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

TESE DE DOUTORADO

Alelopatia: um possível fator relevante em comunidades vegetais campestres e um caminho alternativo no manejo de plantas daninhas?

ELIANE REGINA DA SILVA

Porto Alegre, março, 2018

Alelopatia: um possível fator relevante em comunidades vegetais campestres e um caminho alternativo no manejo de plantas daninhas?

Eliane Regina da Silva

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

Orientador: Prof. Dr. Geraldo Luiz Gonçalves Soares

Co-orientador: Prof. Dr. Gerhard Ernst Overbeck

Comissão examinadora:
Profa. Dra. Sandra Cristina Müller
Profa. Dra. Ângela Pawlowski
Profa. Dra. Paula Novaes

Porto Alegre, março, 2018

Ciência é desafio
cujo fio da meada
só tem começo

Ciência é um chamado
é projeto de vida
é semeadura

E eis que germina a descoberta:
do quão pouco é viver só dos frutos
quando o horizonte é colheita.

Geraldo L. G. Soares

AGRADECIMENTOS

Há tanto para agradecer que esta poderia ser a maior seção da tese. Mas tentando ser breve, agradeço primeiramente ao Geraldo, *the boss*, por ser um orientador tão humano. Obrigada pela amizade e por se preocupar mais com a minha felicidade do que com o meu currículo; isso ajudou a tornar meu doutorado mais tranquilo e também mais produtivo. Sou grata a ti e ao meu co-orientador Gerhard por me motivarem, pela paciência, pelo tempo, pelos ensinamentos, e por serem pessoas tão éticas. Gerhard, obrigada por me estimular a fazer o que tem que ser feito, e dizer que sim eu consigo, e rápido inclusive. Isso foi fundamental, me ajudou a não procrastinar e a acreditar mais em mim.

Obrigada pela parceria meus amigos do LEQTAX, e pelo tanto que me ajudaram em laboratório e em campo. Se não fosse por esse apoio eu estaria até hoje contando plantas germinadas ou cortando EVA. Agradeço em especial ao Luis, com quem pude contar inúmeras vezes do começo ao fim da tese, e também à Cíntia, ao Diego, ao Matias, à Adriana e à Vanessa. Obrigadão Ângela, minha ex-colega de lab e colega de pesquisa pra vida, por me socorrer nas dúvidas de química milhões de vezes e pela parceria sempre, nos eventos, nos artigos e nas indiadas. Agradeço a Ângela e também à Diana por me ajudarem a dar os primeiros passos nos trabalhos com óleo essencial, *los aceites que tanto me encantan*. Obrigada meus amigos do LEVCAMP pela ajuda e parceria, especialmente a Cleusa, o Léo e o Fábio que me deram um baita apoio pra identificar as plantas.

Quiero agradecer a mis supervisores por todo su cariño, apoyo y conocimientos transmitidos durante la estancia en Cádiz. Asímismo, al profesor Francisco A. Macías por recibirme en su laboratorio con los brazos abiertos, por brindarme la oportunidad de aprender y trabajar en su grupo (Alelopatía en plantas superiores y microorganismos). Por otro lado, agradecer al profesor José Manuel Igartuburu (Buru), el Maestro Yoda del gases-masas, por su paciencia, por enseñarme tanto de química, de creatividad y de ciencia. Gracias a él tuve la experiencia de hacer ciencia de la manera más pura, con ilusión y hacer que todo fuera más sencillo. Fue una experiencia que nunca olvidaré.

Gracias a todas las personas que forman parte del grupo de alelopatía de Cádiz por hacerme sentir parte de la familia, por su amistad y cariño. Convivir con esta familia tan alegre fue un regalo de cada día. Gracias a los amigos que tanto me enseñaron en el laboratorio, en especial a David, Maite G., Gustavo y Rosa, aunque podría citar los nombres de tantas personas más... Gracias a los amigos del INBIO por ayudarme tanto con los bioensayos, a Leticia, Maite A., Maite J. y Javi. A Maite J., la mejor diseñadora gráfica de Andalucía, agradezco también por las portadas de la tesis. Gracias Sandra por la amistad

desde antes de llegar a Cádiz, hasta siempre, mi hermanita tan querida, y a todos los amigos españoles y brasileños que me hicieron sentir que en Cádiz estaba como en casa.

Sou grata ao PPG Botânica UFRGS pelo apoio e pela infraestrutura e a todos os professores que participaram de minha formação. Agradeço aos laboratórios da UFRGS que abriram suas portas para o uso de equipamentos: Centro de Ecologia (obrigada Armando), Laboratório de Fisiologia Vegetal, Laboratório de Anatomia Vegetal, e Laboratório de Ecofisiologia Vegetal. Agradeço à Joséli por toda a colaboração e aos laboratórios da UCS - Laboratório de Extração de Óleos Essenciais e Laboratório de Biotecnologia de Produtos Naturais e Sintéticos. Obrigada ao LETS da UFG pela acolhida.

Agradeço à CMPC Celulose Riograndense pelo financiamento dos experimentos em campo, e especialmente ao Elias Araújo por todo o apoio logístico. Obrigada a todos os funcionários que me acompanharam em campo e que trabalharam muito. Sou grata pela dedicação e capricho na montagem das parcelas, por toda ajuda que me deram e pela troca de ideias. Agradeço ao CNPq pela bolsa de doutorado (processo 141533/2014-5) e à CAPES pela bolsa de doutorado sanduíche no exterior (processo 88881.132747/2016-01).

Obrigada Paula, Sandra, Ângela e Ingrid Barros por aceitarem avaliar esta tese. Agradeço muito mesmo por disporem seu tempo para ler a tese - e certamente contribuir para melhorá-la - e para discutir ciência. Agradeço à Raquel Paulsen pela foto de capa da tese. Obrigada a todos que de alguma forma me ajudaram com algo, seja em uma análise estatística, uma informação, o uso de um programa ou um equipamento, uma troca de ideias. Obrigada também aos que não atrapalharam; acreditem, isso é importante.

Agradeço a todos os amigos que estiveram ao meu lado e me trouxeram alegria ao longo destes quatro anos. Agradeço especialmente aos colegas do PPG Botânica, e à Lu e à Cleu, as irmãs que a pós-graduação me trouxe. Por fim, agradeço muito à minha família e ao meu companheiro pelo apoio incondicional e por acreditarem tanto em mim. Muito obrigada mãe por me dar a oportunidade de estudar, de me tornar bióloga, de aprender idiomas; sem isso, não haveria doutorado.

"Yo tengo tantos hermanos
Que no los puedo contar
En el valle, la montaña,
en la pampa y en el mar…
Y así seguimos andando
Curtidos de soledad
Nos perdemos por el mundo
Nos volvemos a encontrar."

Atahualpa Yupanqui

RESUMO

A alelopatia pode desempenhar um papel relevante na dinâmica de campos e também no manejo de plantas daninhas em pastagens. Na região dos campos do sul do Brasil, monoculturas de *Eucalyptus* têm sido plantadas. A vegetação é escassa sob os plantios, o que pode estar associado à alelopatia. Em uma perspectiva aplicada, aleloquímicos de *Eucalyptus* poderiam ser potencialmente empregados como herbicidas naturais. Esta tese tem como objetivo avaliar se a alelopatia pode ser um fator determinante na estruturação da vegetação campestre, e se pode consistir em uma potencial ferramenta no controle de plantas daninhas. No capítulo I, uma revisão sistemática foi feita, visando evidenciar tendências gerais, antigas e atuais na pesquisa sobre alelopatia em ecossistemas campestres, com foco nos métodos utilizados. O capítulo II objetivou avaliar a fitotoxidez do extrato aquoso e do óleo essencial das folhas da serapilheira de *Eucalyptus saligna* Sm. sobre espécies campestres em laboratório. No capítulo III, os efeitos das folhas da serapilheira de *E. saligna* sobre espécies campestres foram investigados em plantios, bem como se esses efeitos estavam relacionados à alelopatia. O capítulo IV visou avaliar o potencial bioherbicida do óleo essencial das folhas da serapilheira de *E. saligna* e determinar quais componentes estavam associados a sua fitotoxidez. No capítulo I, evidenciou-se a potencial relevância da alelopatia na dinâmica de campos e no manejo de plantas daninhas em sistemas cultivados. Além disso, observou-se que os trabalhos recentes sobre alelopatia melhoraram em alguns aspectos de desenho experimental, mas não em outros, e que há inconsistência na terminologia utilizada. No capítulo II, observou-se que as folhas da serapilheira de *E. saligna* apresentaram substâncias fitotóxicas que geraram estresse oxidativo e levaram a danos nas membranas, afetando a germinação e o crescimento de plântulas. No capítulo III, evidenciou-se que as folhas da serapilheira inibiram a vegetação campestre em plantios de *E. saligna*, mas os efeitos foram principalmente físicos, e efeitos alelopáticos não foram detectados. No capítulo IV, foi demonstrado o potencial do óleo essencial de *E. saligna* como herbicida natural. O óleo foi mais fitotóxico que seus componentes majoritários, mas isso variou com o método e com as espécies receptoras. Esta tese evidenciou que generalizações sobre fitotoxidez e alelopatia devem ser evitadas. Conclui-se que a alelopatia possui um potencial maior como alternativa no manejo de plantas daninhas do que como um fator atuante sobre o estabelecimento e desenvolvimento de espécies vegetais campestres. Um maior conhecimento sobre alelopatia e seus mecanismos pode levar a avanços na ciência e em áreas aplicadas.

Palavras-chave: Campos Sulinos; crescimento de plantas; desenho experimental; *Eucalyptus saligna*; fitotoxidez; germinação; monoterpenos.

ABSTRACT

Allelopathy may play a relevant role in dynamics of grasslands, and also in weed management in pastures. In Southern Brazilian grasslands region, *Eucalyptus* monocultures have been planted. Vegetation is scarce under plantations, which may be associated with allelopathy. In an applied perspective, *Eucalyptus* allelochemicals may be potentially employed as natural herbicides. This thesis aimed to evaluate if allelopathy may be a key factor shaping grassland vegetation, and if it consists in a potential tool for weed control. In chapter I, a systematic review was conducted, in order to evidence general, old and current trends in allelopathy research in grassland ecosystems, focusing in used methods. Chapter II aimed to assess the phytotoxicity of *Eucalyptus saligna* Sm. leaf litter aqueous extract and essential oil on grassland species, in laboratory conditions. In chapter III, effects of *E. saligna* leaf litter on grassland species were investigated in plantations, as well as if these effects were related to allelopathy. Chapter IV aimed to evaluate the bioherbicide potential of *E. saligna* leaf litter essential oil, and to determine which compounds were related to the oil phytotoxicity. In chapter I, the potential relevance of allelopathy was evidenced in dynamics of grasslands and in weed management in cultivated systems. Moreover, the review demonstrated that allelopathy research has improved in some experimental design issues, but not in others, and inconsistency in terminology was observed. In chapter II, *Eucalyptus saligna* leaf litter showed phytotoxic compounds that generated oxidative stress and led to membrane damage, affecting seeds and seedling growth. In chapter III, leaf litter was evidenced to inhibit grassland species in *E. saligna* plantations, but effects were mainly physical, and allelopathic effects were not detected. In chapter IV, the essential oil showed potential as a natural herbicide. In general, *E. saligna* essential oil was more phytotoxic than its major compounds, but this varied according to the method and the recipient species. This thesis evidenced that generalizations should not be made, neither about phytotoxicity, nor about allelopathy. In conclusion, allelopathy showed greater potential as a tool for weed management than as a relevant factor influencing establishment and development of grassland vegetation. A better knowledge about allelopathy and its mechanisms may lead to advances in science and in applied fields.

Keywords: Campos Sulinos; *Eucalyptus saligna*; experimental design; germination; monoterpenes; plant growth; phytotoxicity.

RESUMEN

La alelopatía puede tener un papel relevante en la dinámica de pastizales y también en el control de malas hierbas en campos artificiales. En la región de los campos del sur de Brasil, se han plantado monocultivos de *Eucalyptus*. Debido a que la vegetación es escasa en esas plantaciones, puede relacionarse con la alelopatía. En una perspectiva aplicada, aleloquímicos de *Eucalyptus* podrían ser potencialmente empleados como herbicidas naturales. Esta tesis tiene como objetivo evaluar si la alelopatía puede ser un factor determinante en la estructuración de pastizales y si puede consistir en una herramienta potencial para el control de malas hierbas. En el capítulo I, se realiza una revisión sistemática con el fin de mostrar tendencias generales, antiguas y actuales en la investigación sobre alelopatía en pastizales, con especial interés en los métodos utilizados. El objetivo del capítulo II es la evaluación de la fitotoxicidad del extracto acuoso y del aceite esencial de las hojas del mantillo de *Eucalyptus saligna* Sm. sobre especies campestres en laboratorio. En el capítulo III, los efectos de las hojas de *E. saligna* sobre especies campestres fueron investigados en plantaciones, así como si esos efectos estaban relacionados con la alelopatía. El capítulo IV se centra en evaluar el potencial bioherbicida del aceite esencial de las hojas de *E. saligna*, y determinar cuáles componentes están asociados a su fitotoxicidad. En el capítulo I, se destaca la importancia de la alelopatía en la dinámica de las praderas y en el control de malas hierbas en sistemas cultivados. Además, se ha observado que los estudios más recientes en esta área mejorarán en algunos aspectos de diseño experimental, pero no en otros y que no hay consistencia en la terminología usada. En el capítulo II, las hojas de *E. saligna* presentaron sustancias fitotóxicas que generaron estrés oxidativo y causaron daños en las membranas, afectando a la germinación y el crecimiento de plántulas. En el capítulo III, las hojas del mantillo inhibieron las plantas campestres en plantaciones de *E. saligna*, pero los efectos fueron principalmente físicos y no se detectaron efectos alelopáticos. En el capítulo IV, el aceite esencial de *E. saligna* presentó potencial como herbicida natural. En general, el aceite fue más fitotóxico que sus componentes mayoritarios, pero eso ha cambiado con los métodos y con las especies receptoras. Esta tesis demostró que no deben generalizarse sobre la fitotoxicidad y la alelopatía. Se concluye que la alelopatía tiene un potencial mayor como alternativa en el control de malas hierbas que como un factor actuante sobre el establecimiento y desarrollo de especies campestres. Un mayor conocimiento sobre la alelopatía y sus mecanismos puede conducir a avances en la ciencia y en áreas aplicadas.

Palabras-llave: Campos Sulinos; crecimiento de plantas; diseño experimental; *Eucalyptus saligna*; fitotoxicidad; germinación; monoterpenos.

SUMÁRIO

1 INTRODUÇÃO GERAL

1.1 Alelopatia

A alelopatia consiste em uma interação interespecífica ou intraespecífica, na qual um organismo afeta outro através da produção e emissão de substâncias químicas no ambiente. Essa interação envolve pelo menos um organismo doador (que produz os aleloquímicos) e um organismo receptor (que sofre os efeitos), sendo que a ação de aleloquímicos pode também ser indireta, mediada por microrganismos (Rice 1984). Efeitos alelopáticos podem ser positivos ou negativos, e envolver diversos tipos de organismos, tais como plantas, bactérias, algas, fungos, esponjas e corais (IAS 1996, Granéli & Pavia 2006). Em relação a plantas, aleloquímicos podem ser emitidos através de lixiviação por água, exsudação de raízes, volatilização e decomposição de resíduos vegetais (De Albuquerque et al. 2011).

Estudos de alelopatia têm sido conduzidos utilizando diferentes abordagens, sendo basicamente direcionados para ecossistemas naturais ou para cultivos agrícolas. Os primeiros registros de possíveis efeitos alelopáticos encontrados na literatura são para espécies agrícolas, nos quais Theophrastus (300 a.C.) e Plínio II (1 d.C.) observaram que certas plantas cultivadas “faziam o solo adoecer” (Rice 1984, Weston 2005). O conhecimento sobre alelopatia tem sido considerado na rotação de culturas e na utilização de plantas de cobertura (Soltys et al. 2013). Além disso, a aplicação da alelopatia no melhoramento de plantas tem sido visada, buscando-se a seleção de cultivares com maior atividade alelopática (Worthington & Reberg-Horton 2013). Outra aplicação potencial de aleloquímicos é no desenvolvimento de biocidas naturais, que podem ser provenientes de extratos, óleos essenciais ou de seus componentes (Dayan et al. 2009). No Brasil, embora alguns produtos naturais já sejam empregados para combater pragas animais em cultivos (ex. óleo de Nim - *Azadirachta indica* A.Juss.), se desconhece a utilização de herbicidas naturais.

Em relação à abordagem ecológica, a alelopatia foi evidenciada para poucas espécies até o momento. No Brasil, ainda não há estudos que tenham de fato demonstrado a ocorrência desse fenômeno na natureza, levando em conta os critérios estabelecidos na literatura (Reigosa et al. 2013). Entretanto, o mito de que certas plantas impedem outras de crescerem em suas proximidades devido à produção de substâncias químicas tem sido comumente disseminado, tanto na literatura quanto na cultura popular, para espécies que se tornam dominantes em algum local. É comum encontrar afirmações de (provável) alelopatia, quando ocorre a combinação de dois fatores: há pouca vegetação próximo/sob uma planta e

não se sabe o porquê; e um estudo de fitotoxidez em laboratório demonstra que o extrato dessa planta inibe a germinação ou o crescimento de outras. Contudo, no geral, qualquer planta apresenta fitotoxidez após o tecido vegetal passar por algum tipo de extração, em alguma concentração (Harper 1994); isso não significa que a maioria das espécies sejam alelopáticas.

Desde que os estudos de alelopatia surgiram, tem havido muito ceticismo sobre a importância desse fenômeno, devido principalmente ao fato que os ensaios em laboratório não se aproximavam do que poderia ocorrer na natureza e que avaliações em campo eram escassas. A fim de melhorar esse cenário, vários artigos de revisão apresentaram recomendações sobre questões importantes que deveriam ser consideradas no desenho experimental de investigações de alelopatia (Inderjit & Dakshini 1995, Inderjit & Weston 2000, Inderjit & Weiner 2001, Inderjit & Callaway 2003, Inderjit & Nilsen 2003). Na revisão recente de Reigosa et al. (2013), alguns aspectos gerais sobre estudos de alelopatia foram quantificados, sendo observado que muitas pesquisas não utilizavam metodologias adequadas. No entanto, esse trabalho apenas abrangeu investigações realizadas no Brasil, e sem diferenciar em que época foram feitas. Nenhuma revisão sobre alelopatia até o momento quantificou se os estudos recentes têm sido mais robustos e têm utilizado métodos mais adequados, e se com isso a área tem evoluído. Além disso, tem-se observado inconsistência na terminologia empregada na área, e sobre como determinar se uma espécie é alelopática, sendo necessário o esclarecimento dessas questões.

