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INSTITUTO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA

VALERÍ SCHMIDT DA SILVA

TESE DE DOUTORADO

POTENCIAL ALELOPÁTICO DO ÓLEO ESSENCIAL DE ESPÉCIES DE *Heterothalamus* Less. (ASTERACEAE) NATIVAS NO RIO GRANDE DO SUL

Orientador: Prof. Dr. Geraldo LG Soares
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PORTO ALEGRE – RS
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Tese apresentada ao Programa de Pós-Graduação em Botânica da Universidade Federal do Rio Grande do Sul como um dos requisitos para obtenção do grau de Doutor em Ciências: Botânica.

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SUMÁRIO

RESUMO- As Asteraceae constituem o *taxon* mais numeroso dentro das angiospermas eudicotiledôneas, compreendendo cerca de 1.100 gêneros e de 25.000 espécies. São plantas de hábito extremamente variado, incluindo principalmente pequenas ervas ou arbustos e, raramente, árvores. Esta família possui um enorme sucesso na produção de metabólitos que atuam na defesa química e esse seria um dos motivos de sua distribuição cosmopolita. As plantas dessa família são extensivamente estudadas quanto à sua composição química e atividade biológica, algumas tendo servido de base para o desenvolvimento de novos fármacos e defensivos agrícolas. *Heterothalamus* apresenta espécies com elevado potencial de colonização de áreas degradadas pela interferência antrópica. Entretanto, é desconhecido o(s) mecanismo(s) responsável (eis) pela capacidade de competição e invasora dessas espécies vegetais. Neste trabalho foi testada a hipótese que produtos voláteis do metabolismo secundário de *Heterothalamus psiadioides* Less. e *H. alienus* (Spreng.) O. Kuntze produzem modificações no padrão de germinação e desenvolvimento de *Lactuca sativa* L. e *Allium cepa* L. O óleo essencial foi extraído das folhas por hidrodestilação e a análise foi realizada por cromatografia gasosa e espectrometria de massa. Os bioensaios utilizaram como espécies teste o alface (*Lactuca sativa* L.) e cebola (*Allium cepa* L.) e empregaram óleo puro e soluções de 10 e 1% (v/v) em caixas de germinação. Os parâmetros avaliados foram porcentagem de germinação, velocidade de germinação, medida da raiz e da parte aérea, índice mitótico, metafásico, anormalidades cromossômicas, alterações anatômicas e integridade de membrana. Os resultados mostraram uma redução na germinação de alface tratadas com óleo essencial de *H. psiadioides.* Além disso, uma redução na velocidade de germinação e no crescimento de alface e cebola submetidos a ambos os óleos essenciais foi observada. Os óleos essenciais mostraram dezessete componentes em comum, sendo os monoterpenos os compostos majoritários e o β-pineno o composto em maior quantidade encontrado em ambos os óleos. A alface submetida aos óleos de *H. psiadioides* e *H. alienus* reduziram o índice mitótico para todos os tratamentos utilizados. Cebola submetida ao óleo de *H. psiadioides* apresentou uma diminuição no índice mitótico, as tratadas com *H. alienus* apresentaram diminuição no índice mitótico em 89% na maior concentração utilizada. Foram vistas também anormalidades cromossômicas como aderência, c-mitose, micronúcleos, pontes em anáfase em raízes das espécies testadas submetidas aos óleos essenciais de *Heterothalamus*. A análise das alterações anatômicas mostrou danos nas estruturas e grande dano a membrana em plântulas da raiz de alface e cebola na maior concentração de óleos essenciais utilizada. O β-pineno apresentou atividade biológica, mas não correlacionado com os óleos essenciais. A atividade observada pelos os óleos essenciais parece ser resultado da atividade de outro componente ou de uma interação sinérgica entre compostos dos óleos essenciais.

ABSTRACT- The Asteraceae are the most numerous taxon within the eudicots angiosperms, comprising about 1,100 genera and 25,000 species. The plants are extremely varied in habit, mainly including herbs or small shrubs and rarely trees. This family has great success in the production of metabolites that work in chemical defense and that would be one of the reasons for its cosmopolitan distribution. Species of this family are extensively studied about their chemical composition and biological activity, some of them having served as the basis for the development of new drugs and agrochemicals. *Heterothalamus* species can show high potential for colonization of areas degraded by human interference. However, it is unknown what mechanism responsible for competing and invasive capacity of this plant species. In this work we tested the hypothesis that volatile products of secondary metabolism of species tested produce changes in germination pattern and development of *Lactuca sativa* L. and *Allium cepa* L. The essential oil was extracted from the leaves by hydrodistillation and the analysis was performed by gas chromatography and mass spectrometry. The bioassays used as test species lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.). Treatments employed pure oil, 10% and 1% aqueous solution (v/v) in germination boxes. The parameters evaluated were the percentage of germination, speed of germination, measured the seedling's root and shoot length, mitotic index, metaphasic index, chromosomal abnormalities, anatomical changes and membrane integrity. The results showed a reduction in germinability of lettuce treated with essential oil of *H. psiadioides.* Additionally a reduction in speed of germination and in growth of lettuce and onion submitted to both essential oils was verified. The essential oils of the *Heterothalamus* species investigated had seventeen components in common, and β-pinene was the major compound for both oils. Lettuce seeds germinated with essential oils from *H. psiadioides* and *H. alienus* had a reduced mitotic index (MI) for all treatments. Onion seedlings treated with *H. psiadioides* oil showed a decrease in MI, and seedlings treated with *H. alienus* oil showed a decrease in MI of 89% in the most concentrated treatment. Analysis of the results showed chromosomal abnormalities, including stickiness, c-mitosis, micronuclei and anaphase bridges in lettuce and onion root tips exposed to essential oils of *Heterothalamus*. Lettuce and onion seeds which germinated with essential oils from *H. psiadioides* and *H. alienus* had structural alterations in root anatomy, increased membrane damage and change features of early stages of root development. Beta-pinene showed biological activity, but not correlated to the essential oils. The activity observed for the essential oils should be a result of the activity of other component or of a synergic interaction among some compounds of the essential oils.

INTRODUÇÃO GERAL

As Asteraceae constituem o *taxon* mais numeroso dentro das angiospermas eudicotiledôneas, compreendendo cerca de 1.100 gêneros e de 25.000 espécies. São plantas de hábito extremamente variado, incluindo principalmente pequenas ervas ou arbustos e, raramente, árvores (Heywood 1993). Asteraceae (Compositae) é a maior família de Magnoliopsida, com cerca de 23.000 espécies conhecidas, agrupadas em 1.535 gêneros e 17 tribos (Bremer 1994; Judd et al. 1999).

Para Almeida-Cortez *et al.* (2004), Asteraceae é uma das famílias com maior sucesso na produção de metabólitos que atuam na defesa química e esse seria um dos motivos de sua distribuição cosmopolita (Almeida-Cortez *et al.*, 2004). As plantas dessa família são extensivamente estudadas quanto à sua composição química e atividade biológica, algumas tendo servido de base para o desenvolvimento de novos fármacos e defensivos agrícolas (Verdi *et al.* 2005)

A subtribo *Baccharidinae* Hoffmann, composta pelos gêneros *Baccharidastrum* Cabrera, *Baccharis* L. e *Heterothalamus* Less, constitui-se em sua maioria de plantas ruderais e/ou invasoras de pastagens, comportando-se como plantas agressivas durante sua instalação, especialmente na ocupação de áreas perturbadas. Essas plantas ocorrem em formações densas e muitas vezes dominantes. Historicamente, investigações de processos alelopáticos são iniciadas a partir de observações de campo, as quais sugerem uma modificação no padrão de vegetação (Einhellig *et al.* 2002). Em muitos ecossistemas, as plantas tendem a se estabelecer em populações densas e homogêneas, o que é atribuído entre outros fatores, à liberação de substâncias fitotóxicas, dificultando ou não permitindo o estabelecimento de outras espécies na proximidade dessas populações (Castro *et al.* 2002). No Brasil o maior centro de dispersão desta subtribo está localizado na Região Sul (Barroso & Bueno 2002).

Heterothalamus é composto por três espécies (*Heterothalamus psiadioides* Less., *H. alienus* (Spreng.) O. Kuntze e *H. rupestris* Deble , A.S. Oliveira & Marchiori). Essas espécies são arbustos de mais ou menos 0,30 – 2 m de altura, ramificados, com folhas alternas densamente pontuadas de glândulas (Barroso & Bueno 2002).

Heterothalamus psiadioides é um arbusto que tem registro apenas em Santa Catarina e no Rio Grande do Sul, possivelmente sendo endêmica desses estados brasileiros. Há registros que no Rio Grande do Sul esta espécie esteja se tornando pioneira antrópica na região de Porto Alegre formando associações densas, principalmente em lugares devastados e em beiras de estradas (Barroso & Bueno 2002).

A outra espécie deste gênero com ocorrência no Rio Grande do Sul é *H. alienus* que se distribui de Santa Catarina até o Uruguai e a Argentina. É um arbusto de mais ou menos 0,5-1 m de altura, folhas de margens inteiras, com 1,5-2 cm de comprimento e glabras. Sua ocorrência está associada com solos rasos em campos naturais ou em margens de rios (Barroso & Bueno 2002).

Heterothalamus apresenta espécies com elevado potencial de colonização de áreas degradadas pela interferência antrópica. Entretanto, é desconhecido o(s) mecanismo(s) responsável (eis) pela capacidade de competição e invasora dessas espécies vegetais.

A alelopatia pode ser definida como um processo pelo qual produtos do metabolismo secundário de uma determinada espécie vegetal são liberados, afetando a germinação e o desenvolvimento de outras espécies vegetais sintópicas (Soares & Vieira 2000). O termo alelopatia engloba todos os tipos de interações químicas entre plantas e microorganismos locados no reino vegetal. Embora já conhecido há longo tempo (século 5 A.C. – Demócritos, século 3 A.C. – Theophrastus) somente a partir da década de 60 é que este fenômeno vem sendo cada vez mais reconhecido como um importante mecanismo ecológico e agrícola.

Whittaker e Fenny (1971) estudaram as interações bioquímicas entre plantas. Sua pesquisa definiu aleloquímicos como todas as interações químicas entre os organismos. Elroy L. Rice (1984) incluiu todos os efeitos diretos positivos e negativos de uma planta ou de um microorganismo sobre outras plantas liberando substâncias químicas para o ambiente.

Os aleloquímicos são encontrados nos vegetais, distribuindo-se em todos os seus órgãos. A liberação dos aleloquímicos para a rizosfera pode ocorrer através da lixiviação das folhas e partes aéreas, como foi descrito para *Pteridium aquilinum* (L.) Kuhn, onde a liberação de aleloquímicos é feita por lixiviação de suas frondes sendo capaz de reduzir a germinação e o crescimento de outras espécies (Stewart, 1975).

Outro tipo de liberação de aleloquímicos para o ambiente é a volatilização de compostos químicos. Isso é observado em plantas de cravo-da-índia (*Syzygium aromaticum* (L.) Merr. & Perry) que liberam o eugenol, um derivado fenólico volátil com ação alelopática (Mazzafera 2003). Em raízes e materiais vegetais em decomposição também pode ocorrer exsudação de compostos para o ambiente (Weir *et al.* 2004). Um exemplo disso é o que acontece com o centeio (*Secale cereale* L.), um cereal de inverno que exsuda vários compostos pelas raízes ou pela decomposição de suas palhas (Souza & Furtado 2002).

De acordo com Rizvi and Rizvi (1992) os aleloquímicos podem afetar estruturas citológicas, ultraestruturais e hormônios alterando tanto suas concentrações quanto o balanço entre os diferentes hormônios. Também pode afetar a permeabilidade de membranas, absorção de minerais, movimentos dos estômatos, síntese de proteínas, atividade enzimática, relações hídricas, condução e materiais genéticos induzindo alterações no DNA e RNA.

A alelopatia, ao contrário da competição, se caracteriza pela introdução de substâncias produzidas pelo metabolismo secundário no meio ambiente (Rice 1974; Putnam & Tang 1986). De acordo com Einhellig uma característica da alelopatia, que a diferencia de outras interações biológicas, é que a atividade ocorre depois que os aleloquímicos são liberados no ambiente (Einhellig, Reigosa *et al.* 2002).

Os efeitos alelopáticos são mediados por substâncias que pertencem a diferentes categorias de metabólitos secundários. Os recentes avanços na química de produtos naturais, por meio de métodos

modernos de extração, isolamento, purificação e identificação, têm contribuído bastante para um maior conhecimento desses compostos secundários, os quais podem ser agrupados de diversas formas (Ferreira 2004). Embora as substâncias alelopáticas tenham sido comumente encontradas em extratos e resíduos das plantas, algumas foram encontradas em exsudatos de plantas vivas e gases voláteis liberados através de folhas e rizomas (Souza Filho & Alves 2002).

Óleos essenciais são líquidos aromáticos que se evaporam quando expostos ao ar. São obtidos de várias partes vegetais principalmente por hidrodestilação. Constituem-se em misturas de substâncias divididas em dois grupos principais: derivados terpenoídicos (principalmente mono- e sesquiterpenóides) e/ou derivados fenilpropanoídicos (principalmente ésteres, aldeídos derivados do ácido cinâmico) (Wink 1990). Os óleos essenciais distribuem-se amplamente nas plantas floríferas, especialmente nas famílias Asteraceae, Laminaceae, Lauraceae, Myrtaceae, Rutaceae e Apiaceae (Alonso 1998). Sua função envolve principalmente sinais de comunicação química (semioquímicos) entre plantas e a biota associada e atuam como componentes do sistema de defesa química vegetal.

Assim como *Salvia* L. (Lamiaceae), *Eucalyptus* L'Her. (Myrtaceae) e *Artemisia* L. (Asteraceae) liberam produtos voláteis como canfeno, dipentano, pineno que inibem o desenvolvimento de outras plantas (Almeida 1988). Das pesquisas com Asteraceae, Dias (2000) verificou que o óleo essencial obtido das flores de *Aster lanceolatus* Kuntze demonstrou ser capaz de inibir a germinação e crescimento de *Lactuca sativa* L. Apesar de ser reportado na literatura o potencial alelopático dos óleos essenciais a maior parte dos trabalhos é relacionada com extratos aquosos como em carqueja (*Baccharis trimera* (Less.) D.C.) que foram observados retardo na germinação de sementes de tomate. Foi observado que este extrato provocava também alterações no tempo e na velocidade média de germinação. Além disso, a aplicação em cobertura do resíduo do extrato aquoso bruto da parte aérea ou da planta picada, assim como plantas incorporadas no substrato, reduziu significativamente a emergência e o número de folhas de espécies de *Cyperus* L.(Cyperaceae) (Castro & Ferreira 2000).

Observações em formações vegetais onde ocorrem espécies da subtribo *Baccharidinae,* sugerem o seu grande potencial alelopático; pois tais espécies apresentam comportamento em conformidade com a emissão de substâncias fitotóxicas para o ambiente, uma vez que forma populações densas e apresenta sinais de inibição ao desenvolvimento de outras espécies vegetais em condições de campo (Carreira 2007).

Atualmente, a maioria dos estudos sobre efeito alelopático se restringe a ensaios de laboratório e/ou casa de vegetação, onde é verificado simplesmente os efeitos dos extratos e substâncias ativas fixas (não voláteis) sobre a germinação e sobre o desenvolvimento de espécies alvos. Análises que permitam inferir sobre a natureza da atividade fitotóxica das substâncias potencialmente alelopáticas são raros, e permitem um avanço significativo na compreensão do papel das substâncias do metabolismo secundário nas interações entre espécies vegetais. É importante ressaltar que é praticamente desconhecido o potencial alelopático e a natureza do efeito fitotóxico de substâncias que compõem óleos essenciais.

O presente trabalho teve como objetivo contribuir de maneira significativa para o preenchimento dessa lacuna no conhecimento científico do fenômeno químico-ecológico denominado alelopatia.

2. HIPÓTESE - Foi testada a hipótese que produtos voláteis do metabolismo secundário das espécies estudadas produzem modificações no padrão de germinação, desenvolvimento, alterações citogenéticas, na estrutura anatômica da raiz e na integridade de membranas de plantas teste padrão (alface e cebola).

3. OBJETIVOS

- Avaliar a composição química dos óleos essenciais de *H. psiadioides* e *H. alienus*;
- Avaliar o efeito do óleo essencial de *H. psiadioides* e *H. alienus* e do composto majoritário dos óleos (β-pineno) sobre a germinação e sobre o desenvolvimento inicial de espécies cultivadas;
- Verificar a ocorrência de alterações citogenéticas em raízes de espécies vegetais cultivadas tratadas com β-pineno e com o óleo essencial de *H. psiadioides* e *H. alienus*;
- Verificar a ocorrência de alterações anatômicas em raízes de espécies vegetais cultivadas tratadas com β-pineno e com o óleo essencial de *H. psiadioides* e *H. alienus*;

4. MATERIAL E MÉTODOS

4.1. Coleta do Material Vegetal, Extração e Análise dos óleos Essenciais: Folhas de *Heterothalamus psiadioides* e *H. alienus* foram coletadas nas cidades de Porto Alegre e Bagé (Rio Grande do Sul – Brasil). *H. psiadioides* (Figura 1A) foram coletadas em vegetação natural no Morro Santana (30°3'S, 51°7'W) na cidade de Porto Alegre e *H. alienus* (Figura 1B) ao longo de uma rodovia em Bagé (31°18'41"S, 53°56'17"W). As espécies de *H. psiadioides* (153122) e *H. alienus* (153825) foram identificadas e exsicatas foram depositadas no Herbário ICN da Universidade Federal do Rio Grande do Sul (UFRGS).

