

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA

DISSERTAÇÃO DE MESTRADO

Fitotoxicidade do óleo essencial de *Heterothalamus psiadioides* Less.
sobre *Arabidopsis thaliana* (L.) Heynh

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Porto Alegre, abril, 2014

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Botânica, do Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como parte dos requisitos para obtenção do título de Mestre em Botânica.

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Porto Alegre, abril, 2014

AGRADECIMENTOS

Agradeço ao meu orientador Geraldo L. G. Soares por todo o auxílio científico e psicológico que me deu nesses dois anos. Sempre preocupado com meu estado mental – o que é raro no meio acadêmico! – fazia tudo o que estivesse ao seu alcance para tornar nosso relacionamento o melhor possível.

Agradeço aos amigos e parentes (impossível mencionar todos aqui) pelo apoio psicológico, pelas festas e conversas. Especialmente aos meus pais Milton e Carmen, por rezarem constantemente pelo bom andamento dos meus estudos e pela minha segurança na “enorme” Porto Alegre! Agradeço também ao meu esposo Luís F. Zanchet que sempre foi compreensivo em relação aos meus objetivos de vida e suportou ficar a 450 km de distância. Nosso amor me motiva a enfrentar qualquer dificuldade.

Agradeço à Cleusa V. Ely e Fernanda M. Nogueira, grandes parceiras de lar, pelo apoio psicológico e pelas conversas filosóficas sobre os mais variados assuntos e é claro, a respeito das nossas pesquisas. Novamente, agradeço à Cleusa pelo auxílio na coleta de *Heterothalamus psiadioides*.

Agradeço aos colegas de Laboratório de Ecologia Química e Quimiotaxonomia por todo apoio moral e técnico! Esta conquista vem do empenho de cada constituinte do laboratório. À Luíza Machado pelo auxílio com os materiais para os experimentos e no cuidado com as plantas de *Arabidopsis thaliana*, também chamadas carinhosamente como “arabis”. À Ângela Pawlowski pelo auxílio com as técnicas para montagem dos experimentos, discussões, revisões de texto e tudo mais que se possa imaginar. Sem a ajuda dela essa dissertação teria apenas metade do conteúdo que tem atualmente. Em especial à Ane, ou melhor, Eliane R. da Silva pela amizade constante desde a graduação. Obrigada por ter me ajudado a encontrar o nosso orientador e ainda, por revisar muito criticamente cada texto que lhe mandei, por compartilhar ideias, anseios e metas ao longo desses dois anos. Enfim, com essas pessoas eu obtive um enorme crescimento tanto pessoal como profissional e sou muito grata.

À banca examinadora por aceitarem participar desta defesa, e antecipadamente pelas sugestões que sem dúvida enriquecerão esta dissertação.

Agradeço à CAPES pela bolsa e ao PPG Botânica pela minha formação como mestre.

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RESUMO

O arbusto *Heterothalamus psiadioides* Less. (Asteraceae) ocorre no sul do Brasil e no Uruguai, se estabelecendo frequentemente em formações densas. Esse padrão de estabelecimento pode estar relacionado a efeitos alelopáticos dos voláteis liberados por essa planta. Portanto, este trabalho objetivou investigar os efeitos do óleo essencial de *H. psiadioides* sobre a germinação, crescimento inicial, enraizamento adventício e desenvolvimento vegetativo e reprodutivo de *Arabidopsis thaliana* (L.) Heynh., planta-alvo desse estudo. Além disso, a caracterização química do óleo essencial foi realizada. Para avaliar efeitos sobre a germinação e o crescimento inicial, sementes e plântulas de *A. thaliana* foram expostas aos voláteis de *H. psiadioides*, cuja composição química foi analisada através de cromatografia em fase gasosa associada à espectrometria de massas. Para avaliar efeitos sobre o enraizamento adventício, plântulas de *A. thaliana* tiveram suas raízes primárias cortadas, gerando microestacas que foram expostas aos voláteis. Além disso, tratamentos para recuperação dos efeitos causados pelos voláteis foram utilizados – com adição de auxina, citocinina e antioxidante ao meio de cultura. No experimento que testou os efeitos dos voláteis sobre o desenvolvimento reprodutivo e vegetativo da planta-alvo, as plantas foram expostas aos voláteis dentro de câmaras de vidro. Após a exposição e quando as plantas senesceram, parâmetros vegetativos e reprodutivos foram avaliados. A germinação e o crescimento inicial da planta-alvo foram afetados negativamente pelos voláteis de *H. psiadioides*. Da mesma forma, o enraizamento adventício das plantas foi afetado. Microestacas da planta-alvo expostas aos voláteis demoraram mais tempo para enraizar, desenvolveram menos raízes e essas eram mais curtas do que as microestacas controle. Estresse oxidativo foi detectado nas microestacas expostas aos voláteis; além disso, hormônios e antioxidante não reverteram os efeitos fisiológicos negativos causados pelos mesmos. A avaliação do desenvolvimento vegetativo e reprodutivo das plantas expostas aos voláteis nas câmaras de vidro permitiu detectar efeitos negativos dos voláteis apenas em um parâmetro relacionado ao crescimento, que foi mensurado logo após o período de exposição. Nenhum outro parâmetro diferiu significativamente entre plantas expostas aos voláteis e não expostas. Esses resultados indicam que apesar dos drásticos efeitos demonstrados pelos voláteis sobre sementes e plântulas, efeitos pouco relevantes foram encontrados para o desenvolvimento vegetativo e reprodutivo. Portanto, possivelmente o potencial alelopático de *H. psiadioides* pode ser menor do que o esperado, já que plantas juvenis a adultas são capazes de recuperar-se dos efeitos dos voláteis dessa planta.

Palavras-chave: voláteis; desenvolvimento inicial, vegetativo e reprodutivo; fitotoxidez

ABSTRACT

The shrub *Heterothalamus psiadioides* Less. (Asteraceae) occurs in Southern Brazil and Uruguay, where it often establishes in dense populations. This pattern of establishment may be related to allelopathic effects of the volatiles released by this plant. Therefore, this study aims to investigate the effects of the volatile essential oil of *H. psiadioides* on germination, early growth, adventitious rooting and vegetative and reproductive development of *Arabidopsis thaliana* (L.) Heynh., the target plant of this study. Furthermore, the chemical characterization of the essential oil was performed. To assess effects on germination and early growth, seeds and seedlings of *A. thaliana* were exposed to *H. psiadioides* volatiles, which had the chemical composition analyzed by gas chromatography coupled to mass spectrometry. To evaluate effects on adventitious rooting, seedlings of *A. thaliana* had the primary roots excised, generating microcuttings that were exposed to the volatiles. Moreover, treatments to recovery the effects caused by the volatiles were used – with addition of auxin, cytokinin and antioxidant in the culture medium. In the experiment that tested the effects of the volatiles on the vegetative and reproductive development of the target plant, plants were exposed to the volatiles inside of glass chambers. After the exposition and at senescence, vegetative and reproductive parameters were evaluated. The germination and early growth were negatively affected by the volatiles. Adventitious rooting was similarly affected. Microcuttings of the target plant exposed to the volatiles were slower to root, less developed and had shorter roots in relation to control microcuttings. Oxidative damage was detected in the microcuttings exposed to the volatiles; furthermore hormones and antioxidant did not revert negative physiological effects caused by the oil. The evaluation of vegetative and reproductive development of plants exposed to the volatiles inside of glass chambers allowed to detect negative effects just in a parameter measured immediately after removal plants from the chambers. Other parameters did not differ between plants exposed to the volatiles and plants not exposed. These results indicate that despite of drastic effects showed by the volatiles on seeds and seedlings, effects of little relevance were found in vegetative and reproductive development. Therefore, it is possible that allelopathic potential of *H. psiadioides* may be lower than expected, since juvenile and adult plants are able to recover from the effects of the plant volatiles.

Keywords: volatiles; early, vegetative and reproductive development; phytotoxicity

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1 INTRODUÇÃO

1.1 Alelopatia

A alelopatia era definida previamente como interações químicas com a liberação de metabólitos secundários que possuem efeitos tanto benéficos quanto prejudiciais entre plantas e também com os microorganismos (Molisch 1937 apud Rice 1974). Rice na primeira edição do seu livro “Allelopathy” (Rice 1974) definiu a alelopatia similarmente a Molisch, porém excluindo os efeitos benéficos das interações, o que foi reiterado na segunda edição do mesmo (Rice 1984). Já para a Sociedade Internacional de Alelopatia em 1996, esse fenômeno foi definido como “qualquer processo com liberação de metabólitos secundários produzidos por plantas, algas, bactérias e fungos que pode influenciar o crescimento e desenvolvimento dos sistemas biológicos e agriculturáveis”.

Aleloquímicos são substâncias provenientes do metabolismo secundário das plantas e podem estar envolvidos em interações químicas entre plantas. Essas interações ocorrem através da liberação de aleloquímicos por uma planta, que podem causar efeitos prejudiciais para o desenvolvimento de outras plantas nas proximidades (Lambers et al. 2008). As interações alelopáticas entre plantas ocorrem quando há liberação de substâncias fitotóxicas no ambiente e quando as mesmas podem ser detectadas em campo e outros fatores bióticos ou abióticos não sejam responsáveis por elas (Wardle et al. 1998). Os aleloquímicos consistem de uma ampla gama de substâncias tais como compostos fenólicos, alcaloides, glicosídeos cianogênicos, flavonoides e compostos aromáticos (Lambers et al. 2008). A liberação dos aleloquímicos no ambiente pode ocorrer através de volatilização, exsudação radicular, lixiviação e decomposição de material vegetal (Macías et al. 2007). Chegando ao solo ou ao ar, os aleloquímicos podem causar efeitos alelopáticos como implicações sobre comunidades, afetando a diversidade de plantas, densidade e sua dominância em um ambiente (Chou 1999).

Devido à diversificada constituição química dos aleloquímicos seus mecanismos de ação também apresentam variação. Eles podem prejudicar a divisão, alongamento e ultraestrutura celular, alterar os níveis hormonais e a síntese de moléculas tais como o DNA e o RNA, a permeabilidade da membrana celular, a captação mineral e de água, podem inibir ou estimular a atividade de enzimas (Rice 1984), podem alterar o rendimento fotossintético e produzir altos níveis de espécies reativas de oxigênio (EROs), assim como outros efeitos mediados por essas moléculas, como por exemplo a morte celular programada (Weir et al. 2004). Já a concentração dos aleloquímicos nos tecidos da planta e, conseqüentemente, a sua emissão podem variar de acordo com a fase fenológica da planta, incidência de luz, estresse hídrico, poluição, disponibilidade de nutrientes no solo e herbivoria (Lambers et al. 2008).

1.2 Óleos essenciais e sua importância em interações alelopáticas

Óleos essenciais são produtos naturais produzidos pelas plantas que consistem de misturas complexas de substâncias tais como mono e sesquiterpenos e fenilpropanoides entre outras substâncias lipofílicas de baixo peso molecular. Sua produção pode variar de acordo com a temperatura, insolação, frequência e magnitude de precipitações, qualidade do solo e pH, estresse hídrico, estresse por herbivoria e micro-organismos patógenos. Já o armazenamento dos óleos essenciais pode ser feito em diferentes estruturas como os idioblastos (células secretoras), tricomas glandulares, ductos e cavidades (Baser & Buchbauer 2010).

