-erythritol-4-phosphate*"), ativada nos plastídeos. Qualquer uma das rotas conduz à formação de estruturas com cinco unidades de carbono, que podem ser o difosfato de isopentenila (IDP, do inglês "*isopentenyl diphosphate*") ou seu isômero alílico difosfato de dimetilalila (DMADP, do inglês "*dimethylallyl diphosphate*"). Nas duas rotas, o IDP e o DMADP são usados pela prenil transferase em reações de condensação para a formação de difosfato de geranila (GDP, do inglês "*geranyl diphosphate*"), precursor de monoterpenos, e difosfato de farnesila (FDP, do inglês "*farnesyl diphosphate*"), precursor de sesquiterpenos (AHARONI *et al.*, 2005).

1.2.1 Obtenção e caracterização química dos óleos essenciais

Sob o ponto de vista da obtenção, um óleo essencial é definido como sendo o produto obtido dos processos de hidrodestilação, destilação a vapor (arraste a vapor), destilação seca ou ainda por um processo mecânico sem aquecimento (para frutos de *Citrus*) de uma planta ou de parte dela (RUBIOLO *et al*., 2010).

A análise de um óleo essencial geralmente envolve a separação, a identificação e a quantificação dos seus componentes. A cromatografia gasosa monodimensional (1D-GC, do inglês "*one-dimensional gas chromatography*") é uma técnica indicada para a separação dos óleos essenciais devido a volatilidade e a polaridade dos seus constituintes (RUBIOLO *et al*., 2010). De acordo com Stashenko e Martínez (2010), existem duas estratégias envolvendo 1D-GC para a identificação de substâncias. A primeira diz respeito ao uso de substâncias padrão. Entretanto, sendo os óleos essenciais misturas de muitos compostos, essa abordagem se torna inviável. A segunda refere-se à identificação tentativa das substâncias. Nessa abordagem, utilizam-se os índices de retenção, geralmente obtidos por cromatografia gasosa acoplada a um detector de ionização em chama (GC-FID, do inglês "*gas chromatography - flame ionization detector*") em conjunto com os espectros de massas experimentais obtidos por cromatografia gasosa acoplada a espectrometria de massas (GC-MS, do inglês "*gas chromatography – mass spectrometry*"). Esses dados são comparados com os presentes em bases de dados, os quais foram obtidos em colunas de polaridade ortogonal (polar e apolar) e de espectros de massas de substâncias padrão.

Para aumentar o grau de confiabilidade na comparação dos dados de retenção cromatográfica e permitir a identificação tentativa dos analitos, Kovats introduziu um sistema de índices de retenção, denominados índices de Kovats (KI), que se baseiam na medição dos tempos de retenção relativos com respeito aos de uma série homóloga de alcanos lineares corrida sob as mesmas condições experimentais que as da amostra. Entretanto, a análise cromatográfica de misturas requer uma programação de temperatura para a separação efetiva dos componentes dessa mistura. Assim, a equação para o cálculo do índice de Kovats foi modificada a fim de se obter o cálculo dos índices lineares de retenção ou os índices de retenção com programação linear de temperatura (LTPRI, do inglês "*linear temperature programmed retention index*"), que são calculados de acordo com a equação:

$$LTPRI = 100n + 100(t_{RX}-t_{Rn}/t_{RN}-t_{Rn}), \text{ onde:}$$

n= número de átomos de carbono do alcano linear que elui antes do analito; t_{RX}= tempo de retenção do analito; t_{RN}= tempo de retenção do alcano que elui

imediatamente após o analito; t_{Rn}= tempo de retenção do alcano que elui imediatamente antes do analito (STASHENKO; MARTÍNEZ, 2010).

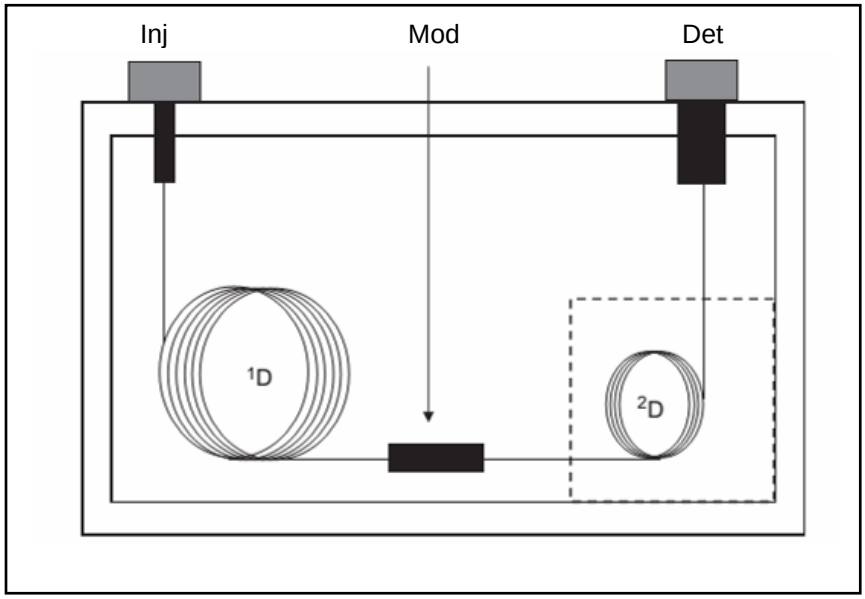
Conforme citado anteriormente, os óleos essenciais são constituídos principalmente por terpenoides, muitos dos quais apresentam estruturas isoméricas cíclicas ou lineares, vários graus de insaturações, substituições e oxigenações, que produzem espectros de massa semelhantes ou idênticos, normalmente cromatograficamente associados dentro de grupos complexos em uma janela estreita de tempo de retenção. Assim, semelhanças nos tempos de retenção de muitos compostos causam a aglomeração destes dentro de uma pequena região cromatográfica (SILVA *et al.*, 2008). Além disso, outra dificuldade encontrada na separação se traduz nas conhecidas e muito frequentes coeluições. As coeluições dos componentes da mistura são impossíveis de detectar e identificar através de 1D-GC, onde apesar do uso de complexos processos de deconvolução, muitas das co-eluições permanecerão invisíveis para o operador. Outro problema relacionado resulta da vastíssima gama de concentrações em que os compostos voláteis se encontram presentes nas variadas matrizes. Consequentemente, componentes traço podem não ser detectados se coeluirem com compostos de concentração mais elevada, não sendo raro que compostos presentes numa concentração reduzida constituam os compostos ativos do ponto de vista biológico para uma dada matriz (MATEUS *et al.*, 2004).

A 1D-GC tem contribuído para o desenvolvimento da ciência dos óleos essenciais nas áreas da fitoquímica, quimiotaxonomia, pesquisa olfatória, bioquímica, pesquisa de interações planta-inseto, na busca por novas fontes de compostos odoríferos para a indústria, e no controle de qualidade dos óleos essenciais. Milhares de aromas e fragrâncias derivadas de materiais vegetais foram identificados, mas existe a necessidade de realizar uma melhor caracterização da composição química dos óleos essenciais (SHELLIE, 2009). Nesse sentido, a cromatografia gasosa bidimensional (2D-GC, do inglês *"two-dimensional gas chromatography"*) vem crescendo rapidamente no mundo todo devido a riqueza de informação analítica obtida. A 2D-GC é caracterizada pelo uso de duas colunas, cujos mecanismos são independentes ou quase independentes um do outro (ortogonais), sendo preservada a separação de cada coluna individual até o fim do processo. Na 2D-GC abrangente (GC×GC, do inglês "*comprehensive two-dimensional gas chromatography*"), todo o efluente da primeira dimensão ou uma

parte suficientemente representativa deste é introduzido na segunda dimensão, sendo que as análises da primeira e segunda dimensão se processam simultaneamente e o tempo total de análise equivale ao tempo empregado para uma análise monodimensional (ZINI, 2009).

Na GC×GC, a primeira coluna apresenta dimensões convencionais e a outra é mais curta, o que proporciona rápida eluição, havendo um modulador entre elas (Figura 4). O sistema de modulação entre as duas colunas promove a amostragem da banda cromatográfica que elui da primeira dimensão, sendo esta banda direcionada para uma rápida separação na segunda dimensão.

Figura 4 – Esquema básico para cromatografia gasosa bidimensional abrangente (GC×GC). Toda a amostra elui através de ambas as colunas e apenas um detector é utilizado. Linhas pontilhadas: forno secundário opcional, colocado dentro do forno principal, para controle independente da temperatura da segunda coluna. Inj = injetor; Det = detector; Mod = modulador

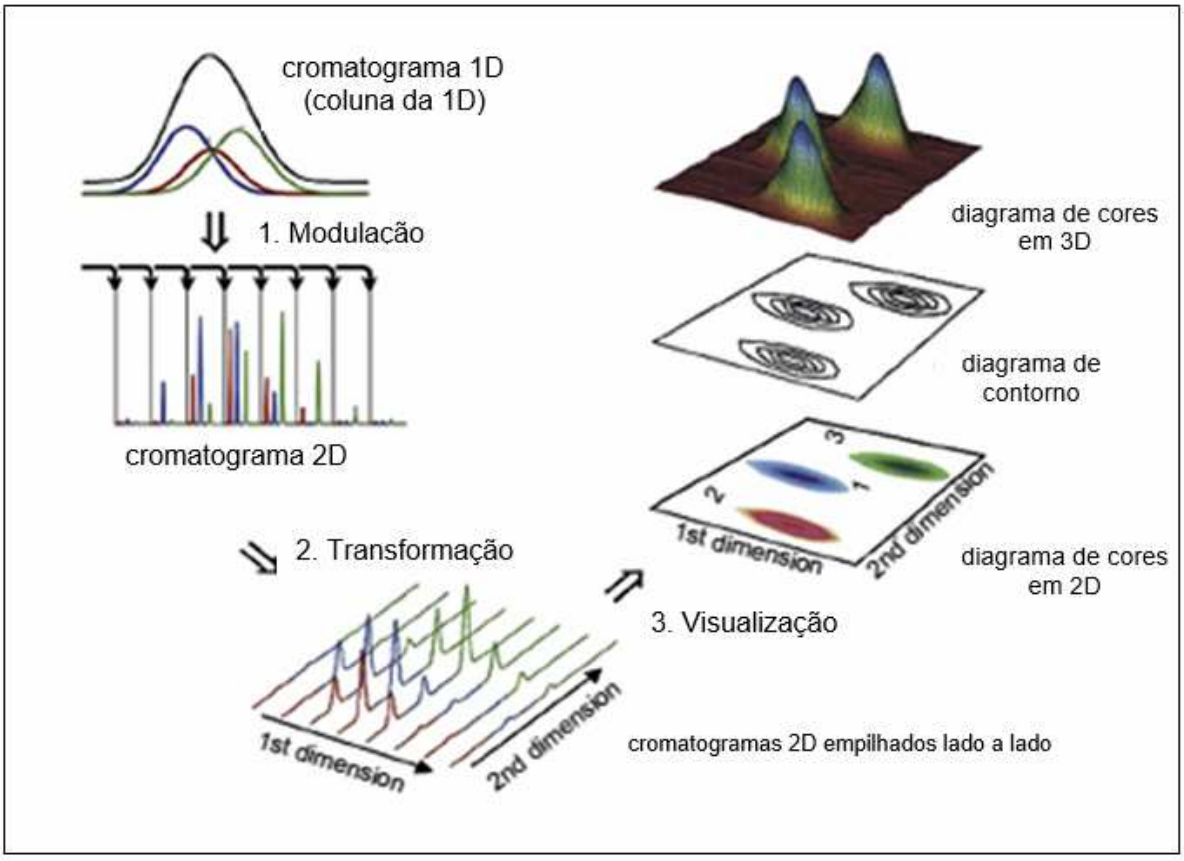


Fonte: Adaptado de Ramos e Brinkman (2000).

Em uma análise GC×GC, a separação na primeira dimensão, que é realizada em uma coluna capilar de baixa polaridade ou apolar, será predominantemente baseada em forças dispersivas, proporcionando essencialmente uma separação pela volatilidade/ponto de ebulição dos constituintes da amostra. As separações na segunda dimensão ocorrerão principalmente devido a interação específica com a fase estacionária, por exemplo, ligação de hidrogênio, interações dipolo-dipolo, etc.

(SHELLIE; MARRIOTT, 2003). Entretanto, mesmo que o mecanismo predominante para todas essas fases estacionárias da segunda dimensão não seja a volatilidade, este parâmetro também contribui para a separação dos compostos. As análises de cada fração individual proveniente da coluna da primeira dimensão são rápidas na coluna da segunda dimensão, sendo consideradas isotérmicas, onde não há a contribuição da volatilidade dos compostos no processo de separação. Isso significa que apenas as interações específicas características da coluna polar predominarão como mecanismo de separação na segunda dimensão (ZINI, 2009). A apropriada programação de temperatura da primeira e segunda colunas produz mecanismos de separação ortogonais, e os componentes se distribuem no plano 2D de acordo com suas propriedades químicas e físicas (VENKATRAMANI *et al.*, 1996). A figura 5 apresenta o processo de geração e a visualização dos dados em GCxGC.

Figura 5 – Geração e visualização dos dados em GCxGC



Fonte: Adaptado de Adahchour *et al.* (2006).

O primeiro trabalho publicado aplicando a técnica da GC×GC na caracterização química de óleos essenciais data de 2000 no qual Dimandja e

colaboradores, analisando os óleos essenciais de hortelã e menta, verificaram um aumento de 2 a 3 vezes no poder de separação ao utilizar a GC×GC, comparada com a 1D-GC. Desde então, a GC×GC tem se revelado útil para a alta resolução de análises de óleos essenciais.

O aumento da capacidade de pico, a velocidade da análise e a sensibilidade da GC×GC mostraram-se vantajosos na identificação de similaridades e diferenças nos óleos essenciais (SHELLIE; MARRIOTT, 2003). A capacidade de pico pode ser definida como o número máximo de compostos que podem ser colocados lado a lado em um espaço de separação (cromatograma) com uma dada resolução em um determinado intervalo de tempo (ZINI, 2009). Estudos demonstram o aumento da capacidade de pico através da técnica da GC×GC (BAHARUM *et al.*, 2010; PRIPDEEVECH *et al.*, 2010).

Além disso, conforme citado anteriormente, os componentes se distribuem no plano 2D de acordo com suas propriedades químicas e físicas. Dessa forma, obtêm-se um cromatograma no qual os analitos se agrupam em bandas ou *clusters* no espaço de separação, de acordo com suas propriedades moleculares, efeito este chamado de estrutura cromatográfica. Este fenômeno de estruturação cromatográfica é uma ferramenta valiosa para a identificação de compostos desconhecidos ou para sua classificação dentro de um grupamento químico (ZINI, 2009).

1.3 Alelopatia, potencial alelopático e fitotoxidez

O termo alelopatia foi definido por Molisch em 1937 como sendo uma ciência nova relacionada às interações bioquímicas inibitórias e estimulatórias entre duas espécies vegetais (ALLELOPATHY JOURNAL, s/d). Posteriormente, outras definições surgiram como a de Rice (1984), que considera alelopatia como sendo qualquer efeito direto ou indireto, danoso ou benéfico, que uma planta exerce sobre outra pela produção de metabólitos secundários. Em 1996, a Sociedade Internacional de Alelopatia ampliou esse conceito e definiu alelopatia como qualquer processo envolvendo metabólitos secundários produzidos por plantas, microorganismos, vírus e fungos que influenciam o crescimento e o desenvolvimento de sistemas biológicos e agrícolas (ALLELOPATHY JOURNAL, s/d).

Segundo Bourgaud e colaboradores (2001), o conceito de metabólito secundário foi criado por Kossel em 1891, diferenciando-os dos metabólitos primários. Os metabólitos secundários vegetais são comumente classificados de acordo com sua rota biossintética. Três grandes famílias de moléculas são geralmente consideradas: compostos fenólicos, terpenoides e alcaloides. Os produtos do metabolismo secundário, embora não sejam necessariamente essenciais para o organismo produtor, garantem vantagens para sua sobrevivência e para a perpetuação de sua espécie em seu ecossistema (SANTOS, 2007). Tais substâncias podem ser liberadas no ambiente na fase aquosa no solo ou substrato pela lixiviação das partes aéreas das plantas, pela decomposição da serrapilheira acumulada no solo, ou pela exsudação de raízes, e também na fase gasosa, como voláteis que se dispersam na atmosfera que cerca as plantas terrestres (WEIR *et al.*, 2004).

Reese (1979 *apud* LOVETT *et al.*, 1989) utilizou o termo aleloquímico para descrever as substâncias químicas não nutricionais produzidas por um organismo que afetam o crescimento, o comportamento ou a biologia populacional de outras espécies. Estudos envolvendo os efeitos inibitórios dos aleloquímicos sobre espécies vegetais geralmente avaliam o efeito de extratos vegetais no desenvolvimento inicial de plantas em bioensaios de laboratório. Nesses trabalhos, diversas vezes o efeito observado é denominado como sendo alelopático. Entretanto, experimentos em laboratório demonstram o efeito fitotóxico das substâncias testadas, e não o efeito alelopático. De acordo com a Scognamiglio e colaboradores (2013), a alelopatia “é um fenômeno complexo, com várias nuances e uma teia de interligações com um número de processos ecológicos e fisiológicos”. A determinação da fitotoxidez pode ser considerada o primeiro passo na identificação de aleloquímicos, mas não é suficiente para indicar que uma interação alelopática foi determinada. Desse modo, no presente estudo dá-se preferência à utilização dos termos ‘efeito fitotóxico’ para caracterizar a atividade dos extratos vegetais testados nos bioensaios e ‘potencial alelopático’ para referir-se ao possível efeito que essas substâncias produzidas por uma planta podem apresentar sobre outras plantas, quando no ambiente.

Nos estudos de fitotoxidez, geralmente testam-se os compostos de uma espécie vegetal sobre o desenvolvimento de outra (exceto nos estudos de autotoxidez), sendo essa segunda denominada espécie alvo. De acordo com

Ferreira (2004), a alface (*Lactuca sativa* L.) é a planta comumente utilizada como espécie alvo em estudos de potencial alelopático devido ao fato dela requerer um pequeno período tanto para a sua germinação (24 a 48 horas) quanto para o seu crescimento.

Arabidopsis thaliana (L.) Heynh. (Brassicaceae) é uma espécie modelo para estudos bioquímicos, fisiológicos e moleculares devido ao seu ciclo de vida curto, fácil transformação genética, genoma completamente sequenciado, produção de grande número de sementes e disponibilidade de diversos mutantes pelo menos parcialmente caracterizados (CORREA *et al*., 2012). Além disso, a espécie também é utilizada em estudos relacionados à avaliação fitotóxica uma vez que ela é sensível a uma variedade de aleloquímicos e proporciona resultados reproduzíveis e confiáveis (PENNACCHIO *et al.*, 2005).

1.4 Abordagens utilizadas no estudo do potencial alelopático de espécies vegetais

1.4.1 Abordagem convencional: germinação e crescimento

O potencial alelopático das substâncias é convencionalmente verificado testando a sua influência sobre os processos de germinação de sementes e crescimento de plântulas. Os efeitos são geralmente avaliados sobre a germinabilidade e a viabilidade das sementes, a morfologia e o comprimento da raiz e da parte aérea e também através da medida do peso de certas partes da planta (GNIAZDOWSKA; BOGATEK, 2005).