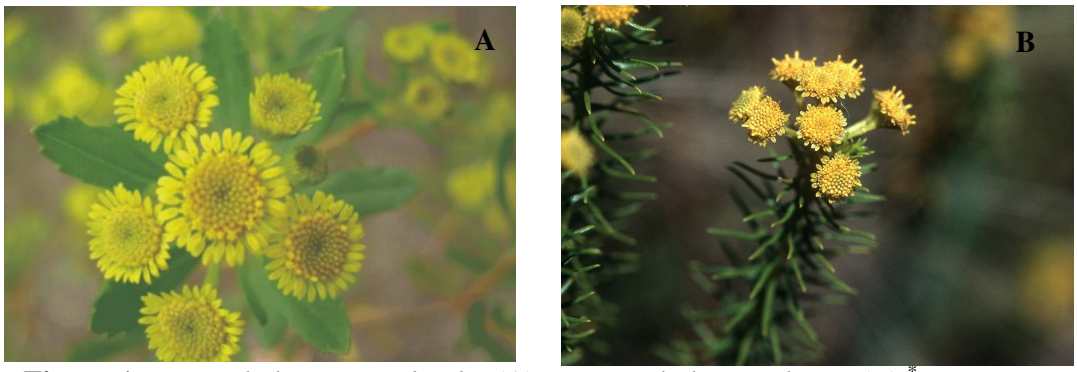

Figura 1. *Heterothalamus psiadioides* (A) e *Heterothalamus alienus* (B).[*]

A caracterização química dos óleos essenciais obtidos foi realizada na Central Analítica do Departamento de Química, IQ/UFRGS. As análises cromatográficas foram realizadas em cromatógrafo

[*] Fonte: (A)http://www6.ufrgs.br/fitoecologia/florars/index.php?pag=buscar_mini.php (B) https://sites.google.com/site/asteraceaegroup/esp%C3%A9ciescampestresrs.

gasoso com detector de ionização em chama (GC-FID) (GC17A, Shimadzu – Figura 3A) e um cromatógrafo gasoso acoplado a um espectrômetro de massas (GC-MS) (GCMS-, Shimatdzu – Figura 3B). Duas colunas capilares foram utilizadas sob as seguintes condições: (i) OV-5 (30 m x 0.25 mm x 0.25 mm, Ohio Valley, Marietta, Ohio, USA) o forno programado de 60ºC a 250ºC a 3ºC min^{-1}. (ii) (ii) DB-WAX (30 m x 0.25 mm x 0.25 mm, J&W Scientific, Folsom, CA, USA) o forno programado de 40ºC a 220ºC a 3ºC min^{-1}. A temperatura do injetor e do detector foram mantidos a 250ºC para OV5 e a 220ºC para DBWAX, sendo utilizado hélio como gás de arraste em um fluxo de 1,0 $ml.min^{-1}$.

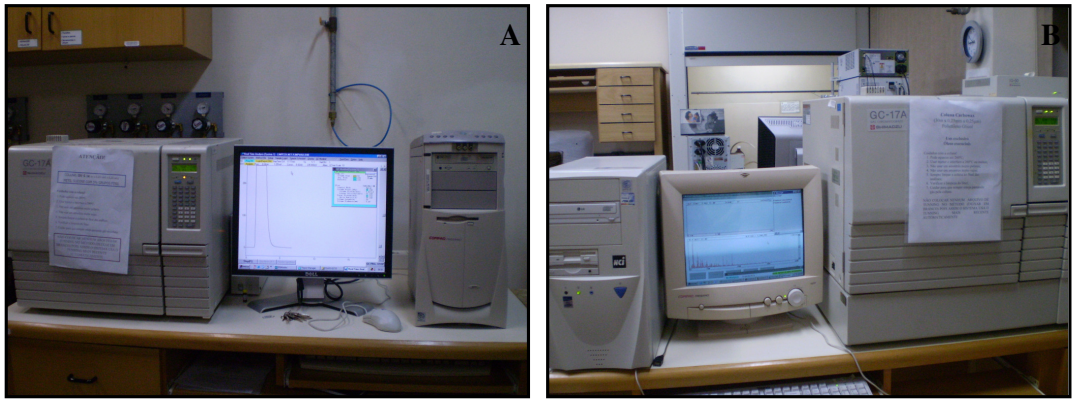

Figura 2. Equipamentos utilizados para a caracterização química. Cromatógrafo gasoso acoplado a detector de ionização em chama (GC-FID) (A). Cromatógrafo gasoso acoplado a espectrômetro de massas (GC-MS) (B).

A determinação estrutural dos componentes do óleo foi feita a partir da comparação dos dados de fragmentação das substâncias com as existentes no banco de dados disponível (Wiley 6ªed) e, eventualmente pela comparação dos dados de fragmentação com dados obtidos na literatura especializada (Adams, 1995).O composto majoritário dos óleos caracterizados quimicamente, o β-pineno, foi adquirida no mercado na forma semi-purificada.

4.2. Potencial Alelopático: Os bioensaios realizados para avaliar a atividade alelopática do óleo essencial de *Heterothalamus psiadioides* e de *H. alienus* incluíram a avaliação dos efeitos sobre a germinação, através da inibição e estímulo, e o crescimento/desenvolvimento através da medida do alongamento da raiz primária/adventícia e do hipocótilo/coleóptilo das espécies-alvo (Inderjit Dakshini & Einhellig 1995; Rice 1974). Os estudos foram feitos com diásporos de espécies cultivadas: alface (*Lactuca sativa* L. cv. Grand rapids) e a monocotiledônea cebola (*Allium cepa* L. cv. Baia Periforme).

4.2.1. Ensaios de germinação e crescimento: Foram utilizadas caixas gerbox forradas com uma camada de papel filtro umedecido com 7 mL de água destilada, sobre os quais foram semeadas 50 diáporos de alface (*Lactuca sativa* L.) e cebola (*Allium cepa* L.) (Figura 3A). Na tampa da caixa foi colado, com

auxílio de fita dupla face, um pedaço de algodão no qual foi adicionado 100 µL de cada diluição de óleo essencial testado, sendo utilizada como controle água destilada (Figura 3B). Foram testados os tratamentos 100% (óleo puro), 10% e 1%. Para os tratamentos de 1% e 10% foi realizada uma diluição a partir do óleo essencial puro com o auxílio de uma solução de Tween 20 a 1%. As caixas foram envoltas com filme plástico, de modo a minimizar a perda dos compostos voláteis. Os testes de germinação foram conduzidos em sala de cultivo com temperatura de 23 ºC ± 2ºC e fotoperíodo de 12 horas. O suprimento de luz foi proporcionado por lâmpadas fluorescentes (20 W).

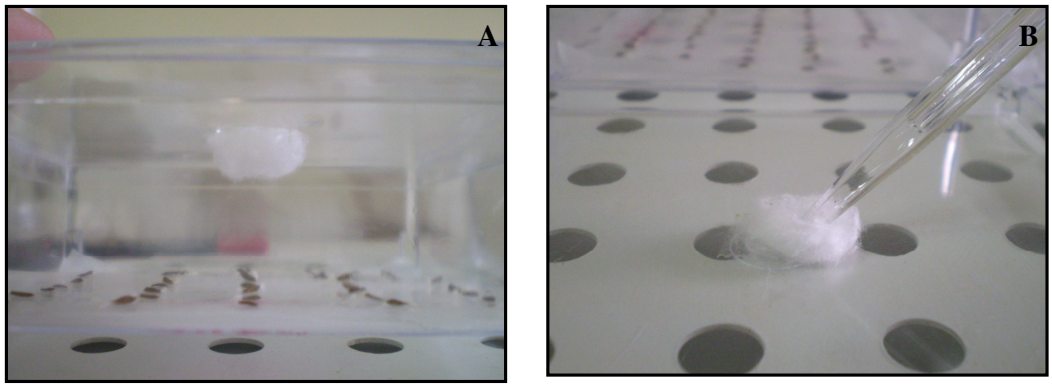

Figura 3. Montagem do experimento de germinação.

Para o ensaio de germinação, os diásporos foram semeados e em seguida, adicionados o óleo em cada caixa gerbox, sendo realizada a contagem dos diásporos germinados diariamente para cálculo do índice de velocidade de germinação (IVG), e, ao final de 72 horas, a porcentagem final de germinação para cálculo da germinabilidade (Ferreira 2004). Já para o ensaio de crescimento do hipocótilo/coleóptilo e raiz primária/adventícia, os diásporos foram semeados e, após 24 horas, quando já se encontravam germinados, foi adicionado o óleo em cada uma das placas.

Foram considerados germinados os diásporos que produziram raiz primária com pelo menos 2 mm de comprimento. O crescimento das plântulas foi avaliado por meio das medidas do hipocótilo/coleóptlio e raiz primária/adventícia obtidas com o auxílio de papel milimetrado.

Os resultados foram submetidos à análise de variância (ANOVA) e quando ocorreram diferenças entre os grupos foi realizado o teste de Tukey. Quando os pressupostos de normalidade e homogeneidade não foram atingidos foi realizado o teste de Kruskal-Wallis como alternativa para a Anova e teste de Dunn como alternativa para o teste de Tukey. Para as análises estatísticas foi utilizado o programa Bioestat 5.0.

4.3 Análises de Alterações Citogenéticas: Sementes de *Lactuca sativa* e *Allium cepa* foram germinadas em caixas gerbox com atmosfera saturada com os óleos essenciais de *Heterothalamus psiadioides* e *H. alienus* como o descrito no item 3.2.1. Além de alterações na morfologia dos cromossomos foi analisada a indução de variações no índice mitótico e metafásico das plântulas obtidas. O tempo após a germinação variou de espécie para espécie e foi determinado quando as raízes da espécie tratada

atingiram entre 0,5 e 1,0 cm de comprimento. As raízes obtidas foram fixadas em uma mistura de etanol e ácido acético na proporção 3:1 (Figura 5A). Após um período de 24h de pós-fixação as raízes foram submetidas à hidrólise com HCl 5N (temperatura ambiente) e à coloração pelo método de Feulgen (Aguiar-Perecin & Vosa 1985). As lâminas com as células meristemáticas coradas foram analisadas em fotomicroscópio Zeiss Axioplan®.

Os índices mitótico (IM) e metafásico (IMet) foram determinados nos controles e nas raízes tratadas, sendo estimados de acordo com as seguintes fórmulas:

IM = número de células em divisão/número total de células meristemáticas*100.
IMet = número de células em metáfase/número total de células meristemáticas*100.

Para a avaliação genotóxica foi investigada a ocorrência das seguintes alterações mitóticas: pontes (anafásicas e telofásicas), aderências, fragmentos cromossômicos, c-mitoses, anáfases multipolares, retardatários e micronúcleos.

4.4 Avaliações Anatômicas: Raízes primárias ou adventícias conforme a espécies testada no ensaio de potencial alelopático (3.2.1) foram fixadas em solução de formaldeído 1% e glutaraldeído 4% em tampão fosfato pH 7,2 desidratados em série etílica, infiltrados e emblocados em historesina (Figura 6A-B). Secções anatômicas foram obtidas em micrótomo rotativo (0,6 µm) (figura 6C) corados com azul de toluidina (Figura 6D) (O'Brien *et al.*, 1964).

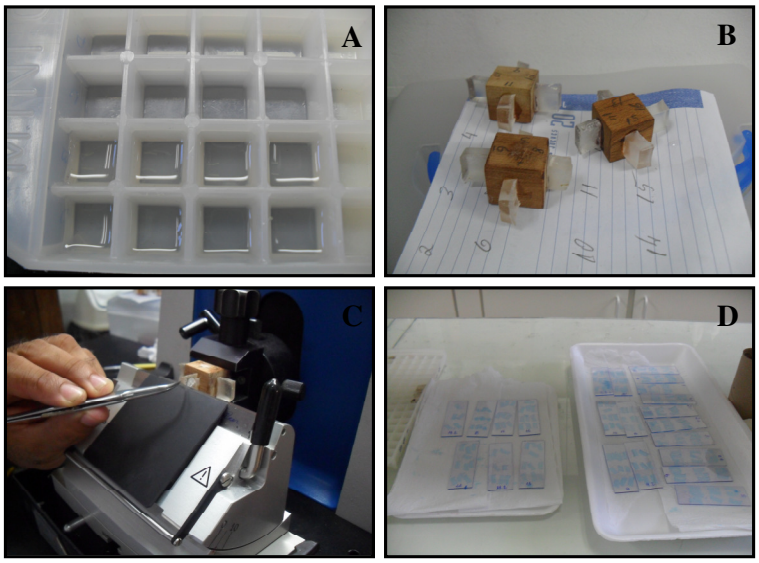

Figura 6. Metodologia para análises anatômicas.

Histometria – Às análises estruturais das raízes foram adicionados dados histométricos e citométricos. Esses dados foram obtidos a partir de fotomicrografias de secções transversais da região da raiz (1 cm a partir do ápice radicular), a mensuração foi feita aleatoriamente. Foi avaliada a área do tecido (n=10) e a

área de células do cilindro vascular, córtex e epiderme (n=15). As medidas foram realizadas usando o programa AxionVision (Zeiss Imaging Systems – versão 4.7.2).

4.5. Avaliação da Integridade das Membranas

A fim de avaliar o vazamento relativo de eletrólitos, 50 mg de raízes foram lavados e incubados em água destilada a temperatura ambiente por 24 horas (Figura 6A). Após esse período a condutividade elétrica da solução foi medida com o auxílio de um condutivímetro (Figura 6B-C). As raízes foram então congeladas (Figura 6D) e em seguida imersas em água destilada por mais 24 horas e medida a condutividade elétrica novamente (Pinheiro & Fletcher, 1994).

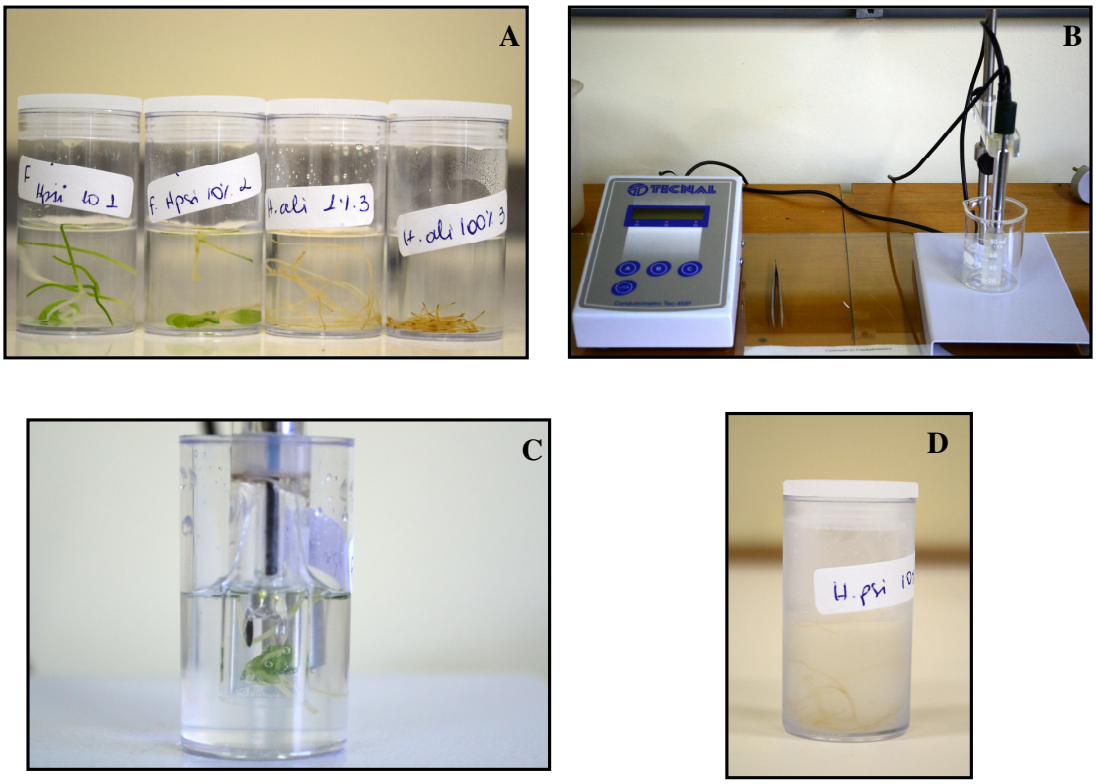

Figura 6. Metodologia Integridade de membrana.

O vazamento relativo de eletrólitos foi medido de acordo com a seguinte equação:

$$REL\ (\%) = (C_1 / C_{1+} C_2).100$$

Onde: C1 = Condutividade medida durante as primeiras 24 h
C2 = Condutividade medida após as 24 h seguintes.

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ARTIGO 1

Chemical composition and phytotoxic potential from two species of *Heterothalamus* Lessing (Asteraceae)

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Chemical composition and phytotoxic potential from two species of *Heterothalamus* Lessing (Asteraceae)

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ABSTRACT – *Heterothalamus* species can produce large amounts of essential oils and they have high potential for colonization of areas degraded by human interference. The aim of this work was a chemical characterization and determination of phytotoxic potential of essential oil of *Heterothalamus psiadioides* and *H. alienus.* The essential oil was extracted from the leaves by hydrodistillation and the analysis was performed by gas chromatography and mass spectrometry. The bioassays used as test species lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.). Treatments employed pure oil, 10% and 1% aqueous solution (v/v) in germination boxes. The parameters evaluated were the percentage of germination, speed of germination and measured the seedling's root and shoot length. A reduction in germinability of lettuce treated with essential oil of *H. psiadioides* was observed. Additionally a reduction in speed of germination and in growth of lettuce and onion submitted to both essential oils was verified. The essential oils of the Heterothalamus species investigated had seventeen components in common, and β-pinene was the major compound for both oils. Beta-pinene showed biological activity, but not correlated to the essential oils. Phytotoxicity observed in essential oils can be due to a result of the activity of other component or due to a synergic interaction of some compounds of the essential oils.

Key words: *Heterothalamus psiadioides, Hetherothalamus alienus,* Asteraceae, essential oil, germination; seedling growth

1. **Introduction**

The Asteraceae family includes many useful species (medicinal, agricultural, industrial, etc.), and their ethnobotanical use has supported a lot of people all over the world. Today 40 genera are relevant for human and animal feeding, as sources of sweeteners, insecticides and essential oils (Del Vitto and Petenatti, 2009).

Heterothalamus psiadioides Less and *Heterothalamus alienus* (Sprengel) O. Kuntze of Asteraceae family are found in South America and are used in Argentinean and Brazilian folk medicine. *Heterothalamus psiadioides* is employed as antipyretic, antidote for snake bites, anti-inflammatory (Suyenaga *et al*., 2004), febrifuge, skin remedy (Biavatti *et al.*, 2007) and against renal affections (Palacios *et al.*, 2007). Internal and external use of *Heterothalamus alienus* as stimulant and also against fever has been reported (Rücker *et al.*, 1996). It is also utilized against *Apis mellifera* L. pests (Ruffinengo *et al*., 2006), as it presents antiviral and antifungal properties (Duschatzky *et al.*, 2007). Both species are recognized as major producers of essential oils with potential biological activity.

Heterothalamus psiadioides had been registered in Santa Catarina and Rio Grande do Sul, possibly being endemic of these states. In Rio Grande do Sul this species is becoming a pioneer in regions of Porto Alegre where exists anthropic pressure, forming stands, mostly in waste places and roadsides (Barroso and Bueno, 2002). *Heterothalamus alienus* grows in the south of Brazil, northern and central Argentina and Uruguay (Duschatzky *et al*., 2007) and in Argentina shows the formation called "romerillal" in the hills with lots of wind and shallow soil (Grosso, 2007). The "romerillal" is named after *H. alienus* folk's name, which is romerillo in Argentina, where it forms dense associations with many individuals.

The vegetation of a particular area can have a succession model conditioned to preexisting plants and also influenced by the compounds they release into environment (Ferreira, 2004). In this work, it has been observed that *H. psiadioides* and *H. alienus* form populations where they are predominant over other species, and it is well known that field observations may be preliminary evidence of allelopathic activity. Allelopathy can be defined as a positive or negative interference of secondary metabolic compounds produced by plants and released into the environment (Rice, 1974).