A volatilização dos óleos essenciais ocorre facilmente em condições naturais já que são constituídos por compostos de baixo peso molecular. Entretanto, monoterpenos são metabólitos em geral mais voláteis do que sesquiterpenos (Baser & Buchbauer 2010). Outro mecanismo de entrada de substâncias contidas em óleos essenciais no ambiente é via solubilização desses metabólitos pela chuva e orvalho, que podem levá-los até o solo. A solubilidade de óleos essenciais em água depende da proporção de derivados oxigenados, o que interfere diretamente na polaridade da mistura, ou seja, quanto maior a polaridade dos constituintes, mais solúvel pode ser o óleo essencial (Guenter 1972). Essa regra também é válida para monoterpenos, quando estudados separadamente (Weidenhamer et al. 1993).

Tendo em vista que as substâncias fitotóxicas dos óleos essenciais podem ser volatilizadas ou parcialmente miscíveis em água, plantas que os produzem podem apresentar potencial alelopático. Quando os voláteis são carregados pelo ar e entram em contato com as folhas de plantas próximas à planta emissora, podem entrar pela epiderme e estômatos, entrando nas células (Rice, 1984). Lixiviados pela água da chuva ou orvalho, os voláteis são levados até o solo, onde podem ser integrados à solução do solo e absorvidos pelas raízes de plantas próximas (Singh et al. 2003). Os principais efeitos sobre plantas relacionados com a ação dos terpenos são na germinação e crescimento inicial (Graña et al. 2013, Singh et al. 2002, Singh et al. 2006, Singh et al. 2009), inibição de índices mitóticos (Graña et al. 2013, Nishida et al. 2005), alteração no transporte hormonal, desorganização da ultraestrutura celular (Graña et al. 2013), redução da biomassa seca (Singh et al. 2002, Singh et al. 2009), diminuição do conteúdo de clorofila, redução da respiração celular (Singh et al. 2002), indução de estresse oxidativo (Singh et al. 2006, Singh et al. 2009) e diminuição da síntese de DNA (Nishida et al. 2005).

1.3 *Heterothalamus psiadioides* Less.

O arbusto da família Asteraceae, *Heterothalamus psiadioides* Less., é uma planta perene que cresce no sul do Brasil e no Uruguai (Deble et al. 2005). A planta tem em média 1 m de altura, com flores numerosas e pequenas sementes, florescendo de agosto a dezembro (Barroso et al. 1999). Na região dos Campos sulinos, *H. psiadioides* se estabelece em manchas densas e esse padrão de estabelecimento da população pode ser devido a vários fatores, dentre eles porque os frutos dessa espécie não possuem estruturas de dispersão (e.g. pappus) e por isso as sementes tendem a ser dispersas próximo à planta mãe (Fidelis et al. 2010) ou por causa dos aleloquímicos presentes nos voláteis e lixiviados dessa planta. O potencial alelopático de *H. psiadioides* já foi demonstrado por outros estudos que evidenciaram efeitos inibitórios dos voláteis sobre a germinação, crescimento inicial (Silva 2014, Schmidt-Silva 2012) e mitose (Schmidt-Silva et al. 2011) de diferentes espécies-alvo (plantas que são expostas aos aleloquímicos). Portanto, esse estudo valida o conhecimento sobre a fitotoxidez de *H. psiadioides* sobre a germinação e crescimento inicial e também testa o efeito dos voláteis dessa planta sobre o enraizamento adventício, processo do desenvolvimento vegetal que ainda não havia sido investigado para estudos de fitotoxidez e atualmente é estudado pelo nosso grupo de pesquisa. Além disso, uma nova contribuição é feita aos estudos de fitotoxidez: o efeito de substâncias fitotóxicas voláteis sobre o desenvolvimento vegetativo e reprodutivo de uma planta-alvo.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar o potencial alelopático dos voláteis do óleo essencial de *H. psiadioides* sobre a germinação, crescimento inicial, enraizamento adventício e desenvolvimento vegetativo e reprodutivo da planta alvo *Arabidopsis thaliana* (L.) Heynh., utilizada como planta modelo nesse trabalho.

2.2 Objetivos Específicos

- Determinar os efeitos do óleo essencial de *H. psiadioides* sobre a germinação e crescimento inicial da planta-alvo *A. thaliana* e realizar a caracterização química do óleo essencial.
- Avaliar efeitos sobre o enraizamento adventício da planta-alvo exposta aos voláteis do óleo essencial de *H. psiadioides*.
- Investigar os efeitos dos voláteis do óleo essencial de *H. psiadioides* sobre parâmetros vegetativos e reprodutivos da planta-alvo exposta aos voláteis em diferentes fases do seu desenvolvimento.

3 ARTIGO I

Phytotoxic activity and chemical composition of *Heterothalamus psiadioides* (Asteraceae) essential oil¹

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Highlights

Heterothalamus psiadioides is an Asteraceae shrub of South Brazilian grasslands.

H. psiadioides essential oil has phytotoxic activity.

Germination and early growth are severely affected by *H. psiadioides* essential oil.

The major chemical compound of the *H. psiadioides* essential oil is β -Pinene.

¹Este trabalho será submetido à Revista South African Journal of Botany

Abstract

Volatile compounds are relevant to chemical interactions among plants, as allelopathy. *Heterothalamus psiadioides* Less. an Asteraceae shrub, produces a range of these compounds that may confer advantages to its establishment in South Brazilian grasslands. Thus, we evaluated the phytotoxicity of *H. psiadioides* essential oil on germination and early growth of *Arabidopsis thaliana* (L.) Heynh. Bioassays were conducted with seeds and seedlings of *A. thaliana* growing on culture medium and exposed to the oil (5, 10, 15, 20 e 25 μ L) inside of Petri dishes. The parameters evaluated were: germination rate, speed of accumulated germination, root and shoot length. Besides, we assess the chemical composition of *H. psiadioides* essential oil, to know which substances may be involved in its effects. All measured parameters were significantly affected by the oil. Germination rate was extremely low 53.91%, 33.92%, 16.52%, 14.79%, and 12.18% and speed of accumulated germination was delayed in 81.52%, 87.89%, 94.35%, 95.79% and 94.16% at respectively, 5, 10, 15, 20 and 25 μ L of oil. Root and shoot length were reduced until 96% and 75.61%, respectively. The chemical composition of the oil showed that it is mainly characterized by monoterpenes hydrocarbons and β -pinene (44.65%) was the major compound. Therefore, the results indicate that the volatiles emitted by the shrub *H. psiadioides* exhibit allelopathic potential and are able to affect germination and early growth of target plants. Thus, these compounds may also affect co-occurring plants, which may explain its pattern of establishment.

1 Introduction

Allelopathy consists in the release of secondary metabolites by a plant into the environment, causing negative effects on other plants (Lambers et al., 1998). These molecules, also referred as allelochemicals, are released into the environment through volatilization, root exudation, leaching and decomposition of plant material (Macías et al., 2007). Shrubs of the Asteraceae family are known to produce volatiles that may be involved in allelopathic interactions (e.g. Ibáñez and Zoppolo, 2011; Li et al., 2011). Essential oils are mixtures of low weight terpenes produced through secondary metabolism. These compounds can vary in a broad range of volatility and lipophilicity according to their molecular weight and polarity (Langenheim, 1994). In natural conditions, these compounds are released by volatilization or carried into the soil by rainfall and dew, because their relative water solubility (Weidenhamer et al., 1993).

Heterothalamus psiadioides Less. is an Asteraceae shrub that grows in south of Brazil and Uruguay (Deble et al., 2005) forming dense and dominant populations. This landscape pattern may

indicative that this species release phytotoxic compounds in the surrounding environment. The phytotoxic effects of volatiles released directly from fresh and dry leaves of this species have been recently studied by our research group (Silva et al., 2014) and showed negative effects on germination and early growth of target plants. Furthermore, the essential oil of *H. psiadioides* was able to affect mitotic activity in cells of lettuce and onion root meristem (Schmidt-Silva et al., 2011). Despite of the compounds present in *H. psiadioides* essential oil have already been identified (Suyenaga et al., 2004), the chemical composition of plants can vary according to several factors, as growth stages, harvesting time, edaphic and climatic factors (Batish et al., 2008). Thus, characterization of the essential oil used to perform bioassays is relevant to relate the actual chemical composition with the effects observed. Therefore, we aimed to evaluate if the essential oil of *H. psiadioides* could cause negative effects on germination and early growth of other plants using *Arabidopsis thaliana* (L.) Heynh. as target species. In addition, to verify which substances may be involved in the effects of the oil we also assessed the chemical composition of *H. psiadioides* essential oil.

2 Materials and methods

2.1. Germination and early growth bioassays

To evaluate the phytotoxicity of *H. psiadioides* essential oil, experiments assessing the oil effects on germination and growth of *A. thaliana* were conducted. According to Meinke et al. (1998), *A. thaliana* is a model species for physiological, biochemical and plant development studies, being related by some researches (Pennacchio et al., 2005; Graña et al., 2013) as a sensitive species to this type of assessment. The experiments were performed with seeds of *A. thaliana* wild type (Col-0) originally obtained from the *Arabidopsis* Biological Resource Center (ABRC) and granted by Laboratory of Plant Physiology (UFRGS). Germination and growth bioassays employed Petri dishes containing culture medium with 0.05x MS minerals (Murashige and Skoog, 1962), 30 g.L⁻¹ sucrose and 8 g.L⁻¹ agar, adjusted to pH 5.8 ± 0.1 prior to autoclaving at 121 °C and 1.5 atm for 20 min. Seeds of *A. thaliana* were disinfected by soaking seeds for 1 min in 70% ethanol and 15 min in 1.5% NaClO (v/v) with a drop of commercial detergent, followed by four washes in sterilized distilled water.

For the germination experiment, 30 seeds of *A. thaliana* were sown on culture medium; the plates were wrapped with PVC transparent film and stored for 2 days in darkness at 4 °C, to synchronize seeds germination. Afterwards, *H. psiadioides* essential oil was applied on a piece of cotton fixed with double-sided tape in the inner face of the plate lid. It was done to avoid direct

contact between seeds and the essential oil, allowing the oil to volatilize within the airspace of the plates. The amounts of oil applied were: 5, 10, 15, 20, 25 μL and the control had no oil application, with four replicates. Then, the plates were wrapped and placed in a growth room, where they stayed for 7 days at 21 °C with a photoperiod of 16 h per day and irradiance of 33.16 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided for fluorescent lamps (20 W). The germinated seeds were recorded every 24 h to calculate the speed of accumulated germination (AS), according to the formula: $AS = [N1/1 + N2/2 + \dots + Nn/n]$, where $N1, N2, N3, Nn$ are the cumulative number of seeds which germinate on day 1, 2, 3, ..., n (Anjum and Bajwa, 2005). The germination rate (%) was calculated as the number of germinated seeds at the last day of the experiment in relation to the number of seeds which were put to germinate.

The early growth experiment was conducted similarly to the germination bioassay. Fifteen seeds of *A. thaliana* were sown on culture medium, transferred to growth room for 48 h until the seeds germination and primary root emergence. Then, *H. psiadioides* essential oil was applied (5, 10, 15, 20 and 25 μL), with exception of the control group, and the plates returned to the growth room for 7 days. At the end of this period, photographs were taken and the primary root and shoot length were measured using the software ImageJ 1.45s.

