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Dissertação de Mestrado

Efeito de duas espécies arbustivas sobre a comunidade vegetal: a alelopatia é um fator determinante?

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Efeito de duas espécies arbustivas sobre a comunidade vegetal: a alelopatia é um fator determinante?

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*“While we animals were busy nailing down things like locomotion and consciousness, the plants (...) acquired an array of extraordinary and occasionally diabolical powers by discovering how to synthesize remarkably complicated molecules.”*

*– Michael Pollan, Botany of Desire: A Plant's-eye View of the World*

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## RESUMO

A alelopatia, na qual uma espécie causa efeitos negativos sobre outra através da liberação de substâncias químicas, é um fenômeno que pode afetar comunidades vegetais. Em alguns casos, o estabelecimento de arbustos aromáticos em formações densas, com apenas poucas espécies ocorrendo em suas proximidades, é um padrão que tem sido relacionado aos efeitos fitotóxicos dos voláteis que os mesmos liberam. Em campos dos sul do Brasil, esse padrão de estabelecimento frequentemente ocorre para as espécies arbustivas aromáticas *Heterothalamus psiadioides* Less e *Baccharis patens* Baker, indicando a possibilidade de alelopatia. Dessa forma, o presente estudo teve como objetivo determinar se *H. psiadioides* e *B. patens* são fitotóxicas e se isso implica em efeitos negativos sobre a comunidade vegetal na qual os arbustos ocorrem. Em ensaios em laboratório, os efeitos dos voláteis liberados diretamente das folhas frescas e secas de *H. psiadioides* e *B. patens* foram testados sobre a germinação e o crescimento das plantas-alvo alface e cebola nas quatro estações do ano. Em um estudo em campo, foram avaliadas cobertura e biomassa vegetal, número de mudas, diversidade, riqueza, e variáveis de estrutura da vegetação no entorno de ambas as espécies arbustivas e onde não havia arbustos. Nos bioensaios, os voláteis de *H. psiadioides* e *B. patens* causaram inibição na porcentagem e velocidade de germinação e redução no tamanho da raiz e da parte aérea das plantas-alvo, sendo que folhas frescas apresentaram maior fitotoxidez. Esses parâmetros foram mais afetados por *H. psiadioides* que por *B. patens* em todas as estações, e os efeitos de ambas foram menores no verão. Em relação à investigação em campo, que objetivou avaliar os efeitos da espécie muito fitotóxica *H. psiadioides* sobre a comunidade vegetal, não foram observadas evidências de alelopatia. Houve maior riqueza e diversidade próximo a *H. psiadioides*, devido à menor cobertura de gramíneas dominantes. Variáveis de estrutura da vegetação e intensidade de luz explicaram os padrões observados de forma mais conclusiva que alelopatia. Esse estudo sugere que o potencial alelopático de plantas pode ser superestimado em bioensaios e que estudos em campo são necessários para demonstrar se efeitos alelopáticos são realmente relevantes em comunidades vegetais. Ainda, o trabalho ressalta a importância de conduzir estudos mais realistas em laboratório e de utilizar controles em estudos em campo.

**PALAVRAS-CHAVE:** Arbustos; Bioensaios; Campos Sulinos; Estudo em campo; Potencial alelopático; Voláteis.

## ABSTRACT

Allelopathy, in which a species causes negative effects on another through the release of chemical substances, is a phenomenon that may affect plant communities. In some cases, the establishment of aromatic shrubs in dense stands, with few other species occurring in their surroundings, has been related to the phytotoxic effects of volatiles released by these shrubs. In South Brazilian grasslands, this pattern of establishment often occurs for the aromatic shrub species *Heterothalamus psiadioides* Less and *Baccharis patens* Baker, indicating the possibility of allelopathy. Thus, this study aimed to determine if *H. psiadioides* and *B. patens* are phytotoxic and if this implicates in negative effects on the plant community in which the shrubs occur. In laboratory assays, the effects of the volatiles released directly from fresh and dry leaves of *H. psiadioides* and *B. patens* on germination and growth of the target plants lettuce and onion were evaluated in four seasons. A field study was carried out, in which vegetation cover and biomass, number of seedlings, diversity, richness, and variables of vegetation structure were evaluated in the surroundings of both species and where there were not shrubs. In the bioassays, *H. psiadioides* and *B. patens* volatiles inhibited the germination rate and speed of germination and reduced the root and shoot length of the target plants, with fresh leaves showing higher phytotoxicity. These parameters were more strongly affected by *H. psiadioides* than by *B. patens* in all seasons, and effects of the both shrubs were lower in summer. In relation to the field investigation that aimed to quantify the effects of the highly phytotoxic *H. psiadioides* on the plant community, no evidences of allelopathy were found. Richness and diversity were higher near *H. psiadioides*, due to lower cover of dominant grasses. Variables of vegetation structure and light intensity explained vegetation patterns more conclusively than allelopathy. This study emphasizes that the allelopathic potential of plants can be overestimated in bioassays, and that field studies are necessary to elucidate if allelopathic effects are in fact significant in plant communities. Still, the relevance of conducting more realistic laboratory studies and of using controls in field studies is highlighted.

**KEYWORDS:** Allelopathic potential; Bioassays; Campos Sulinos; Field study; Shrubs; Volatiles.

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# 1 INTRODUÇÃO

## 1.1 Alelopatia

Interações interespecíficas são processos considerados relevantes na estruturação de comunidades vegetais em escala local. Essas interações podem englobar tanto efeitos positivos quanto negativos para uma ou ambas as espécies envolvidas (Bengtsoon et al., 1994; Brooker et al., 2008). Entre as interações negativas, a competição tem recebido considerável atenção na pesquisa (Goldberg e Barton, 1992; Hautier et al., 2009), mas recentemente, a alelopatia vem se tornando também uma área importante de estudo, sendo reconhecida como um mecanismo que pode afetar significativamente comunidades vegetais (Fitter, 2003; Inderjit et al., 2011).

O termo alelopatia foi primeiramente descrito como qualquer interação química positiva ou negativa entre plantas ou entre microrganismos (Molich, 1937 apud Rice, 1984, p.1). Esse conceito foi posteriormente revisto por Rice (1984), no qual tanto efeitos diretos quanto indiretos de substâncias de uma planta (espécie-doadora) sobre outra (espécie-alvo) eram considerados, sendo os efeitos indiretos em geral mediados pela ação de microrganismos. Ao longo do tempo, novas definições foram propostas, como a de Lambers et al. (1998), onde alelopatia consiste apenas no efeito negativo de uma planta sobre o crescimento ou o desenvolvimento de outra através da emissão de substâncias químicas no ambiente; esse será o conceito utilizado no presente estudo.

As substâncias alelopáticas (aleloquímicos) são provenientes do metabolismo secundário vegetal, consistindo principalmente em terpenos, substâncias fenólicas e alcaloides (Reigosa et al., 2013). Essas substâncias podem chegar ao ambiente de diversas formas: lixiviadas da parte aérea de plantas, liberadas durante a decomposição da serapilheira acumulada no solo, exsudadas de raízes, ou ainda, podem ser volatilizadas (Weir et al., 2004).

Certas plantas podem emitir aleloquímicos no ambiente de forma constante, principalmente espécies perenes (Lambers e Colmer, 2005). No entanto, uma ampla gama de fatores bióticos e abióticos pode causar variação na produção e liberação de aleloquímicos. Dentre os fatores bióticos, Karban (2007) demonstrou que danos por herbivoria podem estimular a produção de defesas químicas por plantas, as quais por sua vez causam efeitos negativos maiores sobre a germinação de outras espécies vegetais do que plantas-não-predadas. Assim, a ação de herbívoros pode implicar em aumento no potencial alelopático de certas espécies, sendo o mesmo observado quando plantas são expostas a

patógenos (Heil e Bostock, 2002). Em relação a fatores abióticos, há estudos que demonstram relações tanto positivas quanto negativas entre a produção de metabólitos secundários e variáveis como luz (Behn et al., 2010), nutrientes (Tharayil et al., 2009), e disponibilidade hídrica (Gray et al., 2003). Além disso, é possível que alguns desses fatores, por apresentarem características sazonais, reflitam também variações ao longo do ano na produção de metabólitos secundários (e.g. Cezarotto et al., 2011; Frizzo et al., 2008; Helmig et al., 2013) e, conseqüentemente, no potencial alelopático das plantas que produzem essas substâncias. Entretanto, estudos de alelopatia geralmente são realizados em laboratório a partir de amostras de plantas coletadas em poucos ou em um único momento, deixando assim de considerar essa possibilidade de variação na produção de aleloquímicos.

## 1.2 Volatilização: o caso dos arbustos aromáticos

Efeitos alelopáticos por emissão de voláteis têm sido investigados desde que Muller et al. (1964) observaram que certos arbustos intensamente aromáticos se estabeleciam em agregações densas e homogêneas. No entorno dessas formações eram muito evidentes zonas de inibição, “cinturões” de solo exposto onde outras espécies vegetais não se estabeleciam. Os autores então evidenciaram em ensaios em laboratório o efeito inibitório de voláteis liberados pelas folhas desses arbustos sobre a germinação e a biomassa de espécies-alvo, e relacionaram a fitotoxicidade desses voláteis à ocorrência de alelopatia. Desde então, monodominância e zonas de inibição são fatores que têm desencadeado investigações sobre o envolvimento de atividade alelopática para espécies aromáticas, as quais em sua maioria pertencem às famílias Asteraceae e Lamiaceae (e.g. Ens et al., 2009; Li et al., 2011; Nishida et al., 2005). Essas espécies são em geral produtoras de óleos essenciais, substâncias voláteis que as conferem um odor forte e característico, e que contêm principalmente terpenos em sua constituição química (Bakkali et al., 2008).

Em campos do sul do Brasil ocorrem vassourais, formações densas de espécies arbustivas (Setubal e Boldrini, 2010), sendo esse padrão de estabelecimento um indicativo de que alelopatia possivelmente ocorra. Muitas dessas espécies são da família Asteraceae, como é o caso dos arbustos aromáticos da subtribo Baccharidinae *Heterothalamus psiadioides* Less e *Baccharis patens* Baker. O óleo essencial de *H. psiadioides* apresenta alta fitotoxicidade, tendo causado efeitos inibitórios muito acentuados sobre a germinação e o desenvolvimento inicial de espécies-alvo, mesmo em baixas concentrações (Schmidt-Silva, 2012). Além disso, plantas-alvo sofreram diminuição na atividade mitótica e apresentaram anormalidades

cromossômicas quando expostas ao óleo essencial de *H. psiadioides* (Schmidt-Silva et al., 2011). Em relação a *B. patens*, o presente estudo é o primeiro a investigar o potencial alelopático dessa planta. No entanto, a fitotoxidez de muitas outras espécies de *Baccharis* já foi demonstrada (Céspedes et al., 2002; Ibáñez e Zoppolo, 2011), indicando a possibilidade de que a planta também cause efeitos inibitórios sobre outras espécies vegetais.

### 1.3 Fitotoxidez implica em alelopatia?

Os termos “efeito fitotóxico” e “efeito alelopático” têm sido em muitos casos utilizados de forma confusa na literatura, ou até mesmo como sinônimos ao se referir a efeitos danosos de substâncias de uma planta sobre outra. Para este estudo, propomos a definição de efeito fitotóxico como a ação negativa de substâncias químicas presentes em tecidos de uma espécie vegetal (os quais geralmente passam por algum tipo de maceração ou extração) sobre outra, sendo esse efeito observado experimentalmente e passível de ocorrência na natureza. Efeito alelopático, como já definido anteriormente, consiste na ação negativa de uma espécie vegetal sobre outra através da liberação de substâncias químicas (Lambers et al., 1998), sendo um processo que ocorre efetivamente em condições naturais. Assim, pode-se dizer de uma planta alelopática que a mesma causa efeitos fitotóxicos, mas, de uma planta que apresenta efeitos fitotóxicos, apenas que possui um potencial alelopático.

Ensaio em laboratório são ferramentas importantes para iniciar investigações sobre alelopatia por permitem avaliar efeitos causados apenas por substâncias químicas, sem interferência de outros fatores (Inderjit e Dakshini, 1995). Porém, esses bioensaios são em geral conduzidos de forma irrealista, sendo ainda comum a realização de estudos “*grind and find*” (Inderjit e Nilsen, 2003). Nesses estudos, para maximizar a concentração de substâncias bioativas dos tecidos vegetais, são utilizados solventes orgânicos e procedimentos de extração (Reigosa et al., 2013). Dessa forma, os bioensaios ficam ainda mais distantes de representar interações que ocorreriam em condições naturais, uma vez que praticamente qualquer planta que passe por algum tipo de extração é em certa concentração capaz de gerar produtos tóxicos para alguma espécie vegetal (Harper, 1994). No caso de voláteis, os ensaios que avaliam a fitotoxidez dessas substâncias são em sua maioria realizados utilizando óleos essenciais extraídos de folhas de plantas (e.g. Ibáñez e Zoppolo, 2011; Singh et al., 2009). Nesses ensaios, a concentração de voláteis em que as plantas-alvo são expostas é maior do que ocorreria em condições naturais, possivelmente superestimando o potencial alelopático dessas espécies.