The constituents of essential oils released by a plant can affect both germination and growth of neighboring plants. Allelopathic potential of these volatile compounds has been studied in several plants, like in *Ageratum conizoides* L., *Salvia leucophylla* Greene and *Neepeta X faasaenii* Bergmans that showed strong growth inhibition of seedlings of different species (Eom *et al*., 2006; Nishida *et al*., 2005; Singh *et al.*, 2002).

Essential oils are an important group of natural products which give the characteristic odor of aromatic plants. They comprehend a low weight mixture of monoterpenes, sesquiterpenes and volatile phenilpropanoids that exhibit a wide variety of compounds (Harborne, 1997). These substances also have many biological activities, acting as larvicides, antimicrobial, antiprotozoal, insecticidal, antiulcer, antitumor and allelopathic agents (Bagetta *et al*., 2010; Barney *et al*., 2005; Massignani *et al.*, 2009; Navarro *et al.* 2008; Rafael *et al.*, 2008; Parreira *et al*., 2010).

A previous research has shown that the essential oils of *H. psiadioides* Less. and *H. alienus* (Sprengel) O. Kuntze diminish the size of the lettuce and onion roots, decrease mitotic index, and induce chromosomal abnormalities (Schmidt-Silva et al., 2011). The chemical composition of *Heterothalamus* was reported by Suneyaga *et al.* (2004) who found 24 compounds in *H. psiadioides* essential oil and Ruffinengo *et al.* (2006) that tested the essential oil of *H. alienus* against *Apis* pests. Other components were identified in *H. psiadioides*, Kerber *et al.* (1993) reported two flavonoids (pinostrombine and galagin) and Rücker (1991) reported the isolation of bisabolene-1,4-endoperoxide. Antifungal activity of rutin and two sesquiterpenes (Pacciaroni *et al.*, 2008), diterpene glycosides and peroxides in *H. alienus* fractions (Rücker *et al*., 1996) were also observed.

These previous work showed that the major compound of essential oil for both species of *Heterothalamus* was *β*-pinene (Duschatzky *et al.*, 2007; Suyenaga *et al*., 2004). Alpha-pinene and *β*-pinene are among the most widely distributed monoterpenes in the plant kingdom and are the major constituents of various essential oils. While both optical isomers of α-pinene have been found, β-pinene almost always occurs as the optically levorotatory (-) isomer. The dextrorotatory isomer (+) is apparently of rather limited distribution (Gambliel and Croteau, 1982).

The aim of this study was to analyze the composistion of the essential oils of *H. psiadioides* and *H. alienus* and also evaluate the effect of these oils and of their main component (β-pinene) in seed germination and seedling growth of lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.).

2. Materials and Methods

Heterothalamus psiadioides and *H. alienus* were collected in march-april of 2008 and 2009, in the cities of Porto Alegre and Bagé, state of Rio Grande do Sul, Brazil. Leaves of *H. psiadioides* were colleted in Morro Santana (30° 4'S and 51° 7'W) in the morning in Porto Alegre city, and *H. alienus* were colleted in the evening at the roadside in Bagé (31° 19'S and 54° 6'W). The voucher specimens of *H. psiadioides* (153122) and *H. alienus* (153825) have been deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN).

The essential oils of *H. psiadioides* and *H. alienus* leaves were obtained by hydrodistillation, using a modified Clevenger apparatus for 4 hours. The oil obtained was dried over anhydrous sodium sulphate and stored at -20ºC prior to use. Linear temperature programmed retention indices (LTPRI) of hydrodistilled oil components were calculated, using the retention data of a 1% hexanic solution of *n*-alkanes (C9 to C24, Sigma-Aldrich, Milwaukee, USA), along with retention data of the essential oils compounds (Adams, 1995).

A gas chromatograph coupled to a mass spectrometric detector (GC/MS- Shimadzu QP5050A) and a Shimadzu gas chromatograph with a flame ionization detector (GC-FID – Shimadzu 17A) were employed to perform all chromatographic analyses. Two capillary columns were used under the following conditions: (i) OV-5 (30 m x 0.25 mm x 0.25 mm, Ohio Valley, Marietta, Ohio, USA) with initial oven temperature of 60ºC, reaching 250ºC at 3ºC min^{-1} ; (ii) DB-WAX (30 m x 0.25 mm x 0.25 mm, J&W Scientific, Folsom, CA, USA) with initial oven temperature of 40ºC, reaching 220ºC at 3ºC min^{-1}. Injector and detector temperature were kept at 250ºC for OV5 and at 220ºC for DB-WAX, while helium flow rate was 1.0 mL min^{-1}, and injection was made in split mode.

All the components were tentatively identified through comparison of their LTPRI with those registered in the literature data bases (Adams, 1995). Experimental mass spectra were also compared with the ones stored in MS data bases (Wiley 6th edition) and a minimum similarity match of 85% was required for the tentative identification of essential oils components. Compounds listed in Table 1 presented a minimum signal to noise of 3:1. Relative percentage of each component was obtained directly from chromatographic peak areas, considering the sum of all eluted peaks as a hundred percent.

Fifty lettuce (*Lactuca sativa* L. cv. Grand Rapids) and onion seeds (*Allium cepa* L. cv. Baia Periforme), obtained from commercial dealers, were sown in gerbox boxes lined with a layer of filter paper moistened with 7 mL of distilled water and sealed with a plastic film. The essential oils of *H. psiadioides* and *H. alienus* leaves were obtained by hydrodistillation, using a modified Clevenger apparatus for 4 hours. The oil obtained was dried over anhydrous sodium sulphate (Synth, São Paulo, Brazil) and stored at -20ºC prior to use. Experiments were carried out with pure oil (100%), 10% and 1% aqueous solution (v/v). The aqueous solutions were made with 0.1% solution of Tween 20 and distilled water (1:100, v/v), and distilled water was employed as a negative control. A 100 µL sample of

Heterothalamus psiadioides and *H. alienus* essential oils, and the solutions (1 and 10%) were dropped onto a cotton ball attached to the inner face of the gerbox lid to avoid direct contact between diaspores and essential oil, allowing the oil to volatilize into the space within the box. For the treatment with β-pinene (99% purity, Sigma-Aldrich, Milwaukee, USA), 40 µL was added to each box. Four replicates were performed for each type of experiment.

Two different tests were performed in order to distinguish the effects of essential oils during germination from those during seedling growth. To determine the effects on pre-germination, seeds were sown and then the essential oil was added. To determine the effects on post-germination, seeds were sown and the essential oil was added after 24 hours for lettuce and 72 hours for onion.

The numbers of seeds germinated were counted daily till completion of germination, which occurred in eight days. Germination of seeds was recorded every twelve and twenty four hours for lettuce and onion respectively. Total germination (also known as final germination percentage) and speed of germination index (SGI) were the parameters selected for this study and were calculated according to Labouriau (1983). The length of shoot and root were measured (cm) in order to determine the growth of tested seedlings.

The statistical analyses were made by comparing the treatment group with the control group using one-way ANOVA and post-hoc Tukey's, whenever the data met requirements of normality and homogeneity of variance. Nonparametric Kruskal-Wallis' test followed by Dunn's test for multiple comparisons were used if data did not follow a normal distribution. Differences were considered significant at $P<0.05$.

3. Results

Chemical composition of the essential oils and the percentage of each essential oil component are shown in Table 1. The major components in both essential oils was β-pinene (43.17-40.80%). Seventeen compounds have been found in both essential oils: α-pinene (3.99-4.24%), sabinene (0.21%), myrcene (3.09-1.09), limonene (6.82-6.86%), (*E*)-β-ocimene (4.94%-5.97%), γ-terpinene (0.24-0.03%), terpinolene (2.58-2.06%), linalool (0.86-0.17%) (*E*)-cariophylene (0.25-0.89%), aromandendrene (0.38-0.43%), α-humelene (1.14-0.72%), valencene (0.49-7.98%), germacrene-D (3.88-3.26%), β-selinene (0.45-0.12%), γ-cadinene (1.19-1.30), δ-cadinene (0.25-0.08%) e spathulenol (0.50-0.71%).

Regarding the effects of essential oils of *Heterothalamus psiadioides* e *H. alienus* on germinability of lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.), it was found that the use of pure essential oil provided a significant reduction ($p<0.05$) in lettuce germination, reducing it by 42% compared to control (Figure 1 and 2). Conversely, the results obtained when β-pinene was tested have not shown significant differences on germination of lettuce and onion, when compared to control.

The seeds of lettuce and onion showed a reduction on the speed of germination index (SGI) when the pure essential oil was tested (Figure 3 and 4). An inhibition of 49.9 % in SGI of lettuce and of 26.3 %

in SGI of onion were verified when both species underwent treatment with essential oil of *H. psiadioides.* The treatment with β-pinene did not show significant differences on speed of germination of lettuce and onion, when compared to control.

The pure essential oils of *H. psiadioides* and *H. alienus* have influenced the growth of lettuce and onion seedlings. The lettuce seedlings submitted to essential oils of *H. psiadioides* an *H. alienus* showed a reduction on growth in 67 and 92% respectively. Lettuce seedlings in contact with the volatile compounds of *H. psiadioides* showed a reduction of 71 and 76% in hypocotyl and primary root growth, respectively (Figure 5). Seedlings tested with volatiles of *H. alienus* suffered a major reduction in growth, 94 and 91% in hypocotyl and primary root, respectively (Figure 6). In the test with β-pinene we observed an increase of 150% in hypocotyl growth of lettuce (Figure 7). Growth of lettuce primary root submitted to β-pinene did not show differences when compared to control.

A reduction of 82 % in growth was observed in coleoptile and adventitious root of onion when it was submitted to volatiles of *H. psiadioides* (Figure 8). Essential oil of *H. alienus* induced a reduction of 88% and 87% in coleoptile and adventitious root respectively (Figure 9). Growth of onion seedlings treated with β-pinene did not show differences when compared to control (Figure 10).

4. Discussion

Sunyenaga *et al.*(2004) have reported that sesquiterpenes were the major classs of compounds (60.9 %) of two populations of *H. psiadioides* collected in Porto Alegre (Rio Grande do Sul – Brazil) and that germacrene D (17.8%) and biciclogermacrene (15.1%) were their main representatives. In contrast with these former results, it has been found that monoterpenes were the major components of *Heterothalamus psiadioides* essential oil (77.31 % and 43.17 % for β-pinene), while the previously mentioned research reported 11.9% as the percentual chromatographic peak area of β-pinene.

Research on essential oils of leaves and flowers of *H. alienus* (Duschatzky *et al.,* 2007) showed that their major components were β-pinene (35.5% and 65.0%, respectively), spathulenol (10.7% and 3.2%) and germacrene D (6.8% and 2.4%). Although the same major component was found in the essential oil of *H. alienus* essential oil (β-pinene, 40.80%), the second and third main components are different from the ones mentioned by Duschatzky *et al.* (2007), and this variations in composition may be easily explained by differences in climate, soil, seasonal growing and sampling methods.

Potential of allelopathic activity for a certain compound is often verified by testing their influence on seed germinability and seed viability (Gniazdowska and Bogatek, 2005). Effects of allelochemicals on seed germination appear to be mediated through a disruption of normal cellular metabolism rather than through damage of organelles. One of the effects can be in reserve mobilization, a process which usually takes place rapidly during early stages of seed germination and seems to be delayed or decreased under allelopathy stress conditions (Gniazdowska and Bogatek, 2005). For *H. psiadioides* and *H. alienus*, in a

previous work, was observed that essential oils of both species can cause decrease in mitotic activity, increase of chromosomal abnormalities (Schmidt-Silva *et al.*, 2011), and change in anatomical features - increasing the cortex area in lettuce and decreasing the width of onion roots (data unpublished).

Salvia leucophylla, a shrub observed in coastal South California, produces several volatile monoterpenoids (camphor, 1,8-cineole, β-pinene, α-pinene, and camphene) that potentially act as allelochemicals. Camphor, 1,8-cineole and β-pinene inhibited germination of *B. campestris* seeds at high concentrations, whereas α-pinene and camphene did not (Nishida *et al.*, 2005). According to Abrahim (2000) limonene, camphor, and eucaliptol were inactive on maize seed germination, while α-pinene had inhibited it (Abrahim *et al.*, 2000).

Different results obtained indicate that both, concentration of the compound(s) and species tested significantly influence the results achieved. This can be clearly observed in the example of (R)-carvone of mint essential oil, as it inhibits sprouting on potatoes when used in concentrations of 4.5 $\mu l\ l^{-1}$, but it induces sprouting on potato plants when used at a concentration of 0.5 $\mu l\ l^{-1}$ (Teper-Bamnolker *et al.*, 2010). One of the suggested explanation for disruption of seedling growth and development during allelopathy stress is modification in mitochondrial respiration leading to decreased supply of ATP for all energy demanding processes (Gniazdowska and Bogatek, 2005).

Besides growth reduction of seedlings, it was also verified that onion seedlings submitted to essential oils of *H. psiadioides* and *H. alienus* were unpigmented. Decrease in chlorophyll content, followed by reduction of carotenoid concentration was detected in lettuce seedlings treated by artemisinin and some of its sesquiterpene analogs (Dayan *et al.*, 1999). Monoterpenes are allelochemicals that diminish the mitochondrial respiration by increasing rates of electron transport through an alternative pathway. Alpha-pinene decreased the oxygen consumption in *Glycine max* (L.) Merr. cotyledons and increased relative partitioning of electrons to the alternative pathway (Penuelas *et al.*, 1996).

The speed of germination index (SGI) is a measure to evaluate the seedlings vigor. The reduction in seedling vigor can cause progressive loss in productive capacity, with reduction in the uniformity of germination. Results have shown that *H. psiadioides* and *H. alienus* essential oil's reduce the speed of germination and seedling growth of lettuce and onion.

A comparative study performed with the monoterpenes camphor, eucalyptol, limonene, and α-pinene showed that they have weak activities on maize seed germination and primary root growth. Whereas limonene was inactive on maize seed germination and primary root growth, camphor and eucaliptol promoted changes in the fresh and/or dry weight of primary roots without alteration of the percentage of germinated seeds. Alpha-pinene, on the other hand, affected both maize seed germination and primary root growth (Abrahim *et al.*, 2000).

The essential oils of *H. psiadioides* and *H. alienus* species caused structural alterations in roots: lettuce seedlings grown under the effect of the essential oils of *H. psiadioides* and *H. alienus* presented an increase in the area of tissues, while onion seedlings presented reduction in the area of tissues

(Schmidt-Silva *et al.*, 2011). Root growth was inhibited by camphor, 1,8 cineole, β-pinene, α-pinene, and camphene, in a dose-dependent manner, using *Brassica campestris* as the test plant, but hypocotyl growth was largely unaffected (Nishida *et al.*, 2005). Myrcene, limonene, β-pinene had no effect on growth of radicle elongation and shoot lengh, while α-pinene stimulated cress radicle growth (Barney *et al.*, 2005). Vokou (2003) compared the allelopathic potential of 47 monoterpenoids of different chemical groups in *Lactuca sativa* L. They have found that as a group, the hydrocarbons, except for (+)-3-carene, were the least inhibitory. Out of the oxygenated monoterpenoids, the least inhibitory were the acetates; whenever the free hydroxyl group of an alcohol turned into a carboxyl group, the activity of the resulting ester was markedly lower (against both germination and seedling growth). Geranyl acetate, linalool, (+)-3-carene, α-terpinene had effect both in seed germination and seedling growth. α-pinene, β-pinene, α-phellandrene, myrcene, limonene, and camphene did not have any effect neither to seed germination nor to seedling growth (Vokou *et al.*, 2003).

Some plants exposed to monoterpenes vapours have shown internal damage. The absence of a variety of intact organelles and the presence of membrane fragments indicate structural breakdown and decomposition occurring within inhibited roots (Lorber and Muller, 1976). As fatty acids and other lipids are known as structural components of membranes, it is reasonable to suppose that membrane degradation could result in the freeing of lipids within the cytoplasm of targeted cells. Then, the free lipids within the cytoplasm could be target of an oxidative action (Scrivanti *et al.*, 2003).

Barney (2005) attempted to identify the specific monoterpenes associated with the phytotoxicity observed in the presence of mugwort foliage, or soil associated volatiles. None of these terpenoids could account for the phytotoxicity observed in foliar assays. Most of the monoterpenes produced no inhibition of either root or shoot growth, while some stimulated growth at low concentrations. It is possible that the toxicity observed in their initial experiments with mugwort foliage was due to synergistic combinations of phytotoxic terpenes (Barney *et al.*, 2005).

The effect of an essential oil on seed germination and seedling growth is often explained in terms of the individual effects of some main constituents. However, an essential oil is frequently a complex mixture of many compounds in different proportions, and it is often not known whether and how they might interact. In addition, there is considerable variability and it may be seasonal or intraspecific, between different populations of the same species, or even between different individuals of the same population (Vokou *et al.*, 2003). The data showed by Wang (2010) demonstrated that elevation in CO_2 concentration induced the expression of the β-caryophyllene synthase gene and increased the emission of β-caryophyllene in *Mikania micrantha* Kunth. These findings support the hypothesis that emission of biogenic organic compounds (BOC) from plants is particularly sensitive to changes of environmental factors such as CO_2 concentration (Wang *et al.*, 2010).

Allelopathic potential of certain compounds or of a mixture of compounds can also be a strategy used by invasive plant species. Many plants species exude/emit compounds into the surrounding

environment with minor consequences in their native habitat due to a long coevolutionary history (Barney *et al.*, 2009). Due to coevolutionary history, the surrounding biota of the native range tolerate, or overcome these BOC in an evolutionary arms race, thus resulting in predictable ecosystem consequences. However, the same compounds can have unpredictable repercussions in a non native environment inexperienced with a specific BOC or a particular mixture of BOC (Barney *et al*., 2009).

Allelopathy must be recognized as a dynamic process that involves more than just donor and target plants. Variations in the type of soil, water and nutrient availability, previous or companion crops, climate conditions are determinants of the occurrence of effective allelopathic activity (de Albuquerque *et al.*, 2011).

It was proved that the essential oils, in the highest concentration used, from *H. psiadioides* and *H. alienus* cause decreased in germinability, in seeds vigour and in seedling growth of standard plant test (lettuce and onion). The release of chemical compounds that have harmful effects on the members of the recipient plant community (i.e., allelopathy) can be an advantage to plants with high colonization capacity. Studies on this matter have normally relied exclusively on laboratory assays and often use extraordinarily sensitive test species to demonstrate potential phytotoxixity. Therefore, allelopathy in the gas phase has remained a subject for skepticism, and will continue to be so until demonstrated empirically in the field (Barney *et al.*, 2009).

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Table 1. Relative composition of the volatile constituents in the leaves of *Heterothalamus*.