2.2. Essential oil extraction

Plant material of *H. psiadioides* was collected at Morro Santana (30° 03' S, 51° 07' W), a hill that is inserted in a chain of granitic hills in southern Brazil, in the 'Campos sulinos' region (Overbeck et al., 2007). The material was dried at room temperature for 15 days and the essential oil was obtained from leaves by hydrodistillation in a modified Clevenger apparatus (Farmacopeia Brasileira, 2010). Then, anhydrous sodium sulfate was employed to eliminate essential oil humidity, and the oil was stored in ultrafreezer at -80 °C. A voucher specimen of *H. psiadioides* (175007) was collected and deposited in the herbarium of Federal University of Rio Grande do Sul (ICN).

2.3. Oil analysis

The compounds of the extracted essential oil were analyzed by gas chromatography. Chromatographic analysis was performed using a Shimadzu 17A gas chromatograph coupled to a mass spectrometer detector (GC-MS) and a Shimadzu 17A gas chromatograph with a flame ionization detector (GC-FID). A capillary column was used under the following condition: DB-5 (30 m \times 25 μm \times 0.25 μm) with an initial oven temperature of 60 °C, raised by 3 °C/min until reaching a final temperature of 220 °C. Injector and detector temperatures were kept at 220 °C.

Helium flow rate was 1.0 ml/min, and desorption occurred in the split mode (1:10). Linear temperature programmed retention indexes (LTPRIs) were determined from the retention data of an *n*-alkane solution (C7–C40), along with the retention data of volatile compounds from *H. psiadioides* samples. All of the components were tentatively identified through a comparison of their LTPRI with those registered in the literature databases (Adams, 2001). Experimental mass spectra were also compared with spectra stored in mass spectrometry databases (Wiley, 6th edition). The relative percentage of each component was obtained directly from chromatographic peak areas, assuming that the sum of all eluted peaks totaled 100%.

2.4. Statistical analysis

The parameters were measured and treatments were compared by one-way PERMANOVA univariate analysis through randomization tests with 10,000 permutations and including pairwise comparison, using the software MULTIV (Pillar, 2009). PERMANOVA enabled a conventional ANOVA approach to be conducted without transformation on the raw data, since it is not based on assumptions of normality and homogeneity of variances (Anderson, 2001). For all tests, the results were considered statistically significant at $P \leq 0.05$.

3 Results

The essential oil of *H. psiadioides* affected the germination and early growth of *A. thaliana* in all the parameters analyzed (Fig. 1). This target plant presented germination rate of 53.91%, 33.92%, 16.52%, 14.79%, and 12.18% for 5, 10, 15, 20 and 25 μ L of oil, respectively, in relation to the control (Fig. 1 A). The speed of accumulated germination of *A. thaliana* was delayed in all treatments with the oil (Fig. 1 B). This parameter decreased 81.52% at 5 μ L, and the other amounts of oil caused a similar effect, reaching a maximal reduction of 95.79% at 20 μ L. Significant differences were observed for final germination rate and speed of accumulated germination between control, 5 and 10 μ L of oil applied, although treatments with other essential oil amounts (15, 20 and 25 μ L) showed no significant differences

The early growth experiment revealed a yellowish phenotype of cotyledonary leaves of *A. thaliana* seedlings, in treatments from 10 to 25 μ L of *H. psiadioides* essential oil. The seedlings also exhibited a severe shortening of roots and shoots' length. The primary roots length was diminished about 61.71%, 88.57%, 92.86%, 94.00%, and 96.00%, corresponding respectively to 5 μ L, 10 μ L, 15 μ L, 20 μ L and 25 μ L, with the last causing the greatest inhibition (~3.36 cm shorter than the roots of control plants). Shoot length was also affected; control and 5 μ L differed significantly in

relation to other treatments. The treatments 10, 15, 20 and 25 μL did not differ and showed a reduction of the shoot length about 73.17%.

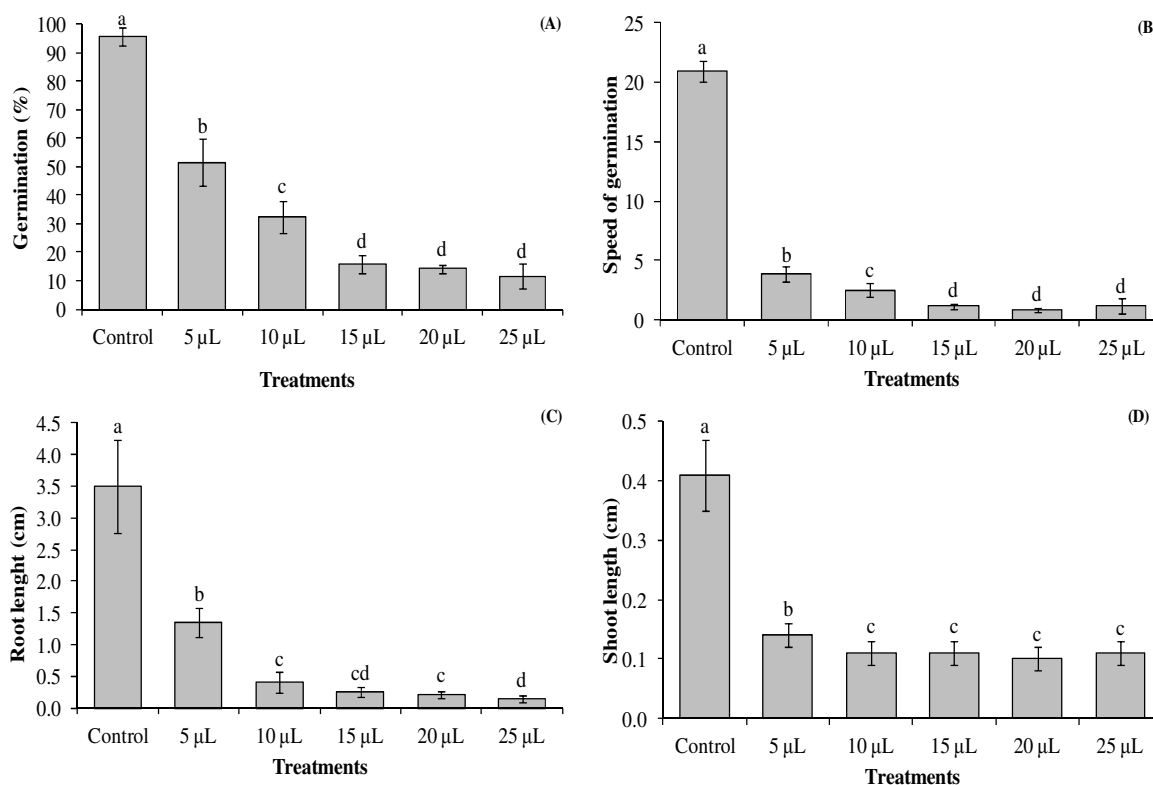


Fig. 1. Effects of different amounts of *Heterothalamus psiadioides* essential oil (5, 10, 15, 20 and 25 μL) on *Arabidopsis thaliana* germination and early growth. (A) Germination rate (%), (B) speed of accumulated germination, (C) root length and (D) shoot length. The data are presented as means \pm standard deviation ($n = 4$). Values with the same letter do not differ significantly, according to PERMANOVA, at $P \leq 0.05$

The chemical composition of *H. psiadioides* essential oil is shown in Table 1. The oil is a mixture of terpenes, mainly constituted by monoterpenes hydrocarbons (76.64%), followed by sesquiterpenes hydrocarbons (14.75%), oxygenated monoterpenes (4.59%) and oxygenated sesquiterpenes (4.02%). The major compound of the oil was the monoterpene β -pinene (44.65%) and other monoterpenes that were present in high quantities were limonene (6.50%) and α -pinene (4.41%). Sesquiterpene fraction had α -curcumene (3.79%) and bicyclogermacrene (2.46%) as the most representative.

Table 1. Chemical composition of *Heterothalamus psiadioides* essential oil.

Compound	LTPRI ^{ht}	LTPRI	Area (%)
<i>Monoterpenes hydrocarbons</i>			
α -Thujene	930	926	0.14
α -Pinene	939	935	4.41
α -Fenchene	953	949	0.23

Camphene	954	956	0.29
Benzaldehyde ^a	960	971	0.08
Sabinene	975	975	0.26
β -Pinene	979	983	44.65
Myrcene	991	991	1.91
N.I.		1014	13.47
α -Terpinene	1017	1018	0.15
Para-cymene	1025	1024	0.79
Limonene	1029	1031	6.50
<i>cis</i> -Ocimene	1037	1035	0.07
<i>trans</i> -Ocimene	1050	1047	2.30
γ -Terpinene	1060	1058	0.19
<i>Oxygenated monoterpenes</i>			
<i>trans</i> -Linalool oxide	1073	1071	0.15
Terpinolene ^b	1089	1089	1.28
Linalool	1097	1099	1.07
Endo-fenchol	1117	1114	0.21
<i>trans</i> -Pinocarveol	1139	1140	0.39
N.I.		1149	0.08
α -Phellandren-8-ol	1170	1159	0.33
Isoborneol	1162	1163	0.16
Terpinen-4-ol	1177	1166	0.35
α -Terpineol	1189	1178	0.61
N.I.		1184	0.20
N.I.		1191	0.51
N.I.		1197	0.38
N.I.		1222	0.08
<i>Sesquiterpene hydrocarbons</i>			
N.I.		1252	1.56
N.I.		1287	0.21
N.I.		1395	0.56
N.I.		1425	0.28
N.I.		1434	0.10
Aromadendrene	1441	1445	0.47
N.I.		1449	0.05
N.I.		1451	0.05
α -Himachalene	1451	1455	0.61
α -Humulene	1455	1459	0.41
N.I.		1465	0.55
N.I.		1467	0.17
γ -Gurjunene	1477	1478	0.27
N.I.		1483	1.45
α -Curcumene	1481	1486	3.79
β -Selinene	1490	1493	0.46
Viridiflorene	1497	1495	0.12
Bicyclogermacrene	1500	1503	2.46
N.I.		1513	0.17
γ -Cadinene	1514	1519	0.33
δ -Cadinene	1523	1528	0.69
<i>Oxygenated sesquiterpenes</i>			
N.I.		1539	0.23
<i>trans</i> -Nerolidol	1563	1565	0.09
Ledol	1569	1575	0.29
N.I.		1584	1.43
Viridiflorol	1593	1590	0.32
N.I.		1599	0.27
N.I.		1603	0.06
N.I.		1611	0.53
N.I.		1616	0.11

N.I.		1638	0.16
N.I.		1644	0.12
α -Eudesmol	1654	1660	0.22
N.I.		1683	0.19

LTPRI^{lit}, linear temperature programmed retention indexes, tabulated (Adams, 2001).

LTPRI, linear temperature programmed retention indexes, calculated.

Relative percentage of each component was obtained directly from chromatographic peak areas, considering the sum of all eluted peaks as 100%.

N.I., not identified.

^a Oxygenated monoterpene.

^b Monoterpene hydrocarbon.