1.2 *Eucalyptus saligna* – uma espécie potencialmente fitotóxica/alelopática

Estudos de fitotoxidez têm sido feitos há algum tempo no Laboratório de Ecologia Química e Quimiotaxonomia (LEQTAX) da UFRGS. Em minha dissertação de mestrado, uma investigação de fitotoxidez em laboratório foi feita e a hipótese de alelopatia foi testada para o arbusto fitotóxico *Baccharis psiadioides* (Less.) Joch.Müll., o qual se estabelece em formações monodominantes e sob o qual se observa solo descoberto (Silva 2014). Embora tudo apontasse para a ocorrência de alelopatia, os padrões de vegetação foram relacionados ao sombreamento exercido pelo arbusto (Silva et al. 2015). Esse estudo indicou que não seria tão simples observar a relevância da alelopatia na natureza. Alguns trabalhos que detectaram alelopatia recentemente relacionaram a sua ocorrência à falta de um histórico de coevolução (ex. Bais et al. 2003, May & Baldwin 2011). Nesses casos, os resultados revelaram que as espécies da comunidade nativa não estavam adaptadas aos aleloquímicos produzidos por espécies exóticas e, por isso, foram mais suscetíveis aos seus

efeitos (hipótese das novas armas, Callaway & Ridenour 2004). Portanto, parece ser mais provável detectar a ocorrência de alelopatia para espécies exóticas.

Efeitos alelopáticos têm sido frequentemente atribuídos para espécies de *Eucalyptus*, uma vez que em plantios dessas espécies, em geral, a cobertura e diversidade de vegetação são reduzidas (ex. Omoro et al. 2010). Essas árvores exóticas têm sido plantadas em ampla escala para atender a demandas econômicas em todo o mundo, inclusive na região do Bioma Pampa. Como os plantios consistem em áreas extensas com a mesma espécie, as chances de alelopatia devem ser maiores do que para espécies distribuídas de forma dispersa pela paisagem. Para espécies de *Eucalyptus*, várias formas de emissão de aleloquímicos são possíveis, tanto a partir de folhas quanto de raízes (Zhang & Fu 2010, He et al. 2014). É provável que o maior potencial de afetar outras plantas seja pela serapilheira do que pelas folhas da copa, uma vez que as folhas da serapilheira permanecem em contato com a água da chuva por maior período. Pelo mecanismo de volatilização, a única forma possível de contato entre os voláteis emitidos e as espécies receptoras seria pela serapilheira. Como uma camada densa de serapilheira se forma sobre o solo nos plantios, a quantidade de aleloquímicos liberados pode ser alta, o que maximiza a possibilidade de alelopatia. Esse conjunto de fatores indica que uma espécie de *Eucalyptus* consiste em um bom modelo de planta doadora.

Na região do Pampa, várias espécies de *Eucalyptus* têm sido cultivadas. As condições de plantio e manejo empregados são similares para as espécies, implicando em quantidade semelhante de serapilheira acumulada sobre o solo. Além disso, a composição química dos óleos essenciais de muitas espécies de *Eucalyptus* costuma ser parecida (Padovan et al. 2014). Dessa forma, é possível que o potencial alelopático das espécies de *Eucalyptus* cultivadas no Pampa seja similar. *Eucalyptus saligna* Sm. foi escolhida como espécie doadora para este estudo por ser uma das mais plantadas na região.

Para determinar se uma espécie é alelopática, primeiramente é preciso demonstrar que a mesma é fitotóxica. Apenas um estudo já havia testado a fitotoxidez do extrato aquoso de *E. saligna* (Lisanework & Michelsen 1993). No entanto, esse trabalho incluía apenas espécies cultivadas como receptoras, e não ficou claro se os efeitos estavam relacionados com os aleloquímicos, já que não havia controle de pH do extrato aquoso, nem caracterização química. Além disso, esse estudo, assim como a maioria das investigações sobre *Eucalyptus*, utilizou folhas verdes coletadas diretamente da copa, e não a serapilheira. Dessa forma, ainda não há estudos que demonstrem se *E. saligna* produz substâncias fitotóxicas com potencial de afetar espécies campestres.

Embora a fitotoxidez de *Eucalyptus* spp. tenha sido demonstrada em muitos estudos, a maioria desses não incluiu avaliações em campo e controles para outros fatores que poderiam causar a supressão da vegetação (ex: Zhang & Fu 2010, Chu et al. 2014). A ocorrência de alelopatia parece ter sido de fato evidenciada apenas para algumas espécies de *Eucalyptus*, em ambientes e condições específicos (Del Moral & Muller 1970, Del Moral et al. 1978). A serapilheira acumulada sobre o solo pode afetar a vegetação não só por fatores químicos, mas também devido à supressão mecânica (Rotundo & Aguiar 2005), ou à redução na incidência de radiação solar (Jensen & Gutekunst 2003). Portanto, estudos em campo são necessários para evidenciar se *E. saligna* é alelopática, estabelecendo controles para efeitos físicos da serapilheira.

Por outro lado, visando uma perspectiva aplicada ao manejo de plantas daninhas, alguns óleos essenciais já têm sido comercializados como herbicidas naturais (Dayan & Duke 2010). Vários estudos têm demonstrado a fitotoxidez de óleos essenciais de *Eucalyptus* spp. sobre plantas daninhas (ex. Batish et al. 2007, Ootani et al. 2017). Nesse sentido, o óleo essencial de *E. saligna* poderia ser investigado como um potencial herbicida natural. Para pesquisas com essa abordagem, também é relevante avaliar quais componentes estão relacionados à fitotoxidez do óleo essencial e se há maior atividade para o óleo ou para um ou alguns de seus componentes apenas.

1.3 Objetivos

Esta tese tem como objetivo avaliar se a alelopatia pode ser um fator determinante no estabelecimento e desenvolvimento da vegetação campestre, e se pode consistir em uma potencial ferramenta no controle de plantas daninhas. Para alcançar esse objetivo, a tese compreendeu um capítulo de revisão sobre estudos de alelopatia em ecossistemas campestres e três capítulos nos quais investigou-se o potencial alelopático e bioherbicida de *E. saligna,* a espécie-modelo desses estudos. A tese apresenta os seguintes objetivos específicos:

- Evidenciar tendências gerais, antigas e atuais na pesquisa sobre alelopatia em ecossistemas campestres, focando nos métodos utilizados e esclarecendo conceitos (Capítulo I);
- Avaliar a fitotoxidez do extrato aquoso e do óleo essencial das folhas da serapilheira de *E. saligna* sobre a germinação, o crescimento inicial, os níveis de peróxido de hidrogênio (H_2O_2) e o vazamento de eletrólitos de membranas de espécies campestres (Capítulo II);
- Determinar os efeitos das folhas da serapilheira de *E. saligna* sobre espécies campestres e, no caso de efeitos, se esses estão relacionados à alelopatia (Capítulo III);

- Avaliar os efeitos fitotóxicos do óleo essencial das folhas da serapilheira de *E. saligna*, especialmente sobre plantas daninhas, e determinar quais componentes estão associados à fitotoxidez do óleo essencial (Capítulo IV).

2 MATERIAL E MÉTODOS

As metodologias utilizadas na tese foram descritas em cada capítulo. No entanto, para a apresentação dos capítulos em formato de artigo, algumas informações precisaram ser sintetizadas. Uma versão mais detalhada dos métodos utilizados será descrita nesta seção, bem como experimentos que se tinha a intenção de incluir na tese, mas que não deram certo. Com isso, pretende-se facilitar a reprodução dos experimentos apresentados, evitar a perda de tempo e recursos na repetição de ensaios que não foram bem-sucedidos, e sugerir ideias para estudos futuros.

2.1 Metodologia do Capítulo II

2.1.1 Extração do óleo essencial e preparo do extrato aquoso

O óleo essencial e o extrato aquoso de *E. saligna* foram obtidos a partir de folhas da serapilheira coletadas em um plantio da espécie, localizado no município de Eldorado do Sul, RS, Brasil (30°18'S 51°62'W, 135 m a.s.l.). O referido plantio pertence à empresa CMPC Celulose Riograndense e foi implantado em 2008. As folhas foram coletadas em agosto de 2014 e eram, portanto, provenientes de árvores com sete anos. As folhas já estavam secas quando coletadas. O óleo essencial foi extraído das folhas através de destilação por arraste a vapor, feita com 5 kg de folhas e utilizando um extrator de inox com uma taxa de fluxo de 3 L/h por 1 hora, apresentando rendimento de 0,56% (peso/volume). A extração do óleo foi realizada na Universidade de Caxias do Sul. Para eliminar resíduos de água no óleo, foi utilizado sulfato de sódio anidro. Para isso, pequenas quantidades de sulfato de sódio foram sendo adicionadas dentro do frasco com óleo essencial e a água se solidificava em contato com o sulfato. Então o óleo essencial foi retirado do frasco. O óleo foi armazenado em ultrafreezer (-80 °C) até o momento da realização dos experimentos.

O extrato aquoso foi preparado com folhas obtidas de dez coletores colocados no plantio (Figura 1). Os coletores eram feitos de madeira, com uma tela de 60 cm^2, profundidade de 10,5 cm e altura de 60 cm. As folhas foram coletadas mensalmente, de dezembro de 2014 até julho de 2015, e foram armazenadas em freezer. O armazenamento em freezer é uma ótima alternativa para casos em que experimentos com extrato precisam ser feitos várias vezes e o local de coleta não é tão próximo. Assim, o extrato foi feito com folhas no mesmo estágio de decomposição (no máximo um mês depois de cair das árvores e evitando coletar as folhas recém-caídas que ainda não estavam secas). Isso é importante pois o estágio de decomposição pode influenciar a quantidade de aleloquímicos nas folhas das plantas (Bernhard-Reversat et al. 2003). Além disso, toda vez que o extrato era

preparado, amostras de folhas coletadas em diferentes meses eram utilizadas. Isso foi feito para evitar possíveis diferenças na produção de aleloquímicos ao longo do ano, como observado para a espécie *Baccharis psiadioides* (Asteraceae) em um trabalho do nosso grupo de pesquisa (Silva et al. 2014). O extrato aquoso era preparado antes de ser utilizado nos experimentos, na proporção de 10 g de extrato para 100 mL de água. As folhas foram picadas grosseiramente e adicionadas à água destilada, onde permaneciam por 72 h em sala de cultivo, devido à temperatura constante da sala (20 ºC). O extrato era passado em papel filtro para reter resíduos sólidos. O extrato bruto (10%) foi diluído em água para as concentrações de 7.5% e 5%.

Figura 1: Coletores instalados em plantio de *Eucalyptus saligna* em Eldorado do Sul, RS, para coleta mensal das folhas da serapilheira utilizadas no preparo do extrato aquoso.

O que não deu certo: (1) Antes de colocar os coletores no plantio, folhas da serapilheira foram coletadas aleatoriamente para fazer o extrato, e alguns ensaios-piloto de germinação foram feitos. A fitotoxidez variou consideravelmente, apresentando em alguns ensaios efeitos inibitórios e em outros não. É possível que o teor de aleloquímicos das folhas tenha variado pelo estágio de decomposição diferenciado. (2) Extração a quente também foi feita: as folhas foram aquecidas em água a 60°C por 60 minutos. O experimento foi totalmente contaminado com fungos logo no dia seguinte. O experimento foi refeito com folhas de diferentes coletas, mas o resultado se repetiu. (3) As folhas foram deixadas inteiras para fazer o extrato, mas as folhas da serapilheira são muito volumosas e 10 g de folhas não ficavam imersas em 100 mL de água. Dessa forma, seria necessário fazer o extrato com uma quantidade menor de folhas e concentração seria bem inferior a 10%. (4) Outra tentativa foi macerar as folhas em um moedor de café para fazer o extrato, mas as folhas praticamente viraram pó e absorveram a maior parte da água adicionada, rendendo

uma pequena quantidade de extrato. (5) As folhas foram deixadas 72 h em água porque apenas 24 h não são suficientes pra extrair os aleloquímicos de *E. saligna* (efeitos fitotóxicos não foram observados); 48 h parece ser suficiente, mas optou-se por deixar 72 h por ter testado mais vezes com esse tempo e não ter visto variação na fitotoxidez.

2.1.2 Espécies receptoras

As espécies receptoras expostas aos aleloquímicos de *E. saligna* foram as Poaceae *Paspalum notatum* Flüggé (grama-forquilha, pensacola) e *Eragrostis plana* Ness (capim annoni), e as Fabaceae *Trifolium repens* L. (trevo branco) e *Lotus corniculatus* L. (cornichão São Gabriel) (Figura 2). Essas espécies representam duas das três famílias com maior riqueza nos campos do sul do Brasil (Bioma Pampa). As espécies foram escolhidas por germinarem rápido, de forma homogênea e em taxas altas.

Apenas *P. notatum* é uma espécie nativa do Pampa, enquanto as três outras espécies são exóticas naturalizadas, ou seja, elas ocorrem nos campos independentemente de ação humana, formando populações estáveis (Schneider 2007). *Paspalum notatum* é uma espécie muito comum nos campos do Pampa, sendo considerada a gramínea perene melhor adaptada ao clima e solo do sul do Brasil (Fontaneli et al. 2012). Além disso, é uma espécie forrageira de elevado valor nutricional para a produção de gado, sendo uma das forrageiras mais cultivadas no país. *Eragrostis plana* é uma espécie exótica invasora nos campos do sul do Brasil (de origem africana), com um alto potencial de expansão, principalmente na América do Sul (Barbosa et al. 2013). Essa planta é muito fibrosa e traz problemas em pastagens para a produção de gado, pois apresenta um baixo valor nutricional e pode causar infecções na gengiva dos animais. *Lotus corniculatus* e *T. repens* são espécies utilizadas como forrageiras invernais em pastagens, apresentando alto valor nutricional para o gado (Fontaneli et al. 2012). Essas espécies podem ser encontradas em campos no Pampa, mas não são consideradas espécies invasoras. Os diásporos de *E. plana* foram coletados na Estação Experimental Agronômica da UFRGS em Eldorado do Sul (30°07'10"S, 51°41'06"W) em março de 2013 e os diásporos das outras espécies foram obtidos de fontes comerciais.

Informações adicionais: O ideal seria ter utilizado somente espécies nativas. Isso é algo que inclusive já nos questionaram em artigos. No entanto, é muito raro encontrar sementes de espécies campestres nativas para compra e há várias dificuldades de utilizar sementes coletadas em campo. Primeiramente, a quantidade de sementes necessárias para experimentos de fitotoxidez é alta (em torno de 4.000 sementes foram utilizadas por espécie para os experimentos descritos a seguir). Além disso, muitas espécies precisam passar por

algum tratamento de quebra de dormência e ainda se manterem viáveis por um tempo. Um exemplo foi a leguminosa *Chamaecrista nictitans* (L.) Moench: um número suficiente de sementes foi coletado e foi trabalhoso tirá-las dos frutos e encontrar a forma adequada de quebrar a dormência das mesmas. A quebra de dormência foi possível colocando as sementes em água a 60 °C por 15 minutos – as sementes absorvem água e logo aumentam de tamanho, para as que não aumentarem o processo pode ser repetido. No entanto, em poucos meses, as sementes perderam a viabilidade e não puderam ser utilizadas nos ensaios. Obter sementes campestres nativas com boa germinabilidade seria algo excelente não apenas para estudos de fitotoxidez.

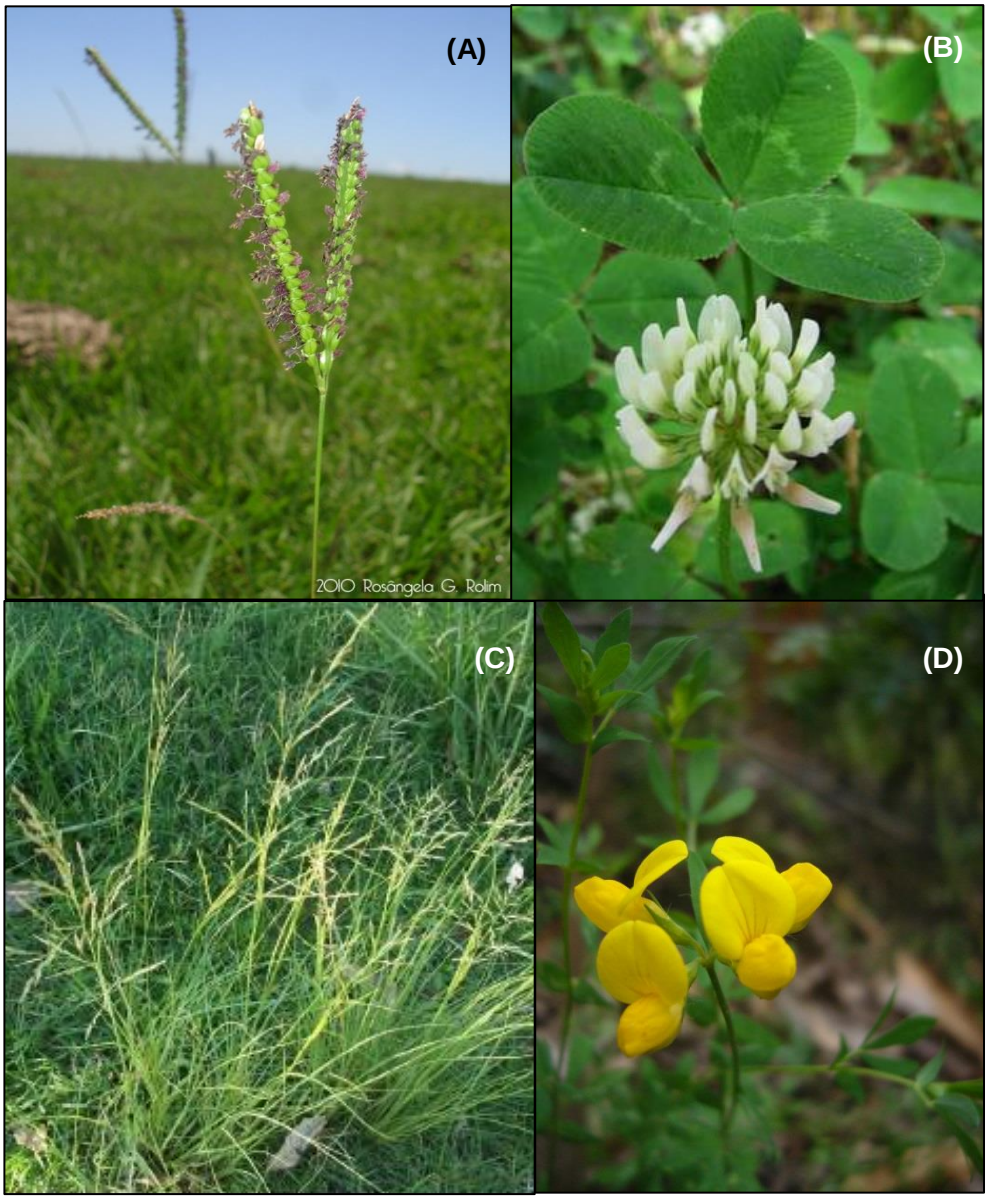


Figura 2: Espécies receptoras expostas aos aleloquímicos de *Eucalyptus saligna*. (A) *Paspalum notatum* (Fonte: http://www.ufrgs.br/fitoecologia/florars/index). (B) *Trifolium repens* (Fonte: http://www.netartsbaytoday.org/). (C) *Eragrostis plana* (Fonte: Gonçalves 2014). (D) *Lotus corniculatus*.