Components	*H. psiadioides*			*H. alienus*	
	LTPRI[#]	LTPRI[1*]	Area (%)	LTPRI[2]	Area (%)
monoterpenes hydrocarbons					
α-pinene	939	936	3.99	952	4.24
camphene	954	953	0.19	-	-
sabinene	975	972	0.21	*	*
β-pinene	979	977	43.17	986	40.80
myrcene	991	991	3.09	993	1.09
α-phellandrene	1003	1002	0.35	-	-
α-terpinene	1017	1020	0.27	-	-
limonene	1029	1039	6.32	1039	6.86
β-phellandrene	1030	1032	0.40	-	-
Δ³-carene	1031	1005	13.73	-	-
(Z)-ocimene	1037	-	-	1045	0.06
(E)-β-ocimene	1050	1054	4.94	1054	5.37
γ-terpinene	1060	1062	0.24	1062	0.03
terpinolene	1089	1081	2.58	1088	0.06
oxygenated monoterpenes					
linalool	1097	1089	0.86	1109	0.17
2-methyl-6-methylene-1,7-octadien-3-one	1117	-	-	1120	0.29
allo ocimene	1132	1132	0.62	-	-
ni†	-	-	-	1194	0.14
ni†	-	-	0.54	-	-
(Z)-sabinene hydrate acetate	1256	-	-	1257	0.77
β-elemene	1338	1335	1.61	-	-
δ-elemene	1338	-	-	1345	0.06
ni†	1375	-	-	1368	0.04
neryl acetate	1362	-	-	1372	0.11
α-copaene	1377	-	-	1375	0.05
geranyl acetate	1381	-	-	1386	10.88
sesquiterpenes hydrocarbons					
β-cubebene	1388	-	-	1389	0.53
β-elemeno	1391	-	-	1402	0.07

[*]LPTRI – Calculated Linear Temperature Programmed Retention Indices calculated. Relative percentage of each component was obtained directly from chromatographic peak areas, considering the sum of all eluted peaks as a hundred percent. LPTRI[#] Calculated Linear Temperature Programmed Retention Indices Tabelated (Adams 2001). † ni - not identified.

Table 1. Cont.

Components		*H. psiadioides*		*H. alienus*	
	LTPRI[#]	LTPRI[1*]	Area (%)	LTPRI[2]	Area (%)
(E)-cariophyllene	1409	1417	0.25	1412	0.89
β-gurjunene	1423	-	-	1422	0.11
α-guaiene	1440	-	-	1427	0.05
aromandendrene	1441	1447	0.38	1432	0.43
ni†	-	-	-	1437	0.09
(Z)-muuroladiene-3,5	1454	-	-	1444	0.08
α-humelene	1455	1450	1.14	1447	0.72
allo-aromadendrene	1460	-	-	1454	0.34
(Z)-Cadinadiene-1(6),4	1477	-	-	1469	0.24
γ-gurjuneno	1477	-	-	1473	0.39
(E)-Muuruoladieno-4(14),5	1485	-	-	1487	0.25
Ar-curcumene	1490	1490	3.74	-	-
valencene	1494	1493	0.49	1496	7.98
α-amorphene	1485	1482	0.44	-	-
germacrene-D	1485	1484	3.88	1478	3.26
β-selinene	1490	1501	0.45	1482	0.12
α-muurulene	1494	-	-	1500	0.27
(E)-β-guaiene	1496	-	-	1507	0.36
γ-cadinene	1500	1517	1.19	1517	1.30
δ-cadinene	1503	1520	0.25	1531	0.08
(E)-calamenene	1514	-	-	1536	0.04
α-cadinene	1523	-	-	1547	0.04
oxygenated sesquiterpenes					
(E)-nerolidol	1529	-	-	1556	0.06
ni†	-	-	-	1563	1.77
spathulenol	1578	1579	0.50	1581	0.71
4-α-copaenol	1591	-	-	1594	0.38
viridiflorol	1593	1590	1.15	-	-
eremoligenol	1631	-	-	1634	1.03
ni†	-	-	-	1645	0.03
α-muurulol	1646	-	-	1649	0.76
ni†	-	-	-	1666	0.02

[*]LPTRI – Calculated Linear Temperature Programmed Retention Indices calculated. Relative percentage of each component was obtained directly from chromatographic peak areas, considering the sum of all eluted peaks as a hundred percent. LPTRI[#] Calculated Linear Temperature Programmed Retention Indices Tabelated (Adams 2001). † ni - not identified.

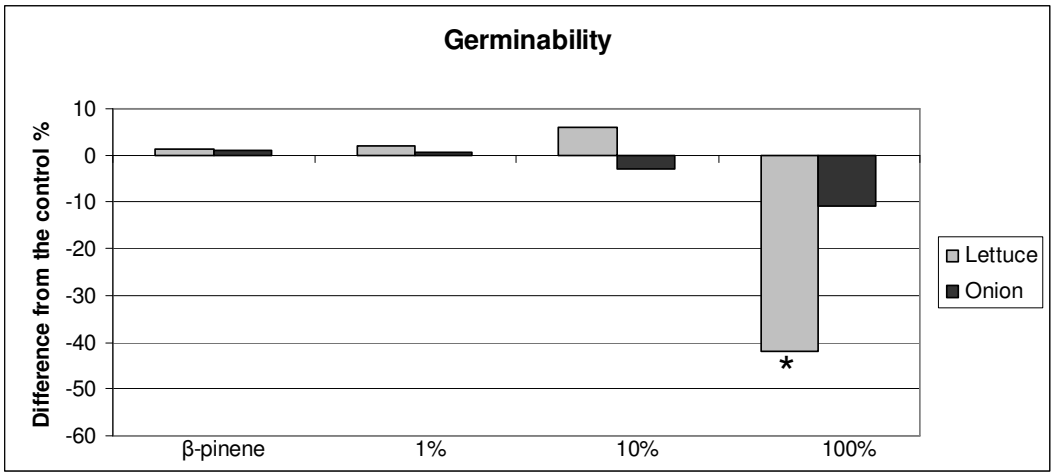

Figure 1. Effect of essential oil of *H. psiadioides* and β-pinene on the germinability of lettuce (*Lactuca sativa*) and onion (*Allium cepa*). Asterisk indicates a significant result (ANOVA and Tukey's test; P = 0.05).

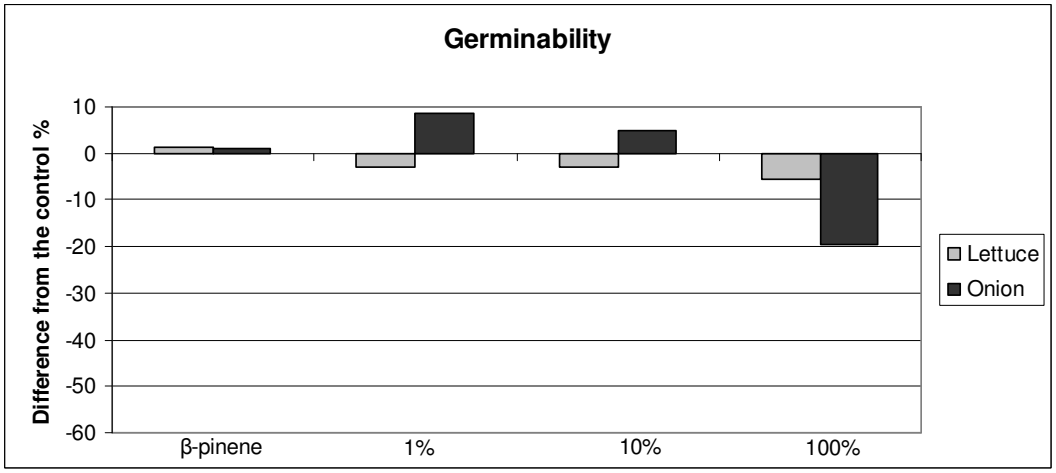

Figure 2. Effect of essential oil of *H. alienus* and β-pinene on the germinability of lettuce (*Lactuca sativa*) and onion (*Allium cepa*). Asterisk indicates a significant result (ANOVA and Tukey's test; P = 0.05).

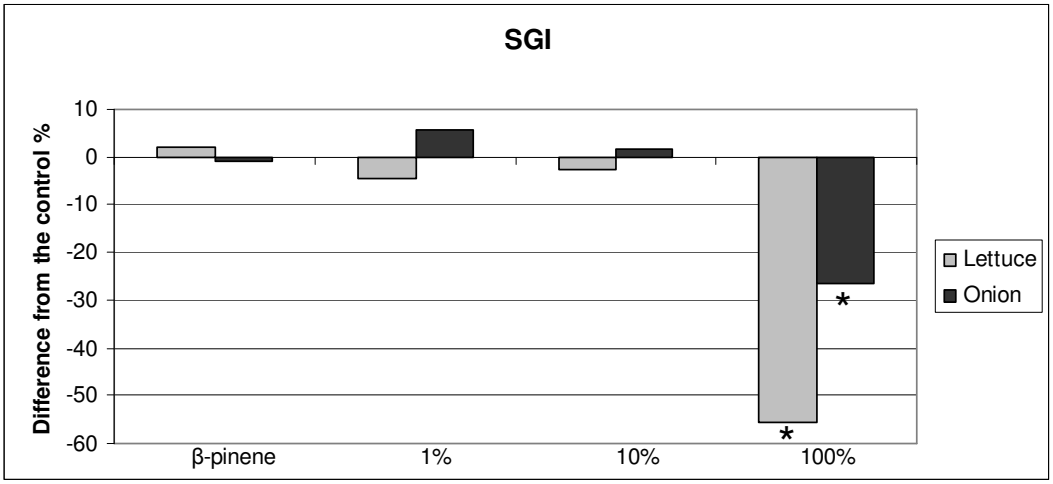

Figure 3. Effect of essential oil of *H. psiadioides* on speed of germination index (SGI) of lettuce (*Lactuca sativa*) and onion (*Allium cepa*). Asterisk indicates a significant result (ANOVA and Tukey's test; P = 0.05).

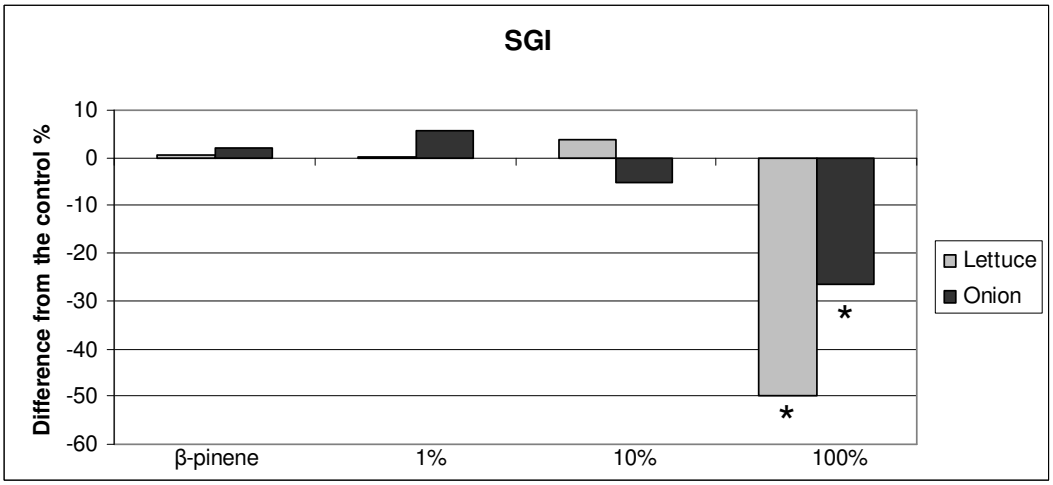

Figure 4. Effect of essential oil of *H. alienus* on speed of germination index (SGI) of lettuce (*Lactuca sativa*) and onion (*Allium cepa*). Asterisk indicates a significant result (ANOVA and Tukey's test; P = 0.05).

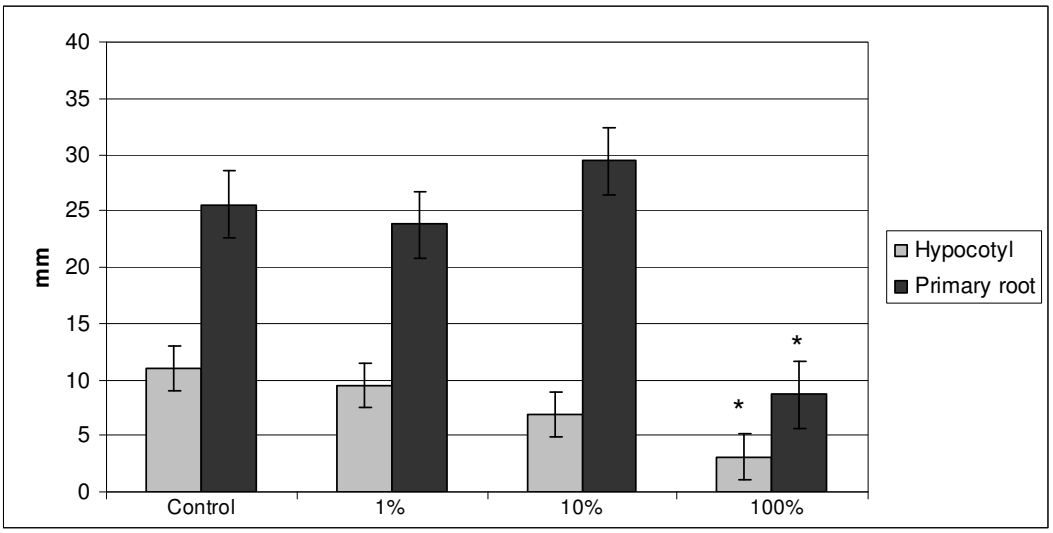

Figure 5. Effect of essential oil of *H. psiadioides* on primary root growth and hypocotyl growth in lettuce. Asterisk indicates a significant result (ANOVA and Tukey's test; $P = 0.05$).

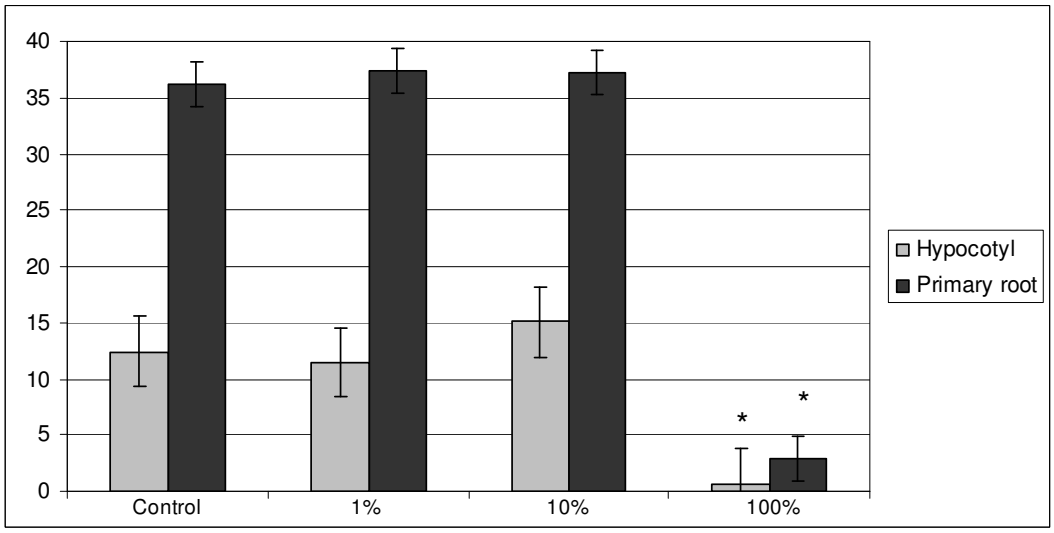

Figure 6. Effect of essential oil of *H. alienus* on primary root growth and hypocotyl growth in lettuce. Asterisk indicates a significant result (ANOVA and Tukey's test; $P = 0.05$).

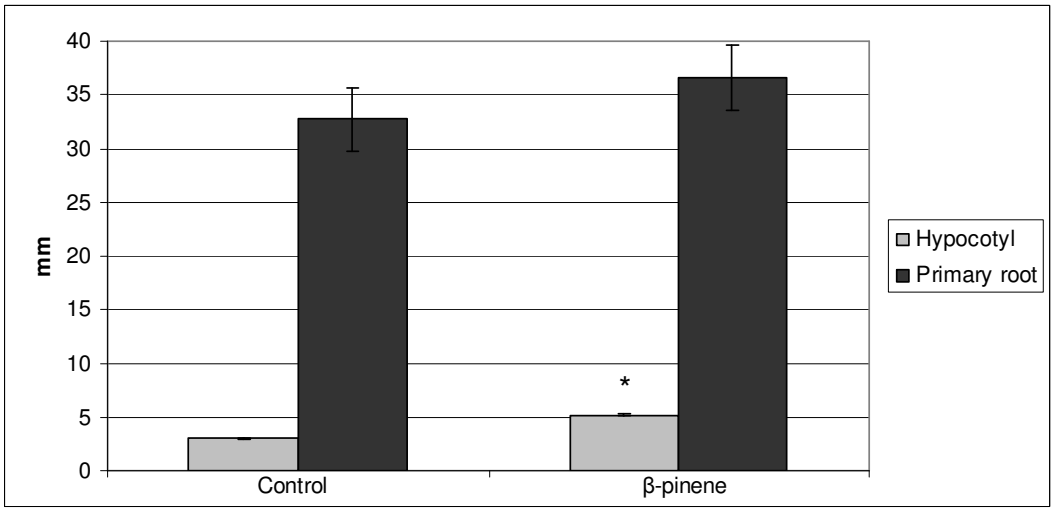

Figure 7. Effect of β-pinene on primary root growth and hypocotyl growth in lettuce. Asterisk indicates a significant result (ANOVA and Tukey's test; $P = 0.05$).

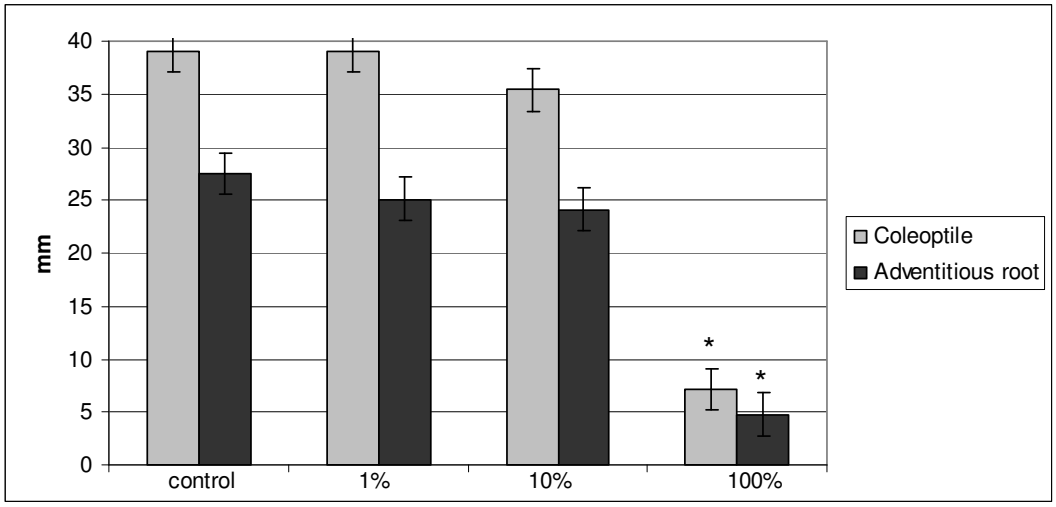

Figure 8. Effect of essential oil of *H. psiadioides* on adventitious root growth and coleoptile growth in onion. Asterisk indicates a significant result (ANOVA and Tukey's test; $P = 0.05$).