Dentre as formas de ação de aleloquímicos, a inibição de espécies vegetais por voláteis é provavelmente o maior alvo de ceticismo quanto à fitotoxidez implicar em alelopatia. Isso se deve principalmente ao fato de que diferente de outros mecanismos onde aleloquímicos podem ser quantificados no solo, como exsudação por raízes (e.g. Bais et al., 2003), a persistência de voláteis no ambiente e sua ação sobre a vegetação podem ocorrer de formas mais complexas e difíceis de mensurar na natureza. Há estudos que sugerem que terpenos sejam carregados pelo ar e se dissolvam diretamente através das membranas das plantas (Muller 1965), ou ainda que sejam lixiviados pela chuva ou neblina e se acumulem no solo, onde podem entrar em contato com as espécies-alvo (Barney et al., 2005; Muller, 1966). Assim, evidências de atividade alelopática de voláteis são em sua maioria resultantes de bioensaios, sem levar em conta importantes fatores que ocorrem em campo e podem ser os reais responsáveis pelos padrões observados. Um exemplo disso é o estudo de Bartholomew (1970), que evidenciou que as zonas de inibição no entorno de agregações arbustivas que haviam sido primeiramente atribuídas à ação de voláteis por Muller et al. (1964), se formavam principalmente devido à atividade de herbívoros, os quais se concentravam no entorno dos arbustos devido à oferta de alimento e abrigo contra predadores. Estudos posteriores argumentaram sobre a relevância da ação de voláteis, havendo uma grande discussão sobre a ocorrência ou não de alelopatia nessas comunidades (Halligan, 1975; Muller et al., 1971). Deste modo, esse representa um dos casos em que a atividade alelopática de substâncias fitotóxicas é duvidosa.

Ainda que em alguns casos ensaios em laboratório sejam conduzidos de forma mais similar a condições naturais, apenas com estudos em campo é possível obter evidências consistentes de alelopatia (Inderjit e Weston, 2000). Nos últimos anos cresceu consideravelmente o número de estudos de alelopatia, tanto no Brasil quanto no mundo (Reigosa et al., 2013), mas a maior parte desses estudos é restrita a bioensaios. Assim, sabe-se que diversas espécies apresentam fitotoxidez, mas dessas, poucas que são realmente alelopáticas. Alguns raros estudos, como os de El-Keblawy e Al-Rawai (2007) e Ridenour e Callaway (2001), convincentemente demonstraram a relevância da alelopatia em comunidades vegetais, onde se observou redução na diversidade e abundância de espécies e mudanças na sua composição no entorno de plantas fitotóxicas. Nesses estudos, os resultados observados em campo refletiram claramente os obtidos com os bioensaios, demonstrando que os padrões de vegetação não podiam ser explicados apenas por outros fatores. Assim, para que a fitotoxidez observada em ensaios de laboratório consista em evidência real de alelopatia, é preciso demonstrar em campo que efeitos alelopáticos, mesmo que não sejam os

únicos a determinar padrões de vegetação, sejam fortes o suficiente para se sobressaírem diante de outros fatores.

#### **1.4 Objetivo e estrutura da dissertação**

Esta dissertação teve como objetivo determinar se duas espécies arbustivas aromáticas nativas no sul do Brasil são fitotóxicas e se isso implica em efeitos negativos sobre a comunidade vegetal em que os arbustos ocorrem, ou seja, se apresentam de fato alelopatia. Para isso, dois estudos foram realizados, um deles em laboratório e o outro em campo, correspondendo, respectivamente, ao Capítulo I e ao Capítulo II que serão apresentados a seguir. No primeiro capítulo, testamos se os voláteis de *H. psiadioides* e *B. patens*, duas asteráceas arbustivas, apresentam efeitos fitotóxicos sobre espécies-alvo em condições de laboratório, e se esses efeitos variam sazonalmente. No segundo capítulo, avaliamos se uma das espécies, cuja fitotoxidez elevada foi demonstrada no primeiro estudo, é capaz de afetar negativamente a comunidade vegetal devido a efeitos alelopáticos, testando assim a relação dos resultados obtidos em laboratório sobre os padrões observados na natureza. Nas considerações finais dessa dissertação, são apresentadas as conclusões do trabalho, suas limitações, e também sugestões para estudos futuros de alelopatia.

# Capítulo I

**Phytotoxicity of volatiles from fresh and dry leaves of two Asteraceae shrubs: evaluation of seasonal effects<sup>1</sup>**

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## **2 CAPÍTULO I: Phytotoxicity of volatiles from fresh and dry leaves of two Asteraceae shrubs: evaluation of seasonal effects**

### **Abstract**

In South Brazilian grasslands, the shrubs *Heterothalamus psiadioides* Less and *Baccharis patens* Baker show a dominance pattern that may be a consequence of allelopathy. In this study, the phytotoxic effects of *H. psiadioides* and *B. patens* and the seasonal variation in these effects were assessed. The effects of the volatiles released directly from fresh and dry leaves of the both species were evaluated on germination and growth of the target plants lettuce and onion in four seasons. The volatiles from *H. psiadioides* and *B. patens* inhibited the germination rate and speed of germination and reduced the shoot and root length of the target plants, with fresh leaves showing greater phytotoxicity. These parameters were more affected by *H. psiadioides* than by *B. patens* in all seasons and both plants showed a decrease in phytotoxicity in summer. It can be concluded that both shrubs possess allelopathic potential, with *H. psiadioides* showing stronger effects. Interseasonal variation of phytotoxicity should be taken into account in studies of potentially allelopathic plants.

*Keywords:* Allelopathy; *Baccharis patens*; Germination; Growth; *Heterothalamus psiadioides*; Seasonality.

### **1. Introduction**

Allelopathy can be defined as any direct or indirect harmful effect by one plant on another through production and release of chemical compounds (allelochemicals), usually secondary metabolites (Andel, 2006; Rice, 1974). In many ecosystems, some plants form dominant stands and this pattern may be related to the release of allelochemicals, reducing or preventing the establishment of other species near them. The shrubs *Heterothalamus psiadioides* Less and *Baccharis patens* Baker (Asteraceae) grow in grasslands and shrublands in southern Brazil and in Uruguay (Deble et al., 2005), where they often establish in dense populations, forming shrublands (Setubal and Boldrini, 2010), thus indicating the possibility of allelopathic interactions with nearby species. However, it has not been investigated which factors are responsible for these patterns, and almost nothing is known about the allelopathic

potential of these species; there is only a study reporting the phytotoxic effects of *H. psiadioides* essential oil (Schmidt-Silva, 2012).

The most promising way to assess the phytotoxicity of *H. psiadioides* and *B. patens* should be to evaluate their volatiles, as the phytotoxic effects of other aromatic shrubs have been mainly related to volatiles release (Ibáñez and Zoppolo, 2011; Muller et al., 1964). Besides, recent studies revealed that the volatile essential oil of *H. psiadioides* is able to affect cell division in target species (Schmidt-Silva et al., 2011). To better assess allelopathic potential of plants, it is advantageous to use methods where emission of volatiles occurs in a way similar to processes actually taking place in the plant community. In this sense, volatiles from senescent leaves of plants may affect germination and development of other species when they are on the soil or incorporated into it (Huang et al., 2013); therefore, to evaluate volatiles released directly from dry leaves of plants seems to be an interesting approach. Nevertheless, the development of neighboring species may be also affected by volatiles from leaves that are still on the plant (Muller et al., 1964). The use of fresh leaves thus could be better to characterize potentially phytotoxic plants, as studies reported that the release of phytotoxic substances decreases along the decomposing process (Huang et al., 2013; Wilt et al., 1993).

The majority of studies consider only one point of time for testing the allelopathic potential of a plant. However, the production of secondary metabolites may vary during the year (Frizzo et al., 2008; Helmig et al., 2013) and this should be reflected by seasonal changes in plant phytotoxicity. This seasonal variation in defensive chemistry is mainly related to the environmental conditions that plants are growing in: biotic and abiotic factors such as the presence of herbivores (Karban, 2007) and pathogens (Heil and Bostock, 2002), temperature (Lur et al., 2009) and precipitation (Gray et al., 2003) vary during the year, and are able to change secondary metabolites synthesis. Assessing this variation is required to characterize the potential phytotoxicity of a plant species and to interpret its effects.

In this study, we aimed to evaluate the phytotoxic effects of *H. psiadioides* and *B. patens* and the seasonal variation of these effects, through a method that assesses the activity of volatiles released directly from fresh and dry leaves of the plants. We tested the following hypotheses: (1) Considering that both species form dense stands, both *H. psiadioides* and *B. patens* present phytotoxicity, affecting germination and growth of other plants; (2) as the release of phytotoxic volatiles must decrease with leaves senescence, the fresh leaves of the plants show stronger effects than the dry leaves; and (3) as allelochemicals production may vary during the year, the phytotoxic effects of *H. psiadioides* and *B. patens* change seasonally.

## 2. Material and methods

### 2.1. Plant collection

Leaves of *H. psiadioides* and *B. patens* were collected in natural grassland vegetation at Morro Santana (30° 03' S, 51° 07' W), a hill in the city of Porto Alegre, Rio Grande do Sul State, Brazil, where they occur with high frequency and abundance values (details on the grassland vegetation on Santana hill: Overbeck et al., 2006). Voucher specimens of *H. psiadioides* (register number 175007) and *B. patens* (register number 192266) were deposited in the herbarium ICN of the Federal University of Rio Grande do Sul.

### 2.2. Germination and growth assays

The effect of the volatiles from *H. psiadioides* and *B. patens* was evaluated on the germination and growth of the target species lettuce (*Lactuca sativa* L. cv. Grand Rapids) and onion (*Allium cepa* L. cv. Baia Periforme). Both target plants were used in the assays because of the relevance of determining the potential selectivity of the phytotoxic compounds on dicotyledons and monocotyledons (Lotina-Hennsen et al., 2006). Two separate experiments were conducted: the first experiment was designed to test for differences between effects of fresh leaves and dry leaves of both species and the second experiment to evaluate the difference in magnitude of the effects across different seasons.

For the first experiment, dry leaves of *H. psiadioides* and *B. patens* were obtained by collecting fresh leaves and leaving them exposed to natural temperature conditions in the laboratory until they were dried, for about ten days. For the treatment with fresh material, leaves were collected and immediately used in the assays. The control group consisted of absence of leaves.

The treatments were arranged in a completely random design with four repetitions. Each repetition corresponded to 50 diaspores of lettuce or onion, obtained from commercial dealers and sown in a layer of filter paper (11 cm<sup>2</sup>, 0.16 mm thick) moistened with 7 mL of distilled water, inside germination boxes of 11 cm x 11 cm x 4 cm. *Heterothalamus psiadioides* and *B. patens* leaves (5 g) were slightly chopped, wrapped in cloth (tulle) and attached with double-sided tape to the inner face of the box lid without direct contact with the seeds, but allowing the compounds to volatilize into the airspace within the box. The leaves were attached in the corners of the box lid to avoid interfering in light supply for the seeds. The boxes were sealed

with plastic film to minimize loss of the volatiles and were incubated in a germination room at an average temperature of 21 °C, with a daily light/dark cycle of 12 h/12 h. The illumination was provided by white fluorescent lamps (20 W), with an irradiance of 48  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ .

For the germination assay, the diaspores were sown and the treatments were added. The germinated diaspores were recorded every 12 and 24 h, for lettuce and onion, respectively. Speed of accumulated germination (AS) was calculated using the following formula:  $AS = [N1/1 + N2/2 + \dots + Nn / n]$ , where N1, N2, and Nn are the cumulative numbers of seeds that germinate by times 1, 2, ..., n (Anjum and Bajwa, 2005). The final germination percentage of the diaspores (germination rate) was calculated after 96 h (lettuce) and 240 h (onion). For growth experiments, the lettuce and onion diaspores were previously germinated (24 h for lettuce and 96 h for onion) and the treatments were added. The seedlings remained exposed to the volatiles for 72 h. After this period, the length of shoot and root (cm) of 20 seedlings per repetition was measured.

The experiment on differences of effects of the volatiles on germination and growth of the two target species across seasons was conducted at four different times of the year, in winter, spring, summer and autumn (from June 2012 to May 2013), with fresh leaves of *H. psiadioides* and *B. patens*. Only fresh leaves of the plants were used because they were hypothesized to show stronger effects than dry leaves and should allow for a better characterization in the variation in phytotoxic effects of the plants. Methods followed those described above for the first experiment.