Outro foco interessante, ainda considerando a análise de parâmetros morfométricos, é avaliar o efeito dos metabólitos secundários sobre o processo de enraizamento adventício. Os mesmos parâmetros utilizados nas análises de crescimento inicial de plântulas podem ser utilizados também na análise de enraizamento adventício, somando-se ainda parâmetros que avaliem densidade de raízes, tempo de enraizamento, entre outros. São poucos os estudos que abordam a atividade de aleloquímicos na formação de raízes adventícias. Nesse sentido, Batish *et al.* (2008) observaram que o ácido cafeico reduziu o crescimento da raiz e prejudicou a formação de raízes adventícias em *Phaseolus aureus* Roxb.. Kibbler *et al.* (2002) correlacionaram a inibição do enraizamento adventício em *Backhousia*

citriodora F. Muell com a concentração de óleos essenciais da espécie e verificaram que este efeito devia-se aos níveis endógenos de um monoterpeno, o citral.

Entretanto, alterações morfológicas nas espécies expostas aos aleloquímicos são manifestações secundárias de efeitos ocorridos em nível celular. Nesse sentido, a abordagem convencional é um importante ponto de partida para o direcionamento do estudo fitotóxico de aleloquímicos, mas não explica o modo de ação das substâncias.

1.4.2 Abordagem fisiológica, bioquímica e citogenética

De acordo com Rizvi e Rizvi (1992 *apud* FERREIRA; ÁQUILA, 2000), os aleloquímicos podem afetar estruturas citológicas e ultra-estruturais; concentração e balanço de hormônios; constituição e permeabilidade de membranas; absorção de minerais; movimento dos estômatos; síntese de pigmentos e processo de fotossíntese; respiração; síntese de proteínas; atividade enzimática; relações hídricas e condução; e material genético.

A interferência dos óleos essenciais e de seus constituintes sobre diferentes processos nas plantas alvo foram verificados. Dentre eles, pode-se citar o efeito sobre a abertura estomática (SCHULZ *et al*., 2007; RAI *et al*., 2003), a divisão celular (PAWLOWSKI *et al*., 2012; PAWLOWSKI *et al*., 2013; SILVA *et al*., 2011), o conteúdo de auxina (GRAÑA *et al*., 2013) e o conteúdo de fosfoliídeos e esterois (ZUNINO; ZYGADLO, 2005). Na última década, tem-se intensificado o número de publicações apontando o efeito de voláteis sobre enzimas antioxidantes (CHOWHAN *et al*., 2011; MUTLU *et al*., 2011; SINGH *et al*., 2006). Os autores propõem que os óleos essenciais e seus constituintes induzem estresse oxidativo nas plantas alvo, interferindo no desenvolvimento vegetal, resultando na redução do tamanho das plantas. Entretanto, o modo de ação dessas substâncias permanece desconhecido.

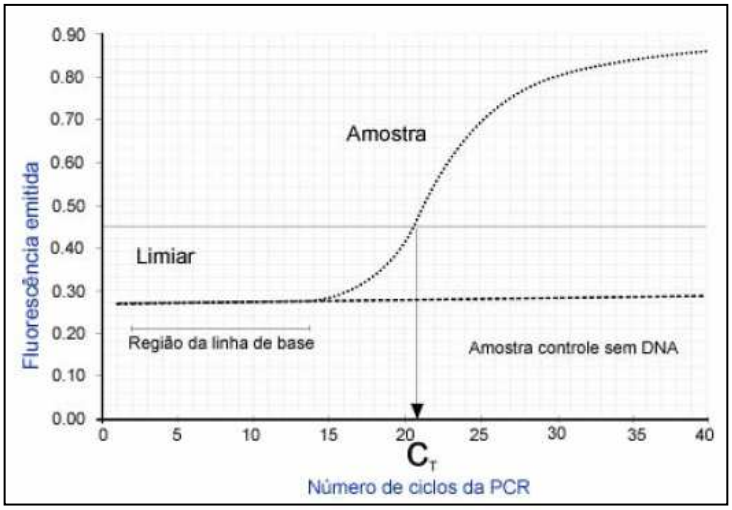
1.4.3 Abordagem molecular

Os avanços na biologia molecular proporcionaram novas ferramentas que podem ser utilizadas para compreender os processos complexos em alelopatia (DUKE *et al.*, 2008). Entretanto, é incipiente a utilização de técnicas moleculares no estudo do efeito fitotóxico de espécies vegetais. Dentre essas técnicas, pode-se citar o uso de microarranjos (GOLISZ *et al.*, 2008) e a reação em cadeia da polimerase

(PCR, do inglês “*polymerase chain reaction”*) (ZHANG *et al.*, 2009; ZHANG *et al.*, 2010).

A PCR serve para amplificar de modo exponencial uma sequência particular de ácido desoxirribonucleico (em inglês *deoxyribonucleic acid*, DNA), sendo uma análise qualitativa. O método baseia-se em ciclos de temperatura, consistindo em repetidos aquecimentos e resfriamentos da reação para desnaturação e replicação enzimática do DNA. A técnica RT-PCR (do inglês *reverse transcription*-PCR) baseia-se na síntese de DNA complementar (em inglês *complementary* DNA, cDNA) a partir de uma amostra de ácido ribonucleico (em inglês *ribonucleic acid*, RNA) em uma reação catalisada pela enzima transcriptase reversa e amplificação por PCR do transcrito de interesse com iniciadores (*primers*) específicos. Por sua vez, a técnica da PCR em tempo real, devido à adição de um fluoróforo, permite detectar o número de cópias, proporcionando a quantificação dos ácidos nucleicos, possibilitando o acompanhamento da reação da PCR (Figura 6). O ponto que detecta o ciclo na qual a reação atinge o limiar da fase exponencial é denominado de *Cycle Threshold* (C_T). A quantificação ocorre de maneira precisa e com reprodutibilidade e se dá baseada no sinal gerado por compostos fluorescentes. A fluorescência aumenta na proporção direta da quantidade de produto da PCR (NOVAIS *et al.,* 2004).

Figura 6 – Curva de amplificação do PCR em Tempo Real. C_T – *Cycle Threshold*. A amplificação mostra 3 fases distintas (1) linha basal: não houve produtos da PCR suficiente para detectar a fluorescência; (2) fase *log*: a quantidade de produtos da PCR dobra a cada ciclo e (3) fase platô: não há mais aumento no número de produtos.



Fonte: Novais *et al.* (2004)

A quantificação de RNA através da PCR em tempo real pode ser feita por dois métodos: a quantificação absoluta e a quantificação relativa. Na quantificação absoluta, uma curva-padrão é construída pela leitura de diversas soluções com concentrações de DNA conhecidas. Os dados obtidos são analisados junto a essa curva, permitindo a quantificação absoluta. Já na quantificação relativa, a amplificação dos genes de interesse é comparada com a de genes denominados genes de referência. Na quantificação relativa, a comparação das amplificações mostra quantas vezes o gene de interesse é mais ou menos expresso que o gene de referência.

Desse modo, a técnica pode ser bastante útil na avaliação da atividade fitotóxica de produtos naturais permitindo comparar a expressão gênica de plantas expostas aos aleloquímicos com a de plantas controle.

2 OBJETIVOS

2.1 Objetivo geral

O objetivo geral do presente estudo foi avaliar o efeito fitotóxico dos voláteis extraídos das folhas de três espécies de *Schinus* sobre o processo de formação de raízes da espécie alvo *A. thaliana*.

Em contraposição à abordagem convencional dos estudos envolvendo o potencial alelopático de metabólitos vegetais, que se baseia na avaliação do efeito fitotóxico dessas substâncias a partir da análise dos resultados de ensaios de germinação e crescimento, o presente estudo apresenta como proposta a utilização de outras metodologias que venham a contribuir com essa área de estudo, a saber, o estudo do enraizamento adventício e a técnica de PCR em tempo real.

2.2 Objetivos específicos

Os objetivos específicos foram:

- realizar a caracterização química dos óleos essenciais de *S. lentiscifolius*, *S. molle* e *S. terebinthifolius* através de cromatografia gasosa bidimensional abrangente;
- avaliar o efeito do óleo essencial de *S. lentiscifolius*, *S. molle* e *S. terebinthifolius* sobre a germinação e o crescimento inicial de *A. thaliana;*
- investigar a expressão relativa de genes através da técnica de PCR em tempo real;
- avaliar o efeito do óleo essencial de *S. lentiscifolius*, *S. molle* e *S. terebinthifolius* sobre o enraizamento adventício de *A. thaliana*;
- verificar se os efeitos induzidos pelos óleos essenciais de *S.lentiscifolius*, *S. molle* e *S. terebinthifolius* no enraizamento adventício de *A. thaliana* poderiam ser revertidos com a adição de fitormônios e de uma substância antioxidante.

3 MATERIAL E MÉTODOS

3.1 Material vegetal

Folhas de *S. lentiscifolius* foram coletadas no município de Encruzilhada do Sul (30° 31' 36.67" S 52° 31' 6.17" W), RS, Brasil, ao longo da BR 471 (próximo ao km 264) enquanto que folhas de *S. molle* e *S. terebinthifolius* foram coletadas nas imediações do Campus do Vale da Universidade Federal do Rio do Sul (UFRGS), no município de Porto Alegre (30° 1' 39.73" S 51° 13' 43.45" W), RS, Brasil. Pelo menos seis indivíduos de cada espécie foram amostrados. As amostras foram identificadas e um voucher de *S. lentiscifolius* (164708), *S. molle* (164709) e *S. terebinthifolius* (164707) foram depositados no Herbário ICN da UFRGS.

Amostras de sementes de *A. thaliana* Columbia (Col-0) tipo selvagem e mutante *sur 1* (do inglês "*superroot 1*") foram gentilmente cedidas pelo professor Dr. Arthur Germano Fett-Netto do Laboratório de Fisiologia Vegetal da UFRGS. Para a obtenção de sementes suficientes para a realização dos experimentos, plantas foram cultivadas em potes plásticos contendo terra de turfa e vermiculita 1:1 (v/v) autoclavada. Tanto na propagação das plantas (Figura 7) quanto na realização dos experimentos, as sementes de *A. thaliana* foram desinfestadas em álcool 70% (v/v) durante um minuto. Após, as sementes foram imersas em solução aquosa de hipoclorito de sódio (NaClO) 2,5% (v/v) com algumas gotas de detergente neutro comercial e agitadas constantemente durante 10 minutos. Depois desse procedimento, as sementes foram lavadas quatro vezes com água destilada deionizada autoclavada. As sementes desinfestadas foram distribuídas no solo úmido e os potes foram armazenados por 48 horas em geladeira (4°C), no escuro, para a quebra de dormência das sementes. Durante o período de crescimento das plantas, foram realizadas regas semanais com solução de 10x de sais MS (MURASHIGE; SKOOG, 1962).

3.2 Obtenção dos óleos essenciais

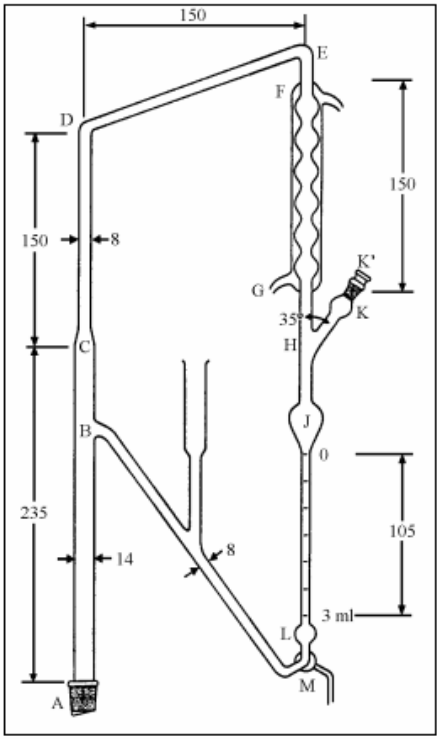
Após a coleta, as folhas das três espécies de *Schinus* estudadas foram dispostas sobre papel pardo e assim mantidas à temperatura ambiente durante 5 dias, para secagem. Os óleos essenciais foram obtidos através da hidrodestilação

do material vegetal em aparelho do tipo Clevenger (Figura 8) (FARMACOPEIA BRASILEIRA, 2010). A extração ocorreu durante 4 horas a partir do momento da ebulição da água destilada, na qual se encontravam imersas as folhas. Os óleos essenciais obtidos foram secos em sulfato de sódio anidro (Na_2SO_4) e armazenados em tubos herméticos sob uma atmosfera inerte, envolvidos em folha de alumínio e armazenado em ultrafreezer (-80°C) até a execução dos experimentos.

Figura 7 – Propagação de *Arabidopsis thaliana*. Plantas em florescimento (A) e com sementes (B).

Fonte: Autoria própria.

Figura 8 – Clevenger utilizado na extração dos óleos essenciais.



Fonte: Farmacopeia Brasileira (2010).

3.3 Caracterização química dos óleos essenciais

A caracterização química dos óleos essenciais obtidos foi realizada na Central Analítica do Departamento de Química, IQ/UFRGS. As amostras foram analisadas por GCxGC-TOF/MS (do inglês "*comprehensive two-dimensional gas chromatography with time of flight mass spectrometer*") utilizando-se as seguintes colunas cromatográficas: DB5 (5% difenil, 95% dimetilpolisiloxano) de 60 m x 0,25 mm x 0,25 µm na primeira dimensão (1D) e DB-17 (50% fenil, 50% metilpolisiloxano) de 2,15 m x 0,18 mm x 0,18 µm na segunda dimensão (2D). A temperatura do forno foi mantida a 50°C durante 5 min e submetida a uma taxa de aquecimento de 4°C/min até 280°C (10 min). A temperatura da segunda coluna foi mantida a 10°C acima da temperatura da primeira. O período de modulação foi de 7s. O injetor foi utilizado no modo split (1:20) e 1 µL de amostra foi injetado na concentração de 2000 mg L^{-1}. As temperaturas de porta de injeção e linha de transferência foram de 280°C e a temperatura da fonte de íons de 250°C. A taxa de aquisição foi de 100 Hz e a tensão do detector de -1.580 V.

Para um composto ser considerado como detectado, foi considerado um valor mínimo de três para a relação sinal/ruído. Um mínimo de 70% de similaridade entre o espectro de massa de uma substância desconhecida e um espectro de massa da biblioteca NIST MS (do inglês "*National Institute of Standards and Technology Mass spectrometry Data Center*") foi considerado como critério para a identificação tentativa da substância. Adicionalmente, foi injetada uma mistura de alcanos lineares (C_6-C_{30}) a 0,1% para a comparação dos LTPRI obtidos experimentalmente com os fornecidos pela literatura (ADAMS, 2001). Os percentuais de cada composto na mistura foram calculados considerando-se o somatório das áreas dos picos cromatográficos equivalente a 100% dos compostos no óleo volátil (método de normalização) (LANÇAS, 1993).

3.4 Ensaios de germinação e crescimento

Todas as etapas de montagem dos experimentos foram realizadas em câmara de fluxo laminar. Nos ensaios de germinação, 30 sementes de *A. thaliana* tipo selvagem desinfestadas foram distribuídas em placas de Petri contendo meio de cultura com 0,05x de sais de MS (MURASHIGE; SKOOG, 1962), 3% (w/v) de

sacarose e 0,8% (w/v) de ágar. O meio foi preparado com água destilada e deionizada e o pH foi ajustado a 5,8 com NaOH e HCl antes de ser autoclavado a 120 °C e 1kgf.cm^{-2} durante 20 minutos. Esse meio de cultivo, deste ponto em diante, será referido como meio padrão. As placas foram seladas com filme de policloreto de vinila (PVC) e armazenadas por 48 horas em geladeira (4°C), no escuro, para a quedra de dormência das sementes. Após, os óleos essenciais nas quantidades de 5 µL, 10 µL, 15 µL, 20 µL e 25 µL foram aplicados em papel filtro (Ø 1,6 cm) fixado na tampa da placa de Petri de modo a evitar o contato direto do óleo com as sementes. As placas foram vedadas com filme PVC a fim de criar uma atmosfera saturada pelos voláteis. Cada tratamento foi realizado em 4 repetições. A ausência de aplicação de óleo caracterizou o grupo controle. Os testes de germinação foram realizados em sala de cultivo sob temperatura média de 20°C e fotoperíodo de 16 horas de luz. O suprimento de luz foi proporcionado por lâmpadas fluorescentes (20 W).

A avaliação do efeito dos tratamentos sobre a velocidade de germinação foi realizada através da contagem periódica do número de sementes germinadas a cada 24 horas. O índice de velocidade de germinação (IVG) foi calculado a partir da seguinte fórmula: IVG = G1/N1+G2/N2+...+Gn/Nn, onde G = número de diásporos germinados; N = número de dias ou horas após a semeadura (ANJUM; BAJWA, 2005). A porcentagem final de germinação foi calculada ao final do experimento considerando o número de sementes germinadas em relação ao total de sementes.

Para avaliar o efeito dos voláteis sobre o crescimento inicial das plântulas, após a quebra de dormência, as sementes de *A. thaliana* foram pré-germinadas durante um dia em sala de cultivo. Assim, após a protusão da raiz, os óleos essenciais foram aplicados conforme explicado anteriormente. As placas de Petri foram orientadas verticalmente em grades para facilitar a análise das plântulas (Figura 9). Após um período de exposição de sete dias, fotografias foram tiradas e a medida do comprimento da parte aérea e da raiz foram feitas com o *software* Image J 1.45s.

3.5 PCR em tempo real

Para a análise da PCR em tempo real, os procedimentos foram similares aos apresentados para a avaliação do efeito dos voláteis sobre o crescimento inicial. A menor quantidade de óleo utilizada nos experimentos de crescimento (5 µL) foi

escolhida para este experimento. As plântulas permaneceram expostas aos voláteis durante 24 horas. As raízes foram coletadas (3 repetições de 25 mg) e imediatamente congeladas em nitrogênio líquido, sendo posteriormente armazenadas em ultrafreezer (-80°C). O RNA foi extraído utilizando-se um reagente de purificação de RNA vegetal (Invitrogen), seguido por tratamento com DNAase I (Invitrogen). Após a extração, o RNA foi quantificado no equipamento NanoDrop. Um µg foi utilizado para a síntese de cDNA catalisada pela enzima transcriptase reversa (M-MLV, Invitrogen). Os genes de interesse e os de referência, bem como a sequência dos *primers* utilizados na amplificação por PCR em tempo real são apresentadas na Tabela 1. As sequências utilizadas podem ser encontradas na base de dados *The Arabidopsis Information Resource* (TAIR; www.arabidopsis.org) sob os seguintes números de acesso: ANP1 (AT1G09000), CDKB1;1 (AT3G54180), Act (AT3G18780) e eEF1α (AT5G60390).

Figura 9 - Ensaio de germinação e crescimento. Disposição das placas de Petri.

Fonte: Autoria própria.