4 Discussion

The germination rate of *A. thaliana* seeds is extremely reduced by *H. psiadioides* essential oil as well as the speed of germination. The mobilization of reserves can be a physiological phase of the germination wherein phytotoxic compounds may act slowing down this process (Gniazdowska and Bogatek, 2005). The data collected in the present study demonstrate a pattern of dose-dependent effect according to the amount of oil applied; however, from 15 to 25 μ L the effects on germination stabilized. Thereby, it is demonstrated the strong phytotoxic potential of essential oils on germination, which according to Bewley (1997) has a very important role: to ensure perpetuation of the species; thus, any adversity in this phase may be prejudicial for maintenance of a plant population.

In our research the root and shoot length are very affected, with a pronounced reduction in length of both measurements. Despite of the small amounts used (5 to 25 μ L), the exposure to the oil was detrimental to germination and early growth of *A. thaliana*. Higher quantities of *H. psiadioides* essential oil (100 μ L) were used in a study that demonstrates inhibition of the mitotic activity and induction of chromosomal abnormalities in lettuce and onion (Schmidt-Silva et al., 2011). Thus, small amounts of oil probably could exhibit the same mitotic effects than that displayed by higher amounts. Moreover, the morphological effects of shoots and roots shortening in early growth of *A. thaliana* can be assigned to the capacity of *H. psiadioides* essential oil to cause damages on mitosis, as demonstrated by Schmidt-Silva et al., (2011). Monoterpenoids can also reduce the mitotic index of root meristem, probably because its activity inhibits both cell-nuclear and organellar DNA synthesis (Nishida et al., 2005). It was also demonstrated that α -pinene, a volatile monoterpene, inhibits root growth of a target plant by inducing oxidative stress measured in terms of increased lipid peroxidation, H₂O₂ accumulation and membrane disintegration (Singh et al., 2006). These alterations from different physiological mechanisms certainly interfere and commit the growth and development of plants exposed to phytotoxic compounds. Roots shortening may be detrimental to the competitive vigor of the plant, because the roots have the important function of capture water and mineral nutrients.

Another indispensable characteristic for plant growth is leaves pigmentation for photosynthesis occurrence. We observed that cotyledons of *A. thaliana* seedlings became yellowish throughout time in treatments with volatiles exposure (from 10 to 25 μL). This effect is possibly caused by chlorophyll degradation or inhibition of its synthesis. Disorders on photosynthesis are one of the most frequently observed physiological effects of many phytotoxic compounds (Gniazdowska and Bogatek, 2005).

In the present study monoterpenes hydrocarbons were the most abundant chemical category of *H. psiadioides* essential oil and the major compound of the oil was the monoterpene β -pinene. Variation in phytotoxicity among the compounds of essential oils may also be observed. Vaughn and Spencer (1993) reported that several monoterpenoids with oxygen-containing functional groups were more inhibitory to germination than hydrocarbons. Moreover, allelopathic interactions are usually not due to a single compound but rather to a pool of several allelochemicals acting synergistically (Reigosa et al., 1999). As an example, Schmidt-Silva et al. (2011) studied the cytotoxic effects of *H. psiadioides* essential oil in cytogenetic evaluations comparing with the effect of an isolated compound of the essential oil, β -pinene (the major compound). The authors verified more severe effects of the oil on mitosis of root meristematic cells of onion and lettuce than when the isolated compound β -pinene was applied. Ultimately, the effects tested for *H. psiadioides* in the present study and also in works with plant material of the same population evaluated here (E.R. Silva, G.E. Overbeck, G.L.G. Soares submitted, Schmidt-Silva et al., 2011) may not be related to exactly the same compounds reported for the oil by Suyenaga et al. (2004).

Volatile compounds arrive to target plants tissues generally using air as the carrier until leaves, where they enter through the epidermis or stomata, spreading into mesophyll cells and to other cells via plasmodesmata (Rice, 1984). Moreover, the essential oils also can penetrate the soil – through rainfall and similar phenomena – and cause negative effects such as observed by Dudai et al. (1999). The authors added to the soil essential oils that demonstrated inhibition of target plants germination and showed a tendency of increased germination with increasing the depth of sowing. Organic matter from plants also constitutes a source of phytotoxic compounds that are incorporated to the soil (Macías et al., 2007). Singh et al. (2009) found in the soil from underneath *Artemisia scoparia* Waldst. et Kit., a shrub of the Asteraceae family, a mixture of mono- and sesquiterpenes. Thus, the upcoming investigations with *H. psiadioides* essential oil could assess its action with more realistic experimental conditions, in order to show possible allelopathic effects that explain its pattern of occurrence besides phytotoxicity recorded in this study.

The present research demonstrates severe effects of *H. psiadioides* essential oil on seeds and seedlings. Small amounts of oil disturbed the rate and speed of accumulated germination and reduced root and shoot growth; however it was observed a dose-dependent effect, with the most

negative effects with increasing amounts of oil. These effects may also be observed with other target plants as well as may affect these phases of development of species that co-occur with *H. psiadioides*. Therefore, *H. psiadioides* essential oil compounds may interact in different locations inside plants cells, affecting many physiological functions.

Acknowledgments

The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) for financial support and the staff of Laboratório de Fisiologia Vegetal (UFRGS) and Laboratório de Química Analítica Ambiental e Oleoquímica (LAAO-UFRGS).

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4 ARTIGO II

Phytotoxic effects of *Heterothalamus psiadioides* (Asteraceae) essential oil on adventitious rooting¹

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¹Este trabalho será submetido à Revista Acta Physiologiae Plantarum

Abstract Essential oils have many functions in plants development; among them, they take part in plant-plant interactions, as allelopathy. The phytotoxicity of the essential oil of *Heterothalamus psiadioides*, an Asteraceae shrub that has been recently investigated regarding its allelopathic potential, was evaluated on *Arabidopsis thaliana* adventitious rooting. This development process has demonstrated great sensibility to phytotoxic essential oils and has been primarily studied by our research group. The essential oil of *H. psiadioides* showed highly negative effects on *A. thaliana* wild type (WT) adventitious rooting in very small amounts. As the hormone auxin has an important role on adventitious rooting, *A. thaliana* mutant *superroot* (*sur1*) that overproduces indole-3-acetic acid (IAA) was tested to verify if it could better respond to the oil than WT. The mutant had a WT-like rooting response and the expression of its phenotype was not evident in treatments that had oil application. The essential oil also induced the generation of H₂O₂, a reactive oxygen species (ROS) in high levels and even trying to recover these effects with the aid of the antioxidant Trolox® the attempt was unsuccessful. Increasing levels of H₂O₂ may affect leaves pigmentation of WT microcuttings and auxin levels of both WT and *sur1* microcuttings. Since the expression of auxin-responsive genes is decreased by H₂O₂ treatment via mitogen-activated protein kinase activation (MAPK), microcuttings growth and rooting are impaired causing altered development pattern.

Keywords allelopathic volatiles, roots, oxidative damage, depigmentation, auxin

Introduction

Volatile substances play many roles in the development of plants, such as attraction of pollinators, avoidance of predatory insects, and plant-plant interactions (Maffei 2010). Chemical substances as volatile essential oils are complex mixtures of compounds produced by plant secondary metabolism and may be involved in allelopathy (Singh et al. 2009). This phenomenon consists in the release of phytotoxins by a plant into the environment, causing beneficial or negative effects on other plants (Rice 1984). *Heterothalamus psiadioides* Less. is an Asteraceae shrub that grows in the South of Brazil and Uruguay (Deble et al. 2005), often forming dense and dominant populations (Setubal and Boldrini 2010), and bare zones that can be observed near/under the species. This pattern of establishment indicates that *H. psiadioides* may release phytotoxic compounds into the environment and inhibit other species through allelopathy. The compounds of *H. psiadioides* essential oil have already shown phytotoxic effects on cell division (Schmidt-Silva et al. 2011), seed germination and early growth (Schmidt-Silva et al. 2012, Silva et al. 2014). Thus, more detailed investigations are necessary to elucidate the oil phytotoxicity.

Most of phytotoxicity evaluations are restricted to test effects on seed germination or growth of seedlings aerial part and main root. A development process that has also shown to be sensitive to phytotoxic compounds is adventitious rooting. The evaluation of this process in phytotoxic studies has been primarily investigated by our research group, with *Arabidopsis thaliana* (L.) Heynh. as the target species (Pawlowski Â, Kaltchuk-Santos E, Zini CA and Soares GLG, submitted). This species is recognized as a model plant for development, biochemical and physiological studies. Adventitious rooting evaluations are performed with *A. thaliana* and the conclusions achieved from it, in general, are valid to other plants (Falasca et al. 2004). Adventitious roots emerge from organs such as hypocotyl, stem, roots and leaves. This rooting system is important to asexual reproduction and consists in the main system of monocotyledons rooting (Osmont et al. 2007). Adventitious roots are also part of the normal rooting pattern of dicot plants, in which are formed through the secondary tissue of roots (Paolillo Jr and Zobel 2002) and when these plants are submitted to flooding (Kozlowski 1984).

The formation of adventitious roots comprises three phases in dicots: induction, initiation and expression. The induction phase comprises molecular and biochemical events with no noticeable changes, i.e., without roots appearance. The initiation phase has cell divisions and root primordia organization and finally, the expression phase is characterized by roots emergence. Many factors are involved in adventitious roots formation such as temperature, light conditions, sugars, mineral salts and hormones (Li et al. 2009). The phases of adventitious rooting are mainly regulated by auxin, which accumulates when dicots suffer some kind of wounding , triggering the development of adventitious roots in the base of the wounding (Agulló-Antón et al. 2013).

Hormones levels are also affected by oxygen reactive species (ROS) (Gidrol et al. 1994, Kovtun et al. 2000), which include the following molecules: singlet oxygen ($^1\text{O}_2$), superoxide radical anion ($\cdot\text{O}_2^-$), hydroxyl radical ($\cdot\text{OH}$) and hydrogen peroxide (H_2O_2). Their generation is an inevitable effect of the oxidative cellular metabolism (Kuźniak and Urbanek 2000). At normal levels, ROS display signaling functions, but at higher amounts, they are toxic. Molecules of H_2O_2 , as an example, are important regulators of physiological processes such as acquiring resistance, cell wall strengthening, senescence, phytoalexin production, photosynthesis, stomata opening and the cell cycle (Petrov and Breusegem 2012). H_2O_2 also has the property of increasing fresh weight and number of adventitious roots in *Cucumis sativus* L., functioning as a signaling molecule (Li et al. 2007). However, when generated in higher quantities within the plant, H_2O_2 indicates stress conditions and requires a control of its concentration in cells (Petrov and Breusegem 2012). H_2O_2 works as a negative regulator of auxin genes, serving as a molecular link between oxidative damage and growth reduction (Kovtun et al. 2000). Furthermore, production of ROS has been proposed as

one of the major mechanisms of phytotoxins action (Weir et al. 2004) and molecules of H₂O₂ are known to cause programmed cell death in allelopathic interactions (Bais et al. 2003).

In this study, we aimed to evaluate the effects of *H. psiadioides* essential oil on *A. thaliana* wild type (WT) and mutant *superroot* (*sur1*) adventitious rooting. This mutant overproduces free and conjugated indole-3-acetic acid (IAA), which confers excessive lateral and adventitious roots production (Boerjan et al. 1995). In addition, we investigated the generation of H₂O₂ molecules, a reactive oxygen species (ROS), and evaluated if the addition of the antioxidant Trolox® would allow plants to recover from damages caused by the oil.