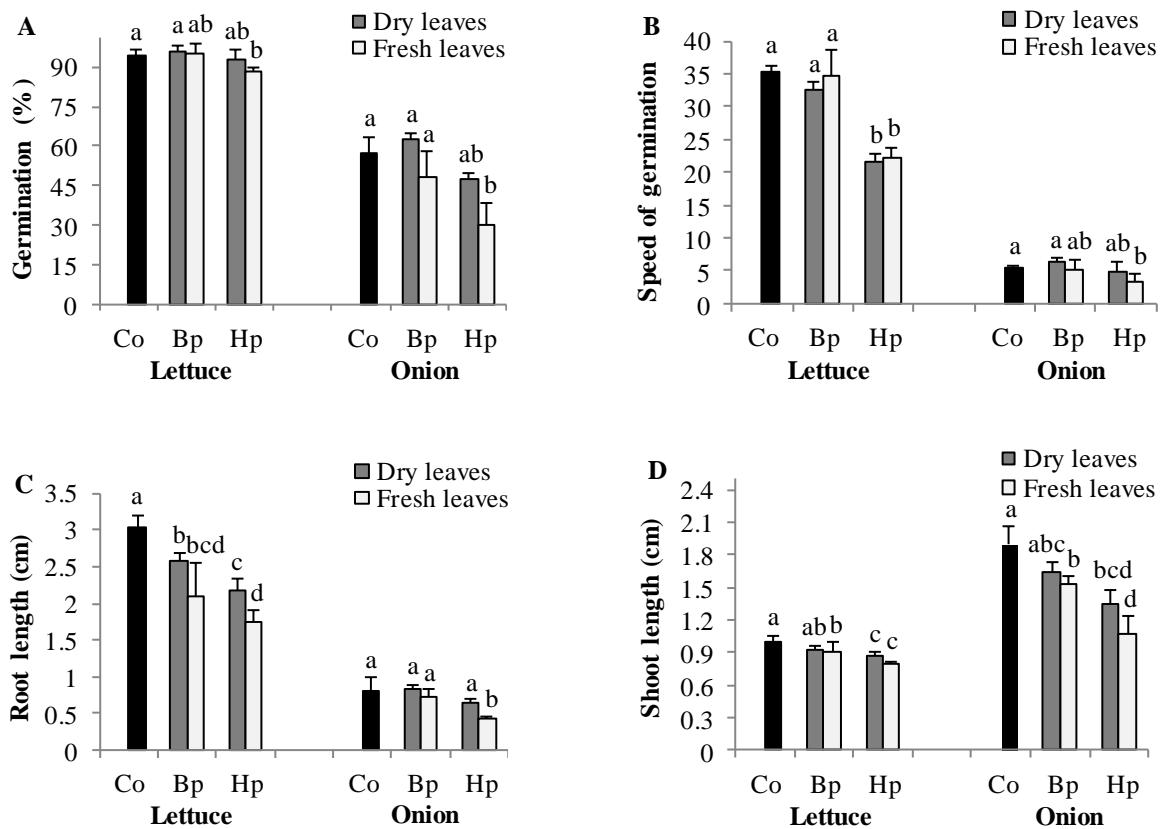
### 2.3. Statistical analysis

The parameters analyzed (germination rate, speed of germination, length of shoot and length of root) in the experiment with fresh and dry leaves of *H. psiadioides* and *B. patens* were submitted to one-way PERMANOVA univariate analysis, comparing all the treatments by randomization tests with 10,000 permutations and including pairwise comparison. In the second experiment, the seasonal variation in these parameters was evaluated, through two-way PERMANOVA that tested the interaction between treatments and seasons, also using randomization tests. Still, to test for differences between treatments specifically in each season, groups were compared through one-way PERMANOVA, including pairwise comparison. PERMANOVA enabled a conventional ANOVA approach to be conducted without transformation on the raw data, since it is not based on assumptions of normality and homogeneity of variances (Anderson, 2001). The analysis used Euclidean distances and

considered a significance level of  $p < 0.05$ . The tests were performed using the software MULTIV (Pillar, 2009).

### 3. Results

In the germination bioassays with fresh and dry leaves of the plants, the volatiles from fresh leaves of *H. psiadioides* caused a significant, though not always very strong, reduction on the speed of germination and germination rate of lettuce (37% and 7%, respectively, when compared to germination rate of the control treatment) and of onion (37% and 47%, respectively). The dry leaves of *H. psiadioides* only reduced the speed of germination of lettuce (39%). Fresh and dry leaves of *B. patens* did not cause any significant effect on lettuce and onion germination (Fig. 1-A and 1-B).



**Fig. 1.** Germination rate (A), speed of germination (B), root length (C) and shoot length (D) of lettuce and onion exposed to the volatiles from *B. patens* (Bp) and *H. psiadioides* (Hp) and control group (Co). Data presented as mean and standard deviation. Treatments sharing the same letter do not differ significantly according to one-way PERMANOVA for the same target species, at  $p < 0.05$  level.

The effects of the volatiles from *H. psiadioides* and *B. patens* on seedlings growth are shown in Fig. 1-C and 1-D. The dry leaves of both shrubs caused lower inhibition on growth of the target plants in relation to the fresh leaves, as shown in the germination assays. The fresh leaves of *H. psiadioides* caused the greatest reduction on shoot and root length of the target plants (21% and 52% for lettuce and 43% and 46% for onion, respectively). The dry leaves of *H. psiadioides* reduced shoot and root length of lettuce (13% and 28%) and shoot length of onion (28%). *B. patens* fresh leaves also caused negative effects on the shoot and root length of lettuce (11% and 32% of reduction) and shoot of onion (13% of reduction). The dry leaves of *B. patens* only reduced lettuce root (15%) and onion shoot length (14%).

In relation to the experiment on differences of effects of the plants volatiles across seasons, the interaction between treatment and season was significant in almost all the parameters evaluated, except for the speed of germination and the root length of onion, i.e. differences among treatments vary according to the season (Table 1). In winter, *H. psiadioides* caused a pronounced inhibition in all the parameters and in spring and autumn almost all the parameters were also substantially affected by the plant volatiles. In summer, however, only the parameters related to seedling growth were considerably inhibited by *H. psiadioides* volatiles. The seasonal pattern in the effects of *B. patens* on the target plants was similar to the pattern observed for *H. psiadioides*: the inhibition by *B. patens* volatiles was pronounced in some parameters in winter, spring and autumn. Nevertheless, almost only the parameters related to seedling growth were considerably affected by the plant. In summer, the inhibition by *B. patens* was not pronounced for any parameter. Besides, the inhibition in almost all the parameters was greater when the target plants were exposed to *H. psiadioides*; the reduction by *B. patens* was only slightly greater on the speed of germination of lettuce in spring and summer, but in these cases the inhibition was not substantial and does not consist in considerable difference between the treatments.

**Table 1.** Inhibition (%) of germination rate, speed of germination, root length and shoot length of lettuce and onion exposed to the volatiles from fresh leaves of *H. psiadioides* (Hp) and *B. patens* (Bp) in the four seasons of the year.

Season	Treatment	Germination assays - inhibition (%)				Growth assays - inhibition (%)			
		Germination rate		Speed of germination		Shoot length		Root length	
		Lettuce	Onion	Lettuce	Onion	Lettuce	Onion	Lettuce	Onion
Winter	Hp	7 <sup>a</sup>	47 <sup>a</sup>	37 <sup>a</sup>	37 <sup>a</sup>	21 <sup>a</sup>	23 <sup>a</sup>	52 <sup>a</sup>	20 <sup>a</sup>
	Bp	0	15	02	08	11	13 <sup>a</sup>	32 <sup>a</sup>	14
Spring	Hp	2	25 <sup>a</sup>	04	31 <sup>a</sup>	32 <sup>a</sup>	43 <sup>a</sup>	42 <sup>a</sup>	46 <sup>a</sup>
	Bp	0	08	10	15	24	19 <sup>a</sup>	19 <sup>a</sup>	10
Summer	Hp	0	10	01	10	03	21 <sup>a</sup>	30 <sup>a</sup>	21 <sup>a</sup>
	Bp	0	04	09	03	00	08	15	06
Autumn	Hp	6	12	25 <sup>a</sup>	07	11 <sup>a</sup>	19 <sup>a</sup>	33 <sup>a</sup>	26 <sup>a</sup>
	Bp	2	11	21 <sup>a</sup>	07	00	11 <sup>a</sup>	23	14
Treatment		0.009	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Season	P-value	0.04	<0.001	<0.001	<0.001	<0.001	<0.001	0.02	<0.001
Treatment*Season		0.01	<0.001	<0.001	0.5	<0.001	<0.001	<0.001	0.18

(<sup>a</sup>)Significant differences in relation to control (no treatment), according to PERMANOVA, with  $p < 0.05$ .

#### 4. Discussion

The results of the assays confirmed our hypothesis that both *H. psiadioides* and *B. patens* present phytotoxicity, affecting germination and growth of seedlings. A greater phytotoxic effect of *H. psiadioides* compared to *B. patens* was observed. The volatiles released from *H. psiadioides* negatively affected all the parameters evaluated in the germination and growth assays. *Baccharis patens* affected only the parameters related to growth (shoot and root length), which are usually more sensitive to phytotoxic effects than parameters related to germination (speed of germination and germination rate) (Chon and Nelson, 2010). Besides, the inhibition on the target plants was stronger when they were exposed to *H. psiadioides* leaves. The differences in the effects of *H. psiadioides* and *B. patens* are likely associated to the amount of phytotoxic volatiles released by them and to differences in their chemical composition. Most of the volatiles in these plants are in the essential oil of their leaves, which showed 1.92% and 0.2% yield for *H. psiadioides* and *B. patens*, respectively (unpublished data). Therefore, *H. psiadioides* is able to release a larger amount of volatiles than *B. patens*. The major compounds of *H. psiadioides* essential oil,

obtained from the same population studied in this article, are monoterpenes hydrocarbons (77.31%), mainly  $\beta$ -pinene (43.17%),  $\Delta^3$ -carene (13.73%) and limonene (6.32%) and although this oil affected germination and growth of other plants,  $\beta$ -pinene, the major compound, did not show phytotoxicity (Schmidt-Silva, 2012). Therefore, the phytotoxic effects of *H. psiadioides* must result from interactive effects of several compounds, as assumed for the effects of plants allelochemicals in field conditions (Inderjit et al., 2011). The essential oils of several *Baccharis* species have already been characterized (Frizzo et al., 2008; Simões-Pires et al., 2005), and although the species share most of the compounds, the major ones vary among them: while monoterpene hydrocarbons are the predominant fraction in some species, in others oxygenated sesquiterpenes or oxygenated monoterpenes are more significant. The chemical composition of *B. patens* has not been described up to now. Thus, further studies characterizing the volatile compounds of *B. patens* essential oil will shed light on the phytotoxic activity of this species.

The action of allelochemicals on germination and growth of plants is diverse and affects different physiological functions, as cell division, metabolic processes, photosynthesis and respiration. A mechanism related to modification in cell division was already reported for *H. psiadioides*: the essential oil of this plant caused a decrease in mitotic activity and an increase of chromosomal abnormalities in target plants (Schmidt-Silva et al., 2011). Besides, effects of allelochemicals on seed germination appear to be mediated mainly through a disruption of normal cellular metabolism. One of the effects can be in reserve mobilization, a process that usually takes place rapidly during early stages of seed germination and seems to be delayed or decreased by phytotoxicity (Gniazdowska and Bogatek, 2005). Seedlings growth is usually affected by disturbances of photosynthesis (Qian et al., 2009) and respiration (Hejl and Koster, 2004; Mushtaq et al., 2013), which are some of the most observed physiological effects of many allelochemicals.

Considering the differences on the phytotoxicity of fresh and dry leaves of *H. psiadioides* and *B. patens*, our hypothesis that the fresh leaves of the plants present stronger effects than the dry leaves was confirmed. This indicates that in natural ecosystems the phytotoxicity of the shrubs leaves must decrease until they are incorporated into the soil. Besides, due to the low phytotoxicity of the dry leaves of *H. psiadioides* and *B. patens*, they would need to drop large amounts of leaves on the soil to affect germination of other species; as these plants are not deciduous, this is unlikely to occur. Thus, it seems difficult that the volatiles from these shrubs are able to affect the germination of other species in plant communities. On the other hand, the strong effects of the fresh leaves of the shrubs, mainly *H.*



*psiadioides*, on growth of the target plants suggest that the volatiles may affect the development of other neighboring species when the leaves are still on the plants, as assumed for other aromatic shrubs which were reported to affect nearby species in field conditions (Ibáñez and Zoppolo, 2011; Muller et al., 1964).

The variation in magnitude in the phytotoxic effects of *B. patens* and *H. psiadioides* according to the period that they were collected confirmed our hypothesis that the plants phytotoxicity changes seasonally. The plants showed generally lower phytotoxicity in summer than in the other seasons – a pattern that must be related to seasonal fluctuations of environmental parameters and trade-offs between primary and secondary metabolism. In summer, plants are exposed to higher temperatures and solar radiation, which were reported to have an inverse relation with secondary metabolite accumulation in plant tissues (Lur et al., 2009; Yu et al., 2003). Besides, plants growth increases in summer; studies revealed trade-offs between primary biological functions of plants, as growth, and resources allocation for chemical defense (Donaldson et al., 2006; Züst et al., 2011). However, other factors, such as interactions with pathogens (Heil and Bostock, 2002) and herbivores (Karban, 2007) or water (Gray et al., 2003) and nutrients (Tharayil et al., 2009) availability are also able to change secondary metabolite production and consequently the plants phytotoxicity. The variation in the phytotoxic effects of *H. psiadioides* and *B. patens* evidences the importance of conducting allelopathy studies along different seasons; if this variation is not considered, the results may not reflect the potential phytotoxicity of a plant species correctly or, on the other hand, the phytotoxic effects may be overestimated.