Tabela 1 – Sequências de primers utilizados na amplificação por PCR em tempo real

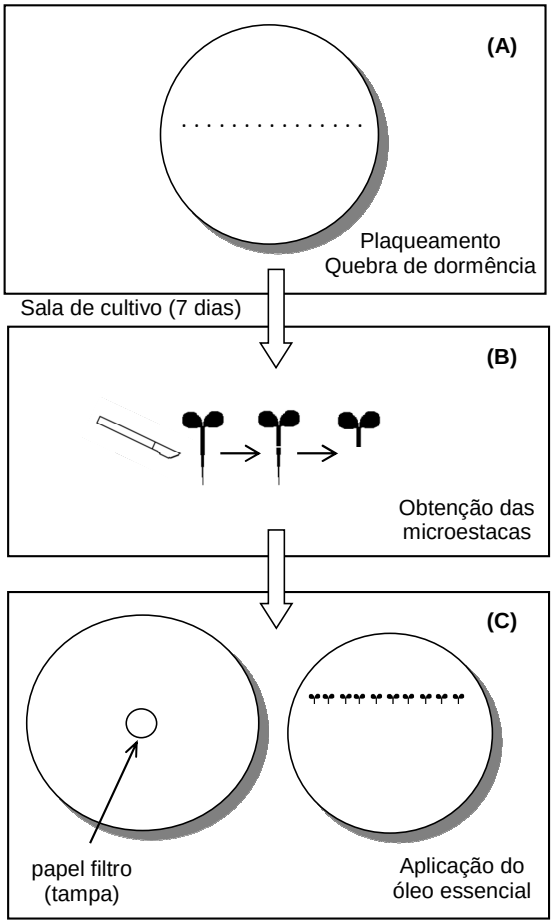
Símbolo	**Nome**		**Sequência dos *primers* (5'-3')**
Act	Actin	F	GCACCCTGTTCTTCTTACCG
		R	AACCCTCGTAGATTGGCACA
eEF1α	elongation factor 1-alpha	F	TGAGCACGCTCTTCTTGCTTTCA
		R	GGTGGTGGCATCCATCTTGTTACA
ANP1	*Arabidopsis* NPK1-like protein kinase 1	F	TTGCATCACCAGGAAAATGA
		R	TTCAGACCAAACCTCGTGTCT
CDK B1;1	cyclin dependent kinase B1;1	F	AGCCGAAAGAATTTCAGCAA
		R	TTCACCAAGACGATGACAACA

Fonte: Autoria própria.

3.6 Ensaios de enraizamento adventício

No ensaio de enraizamento adventício, foram utilizadas sementes de *A. thaliana* tipo selvagem e mutante *sur1*. Quinze sementes foram distribuídas em placas de Petri contendo meio padrão e, após a quebra da dormência, as placas permaneceram na sala de cultivo durante sete dias. As raízes foram cortadas para a obtenção das microestacas e os óleos essenciais foram aplicados no papel filtro nas quantidades de 1 µL, 2 µL, 3 µL, 4 µL e 5 µL. Cada tratamento foi realizado em quatro repetições, cada repetição com dez microestacas. Imediatamente após a aplicação do óleo, as placas retornaram à sala de cultivo, onde permaneceram por mais sete dias (Figura 10).

Figura 10 – Experimento de enraizamento. Sementes de *Arabidopsis thaliana* foram distribuidas sobre meio de cultivo em placas de Petri e estratificadas (A). Após sete dias, as microestacas foram obtidas (B) e os óleos essenciais de *Schinus* foram aplicados em papel filtro fixado na tampa da placa de Petri (C).



Fonte: Autoria própria.

Os parâmetros avaliados foram: tempo médio de enraizamento (TME), porcentagem de microestacas enraizadas, número de raízes (raízes por microestaca enraizada) e comprimento médio da maior raiz (CR). Para calcular o TME, as microestacas foram avaliadas a cada 24 horas em uma forma binária (enraizadas ou não enraizadas). Para serem consideradas enraizadas, o critério adotado foi a presença de pelo menos uma estrutura cilíndrica esbranquiçada visível com comprimento a partir de 1 mm. O TME foi calculado baseado no conceito de tempo médio de germinação (LABOURIAU; OSBORN, 1984). Ao final do tempo de exposição aos voláteis, foi contado o número de raízes por microestaca enraizada. Além disso, fotografias foram retiradas para a realização da medida do CR utilizando o *software* ImageJ 1.45s.

Adicionalmente, para avaliar se uma auxina, uma citocinina ou um antioxidante contribuíam para as microestacas recuperarem-se dos efeitos causados pelos óleos essenciais, 1 µM de ácido indol-acético (AIA), 0,5 nM de cinetina ou 10 µM de Trolox® foram adicionados ao meio padrão. Nesses experimentos, apenas a linhagem selvagem de *A. thaliana* e a quantidade de 5 µL de óleo essencial foram utilizados.

3.7 Detecção histoquímica de peróxido de hidrogênio

O acúmulo de peróxido de hidrogênio (H_2O_2) após a exposição das micoestacas aos óleos essenciais foi detectado pelo método de coloração com 3,3'-diaminobenzidina (DAB) (THORDAL-CHRISTENSEN *et al.*, 1997). Após sete dias de exposição, as microestacas de *A. thaliana* tipo selvagem foram mergulhadas em uma solução de DAB 1 $mg.mL^{-1}$ DAB, pH 3.8, durante 2 h. A reação foi parada através do branqueamento das microestacas em etanol 95% em ebulição, durante 10 min. As microestacas foram fotografadas em microscópio estereoscópico, mergulhadas em etanol 95% à temperatura ambiente, e o acúmulo de H_2O_2 nos cotilédones de *A. thaliana* foram observados como manchas de cor marrom.

4 RESULTADOS E DISCUSSÃO

Os resultados obtidos no presente estudo e a discussão foram organizados na forma de três artigos, os quais estão formatados cada qual segundo as normas das revistas às quais serão submetidos.

O artigo 1 apresenta o efeito fitotóxico dos óleos essenciais das três espécies de *Schinus* através da abordagem convencional, avaliando efeitos sobre a germinação e o crescimento inicial de *A. thaliana*. Além disso, inclui os resultados obtidos com a técnica de PCR em tempo real e a caracterização química por cromatografia gasosa bidimensional. Esse trabalho foi redigido conforme as normas do periódico *Plant Growth Regulation*.

O artigo 2 trata do efeito fitotóxico do óleo essencial de *S. terebithifolius* sobre o enraizamento adventício de duas linhagens de *A. thaliana*: selvagem e mutante *sur1*. Nesse artigo, também são apresentados os resultados obtidos quando uma auxina e um potente antioxidante (Trolox®) foram adicionados ao meio de cultivo. Esse trabalho será submetido à revista *Acta Physiologiae Plantarum*.

Por fim, o artigo 3 também aborda o efeito fitotóxico sobre o processo de enraizamento adventício de *A. thaliana*, nesse caso, avaliando os óleos essenciais de *S. lentiscifolius* e *S. molle*. O efeito da adição de uma citocinina e de um antioxidante na recuperação dos efeitos fitotóxicos dos óleos essenciais foram estudados. Esse trabalho foi redigido nas normas do periódico *Biologia Plantarum*.

4.1 *Schinus* essential oils: phytotoxic effects on *Arabidopsis thaliana* and chemical composition by GC×GC-TOFMS

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Abstract A greater number of *Schinus* essential oils compounds was tentatively identified by GC×GC/TOFMS. Eighty-six, seventy-two and eighty-eight components were identified in the *S. lentiscifolius*, *S. molle* and *S. terebinthifolius* essential oil samples, respectively. Compounds separation due to selectivity of the ^{2}D may be observed. Considering phytotoxic effects, all essential oils in all quantities tested (5 µL, 10 µL, 15 µL, 20 µL and 25 µL) affected both germination and seedling growth of *A. thaliana*. Parameters evaluated included germination rate, speed of accumulated germination, shoot and root length. Considering RT-qPCR, no diferences were observed on ANP1 and CDK B1;1 expression after *Schinus* volatiles exposure. Results suggest that phytotoxic effects of *Schinus* essential oils, in quantities studied, seems to be explained in terms of cellular damage rather than by induction of stress-inducible genes.

Keywords *Schinus lentiscifolius*; *Schinus molle*; *Schinus terebinthfolius*; germination; initial growth; RT-qPCR

Introduction

Commonly known as pepper trees, *Schinus* L. spp. (Anacardiaceae) are characterized by the production of various secondary metabolites, including essential oils, also designated as volatile oils. *Schinus* essential oils show a wide spectrum of ecological interactions such as phytotoxicity (Zahed et al. 2010; Scrivanti et al. 2003). Initial evaluation of phytotoxic activity involves analysis of allelochemical interference on germination and initial growth of target species. Inhibitory effects on these processes reflect allelochemical action on different cellular levels. In this way, essential oils constituents can interfere on respiration (Abrahim et al. 2000),

phospholipid fatty acid content (Zunino and Zygadlo 2005), chlorophyll, protein and carbohydrates contents (Chowhan et al. 2011). Moreover, many authors focused on reactive oxygen species (ROS) metabolism and ROS-scavenging enzymes in target species, and results have demonstrated that essential oils act as oxidative stress inducers (Lara-Nuñez et al. 2006; Oracz et al. 2007; Singh et al. 2009; Mutlu et al. 2011; Kaur et al. 2012).

Plants have developed a complex signaling network that senses and protects them from different abiotic and biotic stresses. Induction and accumulation of ROS is a general plant response in this defense mechanism (Kovtun et al. 2000). The extent to which ROS accumulate is determined by the antioxidative system, which enables organisms to maintain proteins and other cellular components in active state for metabolism. ROS–antioxidant interaction acts as a metabolic interface for signals derived from metabolism and from environment that modulates the appropriate induction of acclimation processes or, alternatively, execution of cell death programs (Foyer and Noctor 2005). These acclimation processes involve expression of a number of genes and signaling transduction pathways. Cells have evolved strategies to utilize ROS as environmental indicators and biological signals that activate and control various genetic stress response programs (Apel and Hirt 2004).

In plants, mitogen-activated protein kinases (MAPKs) signaling appears to involve cross-talk with a variety of stress responses and developmental processes forming complex interconnected networks. MAPKs signaling controls diverse cellular functions that include cell division, hormone signaling, and response to abiotic stress and pathogens (Taj et al. 2010). In order to explain the mode of action of the allelochemical 2(3*H*)-benzoxazolinone, Sánchez-Moreiras and Reigosa (2005) proposed that this substance induced the accumulation of free radicals, such as H_2O_2, which could activate a MAPK pathway, resulting in the induction of stress-inducible genes and the inhibition of auxin-inducible genes. The consequences of this mechanism would result in decreased plant growth and development.

Arabidopsis thaliana (L.) Heynh. is a model plant for several studies including phytotoxicity as it is sensitive to a variety of allelochemicals, is readily available, affordable, and produces reproducible and reliable results (Pennacchio et al. 2005). Furthermore, this species allows molecular studies to be performed, contributing to a better understanding of natural products action mechanisms. Nishihama et al. (1997) demonstrated that the ANP (*Arabidopsis* NPK1-like protein kinase, in which NPK is a

Nicotiana protein kinase), a MAP kinase kinase kinase (MAPKKKs) from *Arabidopsis*, is induced by H_2O_2 and activates a specific class of stress-induced MAPKs. However, the effects of allelochemicals on this pathway is still not completely understood, and further studies are necessary.

Some studies attempted to relate observed effects on root length to cell division processes (Sánchez-Moreiras et al. 2006, Schmidt-Silva et al. 2011). Previous works demonstrated that *Schinus* essential oil causes cytotoxic and genotoxic effects on onion and lettuce root meristems, decreasing mitotic index (Pawlowski et al. 2012, 2013). Krysan et al. (2002) observed that ANP positively regulates cell division and growth and may negatively regulate stress responses. Furthermore, Sasabe et al. (2011) proposed that cyclin-dependent kinases (CDKs) might control the transition from early mitosis to cytokinesis repressing the activation of the MAPK cascade.

The present study was performed to investigate the phytotoxic effect of *Schinus lentiscifolius* March., *S. molle* L. and *S. terebinthifolius* Raddi essential oil on *A. thaliana* germination and initial growth. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was carried out to analyze the effect of *Schinus* essential oils on ANP1 and CDKB1;1 expression. Essential oils characterization was performed by comprehensive two-dimensional gas chromatography.

Materials and methods

Plant material

Schinus lentiscifolius leaves were sampled in natural vegetation in Encruzilhada do Sul City (30° 31' 36.67" S, 52° 31' 6.17" W) and *S. molle* and *S. terebinthifolius* leaves were sampled in natural vegetation in Porto Alegre (30° 1′89 39.73″ S 51° 13′ 43.45″ W), Rio Grande do Sul state, Brazil. At least six plants of each species were sampled. Samples were identified and a voucher of *S. lentiscifolius* (164708), *S. molle* (164709) and *S. terebinthifolius* (164707) was deposited in the herbarium ICN of the Universidade Federal do Rio Grande do Sul, Brazil. Seeds of *A. thaliana* Columbia (Col-0) wild type (WT) were kindly provided by Dr Arthur Germano Fett-Netto, Universidade Federal do Rio Grande do Sul, Brazil.

Essential oil extraction and analysis by comprehensive two-dimensional gas chromatography

Sampled leaves were dried at room temperature, fragmented and subjected to hydrodistillation for 4 h in a Clevenger apparatus (Brazilian Pharmacopea 2010). Anhydrous sodium sulfate was used to remove any trace of water from oils. Essential oil of each *Schinus* species were sealed under inert atmosphere, stored in a sealed vial wrapped in aluminum foil and stored in an ultrafreezer (–80 °C) until required.

Essential oil samples were analyzed by comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOFMS). The column set used in essential oils analysis consists of a DB-5 column (5% diphenyl-95% dimethylpolysiloxane) of 60 m x 0.25 mm x 0.25 µm in first dimension (^{1}D) serially coupled with a DB-17 column (50% phenyl-50% methylpolysiloxane) of 2.15 m x 0.18 mm x 0.18 µm in second dimension (^{2}D). After optimization, the best analytical conditions for GC×GC/TOFMS were: 1 µL of sample in concentration of 2000 mg L^{-1} in split injection mode (1:20), oven temperature maintained at 50 °C for 5 min and subjected to a heating rate of 4 °C/min to 280 °C (10 min). Temperature difference between the ovens (ΔT) was 10 °C and modulation period was 7 s. The injection port and transfer line temperatures was 280 °C, and ion source temperature was 250 °C. Data were collected at a nominal data acquisition rate of 100 Hz. The detector voltage was -1580 V.

Linear Temperature Programmed Retention Indices (LTPRI) were determined employing retention data of an n-alkane solution (C_8–C_{24}), along with retention data of volatile compounds of *Schinus* samples. LTPRI values were obtained using the Van den Dool and Kratz (1963) equation and were calculated for all detected components for the ^{1}D. A minimum value of three for signal to noise ratio was used to consider a compound as detected. The mass spectra were then compared with Adams (2001; 2007) database and the NIST MS library mass spectrum. Relative percentage of each component was obtained directly from chromatographic peak areas, considering the sum of all eluted peaks as a hundred percent (normalization method).

Germination and growth assays

Seeds of *A. thaliana* were surface sterilized and thirty seeds were sown in Petri dishes containing 3% sucrose, 0.8% (w/v) agar and 0.1x MS minerals (Murashige and Skoog 1962). pH media were set with NaOH and HCl to 5.8 and media were autoclaved at 120 °C and 1kgf.cm^{-2} for 20 min. Seeds were stratified for 2 days at 4 °C under continuous darkness. Then, *S. lentiscifolius*, *S. molle* and *S. terebinthifolius* essential oils were pipetted on filter paper (Ø 1.6 cm) attached to the inner face of the Petri dish. This technique aims to oil volatilization into the airspace, avoiding direct contact between seeds and essential oils. Quantities of essential oils pipetted were: 5 µL, 10 µL, 15 µL, 20 µL and 25 µL. Control was the treatment without application of essential oils. Petri dishes were transferred to a controlled environmental chamber at 20 °C ± 2 °C, 16 h photoperiod and irradiance of 37 µmol.m^{-2}.sec^{-1} provided by white fluorescent lamps. Parameters examined included germination rate (%) and speed of accumulated germination (AS), calculated according to Anjum and Bajwa (2005).

In post-germination assays, essential oil was applied 48 h after Petri dishes were transferred to the environmental chamber in order to allow the emergence of the primary root. Seedlings remained exposed to *Schinus* volatiles for 7 days. Photographs were taken to measure shoot and root length using the software ImageJ 1.45s. Ten seedlings were measured per repetition. In both experiments, four repetitions of each treatment (different quantities of essential oil of each *Schinus* species) were performed.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

For RT-qPCR analysis, procedures were similar to that presented for initial growth assays. Five 5 µL of *S. lentiscifolius*, *S. molle* and *S. terebinthifolius* essential oils were applied and seedlings remained exposed to volatiles during 24 h. Thus, seedlings used in this experiment were 3 days old. This was required as CDK B1;1 gene is expressed in light-grown seedlings from 1 up to 7 days after germination with a peak at 2 and 3 days, in actively dividing cells (Yoshizum et al. 1999).

Roots were then collected (3 repetitions of 25 mg material), immediately frozen in liquid nitrogen and stored at -80 °C until RNA isolation. Total RNA was

prepared using Plant RNA Purification reagent (Invitrogen), followed by DNAse I (Invitrogen) treatment. After extraction, RNA quantification was carried out using a NanoDrop. First-strand cDNA synthesis was performed with reverse transcriptase (M-MLV, Invitrogen) using one µg of total RNA. Primer sequences for RT-qPCR amplification are shown in Table 1. Sequence data from this article can be found in The Arabidopsis Information Resource (TAIR; www.arabidopsis.org) under the following accession numbers: ANP1 (AT1G09000), CDK B1;1 (AT3G54180), Act (AT3G18780) and eEF1α (AT5G60390). Act and eEF1α were used as normalization controls.

Table 1 Primer sequences for qRT-PCR amplification

Gene symbol	Gene name		Primer sequence (5'-3')
Act	Actin	F	GCACCCTGTTCTTCTTACCG
		R	AACCCTCGTAGATTGGCACA
eEF1α	elongation factor 1-alpha	F	TGAGCACGCTCTTCTTGCTTTCA
		R	GGTGGTGGCATCCATCTTGTTACA
ANP1	*Arabidopsis* NPK1-like protein kinase 1	F	TTGCATCACCAGGAAAATGA
		R	TTCAGACCAAACCTCGTGTCT
CDK B1;1	cyclin dependent kinase B1;1	F	AGCCGAAAGAATTTCAGCAA
		R	TTCACCAAGACGATGACAACA

Statistical analysis

Comparisons between treatments and control were performed using oneway analyses of variance (ANOVA) and a post hoc Student-Newman-Keuls (SNK) or Tukey test whenever the data satisfied presuppositions of normality and homogeneity of variance. Statistical analyses were performed using SPSS 17.0 software and differences were considered significant at $P \leq 0.05$.

Results

GC×GC analysis provides image patterns that can be correlated to compound class structure (Fig. 1). Sesquiterpenes were the most abundant chemical category in *S. lentiscifolius* essential oil. However, for *S. molle* essential oil, monoterpenes were the most abundant. The chromatographic space was equally divided by mono- and sesquiterpenoids in *S. terebinthifolius* sample. A greater number of compounds was tentatively identified by GC×GC/TOFMS. Eighty-six,

seventy-two and eighty-eight components were identified in the *S. lentiscifolius*, *S. molle* and *S. terebinthifolius* essential oil samples, respectively (Table 2). Compounds separation due to selectivity of the ^{2}D may be observed. Twenty-three, twelve and ten *S. lentiscifolius*, *S. molle* and *S. terebinthifolius* compounds could be separeted in ^{2}D, respectively.