2.1.3 Ensaios de germinação e crescimento de plântulas

Para todos os experimentos, os grupos foram distribuídos de forma aleatória, totalizando quatro repetições (número de placas) por grupo. Nos ensaios em que efeitos do óleo essencial foram avaliados na germinação, cada repetição consistiu em 40 diásporos de uma espécie receptora semeados em uma placa de Petri (9 cm de circunferência) contendo papel filtro umedecido com água destilada. O óleo essencial foi então adicionado a um pedaço de algodão fixado com fita dupla face na tampa da placa, a fim de evitar o contato direto entre os diásporos e o óleo, permitindo a volatilização no espaço dentro da placa (Figura 3-A). Imediatamente, as placas foram vedadas com filme PVC (oito voltas em torno da placa – vedação inadequada poderia ocasionar perda de voláteis). As placas foram mantidas em sala de cultivo com fotoperíodo de 16 h e irradiância de 80,1 µmol m^{-2} s^{-1} fornecida por lâmpadas fluorescentes de 20 W. A sala de cultivo foi mantida a 20 °C, mas para *P. notatum* se utilizou prateleiras na sala que atingem 25 °C, o que torna mais rápida a germinação das sementes da espécie. As quantidades de óleo aplicadas foram 0 (controle), 1, 10, 20, 30 e 50 µL. As sementes germinadas (protusão da raiz) foram contadas durante 20 dias para *P. notatum* e oito dias para as outras espécies. As sementes germinadas de *P. notatum* e *E. plana* foram contadas a cada 24 h, enquanto as sementes de *L. corniculatus* e *T. repens* foram contadas a cada 12 h. A velocidade de germinação acumulada foi calculada de acordo com a fórmula AS=[N1/1+N2/2+…+Nn/n], em que N1, N2, N3, Nn são o número cumulativo de sementes que germinaram na contagem 1,2,3, ..., n (Anjum & Bajwa 2005). A taxa de germinação também foi calculada, consistindo na porcentagem de sementes germinadas até o último dia do experimento.

Para os experimentos nos quais os efeitos do óleo essencial foram testados sobre o crescimento de plântulas, os diásporos foram semeados nas placas e colocados em sala de cultivo até a emergência da raiz e parte aérea das plântulas (144 h para *P. notatum*, 96 h para *E. plana* e 72 h para as Fabaceae). Cada repetição compreendeu 20 plântulas (tamanho similar) transferidas para uma placa e expostas ao óleo essencial de *E. saligna* (0, 1, 10, 20, 30 e 50 µL), nas mesmas condições do ensaio de germinação (Figura 3-B). As placas foram mantidas em sala de cultivo por 96 h, e então as plântulas foram fotografadas e medidas utilizando o programa ImageJ 1.45s. O tamanho da raiz e o da parte aérea foram medidos separadamente. Para as espécies de Poaceae, considerou-se a parte aérea completa e a soma do tamanho de todas as raízes adventícias e, para as Fabaceae, considerou-se a parte aérea, exceto as folhas cotiledonares, e a raiz primária.

Para os experimentos em que os efeitos do extrato aquoso de *E. saligna* foram testados sobre a germinação e no crescimento de plântulas, os métodos foram similares aos descritos para os ensaios com o óleo essencial, mas sem adição do óleo. O extrato (5%,

7,5% e 10%) foi utilizado para umedecer papel filtro nas placas (8 mL) (Figura 3-C, D). Água destilada foi utilizada para o grupo controle. Para *P. notatum*, o qual demora aproximadamente o dobro do tempo para a estabilização da germinação em relação às outras espécies-receptoras, após uma semana um novo extrato foi feito e as sementes foram transferidas para placas contendo esse extrato. Isso foi feito para evitar que a possível degradação do extrato interferisse nos resultados.

Informações adicionais: O tempo do ensaio de germinação para cada espécie foi baseado em ensaios-piloto, em que se observou que após aquele número de dias não havia mudança no número total de sementes germinadas e que provavelmente não haveria mais germinação se o período do experimento fosse extendido. Para as espécies de Fabaceae foram feitas duas contagens por dia porque assim que as primeiras sementes germinavam o número aumentava depressa. Dessa forma, a menor diferença de tempo entre as contagens permitiu caracterizar melhor mudanças na velocidade de germinação.

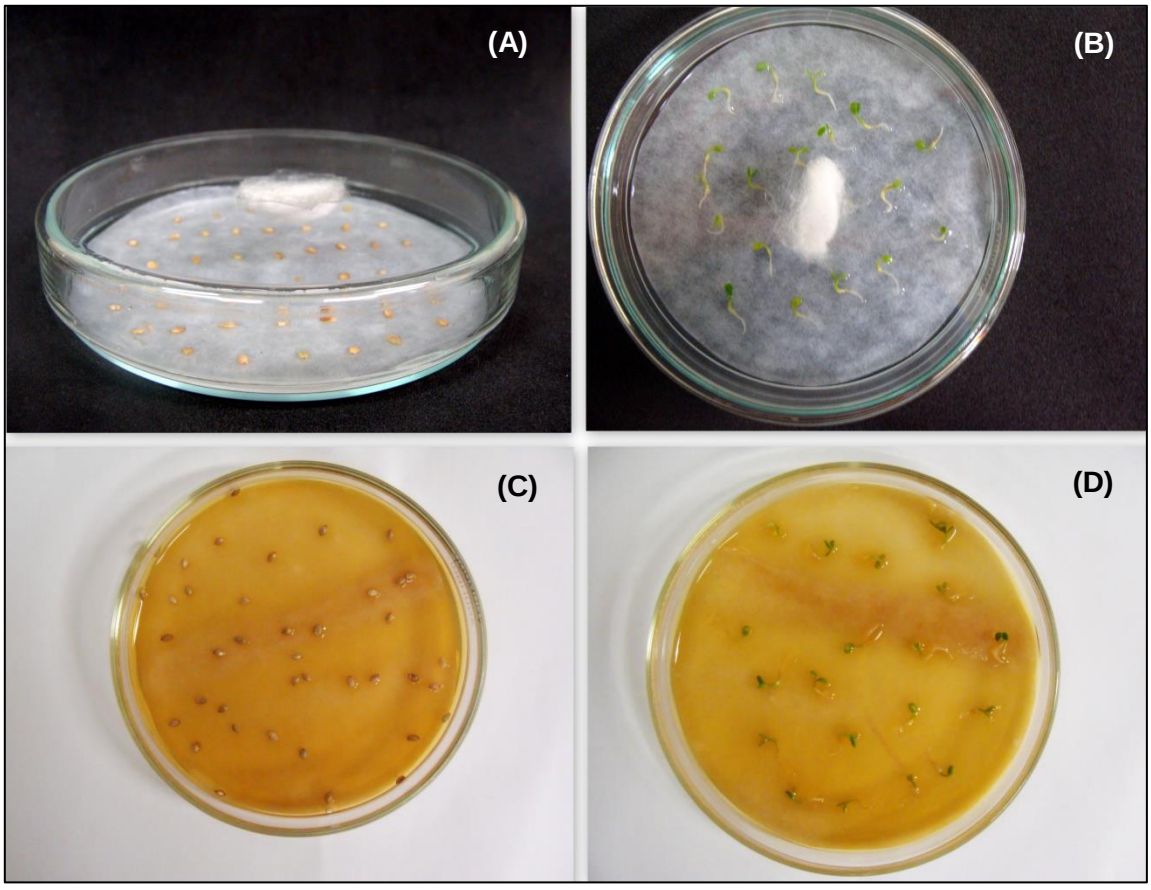


Figura 3: Método de exposição de espécies-receptoras ao óleo essencial e ao extrato aquoso das folhas da serapilheira de *Eucalyptus saligna*, em ensaios de germinação e crescimento inicial utilizando placas de Petri de 9 cm de circunferência. (A) e (B) Ensaio de germinação e crescimento, respectivamente, utilizando óleo essencial. (C) e (D) Ensaio de germinação e crescimento, respectivamente, utilizando extrato aquoso.

2.1.4 Detecção de peróxido de hidrogênio (H_2O_2) em plântulas

Os efeitos do óleo essencial e do extrato aquoso de *E. saligna* sobre o acúmulo de H_2O_2 em plântulas foi avaliado ao final dos experimentos de crescimento. Para a detecção, utilizou-se um método histoquímico adaptado de Thordal-Christensen et al. (1997) que emprega o reagente DAB (3,3′-diaminobenzidina). Após o ensaio de crescimento, seis plântulas por grupo foram separadas. Uma solução de DAB foi preparada na proporção de 1 mg de reagente para 1,5 mL de água (na técnica original é utilizado 1 mg/mL, mas utilizou-se mais porque a água evapora). A solução foi aquecida a 80 °C por 20 min. Após esfriar, o pH da solução foi ajustado para 3,8 (utilizando 1M HCl), o que é necessário para solubilizar melhor o DAB. Em seguida, as plântulas foram colocadas na solução de DAB por 1 h e 15 min. Esse tratamento revela uma coloração amarronzada nas plântulas devido à reação de polimerização do DAB com H_2O_2. Após esse período, as mesmas foram colocadas em álcool etílico a aproximadamente 50 ºC até que perdessem a pigmentação e restasse apenas a coloração da reação com o DAB, o que levou em torno de 3 h. O acúmulo de H_2O_2 foi analisado na parte aérea de *P. notatum*, *T. repens* e *L. corniculatus* considerando a parte aérea completa, incluindo as folhas cotiledonares no caso das Fabaceae. *Eragrostis plana* apresentou manchas escuras (necrose) na parte aérea após exposição ao extrato de *E. saligna*. Como isso poderia mascarar os resultados, a espécie não foi incluída nesse experimento.

A reação ao DAB tem sido utilizada como uma ferramenta qualitativa para caracterizar a produção de espécies reativas de oxigênio (EROs) em plantas, sendo a coloração visível a olho nu classificada de “não visível” a “forte”. Porém, como a visibilidade da reação aumenta com a quantidade de H_2O_2 (Thordal-Christensen et al. 1997), uma abordagem quantitativa foi utilizada para caracterizar de forma mais precisa diferenças na coloração. Primeiramente, as plântulas foram fotografadas com uma câmera digital (Leica® DFC290 HD) integrada a uma lupa (Leica® S6D, aumento 6,3 x). Esses equipamentos foram também integrados a uma fonte de luz (Leica® L2), com a mesma regulação de luz para todos os grupos de cada espécie receptora, e a um computador. O mesmo padrão de exposição foi colocado para todas as fotos, utilizando o programa Leica Application Suite (LAS v. 3.8) (exposure= 66,0, gain=1,7, saturation=1,3, gamma=0,78). Na falta desses equipamentos para tirar fotos padronizadas, uma alternativa é utilizar um cartão de referência para balanço de branco (colocar o cartão ao lado do que vai ser fotografado). A coloração das plântulas foi então determinada a partir das fotos utilizando o programa AxioVision (Rel. 4.9., Zeiss). A parte aérea de cada plântula foi marcada, e valores de vermelho, verde e azul (padrão RGB – red, green e blue) foram determinados para a área total e somados. Assim, um valor médio de coloração foi obtido para cada plântula. Isso permitiu que se considerasse tanto a área

manchada da plântula quanto a intensidade de cor das manchas. Valores menores de RGB caracterizam coloração mais escura, e com isso uma quantidade maior de H_2O_2 pode ser assumida.

Sugestões: (1) Seria interessante avaliar também os níveis de H_2O_2 nas raízes das plântulas. No entanto, as plântulas expostas ao óleo ficaram tão frágeis que praticamente desmanchavam ao manuseio, impossibilitando a análise nas raízes. (2) Há outras técnicas quantitativas para avaliar diferenças em níveis de H_2O_2, como por exemplo, as que envolvem medição de absorbância (Velikova 2000). Neste estudo, não foi possível utilizar esse método pois o mesmo requer uma quantidade bem maior de biomassa. Sugiro que estudos futuros, nos quais mais biomassa de plântulas possa ser obtida, utilizem um método quantitativo em adição ao utilizado, a fim de determinar se diferenças entre os grupos são detectadas por ambas as abordagens da mesma forma.

2.1.5 Efeitos sobre a integridade de membranas das plântulas

Os efeitos do óleo essencial e do extrato aquoso de *E. saligna* sobre a integridade das membranas foram avaliados através da medição do vazamento de eletrólitos das membranas das plântulas. Ao final do experimento de crescimento, plântulas de cada placa (10 g) foram colocadas em tubos com água destilada (50 mL). Como a biomassa de *E. plana* não foi suficiente, os ensaios foram feitos apenas com *P. notatum*, *T. repens* e *L. corniculatus*. Os tubos foram deixados em sala de cultivo por 24 h (para mantê-los em temperatura constante) e a condutividade elétrica do meio (CE1) foi medida utilizando um condutivímetro (Tecnal®, TEC-4MP). As amostras foram congeladas para liberar todos os eletrólitos (o tempo que permanecem congeladas é indiferente), descongeladas, mantidas em sala de cultivo por 24 h, e a condutividade elétrica do meio (CE2) foi medida. O vazamento relativo de eletrólitos (VRE) foi calculado de acordo com a fórmula: VRE (%)=[CE1/(CE1+CE2)]*100.

Informações adicionais: (1) No caso de não ser possível obter 10 g de biomassa por placa, pode-se usar uma quantidade menor, pois o que importa é utilizar 1 g de plantas pra cada 5 mL de água. É preciso apenas ter o cuidado de usar uma quantidade na qual o eletrodo do condutivímetro fique submerso. Também é importante ter cuidado para não danificar as plântulas durante o manuseio. (2) Se for possível obter mais biomassa, raiz e parte aérea podem ser separadas para avaliar vazamento de eletrólitos de ambas as partes.

2.1.6 Caracterização química do óleo essencial

A caracterização química do óleo de *E. saligna* foi feita utilizando um cromatógrafo gasoso acoplado a um espectrômetro de massas (GC-MS) e um cromatógrafo gasoso com detector de ionização em chama (GC-FID) (Hewlett Packard 6890). As análises utilizaram duas colunas capilares HP-Innowax (GC-FID: 30 m x 320 µm x 0,50 µm; GC-MS: 30 m x 250 µm x 0,50 µm), (Hewlett Packard, Palo Alto, USA), com temperatura inicial de 40 °C (8 min) até 180 °C, a 3 °C/min, 180-230 °C a 20°C/min, 230 °C (20 min). As temperaturas do injetor e da interface foram mantidas a 250 °C para o GC-FID e a 280 °C para o GC-MS. O GC-FID utilizou H_2 como gás de arraste e o GC-MS utilizou He, com razão de fluxo de 1,0 mL/min. A dessorção ocorreu no modo split (1:10) e a energia de ionização foi de 70 eV.

Todos os componentes foram tentativamente identificados através de comparação do espectro experimental com espectros de base de dados do espectrômetro de massas (Wiley 275), e também com espectros registrados na literatura (Adams 2001). A porcentagem relativa de cada componente foi obtida diretamente das áreas dos picos cromatográficos, assumindo que a soma de todos os picos eluídos foi 100%.

2.1.7 Controle de pH e de osmolaridade do extrato aquoso

Mudanças no pH e na osmolaridade de extratos de plantas podem afetar espécies-receptoras e, com isso, efeitos de extratos podem ser erroneamente atribuídos a aleloquímicos (Chou & Young 1974, Wardle et al. 1992). Dessa forma, efeitos do pH e da osmolaridade do extrato aquoso de *E. saligna* foram avaliados. O pH do extrato foi medido com um pHmetro (Sanxin® PHS-3D) e a osmolaridade foi medida com um osmômetro de pressão de vapor (Vapro® 5520). O extrato bruto (10%) apresentou osmolaridade 12% superior a da água e pH 25% menor que da água. Para controlar a influência desses fatores, os seguintes grupos foram estabelecidos: controle de pH, consistindo em uma mistura de água e HCl 1M ajustada ao mesmo pH do extrato bruto; e controle de osmolaridade, consistindo em uma mistura de água e polietilenoglicol (PEG 400) na mesma osmolaridade do extrato bruto. Efeitos dos controles de pH e de osmolaridade foram testados na germinação, crescimento de plântulas, níveis de H_2O_2 e vazamento de eletrólitos das plântulas, para todas as espécies-receptoras.

Sugestão: Para facilitar, é possível fazer um tratamento único (com pH e osmolaridade controlados) e comparar com o controle.

2.1.8 Total de fenóis do extrato

O total de fenóis no extrato aquoso de *E. saligna* (5, 7,5 e 10%) foi determinado utilizando o método Folin-Ciocalteu, como descrito em Singleton & Rossi (1965). Uma versão detalhada do método pode também ser encontrada em Waterman & Mole (1994). Uma adaptação foi feita no método a fim de utilizar uma quantidade menor de reagentes. O extrato (100 µL) foi adicionado em um tubo Falcon, seguido por 7 mL de água destilada e 500 µL do reagente Folin-Ciocalteu. Após 5 min, 1,5 mL de uma solução de Na_2CO_3 (20 g de Na_2CO_3 : 100 mL de água, usar um agitador para diluir) foi adicionado à mistura. Em seguida, água foi adicionada até completar 10 mL. Os tubos eram tampados e mexidos quando cada item era adicionado. Os testes foram feitos em triplicata (três tubos por tratamento). Todo o experimento foi feito no escuro (os tubos foram previamente envoltos em papel alumínio e as luzes do laboratório estavam apagadas até mesmo durante a montagem do experimento). As amostras foram mantidas no escuro a temperatura ambiente por 2 h e a absorbância foi lida em 760 nm, utilizando um espectrofotômetro (Biospectro® SP-220). O ácido tânico foi utilizado como fenólico padrão para preparar uma curva de calibração nas concentrações de 0,05; 0,2; 0,5; 0,8; 1,1; 1,4; 1,7; 2,0; 2,3 mg/mL em água. O total de fenóis foi expresso como mg equivalentes de ácido tânico/ mL de extrato. Para esse cálculo, os valores de absorbância das amostras de ácido tânico foram plotados em um gráfico, no qual uma linha de tendência e a equação da reta foram inseridas (**Figura 4**). A equação da reta foi então calculada (y=ax+b) para chegar ao valor em mg equivalentes de ácido tânico/ mL de extrato (x), na qual y é a absorbância da amostra de extrato.

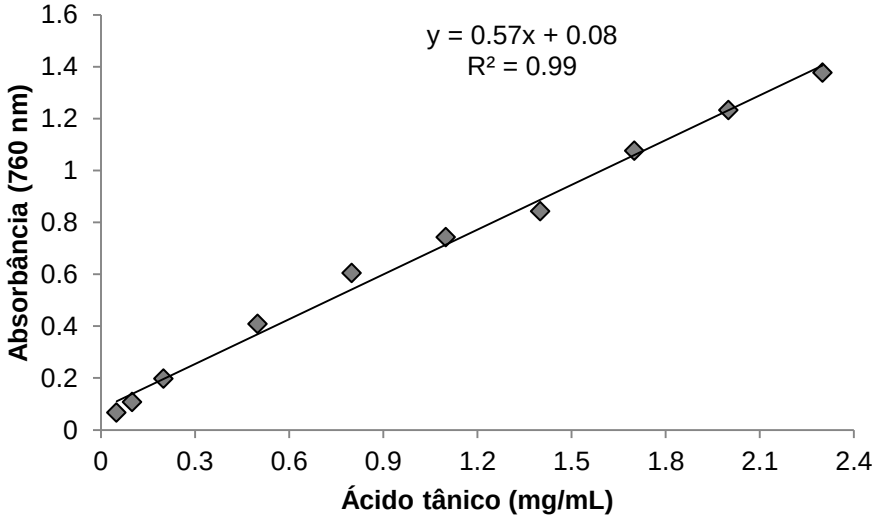


Figura 4: Curva de calibração de ácido tânico, fenólico de referência utilizado para expressar o total de fenóis do extrato aquoso de *Eucalyptus saligna* em mg equivalentes de ácido tânico/ mL de extrato.