The method used in this study was effective to determine volatiles phytotoxicity even though only a small amount of plants leaves was used. The similar pattern of phytotoxicity observed for both shrubs at all times when the assays were conducted, i.e. *H. psiadioides* showing stronger effects than *B. patens*, indicates the efficacy of the technique. Besides, an advantage of the method is that it allows carrying out phytotoxicity studies without interferences, as shown in other methods frequently used: extracts, for example, can inhibit test species through altering pH and osmotic potential (Wardle et al., 1998), masking the true phytotoxic effects of a plant. Assays with essential oils, one of the most used ways to characterize volatiles phytotoxicity (e.g. Pawlowski et al., 2012; Zahed et al., 2010), also show a constraint: the concentration of these substances is much greater than what would be naturally volatilized by plants. However, even though we can assume that our method should be more similar to natural emission of volatiles, it must be considered that the interactions between plants and allelochemicals in the plant community occur in a more dynamic

ecological situation and that phytotoxic effects observed in laboratory conditions may not be relevant in the nature. Many plants release compounds into the surrounding environment with minor consequences in their native habitat due to a long coevolutionary history with other plants (Thorpe et al., 2009). Besides, there are several biotic and abiotic factors that are able to change allelopathic activity (Inderjit et al., 2011).

## 5. Conclusions

The shrubs *H. psiadioides* and *B. patens* show phytotoxicity, which is greater for *H. psiadioides*. The phytotoxic effects of both species differ in magnitude across seasons, evidencing the relevance of considering this variation in allelopathy studies. The strong effects of the fresh leaves of these shrubs indicate that the volatiles may affect the development of other neighboring species when the leaves are still on the plants; thus, allelopathy is a possible mechanism involved in the dominance pattern shown by *H. psiadioides* and *B. patens*. However, additional studies under field conditions are needed to evaluate if these shrubs, through allelopathic interactions, are effectively able to affect other species in plant communities.

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# Capítulo II

**Does a highly phytotoxic species really affect a plant community  
through allelopathy?<sup>2</sup>**

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<sup>2</sup>Este trabalho será submetido à revista Plant Ecology

### **3 CAPÍTULO II: Does a highly phytotoxic species really affect a plant community through allelopathy?**

#### **Abstract**

Phytotoxicity has often been proposed as evidence of allelopathy. However, studies on allelopathic effects are mostly restricted to the laboratory, without investigating their relevance in plant communities. In South Brazilian grasslands, *Heterothalamus psiadioides*, a shrub establishing in dense stands, has shown strong phytotoxicity in laboratory assays. We conducted a field study to evaluate if this species also negatively affects a plant community through allelopathy. At a shrubland site, we established plots with *H. psiadioides*, control plots with *Baccharis patens*, a shrub with low phytotoxicity, and plots without shrubs. We assessed plants cover, biomass, number of seedlings, diversity, richness, and variables of vegetation structure. Our study did not show evidences of plant inhibition near the phytotoxic species. We observed higher plant richness and diversity in plots with *H. psiadioides* than in control groups. The highest diversity near *H. psiadioides* was associated with greater forb cover, due to lower cover of dominant grasses. Variables of vegetation structure and differences in light incidence accounted for vegetation patterns more than allelopathic activity. Therefore, our study showed that the allelopathic potential of the species evaluated was overestimated under laboratory conditions. We emphasize the relevance of conducting allelopathy studies in the field and of having a similar non-phytotoxic species as control in this kind of study, as results based only on laboratory assays and visual evidence of patterns in the field may be misleading and overestimate the role of allelopathy in plant communities.

**Keywords** Allelopathic potential, Diversity, Plant volatiles, Shrub, Campos Sulinos.

#### **Introduction**

Allelopathy, in which one plant produces phytotoxic chemicals that suppress the growth and establishment of other neighboring plants, has often been proposed to influence plant populations and communities (e.g. Bais et al. 2003; El-Keblawy and Al-Rawai 2007; Li et al. 2011). This interference mechanism can be defined as a harmful effect of a donor plant on a target plant through the production of secondary metabolites (allelochemicals) that escape into the environment (Lambers et al. 1998). Allelopathic activity has been shown to reduce

vegetation diversity and abundance and change species composition in plant communities, e.g. for *Centaurea maculosa* (Ridenour and Callaway 2001) and *Prosopis juliflora* (El-Keblawy and Al-Rawai 2007).

The occurrence of allelopathy has been suggested, based on laboratory or field studies, for many aromatic shrub species, such as *Artemisia frigida* (Li et al. 2011), *Salvia* spp. (Muller et al. 1964; Nishida et al. 2005), and *Chrysanthemoides monilifera* (Ens et al. 2009a; Ens et al. 2009b), where the inhibitory effects observed have been mainly related to emission of volatile substances. Most phytotoxic volatiles are terpenes, which can inhibit plants using the air as the carrier, forming “terpene clouds” that directly dissolve in lipophilic cell structures of target plants (Muller 1965). Despite their volatility, some terpenes (mainly monoterpenes) can also show high water solubility (Weidenhamer et al. 1993) and it has been suggested that rainfall or dew may transport these compounds to the soil surface (Muller et al. 1964; Barney et al. 2005). Still, due to the great affinity of monoterpenes for organic matter, they can be adsorbed in the soil that comes into contact with target plants. Concentrations of these substances can be higher near the donor plants and can stay in soil for considerable periods, mainly under conditions of high temperatures and dry soils (Muller 1966). Moreover, phytotoxicity of terpenes can be stronger on some species than others (Barney et al. 2005), and effects of aromatic plants may vary among target species.

The allelopathic potential of *Heterothalamus psiadioides* Less, an Asteraceae shrub, has been recently investigated. This shrub grows in grasslands and shrublands in southern Brazil and Uruguay (Deble et al. 2005), where it often establishes dense populations (Setubal and Boldrini 2010). The species produces large amounts of essential oil, which is easily noticeable because of the strong scent of the volatile substances of the oil near *H. psiadioides*. Laboratory studies have provided strong evidence of the phytotoxicity of the shrub: the essential oil of this species, which possesses monoterpenes as the major compounds, affected germination and growth of target plants even at very low concentrations (Schmidt-Silva 2012). The volatiles released directly from *H. psiadioides* leaves also affected germination and initial growth of other plants (Silva et al. 2014). *Heterothalamus psiadioides* essential oil also caused a decrease in mitotic activity and an increase of chromosomal abnormalities in target plants, a possible mechanism related to the plant phytotoxicity (Schmidt-Silva et al. 2011). Furthermore, as *H. psiadioides* is an evergreen shrub with large quantities of foliage, the species should be able to release phytotoxic volatiles constantly, which increases the possibility of occurrence of allelopathy (Lambers and Colmer 2005).



Even though phytotoxicity has often been proposed as evidence of allelopathy, most studies are restricted to the laboratory, and little effort is made to investigate the relevance of results from laboratory experiments in field conditions (Inderjit and Weston 2000). Apparent negative effects on vegetation near a plant species that establishes in dense stands is frequently followed by phytotoxicity studies, in which inhibitory effects are almost always observed. In this sense, Harper (1994) states: “almost all species can, by appropriate digestion, extraction, and concentration be persuaded to yield a product that is toxic to one species or another.” However, after phytotoxicity evaluations, usually no study is carried out in the field to assess if negative effects on vegetation are really observed near the phytotoxic species, except for few studies, as the ones performed by Ridenour and Callaway (2001) and El-Keblawy and Al-Rawai (2007). Thus, in many cases allelopathy has been claimed as an important determinant of patterns in plant communities, even though this had not been supported by empirical field evidence.

The difficulty of establishing appropriate controls has been one of most critical problems in laboratory and field studies on allelopathy (Inderjit and Nilsen 2003). In the case of field studies, this may result in doubtful inferences that effects of a species on a plant community are due to allelopathic activity, as in some cases other factors related to the supposedly allelopathic plant were responsible for inhibition patterns, as for example, feeding activity by animals (Bartholomew 1970). Hence, we propose that species without phytotoxicity, but otherwise as similar as possible, e.g. regarding plant architecture, that co-occur with the species with allelopathic potential can be used as control for a more accurate evaluation of allelopathic effects than when using only absence of the plant under study. In the case of *H. psiadioides*, the shrub *Baccharis patens* Baker may be considered as appropriate control. The species is similar in height and habit to *H. psiadioides*, with also phylogenetic proximity, belonging to the same subtribe within the family (Barroso and Bueno 2002), but with low phytotoxicity. In laboratory assays, the volatiles from *B. patens* affected the most sensitive parameters in target plants only very slightly, and this low inhibition was not even observed at all times the assays were conducted, while *H. psiadioides* effects were stronger and constant (Silva et al. 2014). The study system proposed thus assumes that as both species are analogous in important aspects, if inhibitory effects are only observed close to the phytotoxic species and not to the control species, allelopathic effects would be evidenced.

In this study, we aimed to evaluate if a species that had been shown to be highly phytotoxic in laboratory assays does in fact negatively affect a plant community due to allelopathy, with *H. psiadioides* as the model species. We tested the following hypothesis: 1)

as *H. psiadioides* has been shown to cause severe inhibition on germination of target plants in laboratory experiments, plant recruitment and establishment near *H. psiadioides* is reduced and thus richness, diversity, and seedling number are lower near the shrub; 2) due to the negative effects of *H. psiadioides* on growth of target plants, vegetation cover and biomass are lower near *H. psiadioides*; 3) as allelochemicals can affect some species more than others, effects of *H. psiadioides* vary in magnitude among plant groups. To test these hypotheses, we conducted a field study in a shrubland area in South Brazil. We evaluated the plant community structure in plots with *H. psiadioides* and also in controls: plots with *B. patens* (a practically non-phytotoxic shrub similar to *H. psiadioides* regarding plant habit and height), and plots without any shrub.

## Methods

### Study site

We conducted the study on Morro Santana (30° 03' S, 51° 07' W, max. alt. 311 m a.s.l.), which is inserted in a chain of granitic hills in southern Brazil, in the 'Campos Sulinos' region (Overbeck et al. 2007). The hill is covered by forests (Atlantic Forest) predominantly on the southern slopes, grasslands on the northern slopes and by a mosaic of forest and grassland at the top (Müller et al. 2012). Often, grasslands in Morro Santana occur in a heterogeneous mosaic with small forest patches and shrublands. Grassland vegetation on the hill can be considered very species-rich in comparison with other vegetation types in the region or with other grassland ecosystems worldwide: a total of 430 species were identified so far, from an estimated pool of 450-500 species, in an area of 220 ha (Overbeck et al. 2006).

Climate in the region is subtropical humid without dry season (Köppen's Cfa), with a mean annual temperature of 19.5 °C, and mean precipitation of 1309 mm yr<sup>-1</sup> (Maluf 2000). The soils are characterized by a horizon A rich in clay and Acrisol is the predominant soil type (Streck et al. 2002). Hot summers with longer periods of rather low precipitation may create conditions that positively influence emission quantities of volatiles and the association of these substances with soil particulates (Muller 1966).

The grassland areas at Morro Santana are ungrazed and are subject to frequent burning (mostly anthropogenic), affecting differently sized patches of grassland every year. This study was conducted in a shrubland area at the top of the hill, at a site where the last burn occurred

more than five years ago. At this site, *H. psiadioides* and *B. patens* are the predominant shrub species, occurring with high abundance values and shaping mixed and dense stands.

#### Data collection

To assess the effects of *H. psiadioides* on the plant community, we selected three groups of plots in the shrubland area: 1) sampling units of 0.5 m by 0.5 m with 50-70% cover of *H. psiadioides* (Hp). 2) Sampling units of the same size with *B. patens* cover in the same range of plots of the first group (50-70%) (Bp). The sampling units with *B. patens* were considered a first control group, as this shrub is similar to *H. psiadioides* in height (70-130 cm for both species in the study site) and leaf density, but with low phytotoxicity. 3) Sampling units with no shrubs (with 5% of woody species at most) that constituted another control (NS). In total, ten sampling units per group (Hp, Bp, and NS) were randomly distributed at the study site. The fire history was the same for all plots.

For each sampling unit, we identified all plant species, estimated their cover using the Londo (1976) decimal scale, and registered the number of seedlings. We also recorded the following variables of vegetation structure in the units: vegetation height (maximum), herbaceous cover (< 30 cm height), canopy cover (> 30 cm height), cover of standing dead biomass (principally dry leaves of tussock grasses still attached to the plant), and bare soil. These variables were also recorded using the Londo scale, except for vegetation height. After vegetation sampling, all live biomass was clipped at the ground level in five plots per group. We separated biomass in graminoids (Poaceae and Cyperaceae species), forbs (herbaceous non-graminoid), and shrubs (including all woody plants) and dried at 60 °C until constant weight.