All essential oils tested affected both germination and seedling growth of *A. thaliana*. For the three *Schinus* volatiles tested, the lowest amount of oil applied (5 µL), reduced about 30% the germination percentage of *A. thaliana* compared to control group (Fig. 2). For *S. lentiscifolius* and *S. molle* essential oils, amounts from 15 µL reduced about 50% the same parameter. However, for *S. terebinthifolius*, the same reduction was observed from 10 µL.

Schinus volatiles also affected the speed of accumulated germination (AS) (Table 3). A dose-dependent effect was observed regarding *S. lentiscifolius* and *S. molle* treatments. Compared to control group, 5 µL, 10 µL, 15 µL, 20 µL and 25 µL treatments with *S. lentiscifolius* essential oil reduced the AS by 55%, 67%, 74%, 77% and 78%, respectively, with no difference among the last three treatments. Similar reduction was observed for *S. molle* treatments. For *S. terebinthifolius* essential oil, application of 5 µL and 10-25 µL reduced by 65% and 75% the AS when compared to control group.

A. thaliana initial growth was also significantly reduced by *Schinus* essential oils. The three essential oils tested reduced shoot length in a very similar manner (Fig. 3a). Compared to the control group, 5 µL of any essential oil tested reduced shoot length by 53%. Volumes equal or higher than 10 µL reduced shoot length by 60%, except for treatment with 10 µL of *S. molle*, which showed lower effect (56%) and 25 µL of *S. lentiscifolius*, which showed higher effect (63%) on this same parameter.

Inhibitory effect on root length (Fig. 3b) was more pronounced than on shoot length. Compared to control group, *S. lentiscifolius* essential oil affected *A. thaliana* root length by 47% (when applied 5 µL of oil) up to 61% (when applied 20 or 25 µL of oil). Treatments with *S. molle* and *S. terebinthifolius* demonstrated a dose-dependent effect. Application of 5 µL of *S. molle* or *S. terebinthifolius* essential oil reduced root length by 27% and 30%, respectively. *Schinus molle* reduced this parameter by 48%, 68% and 85% with 10 µL, 15-20 µL, and 25 µL, respectively. *S. terebinthifolius*

essential oil reduced by 52% and 81% *A. thaliana* rooth length when 10 µL and up to 15 µL were applied, respectively.

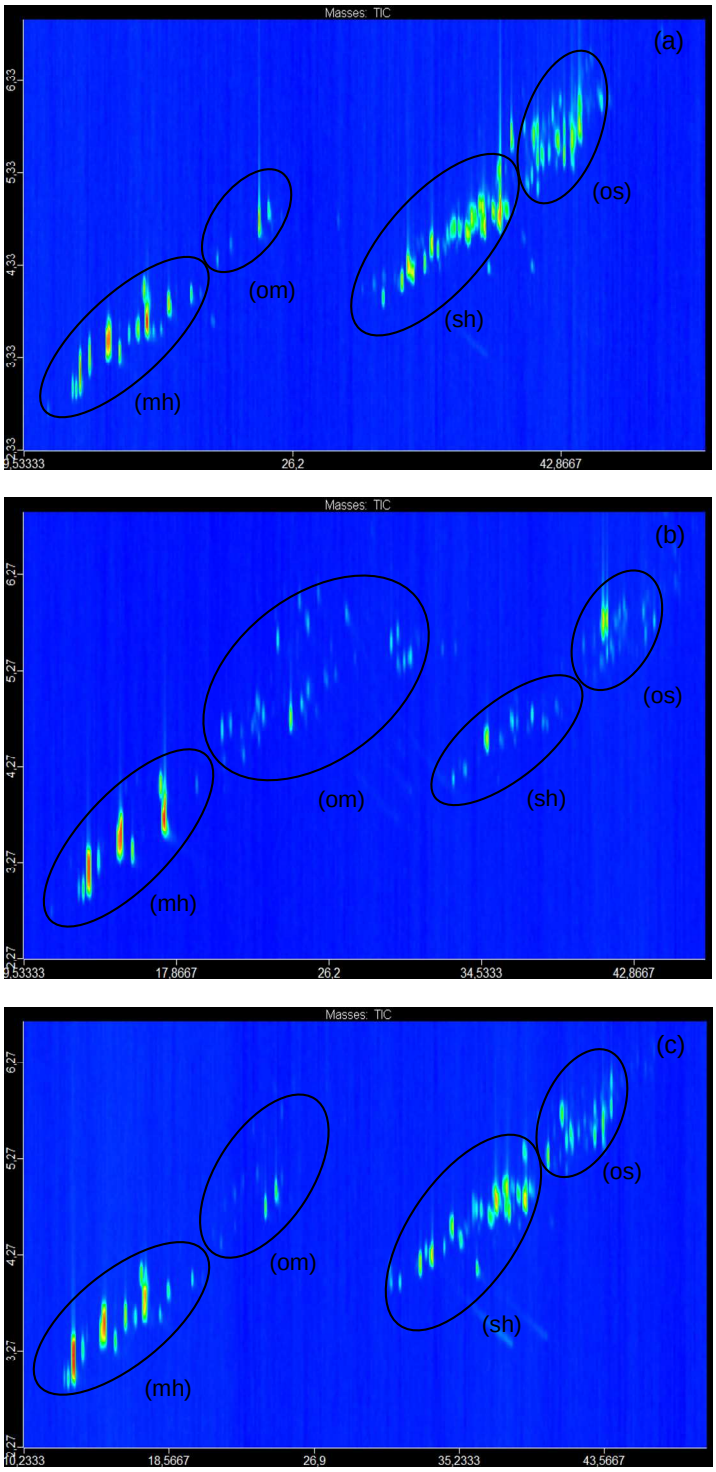

Fig. 1 GC×GC/TOFMS total ion current chromatogram (TIC) data colour plot of *Schinus lentiscifolius* (a), *S. molle* (b) and *S. terebinthifolius* (c) essential oils, showing the distribution of classes of compounds in diffe-rent regions of the chromatogra-phic space, using a non-polar×polar column set. (mh) mo-noterpenic hydrocarbons; (om) oxygenated monoterpenes; (sh) sesquiterpenic hydrocarbons; (os) oxygenated sesquiterpenes

Table 2 Tentative identification of *Schinus* essential oils compounds using GC×GC/TOFMS

Compounds	LTPRI[#]	*S. lentiscifolius* Retention time ^{1}D (min)	^{2}D (s)	LTPRI	Area (%)	*S. molle* Retention time ^{1}D (min)	^{2}D (s)	LTPRI	Area (%)	*S. terebinthifolius* Retention time ^{1}D (min)	^{2}D (s)	LTPRI	Area (%)
Monoterpenes													
Tricyclene	927	12.57	3.01	922	0.34	12.57	3.00	922	0.30	12.57	3.00	922	0.20
α-Thujene	930	12.80	2.99	928	0.37	12.80	3.00	928	1.13	12.80	2.99	928	0.59
α-Pinene	939	13.03	3.18	933	4.71	13.03	3.23	933	24.25	13.03	3.15	933	10.44
Camphene	954	13.62	3.34	947	2.28	13.62	3.27	947	0.87	13.62	3.30	947	0.82
Thuja-2,4(10)-diene	960									13.85	3.44	963	0.07
Sabinene	975	14.67	3.54	972	2.95	14.67	3.54	972	7.47	14.67	3.47	972	2.10
β-Pinene	979	14.90	3.54	978	7.66	14.90	3.60	978	11.48	14.90	3.57	978	13.23
Myrcene	991	15.48	3.40	992	1.26	15.48	3.41	992	2.89	15.48	3.39	992	1.03
2-Pentyl-furan[1,a]	988	15.48	3.69	992	0.03								
Dehydro-1,8-cineole[1]	991					15.48	3.85	992	0.01				
α-Phellandrene	1003	16.07	3.56	1005	0.27					16.07	3.65	1005	2.92
Pseudolimonene	1004					16.07	3.56	1005	0.03				
α-Terpinene	1017	16.65	3.66	1018	1.62					16.65	3.62	1018	0.47
para-Cymene	1025	17.00	4.08	1025	1.40	17.00	4.09	1025	3.22	17.00	4.08	1025	1.55
Limonene	1029	17.23	3.72	1030	8.45	17.12	3.75	1028	16.15	17.12	3.72	1028	3.43
β-Phellandrene	1030	17.12	3.79	1028	3.80					17.23	3.86	1030	5.25
1,8-Cineole[1]	1031	17.35	3.93	1033	0.22	17.35	3.94	1033	0.13	17.23	4.04	1030	0.61
cis-Ocimene	1037	17.58	3.64	1037	0.15					17.58	3.64	1037	0.05
trans-Ocimene	1050	18.05	3.65	1048	0.12					18.05	3.66	1048	0.30
γ-Terpinene	1060	18.52	3.94	1058	2.24					18.52	3.91	1058	0.64

cis-Sabinene hydrate[1]	1070	18.98	4.09	1067	0.01	18.98	4.09	1067	0.17					
para-Mentha-3,8-diene	1073					19.22	4.38	1073	0.02					
Terpinolene	1089	19.92	4.03	1088	0.50					19.92	4.03	1088	0.30	
para-Cymenene	1091	20.03	4.66	1090	*					20.03	4.67	1090	0.01	
Oxygenated monoterpenes														
α-Pinene oxide	1099					20.38	4.66	1097	0.50					
Linalool	1097	20.50	4.03	1100	0.05	20.50	4.04	1100	0.02	20.50	4.03	1100	0.04	
trans-Sabinene hydrate	1098	20.50	4.34	1100	0.01	20.50	4.36	1100	0.12					
1,3,8-para-Menthatriene						20.85	4.72	1107	0.32					
α-Fenchol	1117	21.20	4.46	1115	0.01					21.08	4.50	1112	0.02	
β-Thujone	1114	21.32	4.58	1118	0.01									
trans-para-Mentha-2,8-dien-1-ol	1123					21.43	4.64	1120	0.10					
cis-para-Menth-2-en1-ol	1122	21.55	4.40	1122	0.11	21.55	4.41	1122	0.18	21.55	4.40	1122	0.06	
α-Campholenal	1126									21.78	4.83	1127	0.02	
1-Terpineol	1134	22.37	4.54	1140	0.07									
cis-Limonene oxide	1137					22.02	4.70	1133	0.12					
trans-Limonene oxide	1142					22.25	4.69	1137	0.17					
cis-para-Mentha-2,8-dien-1-ol	1138									22.37	4.58	1140	0.04	
trans-Pinocarveol	1139					22.25	4.95	1137	0.45	22.37	4.91	1140	0.10	
Nopinone	1140					22.25	6.09	1137	0.03					
cis-Verbenol	1141					22.60	4.80	1145	0.40					
Camphene hydrate	1150									22.72	4.97	1148	0.02	
Sabina ketone	1159					23.30	5.87	1160	0.04					
Pinocarvone	1165					23.42	5.60	1163	0.50	23.42	5.59	1163	0.07	
Borneol	1169					23.65	4.94	1167	*	23.65	4.93	1167	0.01	
α-Phellandren-8-ol	1170									23.65	5.13	1167	0.08	
Unknown						23.77	4.97	1170	0.05					

Isopinocamphone	1175	24.00	5.51	1175	*	24.00	5.50	1175	0.03	24.00	5.51	1175	0.01
Terpinen-4-ol	1177	24.12	4.82	1178	3.12	24.12	4.79	1178	3.14	24.12	4.78	1178	1.36
para-Cymen-8-ol	1183	24.58	5.57	1187	0.01	24.47	5.62	1185	0.05	24.47	5.63	1185	0.01
Cryptone	1186					24.58	6.01	1187	0.21	24.58	6.01	1187	0.03
α-Terpineol	1189	24.70	4.93	1190	0.50	24.70	4.93	1190	0.30	24.70	4.95	1190	1.45
cis-Piperitol	1196	24.93	4.73	1195	0.03	24.93	4.74	1195	0.08	24.93	4.74	1195	0.01
Myrtenol	1196					25.05	5.09	1197	0.29	25.05	5.08	1197	0.06
Myrtenal	1196					25.05	5.77	1197	0.41	25.05	5.77	1197	0.07
trans-Piperitol	1208	25.52	4.85	1208	0.03	25.52	4.87	1208	0.08	25.52	4.86	1208	0.02
Verbenone	1205					25.63	6.09	1211	0.19	25.75	6.03	1213	0.03
trans-Carveol	1217					26.10	5.17	1221	0.21	26.10	5.16	1221	0.01
Unknown						26.33	5.54	1226	0.05				
cis-Carveol	1229					26.57	5.24	1232	0.09				
Carvone	1243					27.15	5.88	1245	0.39				
Unknown						27.62	5.41	1255	0.05				
trans-Pinocarvyl acetate	1298					29.60	5.58	1300	0.53				
cis-Pinocarvyl acetate	1312					29.95	5.36	1308	0.24				
Unknown						29.95	5.68	1308	0.34				
Unknown						30.30	5.38	1316	0.31				
Unknown						30.65	5.43	1324	0.52				
Unknown						31.12	5.60	1335	0.07				
Sesquiterpenes													
δ-Elemene	1338	31.23	4.15	1338	0.07					31.35	3.98	1341	0.29
α-Cubebene	1351	31.82	3.99	1351	0.41	31.82	3.98	1351	0.02	31.82	3.99	1351	0.30
α-Longipinene	1353	31.82	4.22	1351	0.07								
cis-Carvyl acetate[2]	1368					32.40	5.51	1365	0.09				
Cyclosativene	1371	32.52	4.2	1368	0.04					32.52	4.19	1368	0.01

α-Copaene	1377	32.98	4.18	1378	1.55	32.98	4.16	1378	0.28	32.98	4.20	1378	3.02
Unknown						33.10	5.55	1318	0.07				
β-Bourbonene	1388	33.33	4.35	1386	3.42	33.33	4.31	1386	0.03				
Nepetalactone										33.22	4.32	1386	0.34
β-Cubebene	1388	33.57	4.27	1392	2.02								
β-Elemene	1391					33.68	4.25	1395	0.24	33.68	4.30	1395	4.30
α-Gurjunene	1410	34.38	4.37	1411	0.89	34.38	4.36	1411	0.02	34.38	4.36	1411	0.25
Unknown										34.73	4.56	1420	0.60
β-Caryophyllene	1419	34.85	4.58	1423	2.68	34.85	4.58	1423	4.07	34.85	4.57	1423	2.43
β-Gurjunene	1434	35.20	4.51	1431	0.58								
γ-Elemene	1437									35.32	4.44	1434	0.74
trans-α-Bergamotene	1435					35.43	4.15	1437	0.02	35.43	4.15	1437	0.01
α-Guaiene	1440	35.55	4.34	1440	*								
Aromadendrene	1441	35.67	4.52	1443	0.11	35.55	4.58	1440	0.19	35.67	4.53	1443	0.21
Unknown		35.78	4.67	1446	0.27								
cis-Muurola-3,5-diene	1450	36.02	4.71	1451	0.68								
α-Himachalene	1451									36.02	4.84	1451	0.52
trans-β-Farnesene	1457	36.25	4.13	1457	0.01					36.25	4.14	1457	0.94
α-Caryophyllene	1455	36.25	4.75	1457	1.02	36.25	4.75	1457	0.57	36.25	4.75	1457	0.36
Alloaromadendrene	1460	36.48	4.75	1463	0.73	36.48	4.74	1463	0.16	36.48	4.75	1463	0.52
γ-Gurjunene	1477	37.07	4.67	1477	2.72	37.07	4.70	1477	0.09	36.95	4.70	1474	1.45
trans-Cadina-1(6),4-diene						37.07	4.91	1477	0.02				
Germacrene D	1485	37.30	4.85	1483	1.96	37.30	4.82	1483	0.49	37.30	4.85	1483	7.90
β-Selinene	1490	37.53	4.87	1489	0.84								
trans-Muurola-4(14),5-diene	1494	37.77	4.99	1494	1.82					37.77	4.99	1494	1.56
10,11-epoxy-Calamenene[2]	1492	37.77	5.78	1494	0.02								
Viridiflorene	1497	37.88	4.75	1497	1.29	37.88	4.74	1497	0.20	37.88	4.76	1497	1.22
Bicyclogermacrene	1500	38.00	4.96	1500	2.32					38.00	4.97	1500	4.55
α-Muurolene	1500	38.12	4.76	1503	2.31	38.12	4.72	1503	0.14	38.12	4.73	1503	1.40

Unknown										38.23	5.13	1506	0.01
α-Farnesene	1506	38.35	4.31	1509	0.13								
Unknown										38.35	4.97	1509	0.33
β-Bisabolene	1506					38.35	4.42	1509	0.01				
Germacrene A	1509	38.35	4.99	1509	0.16								
cis-γ-Bisabolene	1514									38.70	4.59	1518	0.07
γ-Cadinene	1514	38.70	4.94	1518	1.63	38.58	4.92	1515	0.13	38.58	4.93	1527	1.15
δ-Cadinene	1523	39.05	4.82	1527	6.32	38.93	4.83	1524	0.03	39.05	4.84	1527	5.37
trans-Calamenene	1529	39.05	5.37	1527	1.81	38.93	5.34	1524	0.04	38.93	5.36	1524	0.47
trans-Cadina-1(2),4-diene	1535	39.40	4.95	1536	0.56					39.28	4.98	1533	0.54
Unknown		39.40	5.39	1536	0.06								
α-Cadinene	1539					39.52	4.92	1539	0.01				
Selina-3.7(11)-diene	1547									39.75	4.95	1545	0.01
α-Calacorene	1546	39.75	5.81	1545	1.90					39.75	5.70	1545	0.07
cis-Calamenene	1540	39.87	5.32	1548	0.03								
Unknown		39.98	4.97	1552	0.03	40.10	5.55	1555	0.16				
Germacrene B	1561	40.33	5.28	1561	0.04					40.33	5.31	1561	1.97
1-*nor*-Bourbonanone[2]	1563	40.45	6.22	1564	0.03								
trans-Nerolidol[2]	1563	40.57	4.62	1567	0.05					40.57	4.62	1567	0.05
β-Calacorene	1566	40.57	5.83	1567	0.19					40.57	5.83	1567	0.03
Oxygenated sesquiterpenes													
Ledol	1569	40.80	5.2	1573	0.23	40.68	5.23	1570	0.04	40.68	5.24	1570	0.06
Unknown										40.68	5.54	1570	0.06
cis-dihydro-Apofarnesol	1573	41.03	4.33	1579	0.10								
Germacrene D-4-ol	1576	41.03	5.3	1579	0.21					41.03	5.30	1579	0.08
Spathulenol	1578	41.15	5.74	1582	1.38	41.15	5.82	1582	7.13	41.15	5.74	1582	1.39
Gleenol	1587	41.38	5.17	1588	0.19								

Unknown										41.38	5.16	1588	0.06
Globulol	1585	41.38	5.47	1588	0.82	41.38	5.48	1588	0.27	41.38	5.44	1588	0.51
Caryophyllene oxide	1583	41.38	5.8	1588	0.88	41.38	5.82	1588	5.05				
Viridiflorol	1593					41.62	5.54	1594	0.34	41.62	5.56	1594	1.16
Unknown		41.73	5.43	1597	0.36								
Unknown		42.08	5.55	1606	0.34								
Unknown		42.08	5.89	1606	0.06	42,08	5,89	1606	0.22	42.08	5.55	1606	0.22
1,2 epoxi-Humulene	1608	42.32	5.96	1613	0.33	42.32	5,96	1613	0.35	42.32	5.96	1613	0.02
Unknown										42.43	5.64	1616	0.10
1,10-di-epi-Cubenol	1619	42.55	5.44	1619	0.07					42.55	5.44	1619	0.09
Unknown		42.67	5.64	1623	1.78								
trans-Isolongifolanone	1627	42.78	6.12	1626	0.12					42.78	5.59	1626	0.16
1-epi-Cubenol	1629	43.02	5.54	1632	1.31					43.02	5.52	1632	0.85
Unknown		43.02	5.80	1632	0.67					43.02	5.80	1632	0.40
γ-Eudesmol	1632									43.13	5.64	1635	0.11
epoxi-Alloaromadendrene						43.37	5.94	1642	0.25				
τ-Cadinol	1640					43.48	5.59	1645	0.23	43.48	5.64	1645	2.36
α-Muurolol	1646	43.48	5.73	1645	3.50								
δ -Cadinol										43.60	5.77	1648	0.30
α-Cadinol	1654	43.95	5.86	1658	2.52	43.95	5.80	1658	0.34	43.95	5.81	1658	0.72
α-Eudesmol	1654	43.95	6.1	1658	1.14								
Selin-11-en-4-α-ol	1660									43.95	6.08	1658	0.54
cis-Calamenen-10-ol	1661	44.18	6.39	1665	0.03								
trans-Calamenen-10-ol	1669	44.42	6.51	1671	0.05								
14-hydroxy-9-epi-β-Caryophyllene	1670	44.53	6.03	1674	0.14					44.53	6.11	1674	0.08
Cadalene[3]	1677	44.65	6.61	1677	0.07					44.65	6.60	1677	*
Unknown										45.12	6.21	1690	0.03
Unknown		45.35	6.09	1697	0.47								
Eudesm-7(11)-en-4-ol	1700									45.47	6.31	1700	0.04

Unknown	45.82	6.14	1710	0.10					
Unknown							45.93	6.22	1713	0.04
Unknown							46.17	6.22	1720	0.03
Total of no identified compounds				4,58		1.84				1.88

[1] Oxygenated monoterpene; [2] Oxygenated sesquiterpene; [3] Sesquiterpene.
* Area < 0.01
[1]D – first dimension; [2]D – second dimension
LTPRI: linear temperature programmed retention indices calculated
LTPRI[#]: reference linear temperature programmed retention indices (Adams 2001); [a] LTPRI[#] according Adams (2007).