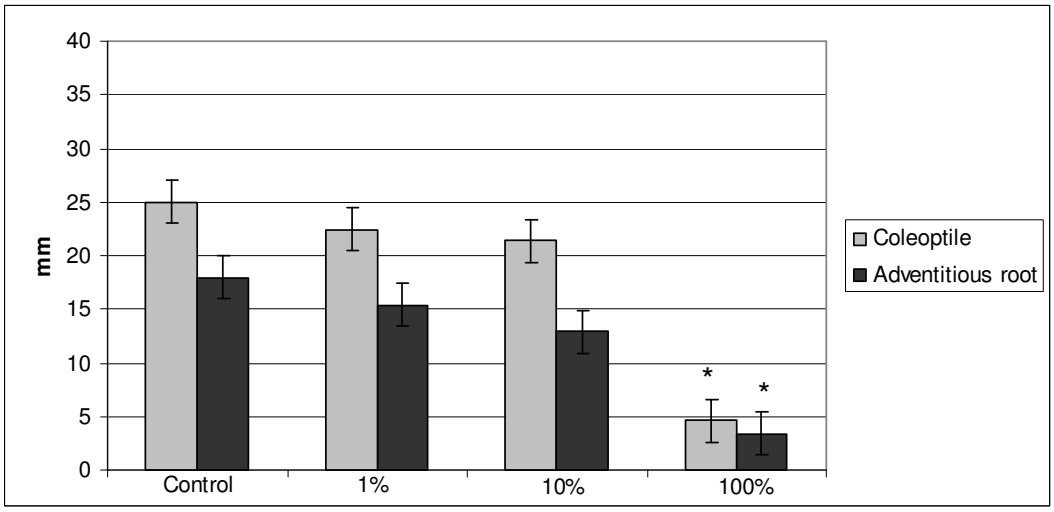

Figure 9. Effect of essential oil of *H. alienus* on adventitious root growth and coleoptile growth in onion. Asterisk indicates a significant result (ANOVA and Tukey's test; $P = 0.05$).

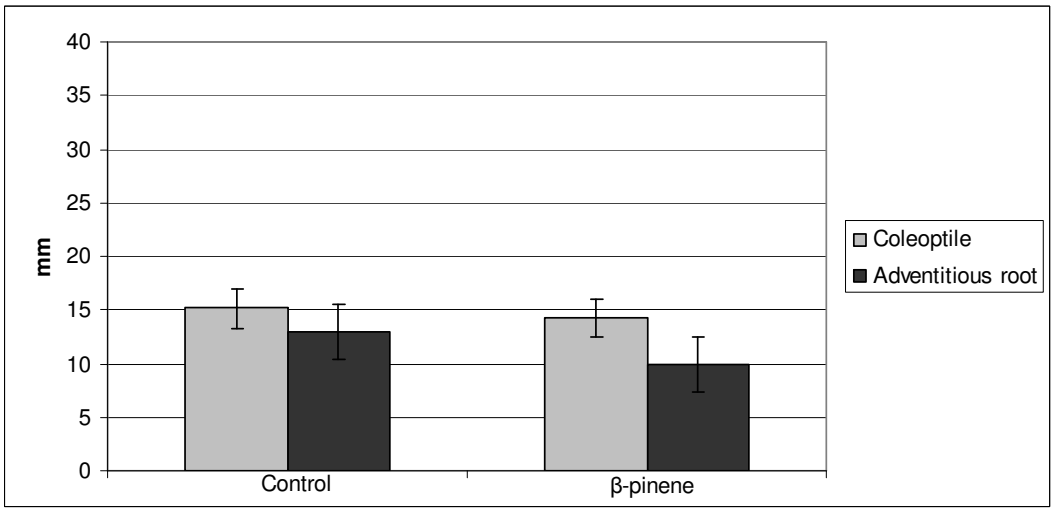

Figure 10. Effect of β-pinene on adventitious root growth and coleoptile growth in onion. Asterisk indicates a significant result (ANOVA and Tukey's test; $P = 0.05$).

ARTIGO 2

Cytotoxicity of essential oils from two species of *Heterothalamus* (Asteraceae)

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Cytotoxicity of essential oils from two species of *Heterothalamus* (Asteraceae)

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Essential oils are widely found in plant species. They can be involved in a variety of ecological interactions and may act as inhibitors of germination, suppressing root apical-meristem growth in some species. The present study aimed to determine the potential cytotoxicity of essential oils from leaves of *Heterothalamus psiadioides* Less. and *H. alienus* (Sprengel) O.Kuntze on root tips of lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.). Lettuce seeds germinated with essential oils from *H. psiadioides* and *H. alienus* had a reduced mitotic index (MI) for all treatments. Onion seedlings treated with *H. psiadioides* oil showed a decrease in MI, and seedlings treated with *H. alienus* oil showed a decrease in MI of 89% in the most concentrated treatment. Analysis of the results showed chromosomal abnormalities, including stickiness, c-mitosis, micronuclei and anaphase bridges in lettuce and onion root tips exposed to essential oils of *Heterothalamus*.

Introduction

Asteraceae is one of the most successful families for secondary metabolite production that acts in chemical defence, which may be one of the reasons for its cosmopolitan distribution (Verdi *et al.* 2005). Asteraceae presents many constituents that have been studied for their antimicrobial characteristics, cytotoxicity, genotoxicity and allelopathy (Baser et al. 2006; Fukuda *et al.* 2006; Iganci *et al.* 2006; Dias *et al.* 2009).

Heterothalamus psiadioides (alecrim-do-campo, vassoura, erva-formiga) and *Heterothalamus alienus* (alecrim-do-campo) are shrubs of the Asteraceae family (Barroso and Bueno 2002). The following three species are recognised in the *Heterothalamus* genus: *H. psiadioides* is found in southern Brazil and Uruguay, *H. alienus* grows in southern Brazil, Uruguay and central Argentina and *H. rupestris* is found in southern Brazil (Pacciaroni *et al.* 2008). The genus generally occurs in shallow rock soils, often found along roadsides (Barroso and Bueno 2002).

These species are used in South America as folk medicine. *H. psiadioides* is used as an antipyretic, antidote for snake bites, anti-inflammatory (Suyenaga et al. 2004), febrifuge, and is potentially used for skin remedies (Biavatti *et al.* 2007) and against renal diseases (Palácios *et al.* 2007). *H. alienus* has been reported as a decoction and as a powder of leaves used internally and externally against fever and as a stimulant (Rücker *et al.* 1996). *H. alienus* is also used against *Apis mellifera* L. pests (Ruffinengo et al. 2006), having antiviral and antifungal properties (Duschatzky *et al.* 2007). Both species are recognised as major producers of essential oils with potential biological activity.

Essential oils are mixtures of volatile compounds produced by plants, and terpene derivatives are generally their main constituents (Bakkali *et al.* 2008). As the largest group of secondary metabolites among plants, terpenes show a wide spectrum of biological properties, including chemical defence and allelopathy (Ding *et al.* 2010). One of the most common examples is artemisin, a sesquiterpene from *Artemisia annua* L., which affects root growth and leaf chlorophyll content and is accompanied by a reduction in mitosis. It also seems to affect formation of microtubule-organising centres (Dayan *et al.* 1999).

Previous work showed that the major compound for both species of *Heterothalamus* was β-pinene (Suyenaga *et al.* 2004; Duschatzky *et al.* 2007). Alpha-pinene and β-pinene are among the most widely distributed monoterpenes in plant species and are the major components of various essential oils. Whereas α-pinene occurs in both isomers, β-pinene almost always occurs in the optically levorotatory (–) isomer, the dextrorotatory isomer (+) apparently being of limited distribution (Gambliel and Croteau 1982).

Most plant species produce compounds that might be toxic to other plant species at some concentration (Harborne 1994). The concept of allelopathy involves compounds that function, at least partially (in plant–plant interactions), as a phytotoxin where a donor plant adversely affects a target plant, providing an advantage for the donor plant (Dayan and Duke 2009). Historically, investigations of allelopathy have originated from field observations. In Rio Grande do Sul, a state of Brazil, *H. psiadioides* is becoming a pioneer in regions of Porto Alegre where anthropic pressure (the pressure imposed on the environment by the socioeconomic conditions of human settlement) exists, forming associations with individuals of these species. Allelopathy may be one of the mechanisms by which these plants become a dominant species.

Cell growth in plants depends on a normal mitotic process. The growth-limiting effects of various kinds of stress have been reported as factors in the control of cell division (Ding *et al.* 2010). The uniform division of all cell components allows a balanced growth for the organism (Reigosa *et al.* 2006). The control of cell-cycle progression and the number of dividing cells will define the plant's growth and morphogenesis, and any alteration of the precise timing and order of these events will act as a critical factor in plant development (Reigosa *et al.* 2006). Plant development has decisive stages that are very vulnerable, such as seed germination, which is important to take into account when seedling growth is

studied. Seed germination is the process by which the plant embryo resumes growth after a period of quiescence. Under favourable conditions, the rapid growth of the embryo culminates in rupture of covering layers and emergence of the radicle, which is considered the completion of germination. At this stage, the fate of individual embryo cells in re-entering the cell cycle or remaining arrested is decisive in determining seedling formation. The development of the plant and its functionality depend on the embryo cells' capacities to resume division and differentiate (Barrôco *et al.* 2005).

Mitotic activity (measured by mitotic index, MI, which is an index of %active dividing cells), alterations in the mitotic phase and individual cell abnormalities are key parameters by which plant growth may be evaluated (Teerarak *et al.* 2010). Onion and lettuce tests are standard techniques for a cytogenetic assay in environmental monitoring because of the fact that data obtained from these plants show correlation with mammalian and non-mammalian test systems (Constantin and Owens 1982).

Because of the well known biological activities of several terpenoid compounds, and also the potential allelopathic activity of *H. pisiadioides* and *H. alienus*, the aim of the present study was to evaluate the role of the essential oils of both species in their interactions with other plant species. The present study also included tests with the major component of these essential oils in an attempt to reveal its participation in the allelopathic potential of both species. The cytotoxic and genotoxic activities were investigated to better understand the chemoecological interactions of such essential oils and their mode of action.

Material and methods

Plant collection

Heterothalamus psiadioides and *H. alienus* were collected in 2008 and 2009, in the cities of Porto Alegre and Bagé, state of Rio Grande do Sul, Brazil. *H. psiadioides* leaves were collected in natural vegetation on a hill in Morro Santana (30°4′S, 51°7′W) in the city of Porto Alegre, and *H. alienus* leaves were collected along a roadside in Bagé (31°19′S, 54°6′W). Voucher specimens of *H. psiadioides* (153122) and *H. alienus* (153825) have been deposited in a herbarium in the Federal University of Rio Grande do Sul (ICN).

Plant bioassays

Fifty lettuce (*Lactuca sativa* cv. Grand Rapids) and onion seeds (*Allium cepa* cv. Baia Periforme), obtained from commercial dealers, were sown in a layer of filter paper moistened with 7 mL of distilled water, inside germination boxes, which were sealed with a plastic film. The essential oils of *H. psiadioides* and *H. alienus* leaves were obtained by hydrodistillation, using a modified Clevenger apparatus (Gottlieb and Taveira- Magalhães 1960) for 4 h. The oil obtained was dried over anhydrous sodium sulfate and stored at –20°C before use. Experiments were carried out with pure oil (100%), and 10% and 1% aqueous solution (v/v). The aqueous solutions were made with 0.1% solution of Tween 20

and distilled water (1:100, v/v), and distilled water was employed as a negative control. A 100-µL sample of *H. psiadioides* and *H. alienus* essential oils, and the solutions (1% and 10%) were dropped onto a cotton ball attached to the inner face of the gerbox lid to avoid direct contact between diaspores and essential oil, allowing the oil to volatilise into the airspace within the box. For the treatment with β-pinene, 40 µL of β-pinene was added to each box. Four replicates were performed for each type of experiment.

Two different tests were performed to distinguish the effects of essential oils during germination from during seedling growth. To determine the effects on pre-germination, seeds were sown and the essential oil was added immeadiately after sowing. To determine the effects on post-germination, seeds were sown and the essential oil was added after 24 h for lettuce and after 72 h for onion.

Measurement of MI, metaphase index (MetI) and chromosome abnormalities

Primary roots of lettuce and onion seedlings, after being treated with *H. psiadioides* and *H. alienus* essential oils, were fixed in freshly prepared ethanol–acetic acid (3:1,v/v) for 24 h at room temperature, and then stored at –18°C until further treatment. Roots were hydrolysed for 10–15 min in 5 N HCl, followed by rinsing with distilled water (three or four times). The next step was their incubation in Schiff's reagent for 1–2 h. Root tips (1 mm) were excised and the meristems were squashed under a coverslip to separate the cells. There were four replicates for each treatment (*H. psiadioides*, *H. alienus*, β-pinene (99% purity, Sigma-Aldrich, São Paulo, SP, Brazil) and control) and four roots were scored for each replicate. MI and MetI were calculated as the ratio of dividing or metaphasic cells to examined cells; 8000 cells were analysed per treatment. The frequency of each mitotic phase was calculated as the percentage of cells in that phase of total dividing cells counted during mitosis in the treatment, whereas chromosomal abnormalities were calculated as the percentage of abnormal cells of total cells examined (Glinska Bartczak *et al.* 2007).

Statistical analysis

Comparisons between treatments and control were performed using one-way ANOVA and *post hoc* Tukey's tests whenever the data met the assumptions of normality and homogeneity of variance. A non-parametric Kruskal–Wallis test, followed by a Dunn's test for multiple comparisons, was employed if data did not follow a normal distribution. Differences were considered significant at $P = 0.05$.

Results

Cytogenetic analysis

The results of MI and MetI of lettuce are presented in Figs 1 and 2. In lettuce treated with the essential oil from *H. psiadioides*, the decrease in MI for all treatments varied from 37% to 57%. For roots treated with the oil from *H. alienus*, the decrease in MI varied from 40% to 59% for the highest concentrations for pre- and post-germination, respectively. Beta-pinene did not affect MI, but increased MetI (Fig. 3).

In onion, the strongest inhibition in MI was seen at high concentrations of oil (Fig. 4). Onion seedlings subjected to pre- and post-germination treatment with the essential oil of *H. psiadioides* showed >80% decrease in MI. Onion seedlings treated with *H. alienus* during pre-germination showed a decrease in MI of ~67% and 90% in treatments with 10% and 100% oil, respectively. Seedlings treated with the same essential oil in highest concentrations during post-germination showed inhibition of nearly 92% in MI. The volatile oils of both species also reduced the metaphasic index of onion-root meristems. In onion treatments, where a decrease was observed in MI, there was also a similar decrease in the MetI (Fig. 5). Beta-pinene did not have an effect on MI or MetI (Fig. 6).

The results of the rate of mitotic stages in *A. cepa* and *L. sativa* can be seen in Figs 7–9) The proportion of metaphasic cells increased whereas that of prophasic cells decreased in all treatments for lettuce. When this happened, the major abnormality that occurred was sticky chromosomes.

Onion root samples treated with *H. psiadioides* oil, at the highest concentration, had a reduced proportion of prophasic cells during pre-germination and a reduced proportion of metaphasic cells during post-germination. The proportion of cells in anaphase–telophase decreased under the same conditions for both essential oils tested. When treated with *H. alienus* essential oil at the highest concentrations, proportions of both prophasic and metaphasic cells increased in onion roots. The proportion of prophasic cells decreased, and those in metaphase and anaphase–telophase increased in lettuce seeds exposed to β-pinene. Onions treated with β-pinene during pre-germination showed a reduced proportion of cells in prophase and an increased proportion in metaphase.

The cytogenetic alterations can be seen in Figs 10–12. Essential oils of *Heterothalamus psiadioides* and *H. alienus* induced chromosome alterations in almost all treatment groups. The exception was the treatment in which the mitotic activity, as indicated by low MI, was strongly inhibited. The results in Tables 1 and 2 indicate that essentials oils from *H. psiadioides* and *H. alienus* induced a wide range of mitotic abnormalities in root tips of *L. sativa*. These essential oils caused, more often, C-mitosis, stickiness and micronuclei. Other abnormalities were also observed, such as non-oriented chromosomes at metaphase, multipolar anaphase, anaphase bridges and telophase bridges.

The results in Tables 3 and 4 indicate that *H. psiadioides* and *H. alienus* also induced mitotic abnormalities in root tips of *A. cepa*. In this case, the most frequent abnormalities were stickiness, anaphase bridges and micronuclei. Other abnormalities included delay of chromosomes in anaphase, chromosome breaks at anaphase and telophase bridges. Beta-pinene caused chromosome abnormalities in lettuce, but not in onion root tips.

Discussion

The higher concentrations of all essential oils caused inhibition of the mitotic activity. Consistent results were found between inhibition of root growth (V Schmidt-Silva, A Pawlowski, MH Reis, CA Zini,

GLG Soares, unpubl. data) and a decrease in MI at the highest essential-oil concentrations tested. The MI for treated roots was significantly lower than that for the control, indicating alterations in the growth and development of exposed plants as a result of chemical action (Hoshina and Marin-Morales 2009).

There are few reports describing consistent cytotoxic or genotoxic activities of essential oils. Several studies with essential oils or their main components have demonstrated that most do not induce nuclear mutations (Bakkali *et al.* 2008). Knoll *et al.* (2006) showed that *Pterocaulon polystachyum* D.C. infusions present cytotoxic and anti-proliferative activity, decreasing the mitotic index. Conversely, Burim *et al.* (1999) reported that Glaucolide B (160–640 mg/kg), a sesquiterpene that is a characteristic of the *Asteraceae* family, does not increase the frequency of chromosomal aberrations in mouse bone-marrow cells nor does it affect cell division. Peres *et al.* (2007) suggested that plants, because of their sessile lifestyle, have developed mechanisms that allow them to adjust their cell cycle in response to the environment. Both biotic and abiotic stress stimuli can affect plant growth negatively through the inhibition of the cell-cycle machinery. There are several ways in which the cell cycle can be affected. Perception of biotic and abiotic stress signals activates signalling cascades that triggers ion fluxes, kinase cascades, the generation of reactive oxygen species and accumulation of antimitogenic hormones, such as abscisic acid and jasmonic acid. These signalling molecules might stimulate cell-cycle checkpoints, resulting into an impaired G1-to-S transition, the slowing down of DNA replication, and/or delayed entry into mitosis (Peres *et al.* 2007).