In addition, the intensity of light under *H. psiadioides* and *B. patens* canopies was evaluated to test if the light available for plants under these shrubs was similar. We measured light in plots with *H. psiadioides* and with *B. patens*, considering cover values between 50% and 70% for both shrubs (50 plots per group), using a Lux meter. In each plot, five measurements were done at 30 cm from the ground level, as under this height shade could result from herbaceous vegetation, and thus only shade caused by the shrubs was taken into account. The mean value of these five measurements was calculated for analysis.

## Statistical analysis

We submitted the matrix of species cover per plot to ordination and cluster analysis to detect vegetation patterns. Results were submitted to bootstrap resampling (10,000 iterations) to verify stability of the ordination axes (Pillar 1999) and cluster groups sharpness. As ordination method, we used principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity measure. For cluster analysis, sum of squares was used as the clustering criterion. Differences in the species composition between groups were evaluated by multivariate analysis of variance (MANOVA) with randomization. This analysis, as well as all the following analyses that used randomization tests presented hereafter, were based on Bray-Curtis dissimilarity measure between sampling units.

To evaluate if *H. psiadioides* affects the establishment of other plants, we tested if species richness, diversity, and seedling number differed between groups. First, we calculated diversity and richness indexes based on the Rényi generalized entropy (Anand and Orłóci 1996). Rényi's entropy of  $\alpha$  orders provides a profile of the most widely used diversity indices, as Shannon ( $\alpha = 1$ ) and Simpson ( $\alpha = 2$ ). For  $\alpha = 0$ , the entropy value does not take into account the variation in the proportion of different species in a given community, and behaves as a richness index. According to Anand and Orłóci (1996) the equitability effect is only stabilized when an  $\alpha$  order much higher than Shannon's is used, and a good  $\alpha$  value suggested was 12. Thus, we used the  $\alpha$  orders 0 (species richness index), 1, 2 and 12 (diversity indexes). Then, we compared the values obtained for each  $\alpha$  order between groups by ANOVA with randomization. We evaluated differences in the number of seedlings between groups by the same method.

To test the hypothesis that vegetation cover and biomass are lower below/near *H. psiadioides*, the relative cover of all species was first summed into graminoid, forb and shrub components, the same criteria established for plants biomass. Then, differences between groups regarding cover data and biomass values were evaluated through ANOVA with randomization.

We also grouped plants according to their life form, to evaluate the possibility of more specific effects of *H. psiadioides*. The species cover was summed into the following categories, modified from the classification developed by Ferreira et al. (submitted) specifically for the Campos Sulinos region: therophytes, evergreen tussocks (principally tussock grasses), prostrate evergreens, evergreen forbs, evergreen subshrubs, evergreen shrubs, succulents, prostrate rosette evergreens, erect rosette evergreens, and geophytes.

Differences between groups were evaluated using MANOVA with randomization. Moreover, the total cover for each category was separately compared between groups through ANOVA with randomization, except for succulents and therophytes, which occurred only in 3% of the sampling units and hence were not included in analyses.

To test if other variables could account for the patterns observed in the plant community we evaluated differences in variables of vegetation structure and also the influence of these variables on the vegetation community. Differences in values for each variable of vegetation structure (vegetation height, canopy cover, herbaceous cover, standing dead biomass, and bare soil) were compared between groups by ANOVA with randomization. Moreover, we evaluated the influence of variables of vegetation structure (explanatory variables) on richness ( $\alpha = 0$ ), diversity ( $\alpha = 1, 2, 12$ ), number of seedlings, and cover of life forms groups that significantly differed between groups (response variables), using multiple regressions. For models simplification, we used an approach based on Akaike's Information Criterion (AIC) (Akaike 1974). AIC is a minimum information theoretic criterion that trades off bias (decreasing with the number of parameters in a model) with variance (increasing with the number of parameters in a model) to identify the most parsimonious model, which is the one with a minimal value of AIC (Johnson and Omland 2004). The model with the smallest AIC was considered to have the best-fit, and thus the set of explanatory variables in this model was used in the regression, for each response variable.

To evaluate if vegetation under *H. psiadioides* and *B. patens* were exposed to the same shading levels, we tested for differences in light intensity under the canopy of both shrubs by ANOVA with randomization. Before analysis, we converted data from lux to photons, according to Thimijan and Heins (1983).

All the randomization tests were conducted with 10,000 iterations. When analyses of variance indicated significant differences between groups, we performed contrast analyses for a pairwise comparison (Pillar and Orlóci 1996). We considered a significance level of  $p < 0.05$ .

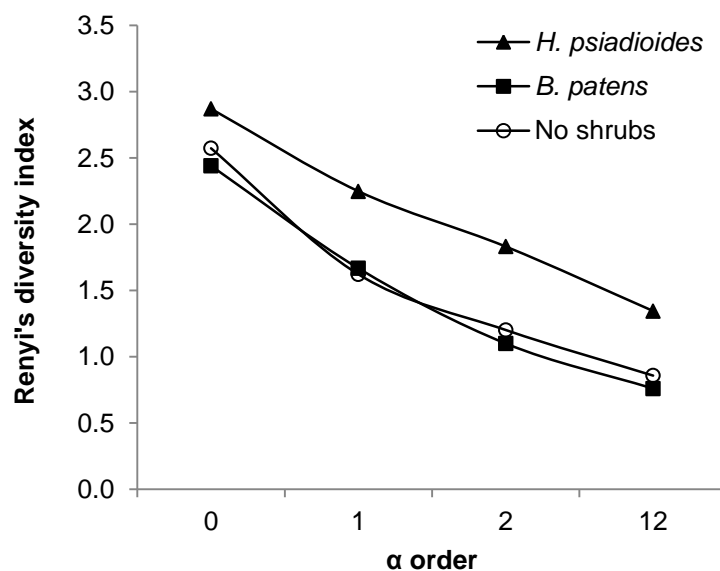
## Results

In the 30 sampling units of our study, a total of 115 (morpho-)species were recorded (not including *H. psiadioides* and *B. patens*). Of these, 2 could only be identified to the family level, and 9 to the genera level, resulting in 104 identified species, 84 genera and 27 families (see Appendix 1 for list). The highest number of genera and species were recorded for

Asteraceae (17 genera; 30 species), followed by Poaceae (17 genera; 25 species). The species with highest cover in each group of plots were predominantly from the Poaceae family. In Hp, the species with higher cover was *Aristida laevis* (11%), followed by *Piptochaetium montevidense* (9%), *Aristida flaccida* (8%), and *Schizachyrium tenerum* (8%). In Bp, *A. flaccida* (14%) had higher cover, followed by *Leptocoryphium lanatum* (11%), *A. laevis* (11%), and *S. tenerum* (7%). In NS, *S. tenerum* (23%) was the species with higher cover, followed by *Aristida jubata* (22%), *A. laevis* (10%) and *L. lanatum* (9%).

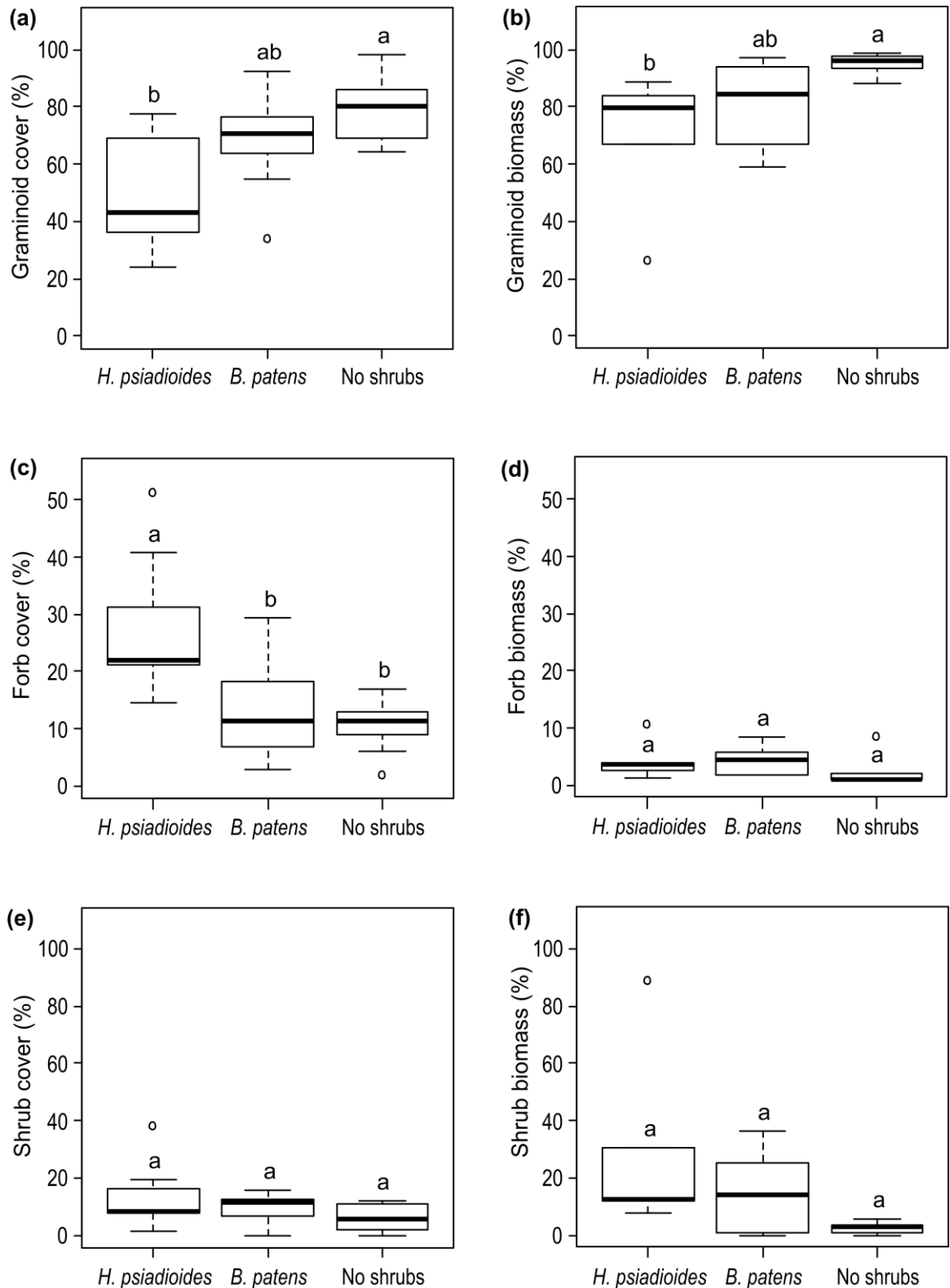
The exploratory ordination and cluster analyses did not indicate a clear separation of groups. Explanatory power of PCoA axes using species data of all sampling units was low (15.9%, 12.5% and 11% for axes 1, 2 and 3, respectively; diagram shown in Appendix 2). Cluster analysis did not denote the existence of sharp groups among the sampling units (diagram not presented). In relation to species composition, the groups did not differ significantly ( $p = 0.07$ ).

Richness and diversity, based on Rényi's entropy, were greater in Hp compared with Bp for all orders of  $\alpha$  ( $p < 0.01$ ), and with NS for the entropy order 2 ( $p = 0.05$ ) (Figure 1). Although the number of seedlings was not significantly different between groups ( $p = 0.7$ ), NS showed a mean seedlings value of  $1.6 \pm 1.2$  (followed by standard deviation), almost 50% lower than Hp ( $3.0 \pm 2.1$ ) and Bp ( $2.8 \pm 2.1$ ).



**Fig. 1** Diversity profiles of vegetation based on Rényi's entropy, in plots with *H. psiadioides* as the dominant shrub (50-70% cover), with *B. patens* as the dominant shrub, and in plots without shrubs. The  $\alpha$  order 0 corresponds to species richness,  $\alpha = 1$  to Shannon's index,  $\alpha = 2$  to Simpson's index, and  $\alpha = 12$  is the order in which equitability effect should stabilize.

We observed differences in relative vegetation cover and biomass for graminoid and forb components. Graminoid cover and biomass were lower in Hp than in NS ( $p = 0.005$  and  $p = 0.02$ , respectively) (Figure 2-a and 2-b). Forb cover was higher in Hp than in NS ( $p < 0.001$ ) and Bp ( $p = 0.008$ ), although forb biomass did not differ between groups ( $p = 0.2$ ) (Figure 2-c and 2-d). Shrub cover and biomass did not differ significantly between groups (Figure 2-e and 2-f). Forbs represented most of the species recorded (52.1%), followed by graminoids (28.7%) and shrubs (17.4%).



**Fig. 2** Mean relative cover and biomass of plants in plots with *H. psiadioides* as the dominant shrub (50-70% cover), with *B. patens* as the dominant shrub, and in plots without shrubs. Cover and biomass values are presented for graminoids (a and b, respectively), forbs (c and d) and shrubs (e and f). Groups sharing the same letter do not differ significantly according to ANOVA with randomization, at  $p < 0.05$  level. Please note different scale of y-axis.