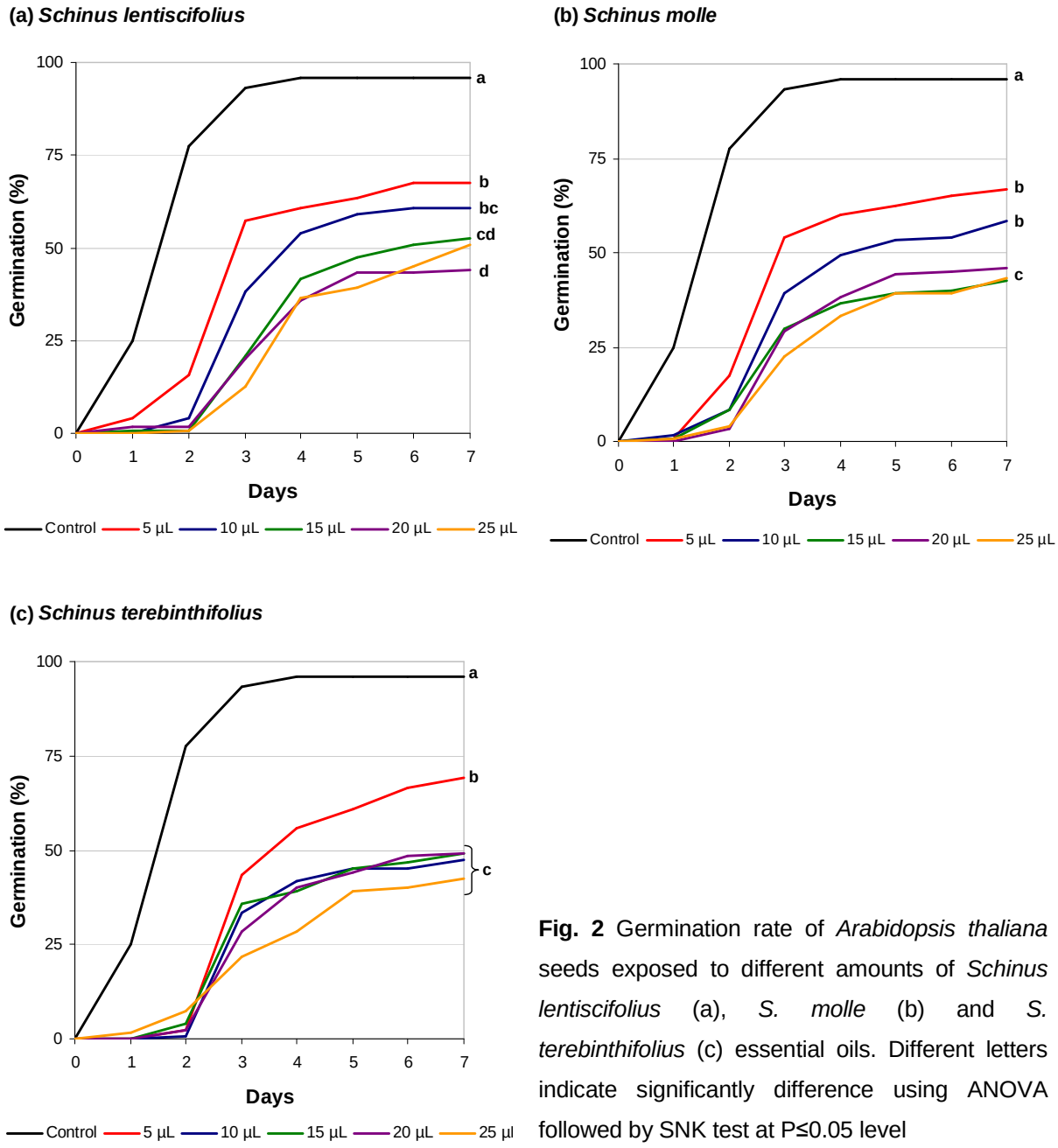

Fig. 2 Germination rate of *Arabidopsis thaliana* seeds exposed to different amounts of *Schinus lentiscifolius* (a), *S. molle* (b) and *S. terebinthifolius* (c) essential oils. Different letters indicate significantly difference using ANOVA followed by SNK test at P≤0.05 level

RT-qPCR results showed that ANP1 and CDK B1;1 expression were not affected by *Schinus* volatiles except for *S. molle* treatment, where it was observed an inhibition of ANP1 expression using eEF1 as normalizer (Fig. 4).

Discussion

Comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC/TOFMS) is a powerful analytical tool for the

analysis of complex matrices due to its high peak capacity, selectivity, and sensitivity (Shellie and Marriott 2003). Essential oils are extremely complex in composition, belonging to different chemical classes, mainly mono- and sesquiterpenoids (Adorjan and Buchbauer 2010; Sangwan et al. 2001). The identification of these compounds is difficult as some of them present similar mass spectra and many co-elutions may occur, preventing a correct identification and quantification process. GC×GC/TOFMS technique shows potential for separation and tentative identification of components of essential oils that exhibit moderate complexity in one-dimensional gas chromatography/mass spectrometry (1D-GC/MS), as *Schinus lentiscifolius*, *S. molle* and *S. terebithifolius* essential oils. Compounds that co-elute in the first chromatographic dimension (^{1}D) could be separated in the second chromatographic dimension (^{2}D) when GC×GC/TOFMS was employed. Several studies suggested that effects observed on target species can be explained as a result of the action of the major component of an essential oil. However, some researches showed that inhibitory effects were more pronounced when target species have been exposed to essential oils than when exposed to a purified component of essential oil (Kong et al. 1999; Schmidt-Silva et al. 2011). Thus, GC×GC/TOFMS is an important tool, to clarify the composition of such complex mixtures, which may contribute to a better understanding about the biological activity of essential oils.

Table 3 Speed of accumulated germination (AS) of *Arabidopsis thaliana* exposed to different quantities of *Schinus lentiscifolius* (Slent), *S. molle* (Smoll) and *S. terebinthifolius* (Stere) essential oils

	AS (mean ± S.D.)		
	Slent	**Smoll**	**Stere**
Control	17.2 ± 2.0 [a]	17.2 ± 2.0 [a]	17.2 ± 2.0 [a]
5 µL	7.8 ± 0.7 [b]	7.2 ± 1.5 [b]	6.1 ± 0.1 [b]
10 µL	5.6 ± 0.9 [c]	5.8 ± 0.6 [bc]	4.3 ± 0.8 [b]
15 µL	4.4 ± 1.2 [c]	4.3 ± 0.8 [c]	4.6 ± 0.9 [b]
20 µL	4.0 ± 0.9 [c]	4.2 ± 0.4 [c]	4.3 ± 0.9 [b]
25 µL	3.8 ± 0.5 [c]	3.9 ± 0.5 [c]	4.1 ± 1.2 [b]

Means within a column followed by different letters are significantly different using ANOVA followed by SNK test (P≤0.05)

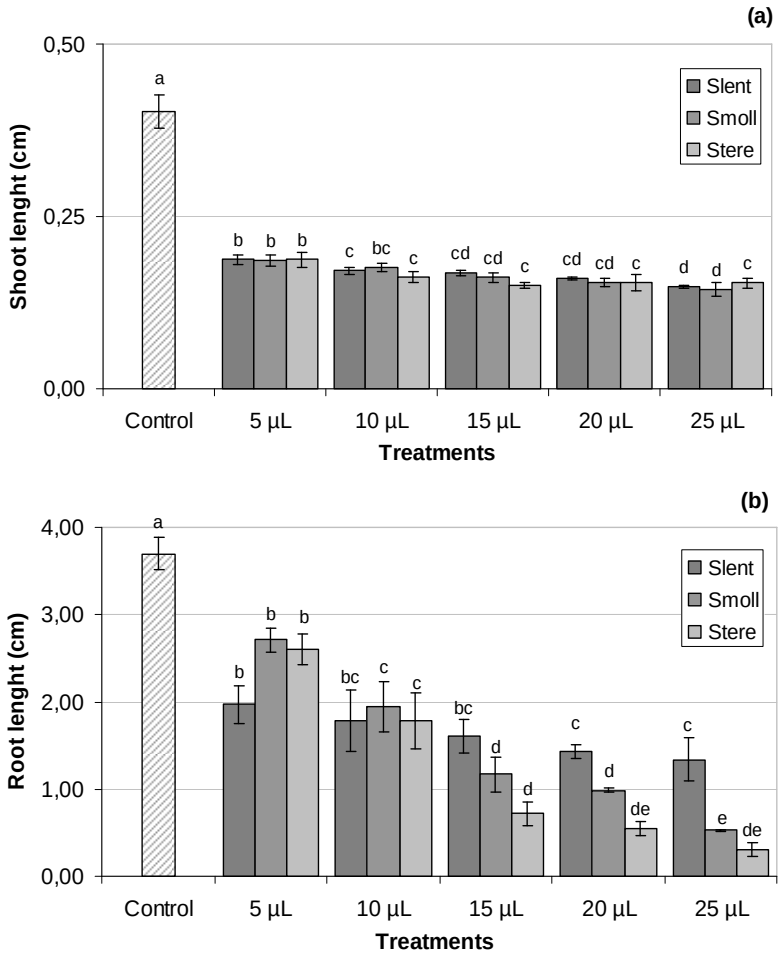

Fig. 3 Phytotoxic effects of different quantities of *Schinus lentiscifolius* (Slent), *S. molle* (Smoll) and *S. terebinthifolius* (Stere) essential oils on shoot length (a) and root length (b) of *Arabidopsis thaliana*. Bars with different letters are significantly different using ANOVA followed by SNK test at P≤0.05 level for the same *Schinus* essential oil

Previous studies have demonstrated that *A. thaliana* is sensitive to different types of natural compounds, including terpenoids (Reigosa and Pazos-Malvido 2007; Zhao et al. 2009; Grana et al. 2013). According to Ranal and Santana (2006), the germination capacity of one seed, based on a binary answer (germinated/non germinated), is one qualitative attribute of the germination process, converted in a quantitative attribute. In the present study, when *A. thaliana* seeds where exposed to volatiles, it was not observed a clear dose-dependent effect on the germination process. Different interference mechanisms were proposed to explain allelochemicals effects on germination process of target species. Allelochemicals modes of action on germination may be related to generation of ROS-induced oxidative stress and restriction in seed reserve mobilization (Bogatek and

Gniazdowska 2007; Oracz et al. 2007). Kato-Noguchi et al. (2013) suggested that momilactone A and B could inhibit the germination of *A. thaliana* seeds by inhibiting the degradation process of cruciferin, an essential step during germination whose product is used by the germinating seedling as an initial source of nitrogen.

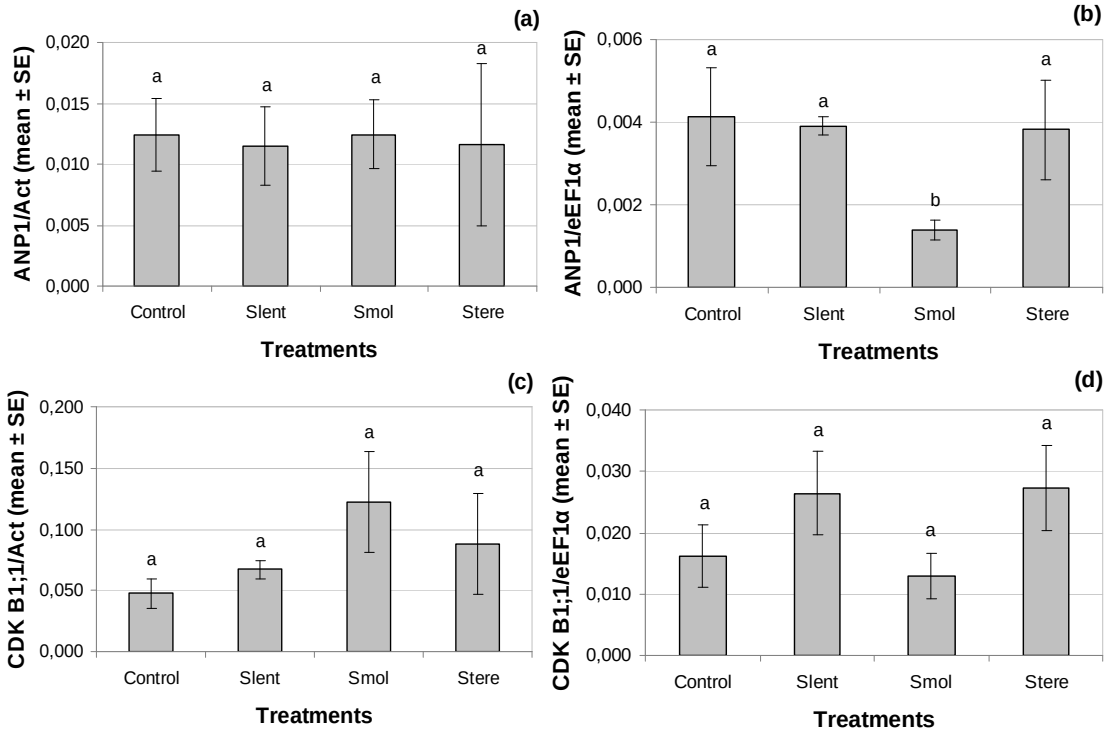

Fig. 4 Relative expression profile (actin and eEF1α as reference genes) of ANP1 (a and b) and CDKB1;1 (c and d) genes after 24 h of *Schinus lentiscifolius*, *S. molle* and *S. terebinthifolius* essential oil exposition. Bars with different letters are significantly different using ANOVA followed by Tukey HSD test at P≤0.05 level

It was evidenced a dose-dependent effect from 5 to 25 µL of essential oil on initial growth. In a previous experiment (data not show), 100 µL of the three *Schinus* essential oil tested reduced about 95% the root length of *A. thaliana*. The same quantity applied of *S. lentiscifolius*, *S. molle* and *S. terebinthifolius* essential oils inhibited *A. thaliana* shoot length on 60%, 70% and 80%, respectively. Phytotoxic effects of *Schinus* volatiles on *A. thaliana* growth was higher than effects observed on *Lactuca sativa* L. (Pawlowski et al. 2012, 2013), a species commonly used in phytotoxic experiments. Therefore, *A. thaliana* demonstrated to be an effective and very sensitive species that can be use in phytotoxic studies, particularly when effects are evaluated on root growth.

Modifications in plants growth in response to natural products can be explained by alterations of cell molecular biology, ultrastructure as well as biochemical and physiological processes (Gniazdowska and Bogatek 2005). *In-situ* localization of H_2O_2 on *A. thaliana* tissues was detected after *Schinus* essetial oils exposure (unpublished data). Redox signaling is involved in fine-tuning of many specific metabolic reactions and developmental processes as well as in defense mechanisms (Dietz 2003). H_2O_2 is an active signaling molecule, and its accumulation (oxidative stress) leads to a variety of cellular responses. Plant responses to H_2O_2 are clearly dose dependent. High concentrations of H_2O_2 induce cellular damage that can result in cell death. Dose-dependent effects observed in the present study suggest that phytotoxic effects of *Schinus* volatiles can be related with ROS damage. As essential oils are constituted by lipophilic substances, their constituents entry into the plant cell by penetration through the cell wall and cell membrane (Mutlu et al. 2011) or by stomata. These substances act synergistically probably by inducing ROS production, resulting in cellular damage.

On the other hand, at low concentrations, H_2O_2 aids in cellular defense, provides tolerance against stress, blocks cell cycle progression and acts as a developmental signal for the onset of secondary wall differentiation (Kovtun et al. 2000; Stone and Yang 2006). H_2O_2 is produced by several environmental and developmental stimulants and can act as a signaling molecule that regulates plant development and stress tolerance and programmed cell death, a hypersensitive response to pathogens and allelopathic plant-plant interactions (Taj et al. 2010). It was expected that the lowest essential oil quantity tested demonstrated phytotoxic effects by inducing ANP expression and repressing CDK B1;1 one. According to previous results and the present ones, phytotoxic effects of *Schinus* essential oils, in quantities studied, seems to be explained in terms of cellular damage rather than by induction of stress-inducible gene. *Schinus* essential oils, under the presented experimental conditions, did not affected ANP1 and CDK B1;1. Difference observed on ANP1 expression on *S. molle* treatment is probably spurious, given that using Act as normalized there was not difference, and that other essential oils did not show effect on gene expression. Exact mechanisms that *Schinus* essential oils trigger in *A. thaliana* germination and growth process remains an open question.

In conclusion, results demonstrated the phytotoxic effects of *Schinus* essential oils on germination and early growth of *A. thaliana*. Volatiles interference on ANP1

pathway can not be discarded. Often, studies involving the phytotoxic activity of allelochemicals are considered promising only when drastic effects are observed. However, when the aim is to understand the mode of action of these substances, in this special case, testing lower concentrations than those that cause harmful effects may represent an interesting alternative, in particular in ANP1 pathway, that is activated by ROS. Further research should consider this when evaluating allelochemicals modes of action at the molecular level.