Materials and methods

Extraction of the essential oil

Leaves of *H. psiadioides* were collected at Morro Santana (30° 03' S, 51° 07' W), located in the city of Porto Alegre, Rio Grande do Sul State, Brazil. The material collected was dried at room temperature and the essential oil was obtained from the leaves by hydrodistillation in a modified Clevenger apparatus (Farmacopeia Brasileira, 2010) and stored at -80 °C.

Plant material and culture conditions

Seeds of *A. thaliana* wild type (WT) Col-0 and of the mutant *superroot* (*sur1*) Col CS8156 were obtained from *Arabidopsis* Biological Resource Center (ABRC) and granted by Laboratório de Fisiologia Vegetal (Universidade Federal do Rio Grande do Sul). The seeds were disinfected by soaking seeds for 1 min in 70% ethanol and 15 min in 1.5% NaClO (in water) with a drop of commercial detergent, followed by four washes in autoclaved distilled water. Ten seeds were plated in each Petri dish on medium containing 0.05x MS minerals (Murashige and Skoog 1962), 30 g.L⁻¹ sucrose and 8 g.L⁻¹ agar, adjusted to pH 5.8 ± 0.1 prior to autoclaving at 121°C and 1.5 atm for 20 min. The dishes were stored for 2 days in darkness at 4 °C, to synchronize seeds germination, and then placed in a growth room at 21 °C/16-h photoperiod provided for fluorescent lamps (20 W). The Petri dishes were vertically oriented and *A. thaliana* plants grew for 7 days. After this period the primary root of *A. thaliana* seedlings were excised, generating microcuttings. The microcuttings were transferred to new plates containing basal or specified culture medium to evaluate adventitious roots formation in two sets of bioassays.

Bioassay 1: Effects of *H. psiadioides* essential oil on *A. thaliana* adventitious rooting

The essential oil of *H. psiadioides* was applied on a piece of cotton fixed with double-sided tape in the inner face of the Petri dish, to avoid direct contact between seeds and the essential oil, but allowing the oil to volatilize into the airspace within the Petri dish. The amount of oil applied for bioassay 1 was 1, 2, 3, 4 and 5 μL and control consisted in no oil application. The treatments were constituted by four repetitions, with 10 microcuttings each. Then, the Petri dishes returned to the growth room, with irradiance of $42.94 \mu\text{mol m}^{-2} \text{s}^{-1}$, for more 7 days and the microcuttings were daily monitored to verify the adventitious roots emergence. At the end of this period, photographs were taken and the roots were measured and counted.

The effects of the oil were also tested on a mutant plant of *A. thaliana*, the *surl1*, which contains increased IAA concentrations in its tissues (Boerjan et al. 1995), the main hormone involved in adventitious rooting (Agulló-Antón et al. 2013). With this experiment we wanted to detect if this mutant was able to overcome the effects caused by the oil. To assess this, we designed this experiment with the same conditions designed to *A. thaliana* WT.

Bioassay 2: Recovery attempt with Trolox®

This bioassay used medium supplemented with 10 μM Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The concentration mentioned was tested and showed the best performance – mainly related to the number and length of adventitious roots – in plants not treated with *H. psiadioides* essential oil (data not shown). Thus, it was tested if the treatment with Trolox® would allow the microcuttings exposed to *H. psiadioides* essential oil to recover from its effects and show a phenotype similar to that exhibited by control microcuttings. The same methods described for bioassay 1 were followed, using just seeds of *A. thaliana* WT. However, the treatments with oil application only had 5 μL of *H. psiadioides* essential oil, with exception of controls (C and TC). Therefore, the treatments were: control (C), oil application (O), medium plus Trolox® control (TC), and medium plus Trolox® and oil application (TO). The maximum amount of oil (5 μL) was used because it was the treatment with greater damage to the microcuttings. This bioassay was conducted with $78.93 \mu\text{mol m}^{-2} \text{s}^{-1}$ of irradiance and the treatments were constituted by five repetitions, with 10 microcuttings each.

Histochemical detection of H₂O₂

After the period of exposure to the oil, presence of H₂O₂ in *A. thaliana* microcuttings was detected by employing 3,3-diaminobenzidine (DAB), using a method adapted from Thordal-Christensen et al. (1997). The microcuttings were dipped in 1 mg/mL solution of DAB, pH 3.8, for 1 h. Then, the plant material was immersed in boiling ethanol (96%) at 25 °C, for 10 min. This treatment decolorized the microcuttings and revealed brown spots caused by the polymerization reaction of DAB with H₂O₂, which indicates presence of ROS. Then, the microcuttings were photographed in fresh ethanol.

Rooting measurements and statistical analysis

The measurements performed in the experiments were: percentage of rooted microcuttings (%R), mean rooting time (RT), mean number of roots per rooted microcutting (NR) and mean length of longest root in cm (LR). RT had one daily observation for bioassay 1 and two daily observations for bioassay 2, during 7 days. RT was calculated as described for mean of germination time in Harrington (1963). To measure LR, the software ImageJ 1.45s was used.

For bioassay 1, the parameters were measured and the inhibition or stimulation percentage – negative or positive values in relation to the control group – was calculated according to the formula: Inhibition or stimulation percentage = [(treatment-control)/control]*100. The parameters evaluated were then compared between groups (control, 1, 2, 3, 4, and 5 µL of the oil) using univariate analysis of variance with randomization (PERMANOVA). To test for differences in effects of *H. psidioides* essential oil on WT and *sur1*, values of inhibition/stimulation percentage were compared for each concentration of the oil using the same method. For bioassay 2, the parameters were measured and differences between groups were tested also using PERMANOVA. This randomization test enabled a conventional ANOVA to be used without transformation on the raw data, as it is not based on assumptions of normality and homogeneity of variances (Anderson, 2001). The analysis used Euclidean distances and was conducted with 10,000 bootstrap iterations, considering a significance level of $p < 0.05$. When analyses of variance indicated significant differences between groups, contrast analyses were performed for pairwise comparisons (Pillar and Orlóci 1996).

Results

Bioassay 1: Effects of *H. psiadioides* essential oil on *A. thaliana* adventitious rooting

The essential oil of *H. psiadioides* interfered in the adventitious rooting of *A. thaliana* WT. Almost all the parameters analyzed were negatively affected by the oil and presented a gradient of severity with the increasing amount of oil (Table 1). The strongest effects were demonstrated in 5 μ L treatment, which severely reduced the rooting time, number and length of adventitious roots produced. %R was not affected by *H. psiadioides* essential oil application. The oil significantly delayed RT of *A. thaliana* microcuttings, mainly in the treatments 4 and 5 μ L, which started to root about two days after control. NR had greater inhibition in the treatment of 5 μ L of oil in relation to the control. LR was similar only between control and 1 μ L of *H. psiadioides* essential oil. The treatments 2, 3, 4 e 5 μ L of oil differed statistically between each other and the LR of microcuttings was drastically reduced in relation to the control.

In view of the severe effects demonstrated by the oil on *A. thaliana* WT adventitious rooting, we tested if the oil could impair the adventitious rooting of the mutant *sur1*. According to the results obtained for *sur1* and the comparison of this with WT (Table 1), both were similarly affected by *H. psiadioides* essential oil, although some parameters have differed statistically; thus, further experiments used only *A. thaliana* WT.

Table 1 Effects of different amounts of *Heterothalamus psiadioides* essential oil on *Arabidopsis thaliana* WT and mutant *sur1* adventitious rooting. The data are presented as inhibition/stimulation percentage in relation to the control group (n = 4, with 10 microcuttings each) for WT and *sur1*, separately. Values with the same letter do not differ significantly and values with “a” do not differ from the control, according to PERMANOVA, at $p \leq 0.05$. (*) Significant differences in inhibition/stimulation percentage between WT and *sur1* for each concentration of the oil. The parameters analyzed were percentage of rooted microcuttings (%R), mean rooting time (RT), mean number of roots per rooted microcutting (NR) and mean length of longest root (LR).

	Treatment	% R	RT	NR	LR
WT	1 μ L	0 a	+1 ab	+16 ab	-11 a
	2 μ L	0 a	-12 c	+21 ab	-48 b
	3 μ L	0 a	-12 ac	-9 ac	-58 c
	4 μ L	-3 a	-41 d	-23 ac	-84 d
	5 μ L	-15 a	-58 d	-41 d	-94 e

<i>sur1</i>	1 μ L	0 a	-8 ab	-10 ab	-27 b
	2 μ L	0 a	-7 ab	-1 ac	-43 b
	3 μ L	0 a	-13 b	-35 d	-63 c
	4 μ L	0 a	-17 bc	-29 abd	-68 c
	5 μ L	-5 a	-29 c	-43 d	-83 d
WT x <i>sur1</i> <i>p</i> values	1 μ L	1	0.06	0.03 *	0.14
	2 μ L	1	0.11	0.09	0.31
	3 μ L	1	0.94	0.03 *	0.60
	4 μ L	1	0.03 *	0.45	0.11
	5 μ L	1	0.03 *	0.80	0.03 *

Leaves of WT microcuttings that were exposed to *H. psiadioides* essential oil became whitish (Fig. 1A-F). This effect was noticeable from 3 to 5 μ L of the oil in the cotyledonary leaves. Thus, oxidative damage was hypothesized to be the cause of pigmentation loss in the microcuttings leaves.

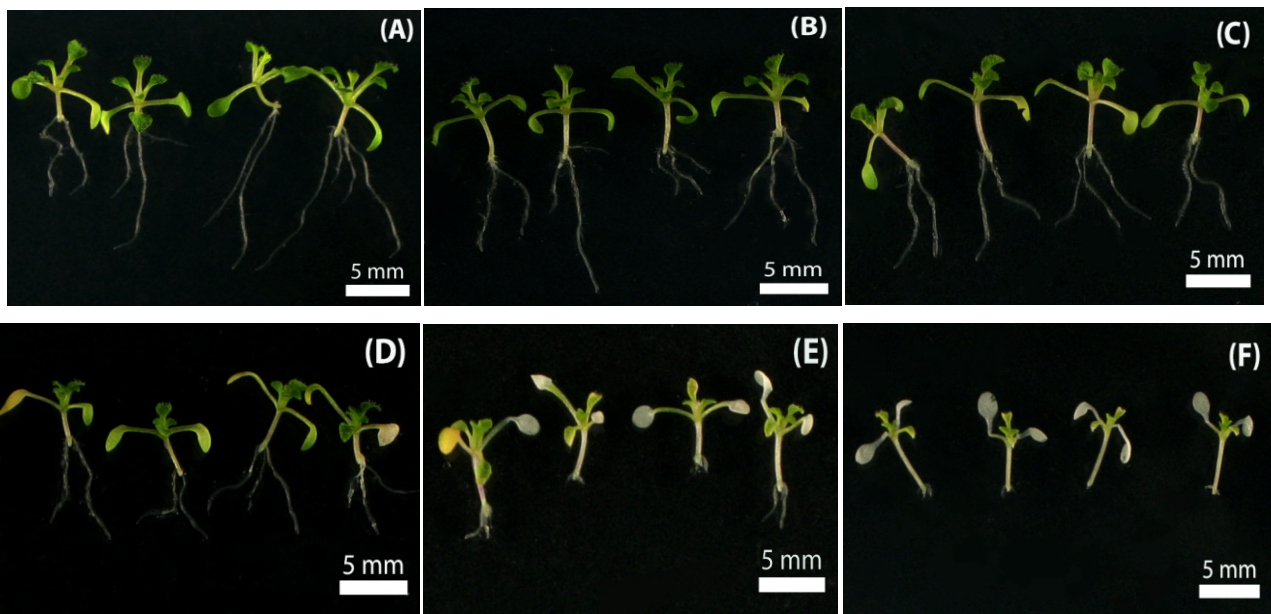


Fig. 1 Effect of *Heterothalamus psiadioides* essential oil on leaves of *Arabidopsis thaliana* WT microcuttings. (A) Control; (B) 1 μ L; (C) 2 μ L; (D) 3 μ L; (E) 4 μ L and (F) 5 μ L of oil

Oxidative damage

After noticing a bleaching in cotyledonary leaves of *A. thaliana* microcuttings treated with more than 3 μ L of oil in bioassay 1, we assigned this phenotype to ROS generation. The reaction of DAB with H_2O_2 revealed brown spots that indicate oxidative damage. The bioassay 1, with different quantities of *H. psiadioides* essential oil, demonstrated an increased staining with higher

amounts of oil (Fig. 2A-F). The bioassay 2, using Trolox®, also demonstrated brown coloring in treatments exposed to the oil (Fig. 3A-D).