When species were grouped according to their life form, Hp differed from Bp ( $p = 0.01$ ) and also from NS ( $p = 0.002$ ). When analysing life forms separately, differences were significant only for tussocks and evergreen forbs (Table 1). Hp showed lower tussocks cover compared with Bp (27% lower) and with NS (34% lower). In relation to evergreen forbs, however, the opposite pattern was observed: their cover in Hp was 56% higher than in Bp and 68% higher than in NS.

**Table 1** Mean relative cover of plants grouped according to their life form in plots with *H. psiadioides* as the dominant shrub (50-70% cover), with *B. patens* as the dominant shrub and in plots without shrubs. Groups sharing the same letter do not differ significantly according to ANOVA with randomization, at  $p < 0.05$  level. Succulents and therophytes were not analysed, as present only in 3% of the sampling units.

Life form	Mean relative cover (%)		
	<i>H. psiadioides</i>	<i>B. patens</i>	No shrubs
Evergreen tussocks	53.73 a	69.45 b	80.16 b
Prostrate evergreens	10.92 a	7.53 a	5.81 a
Evergreen forbs	14.57 a	6.31 b	4.40 b
Evergreen subshrubs	5.71 a	7.03 a	2.91 a
Evergreen shrubs	6.89 a	5.06 a	4.31 a
Prostrate rosette evergreens	3.44 a	0.26 a	0.67 a
Erect rosette evergreens	3.06 a	3.18 a	0.96 a
Geophytes	0.27 a	0.20 a	0.34 a

In relation to the variables of vegetation structure, some significant differences were observed, and some of these variables showed influence on descriptive variables of vegetation community. Herbaceous cover and standing dead biomass were lower in Hp and in Bp, while bare soil had higher values in these groups than in NS, with greater differences between Hp and NS (Table 2). Regressions showed that standing dead biomass had a significant negative effect on richness ( $\alpha = 0$ ) and diversity ( $\alpha = 1, 2, 12$ ) (Table 3). For  $\alpha = 2$  and  $\alpha = 12$ , canopy cover and herbaceous cover were also relevant in the model, with a positive influence, although not significant, of canopy cover and a negative influence, but only significant for  $\alpha = 12$ , of herbaceous cover. Number of seedlings was negatively affected by herbaceous cover. Moreover, in relation to life forms groups that differed between groups, tussocks cover was negatively influenced by canopy cover and positively by herbaceous cover, while cover of evergreen forbs was positively influenced by bare soil.

**Table 2** Variables of vegetation structure in plots with *H. psiadioides* as the dominant shrub (50-70% cover), with *B. patens* as the dominant shrub, and in plots without shrubs. Groups sharing the same letter do not differ significantly according to ANOVA with randomization, at  $p < 0.05$  level. Data are expressed as mean  $\pm$  standard deviation.

Variable	<i>H. psiadioides</i>	<i>B. patens</i>	No shrubs
Vegetation height (cm)	110.8 $\pm$ 19.9 a	102.2 $\pm$ 19.8 a	30.9 $\pm$ 6.6 b
Canopy cover (%)	65.3 $\pm$ 15.8 a	67.1 $\pm$ 17.1 a	0.0 $\pm$ 0.0 b
Herbaceous cover (%)	58.8 $\pm$ 17.0 a	66.5 $\pm$ 18.6 a	94.1 $\pm$ 6.0 b
Bare soil (%)	40.0 $\pm$ 11.9 a	32.0 $\pm$ 13.7 a	3.3 $\pm$ 2.6 b
Standing dead biomass (%)	33.3 $\pm$ 9.2 a	43.0 $\pm$ 16.2 a	50.0 $\pm$ 24.2 a

**Table 3** Values obtained for regressions based in AIC models, which tested the influence of variables of vegetation structure (explanatory variables) on descriptive variables of vegetation community (response variables). Explanatory variables consisted in vegetation height, canopy cover, herbaceous cover, bare soil, and standing dead biomass and response variables were richness ( $\alpha = 0$ ), diversity ( $\alpha = 1, 2, 12$ ), number of seedlings, and cover of life form groups that significantly differed between groups (tussocks and evergreen forbs). The model with the smallest AIC was considered the most parsimonious, and the set of explanatory variables in this model was used in the regression, for each response variable.

Response variable	Regression model	AIC	R <sup>2</sup>	$p$ – overall regression	$p$ - each explanatory variable	Regression coefficient (slope)
Reny's entropy - $\alpha = 0$	Standing dead biomass	47.3	0.34	< 0.001	< 0.001	-0.01
Reny's entropy - $\alpha = 1$	Standing dead biomass	47.3	0.53	< 0.001	< 0.001	-0.02
Reny's entropy - $\alpha = 2$	Standing dead biomass + herbaceous cover + canopy cover	43.9	0.49	< 0.001	0.003 0.06 0.18	-0.02 -0.01 0.004
Reny's entropy - $\alpha = 12$	Standing dead biomass + herbaceous cover + canopy cover	50.1	0.41	< 0.001	0.02 0.03 0.09	-0.01 -0.01 0.005
Number of seedlings	Herbaceous cover	51.8	0.15	0.02	0.02	-0.04
Tussocks cover	Canopy cover + herbaceous cover	27.2	0.61	< 0.001	0.006 0.007	-0.01 0.01
Evergreen forbs cover	Bare soil	245.2	0.13	0.03	0.03	0.0003

The intensity of light under the canopy of the two shrub species differed significantly ( $p < 0.001$ ). Mean light intensity under *H. psiadioides* was  $1,194.7 \pm 169.2 \mu\text{mol m}^{-2} \text{sec}^{-1}$ , about 20% lower than under *B. patens*, where the mean value was  $1,489.9 \pm 190 \mu\text{mol m}^{-2} \text{sec}^{-1}$ . Light intensity outside the shrub canopy was  $1,881 \pm 53.7 \mu\text{mol m}^{-2} \text{sec}^{-1}$ .

## Discussion

In this field study, we did not find any evidences of plant inhibition below or near the highly phytotoxic species that served as our model species. No clear pattern in the plant community was evidenced, as ordination and cluster analyses did not denote the existence of groups of plots and as species composition did not differ between Hp, Bp and NS. If *H. psiadioides* did in fact show inhibitory effects, we would expect that at least a considerable number of species would not be able to occur close to the donor species. This should then reflect on community composition and structure, which was not observed. Our hypothesis that near *H. psiadioides* there would be lower plants richness and diversity, as well as fewer seedlings than in plots without the species was not confirmed. We observed, on the contrary, higher species richness and diversity in Hp than in the control groups. These results are our first evidences that allelopathy is no relevant mechanism in this study system.

Our hypothesis that close to *H. psiadioides* lower vegetation cover and biomass would be found was partially confirmed: these effects were only observed for graminoids. Forbs, on the other hand, reached higher cover values in Hp than in Bp and NS. In grasslands around the world, grasses account for most of the plant cover and biomass, while forbs constitute most of richness and show higher dynamics in time (e.g. Uys et al. 2004; Overbeck and Pfadenhauer 2007; Lauenroth and Adler 2008), as observed in this study. When dominance of grasses (or graminoids, if also considering Cyperaceae and Juncaceae) is lower, more forbs can establish and species richness increases (e.g. Overbeck et al. 2005). The low graminoid cover observed in Hp must have allowed more species to establish, mainly forbs, resulting in the highest richness and diversity in plots with the shrub.

Our third hypothesis was that effects of *H. psiadioides* would vary in magnitude among plant groups, and we did in fact observe negative effects near the shrub only for graminoids, as mentioned above, and more specifically for tussocks, as evidenced when using the more refined classification of life forms. These results might suggest that if the species inhibited other plants through allelopathy, the effects would more likely occur in tussocks, in our case, Poaceae and Cyperaceae (monocotyledons), than in species from other plant groups that consist mainly of dicotyledons. Nevertheless, laboratory studies did not provide evidence that monocotyledons are more sensitive to *H. psiadioides*. Studies have shown that *Allium cepa* L. and *Lactuca sativa* L., a mono and a dicotyledon, were inhibited by volatiles from leaves of *H. psiadioides* (Silva et al. 2014) as well as by its essential oil (Schmidt-Silva 2012) in the same magnitude. Still, a study from our research group showed that *H. psiadioides* essential

oil affected germination and growth of *Arabidopsis thaliana* (L.) Heynh., another dicotyledon (Lazarotto 2014), with strong phytotoxic effects on this plant even in quantities of oil much lower than the ones that affected *L. sativa* and *A. cepa*. Moreover, Barney et al. (2005) assessed the phytotoxicity of some terpenes, which included  $\alpha$ -pineno and  $\beta$ -pineno that are some of the major compounds of *H. psiadioides* essential oil (Schmidt-Silva 2012), and also did not observe higher phytotoxic effects on monocotyledons, but indeed the contrary: the volatile terpenes caused greater inhibition on dicotyledons. In our study, we did not observe higher inhibition on this plant group, as the forbs, which represent most of the dicotyledons recorded, reached higher cover values in Hp. Thus, the vegetation patterns observed in this field study do not reflect the results of laboratory experiments that analyse the effect of volatiles of *H. psiadioides* on target species. This is another evidence that allelopathy is not relevant in this community.

The analyses involving variables of vegetation structure and differences in light availability shed some light on the patterns evidenced. We observed a negative influence of standing dead biomass on richness and diversity and also a negative effect, even though weaker, of herbaceous cover on some diversity indexes. Standing dead biomass and herbaceous cover, both mainly influenced by cover of tussock plants, had lower values in Hp, where higher diversity and richness were observed. We also observed that tussocks were negatively influenced by canopy cover, indicating an effect caused by differences in light incidence. A number of studies have shown that graminoid vegetation can be considerably reduced under shrubs due to low sunlight incidence (Köchy and Wilson 2000; Lett and Knapp 2003; Heisler et al. 2004). In fact, we observed that although *B. patens* caused some shading on vegetation, under *H. psiadioides*, where there was less tussocks cover, light incidence was significantly lower than in Bp. In addition, the negative effects of shading are mainly observed on C<sub>4</sub> grasses (Lett and Knapp 2003; Píriz 2011), which tend to have high light compensation points. Indeed, we observed that C<sub>3</sub> tussocks had higher cover in Hp, while C<sub>4</sub> species, which represented most of the tussocks recorded (78%), had lower cover in Hp than in controls (see Appendix 3 for a list of C<sub>3</sub> and C<sub>4</sub> tussocks). Thus, with higher shading under or near *H. psiadioides*, C<sub>4</sub> tussocks did not manage to reach high cover as in NS or Bp, implicating also in less herbaceous cover and less standing dead biomass, which consequently resulted in more bare soil available for recruitment of evergreen forbs and higher diversity in Hp. Hence, even though other factors not evaluated in our study could also be important to explain the patterns in the community, it is important to point out that just one variable (light) already elucidated vegetation patterns more conclusively than allelopathy.

The lack of evidences of allelopathy in the present field study shows that the allelopathic potential of *H. psiadioides* has been overestimated. *Heterothalamus psiadioides* volatiles were highly phytotoxic under laboratory conditions, even at very low concentrations (Schmidt-Silva et al. 2011; Silva et al. 2014). However, it is very difficult to assess allelochemical concentrations in nature, especially for volatiles, which production and emission rates can vary due to several factors (e.g. Kong et al. 2002; Curado et al. 2006; Karban 2007). In laboratory studies, exposure to volatiles is constant and concentrated on few target plants, which are placed in closed settings. Hence, laboratory assays are not able to truly represent complex environments, where allelochemicals effects can be dissolved. Wardle et al. (1996) showed that allelopathic activity is less likely to occur in species-rich ecosystems, as only one species would probably not reach sufficient dominance for its allelochemicals to dominate the environment biochemistry. In our study, *H. psiadioides* does in fact reach high cover values and dominance, but nonetheless, it seems that the quantity of volatiles released by *H. psiadioides* into air and soil is too low to affect the surrounding vegetation. Additionally, it is important to consider that laboratory experiments on *H. psiadioides* phytotoxicity were not conducted using soil as substrate. Some studies have shown the importance of using soil from the natural setting of the study specie, as effects of allelochemicals can be even neutralized due to interactions with biotic and abiotic factors of soil (Inderjit and Weiner 2001; Kaur et al. 2009). Thus, even if concentration of allelochemicals in the environment is not so low, these substances may not manage to persist in it.

In laboratory studies on allelopathy, controls are employed to set aside possible interferences of other factors than allelopathy on the effects observed. This should also be done in field evaluations. In plant communities it has been a challenge to establish appropriate controls, as even in laboratory assays perfect controls are very difficult to obtain (Inderjit & Nilsen 2003). In our study, we used the non-phytotoxic species *B. patens* as a way of control, as it seemed comparable to *H. psiadioides* in height, canopy cover and density, at least visually. We thus expected that if no inhibition of neighbouring plants by *H. psiadioides* was observed, the vegetation close to both shrubs would be similar. However, the patterns observed in Bp were more alike those found in NS, showing that the two species have different effects on the plant community, evidenced by the differences in light intensity below the two shrubs. But even though we did not manage to establish a perfect control, we emphasize the necessity of this approach in allelopathy studies. The use of a control species allowed us to notice the lack of relation between the patterns observed and allelopathic

activity, and also to understand which other factors could influence the plant community (i.e. differences in light availability).