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4.2 Phytotoxic effects of *Schinus terebinthifolius* volatiles: interference on *Arabidopsis thaliana* adventitious rooting

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Abstract This investigation was undertaken to evaluate *Schinus terebinthifolius* essential oil effects on adventitious rooting process using *Arabidopsis thaliana* as target species. Two lineages of *A. thaliana* were used: ecotype Col-0 WT and *sur1* mutant (an auxin-overproducing mutant). Analysing the same rooting parameters after exposure to volatiles, both lineages demonstrated a similar response pattern. The essential oil reduced mean rooting time and root length but did not affect the percentage of rooting or number of roots. Compared with control, the highest quantity of oil tested (5 µL) retarded rooting by approximately one day and reduced the root length by 50%. Additional experiment using *A. thaliana* WT demonstrated that phytotoxic effects caused by volatiles on root length decreased in treatment with the addition of Trolox®, a powerful antioxidant. However, exogenous auxin did not allow

the microcuttings to recover from the effects caused by the essential oil. Hydrogen peroxide accumulation upon volatiles exposure assayed by histochemical detection was evident in *A. thaliana* cotyledons exposed from 2 µL to 5 µL of essential oil. In treatment where Trolox® was added, no hydrogen peroxide was detected. Results indicated that *S. terebinthifolius* essential oil acted more strongly on the process of root elongation than on root formation. The mechanism of the essential oil action may occur by the induction of an oxidative burst leading to accumulation of reactive oxygen species causing secondary effects, such as depigmentation of cotyledons, delay on rooting time and decrease on root length.

Keywords: Essential oil; Trolox; Rooting time; Root length.

Introduction

Schinus terebinthifolius Raddi (Anacardiaceae) is an evergreen, dioecious plant native to South America. It is known by a variety of common names including Brazilian pepper, Christmas-berry, pink-pepper. It produces an abundance of fruits that usually ripen from November to January which are marketed as a substitute for black pepper (Barbosa et al. 2007). The species has been introduced into subtropical areas worldwide, becoming an invasive species. Secondary metabolites produced by *Schinus* spp. may be involved in the mechanism of invading and disrupting natural communities (Morgan and Overholt 2005; Williams et al. 2005).

The essential oil extracted from *S. terebinthifolius* leaves presented high concentrations of monoterpenes and sesquiterpenes hydrocarbons, being α-pinene a major compound (Pawlowski et al. 2012). In nature, essential oils play an important role in the protection of plants as antibacterials, antivirals, antifungals, insecticides and also against herbivores (Bakkali et al. 2008). Several studies have demonstrated that essential oils can affect initial growth of plants (Singh et al. 2002; Zahed et al. 2010) in a chemo-ecological phenomenon known as allelopathy (Rice 1984).

Effects of allelochemical action can be detected at different levels of plant organization: molecular, structural, biochemical, physiological and ecological. Different methods assessing the phytotoxic effects of secondary metabolites have been used, evaluating effects on enzyme activities, cell division, membrane permeability, photosynthesis and respiration (Gniazdowska and Bogatek 2005; Weir

et al. 2004). Moreover, studies have shown that allelochemicals cause oxidative stress in plants, inhibiting germination and root growth through generation of reactive oxygen species (ROS) (Lara-Nupez et al. 2006; Oracz et al. 2007; Singh et al. 2009). Other studies have verified that secondary metabolities induce disturbances in hormonal balance (Bogatek and Gniazdowska 2007; Ishii-Iwamoto et al. 2012; Rentzsch et al. 2012). Despite all these approaches, comprehensive understanding of allelochemical mode of action still remains an open question.

Arabidopsis thaliana (L.) Heynh. is a model plant for biochemical, physiological and developmental studies due to its shortlife cycle and extensive knowledge available on this species, including the complete sequence of its genome and the characterisation of a great quantity of mutants and several ecotypes (The *Arabidopsis* Genome Initiative 2000). This plant is sensitive to a variety of potent allelochemicals and satisfies selection criteria for target species in phytotoxicity studies: be readily available, be affordable, and produce repeatable and reliable results (Pennacchio et al. 2005). Furthermore, this species has been shown to be useful for adventitious rooting studies (Boerjan et al. 1995; Gutierrez et al. 2012).

Phytotoxic effects are commonly tested on germination and seedling growth of target species. A few studies have assessed phytotoxic effects of secondary metabolites on adventitious rooting process (Batish et al. 2008; Lazarotto et al. 2014). Primary root and adventitious root are different in terms of its origins. Primary roots are embryonic and sustained by the quiescent center, also established in the embryo, in the root meristem. Otherwise, adventitious roots are post-embryonic and formed from a small number of differentiated cells situated at the periphery of the vascular tissues (Della Rovere et al. 2013; Laplaze et al. 2007). However, both require an auxin-cytokinin balance for proper root formation (Su et al. 2011). Auxins profoundly influence root morphology, inducing adventitious roots, increasing lateral root production and inhibiting root elongation (Woodward and Bartel 2005). Thus, the present investigation was undertaken to evaluate the effect of *S. terebinthifolius* essential oil on adventitious rooting process using *A. thaliana* as target species. Additionaly, it was evaluated if the addition of an auxin and a powerful antioxidant in the culture medium contribute in recovering microcuttings from the effects caused by *S. terebinthifolius* volatiles. Histochemical detection of hydrogen peroxide was also carried out.

Materials and methods

Essential oil extraction

Schinus terebinthifolius leaves were sampled in natural vegetation in Porto Alegre (30° 1′ 39.73″ S 51° 13′ 43.45″ W), state of Rio Grande do Sul, Brazil. Voucher specimen (164707) was deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN). After sample, leaves were dried at room temperature, fragmented and subjected to extraction. The essential oil was obtained by hydrodistillation from *S. terebinthifolius* leaves, during 4 h, in a modified Clevenger apparatus (Gottlieb and Taveira-Magalhães 1960). Essential oil humidity was eliminate using anhydrous sodium sulfate. The essential oil was stored in airtight tubes, wrapped in aluminum foil and stored in ultrafreezer (-80°C) prior to use.

Microcuttings experiments

Seeds of *A. thaliana* Columbia (Col-0) wild type (WT) and Col mutant CS8156 (*sur1*) were used in microcuttings experiments. *Arabidopsis* mutant designated *sur1* (superroot 1, also identified as rooty – *rty*) overproduces indole-3-acetic acid (IAA) and most of the phenotypic characteristics of this mutant can be explained as being a consequence of an elevated IAA level, as profuse development of adventitious and lateral roots (Boerjan et al. 1995). Seeds were surface sterilized in 70% (v/v) ethanol for 1 min and 2.5% (v/v) NaClO (with a few drops of neutral detergent) for 10 min with constant stirring, followed by four washes in sterile distilled water. Fifteen seeds were sown in Petri dishes containing 3% sucrose, 0.8% (w/v) agar medium and 0.05x MS minerals (Murashige and Skoog 1962), hereafter referred to standard medium. All reagents were analytical grade and the media were prepared with distilled water, followed by autoclaving at 120°C and 1kgf.cm^{-2} for 20 min; pH was set with NaOH and HCl to 5.8 prior autoclaving. Seeds were stratified for 2 days at 4°C under continuous darkness and then germinated in a controlled environmental chamber at 20°C ± 2 °C, 16 h photoperiod and irradiance of 37 µmol.m^{-2}.sec^{-1} provided by white fluorescent lamps. To facilitate analysis of roots, plants were grown on vertically oriented agar plates. After seven days, ten microcuttings were obtained from seedlings. Then, the essential oil (1 µL, 2 µL, 3 µL, 4 µL and 5 µL) was applied on

filter paper (∅ 1.6 cm) attached to the inner face of the Petri dish lid to avoid direct contact between microcuttings and essential oil, allowing the oil to volatilize into the airspace within the dish. Each treatment was performed in four repetitions. Microcuttings remained exposed to volatiles for seven days.

To evaluate if an auxin or an antioxidant allow the microcuttings to recover from the effects caused by the essential oil, standard medium was supplemented with 1 µM of IAA or 10 µM of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, known as Trolox® (T). These concentrations were defined according to previous experiments. Auxin concentration that did not interfere negatively on root length and Trolox® concentration that demonstrated better performance than the control was chosen. Six treatments were conducted: control (standard medium), oil (standard medium; 5 µL of *S. terebinthifolius* essential oil applied as described above), IAA + oil (medium containing 1 µM of IAA; 5 µL of essential oil), T + oil (medium containing 10 µM of Trolox®; 5 µL of essential oil), IAA (medium containing 1 µM of IAA) and T (medium containing 10 µM of Trolox®). In all these treatments microcuttings of *A. thaliana* WT were used.

Parameters examined included mean rooting time (MRT), percentage of rooted microcuttings, number of roots (roots per rooted microcutting) and mean length of longest root (root length). To calculate MRT, microcuttings were evaluated every 24 h. Rooting criterion was the presence of at least one visible whitish cylindrical structure with 1mm length. MRT was calculated based on the concept of mean time of germination (Labouriau and Osborn 1984). In the end of the essential oil exposure period, roots per rooted microcutting were counted and photographs were taken to measure root length using the software ImageJ 1.45s. Data sets were analysed by one-way ANOVA followed by the Tukey's multiple comparison test. Statistical significance of differences between means of the groups was defined as a *P*-value < 0.05.

Histochemical detection of hydrogen peroxide

Hydrogen peroxide (H_2O_2) production upon the essential oil exposure was assayed by the 3,3'-diaminobenzidine (DAB) staining method (Thordal-Christensen et al. 1997). After the exposure period, *A. thaliana* WT microcuttings were dipped in 1 $mg.mL^{-1}$ DAB solution, pH 3.8, for 2 h. Then, the reaction was stopped by

bleaching microcuttings in boiling ethanol (95%) for 10 min. Microcuttings were photographed under a stereomicroscope, in fresh ethanol, and H_2O_2 was visualized as a reddish-brown colouration.

Results

Schinus terebinthifolius volatiles affected both *A. thaliana* WT and *sur1* mutant adventitious rooting process. Regarding MRT, treatments demonstrated a dose-dependent effect (Fig. 1a and 1b). Compared with control, the highest quantity of oil tested (5 µL) retarded rooting by approximately one day. However, in relation to percentage of rooted microccutings, none of the treatments was affected by volatiles. All microcuttings rooted (100%), as in control groups as in treatments of both lineages (WT and *sur1* mutant).

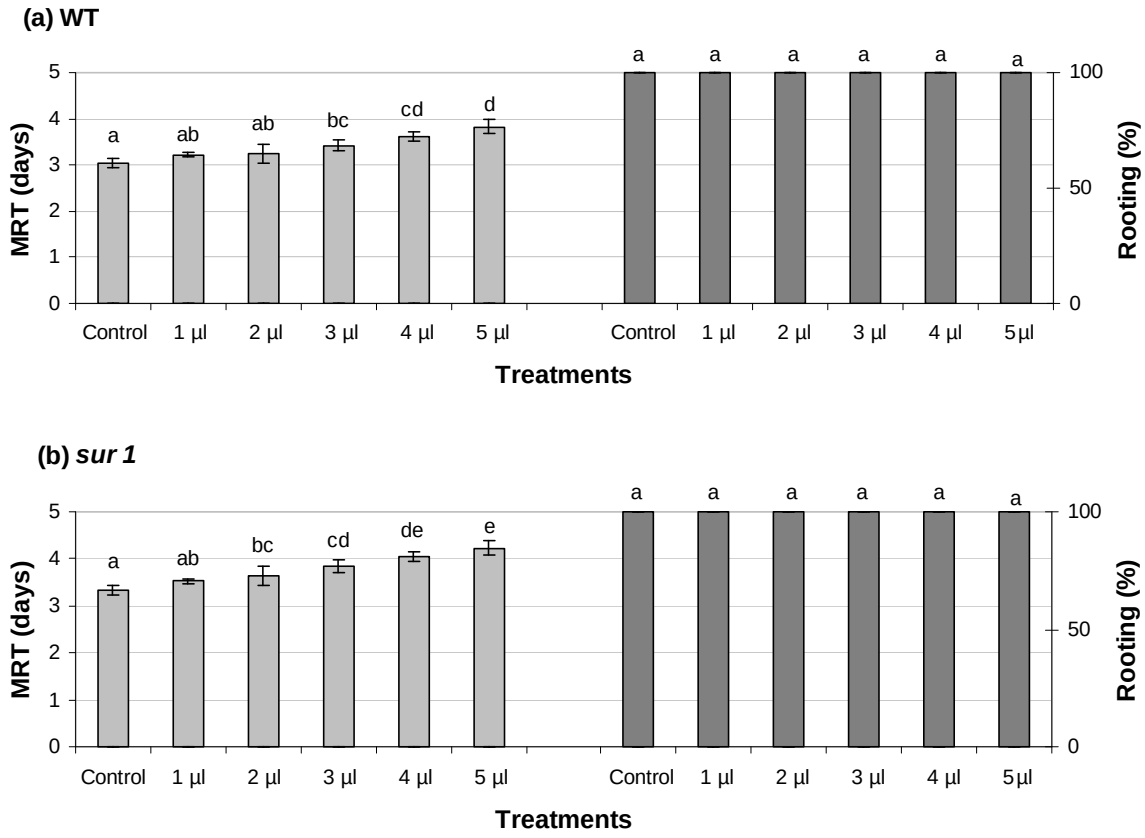

Fig. 1 Mean rooting time (MRT) and percentage of rooting of *Arabidopsis thaliana* Col-0 WT (a) and *sur1* mutant (b) microcuttings exposed to different quantities of *Schinus terebinthifolius* essential oil. Bars with different letters are significantly different according to Tukey's test ($P < 0.05$)

The essential oil did not affect the number of adventitious roots per rooted microcutting in both lineages, but there was a significant inhibitory effect on root length (Fig. 2a and 2b). Compared with control, a root length reduction in WT microcuttings of 25% for 1 µL and 2 µL treatments and about 50% for 3 µL, 4 µL and 5 µL treatments was observed. For mutant lineage, also compared with control, 2 µL of essential oil treatment reduced by 26% the root length and 3 µL, 4 µL and 5 µL treatments reduced about 58% the same parameter.

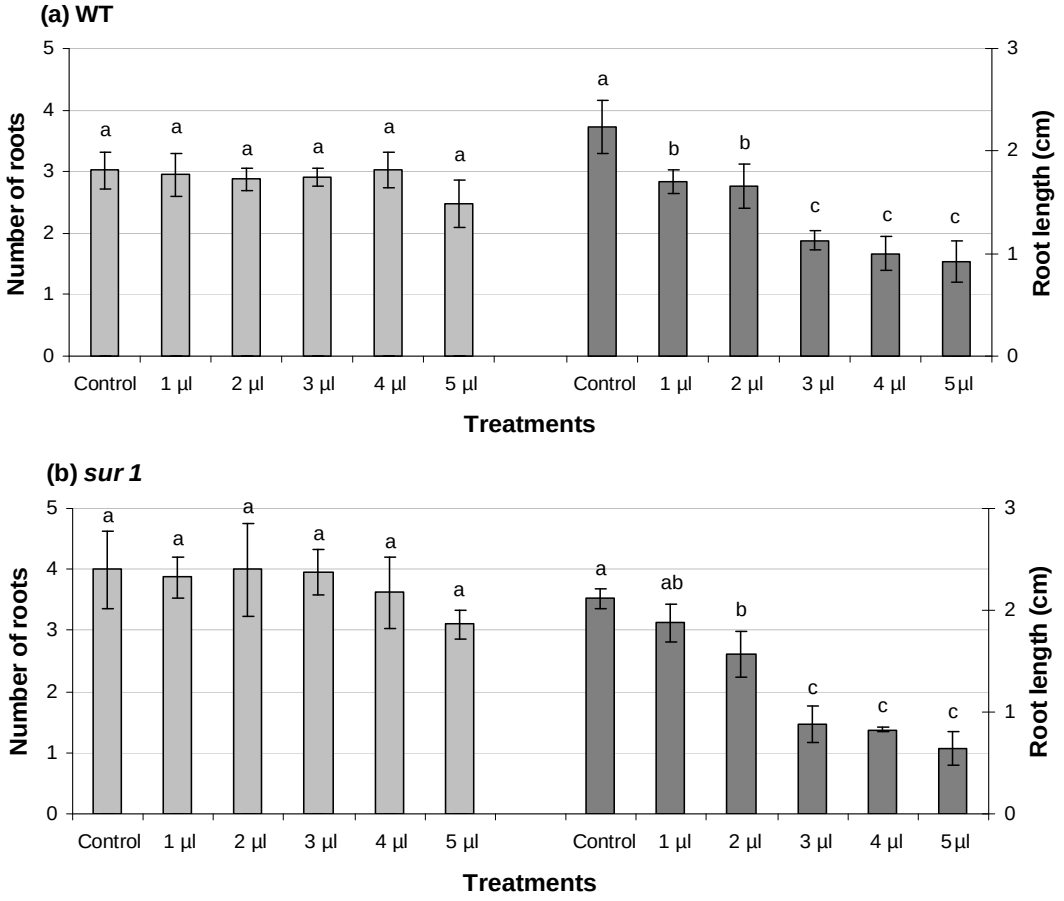

Fig. 2 Mean number of roots and mean root length of *Arabidopsis thaliana* Col-0 WT (a) and *sur1* mutant (b) microcuttings exposed to different quantities of *Schinus terebinthifolius* essential oil. Bars with different letters are significantly different according to Tukey's test ($P < 0.05$)

In experiments that IAA or T were added in the culture medium, IAA treatment did not alter phytotoxic effects caused by volatiles in any parameter. There was no difference in MRT, number of roots and root length between IAA + oil and essential oil treatments (Fig. 3). T + oil did not differ from essential oil treatment regarding MRT and number of roots, but microcuttings recovered in about 12% from the inhibitory effect caused by the essential oil on root length. Considering percentage of rooting,

there was no difference among treatments (100% rooted). IAA and T treatments without application of the essential oil did not affect MRT, percentage of rooting or number of roots of *A. thaliana* microcuttings, but caused differences in root length. IAA treatment did not differ from control or T treatment for this parameter, but the latter differed from control. T treatment exhibited an increasing on root length by 11%.