Informações adicionais: (1) As concentrações de ácido tânico a serem utilizadas para a curva podem variar devido a diferenças no espectrofotômetro, marca do reagente, etc, por isso é importante fazer ensaios-piloto. Não é preciso repetir a curva de calibração cada vez que for fazer o experimento, mas se demorar muito tempo, aí sim é interessante (supondo que com o tempo os reagentes estejam mais velhos ou o espectrofotômetro esteja com algum problema, o que implicaria em resultados diferentes). É importante utilizar uma gama de concentrações que inclua valores de absorbância superiores e inferiores aos do extrato, e obter uma curva de calibração com R^2 alto (pelo menos 0,9, se for baixo significa que as diluições não foram bem feitas). (2) Ácido gálico pode também ser utilizado como fenólico de referência, mas não foi testado.

2.1.9 Avaliação do envolvimento de fenólicos na fitotoxidez do extrato

Para testar a influência de derivados fenólicos na fitotoxidez do extrato aquoso de *E. saligna*, ensaios foram conduzidos expondo as espécies-receptoras ao extrato bruto (10%) e também ao extrato com concentração reduzida de fenólicos. Para reduzir a concentração de fenólicos no extrato bruto, polivinilpolipirrolidona insolúvel (PVPP) foi adicionada ao extrato (1 g : 100 mL). Essa mistura permaneceu 10 min sobre um agitador magnético. Após o PVPP precipitar (15 min), o sobrenadante foi passado por papel filtro para remover partículas remanescentes. O total de fenóis do extrato antes e depois do tratamento com PVPP foi determinado usando o método de Folin-Ciocalteu, como descrito acima. O PVPP foi adicionado novamente ao extrato até que o total de fenóis não mudasse mais, sendo que para esse extrato a adição de PVPP precisou ser feita três vezes. Os efeitos do extrato bruto e do extrato com concentração reduzida de fenólicos (total de fenóis de 30% em relação ao extrato bruto) foram testados na germinação, crescimento inicial, níveis de H_2O_2 e vazamento de eletrólitos das membranas de *P. notatum* e *L. corniculatus*. Os efeitos foram testados apenas em uma Poaceae e em uma Fabaceae porque todas as espécies-receptoras foram afetadas de forma similar pelo extrato bruto de *E. saligna*, ou seja, praticamente todos os parâmetros foram afetados na maior concentração testada.

Informações adicionais/ o que não deu certo: (1) O reagente tem que ser especificamente o PVPP (com o PVP que é hidrossolúvel não é possível). (2) No caso do extrato de *E. saligna,* não foi possível tirar mais de 70% dos fenólicos. Isso ocorreu porque o PVPP não se liga a substâncias com peso molecular igual ou inferior ao do ácido clorogênico. No entanto, é possível que, para extratos de outras espécies de plantas, o PVPP seja ainda mais efetivo caso a composição inclua poucos fenólicos de peso molecular baixo. Essa técnica consiste em uma ótima alternativa para determinar se o efeito de um

extrato está de fato relacionado a fenólicos (apenas presença de fenólicos não significa relação). (3) Houve uma tentativa de utilizar gelatina como alternativa ao PVPP para reduzir a concentração de fenólicos no extrato. Quando a gelatina foi adicionada ao extrato (1 g em 30 mL) o total de fenóis já foi reduzido em 70% (é possível que se a adição fosse repetida diminuiria ainda mais a concentração, mas isso não foi testado). Entretanto, sementes de algumas das espécies-receptoras foram colocadas em placas com gelatina dissolvida em água e em poucos dias as placas ficaram completamente contaminadas com bactérias, e nenhuma semente germinou. O mesmo não ocorreu com o PVPP quando colocado em água: esse reagente é atóxico para sementes e plântulas, e não causa contaminação.

2.1.10 Análise estatística

Para todos os experimentos, os parâmetros avaliados para cada espécie-receptora (taxa de germinação, velocidade de germinação, tamanho da parte aérea, tamanho da raiz, vazamento de eletrólitos e RGB total) foram comparados entre grupos por análise de variância com permutação (PERMANOVA). Quando a PERMANOVA indicou diferença estatística entre grupos, análise de contrastes foi feita para comparação entre pares (Pillar & Orlóci 1996). As análises foram feitas com 10.000 iterações *bootstrap*, e consideraram um nível de significância de $p \leq 0,05$. A distância euclidiana foi utilizada como medida de semelhança e a estatística do teste foi a soma de quadrados entre grupos (Qb; Pillar & Orlóci 1996). As análises foram feitas no programa Multiv (Pillar 2009).

Sugestão: Exceto por trabalhos do nosso grupo (ex. Lazarotto et al. 2014, Silva et al. 2014), desconheço estudos de fitotoxidez que utilizem PERMANOVA para comparar diferenças entre grupos para dados quantitativos. Normalmente se utiliza ANOVA, mas os dados podem não apresentar normalidade e homogeneidade de variância (é comum encontrar trabalhos em que isso é ignorado, mas não deve ser, já que são pressupostos de análises paramétricas). Alternativas para essas situações são transformar os dados (ex. log) ou fazer testes não paramétricos (ex. Kruskal-Wallis). No entanto, há casos em que mesmo transformar os dados não resolve esses problemas. Dessa forma, recomendo muito a utilização da PERMANOVA. A principal vantagem desse tipo de análise é que os testes de aleatorização não são baseados em pressupostos de normalidade e homogeneidade de variâncias (Anderson 2001), sendo sempre adequados para análise dos dados. Com isso, é muito mais simples fazer a análise, e todos os dados são analisados da mesma forma. Esse tipo de análise apresenta muita acurácia quando se utiliza soma de quadrados e distância euclidiana - embora apresente algumas restrições quando se usam outras medidas de distância e estatística - (Pilar 2013). A única restrição que foi observada é no caso de haver

menos de quatro réplicas e diferenças menos substanciais, em que pode ser mais difícil detectar significância, mesmo quando se sabe que é de fato algo significativo (como, por exemplo, no teste de coleóptilo que será descrito no capítulo IV).

2.2 Metodologia do Capítulo III

2.2.1 Áreas de estudo

Neste capítulo, dois experimentos foram realizados em diferentes locais. O experimento 1 foi realizado em plantios localizados no município de São Gabriel, RS, Brasil (30°53'S 54°51'W e 30°49'S 54°54'W, 180 m a.s.l.), na região dos Campos Sulinos (Overbeck et al. 2007). A matriz em torno dos plantios é de vegetação campestre, sendo que pouco dessa vegetação restou na área. Os plantios compreendem outras espécies além de *E. saligna*, como *E. dunni*, *E. benthami* e híbridos. No interior dos plantios, a cobertura vegetal é muito reduzida (Figura 5A). No entorno dos plantios, há predomínio de pequenas propriedades com cultivo principalmente de soja e trigo, em sistema de rotação. A vegetação campestre remanescente pode ser observada em pequenas áreas abandonadas próximas aos plantios ou nas áreas de preservação permanente (APPs) entre os plantios (Figura 5B), sobre as quais não é feito nenhum tipo de manejo. A vegetação campestre também é mantida em algumas poucas propriedades em que há gado/ovelhas.

O experimento 2 foi realizado em Eldorado do Sul, RS (30°18'S 51°62'W, 135 m a.s.l.), no mesmo plantio onde a serapilheira de *E. saligna* foi coletada para os experimentos referentes ao capítulo II. A matriz desse local é florestal, embora a maior parte da área no entorno consista em outros plantios de *E. saligna* e de outras espécies do gênero. É possível observar espécies florestais nos plantios, sendo a cobertura de vegetação visivelmente superior à observada nos plantios em São Gabriel (Figura 5C). Ambos os experimentos foram realizados em plantios de *E. saligna* implantados em 2008, no qual as árvores apresentavam circunferência à altura do peito (CAP) de aproximadamente 23 cm, plantadas em fileiras a uma distância de 2 m x 3 m uma da outra.

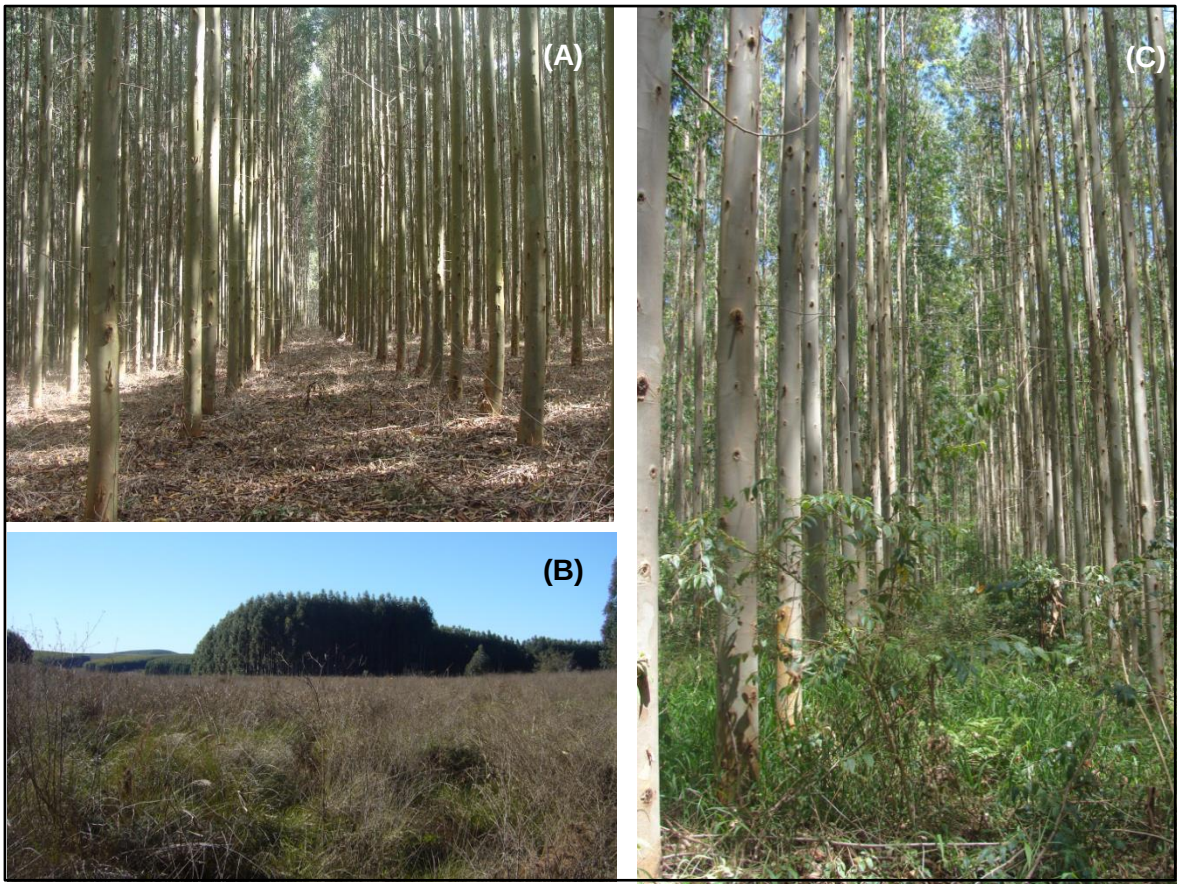


Figura 5. Plantios de *Eucalyptus saligna*. (A) Plantio em São Gabriel – RS (matriz campestre), onde o experimento 1 foi realizado, no qual é evidenciado um padrão de cobertura vegetal reduzida, e (B) área de APP de vegetação campestre entre plantios. (C) Plantio em Eldorado do Sul - RS (matriz florestal) onde foi realizado o experimento 2, evidenciando maior cobertura vegetal em relação à área do experimento1.

2.2.2 Experimento 1: Efeitos da serapilheira de Eucalyptus saligna sobre a comunidade vegetal campestre

O experimento 1 foi delineado para testar os efeitos da serapilheira de *E. saligna* sobre o estabelecimento da vegetação campestre. Esse experimento foi iniciado em janeiro de 2015. Primeiramente, unidades amostrais de 80 x 80 cm foram estabelecidas distantes 40 cm uma da outra, perfazendo seis blocos com quatro unidades por bloco. Desses, três blocos foram situados distantes 200 e 260 m do bloco mais próximo. Há aproximadamente cinco km de distância, os outros três blocos foram situados a 300 e 500 m do bloco mais próximo (Figura 6).

Todas as plantas e a serapilheira foram removidas, sendo feito o corte da biomassa aérea rente ao solo. As parcelas foram então cercadas utilizando tela plástica (30 cm de altura). O tamanho da parcela cercada foi de 1 m x 1 m (unidade experimental), mantendo assim um *buffer* de 10 cm em cada lado das unidades amostrais, minimizando possíveis efeitos do entorno. Quatro grupos foram estabelecidos em cada bloco, consistindo em duas

densidades de serapilheira e em dois controles (Figura 7A). Grupo 1: 100% de serapilheira, consistindo em 720 g de folhas que se encontravam sobre o solo e correspondiam a aproximadamente 7 cm de altura, baseado na média de densidade de folhas encontradas em 1 m² sobre o solo nos plantios. Grupo 2: 50% de serapilheira, consistindo em 360 g de folhas. Grupo 3: 0% de serapilheira, consistindo no controle negativo. Grupo 4: folhas artificiais feitas de etil vinil acetato (EVA), de aproximadamente 16 cm x 4 cm e 1,7 mm de espessura (Figura 7B) (720 g). A serapilheira artificial foi feita para controlar a redução na quantidade de luz sobre o solo e também o impedimento mecânico causado pela serapilheira. O EVA foi utilizado por ser um material não biodegradável que pode ser mantido em campo por um longo período, sendo que uma folha de EVA apresenta peso de 0,38 g, similar ao de uma folha de *E. saligna* (0,31 g). Cada bloco foi cercado com arame, para impedir o acesso pelo gado bovino que ocasionalmente entra nos plantios (Figura 7C).

O estabelecimento da vegetação campestre foi acompanhado trimestralmente (01/2015 – 06/2016) através de estimativa de cobertura total por parcela. A cada acompanhamento, as folhas que caíam sobre as unidades controle eram removidas. Após um ano e meio, todas as espécies foram identificadas em cada parcela, e a cobertura relativa foi estimada através da escala decimal de Londo (1976). A altura da vegetação (máxima) foi aferida a partir da média de cinco medidas, e a cobertura total por parcela foi determinada. Após a amostragem da vegetação, a biomassa aérea vegetal foi coletada. A biomassa foi separada em graminoides (Poaceae e Cyperaceae) e forbs (herbáceas não graminoides), e seca em estufa (60 °C) até massa constante. A radiação fotossinteticamente ativa (PAR) foi medida nas unidades amostrais (a 10 cm de altura) com um quantum sensor (LI-COR LI-250A). Os valores variaram muito em cada bloco (ex. de 25 a 900 µmol m^{-2} s^{-1} ao meio-dia, com variação média de 497 µmol m^{-2} s^{-1}). A PAR foi de 0 µmol m^{-2} s^{-1} sob as folhas de eucalipto e as artificiais, e 1600 µmol m^{-2} s^{-1} fora do plantio (junho, fim do outono).

Figura 6. Blocos de unidades amostrais estabelecidos para o experimento 1 em plantios de *Eucalyptus saligna*, em São Gabriel- RS. No total, seis blocos (P1 - P6) com quatro unidades cada foram estabelecidos.

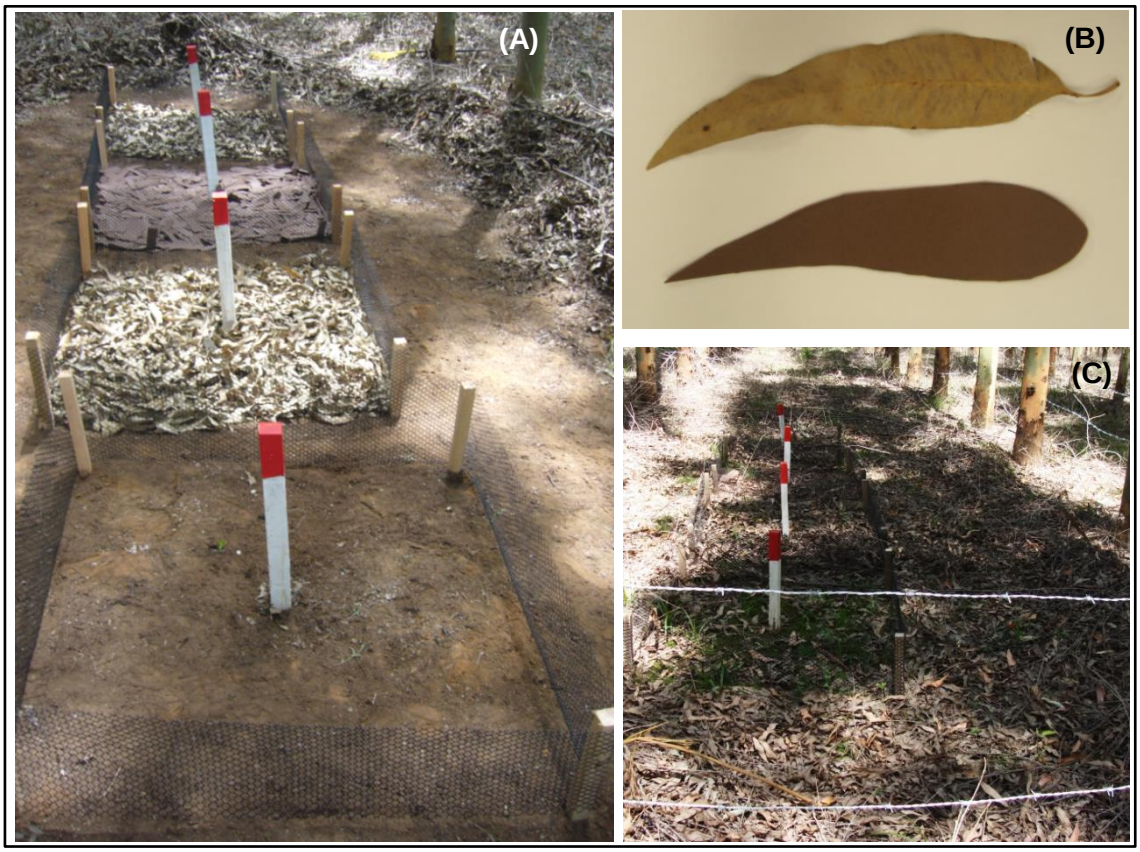


Figura 7. Experimento 1 realizado em plantio de *Eucalyptus saligna* em São Gabriel – RS. (A) Após retirada da vegetação, os seguintes grupos foram estabelecidos (de cima pra baixo): 100% de serapilheira de *E. saligna* (720 g); serapilheira artificial feita de EVA (720 g); 50% de serapilheira (360 g); e 0% de serapilheira. (B) Folha de *E. saligna* e folha artificial feita de EVA. (C) Cerca de arame farpado no entorno de bloco de parcelas, a fim de evitar acesso de gado bovino.

O que não deu certo: A intenção inicial era que neste experimento houvesse blocos também em áreas de campo próximas aos plantios, com os mesmos tratamentos. Antes de adicionar os tratamentos, a vegetação tinha sido caracterizada nas parcelas nos plantios de eucalipto (os dados acabaram não sendo utilizados no artigo) e também em parcelas nas áreas de campo. No entanto, após um mês as folhas e o EVA estavam quase completamente ausentes das parcelas: parte tinha voado para fora, e o que restou estava nos cantos (as folhas estavam bem secas e enroladas). A vegetação já estava bem estabelecida, com cobertura variando de 30 – 70%, as plantas estavam altas e muitas até com flores. Dessa forma, desistimos dessa parte do experimento.