In most systems used in cell-cycle investigation, it seems impossible to distinguish the action mode of essential oils and their targets because cytotoxicity, mutagenicity or anti-mutagenicity are assessed without accounting for possible defects in energy metabolism and respiration as direct or indirect causes (Bakkali *et al.* 2008). How cells determine when to divide is important for understanding the biological processes whereby cell proliferation is manifested, because cells need to accumulate precursors before duplication and cellular metabolism is expected to have an impact on cell division. Mitochondrial processes are fundamental to the control over cell metabolism.

Root growth arises from the proliferation of meristematic cells followed by cellular expansion that results in root elongation (Ding *et al.* 2010). A study using two volatile monoterpenes (cineole and citronellol) on *Ageratum conyzoides* L. has shown that both of them severely affected seedling growth (Singh *et al.* 2002). Five monoterpenoids (camphor, 1,8-cineole, β-pinene, α-pinene and camphene) produced by *Salvia leucophylla* Greene potentially act as allelochemical compounds on *Brassica campestris* L. (Nishida *et al.* 2005). These monoterpenes lowered mitotic index in the root apical meristems, which suggests that the monoterpenoids produced by *S. leucophylla* could interfere with the growth of other plants in its vicinity by inhibiting cell proliferation in the root apical meristems.

Lettuce and onion seedlings treated with higher concentrations of essential oils of *H. psiadioides* and *H. alienus* experienced a reduction in root length that might be consistent with the decrease in MI of root

meristematic cells. When lower concentrations (1% and 10%) were used, there was no reduction in the root length (data unpublished), even though the MI showed a decrease. This may have been the result of increasing the size of the cells in the roots, instead of increasing the number of cells (Harashima and Schnittger 2010).

The cell has mechanisms for monitoring the progress of cell-cycle events and transmitting this information to a cell-cycle control system. The cell-cycle control system progresses through the cell cycle at regulatory transitions called checkpoints (Morgan 2007). There are three major checkpoints, the first of which is called the start or G1/S checkpoint. The second is the G2/M checkpoint, which progresses to entry into mitosis. The third major checkpoint is the metaphase–anaphase transition, which leads to sister-chromatid segregation, completion of mitosis and cytokinesis (Morgan 2007). If the control system detects non-completion of an event, it will delay the initiation of later events. The checkpoint blockage will vary depending on the substance applied and its mode of action. For instance, Metolcarb®, a pesticide, increases the prophase index and simultaneously decreases metaphase, anaphase and telophase indices, which might indicate blockage of a checkpoint between prophase and metaphase (Liman et al. 2010). When analysing the root-tips cells of lettuce in the present study, a pattern for essential oils was perceived in which the number of prophasic cells decreased and that of metaphasic cells increased. In this case, substances in the essential oils of the species tested may have been blocking the cell cycle in the metaphase–anaphase transition, as evidenced by the increase in the proportion of cells in metaphase.

Structural chromosomal alterations may be induced by several factors, such as DNA breaks, inhibition of DNA synthesis and replication of altered DNA (Leme and Marin-Morales 2009). Chromosomes bridges or interchromatid connections are formed by chromatin fibres that join sister chromatids at metaphase and hold the chromatids together until late anaphase or telophase; if these connections become strong, chromatids might break at or near the points of connection at anaphase (Fernandes *et al.* 2007). Chromosomal abnormalities, such as fragments and chromosome losses, can result in micronucleated cells, because both fragments and entire chromosomes cannot be incorporated into the main nucleus during the cell cycle (Fenech 2002). In many chromosomal abnormalities, clastogenic and aneugenic action is observed. Clastogenic action is characterised by the induction of chromosomal breakage during cell division, whereas aneugenic action comprises the inactivation of a cell structure, such as the mitotic spindle, leading to chromosomal losses (Fenech 2002).

The major chromosomal abnormalities observed in lettuce and onion in the present study were adherence, c-mitosis and micronuclei. This provided evidence for more aneugenic effects observed in the present assay. Chromosomal adherence is a common sign of toxic effects and it may cause irreversible effects on the cell, triggering the cell-death process (Fernandes *et al.* 2007). Another aneugenic effect is c-mitosis which provides the complete inactivation of the cell mitotic spindle (Leme and Marin-Morales 2009). Micronuclei are determined from acentric fragments (clastogenic agent) or whole chromosomes

(aneugenic agent) that were not incorporated into the main nucleus during the cell-division cycle (Fenech 2002).

The essential oils have a complex chemical composition and, therefore, the evaluation of their toxicity and possible genotoxic effects is difficult (Bakkali *et al.* 2005). These oils are the sum of different ingredients, mostly terpenes, and therefore, it is necessary to investigate the contribution of the major components of essential oils to their activity (Michaelakis *et al.* 2009). The essential oils tested here induced chromosome abnormalities, with exception of the highest concentration of *H. alienus* essential oil applied pre-germination to the onion. The oils may have induced apoptosis effects because there was a large reduction in MI at the highest concentration of the oils, whereas few abnormalities were found in onion. Apoptosis is a programmed cell death and a highly organised physiological mechanism to destroy injured or abnormal cells (Taraphdar *et al.* 2001). A wide variety of natural substances have apoptotic effects, including constituents of essential oils such as geraniol (Kim *et al.* 2011) and stylosin (Rassouli *et al.* 2011).

The comparison of results of tests with β-pinene and essential oils did not show similarities in MI and MetI. The abnormalities in β-pinene treatment were found in lettuce, but not in onion. In onion, the application of the essential oils at the highest concentration resulted in a large reduction in MI, which did not occur when the the root tips were treated with β-pinene. In some cases, the allelopathic inhibition does not result from the exposure to one single chemical agent, but is a result of using different kinds of allelochemicals together. Biological activity from allelopathic essential oils may not occur merely as a result of the concentration of individual chemicals, but is also influenced by the interaction among the chemicals (Souza Filho 2006).

The results of the present work indicated that essential oils from *H. psiadioides* and *H. alienus* can cause mitodepressive effects and chromosomal abnormalities in onion- and lettuce-root meristems. *Allium cepa* and *Lactuca sativa* tests showed differences in the response to essential oils of *H. psiadioides* and *H. alienus.* These results are explained by the fact that living organisms might have many biological differences, such as distinct metabolic systems, which can lead to a differential response to chemical agents. The test organisms used in the present assays are excellent bioindicators of genotoxic and mutagenic effects of natural chemicals (Leme and Marin-Morales 2009) because both species are used in folk medicine. The effects observed on mitotic activity can also contribute to understanding the effects of the essential oils on the growth of the target species. Studies aimed at determining the synergistic activity exerted by the compounds present in the essential oils can also contribute to understanding of the mode of action of natural products with allelopathic potential and to understanding of ecological relationships among species.

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Table 1. Percentage of lettuce meristematic root cells with chromosome abnormalities as induced by *Heterothalamus* essential oils at different concentrations in pre-germination

Chromosome abnormality	Proportion of cells with chromosome abnormalities (%)							
	Control	β-pinene	*H. psiadioides* 1%	*H. psiadioides* 10%	*H. psiadioides* 100%	*H. alienus* 1%	*H. alienus* 10%	*H. alienus* 100%
Spindle disturbance at late prophase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Micronucleus at prophase	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
C-mitosis	0.00	0.08	0.00	0.00	0.91	0.31	0.40	0.40
Sticky metaphase	0.01	0.21	0.35	0.44	1.14	0.78	0.53	1.08
Non-oriented chromosome at metaphase	0.00	0.05	0.00	0.03	0.00	0.16	0.13	0.19
Multipolar anaphase	0.01	0.01	0.00	0.04	0.10	0.01	0.00	0.00
Anaphase bridges	0.04	0.05	0.00	0.06	0.05	0.02	0.01	0.08
Delay of chromosome at anaphase	0.00	0.00	0.00	0.00	0.05	0.04	0.00	0.03
Chromosome breaks at anaphase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Telophase bridges	0.01	0.01	0.01	0.03	0.00	0.00	0.00	0.10
Delay of chromosome at telophase	0.00	0.00	0.01	0.00	0.04	0.00	0.00	0.04
Chromosome breaks at telophase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Micronucleus at interphase	0.07	0.00	0.14	0.15	0.26	0.19	0.18	0.23
Buds at interphase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 2. Percentage of lettuce meristematic root cells with chromosome abnormalities as induced by *Heterothalamus* essential oils at different concentrations in post germination

Chromosome abnormality	Proportion of cells with chromosome abnormalities (%)							
	Control	β- pinene	*H. psiadioides* 1%	*H. psiadioides* 10%	*H. psiadioides* 100%	*H. alienus* 1%	*H. alienus* 10%	*H. alienus* 100%
Spindle disturbance at late prophase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Micronucleus at prophase	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
C-mitosis	0.00	0.08	0.35	0.31	1.15	0.00	0.05	0.03
Sticky metaphase	0.01	0.13	0.45	0.48	1.16	0.29	0.70	1.05
Non-oriented chromosome at metaphase	0.00	0.03	0.09	0.06	0.20	0.06	0.00	0.06
Multipolar anaphase	0.01	0.01	0.03	0.08	0.15	0.00	0.01	0.00
Anaphase bridges	0.04	0.03	0.04	0.05	0.04	0.08	0.06	0.05
Delay of chromosome at anaphase	0.00	0.00	0.03	0.03	0.00	0.00	0.00	0.00
Chromosome breaks at anaphase	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00
Telophase bridges	0.01	0.00	0.00	0.00	0.03	0.01	0.01	0.04
Delay of chromosome at telophase	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Chromosome breaks at telophase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06
Micronucleus at interphase	0.07	0.00	0.22	0.21	0.13	0.19	0.34	1.46
Buds at interphase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 3. Percentage of onion meristematic root cells with chromosome abnormalities as induced by *Heterothalamus* essential oils in pre germination

Chromosome abnormality	Proportion of cells with chromosome abnormalities (%)							
	Control	β- pinene	*H. psiadioides* 1%	*H. psiadioides* 10%	*H. psiadioides* 100%	*H. alienus* 1%	*H. alienus* 10%	*H. alienus* 100%
Spindle disturbance at late prophase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Micronucleus at prophase	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
C-mitosis	0.00	0.01	0.11	0.06	0.00	0.00	0.00	0.00
Sticky metaphase	0.00	0.00	0.35	0.43	0.15	0.29	0.10	0.09
Non-oriented chromosome at metaphase	0.04	0.01	0.11	0.15	0.03	0.05	0.00	0.00
Multipolar anaphase	0.00	0.05	0.06	0.00	0.00	0.00	0.00	0.00
Anaphase bridges	0.05	0.00	0.04	0.40	0.08	0.50	0.02	0.00
Delay of chromosome at anaphase	0.00	0.02	0.10	0.02	0.00	0.03	0.04	0.00
Chromosome breaks at anaphase	0.00	0.00	0.11	0.17	0.00	0.00	0.00	0.00
Telophase bridges	0.00	0.00	0.14	0.02	0.01	0.04	0.06	0.01
Delay of chromosome at telophase	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00
Chromosome breaks at telophase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Micronucleus at interphase	0.00	0.00	0.14	0.18	0.02	0.09	0.01	0.02
Buds at interphase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 4. Chromosome abnormalities percentage of onion meristematic root cells induced by *Heterothalamus* essential oils in post-germination

Chromosome abnormality	Proportion of cells with chromosome abnormalities (%)							
	Control	β –pinene	*H. psiadioides* 1%	*H. psiadioides* 10%	*H. psiadioides* 100%	*H. alienus* 1%	*H. alienus* 10%	*H. alienus* 100%
Spindle disturbance at late prophase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Micronucleus at prophase	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
C-mitosis	0.00	0.00	0.00	0.00	0.01	0.06	0.10	0.00
Sticky metaphase	0.01	0.02	0.20	0.44	0.08	0.16	0.05	0.21
Non-oriented chromosome at metaphase	0.00	0.00	0.01	0.00	0.06	0.00	0.00	0.04
Multipolar anaphase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Anaphase bridges	0.00	0.02	0.33	0.38	0.04	0.15	0.06	0.05
Delay of chromosome at anaphase	0.00	0.01	0.00	0.19	0.00	0.05	0.03	0.00
Chromosome breaks at anaphase	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00
Telophase bridges	0.05	0.00	0.00	0.04	0.00	0.00	0.00	0.01
Delay of chromosome at telophase	0.01	0.00	0.00	0.03	0.01	0.00	0.00	0.00
Chromosome breaks at telophase	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
Micronucleus at interphase	0.04	0.00	0.24	0.16	0.01	0.21	0.00	0.06
Buds at interphase	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

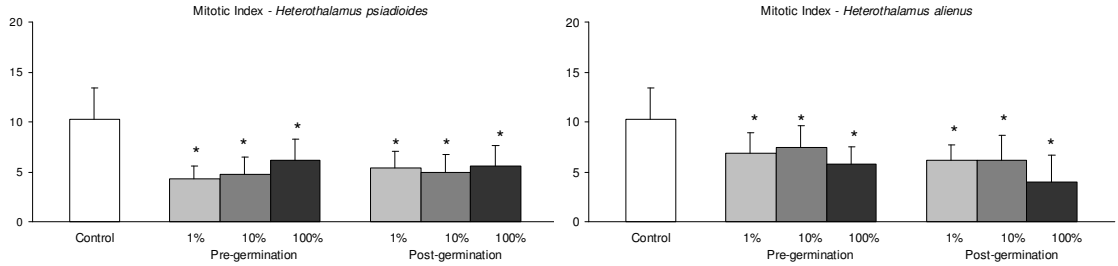

Figure 1. Mitotic index of lettuce meristematic root cells exposed to essential oil of Heterothalamus psiadioides and Heterothalamus alienus. *(Kruskal-Wallis' and Dunn's test; P<0.05).

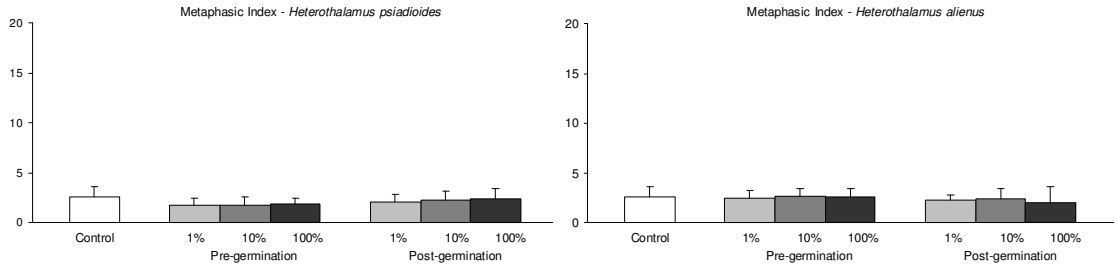

Figure 2. Metaphasic index of lettuce meristematic root cells exposed to essential oil of *Heterothalamus psiadioides and Heterothalamus alienus.* *(ANOVA and Tukey's test; P<0.05).

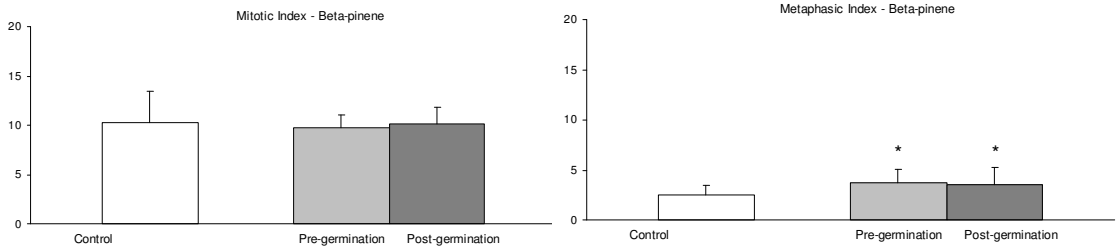

Figure 3. Mitotic and metaphasic index of lettuce meristematic root cells exposed to β-pinene.
*(Kruskal-Wallis' and Dunn's test; P<0.05).

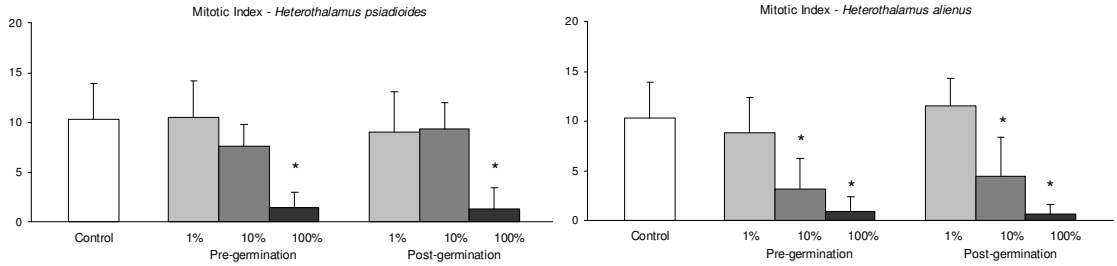

Figure 4. Mitotic index of onion meristematic root cells exposed to essential oil of *Heterothalamus psiadioides and Heterothalamus alienus.* *(Kruskal-Wallis' and Dunn's test; P<0.05).

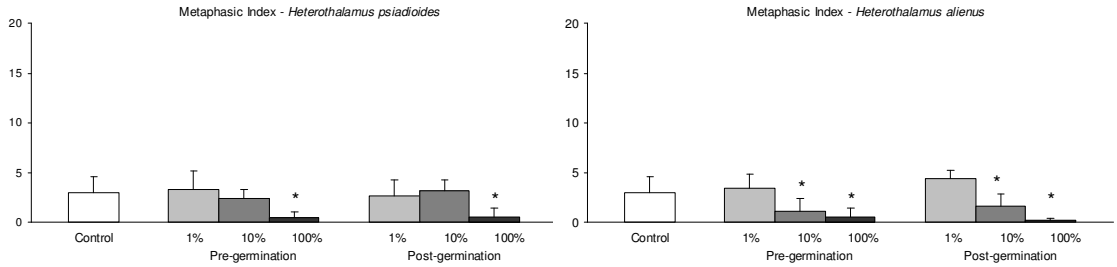

Figure 5. Metaphasic index of onion meristematic root cells exposed to essential oil of *Heterothalamus psiadioides and Heterothalamus alienus.* *(ANOVA and Tukey's test; P<0.05).