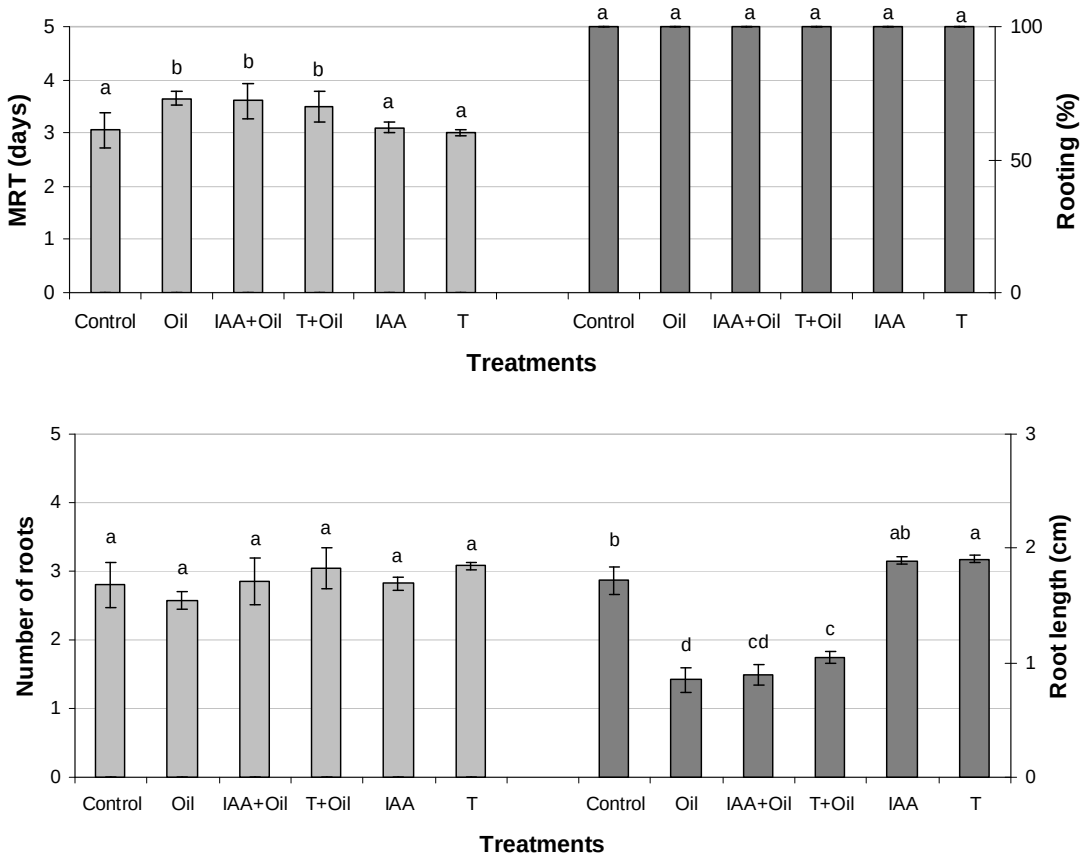

Fig. 3 Mean rooting time (MRT), percentage of rooting, number of roots and root length of *Arabidopsis thaliana* Col-0 WT microcuttings exposed to 5 µL of *Schinus terebinthifolius* essential oil (Oil) in different culture media. IAA = indole-3-acetic acid (1 µM); T = Trolox® (10 µM). Bars with different letters are significantly different according to Tukey's test ($P < 0.05$)

During the experiments, it was observed that cotyledons lost their green color and became yellowish or whitish when exposed to 2 µL, 3 µL, 4 µL and 5 µL of *S. terebinthifolius* essential oil (Fig. 4), indicating that volatiles may be inducing oxidative stress in microcuttings. This observation was confirmed by the positive reaction to DAB, which appeared as a diffuse brownish color in the cotyledon, revealing the formation of H_2O_2 among other free radicals. ROS were more evident in

leaves from 2 µL to 5 µL treatments (Fig. 5). In treatment where T was added, no ROS was detected by DAB reaction (Fig. 6).

Fig. 4 *Arabidopsis thaliana* Col-0 WT microcuttings lost their green color and became yellowish or whitish when exposed to different quantities of *S. terebinthifolius* essential oil. (a) Control, (b) 2 µL, (c) 3 µL, (d) 4 µL, (e) 5 µL

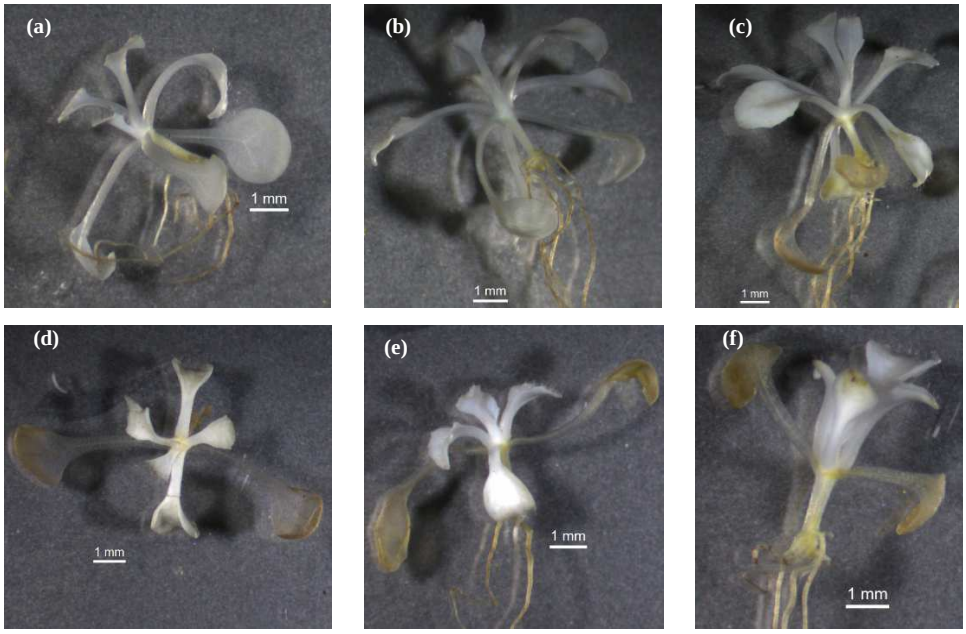

Fig. 5 Localization of hydrogen peroxide (H_2O_2) in *Arabidopsis thaliana* Col-0 WT microcuttings exposed to different quantities of *Schinus terebinthifolius* essential oil. The positive reaction to DAB appeared as a diffuse brownish color in the cotyledon. (a) Control, (b) 1 µL, (c) 2 µL, (d) 3 µL, (e) 4 µL, (f) 5 µL

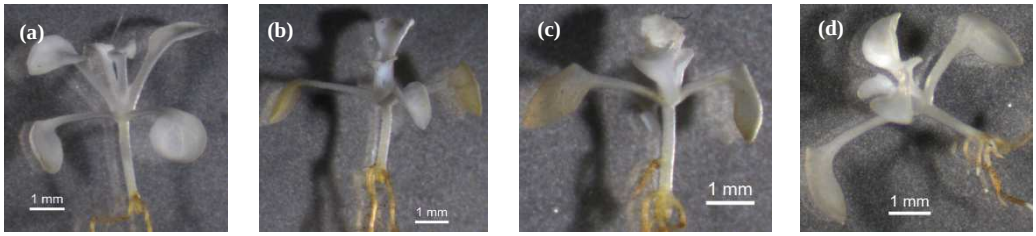

Fig. 6 Localization of hydrogen peroxide (H_2O_2) in *Arabidopsis thaliana* Col-0 WT microcuttings exposed to 5 µL of *Schinus terebinthifolius* essential oil. The positive reaction to DAB appeared as a diffuse brownish color in the cotyledon. (a) Control, (b) essential oil, (c) essential oil and culture medium containing IAA (1 µM), (d) essential oil and culture medium containing Trolox® (10 µM)

Discussion

The present study provides further evidences on phytotoxic activity of *S. terebinthifolius* volatiles on adventitious rooting process. Results indicate that the essential oil acted more strongly on root elongation than on root formation. In *A. thaliana* WT, although MRT did not differ among control, 1µL and 2 µL treatments, seemingly these small amounts of essential oil applied already affected physiological mechanism of root formation, as root length of these treatments differed from control. Bioassay with *sur1* mutant had a WT-like rooting response except in 1 µL treatment, where root length did not differ from control. In a study evaluating distinct modes of adventitious rooting in *A. thaliana*, Correa et al. (2012) observed that auxin-overproducing mutant *sur1* had a WT-like rooting response in de-rooted plant system, which agrees with the results obtained in this study.

Adventitious rooting evaluation after allelochemical exposure in order to comprehend phytotoxic effects on phytohormone balance may present some advantages over primary root analysis. Adventitious rooting allows evaluating root formation with fewer interference. In phytotoxic bioassays, allelochemicals are usually applied on the seed and reported effects may result from a combined action on germination and root protrusion. Application of phytotoxic substances in post-germination experiments is also often. In this case, it is possible to evaluate effects more efficiently on root elongation than on root formation, as post-embryonic root tip is already established. In the present work, adventitious rooting bioassay was useful to evidence that *S. terebinthifolius* essential oil effects were less expressive on root formation than on root elongation. Among phytohormones, auxins play a central role in adventitious root formation (De Klerk et al. 1999; Teale et al. 2005), promoting

lateral root initiation and lateral root primordium development (Fukaki and Tasaka 2009; Péret et al. 2009). According to Della Rovere et al. (2013), *Arabidopsis* adventitious roots originate from cells from the hypocotyl pericycle and establish the quiescent center in the apical meristem similarly as in lateral root primordia. For this, establishment of an auxin gradient is required, that is dependent on auxin transport mediated by auxin efflux PINFORMD (PIN) transmembrane proteins to the pericycle cells. In the present study, IAA treatment did not differ from control. Furthermore, exogenous IAA did not allow the microcuttings to recover from the effects caused by the essential oil. It is possible that this phytohormone requirement was already satisfied on *A. thaliana* microcuttings, what is further supported by the fact that microcuttings reached 100% rooting without exogenous auxin.

Graña et al. (2013) verified that citral, a monoterpene present in essential oils, decreased auxin level in *A. thaliana* roots, although increased auxin in the plant aerial part. The authors suggested that this result indicated that citral interfers in root development problably by the inhibition of polar auxin transport. It is known that essential oils cause membrane disruption, resulting in loss of it integrity (Singh et al. 2009; Poonpaiboonpipat et al. 2013), and this effect can interfere in normal operation of membrane transporters, as auxin carriers. Therefore, according to our results, the delay observed in rooting time when the essential oil was applied may be related to membrane damages, interfering on auxin transport. However, negative effects of volatiles were not so extensive to prevent the establishment of an auxin gradient required for adventitious root formation.

Cotyledons depigmentation observed in microcuttings exposed from 2 µL to 5 µL of *S. terebinthifolius* essential oil is an indicative of oxidative stress. According to Yasar et al. (2008), chlorophyll degradation is a consequence from effects caused by oxidative damage. ROS accumulation induces membrane lipid peroxidation, reducing membrane fluidity and selectivity. Chlorophylls and their binding proteins form complexes into thylakoid membranes and complexes dissociation leads to chlorophyll degradation pathway. Sairam and Srivastava (2000) observed that effects of H_2O_2 treatments on chlorophyll degradation in wheat are dose-dependent. High concentrations of H_2O_2 induce cellular damage that can result in cell death. DAB reaction confirmed that *S. terebinthifolius* volatiles induced H_2O_2 accumulation, which can cause oxidative damage in *A. thaliana* tissues. Moreover, the addition of the antioxidant in standard medium provided greater root length than essential oil

treatment, indicating that the antioxidant Trolox® could minimize effects caused by the essential oil. Plants have evolved non-enzymatic and enzymatic protection mechanisms that efficiently scavenge ROS, responding to oxidative stress (Caverzan et al. 2012; Inzé and Van Montagu 1995). In this way, studies have shown that plants exposed to essential oils or their constituents presented an increase in plant antioxidant activity (Chowhan et al. 2014; Singh et al. 2009). Singh et al. (2006) verified that α-pinene inhibited early root growth and caused oxidative damage in root tissue evidenced by increased malondialdehyde, proline and H_2O_2 contents and by elevated antioxidant enzyme levels. The potential of α-pinene to induce cellular oxidative stress depends on the balance between the generation of ROS and the capacity of the antioxidant defence system (Ishii-Iwamoto et al. 2012). Mutlu et al. (2011) studied the phytotoxic effects of *Nepeta meyeri* Benth. essential oil on germination and antioxidative systems in early seedlings of seven weed species and verified that volatiles increased catalase activity in target species, a known antioxidant enzyme that detoxifies H_2O_2 by breaking it down directly to form H_2O and O_2. In this study, the authors also observed an increase in the H_2O_2 concentration. These studies also demonstrated the increased lipid peroxidation after volatiles exposure, indicating that oxidative stress is responsible by loss of cell integrity.

The generation of ROS has been proposed as a key process shared between biotic and abiotic stress responses (Apel and Hirt 2004; Fujita et al. 2006). Enhanced level of ROS can cause damage to biomolecules such as lipids, proteins and nucleic acids (Sharma et al. 2012). *Schinus* essential oils phytotoxic activity include cytotoxic and mutagenic effects on root meristem of target species (Pawlowski et al. 2012; Pawlowski et al. 2013). Deoxyribonucleic acid (DNA) damages induced by ROS comprise mutagenic alterations, DNA strand breakage and DNA protein crosslinks (Sharma et al. 2012). Considering the wide spectrum of *Schinus* volatiles activity reported in literature and the results presented in this study, phytotoxic effects of *S. terebinthifolius* essential oil on *A. thaliana* adventitious rooting may involve ROS generation and the magnitude of the effects is dose-dependent, interfering in cellular machinery, being evidenced in morphologic level. Effects of ROS accumulation could be detected in short-time effects on rooting time and cotyledon depigmentation and long-time effects on root length. Exogenous antioxidant contributed to a slight recover from the inhibitory effetcs on root elongation, indicating that oxidative stress induced by volatiles was higher than scavenger capacity of *A. thaliana* endogenous

antioxidant system. Phytotoxic effects of *S. terebinthifolius* essential oil on other phytohormones involved in root elongation, as cytokinin, can contribute to the better understanding of volatiles activity on root length.

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4.3 *Schinus lentiscifolius* and *S. molle* essential oils as phytotoxic agents on *Arabidopsis thaliana* adventitious rooting

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Abstract

Essential oils are complex mixtures of volatile components with similar physicochemical characteristics. Phytotoxic effects of *Schinus lentiscifolius* and *S. molle* essential oils were assessed on *Arabidopsis thaliana* adventitious rooting process. Results indicated that volatiles inhibitory activity were more evident on root elongation than on root formation. Volatiles reduced mean rooting time (MRT) and root length, but did not affect the percentage of rooting or number of roots. Both *S. lentiscifolius* and *S. molle* essential oils affected *A. thaliana* microcuttings rooting in a similar manner. Effects caused by the essential oils on MRT decreased in treatments with the addition of kinetin (K). Trolox® (T), a powerful antioxidant, was able to prevent phytotoxic damages on MRT due to *S. lentiscifolius* essential oil. A similar trend was observed to *S. molle* treatment, but differences were not significant. Considering root length, a recovery trend due to K, T and K+T was observed in treatments with *S. lentiscifolius* essential oil, but did not contribute on recovery from damages caused by *S. molle* volatiles. Results indicated that inhibitory activity of *Schinus* volatiles may be explained as a result of multi site action, through reactive oxygen species generation and hormone interference, resulting in phytotoxic effects observed on morphologic level.

Additional key words: volatiles, kinetin, Trolox®, root length.

Abbreviations:

Col-0 – Columbia; CDXs – cytokinin oxidase/dehydrogenases; K – kinetin; MRT – mean rooting time; PIN – PINFORMD; ROS – reactive oxygen species; T – Trolox®; WT – wild type.

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Schinus lentiscifolius March and *S. molle* L. (Anacardiaceae) are species native from South America widely studied due to their medicinal properties related to secondary metabolites production (Gehrke *et al*. 2013, Machado *et al*. 2007). Essential oils and phenolic derivatives are the mainly natural products synthesized by *Schinus* spp. (Santana *et al*. 2012, Zahed *et al*. 2010). Essential oils are often explored for pharmaceutical, industrial and perfume uses (Adorjan and Buchbauer 2010). For the producing organism, these substances present a wide spectrum of biological activities (Bakkali *et al*. 2008), also acting in plant-plant interactions in a phenomenon known as allelopathy (Rice 1984).

Chemically, essential oils are complex mixtures of volatile components with similar physicochemical characteristics (Rubiolo *et al*. 2010). *Schinus lentiscifolius* essential oils are composed mainly by sesquiterpenes (Pawlowski *et al*. 2013, Rossini *et al*. 1996). Otherwise, monoterpenes are the mainly class compound in *S. molle* essential oil (Pawlowski *et al*. 2012, Zahed *et al*. 2010). Despite differences in chemical characterization, volatiles from both species are recognized to interfere on germination and seedling growth of other plants (Pawlowski *et al*. 2012, Pawlowski *et al*. 2013, Zahed *et al.* 2010).

Previous studies demonstrated *S. terebinthifolius* Raddi essential oil was phytotoxic to *Arabidopsis thaliana* (L.) Heynh. adventitious rooting, mainly interfering on root elongation (unpublished data). The present investigation was undertaken to evaluate the phytotoxic effects of *S. lentiscifolius* and *S. molle* essential oils using this same target species. Additionally, the possible recovery capability of microcuttings from the effects of the essential oils was tested by adding a cytokinin and an antioxidant in culture medium.

Schinus lentiscifolius leaves were sampled in natural vegetation in Encruzilhada do Sul City (30° 31' 36.67" S, 52° 31' 6.17" W) and *S. molle* leaves were sampled in natural vegetation in Porto Alegre (30° 1′89 39.73″ S 51° 13′ 43.45″ W), Rio Grande do Sul state, Brazil. At least six plants of each species were sampled. Samples were identified and a voucher of *S. lentiscifolius* (164708) and *S. molle* (164709) was deposited in the herbarium ICN of the Universidade Federal do Rio Grande do Sul, Brazil. The material collected from each species were dried at room temperature, fragmented and subjected to hydrodistillation for 4 h in a Clevenger apparatus (Gottlieb and Taveira-Magalhães 1960). Anhydrous sodium sulfate was employed to eliminate essential oil humidity. Essential oil of each *Schinus* species were sealed

under inert atmosphere and stored in a vial wrapped in aluminum foil in an ultrafreezer (–80 °C) until required.

Seeds of *A. thaliana* Columbia (Col-0) wild type (WT) were surface sterilized and fifteen seeds were sown in Petri dishes containing 3% sucrose, 0.8% (w/v) agar medium and 0.05x MS minerals (Murashige and Skoog 1962), hereafter referred to standard medium. Seeds were stratified for 2 days at 4 °C under continuous darkness and then germinated in a controlled environmental chamber at 20 °C ± 2 °C, 16 h photoperiod and irradiance of 37 µmol m^{-2} sec^{-1} provided by white fluorescent lamps. After seven days, ten microcuttings were obtained from seedlings. Then, the essential oil (1 µL, 2 µL, 3 µL, 4 µL and 5 µL) was applied on filter paper (∅ 1.6 cm) attached to the inner face of the Petri dish lid to avoid direct contact between microcuttings and essential oils. Each treatment was performed in four repetitions. Microcuttings remained exposed to volatiles for seven days.

To evaluate if a cytokinin or an antioxidant allow the microcuttings to recover from the effects caused by essential oils, standard medium was prepared adding 0.5 nM of kinetin (K) or 10 µM of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, known as Trolox® (T). These concentrations were defined according to previous experiments. Kinetin concentration that did not interfere negatively on root formation and Trolox® concentration that demonstrated better performance than the control was chosen. Eigth treatments were conducted: control (standard medium), K (standard medium containing 0.5 nM of kinetin), T (standard medium containing 10 µM of Trolox®), K + T, oil (standard medium; 5 µL of *Schinus* essential oils), K + oil, T + oil, K + T + oil.

Examined parameters included percentage of rooted microcuttings, mean rooting time (MRT), mean length of the longest root (root length) and roots per rooted microcutting (number of roots). Rooting criterion was the presence of at least one visible whitish cylindrical structure with 1mm length. Microcuttings were evaluated every 24 h to calculate MRT, as described in Labouriau and Osborn (1984), based on the concept of mean time of germination. After seven days, roots per rooted microcutting were counted and photographs were taken to measure root length, using the software ImageJ 1.45s. Data sets were analysed by one-way ANOVA followed by the Tukey's multiple comparison test. Statistical significance of differences between means of the groups was defined as a *P*-value ≤ 0.05.