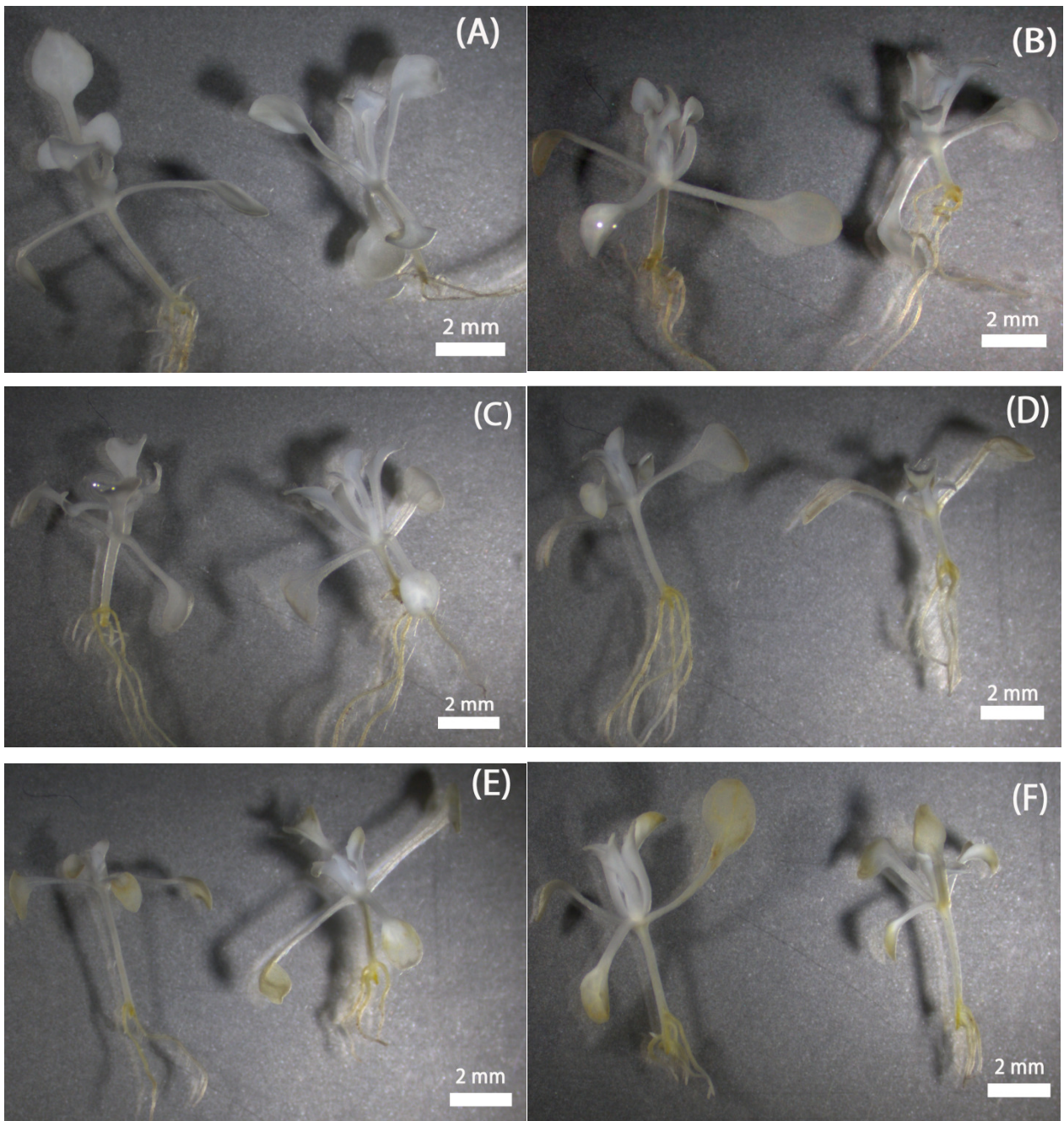


Fig. 2 Detection of H₂O₂ by DAB histochemical assay in *Arabidopsis thaliana* WT microcuttings in bioassay 1, with different quantities of *Heterothalamus psiadioides* essential oil. (A) Control; (B) 1 μ L; (C) 2 μ L; (D) 3 μ L; (E) 4 μ L and (F) 5 μ L of oil

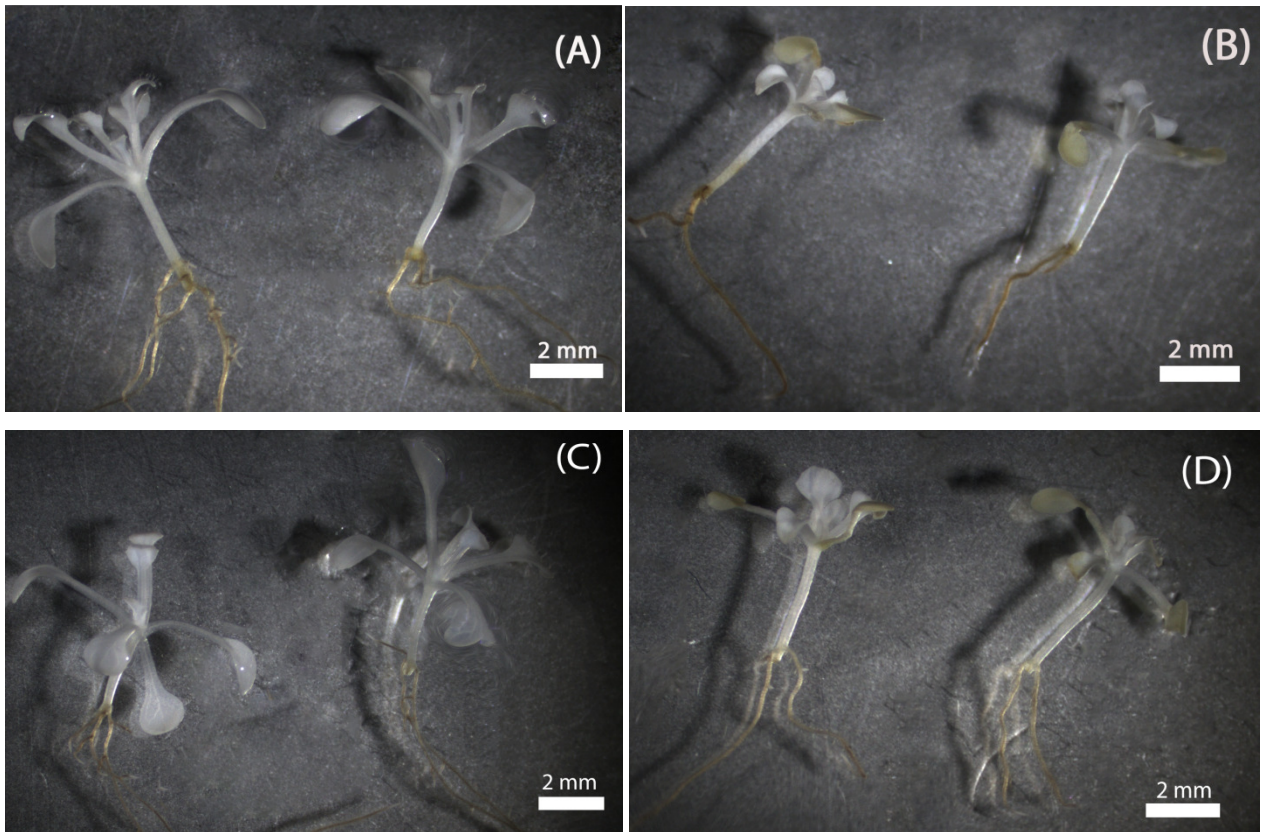


Fig. 3 Detection of H_2O_2 by DAB histochemical assay in *Arabidopsis thaliana* WT microcuttings in bioassay 2, using 5 μ L of *Heterothalamus psiadioides* essential oil. (A) Control -C; (B) oil application -O; (C) medium plus Trolox® control -T; (D) medium plus Trolox® and oil application -TO

Bioassay 2: Recovery attempt with Trolox®

In this bioassay, it was tested if exogenous addition of Trolox® in the culture medium could revert the effects caused by the oil and recover the normal adventitious rooting in *A. thaliana* microcuttings exposed to *H. psiadioides* essential oil. Trolox® did not present action of prevention or recovery from damages. The parameters RT, NR and LR were severely affected by the oil, whereas %R did not differ between all the treatments, despite of the fact that treatments with oil application did not reach 100% of rooting (Fig. 4A). Furthermore, it was presented a delay in roots emergence in these treatments, which was better demonstrated in the RT (Fig. 4B). The treatment with Trolox® (TC) presented an unexpected low NR, which was equal to the treatments with oil application (Fig. 4C); however, this treatment (TC), had the highest value of adventitious roots length (Fig. 4D). LR was the measure most affected by *H. psiadioides* essential oil.