Além disso, os plantios eram cercados de modo que gado não tivesse acesso aos mesmos, portanto parecia não haver necessidade de cercar os blocos. No entanto, seis meses após o início dos experimentos, encontrei gado dentro de um dos plantios. Nessa ocasião, havia algumas parcelas visivelmente mexidas pelo gado (havia também esterco próximo ao bloco), em dois blocos, com a cerca no entorno dessas parcelas retirada. Houve perda de folhas em parcelas com 100% de serapilheira, pisoteio e possível remoção de plantas em parcelas controle. Os blocos foram então cercados com arame farpado, mas no monitoramento seguinte um pequeno animal mexeu em uma das mesmas parcelas controle. Com isso, dois blocos foram removidos das análises, pois possivelmente houve danos a algumas parcelas. Uma árvore caiu sobre uma parcela, mas sem danos consideráveis, e muitas caíram bem perto. Dessa forma, mesmo em um experimento em plantio em uma área cercada, no qual vários fatores podem ser controlados, muitos imprevistos podem ocorrer. Além disso, não esperava que a vegetação levasse tanto tempo para se estabelecer. A ideia tinha sido iniciar o experimento no fim do verão, para que as plântulas que se estabelecessem não estivessem expostas a um calor tão intenso. No entanto, foi no verão seguinte que a maior parte da vegetação se estabeleceu. Como são áreas úmidas (o que notou-se pela quantidade de briófitas e de algumas plantas características de solos úmidos), o calor intenso não foi uma restrição para o estabelecimento das plantas.

A intenção inicial era fazer medidas precisas de luz incidente nas parcelas, o que poderia ser talvez usado como uma variável explanatória nas análises. Porém, a medição de luz foi complicada, uma vez que as árvores são muito flexíveis e os caules e folhas se movem facilmente com o mínimo vento. Com isso, em questão de segundos havia variação muito alta na PAR em um ponto. A presença de nuvens também atrapalha, sendo necessário esperar que elas saiam da frente do sol a cada medida. Em pouco tempo, com o movimento do sol, havia também mudança visível na luminosidade média na parcela. Dessa forma, foi possível apenas estimar valores máximos e mínimos de PAR em cada parcela

considerando apenas um horário do dia. O ideal mesmo seria ter levado o equipamento sempre a campo, até conseguir condições ideais, e repetir isso o máximo de vezes possível.

2.2.3 Experimento 2 – Avaliação dos efeitos da serapilheira de Eucalyptus saligna sobre Paspalum notatum e Lotus corniculatus

O experimento 2 foi delineado para testar os efeitos da serapilheira de *E. saligna* sobre a germinação de sementes e crescimento de espécies campestres. As espécies receptoras semeadas foram *P. notatum* e *L. corniculatus*, representando uma das gramíneas e uma das leguminosas utilizadas nos experimentos de fitotoxidez (Capítulo II). Unidades amostrais de 80 cm x 80 cm foram estabelecidas distantes 40 cm uma da outra, perfazendo seis blocos. Os blocos foram alocados a uma distância mínima de 100 m um do outro, sendo que cada bloco com *L. corniculatus* era mantido há aproximadamente 20 m de um bloco com *P. notatum* (apenas por questão de logística) (Figura 8). Locais onde a vegetação estava mais densa foram evitados, por dificultar a remoção das plantas, que em alguns casos eram arbustivas e arbóreas.

Figura 8. Blocos de unidades amostrais estabelecidos para o experimento 2 em plantios de *Eucalyptus saligna*, em Eldorado do Sul- RS. No total, havia seis blocos de unidades amostrais, onde foram semeadas as espécies *Lotus corniculatus* (Lc1- Lc6) e *Paspalum notatum* (Pn1-Pn6).

Este experimento foi feito em duas etapas. Para a primeira etapa, iniciada em maio de 2015, três unidades amostrais foram estabelecidas por bloco. Todas as plantas e a serapilheira foram removidas, tentando arrancar as plantas por completo. As parcelas foram então cercadas (1 m x 1 m), como descrito no experimento 1. As sementes de

L. corniculatus foram semeadas em cada parcela na quantidade de 1,8 g/m². Essa quantia, que corresponde a aproximadamente 936 sementes, seguiu a recomendação para plantio da espécie. Embora a recomendação seja de 1,2 g/m², por se tratar de sementes peletizadas, as mesmas são mais pesadas e, portanto, optou-se por utilizar um peso maior. De acordo com o fornecedor, a peletização consistiu em inoculação das sementes com a bactéria *Rhizobium* sp., revestimento com carbonato de cálcio e polímeros específicos de dispersão aquosa e tratamento com fungicida Thiram.

As sementes foram cobertas por uma camada fina de solo e regadas com 1,5 L de água. *Paspalum notatum* foi semeada da mesma forma que *L. corniculatus*, na quantidade de 2 g/m², o que corresponde a aproximadamente 1.200 sementes, de acordo com recomendação da Embrapa (Fontaneli et al. 2012). Após a semeadura, os seguintes grupos foram estabelecidos, de forma similar ao experimento 1: Grupo 1: 100% de serapilheira, consistindo em 720 g de folhas que se encontravam sobre o solo. Grupo 2: 50% de serapilheira, consistindo em 360 g de folhas. Grupo 3: 0% de serapilheira, consistindo no controle (Figura 9A). O número de sementes germinadas nas unidades amostrais foi contado em média a cada 45 dias por seis meses (quatro contagens). Essas contagens foram feitas em cinco subparcelas de 20 cm x 20 cm feitas de arame alocadas permanentemente em cada unidade amostral de 80 cm x 80 cm (Figura 9C-D). A cada contagem, as folhas que caíam sobre as unidades controle eram removidas. O número de sementes germinadas, de forma geral, não aumentou a partir da terceira contagem. Após a quarta contagem, aguardou-se 90 dias para garantir que não houvesse mais germinação e então o experimento foi encerrado. Para uma contagem final mais precisa, todas as plantas foram retiradas de cada subparcela e contadas. A parte aérea das plantas das cinco subparcelas foi acondicionada em sacos de papel (um por unidade amostral) para pesagem da biomassa. As plantas foram colocadas em estufa a 60 °C e pesadas até massa constante, o que levou cinco dias. Os valores de PAR variaram muito em todos os blocos (de 41 a 1140 µmol m^{-2} s^{-1} ao meio-dia, variação média de 751 µmol m^{-2} s^{-1} por unidade amostral). A PAR foi de 0 µmol m^{-2} s^{-1} sob folhas de eucalipto e folhas artificiais, e 1800 µmol m^{-2} s^{-1} fora do plantio (medido em agosto, inverno).

Figura 9. Experimento 2 realizado em plantio de *Eucalyptus saligna* em Eldorado do Sul – RS. (A) Fase 1- após retirada da vegetação, os seguintes grupos foram estabelecidos (de cima pra baixo): 0% de serapilheira; 50% de serapilheira de *E. saligna* (360 g); e 100% de serapilheira de *E. saligna* (720 g). (B) Fase 2- após término da fase 1, os seguintes grupos foram estabelecidos (de cima pra baixo): controle com folhas artificiais de EVA (720 g); tela sombrite reduzindo em média 84.6% a incidência de luz; controle já estabelecido na fase 1 do experimento (9 meses sem serapilheira) – controle 1; 100% de serapilheira de *E. saligna*; 0% de serapilheira recém-removida, consistindo nas parcelas em que na fase 1 havia 50% de serapilheira - controle 2. (C) e (D) Subparcelas de 20 cm x 20 cm, no total de cinco subparcelas por unidade amostral, para a contagem plântulas.

A segunda fase (fase 2A) do experimento foi feita nos mesmos blocos da primeira fase, iniciada em fevereiro de 2016. *Paspalum notatum* e *L. corniculatus* foram semeados novamente, como descrito na fase 1, exceto pela quantia de sementes que foi 50% superior a utilizada na fase 1: 2,7 g/m² para *L. corniculatus* e 3 g/m² para *P. notatum*. Nessa fase, cinco grupos foram estabelecidos (Figura 9B). Grupo 1: 100% de serapilheira de *E. saligna*, consistindo nas mesmas parcelas da fase 1, na qual a serapilheira foi retirada para adição das sementes e recolocada. Grupo 2: controle 1- 0% de serapilheira (ausente sobre o solo por nove meses), consistindo no grupo controle estabelecido na fase 1 do experimento. Grupo 3: controle 2 - 0% de serapilheira recém-removida, consistindo nas parcelas em que na fase 1 havia 50% de serapilheira. Grupo 4: serapilheira artificial feita de EVA (720 g). Grupo 5: cobertura com tela sombrite (uma tela de sombrite 75% e duas telas de 50% por parcela), reduzindo em 84,8±9,6% a incidência de luz sobre a unidade amostral. No grupo 1, a serapilheira foi pesada e apresentou valores similares ao inicial (700-800 g), por isso optou-se por manter a mesma serapilheira. No grupo onde antes havia 50% de serapilheira, a quantidade apenas aumentou um pouco ao longo da fase 1 do experimento (400-500 g). Esse controle foi adicionado a fim de detectar se há diferença nos efeitos sobre as plantas em um local sem serapilheira a nove meses, e outro em que a serapilheira acabou de ser removida (podendo assim haver efeitos de aleloquímicos acumulados no solo). Os grupos 4 e 5 foram feitos em novas parcelas adicionadas em cada bloco. O número de sementes germinadas nas unidades amostrais foi contado em média a cada 45 dias por seis meses (quatro contagens), sendo a primeira contagem após um mês, e a última após dois meses, entre 02 - 08/2016, utilizando subparcelas para a contagem, como na fase 1.

A fim de garantir a replicabilidade dos resultados, a fase 2 foi feita novamente (fase 2B), exatamente da mesma forma que a fase 2A, entre 09 e 12/2016. No entanto, um dos tratamentos não foi repetido (controle 2), pois não verificou-se diferença em nenhum parâmetro entre os controles na fase 2A. Como nas fases 1 e 2A a germinação e o crescimento das plantas estabilizou após três meses, optou-se por reduzir o tempo da 2B.

Sugestões: Estudos de alelopatia em campo são fundamentais, mas nem sempre viáveis, pois requerem, em geral, mais recurso financeiro e tempo do que ensaios em laboratório. Contudo, quando não é possível fazer um experimento em campo, existe a possibilidade de simular as condições de campo ao máximo possível em laboratório. Neste estudo, isso não foi possível para o experimento 1, mas foi (pelo menos parcialmente) para o experimento 2 (dados não incluídos no artigo). Solo dos plantios em Eldorado do Sul foi coletado e colocado em potes. Nesses potes, *P. notatum* e *L. corniculatus* foram semeados, e tratamentos semelhantes aos dos experimentos em campo foram adicionados (folhas da serapilheira de *E. saligna*, folhas artificiais, sombreamento utilizando papel pardo, e

ausência de folhas). Os potes foram colocados em sala de cultivo e regados quando necessário por 30 dias. Os resultados foram similares aos observados em campo sobre germinação, biomassa, e mortalidade das plantas. Contudo, não é possível extrapolar que resultados similares se observariam para qualquer espécie doadora, pois as interações em campo podem ser influenciadas por outros fatores bióticos e abióticos. Apesar disso, esses experimentos consistem em algo mais realista do que os ensaios de fitotoxidez padrão em placas de Petri com extratos, podem ser feitos de forma rápida e com um orçamento baixo. Mesmo que os resultados não sejam conclusivos, eles podem dar boas pistas sobre a relevância da alelopatia no ambiente.

2.2.4 Experimento 3 – Avaliação em laboratório da fitotoxidez do solo de plantios de Eucalyptus saligna

O experimento 3 foi realizado a fim de testar se aleloquímicos de *E. saligna* acumularam em solo em níveis fitotóxicos. No plantio em Eldorado do Sul, a serapilheira foi removida e solo foi coletado em 10 cm de profundidade. A coleta foi feita em cinco pontos no plantio e o solo foi misturado. Carvão ativado foi misturado em solo (20 g/kg) (Parepa & Bossdorf 2016), a fim de remover aleloquímicos possivelmente acumulados no solo. Em potes (média de 53 cm de circunferência), foi colocado solo ou solo com carvão ativado (1 kg), com quatro repetições por grupo. Sementes de *P. notatum* e *L. corniculatus* foram semeadas, no total de 30 sementes por repetição. Os potes foram mantidos em sala de cultivo e regados quando necessário para manter a umidade do solo (em média a cada três dias). Após um mês, quando a emergência das plântulas cessou, todas as plantas foram removidas dos potes. As plantas foram contadas e a biomassa aérea foi pesada até massa constante (60 ºC).

Informações adicionais: Algumas ressalvas devem ser feitas sobre experimentos com carvão ativado. No caso deste experimento, as plantas receptoras germinaram e cresceram de forma similar nos tratamentos com e sem carvão. Quando há maior germinação/crescimento em tratamentos com carvão, isso é normalmente tomado como evidência de que o carvão removeu substâncias fitotóxicas. Mas isso pode ser evidência de alelopatia *ou não*. Para certas espécies, e dependendo das características do solo, o carvão pode causar efeitos estimulatórios sobre as plantas por afetar a concentração de certos nutrientes (Lau et al. 2008). Em caso de estímulo em tratamentos com carvão, seria necessário repetir o experimento adicionando carvão em solo onde não há potenciais aleloquímicos, e testar efeitos sobre as espécies receptoras. Se nesse caso não houver diferença entre os parâmetros avaliados, então é possível relacionar os efeitos observados com alelopatia. No entanto, isso é complicado pelo fato de que em um solo diferente

(mesmo que seja, por exemplo, coletado adjacente a um plantio), pode haver diferença na quantidade de nutrientes, matéria orgânica, pH e micro-organismos. Idealmente, poderia ser feita também a caracterização de aleloquímicos no solo antes e após a adição de carvão ativado (embora eu não conheça nenhum trabalho que já tenha feito isso). Outra possibilidade seria, após determinar quais substâncias estão relacionadas à fitotoxidez, determinar se essas substâncias se acumulam no solo próximo à espécie doadora (Jatoba et al. 2016).

Outra questão é que um experimento assim apenas indica efeitos de aleloquímicos que acumularam no solo. No entanto, componentes de óleos essenciais, por exemplo, podem ser degradados muito rápido, em até dois dias (Isman 2000). Com isso, se terpenos são lixiviados da folhagem pela chuva constantemente, e também da serapilheira, e degradam rapidamente, isso estaria sendo desconsiderado. Com esse método, também não é possível simular o fluxo constante de aleloquímicos que podem ser exsudados de raízes. Considerando essas limitações do experimento 2 e 3, para cada forma de emissão de aleloquímicos suspeitada é necessário pensar em uma forma diferente de conduzir os experimentos, e deve-se ponderar que nem tudo que ocorre no campo está sendo simulado.

2.2.5 Análise estatística

Para o experimento 1, a matriz de cobertura de espécies por parcela foi submetida a análise de ordenação para detectar padrões de vegetação. A análise de coordenadas principais foi utilizada (PCoA), baseada na medida de dissimilaridade de Bray-Curtis. Os resultados foram submetidos à reamostragem *bootstrap* (10.000 iterações) para verificar a estabilidade dos eixos de ordenação (Pillar 1999). Para testar os efeitos da serapilheira de *E. saligna* sobre o estabelecimento da vegetação campestre (experimento 1), foram avaliadas diferenças entre grupos na riqueza de espécies, diversidade, composição, biomassa, cobertura e altura da vegetação. Índices de diversidade foram calculados baseados na entropia de Rényi (Anand & Orlóci 1996). A entropia de Renyi de ordens α fornece um perfil dos índices de diversidade mais utilizados, como Shannon (α = 1) e Simpson (α = 2). Para α = 0, o valor de entropia não leva em conta a variação na proporção de diferentes espécies em uma comunidade, e corresponde a um índice de riqueza. O efeito da equitabilidade apenas se estabiliza quando uma ordem α muito mais alta que a de Shannon é utilizada, tal como 12 (Anand & Orlóci 1996). Dessa forma, as ordens α utilizadas foram 0 (índice de riqueza de espécies), 1, 2, e 12 (índices de diversidade). Para testar efeitos mais específicos, as espécies foram separadas em categorias e os valores foram somados por parcela para cada categoria. Essas categorias foram graminoides (Cyperaceae

e Poaceae), forbs (herbáceas não-graminoides), briófitas e arbustos. Porém, diferenças na cobertura de arbustos não foram analisadas porque a única espécie registrada estava presente em apenas 6% das parcelas. Diferenças entre as ordens α, biomassa, altura e cobertura da vegetação (total e por grupo) foram comparadas entre grupos por ANOVA com aleatorização. As diferenças na composição de espécies foram testadas entre grupos por MANOVA com aleatorização.

Em relação ao experimento 2, na primeira parte do experimento, as diferenças entre grupos (100% de serapilheira, 50% de serapilheira e controle) foram avaliadas no número final de plantas e biomassa seca. Na segunda parte do experimento, foram avaliadas diferenças entre grupos (100% de serapilheira, sombreamento, folhas artificiais e controle) na emergência inicial das plantas, número final de plantas, mortalidade e biomassa seca. A emergência inicial foi reportada como o número de plantas na primeira contagem (após três semanas), na qual o maior número de plantas foi obtido para a maioria dos blocos. A mortalidade foi calculada como a diferença entre o número inicial e final de plantas, e expressa como a porcentagem de redução do número inicial. Para o experimento 3, foram comparadas diferenças entre grupos (com e sem carvão ativado) para o número de plantas e biomassa seca. Para o experimento 2 e 3, as diferenças entre grupos foram comparadas por ANOVA com aleatorização, separadamente para *P. notatum* e *L. corniculatus*. Para todos os experimentos, ANOVA (para uma variável) e MANOVA (para conjuntos de variáveis) com aleatorização foram conduzidas utilizando como medida de semelhança a distância euclidiana entre unidades amostrais. Os testes de aleatorização foram conduzidos com 10.000 iterações *bootstrap*. Quando a análise de variância indicou diferenças significativas entre grupos, análises de contraste foram feitas para comparação entre pares. O teste estatístico da ANOVA e da MANOVA foi soma de quadrados entre grupos (Qb; Pillar & Orlóci 1996). Todas as análises foram feitas no programa Multiv (Pillar 2009).

2.3 Metodologia do Capítulo IV

2.3.1 Coleta, extração e caracterização do óleo essencial de Eucalyptus saligna

O óleo essencial de *E. saligna* foi extraído a partir de folhas da serapilheira coletadas em dezembro de 2015, no mesmo plantio onde foram coletadas folhas para a extração do óleo utilizado nos experimentos do capítulo II em 2014. O óleo foi extraído por destilação de arraste a vapor, como descrito no capítulo II. A extração do óleo essencial foi realizada na Universidade de Caxias do Sul.