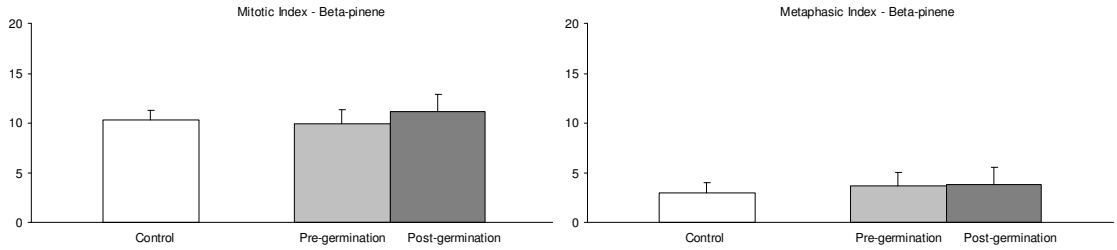

Figure 6. Mitotic and metaphasic index of onion meristematic root cells exposed to β-pinene. *(Kruskal-Wallis' and Dunn's test; P<0.05).

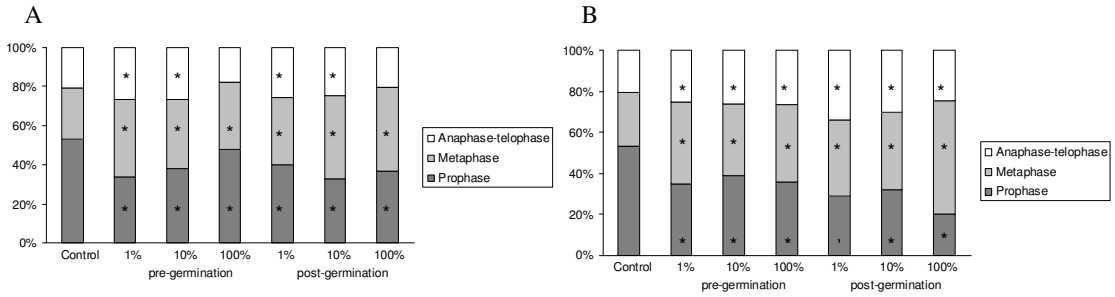

Figure 7. Mitotic phases of lettuce meristematic root cells exposed to essential oil of *Heterothalamus psiadioides* (A) and *Heterothalamus alienus* (B). *(ANOVA and Tukey's test; P<0.05).

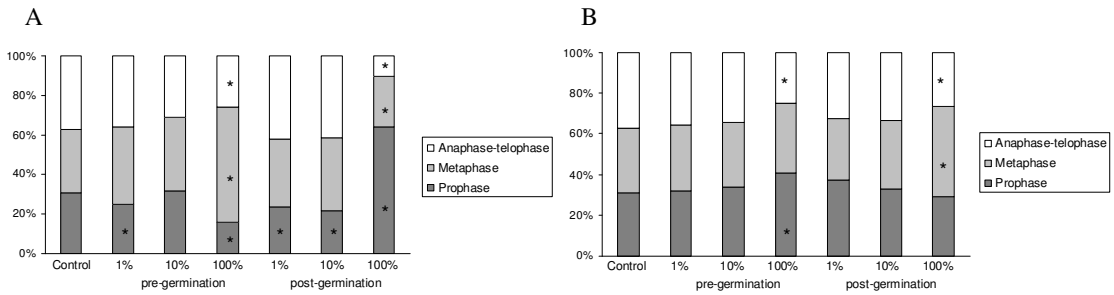

Figure 8. Mitotic phases of onion meristematic root cells exposed to essential oil of *Heterothalamus psiadioides* (A) and *Heterothalamus alienus* (B). *(ANOVA and Tukey's test; P<0.05).

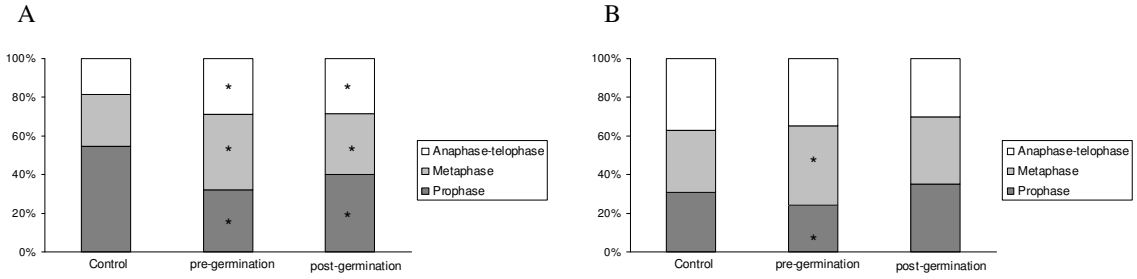

Figure 9. Mitotic phases of lettuce (A) and onion (B) meristematic root cells exposed to β-pinene. *(ANOVA and Tukey's test; P<0.05).

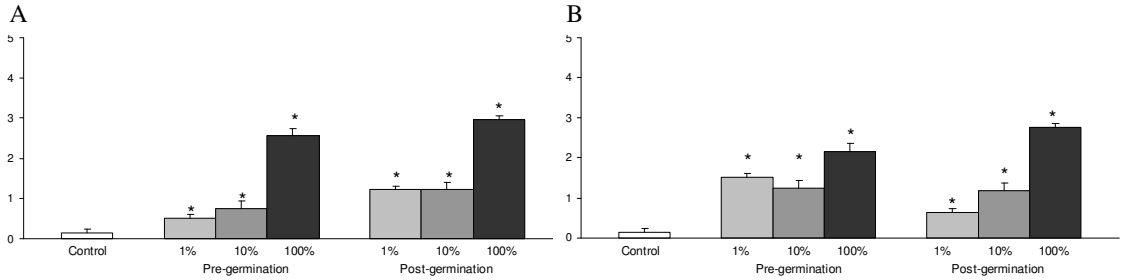

Figure 10. Percentage of chromosomal abnormalities of lettuce meristematic cells induced by *Heterothalamus psiadioides* (A) and *Heterothalamus alienus* (B). *(ANOVA and Tukey's test; P<0.05).

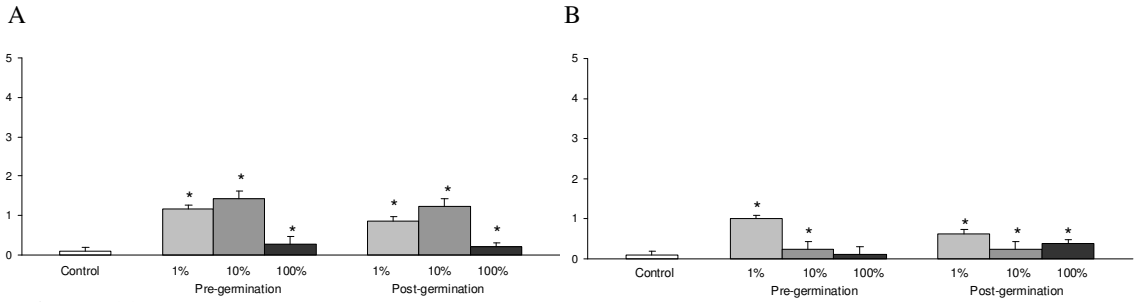

Figure 11. Percentage of chromosomal abnormalities of onion meristematic cells induced by *Heterothalamus psiadioides* (A) and *Heterothalamus alienus* (B). *(ANOVA and Tukey's test; P<0.05).

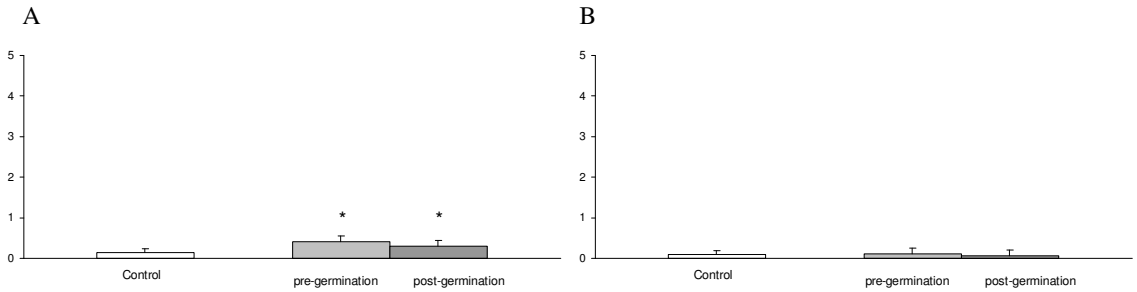

Figure 12. Percentage of chromosomal abnormalities of lettuce (A) and onion (B) meristematic cells induced by β-pinene. *(ANOVA and Tukey's test; P<0.05).

ARTIGO 3

Phytotoxicity of the essential oil of *Heterothalamus* spp.: effects on root anatomy and cell membrane integrity of lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.)

Submetido ao Biochemical Systematics and Ecology

PHYTOTOXICITY OF THE ESSENTIAL OIL OF *Heterothalamus* spp.: EFFECTS ON ROOT ANATOMY AND CELL MEMBRANE INTEGRITY OF LETTUCE (*Lactuca sativa* L.) AND ONION (*Allium cepa* L.)

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Abstract. Essential oils are complex mixtures of various compounds with low molecular weights and diverse chemical structures. They are part of the defence system of plants, acting as allelochemicals in plant-herbivorous, plant-microorganism and plant-plant interactions. The present study aimed is determining the effect of essential oils from leaves of *Heterothalamus psiadioides* Less. and *H. alienus* (Sprengel) O. Kuntze on the membrane integrity and anatomical structure of roots of lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.). Lettuce and onion seeds which germinated with essential oils from *H. psiadioides* and *H. alienus* had structural alterations in root anatomy and increased membrane damage. Analysis of the results showed that the essential oils of *Heterothalamus* change features of early stages of root development, affecting plant development of lettuce and onion.

1. Introduction

Essential oils are an important group of compounds of plants and consist of a broad mixture of low molecular weight compounds: monoterpenes, sesquiterpenes and phenilpropanoids (Vokou *et al.*, 1999). The biological activity of the essential oils in the environment depends on the concentrations of its constituent parts, may have synergistic effects, and act in numerous interactions, including allelopathy.

The concept of allelopathy involves the suppression of neighboring plant growth by the release of toxic compounds (Fitter, 2003). The inhibition can result from the combined action of multiple allelochemicals (Einhellig, 1996) with a primary effect on root growth and root tip ultrastructure (Cruz-Ortega *et al.*, 1998). Allelopathic interaction is a complex process that affects all development and growth aspects (El-Khatib *et al.*, 2004), with many phytotoxins altering the morphology and anatomy of seedlings (Maraschin-Silva and Aquila 2006). Plant development has decisive and very vulnerable stages, such as seedling growth, which must be taken into account when growth is studied.

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Three species are recognized for the *Heterothalamus* genus: *Heterothalamus psiadioides* Less. which occurs in Southern Brazil and Uruguay, *Heterothalamus alienus* (Spreng.) Kuntze which grows in South of Brazil, Uruguay and Central Argentina, and *Heterothalamus rupestris* Deble, A.S Oliveira & Marchiori which is found in Southern Brazil (Pacciaroni *et al.*, 2008). This genus generally occurs in shallow rock soils, often along roadsides (Barroso and Bueno, 2002). Historically, investigations of allelopathy have originated from field observations, which suggest a change in the pattern of vegetation. *H. psiadioides* is becoming a pioneer in regions of Porto Alegre, in Rio Grande do Sul, Brazil (Barroso and Bueno, 2002). Monteiro and Vieira (2002) observed that in many ecosystems plants tend to be established in formations with many individuals and sometimes in homogeneous populations, which is attributed, among other factors, to the release of phytotoxic substances, reducing or preventing the establishment of other species near them.

Heterothalamus psiadioides Less. (alecrim-do-campo, vassoura, erva-formiga) and *H. alienus* (Sprengel) O. Kuntze (alecrim-do-campo) are used in South American folk medicine. *H. psiadioides* is used as an antipyretic, antidote for snake bites, anti-inflammatory (Suyenaga *et al.*, 2004), febrifuge. It is also used for skin remedies (Biavatti *et al.*, 2007) and against renal affections (Palacius *et al.*, 2007). *Heterothalamus alienus* has been reported as a decoction and as leaf powder used against fever and as a stimulant (Rucker *et al.*, 1996). *Heterothalamus alienus* is also used against *Apis mellifera* L. pests (Ruffinengo *et al.*, 2006), and also has antiviral and antifungal properties (Duschatzky *et al*., 2007). Both species are recognized as major producers of essential oils with potential biological activity.

The observation of macroscopic effects by allelochemicals in dose-response bioassays can provide clues about their mode of action (Macías *et al.*, 2008). A previous research has shown that the essential oils of *Heterothalamus psiadioides* Less. and *H. alienus* (Sprengel) O. Kuntze diminish the size of the lettuce and onion roots, decrease mitotic index, and induce chromosomal abnormalities (Schmidt-Silva *et al.*, 2011).

Duschatzky *et al.* (2007) observed that the major compound of essential oil for both species of *Heterothalamus* was β-pinene (Duschatzky *et al.*, 2007; Suyenaga *et al*., 2004). Alpha-pinene and β-pinene are among the most widely distributed monoterpenes in the plant kingdom and are the major constituents of various essential oils.

Due to the well known biological activities of several terpenoid compounds, constituents of the essential oils of *H. pisiadioides* and *H. alienus*, and their potential allelopatic activity, this study aimed is analyzing the effect of the essential oils from these plant species and their major compound (β-pinene) on the membrane integrity and anatomical structure of roots of lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.).

2. Material and Methods

2.1 Plant collection

Leaves of *Heterothalamus psiadioides* and *H. alienus* were collected in 2008 and 2009. *H. psiadioides* samples were collected in a natural vegetation site at Morro Santana, a hill (30° 4'S and 51° 7'W) in the city of Porto Alegre, and *H. alienus* samples were collected along a roadside in Bagé (31° 19'S and 54° 6'W), State of Rio Grande do Sul, Brazil. Voucher specimens of *H. psiadioides* (153122) and *H. alienus* (153825) have been deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN).

2.2 Plant Bioassays

Fifty seeds of lettuce (*Lactuca sativa* L. cv. Grand Rapids) and onion (*Allium cepa* L. cv. Baia Periforme), obtained from commercial dealers, were sown in a layer of filter paper moistened with 7 mL of distilled water, inside germination boxes sealed with plastic film and incubated in a germination room at a mean temperature of 20 ºC under a 12-h photoperiod. Lighting was provided by 20 W fluorescent lamps. The essential oils of *H. psiadioides* and *H. alienus* leaves were obtained by hydrodistillation, using a modified Clevenger apparatus (Gottlieb and Taveira-Magalhães, 1960) for 4 hours. The oil obtained was dried over anhydrous sodium sulphate and stored at -20ºC prior to use. Experiments were carried out with pure oil (100%), 10% and 1% aqueous solution (v/v). The aqueous solutions were made with 0.1% solution of Tween 20 and distilled water (1:100, v/v). Distilled water was employed as a negative control. A 100 µL sample of *H. psiadioides* and *H. alienus* essential oils, and the solutions (1 and 10%) were dropped onto a cotton ball attached to the inner face of the gerbox lid to avoid direct contact with the seeds, but allowing the oil to volatilize into the airspace within the box. For the treatment with β-pinene, 40 µL was added to each box.

2.3 Anatomical analysis

Roots from lettuce and onion were fixed in 1% glutaraldehyde and 4% formaldehyde in phosphate buffer (pH 7.2) (McDowell and Trump, 1976), dehydrated in ethanol series, infiltrated and embedded in Historesin (Reichert-Jung). Anatomical sections were obtained in rotative microtome (6 µm), and stained with 0.05% toluidine blue O in 0.1M phosphate buffer (O'Brien *et al.*, 1964).

2.4 Histometry

To the structural analysis of roots of lettuce and onion, histometric and cytometric data were added. These data were obtained from photomicrographs of transverse sections of the root (1 cm from the root cap), the measurements were done randomly. The tissue area (n=10) and the area of cells in the vascular

cylinder, cortex and epidermis (n=15) were evaluated. Measurements were performed using the graphics program Axion Vision, Zeiss Imaging Systems, version 4.7.2.

2.5 Relative electrolyte leakage - In order to evaluate the relative electrolyte leakage, 50 mg from roots and leaves for lettuce and seedlings for onion were washed and incubated in distilled water. For onion, the whole seedling was used because of the damage the essential oil caused in root and shoot length, being impossible to get enough weight for the assay. After 24 h of incubation at room temperature, the electrical conductivity of the solution was measured in a conductivity meter (Tecnal Digital Conductivimeter, TEC-4MP). The roots were then frozen, defrost and left at room temperature for over 24 h, after which the electrical conductivity was measured again. The relative electrolyte leakage (REL) was calculated according to the following equation:

$$REL\ (\%) = (C_1 / C_{1+} C_2).100$$

Where: C_1 = Conductivity measured during the first 24 h

C_2 = Conductivity measured during the following 24 h.

3. Results

3.1 Anatomy

Allelochemicals caused anatomical defects on lettuce and onion roots.

3.1 *Anatomical effects on lettuce*

The control plants had uniseriate epidermis, differentiated exodermis, 4-layered cortex with thin-walled cells, and vascular cylinder with diarch xylem (Figure 1A). The beta-pinene and the essential oil of *Heterothalamus psiadioides* (1%) did not cause any differences in the anatomy of onion root tips.

The essential oil of *H. psiadioides* (10%) caused no alteration in epidermis and exodermis, but the cortex was 8-layered formed by divisions of endodermal cells, some with necrosis, and evident intercellular spaces. Vascular cylinder had diarch xylem, differentiated metaxylem and undifferentiated phloem (Figure 1B). When the essential oil of *H. psiadioides* (100%) was applied the epidermis collapses, the exodermis is indistinct, the cortex was 7-8 layered with intermediate cells larger than the others, some with necrosis, and reduced cellular spaces. Vascular cylinder protoxylem was diarch and the phloematic portion was poorly differentiated when compared to the plants submitted to 10% (Figure 1C).

The plants submitted to the essential oil of *Heterothalamus alienus* at the concentrations of 1% and 10% had uniseriate epidermis, indistinct exodermis, 5-layered cortex with elongated cells and reduced intercellular spaces. Vascular cylinder with diarch xylem and differentiated phloem (Figure 1D;

1E). Plants submitted to the essential oil of *H. alienus* (100%) maintained the uniseriate epidermis, and indistinct exodermis. The cortex was 7-8 layered, the vascular cylinder had diarch xylem and more developed phloematic portion. The treatments with the highest concentrations caused the accumulation of different cell contents in the outer layers (epidermis and exodermis) (Figure 1F).

3.2 Anatomical effects on onion

The control plants had uniseriate epidermis, indistinct exodermis, 6-7 layered cortex, and vascular cylinder with diarch protoxylem with a central metaxylem element (Figure 2A). The beta-pinene and the essential oil of *Heterothalamus psiadioides* (1%) did not cause any differences in the anatomy of onion root tips.