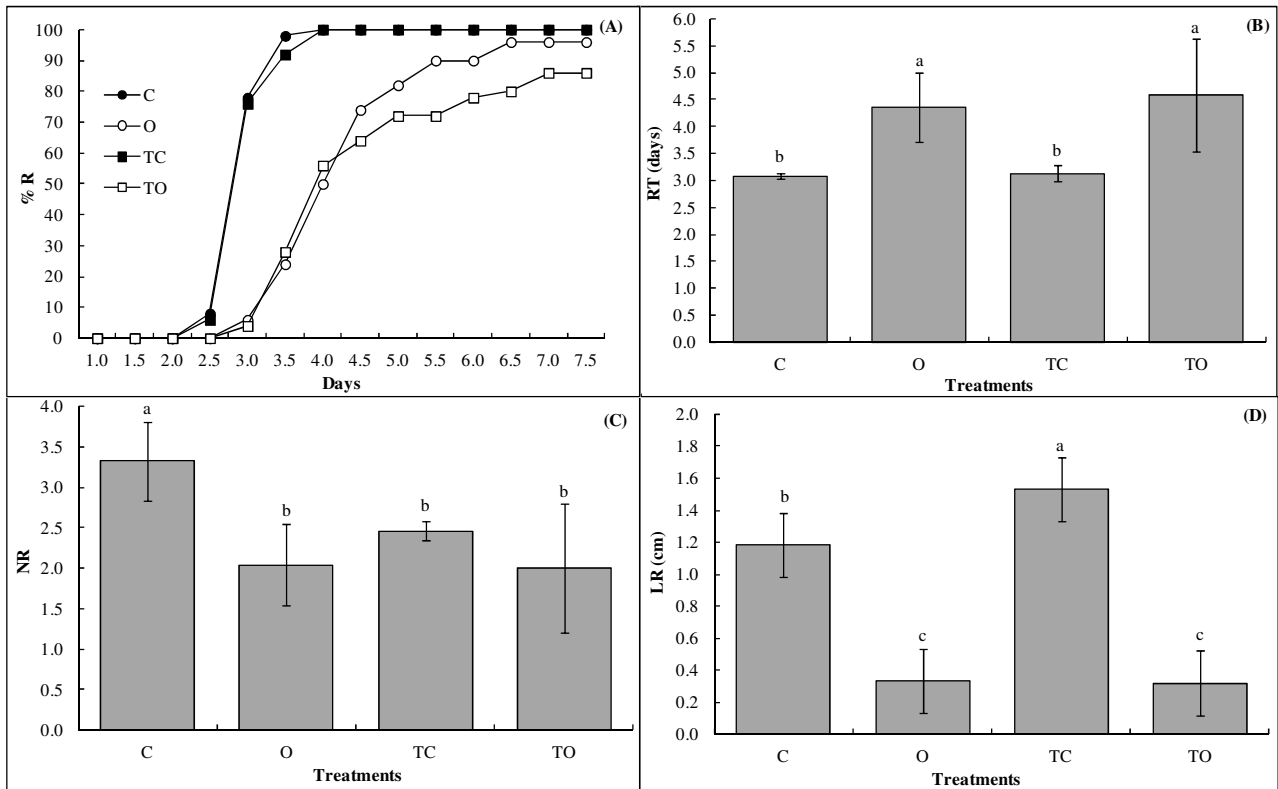


Fig. 4 Effects of 5 µL of *Heterothalamus psiadioides* essential oil and exogenous supplement of Trolox® on adventitious rooting measures of *Arabidopsis thaliana* WT. The treatments were: control (C), oil application (O), medium plus Trolox® control (TC), medium plus Trolox® and oil application (TO). The measures shown are percentage of rooted microcuttings (%R), mean rooting time (RT), mean number of roots per rooted microcutting (NR) and mean length of longest root (LR). The data are presented as mean ± standard deviation (n = 5, with 10 microcuttings each). Values with the same letter do not differ significantly, according to PERMANOVA, at $p \leq 0.05$

Discussion

We demonstrated that *H. psiadioides* essential oil causes negative effects on adventitious rooting of *A. thaliana*, in very small amounts of oil (from 1 to 5 µL). Despite the severe reduction of some parameters, %R was not significantly affected by the oil. Thus, it is possible that the action of the oil does not prevent the formation, but interferes on cell division and consequently in the number and the growth of adventitious roots. In view of the numerous modes of action of phytotoxic compounds, it is difficult to know the exact mechanism whereby *H. psiadioides* essential oil affects *A. thaliana* adventitious rooting. Thus, it was investigated the presence of oxidative damage, and H₂O₂ was detected in leaves tissues of *A. thaliana*. Furthermore, it was tested if exogenous addition of the antioxidant Trolox® would allow plants to recover from the effects on adventitious rooting; however, no significant result was obtained. Different concentrations of

Trolox® were tested and the one that demonstrated better performance than the control was chosen. However, this concentration (10 µM) was not efficient to revert the oil effects. Dayan et al. (2000) found that optimum recovery may not be detectable when high concentration of a phytotoxin is used. Therefore, it is possible that 5 µL of *H. psiadioides* essential oil could be a high amount for *A. thaliana* microcuttings recover from its effects, mainly because of the oxidative damage. At lower concentrations of a phytotoxin the antioxidative mechanism may revert this process; however, at higher concentrations oxidative burst is unavoidable (Batish et al. 2006). The recovery also depends of the exposure time; a long-term exposure to the allelochemicals causes severe cellular damage related to increase of ROS production in time (Oracz et al. 2007, Sánchez-Moreiras et al. 2011).

A strict control of ROS levels is essential to prevent their toxicity and to ensure an accurate execution of their signaling functions (Petrov and Breusegem 2012). During periods of biotic or abiotic stress, ROS levels can increase excessively, leading to an oxidative stress state (Apel and Hirt 2004). Molecules of H₂O₂ were detected in whitish leaves of *A. thaliana* microcuttings that were exposed to the oil. Many types of stress induce H₂O₂ accumulation and lipid peroxidation, causing leaves depigmentation. Oxidative damage may decrease chlorophyll content and chlorophyll fluorescence (Niu et al. 2013, Sánchez-Moreiras et al. 2011), impact chlorophyll biosynthesis in cotyledons (Aarti et al. 2006) and may also cause necrosis induced by early senescence process in oldest leaves (Sánchez-Moreiras et al. 2011).

High levels of H₂O₂ are also involved in some processes such as induction of cell death (Bais et al. 2003), decrease of root growth (Singh et al. 2009) and alteration in hormone levels (Prasad and Subhashini 1994, Graña et al. 2013) in allelopathic interactions. As plant hormones are required in small concentrations to regulate plant processes (Tukey et al. 1954) and auxin is the major growth-promoting hormone involved in lateral and adventitious rooting (Fabijan et al. 1981), it was not expected that microcuttings of the mutant *sur1*, an IAA overproducer, were not able to recover from the effects caused by *H. psiadioides* essential oil. Thus, we consider the possibility of alteration on auxin levels by some mechanism of the oil action. Possibly, the increasing levels of hydrogen peroxide (H₂O₂) molecules, as demonstrated with H₂O₂ detection at increasing oils amounts (from 1 to 5 µL), can affect auxin levels. It is well known that expression of auxin-responsive genes is decreased by H₂O₂ treatment via mitogen-activated protein kinase activation (MAPK). The activated MAPK cascade activates stress-response genes that protect plants from diverse environmental stresses and represses auxin-inducible promoters (Kovtun et al. 2000). Thus, prolonged stress exposure leads to altered growth patterns such as compact growth and reduced cell division. The integration of ROS with auxin signaling networks, triggered by environmental factors, is known as the stress-induced morphogenic response (Potters et al. 2009). In our study we conclude that *H. psiadioides* essential oil negatively affects *A. thaliana* adventitious rooting. The effects of

the oil are observed even in very small amounts, and in a dose-dependent manner. The mechanism of the oil action may occur via ROS generation, also causing secondary effects, as depigmentation of cotyledonary leaves and a possible alteration on auxin levels. In the highest concentration tested, even with exogenous addition of an antioxidant, effects on adventitious rooting could not be reverted, indicating that the oxidative damage was too severe. However, even though we can assume a possible mechanism by which effects of the volatile oil occurred in adventitious rooting, this is still a new development process to be investigated in phytotoxicity studies and requires more exploratory researches to be elucidated.

Author contribution The work presented here was carried out in collaboration between all authors. Diana Carla Lazarotto designed experimental setup, conducted the laboratory experiments, analyzed the data and wrote the paper. Ângela Pawlowski co-worked in laboratory, interpreted, discussed and made a critical revision of the manuscript. Eliane Regina da Silva discussed the results and made a critical revision of the manuscript. Joséli Schwambach and Geraldo Luiz Gonçalves Soares discussed the results and revised the manuscript. All authors declare that they have no conflict of interest.

Acknowledgments We thank the Brazilian Funding agency Comissão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted to the first author, and the Laboratório de Fisiologia Vegetal (UFRGS).

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5 ARTIGO III

New insights for volatile allelochemicals: evaluation of effects on vegetative and reproductive development of juvenile and adult plants¹

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¹Este trabalho será submetido à Revista Journal of Chemical Ecology na forma de Rapid Communication

Abstract In South Brazilian grasslands, *Heterothalamus psiadioides*, an Asteraceae shrub that often establishes in dense populations, has been studied by the allelopathic potential of its volatiles. Allelopathy of volatile compounds, such as essential oils, has been restricted to evaluate effects on germination and seedlings growth, as occurs for *H. psiadioides*. Thus, we evaluated the effects of *H. psiadioides* essential oil on juvenile (before bolting) and adult (after bolting) *Arabidopsis thaliana*

plants, which were exposed to the volatiles inside of glass chambers. After exposure, parameters related to vegetative and reproductive development of the plants were measured. Some effect was just found immediately after to the volatiles exposure, which indicates that, in the course of time, plants recovered from physiological effects and continued their normal growth. Because of that, the essential oil did not affect most parameters evaluated. This suggests that effects of volatiles on early growth found in previous studies may be more expressive than on adult plants, possibly because of leaves fragility in early phases. Therefore, studies that evaluate just these phases may provide an incomplete knowledge about the phytotoxicity of plants. The present research is the first to investigate allelopathic effects of volatiles on fully developed plants.

Keywords essential oil, phytotoxicity, fully developed plants, recovery

Introduction

Allelopathy is a phenomenon that consists in the release of phytotoxins by a plant into the environment, causing negative or beneficial effects on other plants (Rice 1984). Asteraceae shrubs produce a range of compounds that may be involved in allelopathic interactions (e.g. Li et al. 2011). *Heterothalamus psiadioides* Less., an Asteraceae shrub, grows in southern Brazil and Uruguay (Deble et al. 2005), forming dense and dominant populations. This landscape pattern may indicate that this species releases phytotoxic compounds in the surrounding environment. Furthermore, previous studies of our research group have demonstrated negative effects of volatiles from *H. psiadioides* on germination and early development of target plants (Schmidt-Silva et al. 2011; Silva 2014).

Allelopathic inhibitory effects are generally observed for volatiles, but these effects are only assessed on early phases of target plants life, as germination and seedlings growth. Thus, the evaluations are not continued, and the effects found on early phases may not have relevant consequences on plants development and reproduction. There are few studies that assess effects of allelochemicals on reproductive development of plants (e.g. Cipollini and Dorning 2008), but there are no studies evaluating the effects of volatiles on fully developed plants, as juveniles and adults. If volatile allelochemicals are able to affect reproduction of plants, then they must have very important trans-generation consequences on plant community. Thus, we aimed to investigate the effects of volatiles on vegetative and reproductive development of plants. For this study, we used *H. psiadioides* as the model species, testing the effects of its volatiles on the target species *Arabidopsis thaliana* (L.) Heynh.

Methods and Material

Leaves of *H. psiadioides* were collected at Morro Santana hill (30° 03' S, 51° 07' W), which is inserted in a chain of granitic hills in southern Brazil, in the 'Campos sulinos' region (Overbeck et al. 2007). Essential oil extraction was made by hydrodistillation as described in Schmidt-Silva et al. (2011).

Plant material and culture conditions For the assays, we used *A. thaliana* wild type (Col-0) as the target species. Seeds were sown in pots, containing a substrate of peat and vermiculite (1:1). The pots were transferred to a growth room (21 °C, 16/8 h day/night, irradiance of 52.75 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Distilled water was used to irrigate the substrate, which once a week was fertilized with liquid medium (1 x MS minerals) (Murashige and Skoog 1962). Plants grew under these conditions until they were used in the bioassays.