Phytotoxic effects on *A. thaliana* rooting was observed for both essential oils tested. All microcuttings rooted (100%) in all experiments and it was not observed difference on number of adventitious roots among treatments (Table 1). However, *Schinus* volatiles interfered on *A. thaliana* microcuttings retarding MRT in a dose-dependent manner (Fig. 1*A*). Compared with control, 5 µL of *S. lentiscifolius* retarded rooting by approximately one day. The same delay was observed for *S. molle* essential oil in 4 µL and 5 µL treatments. It was also observed a significant inhibitory effect on root length for both essential oils tested (Fig. 1*B*). Compared with control, 1 µL and 2 µL of *S. lentiscifolius* essential oil reduced by 35% the root length whereas 3 µL, 4 µL and 5 µL treatments reduced the same parameter by 50% and 70%, respectively, with no difference between last two treatments. For 1 µL, 2 µL and 5 µL of *S. molle* essential oil, root length was reduced by 19%, 47% and 74%, respectively.

Table 1. Mean number of roots of *Arabidopsis thaliana* microcuttings exposed to different quantities of *Schinus lentiscifolius* (S lent) and *S. molle* (S moll) essential oils

	Numer of roots (mean ± s.d.)	
Treatments	**S lent**	**S moll**
Control	2.3 ± 0.1 [a]	2.4 ± 0.3 [a]
1 µl	2.5 ± 0.2 [a]	3.0 ± 0.3 [a]
2 µl	2.4 ± 0.4 [a]	2.5 ± 0.5 [a]
3 µl	2.7 ± 0.4 [a]	2.6 ± 0.5 [a]
4 µl	2.5 ± 0.1 [a]	2.6 ± 0.6 [a]
5 µl	2.5 ± 0.4 [a]	2.4 ± 0.2 [a]

Means in a column followed by different letters are different according to Tukey's test ($P \leq 0.05$)

Despite different composition according to the literature, both *S. lentiscifolius* and *S. molle* essential oils affected *A. thaliana* microcuttings rooting in a similar manner. Results indicate that essential oils acted more strongly on root elongation than on root formation. This is in agreement with previous results obtained by our research group evaluating *S. terebinthifolius* volatiles (unpublished data). Regarding to MRT, although no difference was observed between control and 1µL of *S. molle* essential oil, this treatment already affected physiological mechanisms of root formation, as root length differed between treatment and control. It was also observed a cotyledon depigmentation on treated microcuttings (Fig. 1*C*, 1*D* and 1*E*).

Cotyledon became yellowish or withish according to essential oil quantity applied, indicating oxidative damage in a dose-dependent manner.

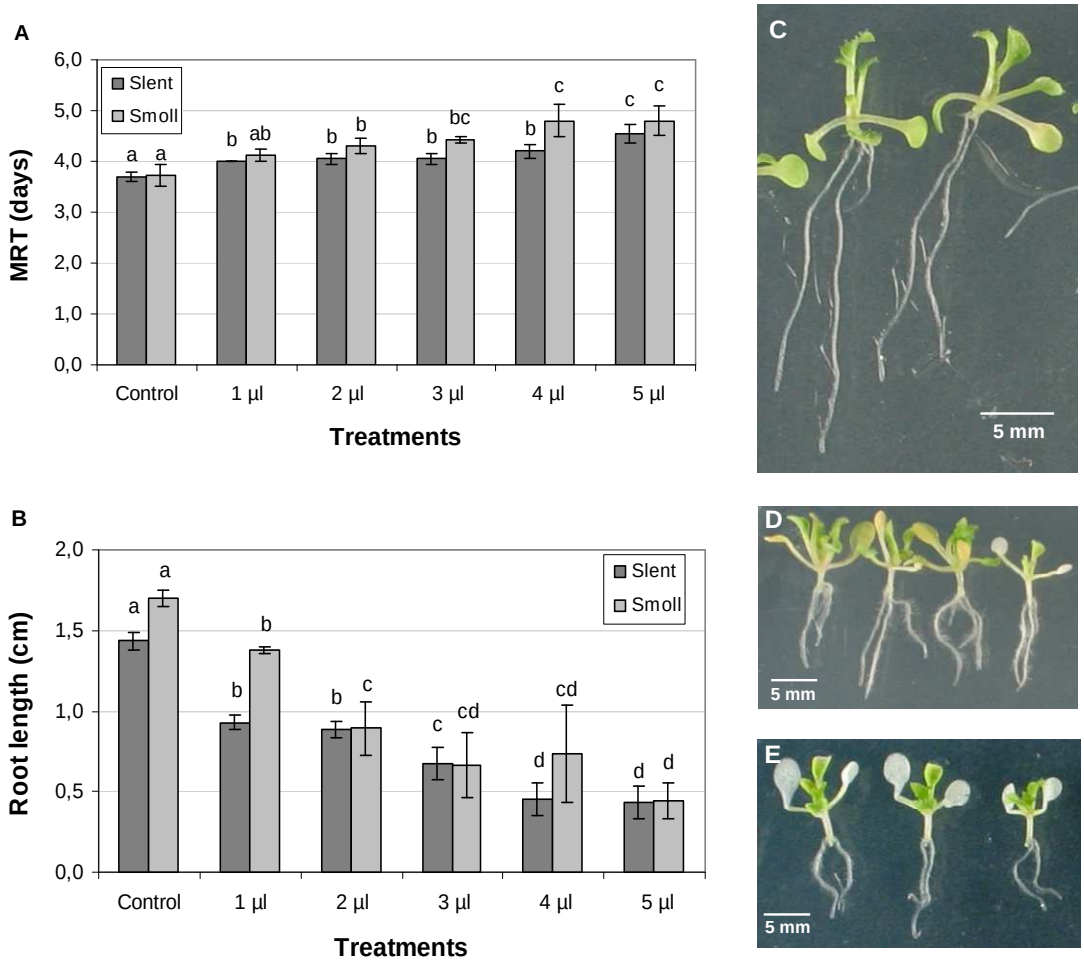

Fig. 1. Mean rooting time (MRT; A) and mean root length (B) of *Arabidopsis thaliana* microcuttings exposed to different quantities of *Schinus lentiscifolius* and *S. molle* essential oils. Cotyledon depigmetation was evidenced on microcuttings exposed to essential oils. Control (C), 2 µL (D) and 5 µL of *S. molle* essential oil (E). Bars with different letters are significantly different according to Tukey's test ($P \leq 0.05$) for the same *Schinus* essential oil

In experiments that K or T were added in the standard medium, *A. thaliana* microcuttings recovered from the phytotoxic effects on MRT when K was applied (Fig. 2*A*). T and K+T also contributed to microcuttings recovery just when *S. lentiscifolius* essential oil was applied. A similar trend was observed to *S. molle* treatment, but differences were not significant. Considering root length, a recovery trend due K, T and K+T was observed in treatments with *S. lentiscifolius* essential oil (Fig. 2*B*). However, exogenous K and T did not recover *A. thaliana* root length when *S. molle* was applied. Once again, all microcuttings rooted (100%) in all experiments

and treatments did not affect the number of adventitious roots per rooted microcutting (Table 2).

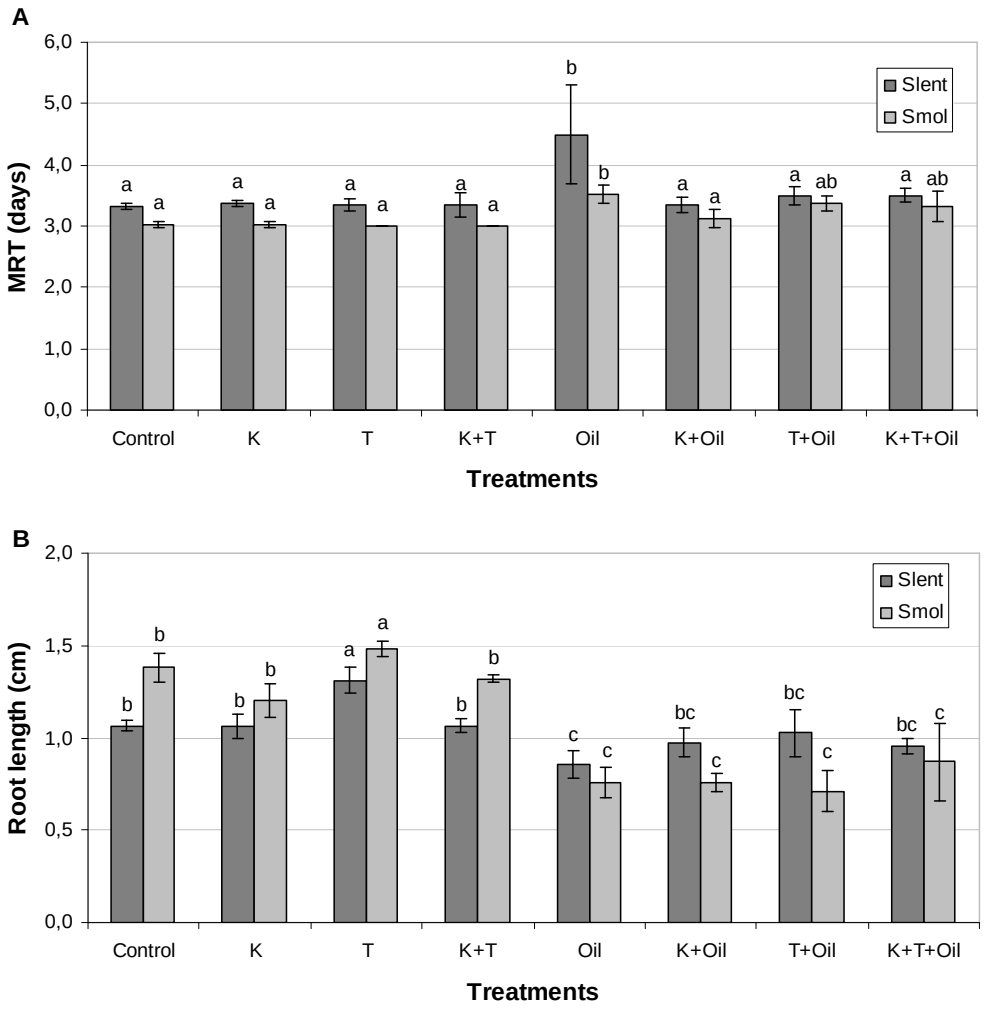

Fig. 2. Mean rooting time (MRT; A) and mean root length (B) of *Arabidopsis thaliana* microcuttings exposed to 5 µL of *Schinus lentiscifolius* or *S. molle* essential oils (Oil). K = kinetin (0.5 nM); T = Trolox® (10 µM). Bars with different letters are significantly different according to Tukey's test ($P < 0.05$) for the same *Schinus* essential oil

Phytotoxic effects observed on morphologic level result from changes in cellular machinery. Different physiological methods assessing the phytotoxic effects of secondary metabolites have been used (Gniazdowska and Bogatek 2005, Weir *et al*. 2004). In recent years, several researches have showed essential oils and their constituents can act as oxidative stress inducers (Chowhan *et al*. 2011, Mutlu *et al*. 2011, Singh *et al*. 2009). Lazarotto *et al*. (2014), evaluating the phytotoxic effects of *Heterothalamus psiadioides* Less. essential oil on *A. thaliana* adventitious rooting, demonstrated that inhibitory effects were due reactive oxygen species (ROS)

generation. In their study, Trolox® did not have effects on prevention or recovery from damages. Similarities and differences observed between the results cited and the present ones can be explained in terms of essential oils chemical composition. *H. psiadioides* essential oil presents high quantities of β-pinene (Lazarotto, 2014), a monoterpene hydrocarbon, whereas *S. molle* presents high quantities of α-pinene (Pawlowski *et al*. 2012), also a monoterpene hydrocarbon. In turn, *S. lentiscifolius* presents high quantities of δ-cadinene (Pawlowski *et al*. 2012), a sesquiterpene hydrocarbon. Despite inhibitory effects observed on *S. lentiscifolius* treatments, it is possible that sesquiterpenes are less phytotoxic than monoterpenes in terms of ROS accumulation, as Trolox® could be efficient to revert negative effects of *S. lentiscifolius* volatiles. Moreover, studies demonstrated that α-pinene (Singh *et al*. 2006) and β-pinene (Chowhan *et al*. 2014) induce oxidative stress in plant tissue.

Table 2. Mean number of roots of *Arabidopsis thaliana* microcuttings exposed to 5 µL of *Schinus lentiscifolius* (S lent) and *S. molle* (S moll) essential oils (Oil). K = kinetin (0.5 nM); T = Trolox® (10 µM)

	Numer of roots (mean ± s.d.)	
Treatments	**S lent**	**S moll**
Control	2.2 ± 0.1 [a]	2.3 ± 0.1 [a]
K	2.0 ± 0.1 [a]	2.4 ± 0.2 [a]
T	2.2 ± 0.2 [a]	2.3 ± 0.2 [a]
K+T	2.1 ± 0.2 [a]	2.4 ± 0.1 [a]
Oil	2.1 ± 0.1 [a]	2.2 ± 0.2 [a]
K+Oil	2.1 ± 0.1 [a]	2.4 ± 0.1 [a]
T+Oil	2.1 ± 0.1 [a]	2.1 ± 0.1 [a]
K+T+Oil	2.0 ± 0.1 [a]	2.1 ± 0.1 [a]

Means in a column followed by different letters are different according to Tukey's test (P ≤ 0.05)

Other studies have verified that secondary metabolites induce disturbances in hormonal balance (Bogatek and Gniazdowska 2007, Graña *et al*. 2013). Several phytohormones pathways are involved in regulation of root development, with auxin and cytokinin being the principal players (Růžička *et al*. 2009). *Arabidopsis* adventitious roots are derived from pericycle cells adjacent to xylem poles (Della Rovere *et al*. 2013). Establishment of an auxin gradient based on auxin transport from the apex to the base is required (Muday and DeLong 2001). Cytokinins are plant hormones that regulate plant cell division (Riou-Khamlichi *et al*. 1999). Exogenous or endogenous modification of cytokinin levels perturb the expression of

several auxin efflux carrier genes from the PINFORMD (PIN) family, preventing the formation of an auxin gradient, disrupting root formation (Růžička *et al*. 2009). In the present work, exogenous kinetin added to the standard medium did not inhibit lateral root formation, indicating that the concentration used was appropriate. Furthermore, kinetin was able to recover phytotoxic effects caused by volatiles on MRT, indicating that essential oils are acting interfering on cytokinin responses. Cytokinin homeostasis is controlled by its catabolism process through cytokinin oxidase/dehydrogenases (CKXs) (Su *et al*. 2011). This enzyme catalyses the oxidation of cytokinin substrates bearing unsaturated isoprenoid side chains, using molecular oxygen as the oxidant (Hare and Van Staden 1994). Previous studies demonstrated that essential oils, including volatiles from *Schinus*, induced cytotoxic and genotoxic effects on primary root meristem (Pawlowski *et al*. 2012, Pawlowski *et al*. 2013, Schmidt-Silva *et al*. 2011). Volatiles interference on cytokinin could explain these alterations on cell division.

Results indicated that *S. lentiscifolius* and *S. molle* essential oils acted as phytotoxic agents on *A. thaliana* adventitious rooting. This study demonstrated that volatiles inhibitory activity can be explained as a result of multi site action. Several studies evaluating different essential oils, each one with specific composition, have demonstrated that they can act inducing oxidative stress. We proposed that the mechanism of *Schinus* volatiles action may occur via ROS generation, interfering, among other possible cellular sites, on cytokinin activity, decreasing root growth.

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5 CONSIDERAÇÕES FINAIS

O presente estudo demonstrou a atividade fitotóxica dos óleos essenciais de *S. lentiscifolius*, *S. molle* e *S. terebinthifolius* sobre a germinação, o crescimento inicial e o enraizamento adventício de *A. thaliana*. A adição de fitormônios e de antioxidantes no meio de cultivo foi uma abordagem nova, proposta pelo nosso grupo de pesquisa. Diversos trabalhos têm apresentado que os óleos essenciais e seus constituintes afetam o desenvolvimento das espécies alvo ao atuarem como indutores de estresse oxidativo. Esses estudos baseiam suas conclusões principalmente em efeitos observados sobre a atividade de enzimas antioxidantes e a produção de espécies reativas de oxigênio (EROs). A metodologia de adição de Trolox® ao meio de cultivo é uma técnica nova que também demonstra que os óleos essenciais interferem no desenvolvimento de outras plantas por esse mecanismo. Além disso, a detecção histoquímica de H_2O_2 evidenciam que os óleos essenciais de *Schinus* induzem a produção de EROs.

A técnica de PCR em tempo real não foi conclusiva para os experimentos realizados, mas não pode ser descartada. A ausência de efeitos não deve ser considerada apenas sob o ponto de vista de ‘obtenção de resultados negativos’ ou discordantes do esperado. Conforme amplamente demonstrado na literatura e também pelos resultados do presente estudo, os efeitos fitotóxicos dos óleos essenciais sobre o fenótipo da espécie alvo está claramente relacionado ao estresse oxidativo. Também conforme consta na literatura, mecanismos celulares de defesa são induzidos por baixas concentrações de peróxido de hidrogênio. Desse modo, o que se pode inferir é que a quantidade de óleo essencial utilizada no experimento, mesmo sendo baixa se comparada aos demais estudos desenvolvidos, ainda é uma quantidade alta e possivelmente atue na produção elevada de EROs que, por sua vez, levam aos danos celulares que resultam em menor crescimento da planta.

Os estudos com aleloquímicos geralmente testam seus efeitos sobre o desenvolvimento da raiz primária das plantas alvo. Muito foi questionado sobre a relevância do presente estudo ao avaliar os efeitos fitotóxicos sobre a formação da raiz adventícia. Obviamente, a formação da raiz primária e da raiz adventícia apresentam peculiaridades em cada um dos processos. Entretanto, ambas necessitam do balanço auxina-citocinina para a correta formação da raiz. Estudar a raiz adventícia com o intuito de compreender o efeito dos voláteis sobre esses

hormônios na formação da raiz é possível ao avaliar o enraizamento adventício, mas impossível ou no mínimo muito complicado ao se considerar a formação da raiz primária pois, para estudá-la, invariavelmente o aleloquímico deverá ser aplicado na semente, e o efeito verificado resultará de uma ação conjunta sobre o processo de germinação e de formação da raiz, envolvendo inclusive outros hormônios. Uma prática recorrente é a aplicação do aleloquímico em experimentos pós-germinação. Nesse caso, o efeito da substância apenas será verificado sobre o crescimento da raiz.

Por fim, de um modo geral, espera-se que os efeitos fitotóxicos sejam sempre danosos e deletérios. E, quanto maior o efeito inibitório, mais crédito o estudo apresenta. Isso é válido quando o objetivo é descobrir novas substâncias com potencial para serem aplicadas no controle de pragas e plantas daninhas. Entretanto, se o objetivo for compreender o modo de ação da substância, testar concentrações de aleloquímicos inferiores, nas quais não sejam evidenciados efeitos deletérios, podem ajudar a elucidar o mecanismo molecular de resposta ao aleloquímico antes de ocorrer o dano, geralmente letal. Tal abordagem permite a compreensão do metabolismo da planta alvo, podendo-se verificar se a planta responde ou não ao produto natural. A toxidez de agentes bióticos e abióticos, orgânicos e inorgânicos em plantas relacionada com a produção de EROs está bem documentada na literatura. Entretanto, o modo de ação desses agentes permanece aberto para discussões.

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