The essential oil of *H. psiadioides* (10%) caused no alteration in epidermis, exodermis, and cortical layers. Vascular cylinder is diarch xylem but two central elements of metaxylem differentiated. Lignification is inhibited (Figure 2B). The essential oil of *H. psiadioides* (100%) cause a reduced volume on epidermal and cortical cells, and cells of epidermis elongated. Vascular cylinder had diarch xylem and metaxylem did not differentiate. The phloematic portion was more developed (Figure 2C).

There were no differences in the anatomy of onion root tips submitted to the essential oil of *H. alienus*.

3.3 Histometry

The results of histometry are presented in Table 1. The areas of the cortex and vascular cylinder increased in roots of lettuce submitted to 10 and 100% essential oils for both species tested. The area of epidermis increased in the highest concentrations used (10 and 100%) for both oils tested, although only the highest concentration of *H. psiadioides* has caused an increase in cell areas.

In onion, the area of the vascular cylinder decreased at all concentrations of both oils tested, and the cell areas decreased in the concentration of 1 and 100% for *H. psiadioides* and of 100% for *H.* alienus. The area of the cortex decreased in the lowest concentration (10%) of the essential oil of *H. psiadioides*. Additionally, cortical cells area decreased in the concentration of 1% for *H. psiadioides* and 100% for *H. alienus*. Epidermis area did not show any differences from the control, although the epidermis cell area had decreased in 1% and had increased in 10% for *H. psiadioides*, and decreased in 10% for *H. alienus*. There were no differences in the cell or tissue areas of onion root tips submitted to β-pinene.

3.4 Membrane Integrity

The results of membrane integrity are presented in Table 2. The highest concentration of both oils increased the relative electrolyte leaking in lettuce roots. For leaves, only the essential oil of *H. alienus* in

its highest concentration caused an increase in electrolyte leakage. For onion, the results were similar. There was also an increase in leakage in onion seedlings submitted to *H. alienus* (10%).

4. Discussion

Allelochemicals typically suppress seed germination, cause injury to root growth and other meristems, and inhibit seedling growth. Division and expansion of meristematic and non-meristematic cells are not alternative or sequential, but interdependent processes which can modify the rate of plant organs growth (Beemster and Baskin, 2000). Consequently, higher plants control the rate of their organs growth by adjusting the rate and duration of cell division and expansion (Beemster and Baskin, 2000). According to Al-Wakeel *et al.* (2007), the inhibition of cell elongation can be related to the direct action of allelochemicals, which interfere in the process of cell division and thus alter the balance of different hormones. They can also generate a combined effect of the retardation of auxin-induced growth (Hoshi *et al.*, 1994) and the destabilization of cell walls (Gonzales and Rojas, 1999).

In the present study, the essential oils of *H. psiadioides* and *H. alienus* caused structural and size-related antagonic alterations in the structure of roots of the two model plants, lettuce and onion. Lettuce seedlings grown under the effect of the essential oils of *H. psiadioides* and *H. alienus* had an increase in the area of tissues, while onion seedlings had a reduction in the area of tissues. This apparent contradictory effect to the essential oils can be explained by physiological differences between the responses of monocots and dicots to allelochemicals. In a previous research (unpublished data), it was found that these essential oils caused a decrease in root and shoot seedling length, and a decrease in the mitotic index either in lettuce or onion root tips, with a strongest decrease in onion (Schmidt-Silva *et al.*, 2011). These results indicate that even with contrasting effects, the essential oils tested herein did affect the model species.

Our data are also in accordance with Burgos *et al.* (2006) who showed that DIBOA, an allelopathic agent found in maize, increased cell width and reduced cell elongation, along with additional differentiation of cortical cell columns, which resulted in reduced root elongation and increased root diameter. Another allelochemical umbelliferone, commonly found in grasses, also decreased cell elongation and increased radial cell expansion in *Cucumis sativus* L. roots (Jankay and Muller, 1976). These effects were considered by Hoshi *et al.* (1994) as an effect of retardation of auxin-induced growth which may be the explanation for the results obtained herein.

Other possible explanations come from Chon *et al.* (2005) who reported that allelochemicals like coumaric acid and aqueous leachates of alfafa leaves, caused an increase in alfafa root diameter due to an expansion of the vascular cylinder and cortical cell layers. The authors explained that coumaric acid inhibited cell division, and consequently the thickness of seminal roots. These roots were abnormally

enlarged due to the inhibition of longitudinal root growth. The transverse growth of the roots was sequentially maintained, while longitudinal growth was inhibited by the extracts and the phenolic compounds. Nevertheless, Muscolo *et al.* (2005) observed another antagonic effect, as phenol solutions inhibited cortex and stele growth, but increased roots elongation in *Pinus pinaster* Aiton seedlings. On the other hand, *Pinus laricio* Santi roots, phenol solution induced a greater growth of cortex and stele compared to the control.

In addition to the structural alterations, lettuce and onion seedlings treated with the essential oils of *H. psiadioides* and *H. alienus* experienced an increase on electrolyte leakage. This is in agreement with earlier reports of the ability of terpenes phytotoxins to increase membrane leakage, damaging root growth. Terpenes are usually highly hydrophobic substances and most of them readily interact with biomembranes. They can increase the fluidly of the membranes and lead to an uncontrolled efflux of ions and metabolites, modulation of membrane proteins and receptors or even to cell leakage, resulting in cell death (Wink, 2010).

Increased conductivity of the bathing medium indicates cellular membrane disruption resulting in excessive solute leakage. The measurement of relative membrane leakage takes into account the primary leakage of an intact tissue and after an injury to the membrane. In the higher levels of the essential oils of *Heterothalamus* species, the difference in values of primary leakage and after a membrane injury was low, which can reflect the damage in the tissues previous to a freezing forced injury. The effects on membrane integrity are generally seen in a time- and dose-dependent manner, with higher concentrations causing more rapidly and severe effects, while lower concentrations exert either nonlethal effects or lethal activity only after a longer exposure time (Hammer and Carson, 2011).

The current results are in agreement with earlier reports that the essential oils and the monoterpenes (citronellal, α-pinene, and (+)-pulegone) cause ion leakage on plant tissues (Maffei *et al.*, 2001; Sigh *et al.*, 2006). Studies with liposome model systems showed that cyclic terpenes hydrocarbons accumulate in the membrane, and cause a loss of membrane integrity and dissipation of the proton motive force (Sikkema *et al.*, 1994). Oka *et al.* (2004) related the ion leakage to the plasmolysis of mesophyll cells, which result in cell destruction. This is consistent with the structural defects caused by the *Heterothalamus* extracts.

It was proved that the essential oils from *H. psiadioides* and *H. alienus* cause anatomical abnormalities and induce membrane damage in onion- and lettuce-root meristems. Further studies may elucidate the mechanism by which the allelopathic compounds of these oils influence plant development and shed further light on their biological activities.

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Table 1. Histometry of lettuce and onion root cells submitted to *Heterothalamus* essential oils at different concentrations. Asterisk indicates a significant result (Kruskal–Wallis and Dunn's test; $P = 0.05$).

	Histometry (μ^2)							
	Control	**β-pinene**	***H. psiadioides* 1%**	***H. psiadioides* 10%**	***H. psiadioides* 100%**	***H. alienus* 1%**	***H. alienus* 10%**	***H. alienus* 100%**
Lettuce/tissue								
Area of vascular cylinder	25.75	34.00	23.03	59.08*	64.73*	21.32	47.13*	85.47*
Area of cortex	157.35	189.02	132.7	487.01*	444.81*	190.00	379.19*	481.07*
Area of epidermis	116.01	164.93	140.00	304.56*	291.46*	107.43	235.03*	616.79*
Lettuce/cell								
Area of vascular cylinder	0.31	0.45	0.30	0.69*	0.68*	0.30	0.67*	0.71*
Area of cortex	2.43	3.48	1.76	6.21*	6.52*	2.48	4.00*	6.00*
Area of epidermis	1.62	2.22	1.39	2.31	3.80*	1.37	1.92	1.75
Onion/tissue								
Area of vascular cylinder	100.85	107.37	74.72*	84.21*	83.55*	85.23*	86.26*	48.47*
Area of cortex	332.37	350.72	259.15*	343.52	346.32	318.07	295.94	295.87
Area of epidermis	58.46	47.55	44.67	52.70	59.83	48.00	48.09	52.43
Onion/cell								
Area of vascular cylinder	1.43	1.11	0.65*	0.93	0.89*	0.69	0.93	0.81*
Area of cortex	12.61	14.15	7.79*	16.46	10.26	9.47	11.26	8.14*
Area of epidermis	4.01	3.74	2.10*	5.34*	4.40	3.39	3.02	3.61

Table 2. Membrane integrity of lettuce and onion submitted to *Heterothalamus* essential oils at different concentrations. Asterisk indicates a significant result (Kruskal–Wallis and Dunn's test; $P = 0.05$).

	Relative Electrolyte Leakage (%)							
	Control	**β-pinene**	***H. psiadioides* 1%**	***H. psiadioides* 10%**	***H. psiadioides* 100%**	***H. alienus* 1%**	***H. alienus* 10%**	***H. alienus* 100%**
Lettuce/shoot								
	16.34	16.13	14.08	17.98	20.51	17.13	14.97	37.10*
Lettuce/root								
	22.82	22.70	20.38	23.11	40.24*	23.63	22.68	48.91*
Onion								
	16.06	12.87	12.64	19.82	45.34*	14.22	35.32*	43.96*

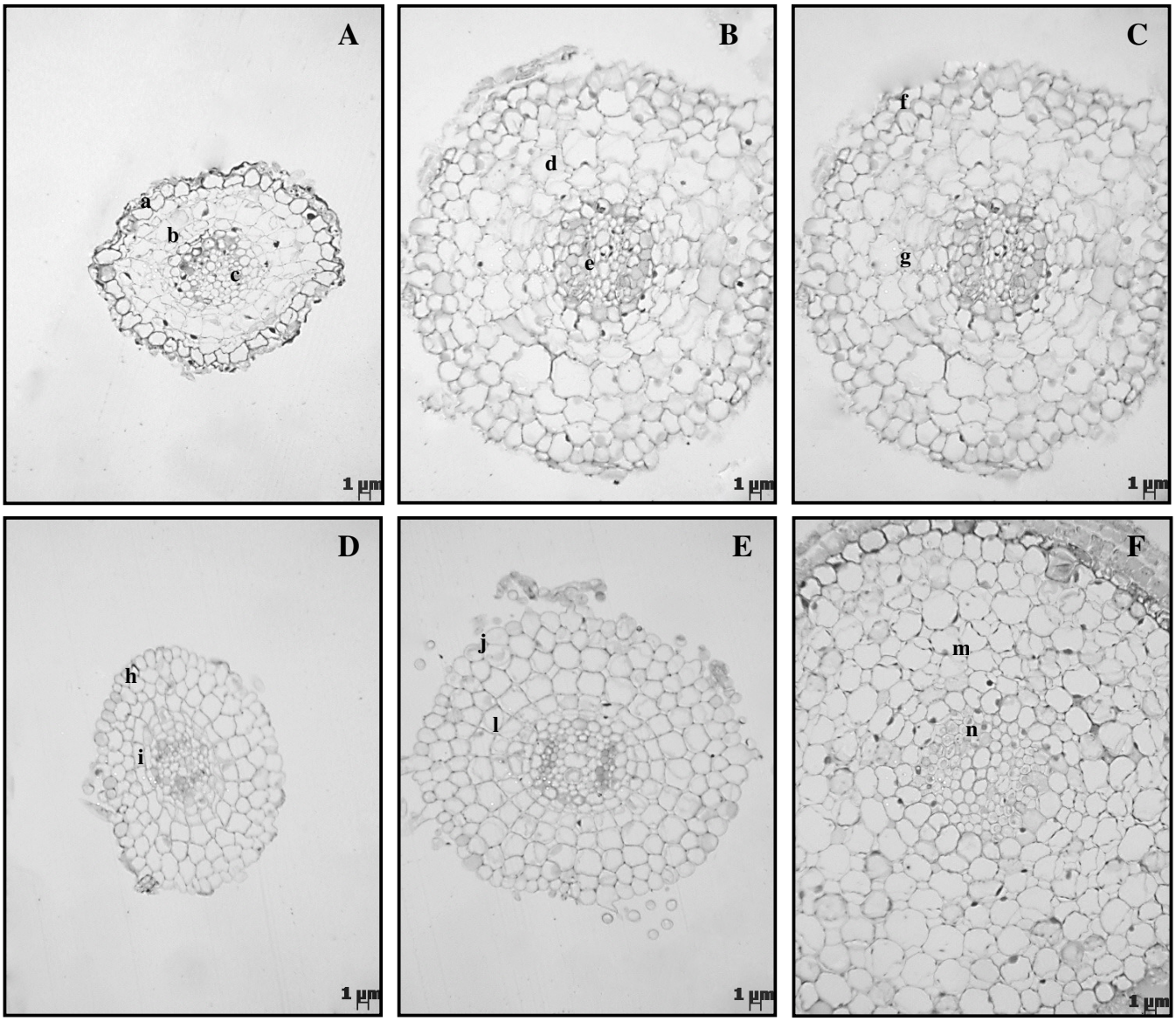

Figure 1. Transverse sections of *Lactuca sativa* L. primary roots. (A) Primary root of the control group (**a-** uniseriate epidermis, **b-** 4-layered cortex, **c-** vascular cylinder with diarch xylem). (B) Primary root of the group submitted to *H. psiadioides* 10% (**d-** 8-layered cortex, **e-** undifferentiated phloem). (C) Primary root of the group submitted to *H. psiadioides* 100% (**f-** collapsed epidermis, **g-** 8-layered cortex). (D) Primary root of the group submitted to *H. alienus* 1% (**h-** uniseriate epidermis, **i-** 5-layered cortex). (E) Primary root of the group submitted to *H. alienus* 10% (**j-** uniseriate epidermis, **l-** 5-layered cortex). (F) Primary root of the group submitted to *H. alienus* 100% (**m-** 7-8-layered cortex, **n-** developed phloematic portion).

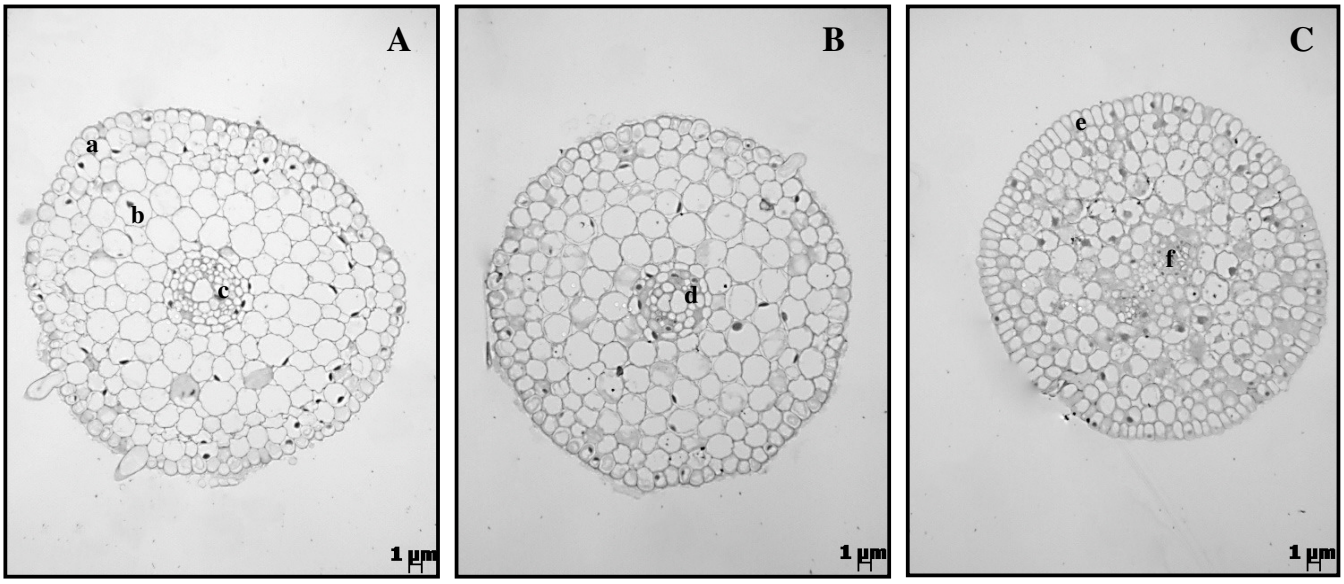

Figure 2. Transverse sections of *Allium cepa* L. adventitious roots. (A) Primary root of the control group (**a-** uniseriate epidermis, **b-** 6-7 layered cortex, **c-** vascular cylinder with diarch protoxylem with a central metaxylem element). (B) Primary root of the group submitted to *H. psiadioides* 10% (**d-** vascular cylinder with diarch protoxylem with two central metaxylem elements). (C) Primary root of the group submitted to *H. psiadioides* 100% (**e-** epidermis elongated, **f-**metaxylem not differentiated).

CONSIDERAÇÕES FINAIS

A alelopatia, supressão do crescimento de plantas por metábolitos secundários (aleloquímicos), têm sido alvo de pesquisas intensas desde meados do século XX. Muito dos trabalhos recentes tem se mostrado insuficientes para comprovar a alelopatia em condições de campo. Deste modo, estudos focados apenas no potecial alelopático têm sido alvo de críticas no que se refere a reprodutibilidade dos resultados obtidos. Entretanto, esses testes de laboratório são amplamente usados nas etapas iniciais de investigação, servindo para indicar o caminho a ser seguido nas fases posteriores do estudo.

Existe um ceticismo exacerbado em torno deste fenômeno quimioecológico. Acredita-se que esse fato decorre da complexidade de fatores que, junto à alelopatia, regem o estabelecimento de espécies vegetais em campo. Obviamente, ainda há um longo caminho a ser trilhado para que se evolua da avaliação dos efeitos fitotóxicos (potencial alelopático) para a comprovação da intreação química entre espécies vegetais nos seus ambientes característicos. Entretanto, os ensaios de laboratório, como os que foram desenvolvidos no presente estudo, são fundamentais para detectar a possibilidade do fenômeno no campo. Além disso, tais estudos podem fornecer dados valiosos (em especial sobre os mecanismos de ação) para o desenvolvimento de estratégias de controle de plantas invasoras, como alternativa ao uso dos herbicidas tradicionais.

As espécies que foram alvo deste trabalho são consideradas pioneiras e provocam inibição no estabelecimento de outras espécies. Nos bioensaios realizados comprovou-se a fitoxidez do óleo essencial de *Heterothalamus psiadioides* e
H. alienus. Portanto, estas espécies são caracterizadas como potencialmente alelopáticas. Este fato é a base para estudos de cunho ecológico que envolvam ensaios de campo.