Experimental design We conducted two experiments with *A. thaliana*, each comprising a different life stage: juvenile and adult plants. Juvenile plants were in vegetative stage, before bolting, with about nine rosette leaves, and had 20 days; adult plants were in reproductive stage, after bolting, with inflorescence stem emerged, and had 35 days. In each experiment 10 plants were exposed to the volatiles, and other 10 plants in the same life stage were not exposed, consisting in the control. Experiments were fully replicated in order to confirm the results. The plants were irrigated with distilled water and placed inside transparent glass chambers (25 x 25 x 45 cm). The essential oil of *H. psiadioides* (1 mL) was applied onto a piece of cotton attached to the inner face of the box lid, allowing the oil to volatilize into the airspace within the box. The chambers were closed and wrapped with PVC transparent film. Control group had exactly the same conditions, but without addition of the oil. Plants were kept 10 days inside the chambers in the growth room when they were removed from the chambers and remained into the growth room. The plants were watered continuously with eventual addition of liquid MS.

At senescence, seeds were harvested and the parameters evaluated were: number of primary stems – including the main stem and other stems originated from rosette –, number of siliques in the main stem, number of total siliques per plant, dry aerial biomass, seed weight per plant, and germination rate of seeds produced (%). To obtain dry aerial biomass, plants were dried in an oven at 70 °C until constant weight. In the experiment with juvenile plants, some additional parameters were measured: rosette diameter and time of first flower to open. Rosette diameter was measured before and after the exposure to the volatiles. Effect on the rosette growth was estimated based on the percentage of the rosette size after exposure to the volatiles in relation to its size before the

exposure (which corresponded to 100%). The time of first flower to open was counted since the day seeds were sown.

To evaluate the germination rate (%), assays were conducted using plates containing culture medium (0.05 x MS minerals, 30 g.L⁻¹ sucrose, 8 g.L⁻¹ agar, pH 5.8 ± 0.1). Twenty seeds of *A. thaliana* were disinfected from each plant, sown in plates (four repetitions) which remained for 7 days in a growth room (21 °C, 16/8 h day/night, irradiance of 78.84 μmol m⁻² s⁻¹). Germination rate (%) was estimated as the percentage of germinated seeds in relation to the number of seeds that were sown.

Statistical analysis The parameters analysed were compared between groups through randomization tests, analogous to *t*-test, with 10,000 permutations. Randomization tests enabled a conventional *t*-test approach to be conducted without transformation on the raw data, since they are not based on assumptions of normality and homogeneity of variances. The analysis used Euclidean distances and considered a significance level of $P \leq 0.05$.

Results

The exposure to *H. psiadioides* volatiles did not affect significantly the target species in most of the vegetative and reproductive parameters measured (Table 1). Differently, rosette diameter of juvenile plants was significantly affected by *H. psiadioides* essential oil ($P < 0.001$) (Fig. 1). Reduction of the rosette diameter in plants treated with the volatiles was about 17.34% in relation to the control group, measured after the exposure to the volatiles. Furthermore, dry aerial biomass of adult plants exposed to the volatiles was 25% higher than control ($P = 0.05$). Germination rate of seeds produced (%) by plants was similar between control and exposed to volatiles; however, seed germination from control plants was lower than the expected (i.e., close to 100%).

Table 1. Vegetative and reproductive parameters of juvenile and adult *Arabidopsis thaliana* plants exposed to the volatiles of *Heterothalamus psiadioides*. The data are presented as mean ± standard deviation (n = 10). Values with the same letter do not differ significantly, according to randomization tests, at $P \leq 0.05$

Measured Parameters	Juvenile plants		Adult plants	
	Control	Volatiles	Control	Volatiles

Number of primary stems	4.60 ± 2.22a	4.10 ± 1.37a	4.50 ± 1.51a	5.10 ± 2.18a
Day of first flower open	40.70 ± 2.58a	40.50 ± 2.80a	–	–
Number of siliques in main stem	415.50 ± 98.63 a	398.70 ± 95.72a	368.30 ± 116.20a	373.50 ± 177.99a
Number of total siliques	555.10 ± 88.30a	565.30 ± 106.98a	509.00 ± 131.57a	525.90 ± 209.64a
Seed weight (g)	0.09 ± 0.02a	0.09 ± 0.04a	0.07 ± 0.02a	0.08 ± 0.04a
Dry aerial biomass (g)	0.50 ± 0.08a	0.49 ± 0.06a	0.30 ± 0.07a	0.40 ± 0.12b
Germination rate (%)	74.00 ± 10.73a	78.00 ± 16.06a	65.00 ± 22.65a	52.00 ± 11.76a

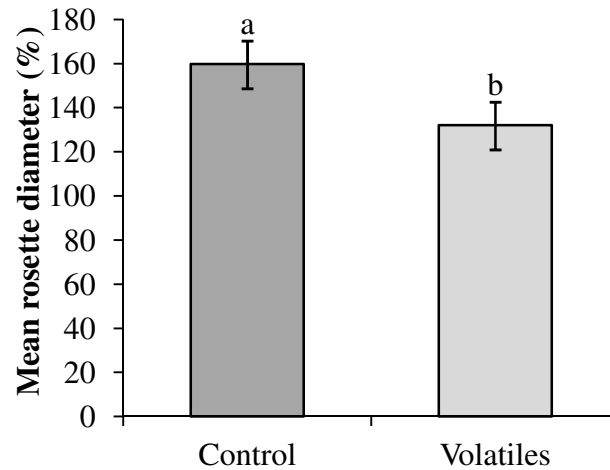


Fig. 1 Mean percentage of rosette diameter of juvenile *Arabidopsis thaliana* plants after exposure to the volatiles of *Heterothalamus psiadioides* (rosette size before exposure was 100%). The data are presented as mean ± standard deviation (n = 10). Values with different letters differ significantly, according to randomization tests, at $P \leq 0.05$

Discussion

The essential oil of this shrub has already demonstrated severe phytotoxic effects on germination and early growth of *A. thaliana* seeds and seedlings, despite of small amounts used (from 5 to 25 μL) (unpublished data). Inhibition of mitotic activity and induction of chromosomal abnormalities on primary root meristem of lettuce and onion was also observed in plants exposed to *H. psiadioides* essential oil (100 μL) (Schmidt-Silva et al. 2011). Thus, the effects of the volatiles on the initial development of plants were more expressive than the effects observed in this study on juvenile and adult plants. This may be due to the fact that the seedlings have thinner epidermis and cuticle than adult plants, enabling the volatile compounds to enter more easily into cells (Rice 1984).

Vegetative parameters affected by *H. psiadioides* essential oil were dry aerial biomass of adult plants and rosette diameter in juvenile plants. The result of biomass was disregard because each experiment was replicated once (data not shown) and this result happened only once.

Regarding the rosette diameter, a result that was confirmed and reproducible, we hypothesize that, in the conditions of this study, effects of the oil are just found immediately after its exposure. In the course of time, plants may have recovered from physiological effects and continued their normal development. Similarly, aqueous extracts of *Lonicera maackii* (Rupr.) Maxim. caused a reduction of the rosette diameter when *A. thaliana* plants were six weeks old; however, even with continuous exposure to the extracts, normal rosette diameter was recovered after some weeks (Cipollini and Dorning 2008). *Heterothalamus psiadioides* volatiles showed a seasonal variation in the magnitude of its phytotoxic effects, and volatiles were highly phytotoxic in most of the seasons (Silva 2014). Volatiles production and emission rates are conditioned by biotic and abiotic factors (Langenheim 1994) and when the emission of *H. psiadioides* volatiles decreases, plants exposed to them may overcome the allelopathic effects. Based in the results of the present study, we suggest that if allelopathic effects indeed occur for this shrub, they may be reversible when interrupted for some period, and the allelopathic potential of *H. psiadioides* would be lower than expected in previous studies.

The present research is the first to investigate the effects of volatile compounds on fully developed plants, such as juveniles and adults. Our study also presents relevant observations on plant grown in soil, since several studies are conducted with filter paper or culture medium, substrates that overestimate the allelopathic potential of plants. However, some adaptations in our methods are necessary, such as implantation of an air circulation system to avoid water condensation inside the chambers and saturation of humidity because of plant transpiration. Another point to consider is that if the exposure to *H. psiadioides* volatiles continued for a longer period or if more than one exposition was done, negative effects would be seen.

Acknowledgments We thank the Brazilian Funding agency Comissão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted to the first author.

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6 CONSIDERAÇÕES FINAIS

Os efeitos fitotóxicos do óleo essencial de *H. psiadioides* já foram demonstrados por estudos anteriores e com diferentes plantas-alvo. No presente trabalho os voláteis do óleo essencial de *H. psiadioides*, mesmo em quantidades muito pequenas, causaram efeitos extremamente deletérios à

germinação e crescimento inicial da planta alvo *A. thaliana*. Dessa forma, acreditamos que os efeitos negativos do óleo essencial de *H. psiadioides*, nessas fases de desenvolvimento, estejam devidamente fundamentados e possam ser generalizados para quaisquer espécies-alvo.

Já o estudo do enraizamento adventício deve ser mais explorado, pois consiste de uma nova forma de avaliação para pesquisas de fitotoxidez. Nesse trabalho, as raízes adventícias da planta-alvo expostas ao óleo foram reduzidas em número e tamanho, e tiveram maior tempo médio de enraizamento. Verificou-se ainda que as microestacas expostas ao óleo essencial de *H. psiadioides* apresentaram-se despigmentadas, fenótipo causado por estresse oxidativo. Na tentativa de recuperar esses efeitos e assim encontrar uma possível causa para os mesmos, os hormônios auxina e citocinina, e o antioxidante Trolox foram utilizados. Entretanto, nenhum desses tratamentos foi capaz de reverter os efeitos causados pelo óleo. Presume-se que altos níveis de EROs foram geradas e isso levou a efeitos secundários como a degradação de auxina, apesar dessa variável não ter sido mensurada.

Em relação à investigação feita com as plantas juvenis e adultas, apenas encontrou-se efeito negativo dos voláteis do óleo essencial de *H. psiadioides* na mensuração do diâmetro da roseta, que aconteceu imediatamente após a exposição aos voláteis. Portanto, é possível que ao longo do tempo as plantas se recuperaram dos efeitos fisiológicos e morfológicos causados pelos voláteis e continuaram seu desenvolvimento normal. Dessa forma, não foram encontrados efeitos negativos em parâmetros reprodutivos, contudo não pode ser descartada a possibilidade de que se fossem realizadas mais exposições aos voláteis, efeitos pudessem ser relatados.

No presente estudo, bem como em estudos prévios, os voláteis de *H. psiadioides* mostraram-se fitotóxicos para a germinação e para plântulas de diferentes espécies-alvo. Entretanto, quando seus efeitos foram testados sobre plantas juvenis e adultas, resultados pouco relevantes para o desenvolvimento vegetativo e reprodução dessas plantas foram encontrados. Por isso, fases iniciais do desenvolvimento de uma planta podem ser mais sensíveis e são estas as fases que a maioria dos estudos de fitotoxidez avalia. Dessa forma, nossos resultados indicam que *H. psiadioides* possui potencial alelopático para afetar plantas vizinhas em campo devido, principalmente, aos efeitos dos voláteis sobre a germinação e crescimento inicial.

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