Our study highlights that even if a plant shows a high allelopathic potential, evidenced in the laboratory, field studies are necessary to elucidate if allelopathy is in fact a relevant process in the plant community. The present study showed that the allelopathic potential of a plant can be overestimated in laboratory assays. Although we can not affirm that any species was affected by the phytotoxic species evaluated herein, an important point is that allelopathic effects, even if they occurred, were only secondary in relation to other environmental variables, and not strong enough to be relevant for community composition and structure. This was evidenced mainly due to the use of a control species, and we therefore suggest the use of a similar non-phytotoxic species as a way of control in allelopathy studies. Our study also points out that the apparent dominance of the supposedly allelopathic species and the occurrence of bare soil under and close to it, which are aspects that in many cases lead to the hypothesis of allelopathy, are not necessary in a causal relation. The affirmation that allelopathy can determine community patterns thus should not be made only based on visual evidences and on laboratory studies. Detailed field investigations are necessary to affirm if allelopathy is a relevant mechanism in the plant community or not.

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**Appendix 1** Mean relative cover of plant species in plots with *H. psiadioides* as the predominant shrub (50-70% cover) (Hp), with *B. patens* as the main shrub in the same cover values (Bp), and in plots with no shrubs (Ns). Species are classified according to their life form: Therophytes (Th); Evergreen tussocks (Et); Prostrate evergreens (Pe); Evergreen forbs (Ef); Evergreen subshrubs (Ss); Evergreen shrubs (Sh); Succulents (Su); Prostrate rosette evergreens (Pr); Erect rosette evergreens (Er); Geophytes (Ge).

Family	Species	Life form	Mean relative cover (%)		
			Hp	Bp	Ns
Acanthaceae	<i>Justicia axillaris</i> Ness. Lindau	Ef	0.50	0.00	0.00
	<i>Stenandrium diphyllum</i> Nees	Pr	0.00	0.00	0.22
Amaranthaceae	<i>Gomphrena graminea</i> Moq.	Ef	0.16	0.00	0.00
	<i>Pfaffia tuberosa</i> Hicken	Ef	0.57	1.02	0.19
Anemiaceae	<i>Anemia tomentosa</i> (Savigny) Sw.	Ef	0.00	0.00	0.20
Apiaceae	<i>Eryngium ciliatum</i> Cham. & Schtdl.	Pr	0.44	0.00	0.00
	<i>Eryngium horridum</i> Malme	Er	1.71	2.60	0.44
	<i>Eryngium sanguisorba</i> Cham. & Schtdl.	Er	0.91	0.20	0.42
Apocynaceae	<i>Mandevilla pinifolia</i> (A.St.-Hil.) Miers	Ef	0.00	0.00	0.09
Araliaceae	<i>Hydrocotyle exigua</i> Malme	Pe	0.61	0.00	0.11
Arecaceae	<i>Butia odorata</i> (Barb.Rodr.) Noblick & Lorenzi	Sh	0.00	0.17	0.00
Asteraceae	<i>Achyrocline satureioides</i> (Lam.) DC.	Ef	1.29	0.00	0.00
	<i>Acmella bellidioides</i> (Smith in Rees) R.K. Jansen	Ef	0.85	0.00	0.42
	<i>Aspilia montevidensis</i> (Spreng.) Kuntze	Pe	0.65	0.81	0.11
	<i>Aspilia pascaliioides</i> Griseb.	Ef	0.00	0.18	0.00
	<i>Baccharis articulata</i> (Lam.) Pers.	Sh	0.00	0.00	0.09
	<i>Baccharis cognata</i> DC.	Sh	0.85	0.71	0.32
	<i>Baccharis dracunculifolia</i> DC.	Sh	0.21	2.91	0.47
	<i>Baccharis trimera</i> (Less.) DC.	Ss	0.48	0.15	0.45
	<i>Campovassouria bupleurifolia</i> (DC.) R.M. King & H. Rob.	Sh	3.02	0.51	0.00
	<i>Chaptalia integerrima</i> (Vell.) Burkart	Pr	0.37	0.00	0.00
	<i>Chaptalia runcinata</i> Kunth	Pr	0.53	0.13	0.20
	<i>Chevreulia acuminata</i> Less.	Pe	0.19	0.13	0.00
	<i>Chevreulia sarmentosa</i> (Pers.) S.F. Blake	Pe	0.11	0.00	0.00
	<i>Conyza primulifolia</i> (Lam.) Cuatrec. & Lourteig	Er	0.45	0.39	0.09
	<i>Criscia stricta</i> (Spreng.) Katinas	Pr	1.69	0.00	0.00
	<i>Eupatorium ascendens</i> Sch. Bip. ex Baker	Ef	4.08	2.62	1.43
	<i>Eupatorium ligulaefolium</i> Hook. & Arn.	Ss	1.36	3.93	0.51
	<i>Eupatorium</i> sp. 1	-	0.00	0.54	0.00
	<i>Eupatorium</i> sp. 2	-	0.00	0.00	0.10
	<i>Eupatorium squarulosum</i> Hook. & Arn.	Ss	0.00	0.34	0.00
<i>Eupatorium tanacetifolium</i> Gillies ex Hook. &	Ef	0.37	0.00	0.00	

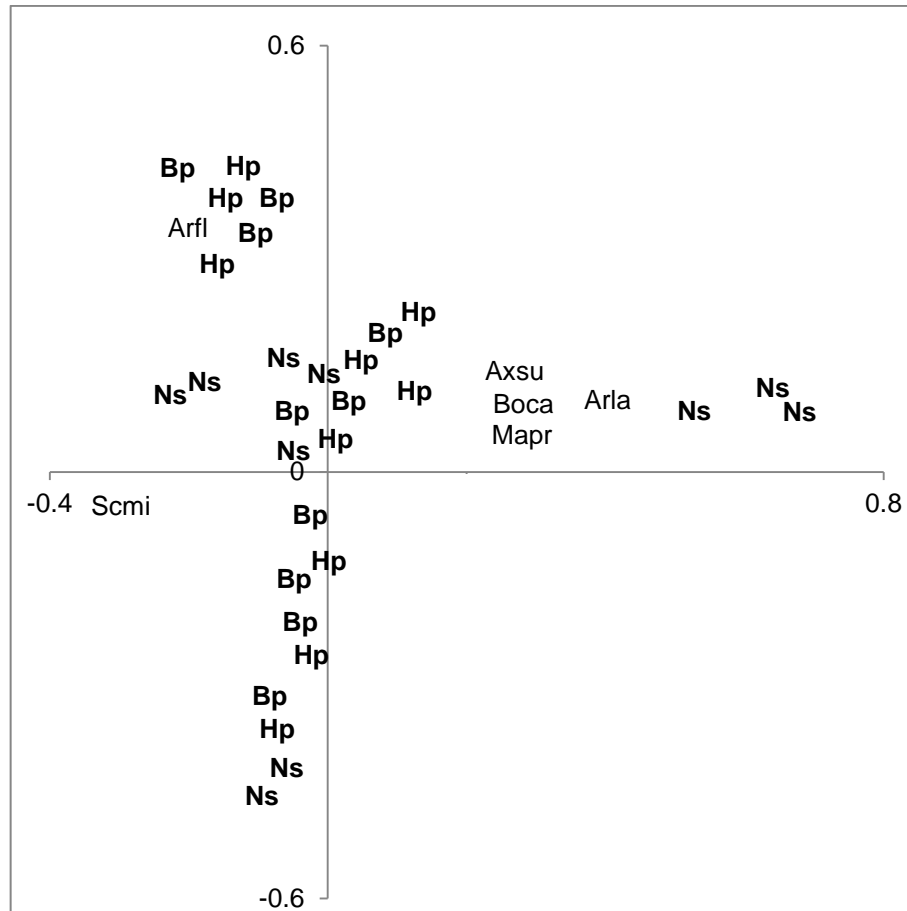
	Arn.				
	<i>Lucilia acutifolia</i> (Poir.) Cass.	Ef	0.00	0.00	0.56
	<i>Lucilia nitens</i> Less.	Ef	0.43	0.00	0.00
	<i>Orthopappus angustifolius</i> (Sw.) Gleason	Pr	0.00	0.00	0.12
	<i>Podocoma hirsuta</i> (Hook. & Arn.) Baker	Pr	0.40	0.00	0.00
	<i>Porophyllum</i> sp.	-	0.20	0.00	0.00
	<i>Pterocaulon rugosum</i> (Vahl) Malme	Ef	0.69	0.35	0.00
	<i>Pteurocaulon</i> sp.	-	0.19	0.00	0.22
	<i>Stenachaenium campestre</i> Baker	Ef	0.19	0.20	0.22
	<i>Vernonanthura nudiflora</i> (Less.) H. Rob.	Ss	0.11	0.00	0.34
Cactaceae	<i>Parodia ottonis</i> (Lehm.) N.P. Taylor	Su	0.41	0.00	0.00
Calyceraceae	<i>Acicarpa</i> sp.	-	0.00	0.00	0.11
Cistaceae	<i>Halimium brasiliense</i> (Lam.) Grosser	Ef	0.67	0.00	0.10
Convolvulaceae	<i>Dichondra sericea</i> SW.	Pe	0.61	0.13	0.32
	<i>Evolvulus sericeus</i> SW.	Pe	1.66	0.67	0.44
Cyperaceae	<i>Bulbostylis capillaris</i> (L.) C.B. Clarke	Et	0.45	0.00	0.22
	<i>Bulbostylis juncooides</i> (Vahl) Kük. ex Osten	Et	3.38	0.40	0.00
	<i>Carex phalaroides</i> Kunth	Pr	0.00	0.13	0.12
	<i>Kyllinga odorata</i> Vahl	Et	0.45	0.00	0.00
	<i>Rhynchospora megapotamica</i> (Spreng.) H. Pfeiff.	Pe	4.50	1.71	1.09
	<i>Rhynchospora setigera</i> Griseb.	Et	0.43	0.20	0.44
	<i>Scleria sellowiana</i> Kunth	Et	0.00	0.00	0.31
Euphorbiaceae	<i>Croton gnaphalii</i> (Baill.) Kuntze	Sh	1.00	0.64	0.41
	<i>Croton lanatus</i> var. <i>tatacuensis</i> (Ahumada) P.E. Berry	Sh	0.18	0.00	0.00
	<i>Euphorbia selloi</i> Klotzsch & Garcke	Pe	0.33	0.00	0.22
Fabaceae	<i>Aeschynomene falcata</i> (Poir.) DC.	Pe	0.30	0.00	0.12
	<i>Centrosema virginianum</i> (L.) Benth.	Ef	0.21	0.00	0.00
	<i>Clitoria nana</i> Benth.	Ef	1.75	0.83	0.56
	<i>Crotalaria tweediana</i> Benth.	Ss	0.00	0.37	0.10
	<i>Desmanthus tatuhyensis</i> Hoehne	Ss	0.30	0.20	0.00
	<i>Desmodium incanum</i> (Sw.) DC.	Pe	0.17	0.00	0.12
	<i>Galactia marginalis</i> Benth.	Ss	0.61	0.73	0.33
	<i>Macroptilium prostratum</i> (Benth.) Urb.	Pe	0.00	0.23	0.00
	<i>Zornia</i> sp.	Ef	0.19	0.00	0.12
Iridaceae	<i>Sisyrinchium vaginatum</i> Spreng.	Ge	0.00	0.00	0.12
Lamiaceae	<i>Glechon ciliata</i> Benth.	Ss	1.37	0.61	0.78
	<i>Peltodon longipes</i> Kunth ex Benth.	Pe	0.17	0.00	0.19
Lythraceae	<i>Cuphea campylocentra</i> Griseb.	Ef	0.32	0.13	0.20