Os procedimentos descritos neste capítulo foram realizados nos laboratórios do grupo de alelopatia da Universidad de Cádiz, Espanha. A caracterização química do óleo de

E. saligna foi feita por cromatografia gasosa acoplada a espectrometria de massas (GC-MS - Bruker Scion 436, USA). O óleo de *E. saligna* extraído em 2014 também foi caracterizado, para avaliar se ambos apresentavam composição química similar, uma vez que a coluna utilizada na análise do capítulo II era diferente. As análises utilizaram uma coluna capilar SPB-5 (30 m x 250 µm x 0,25 µm) (Bruker, USA), com temperatura inicial de 40 °C (10 min) até 180 °C, a 3 °C/min, 180-300 °C a 10 °C/min, 300 °C (5 min). As temperaturas do injetor e da interface foram mantidas a 250 °C. O GC-MS utilizou He como gás de arraste, com razão de fluxo de 1 mL/min. A dessorção ocorreu no modo split (1:100).

Índices de retenção de temperatura linear programada foram determinados a partir de uma solução de n-alcanos (C7-C25), junto com os tempos de retenção dos componentes das amostras de *E. saligna*. Todos os componentes foram tentativamente identificados pela comparação do seu tempo de retenção com o registrado na literatura (Adams 2007 e http://essentialoilcomponentsbygcms.com/list-of-compounds-in-the-essential-oil-components-database/). Além disso, os espectros de massa experimental foram comparados com os espectros armazenados na base de dados do espectrômetro de massas (NIST) e espectros da literatura (Adams 2001). A porcentagem relativa de cada componente foi obtida diretamente das áreas dos picos cromatográficos, assumindo que a soma de todos os picos eluídos foi 100%.

Informações adicionais: As amostras de óleo essencial foram diluídas em hexano (1:9) para injeção no cromatógrafo. A solução de alcanos foi diluída a 100 ppm em hexano. A caracterização química dos óleos essenciais foi feita inicialmente sob as mesmas condições que no capítulo II, utilizando um cromatógrafo que possuía coluna VF-WAX (parecida com a Innowax, ambas tem fase estacionária de polietilenoglicol), e com a mesma rampa de aquecimento (40 °C (8 min) até 180 °C, a 3 °C/min, 180-230 °C a 20 °C/min, 230 °C (20 min)) e modo Split 1:10. Com isso, a composição química do óleo essencial de 2014 foi muito similar à obtida no capítulo II, e também a do óleo de 2015. Para os óleos de 2014 e 2015 foram obtidos, respectivamente, 32 e 30 picos, dos quais 16 e 15 foram identificados. Para tentar obter uma caracterização mais refinada, foi utilizado o cromatógrafo com a coluna SPB-5, que é menos polar (fase estacionária é 5% difenil/ 95% dimetilpolisiloxano), e o split foi modificado para 1:100 e a rampa de aquecimento para como descrito acima, sendo então mais lento o aquecimento a partir de 180 °C. A melhor resolução foi obtida com essas condições (especialmente para os sesquiterpenos), apresentando mais do dobro de picos, e mais do triplo de componentes identificados. No total, os óleos de 2014 e 2015, respectivamente, apresentaram 69 e 65 picos, dos quais 54 e 53 foram identificados. Além disso, o índice de temperatura linear programada não tinha sido calculado na análise do capítulo II, o que implicou na identificação incorreta de alguns

componentes. A única vantagem de usar a coluna mais polar foi conseguir detectar a presença de limoneno, que provavelmente saiu atrás do pico de eucaliptol na análise na SPB-5. Foi possível ter certeza que a identificação do limoneno na Innowax estava correta quando foram obtidas frações do óleo que eram mais pobres em eucaliptol, e o limoneno apareceu. É importante se basear no índice aritmético, reportado na referência e no site descritos acima. O índice logarítmico (Índice de Kovats) descrito em Adams (2001) não se utiliza para temperaturas programadas de coluna, apenas para condições isotérmicas.

2.3.2 Caracterização de voláteis por microextração em fase sólida (SPME) e GC-MS

Os voláteis emitidos das folhas da serapilheira de *E. saligna,* e do óleo essencial durante o experimento de volatilização foram obtidos por SMPE e caracterizados por GC-MS. Primeiramente, a fibra de SPME (fibra PMDS, 100 µm, Supelco) foi limpa, inserindo a agulha no injetor do cromatógrafo gasoso (a 250 °C), expondo a fibra, e mantendo-a por 2 min. Uma análise simples foi feita por GC-MS com a seguinte rampa de aquecimento: 40 °C (2 min) até 300 °C a 33 °C/min (5 min). Com isso, apenas um pico de nitrogênio foi observado. A análise foi repetida, e nenhum pico foi detectado, confirmando que a fibra estava limpa. Folhas da serapilheira de *E. saligna* que estavam congeladas foram utilizadas. Essas folhas haviam sido coletadas no mesmo momento da coleta de serapilheira para extração do óleo essencial (dezembro de 2015). As folhas foram picadas (1 g) e colocadas em um tubo de ensaio de 25 mL fechado com uma tampa de borracha, onde foram mantidas por 24 h. Um pequeno furo foi feito com uma agulha quente na tampa, pelo qual a agulha foi inserida, e então a fibra de SPME foi exposta por 10 min (Figura 10A). A fibra foi retraída e imediatamente inserida no injetor do cromatógrafo gasoso (Figura 10B). A análise foi feita usando a mesma programação de temperatura usada para o óleo essencial. No entanto, a análise começou imediatamente após a injeção (e não após 3 min), e a fibra foi mantida no injetor por 2 min, para que estivesse limpa para a próxima análise. O mesmo procedimento foi repetido mantendo as folhas por 24 h a 45 °C e expondo a fibra dentro do tubo, e por 48 h a 45 °C. Em todos os casos, o perfil do cromatograma foi parecido com o do óleo essencial, mas os picos ficaram pequenos e muitos não puderem ser identificados. Então, a quantidade de folhas foi aumentada para 3 g, as quais foram maceradas no liquidificador para caberem no tubo. A fibra foi exposta da mesma forma, e o procedimento também foi feito mantendo o tubo a 45 °C por 24 h, e então a 65 °C por mais 24 h.

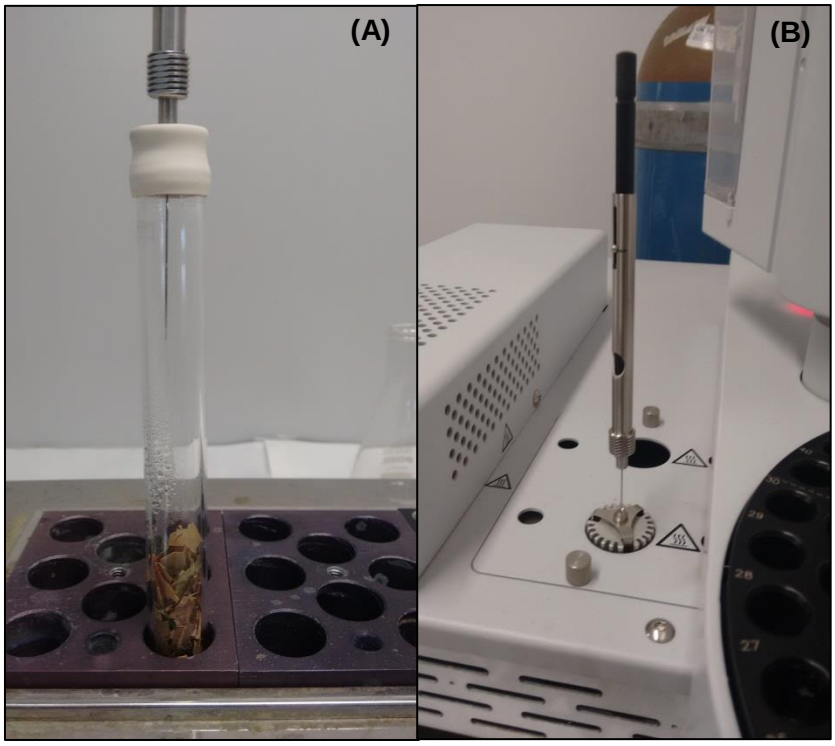


Figura 10. Fibra de SPME exposta a voláteis presentes nas folhas da serapilheira de *Eucalyptus saligna* (A). Após 10 min de exposição da fibra dentro do tubo, a agulha foi colocada no injetor do cromatógrafo gasoso, a fibra foi exposta e mantida no injetor por 2 min (B). A análise química dos voláteis foi então realizada por GC-MS.

Uma simulação de um ensaio de volatilização foi feita para caracterizar os voláteis emitidos a partir do óleo essencial ao longo do período de um experimento. Diásporos de *E. plana* (20) foram semeados em placas de Petri sobre papel filtro umedecido com solução tampão (detalhes desse experimento serão descritos a seguir). O óleo essencial de *E. saligna* (30 µL) foi adicionado em um algodão fixado nas tampas das placas, as quais foram vedadas e mantidas em sala de cultivo (25 °C, 16 h/luz), havendo duas repetições. Previamente, um furo foi feito em cada placa de plástico usando uma agulha quente, e por ele foi inserida a agulha de SPME e a fibra foi exposta por 10 min (Figura 11). A agulha foi colocada no cromatógrafo e a caracterização dos voláteis foi feita como descrita anteriormente. Esse procedimento foi feito 30 min após adicionar o óleo essencial na placa e então a cada 24 h por sete dias. Cada vez que a agulha era retirada da placa, a mesma era fechada (girando a tampa da placa) e vedada com Parafilm®.

Figura 11. Fibra de SPME exposta a voláteis emitidos a partir do óleo essencial de *Eucalyptus saligna* em um experimento de volatilização simulado. O procedimento foi repetido durante sete dias, e a cada dia os componentes absorvidos na fibra foram analisados por GC-MS.

Informações adicionais e o que não deu certo: A caracterização revelou mais componentes utilizando 3 g, mas não houve diferença em relação à temperatura. O tempo que as folhas permaneceram no tubo também não influenciou, pois foram feitas análises 24 h e 72 h depois de colocá-las no tubo a temperatura ambiente, e o resultado foi similar. Em relação ao óleo essencial, inicialmente 30 µL de óleo foram colocados em um tubo de ensaio de 25 mL e a fibra foi exposta por 30 min, mas com isso a fibra captou uma quantidade muito alta de voláteis, e o cromatograma apresentou picos “largos e arrastados”, mais difíceis de identificar, de forma que o ápice do pico ficava mais voltado para a direita, e o valor do tempo de retenção saía mais alto do que o esperado. Isso foi repetido deixando a fibra exposta 10 min e o cromatograma obtido foi bem melhor. Mesmo fazendo com apenas 10 min de exposição da fibra, nas primeiras análises das placas, em que o espaço estava mais saturado de voláteis, os picos saíram dessa forma arrastada. Com isso, os índices de temperatura linear programada calculados tiveram valores mais altos que o descrito na literatura, e acabamos optando por não reportá-los.

Outra ideia foi colocar um padrão interno, a fim de quantificar o que estava volatilizando de fato (já que a quantificação é feita apenas com valores normalizados). Para isso, a ideia foi colocar uma quantidade conhecida (2 µL) de um monoterpeno ou sesquiterpeno que não havia no óleo essencial junto com o óleo no tubo. Dentre os produtos disponíveis no laboratório, procuramos algum que pelo tempo de retenção fosse esperado que eluísse em um local mais “vazio” do cromatograma. Uma tentativa foi utilizar o sesquiterpeno oxigenado farnesol, mas os sesquiterpenos eluem com picos baixos quando obtidos por SPME (por serem menos voláteis que os monoterpenos), e o farnesol simplesmente não apareceu no cromatograma. Outra tentativa foi utilizar o mirceno, que é um monoterpeno hidrocarboneto, mas o pico saiu grande demais e encobriu outros picos.

2.3.3 Ensaio do coleóptilo

O ensaio do coleóptilo consiste em um teste preliminar para verificar se um composto/substância é bioativo. Nesse caso, não significa necessariamente que implique em atividade fitotóxica, mas atividade sobre células (pode, por exemplo, indicar potencial aplicação sobre células cancerígenas).

Para este ensaio, o óleo essencial de *E. saligna* foi diluído em solução tampão com pH = 5,6 (em 1 L de água destilada foi adicionado 2,9 g de fosfato dissódico trihidratado e 10,05 g de ácido cítrico – a quantidade de ácido cítrico pode variar dependendo do pH da água) suplementado com 20 g de sacarose. Dimetilsulfóxido (DMSO) foi adicionado ao tampão (0,5%), a fim de melhor dissolver o óleo. As concentrações de óleo essencial utilizadas utilizadas foram 0,4, 0,2, 0,1, 0,05, 0,025 e 0 mg/mL (controle). O herbicida Logran® foi utilizado como controle positivo nas mesmas concentrações. A concentração inicial (0,4 mg/mL) foi feita pesando o óleo essencial, adicionando a solução tampão, e mantendo em banho de ultrassom por 1 min para uma melhor diluição. As concentrações seguintes foram feitas a partir dessa concentração, com diluições consecutivas em tampão, e a cada diluição utilizando ultrassom. O ensaio foi feito com o óleo essencial de *E. saligna* coletado em 2014 e em 2015. Como os resultados foram similares, a partir de então apenas o óleo de 2015 foi utilizado nos experimentos (tínhamos pouca quantidade do óleo de 2014, por isso visamos utilizar outra amostra nos ensaios).

Sementes de trigo (*Triticum aestivum* L.) foram colocadas para germinar em placas de Petri contendo papel filtro umedecido com água destilada e mantidas em câmara de germinação em ausência de luz (25 °C) por 96 h. Fragmentos de coleóptilo das plântulas foram cortados (4 mm), a dois milímetros do ápice do coleóptilo, usando uma guilhotina Van der Weij (Figura 12A). O processo foi conduzido sob luz verde de segurança (por apresentar valores de radiação fotossinteticamente ativa próximos a zero). Cinco coleóptilos foram colocados em tubos de ensaio contendo 2 mL de cada tratamento, com três repetições por tratamento. Os tubos foram fechados e incubados em agitação constante (6 rpm) em um rotor SC2 Stuart Scientific em ausência de luz (25 °C) por 24 h (Figura 12B). A rotação é importante porque os coleóptilos precisam de certo contato com oxigênio para crescer bem. Após esse período, os coleoptilos foram fotografados e medidos utilizando o programa PhotoMed®. A porcentagem de inibição do crescimento do coleóptilo foi então calculada para cada repetição em relação ao grupo controle, a partir da seguinte fórmula: ((((Ci-Cr)-(Ci-Cc))/(Ci-Cc)) x 100), em que Cr = comprimento inicial do coleóptilo (4 mm), Cr = comprimento médio do coleóptilo na repetição, Cc = comprimento médio do coleóptilo no controle.

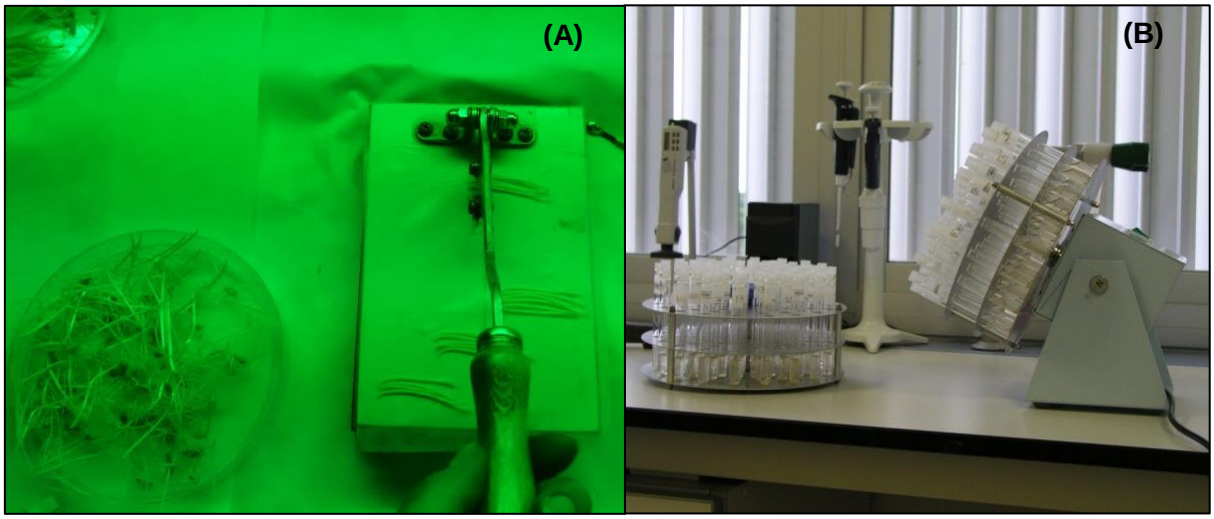


Figura 12. (A) Preparo de coleóptilos de plântulas de trigo com 4 mm de comprimento, a 2 mm do ápice, utilizando guilhotina. (B) Tubos de ensaio colocados em rotor, contendo os coleóptilos expostos aos tratamentos. O rotor foi mantido em câmara de cultivo a 25 °C durante 24 h, em ausência de luz.

Informações adicionais: O ensaio do coleóptilo é um ensaio rápido, simples, e interessante de ser usado quando se tem pouca quantidade do produto a ser testado. Esse ensaio pode indicar quais concentrações serão ativas em testes de fitotoxidez, servindo como um piloto. Pelo que observei (tanto nos meus ensaios com óleo essencial, quanto em ensaios de colegas com produtos distintos), as concentrações que inibem o crescimento de coleóptilo apresentam efeitos similares sobre a germinação e o crescimento de eudicotiledôneas com germinação rápida e homogênea, como alface. Já para gramíneas, concentrações mais altas são necessárias para que efeitos inibitórios sejam observados.

É importante pesar o óleo/componentes ao invés de pipetar quando se realizam ensaios como este, em que pequenas quantidades dos produtos são dissolvidas em água. Isso porque o óleo e os monoterpenos em questão apresentam certa viscosidade, e com isso, por exemplo, 10 µL de óleo essencial pode pesar 9 mg, enquanto 10 µL de α-pineno pode pesar 8 mg, e isso ainda pode variar a cada pipetagem. Para ensaios de volatilização, em que quantidades de óleo são diretamente adicionadas ao algodão nas placas, utilizar peso seria complicado. No entanto, por serem usadas quantidades maiores e com certa diferença entre elas, acredito que essa variação não seja problemática já que o desvio padrão nos parâmetros avaliados costuma ser muito baixo. Um ensaio-piloto foi feito com 0,8 mg/mL, mas em 0,4 já houve inibição de quase 100% no crescimento, então os ensaios posteriores partiram de 0,4 mg/mL. Ensaios também tinham sido feitos com os componentes majoritários do óleo essencial (α-pineno e eucaliptol), mas como não foram obtidas frações suficientes, optou-se por não apresentar esses resultados.

O que não deu certo: Utilizando DMSO em tampão para dissolver o óleo, a solução não ficou translúcida, indicando que não foi uma diluição perfeita. Como uma tentativa de obter melhor diluição, Tween 20 foi utilizado a 0,1%. Para ensaios de volatilização, já utilizei diluição com Tween e funcionou, mas para este tipo de ensaio não deu certo. Isso porque os coleóptilos tiveram seu crescimento inibido no tratamento controle com Tween em relação ao controle sem Tween. Contudo, pode ser que com sementes não ocorresse o mesmo problema (há trabalhos de fitotoxidez que usam Tween diluído em água), mas isso não foi testado. O ensaio do coleóptilo também foi feito sem adicionar DMSO. Os efeitos foram bem similares, mas menos lineares, indicando que o DMSO ajuda um pouco a diluir o óleo, por isso optamos por usá-lo.

2.3.3 Fitotoxidez do óleo essencial de Eucalyptus saligna e seus componentes majoritários

Para compreender a relevância dos efeitos dos componentes majoritários na fitotoxidez do óleo essencial de *E. saligna*, foram feitos experimentos por volatilização, como no capítulo II, e também diluindo o óleo essencial em água. Os ensaios foram feitos com o óleo essencial, eucaliptol, α-pineno e a combinação de eucaliptol e α-pineno proporcional ao encontrado no óleo essencial (54,2% e 45,8%, respectivamente) Os componentes majoritários eucaliptol e α-pineno foram obtidos de fonte comercial (Sigma-Aldrich®).

Para o ensaio de volatilização, efeitos do óleo/componentes foram testados sobre *E. plana*. Apenas uma espécie receptora foi utilizada porque nos experimentos de volatilização do capítulo II, as quatro espécies receptoras foram afetadas de forma similar. Vinte sementes foram colocadas em placas de Petri (5 cm) sobre papel filtro umedecido com uma solução aquosa (em 1 L de água destilada foi adicionado 1,95 g de MES - ácido 2-(N-morfolino) etanossulfônico monohidratado) e 5 g de hidróxido de sódio – quantidade aproximada para obter pH = 6, o que pode variar) contendo DMSO (0,5%), havendo quatro repetições por tratamento. O óleo/componente foi adicionado a um pedaço de algodão fixado com fita dupla face na tampa da placa, a fim de evitar o contato direto entre os diásporos e o óleo, permitindo a volatilização no espaço dentro da placa (como nos experimentos do capítulo II). As placas foram seladas com Parafilm e colocadas em sala de cultivo (25 °C, 16 h/luz). As quantidades de óleo/componente utilizadas foram 30, 20, 10, 5, 1 e 0 µL (controle). Após sete dias as placas foram congeladas e assim mantidas até o momento da análise. O total de sementes germinadas, o tamanho da raiz e da parte aérea foram então medidos utilizando o programa Fitomed®. A taxa de germinação foi calculada como a porcentagem de sementes germinadas.

O experimento em que o óleo essencial foi diluído em água visou explorar a potencial aplicação do óleo essencial com um herbicida natural. As espécies receptoras nesse experimento foram *Lactuca sativa* L., *Amaranthus viridis* L., *E. plana* e *P. notatum*. *Lactuca sativa* (alface) é uma espécie padrão considerada modelo para avaliações de fitotoxidez (Macías et al. 2000). *Amaranthus viridis* é uma espécie daninha em regiões tropicais e subtropicais, e essa espécie exótica ocorre no Brasil em culturas agrícolas e em pastagens. Como mencionado anteriormente, *E. plana* é uma espécie exótica invasora nos campos do sul do Brasil, onde é uma das espécies mais problemáticas em pastagens para produção de gado, enquanto *P. notatum* é uma espécie nativa nos campos e uma espécie desejável em pastagens. Incluir espécies desejáveis no estudo é importante, pois se um produto afetar igualmente todas as espécies significa que sua aplicabilidade é limitada.

Os métodos utilizados no experimento com diluição em água foram similares aos do experimento de volatilização. Porém, o óleo essencial não foi adicionado na tampa da placa, mas diluído em solução tampão (a mesma do experimento de volatilização) com DMSO. Para *L. sativa* e *A. viridis,* as concentrações utilizadas foram as mesmas do experimento do coleóptilo (0,4, 0,2, 0,1, 0,05, 0,025). Essas concentrações foram pouco ativas sobre as gramíneas, então para *E. plana* e *P. notatum* foram utilizadas as concentrações de 1,0, 0,8, 0,6, 0,4 e 0,2 mg/mL. O herbicida Logran foi utilizado como controle positivo nas mesmas concentrações, e o tampão como controle negativo. As placas foram mantidas em sala de cultivo a 25 °C por seis dias para *L. sativa* e *A. viridis*, sete dias para *E. plana* e 14 dias para *P. notatum*. O fotoperíodo foi de 16 h/ luz, exceto para *L. sativa* que foi mantida no escuro.

Informações adicionais: O congelamento das placas ao fim do ensaio é muito interessante porque assim as medidas podem ser feitas a qualquer momento, podendo ficar no congelador por dias ou meses. Além disso, após retirar as plantas do congelador e esperar alguns minutos para que se descongelem, as mesmas ficam maleáveis e é fácil esticá-las para tirar as fotos. Tem-se como padrão no grupo de pesquisa fazer os ensaios em ausência de luz, como foi feito para a alface. No entanto, *E. plana* ficou muito frágil e quebrava facilmente ao manuseio quando o experimento foi feito sem luz, por isso optou-se por usar luz para essa espécie. O experimento de volatilização e de diluição em água foi feito também em ausência de luz para *E. plana* e *A. viridis* com duas concentrações em cada (30 e 10 µL; 1,0 e 0,6 mg/mL), e os resultados foram similares. Com isso, optamos por utilizar luz para as outras espécies também, já que ausência de luz me parece uma condição menos realista. Para *L. sativa*, a ausência de luz foi mantida já que essa é uma espécie padrão, e é interessante ter uma ideia de como seriam os efeitos sobre ela nas mesmas condições que são utilizadas no laboratório, para fins comparativos com outros produtos.

Nos experimentos de volatilização, as concentrações foram mais ativas do que quando os mesmos ensaios foram feitos no capítulo II, pois aqui foram utilizadas placas de Petri bem menores (5 cm ao invés de 9 cm), e 50% menos sementes. Inicialmente, a concentração de 50 µL tinha sido incluída, então o ensaio foi repetido sem essa concentração mais alta e incluindo 5 µL, na intenção de caracterizar melhor até que quantidade se observavam os efeitos de cada tratamento. Dependendo de quão fitotóxico seja um óleo, pode ser necessário diluí-lo para conseguir uma resposta melhor. O herbicida Logran foi utilizado como controle positivo porque era o produto disponível no laboratório e já se sabia como era sua atividade. Mas acredito que em trabalhos com óleo essencial seria bem interessante utilizar cinmethylin, já que é um herbicida derivado do cineol (especialmente no caso de óleos de eucalipto em que 1,8-cineol costuma ser um dos majoritários). Outras gramíneas foram avaliadas também no experimento de diluição em água, *Lolium perenne* L. e *Brachiaria decumbens* Stapf. No caso da invasora exótica *B. decumbens*, as sementes apresentavam uma variação muito grande na germinação (germinavam pouco, e cada uma em um dia diferente, além de crescerem rápido). Então o desvio padrão era tão alto, que só seria possível observar efeitos se os mesmos fossem extremamente fortes. No caso de *L. perenne*, os resultados até foram interessantes, a espécie foi um pouco mais afetada pelo óleo que as outras gramíneas. No entanto, acabamos não incluindo a espécie no artigo porque essa não é uma espécie bem estabelecida no Brasil, é forrageira ou daninha em outros locais. No Brasil, uma espécie do gênero bem estabelecida como forrageira, e também como planta daninha em certas culturas é *Lolium multiflorum* Lam. Mas nos laboratórios em Cádiz não havia sementes dessa espécie, e não tive a ideia de levá-las do Brasil para esses ensaios.

Idealmente, os ensaios de germinação e crescimento deveriam ter sido feitos separados, como no capítulo II. No entanto, dessa forma como é feito nos laboratórios do Grupo de Alelopatía de Cádiz usando o programa é muito mais rápido, tanto os experimentos quanto as medições, pois, ao fazer os experimentos em separado, tem-se o dobro do tempo envolvido, bem como a quantidade de produto utilizado, considerando, ainda, que são duas fases no experimento de crescimento. Eu tinha a intenção de fazer os ensaios exploratórios com várias espécies e testando quais concentrações usar dessa forma mais simples, e depois repetir tudo que tivesse sido mais interessante com germinação e crescimento separado. Mas os seis meses de doutorado sanduíche foram pouco tempo para tudo (nem sequer teria frações suficientes para dois experimentos separados). Em todo o caso, se de fato um dia for possível evoluir para experimentos em campo testando o potencial do óleo como herbicida natural, os resultados podem diferir em solo. Como pré-emergente, o uso do óleo parece pouco provável, já que se decompõe muito rápido no

ambiente; então, teriam que ser feitos experimentos com plantas em estágios de desenvolvimento diferentes. De qualquer forma, recomendo que sempre que possível os experimentos sejam feitos separados, porque senão não há como ter certeza quais são de fato os parâmetros afetados. Os efeitos que parecem ser no crescimento podem ser talvez resultantes de um atraso na germinação (se germina mais tarde, a planta tem menos tempo para crescer até o fim do experimento), além de outras questões, como possíveis alterações no metabolismo das sementes.

2.3.4 Fracionamento do óleo essencial

O fracionamento do óleo essencial foi feito por cromatografia em camada delgada preparativa. Primeiramente, uma cromatografia em camada delgada (CCD) foi feita para determinar qual fase móvel era mais promissora para obtenção de frações. Para isso, o óleo essencial foi misturado em hexano (1:1) e adicionado em uma placa de sílica gel (fase estacionária normal, F_{254}, Merck®). O óleo foi adicionado em dois pontos a 1 cm da base da placa, utilizando um capilar. O solvente (fase móvel) foi colocado em um béquer (a quantidade é indiferente, mas deve ficar abaixo dos pontos onde a amostra foi adicionada), e então a placa foi colocada e o béquer tampado. Quando a fase móvel passou por quase toda a placa (menos o último 0,5 cm), a placa foi observada em UV (254 e 365 nm). Então, a placa foi embebida em um revelador (Oleum - 200 mL de água, 10 mL de H_2SO_4, 40 mL de AcOH) e seca com um secador, revelando o que foi observado no UV. Diferentes fases móveis foram utilizadas, aumentando a polaridade: hexano; 5% de acetato de etila em hexano; 10% de acetato; e 20% de acetato (Figura 13). A fração com 10% de acetato de etila em hexano foi escolhida, a qual revelou aparentemente seis a sete frações.

Para o fracionamento, foi feito algo similar a CCD, mas em escala maior e onde é possível recuperar o produto aplicado: uma CCD preparativa. Placas de sílica gel de vidro (200 mm x 200 mm; F_{254}, fase normal, Merck) foram utilizadas. Primeiramente, as placas foram limpas. Para isso, duas placas foram colocas em uma caixa de vidro onde um pouco de acetato de etila foi colocado (aproximadamente 0,5 cm de altura). Uma folha de papel filtro foi colocada escorada em um dos lados da caixa, o que ajuda a distribuir o acetato de forma homogênea (mas deve ser menor que a altura da placa), e a caixa foi tampada. Após o acetato passar por toda a placa, as mesmas foram secas a temperatura ambiente e o procedimento foi repetido mais duas vezes. É importante marcar qual é o lado de cima da placa, para sempre colocá-la da mesma forma na caixa. Isso foi feito para oito placas. As placas foram secas em estufa a 60 °C (foram deixadas de um dia para outro, já que o procedimento leva o dia todo para quatro placas - mas deixando 1 h seria suficiente para

secar bem a placa). Após secar as placas, a sílica da parte superior das mesmas foi raspada (0,5 cm) - é nessa parte que as impurezas da placa ficam acumuladas.

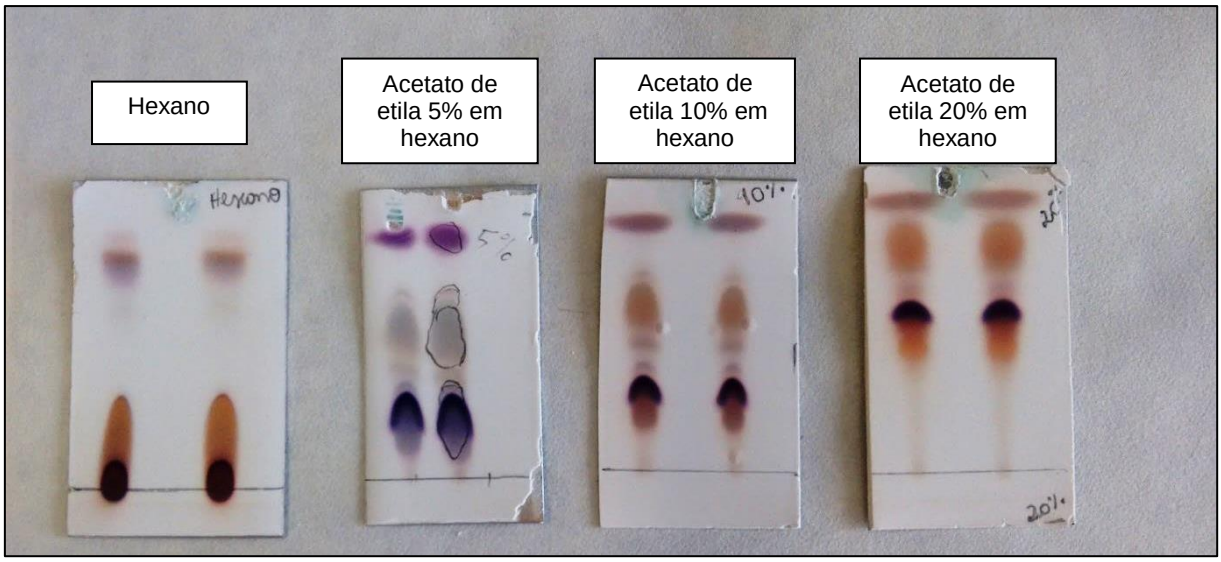


Figura 13. Cromatografia em camada delgada do óleo essencial de *Eucalyptus saligna*, utilizando fase estacionária normal de sílica gel e fase móvel de hexano e acetato de etila, em nível crescente de polaridade da esquerda para a direita.

O óleo essencial diluído em hexano (1:1, 100 µL) foi aplicado a 1 cm da base da placa (linha base) utilizando um aplicador automatizado (Figura 14A). Isso foi feito para garantir uma aplicação homogênea, de forma que depois as frações saíssem retas. A placa foi colocada na caixa de vidro com a fase móvel (uns 0,5 cm de hexano: acetato de etila 9:1) e a caixa foi tampada (Figura 14B). Quando a fase móvel chegou a mais ou menos 1 cm do ápice da placa, a mesma foi retirada da caixa, seca a temperatura ambiente e o procedimento foi repetido. Essa repetição ajudou a separar um pouco melhor as frações. As placas então foram observadas sob luz UV (254 e 365 nm, mas só foi visto algo em 254 nm) (Figura 14C). Ainda sob a luz UV, as frações observadas foram desenhadas com um lápis (um pouco além do limite observado de cada fração pra evitar perda da fração). No total, seis frações foram obtidas. Em seguida, as frações foram removidas com uma espátula bem fina (Figura 14D). A sílica removida foi colocada em um frasco com éter dietílico, separadamente para cada fração. Após, a sílica foi filtrada utilizando papel filtro. A amostra então foi colocada em um balão e seca em rotavapor (a temperatura ambiente, com vácuo, 100 rpm). A quantidade de cada fração foi determinada pesando o balão antes de colocar a amostra, e depois de secá-la. Para saber que a amostra estava seca, o balão foi tirado do rotavapor e pesado algumas vezes até que o peso não mudasse mais. Isso levou apenas alguns minutos, com alguns segundos entre uma pesagem e outra, já que o éter é muito volátil. Uma pequena amostra de cada fração foi retirada (aproximadamente 1 mg), colocada

em 100 µL de éter e caracterizada por GC-MS. Dos 400 µL de óleo essencial aplicados nas placas, 181 mg foram recuperados nas frações (Tabela 1).

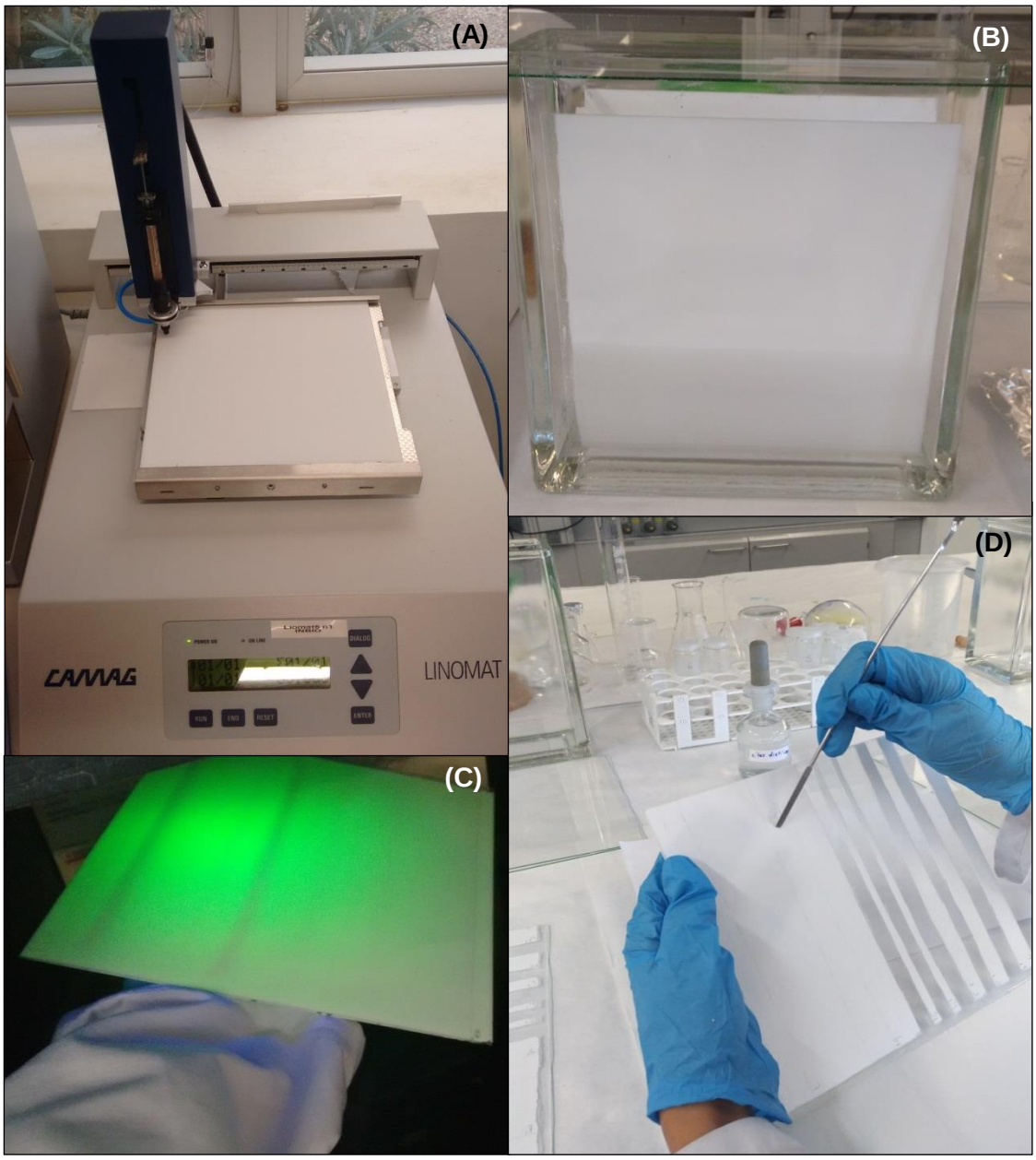


Figura 14. Fracionamento do óleo essencial de *Eucalyptus saligna* por cromatografia em camada delgada preparativa. (A) Injeção automática da amostra de óleo essencial diluída em hexano. (B) Aplicação da fase móvel (hexano: acetato de etila, 9:1). (C) Visualização das frações em 254 nm. (D) Remoção da sílica das placas para a recuperação de cada fração.