	<i>Cuphea glutinosa</i> Cham. & Schltldl.	Ef	0.00	0.55	0.20
Malvaceae	<i>Krapovickasia flavescens</i> (Cav.) Fryxell	Pe	0.00	0.13	0.00
	<i>Sida rhombifolia</i> L.	Ss	0.21	0.35	0.00
	<i>Wissadula glechomifolium</i> (A. St.-Hil.) R.E. Fr.	Ss	1.11	0.00	0.10
Melastomataceae	<i>Tibouchina gracilis</i> (Bonpl.) Cogn.	Ef	0.00	0.26	0.00
Orchidaceae	<i>Habenaria parviflora</i> Lindl.	Ge	0.00	0.00	0.11
	Orchidaceae sp.	-	0.00	0.44	0.00
Oxalidaceae	<i>Oxalis bipartita</i> A. St.-Hil.	Ge	0.16	0.00	0.00
	<i>Oxalis tenerrima</i> R. Knuth	Ge	0.11	0.20	0.11
Poaceae	<i>Andropogon lateralis</i> Nees	Et	0.00	0.80	3.34
	<i>Andropogon ternatus</i> (Spreng.) Nees	Et	0.38	6.24	1.11
	<i>Aristida flaccida</i> Trin. & Rupr.	Et	8.17	14.26	4.56
	<i>Aristida jubata</i> (Arechav.) Herter	Et	0.00	0.00	21.91
	<i>Aristida laevis</i> (Nees) Kunth	Et	11.25	10.72	9.77
	<i>Axonopus siccus</i> (Nees) Kuhlms.	Et	0.19	0.17	0.19
	<i>Axonopus suffultus</i> (Mikan ex Trin.) Parodi	Et	3.76	0.81	1.00
	<i>Danthonia</i> sp.	Et	1.25	0.00	0.19
	<i>Dichanthelium sabulorum</i> (Lam.) Gould & C.A. Clark	Pe	0.30	2.59	0.76
	<i>Elionurus muticus</i> (Spreng.) Kuntze	Et	0.00	7.05	1.11
	<i>Elionurus</i> sp.	Et	1.86	0.00	0.00
	<i>Eragrostis neesii</i> Trin.	Th	0.45	0.00	0.00
	<i>Leptocoryphium lanatum</i> (Kunth) Nees	Et	2.09	11.04	8.75
	<i>Melica brasiliana</i> Ard.	Et	0.00	0.00	0.30
	<i>Paspalum plicatulum</i> Michx.	Et	0.96	0.00	0.00
	<i>Piptochaetium montevidense</i> (Spreng.) Parodi	Et	9.42	3.14	1.42
	Poaceae sp.	-	0.16	0.00	0.00
	<i>Schizachyrium microstachyum</i> (Desv. ex Ham.) Roseng., B.R. Arrill. & Izag.	Et	1.41	0.00	0.00
	<i>Schizachyrium tenerum</i> Nees	Et	8.11	7.44	23.25
	<i>Setaria parviflora</i> (Poir.) Kerguelen	Et	0.00	0.00	0.44
	<i>Setaria vaginata</i> Spreng. var <i>vaginata</i>	Et	0.00	0.23	0.00
	<i>Sorghastrum</i> sp.	Et	0.00	0.67	0.12
	<i>Steinchisma hians</i> (Elliott) Nash	Et	0.00	0.20	0.00
	<i>Stipa tenuiculmis</i> Hack.	Et	0.00	0.17	1.63
	<i>Trachypogon montufarii</i> (Kunth) Nees	Et	0.17	6.03	0.11
Rubiaceae	<i>Borreria capitata</i> (Ruiz & Pav.) DC.	Ss	0.17	0.37	0.30
	<i>Borreria verticillata</i> (L.) G. Mey.	Ef	0.00	0.00	0.11
	<i>Galianthe fastigiata</i> Griseb.	Ef	2.11	0.00	0.00
	<i>Galium humile</i> Cham. & Schltldl.	Pe	0.37	0.00	0.00

	<i>Galium richardianum</i> (Gillies ex Hook. & Arn.) Endl. ex Walp.	Pe	0.17	0.00	0.11
	<i>Relbunium hirtum</i> (Lam.) K. Schum.	Pe	0.00	0.31	0.00
	<i>Richardia grandiflora</i> (Cham. & Schltdl.) Steud.	Pe	0.60	0.34	2.03
Sapindaceae	<i>Dodonaea viscosa</i> Jacq.	Sh	0.60	0.00	0.00
Solanaceae	<i>Calibrachoa ovalifolia</i> Miers	Pe	0.19	0.30	0.10
Verbenaceae	<i>Glandularia marruboides</i> (Cham.) Tronc.	Pe	0.00	0.20	0.09
	<i>Lantana montevidensis</i> (Spreng.) Briq.	Sh	1.02	0.13	3.02
	<i>Verbena ephedroides</i> Cham.	Ef	0.19	0.00	0.00

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(-) Not totally identified species were not classified into categories.



**Appendix 2** Ordination diagram by PCoA of the sampling units with *H. psidioides* as the dominant shrub (50-70% cover) (Hp), with *B. patens* as the dominant shrub (Bp), and in plots with no shrubs (Ns). Bray-Curtis dissimilarity was used as resemblance measure. The ordination axes sum 28.4% of total variation (15.9% and 12.5% for axes 1 and 2, respectively). Variables correlated more than 0.2 were plotted in the diagram: (Arfl = *Aristida flaccida*; Arla = *Aristida laevis*; Axsu = *Axonopus suffultus*; Boca = *Borreria capitata*; Mapr = *Macoptilium prostratum*; Scmi = *Schizachyrium microstachyum*).

**Appendix 3** Mean relative cover of tussock species, separated according to their metabolism ( $C_3$  and  $C_4$ ), in plots with *H. psiadioides* as the dominant shrub (50-70% cover) (Hp), with *B. patens* as the dominant shrub (Bp), and in plots with no shrubs (Ns).

Family	Species	Mean relative cover (%)		
		Hp	Bp	Ns
<i>C<sub>3</sub> metabolism</i>				
Cyperaceae	<i>Rhynchospora setigera</i> Griseb.	0.43	0.20	0.44
Cyperaceae	<i>Scleria sellowiana</i> Kunth	0.00	0.00	0.31
Poaceae	<i>Danthonia</i> sp.	1.25	0.00	0.19
Poaceae	<i>Melica brasiliiana</i> Ard.	0.00	0.00	0.30
Poaceae	<i>Piptochaetium montevidense</i> (Spreng.) Parodi	9.42	3.13	1.42
Poaceae	<i>Stipa tenuiculmis</i> Hack.	0.00	0.17	1.63
<b>Total</b>		<b>11.10</b>	<b>3.51</b>	<b>4.29</b>
<i>C<sub>4</sub> metabolism</i>				
Cyperaceae	<i>Bulbostylis capillaris</i> (L.) C.B. Clarke	0.45	0.00	0.22
Cyperaceae	<i>Bulbostylis juncooides</i> (Vahl) Kük. ex Osten	3.38	0.40	0.00
Cyperaceae	<i>Kyllinga odorata</i> Vahl	0.45	0.00	0.00
Poaceae	<i>Andropogon lateralis</i> Nees	0.00	0.80	3.34
Poaceae	<i>Andropogon ternatus</i> (Spreng.) Nees	0.38	6.23	1.11
Poaceae	<i>Aristida flaccida</i> Trin. & Rupr.	8.17	14.24	4.56
Poaceae	<i>Aristida jubata</i> (Arechav.) Herter	0.00	0.00	21.91
Poaceae	<i>Aristida laevis</i> (Nees) Kunth	11.25	10.70	9.77
Poaceae	<i>Axonopus siccus</i> (Nees) Kuhlm.	0.19	0.17	0.19
Poaceae	<i>Axonopus suffultus</i> (Mikan ex Trin.) Parodi	3.76	0.81	1.00
Poaceae	<i>Elionurus muticus</i> (Spreng.) Kuntze	0.00	7.03	1.11
Poaceae	<i>Elionurus</i> sp.	1.86	0.00	0.00
Poaceae	<i>Leptocoryphium lanatum</i> (Kunth) Nees	2.09	11.02	8.75
Poaceae	<i>Paspalum plicatulum</i> Michx.	0.96	0.00	0.00
Poaceae	<i>Schizachyrium microstachyum</i> (Desv. ex Ham.) Roseng., B.R. Arrill. & Izag.	1.41	0.00	0.00
Poaceae	<i>Schizachyrium tenerum</i> Nees	8.11	7.43	23.25
Poaceae	<i>Setaria parviflora</i> (Poir.) Kerguélen	0.00	0.00	0.44
Poaceae	<i>Setaria vaginata</i> Spreng. var <i>vaginata</i>	0.00	0.23	0.00
Poaceae	<i>Sorghastrum</i> sp.	0.00	0.67	0.12
Poaceae	<i>Steinchisma hians</i> (Elliott) Nash	0.00	0.20	0.00
Poaceae	<i>Trachypogon montufarii</i> (Kunth) Nees	0.17	6.02	0.11
<b>Total</b>		<b>42.63</b>	<b>65.94</b>	<b>75.87</b>



#### 4 CONSIDERAÇÕES FINAIS

Nos ensaios realizados em laboratório, foi demonstrado que *H. psiadioides* e *B. patens*, as duas asteráceas arbustivas estudadas, apresentam fitotoxidez. *Heterothalamus psiadioides* apresentou efeitos fitotóxicos muito acentuados sobre a germinação e o crescimento de espécies-alvo, os quais ocorreram nas quatro estações do ano. *Baccharis patens* também apresentou esses efeitos, porém mais fracos que *H. psiadioides* e menos frequentes. Assim, os bioensaios indicam que embora ambas as espécies possuam potencial alelopático, para *H. psiadioides* esse potencial é consideravelmente maior.

Os efeitos fitotóxicos dos voláteis das duas espécies foram avaliados diretamente a partir das folhas frescas e secas das plantas, sem envolver qualquer tipo de extração. Esse método foi efetivo para determinar o potencial alelopático de *H. psiadioides* e *B. patens*, no qual foi demonstrado que as folhas frescas das plantas são mais fitotóxicas que as secas. Com isso, os efeitos observados resultam de experimentos mais realistas em relação à grande parte dos estudos conhecidos sobre fitotoxidez de voláteis.

Ainda, nos bioensaios foi observada uma variação sazonal nos efeitos fitotóxicos de ambas as plantas; assim, pode-se ressaltar que avaliações de potencial alelopático deveriam ser realizadas a partir de amostras coletadas ao longo de diferentes períodos do ano e não apenas em um único momento, como usualmente ocorre. Além disso, sugere-se que para a realização de estudos mais completos, fatores bióticos e abióticos sejam monitorados ao longo das estações (e.g. temperatura, precipitação, sinais de predação), bem como a caracterização química sazonal dos voláteis emitidos pelas plantas, sendo a microextração por fase sólida em espaço confinado uma boa técnica para este tipo de estudo. Se esses aspectos tivessem sido considerados no presente trabalho, talvez fosse possível aferir de forma precisa sobre que fatores são os principais responsáveis pela variação ao longo do ano na fitotoxidez das espécies investigadas.

Em relação ao estudo em campo, quando avaliado se *H. psiadioides*, devido a sua alta fitotoxidez, afetaria negativamente a comunidade vegetal, não se observaram evidências de efeitos alelopáticos. Na presença do arbusto, por outro lado, o número de espécies por parcela foi elevado em relação aos controles (parcelas sem arbustos e parcelas com *B. patens*, que é pouco fitotóxica), havendo maior cobertura de herbáceas não-graminoides. Isso ocorre devido a menor cobertura de gramíneas dominantes próximo a *H. psiadioides*. Os padrões observados foram melhor explicados por variáveis de estrutura da vegetação e disponibilidade de luz que por alelopatia. Dessa forma, foi demonstrado que o potencial

alelopático de *H. psiadioides* foi superestimado nos ensaios em laboratório. Isso indica que embora os voláteis da planta tenham sido fitotóxicos em concentrações baixas nos ensaios, no ambiente esses voláteis podem estar em concentrações ainda menores.

Por meio desse estudo em campo, pode-se destacar a importância de avaliar se o potencial alelopático de uma planta demonstrado em laboratório realmente tem implicações sobre as interações das espécies na natureza. Apenas com avaliações em campo é possível determinar se uma planta fitotóxica possui efeitos alelopáticos, e se os mesmos são fortes o suficiente para se sobressaírem em uma comunidade vegetal. Além disso, o estabelecimento de controles em campo, como *B. patens* no caso do presente estudo (uma planta semelhante em hábito e altura à *H. psiadioides*, mas praticamente não fitotóxica), pode ser uma importante ferramenta para a compreensão de outros fatores que podem determinar padrões de vegetação além de alelopatia. Em comunidades naturais há vários fatores que podem se sobressair, mascarando efeitos decorrentes de interações químicas, o que torna tão complexo avaliar alelopatia em campo. Assim, talvez uma das melhores formas de trazer respostas sobre a ocorrência de alelopatia seja através de experimentos em campo, algo muito mais próximo a condições naturais que uma sala de cultivo, mas onde vários fatores podem ser controlados.

Por fim, sugere-se que estudos futuros de alelopatia considerem realizar ensaios em laboratório de forma mais próxima a condições naturais, a fim de que os resultados dos mesmos sejam mais possíveis de conectar aos dos estudos em campo. Nesse sentido, alguns aspectos não considerados na maioria dos estudos de alelopatia (e também no presente trabalho) podem tornar os bioensaios mais realistas. Um desses aspectos é a utilização de solo proveniente de onde a planta-doadora ocorre nos experimentos, ao invés de substratos artificiais, já que os aleloquímicos podem interagir com fatores bióticos e abióticos do solo e ter sua ação profundamente modificada. Além disso, deve-se buscar utilizar como espécies-alvo plantas que ocorram nos mesmos ambientes que a espécie-doadora e que sejam comuns nesses locais. A obtenção de espécies-alvo adequadas ainda é uma dificuldade, especialmente no Brasil, por ser necessário plantas que germinem de forma muito rápida e homogênea para os ensaios. Isso praticamente só é possível com espécies cultivadas, que no caso de espécies campestres (e.g. gramíneas e leguminosas), são predominantemente exóticas. Assim, acredita-se que a utilização de métodos mais realistas em ensaios em laboratório, associada à realização de estudos em campo controlando o máximo possível de interferências, trariam resultados mais consistentes sobre a ocorrência efetiva de alelopatia em comunidades naturais.

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