O efeito das seis frações foi testado sobre *A. viridis* e *E. plana*, como descrito anteriormente. O óleo foi diluído em solução tampão com DMSO nas concentrações de 0,4, 0,2, 0,1, 0,05 e 0,025 mg/mL para *A. viridis* e 1,0, 0,6, e 0,2 para *E. plana*. Menos concentrações foram usadas para *E. plana* porque não obtivemos quantidade suficiente de algumas frações.

Tabela 1. Quantidade de frações obtidas a partir do óleo essencial de *Eucalyptus saligna* por cromatografia em camada delgada preparativa.

Fração	Quantidade obtida (mg)
1 (fração mais acima na placa)	25,5
2	23,0
3	40,2
4	24,4
5	35,5
6 (fração mais próxima da base)	32,4
Total	181,0

Informações adicionais: Um ensaio-piloto foi feito para obtenção das frações, no qual as frações de algumas placas foram colocadas em hexano, e de outras em éter. A análise por GC-MS revelou cromatogramas muito similares, então por isso optou-se por diluir em éter, por ser bem mais rápido secar as frações, e com isso haveria menos possibilidade de perder amostra no vácuo do rotavapor. No piloto não foi possível estimar bem quantas placas seriam necessárias para obter uma boa quantidade das frações e havia pouco tempo para fazer muitas mais. Isso porque no piloto fui menos cuidadosa na filtragem e passou sílica para alguns balões, então não foi possível saber exatamente quanto havia em cada fração. Um ensaio de volatilização com essas frações só seria possível se o óleo fosse diluído em tampão, como foi feito neste experimento (a quantidade foi calculada e o tampão com DMSO foi colocado diretamente no balão), e depois pipetado e adicionado ao algodão em diferentes quantidades. Isso porque o óleo não fica acumulado no fundo do balão, mas sim, aderido às paredes. Não sei quantas placas precisariam ser feitas para conseguir que acumulasse no fundo do balão uma quantidade que pudesse ser pipetada pura, mas certamente bem mais que o dobro. Além das seis frações, se pretendia recuperar também uma sétima, na linha base (aparecia uma faixa fraca no UV), mas no GC só se observou poucos picos bem baixos e difíceis de caracterizar, então optamos por não incluí-la.

O que não deu certo: A ideia inicial era obter frações bem separadas, cada uma com uma classe de componentes, mas o resultado foi bem diferente (apesar que mesmo assim os resultados foram bem surpreendentes e interessantes a meu ver). Muitos componentes se repetiram nas frações obtidas, mesmo que em quantidades diferentes, e os monoterpenos hidrocarbonetos que são em geral mais voláteis praticamente desapareceram (mesmo o α-pineno que era um dos majoritários). Fracionamento de óleo essencial não é algo muito comum de encontrar na literatura, mas em alguns casos que parece ter dado

certo (pelo menos não houve tanta perda ou repetição de componentes) foi através de um fracionamento com fluído supercrítico ou de um fracionamento já durante o processo de extração do óleo por destilação. Até considerei usar CO_2 supercrítico, mas os professores da UCA que trabalham com esse tipo de extração já tentaram com alguns óleos essenciais e não conseguiram (como tinha pouco tempo, descartei então a opção). Pensando na destilação, surgiu a ideia de fazer uma microdestilação. Pretendia-se ir aquecendo o óleo aos poucos e os componentes mais voláteis iriam condensar primeiro, então a cada mudança de temperatura novas frações iriam sendo coletadas. Curiosamente, os voláteis não volatilizaram. Depois de várias tentativas, desistimos - *no salió bien*.

Detalhes da microdestilação (Figura 15) - Óleo vegetal foi colocado em um cadinho sobre uma placa de aquecimento. O óleo essencial (2 mL) foi colocado em um balão pequeno. Sobre ele foram colocadas colunas de vidro e a direita um condensador foi conectado, com um sistema de resfriamento (água gelada passava pelo condensador através de uma mangueira conectada a um banho de gelo a 2 °C). Após o condensador havia uma conexão com cinco pequenos balões, a qual deveria ser girada cada vez que uma nova fração fosse coletada para um dos balões. Esse sistema estava conectado com vácuo, que primeiramente foi mantido desligado. A placa foi colocada inicialmente a 60 °C. Após 40 min, o óleo não tinha começado a condensar. A temperatura foi aumentada para 100 °C, e seguia não condensando. Uma das colunas sobre o balão foi removida a fim de que fosse mais rápido, mas não resolveu. Papel alumínio foi colocado em volta do balão e colunas pra manter a temperatura no sistema que vinha antes do resfriamento, mas também não adiantou. Após 1 h a temperatura foi aumentada para 140 °C, e continuava não condensando. Vácuo foi colocado, e então o óleo foi puxado pelo sistema, mas foi tudo muito rápido. O óleo foi coletado em diferentes tubos (6) conforme certa quantidade chegava ao fim do sistema, mas na análise por GC-MS os cromatogramas foram muito parecidos. Muitos componentes desapareceram do óleo essencial, especialmente os sesquiterpenos (praticamente só se observou o α-pineno e o eucaliptol, com alguns monoterpenos oxigenados quase indistinguíveis).

Em uma segunda tentativa, foi colocado vácuo reduzido - no balão onde foi colocado o óleo, uma pipeta pasteur foi conectada com a ponta queimada de forma que ficasse muito fina, pela qual saía o mínimo de ar, e na outra ponta do sistema o vácuo foi ligado. Mas mesmo aumentando até 80 °C, o óleo não condensava, e não queríamos aumentar mais para o óleo não degradar. Então, vácuo normal foi colocado, e partes do sistema foram lavadas com hexano, as quais foram separadas como frações. Cromatogramas similares aos da destilação anterior foram obtidos.

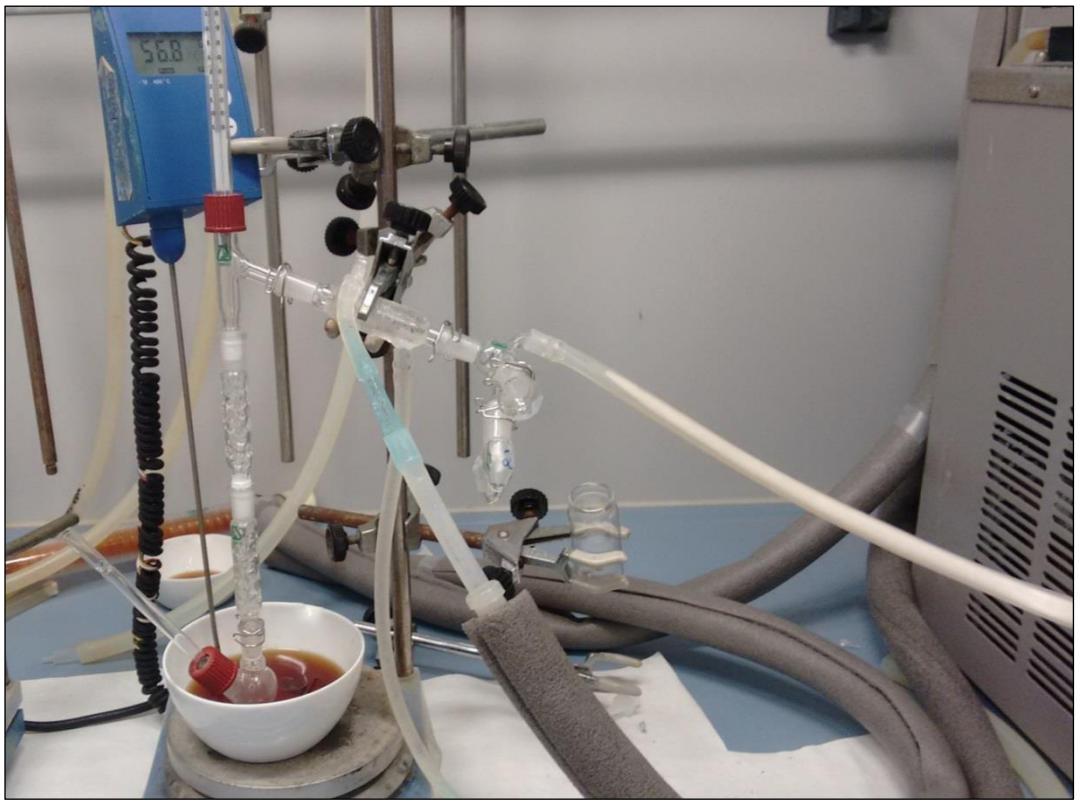


Figura 15. Tentativa de fracionamento do óleo essencial de *Eucalyptus saligna* por microdestilação.

2.3.5 Análise estatística

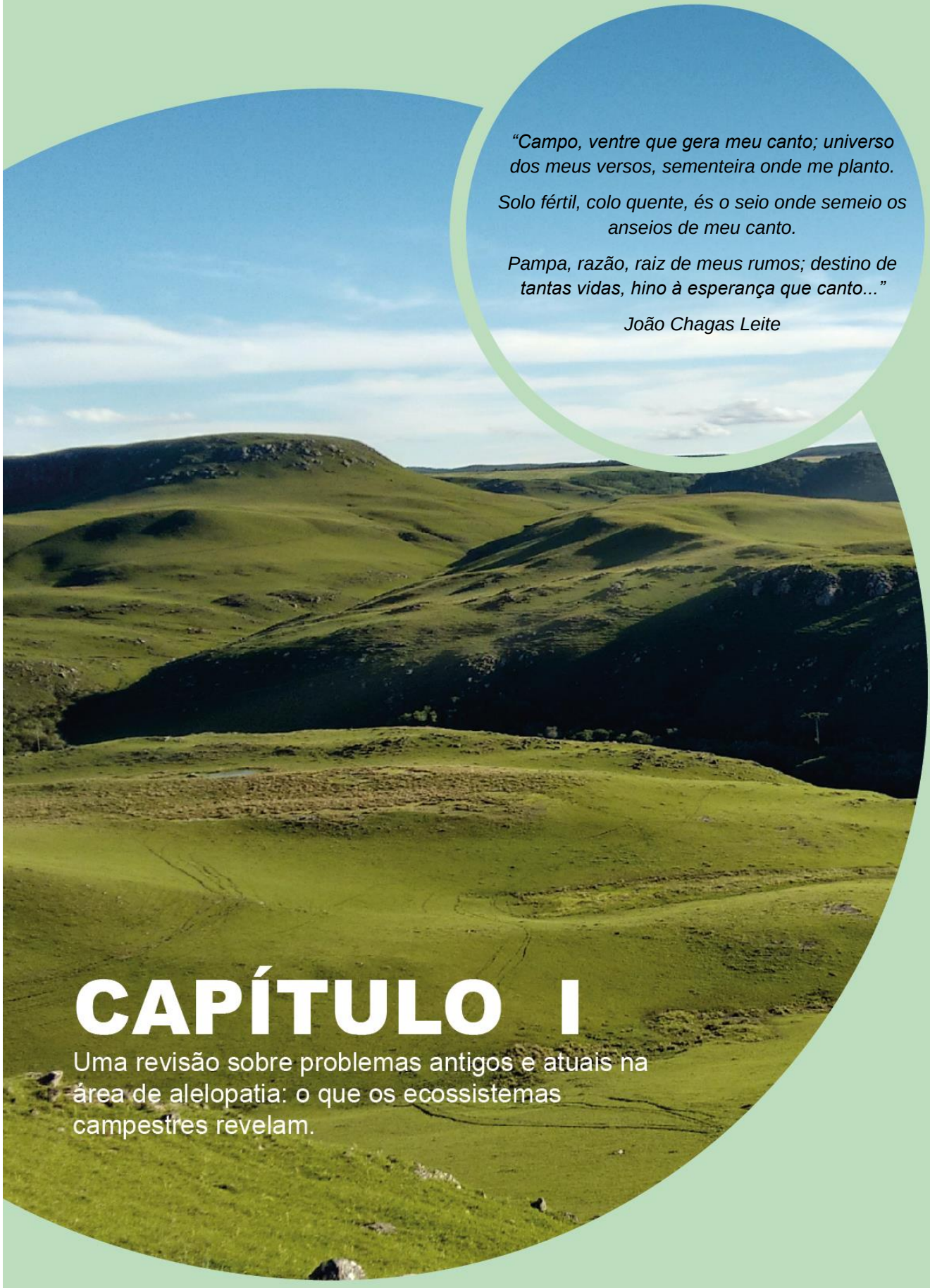
Diferenças entre os parâmetros analisados (tamanho do coleóptilo, taxa de germinação, tamanho da raiz e tamanho da parte aérea) foram comparadas entre os grupos através de análise de variância (ANOVA), para cada espécie. Quando as diferenças foram significativas ($p \leq 0,05$), a ANOVA foi seguida do teste de Tukey para comparação entre pares. Nos casos em que os dados não seguiram os pressupostos de normalidade e homogeneidade de variância (testado pelos testes de Shapiro-Wilk e Lavene, respectivamente), foi utilizada análise de variância com aleatorização (PERMANOVA). Quando diferenças significativas foram observadas, análise de contrastes foi feita para comparação entre pares. A PERMANOVA foi feita com 10.000 iterações *bootstrap* e utilizou distância Euclidiana como medida de semelhança.

“Campo, ventre que gera meu canto; universo dos meus versos, sementeira onde me planto.

Solo fértil, colo quente, és o seio onde semeio os anseios de meu canto.

Pampa, razão, raiz de meus rumos; destino de tantas vidas, hino à esperança que canto...”

João Chagas Leite



CAPÍTULO I

Uma revisão sobre problemas antigos e atuais na área de alelopatia: o que os ecossistemas campestres revelam.

SOMETHING OLD, SOMETHING NEW IN ALLELOPATHY REVIEW: WHAT GRASSLAND ECOSYSTEMS TELL US

Eliane Regina da Silva, Gerhard Ernst Overbeck, Geraldo Luiz Gonçalves Soares

Chemoecology 27(6): 217–231, 2017. doi: 10.1007/s00049-017-0249-x

Resumo

A alelopatia pode ser um fenômeno relevante na dinâmica de campos, um bioma que apresenta uma grande biodiversidade e fornece serviços ecossistêmicos únicos. Pesquisas sobre alelopatia têm sido conduzidas em todo o mundo, e sugestões importantes para melhorar os estudos foram feitas recentemente. Contudo, poucos padrões gerais foram estabelecidos até agora. Nesta revisão, nós reportamos e discutimos pesquisas sobre alelopatia conduzidas em ecossistemas campestres. Nós realizamos uma pesquisa sistemática sobre estudos de alelopatia em campos e avaliamos aspectos descritivos, metodológicos e conceituais em cada artigo. Além disso, nós avaliamos se a qualidade da pesquisa melhorou nos últimos anos. Nós observamos que os estudos investigaram principalmente interações envolvendo espécies herbáceas, em muitos tipos de campos por todo o mundo. Os estudos avaliaram o potencial da alelopatia em estruturar campos naturais e pastagens artificiais, e de aplicar a alelopatia ao desenvolvimento de herbicidas naturais e à restauração de ecossistemas naturais. Nós observamos inconsistência em terminologia e discutimos a definição de alelopatia. Além disso, nós percebemos que recentemente os trabalhos sobre alelopatia melhoraram em algumas questões de desenho experimental, mas não em outras. Isso demonstra que nem todas as recomendações na literatura têm sido consideradas. Por outro lado, métodos inovadores e ferramentas analíticas surgiram. Apesar do progresso lento, a alelopatia tem demonstrado uma potencial relevância na dinâmica de campos, assim como no manejo de plantas daninhas em sistemas cultivados. Dessa forma, um maior conhecimento sobre alelopatia pode levar a avanços na ciência e em áreas aplicadas.

Palavras-chave: desenho experimental; dinâmica de ecossistemas; fitotoxidez; interações entre plantas; manejo de plantas daninhas; terminologia.

Abstract

Allelopathy may be a relevant phenomenon in the dynamics of grasslands, a biome that presents high biodiversity and provides unique ecosystem services. Research on allelopathy in grasslands has been conducted worldwide and important suggestions to improve studies have been made in the recent past. However, few general patterns have been established so far. In this review, we report and discuss allelopathy research conducted in grassland ecosystems. We carried out a systematic search for allelopathy studies in grasslands and assessed descriptive, methodological, and conceptual aspects of each article. We also evaluated if research quality has improved in recent years. We found that the studies investigated interactions mostly involving herbaceous species in many types of grasslands around the world. The studies have assessed the potential of allelopathy in structuring natural grasslands and artificial pastures, and of applying allelopathy to bioherbicide development and to restoration of natural ecosystems. We observed inconsistency in terminology and discussed allelopathy definition. Moreover, we found that in recent years, allelopathy research has improved in some experimental design issues, but not in others. This shows that not all recommendations in literature have been taken into account. Otherwise, innovative methods and analytical tools have emerged. In spite of slow progress, allelopathy has shown potential relevance in dynamics and restoration of grasslands, as well as in weed management in cultivated systems. Thus, a better knowledge about allelopathy can lead to advances in science and in applied fields.

Keywords: ecosystem dynamics; experimental design; plant-plant interactions; phytotoxicity; terminology; weed management.

1 Introduction

Allelopathy has been shown to play a relevant role in inhibiting or promoting plant species establishment and development in many natural and cultivated systems. In natural ecosystems, allelopathic interactions can assume great importance even at large temporal and spatial scales (Inderjit et al. 2011). Furthermore, the use of allelopathy in weed management has been considered promising (Aslam et al. 2017; Belz 2007; Bhowmik and Inderjit 2003; Macías et al. 2007). One of the major expected applications consists in developing natural herbicides (bioherbicides), which could replace synthetic ones (Macías et al. 2001; Soltys et al. 2013). In order to distinguish these contrasting approaches in allelopathy research, Reigosa et al. (2013) proposed a compartmentation in three categories.

These categories include: “allelopathy *sensu stricto*”, which corresponds to studies that aim at elucidating ecological interactions in natural ecosystems; “applied allelopathy”, which follows commercial and economic criteria and focuses on interactions especially between cultivated species; and a third approach that aims at identifying bioactive molecules that could be used to develop new herbicides and plant growth regulators. In another line of work, allelopathy has been evaluated regarding its use in ecological restoration (e.g. Tian et al. 2007).

Research in allelopathy has long faced criticism. One frequently made point is that very often species have been considered allelopathic only based on experiments under controlled settings, without evaluating interactions in the field (Inderjit and Weston 2000). Another reason for criticism is that many laboratory assays have been conducted under conditions that are not close to natural ones. In some excellent reviews, a number of common problems in research methods have been highlighted, such as the use of inadequate substrate, inadequate recipient species and lack of controls (Inderjit and Callaway 2003; Inderjit and Dakshini 1995; Inderjit and Nilsen 2003; Inderjit and Weiner 2001; Inderjit and Weston 2000). These reviews, among others, provided suggestions to improve allelopathy research. In recent years, the amount of allelopathy studies has increased considerably and different methodologies have been used (De Albuquerque et al. 2011; Scognamiglio et al. 2013). However, it is not known if suggestions to overcome problems in experimental design were assimilated to increase research quality.

Grassland ecosystems have been especially in the focus of allelopathy studies (Chou 1999; Lipinska and Harkot 2007; Smith 1999). Prominent examples are the invasive forb *Centaurea stoebe* (May and Baldwin 2011; Ridenour and Callaway 2001) and the aromatic *Artemisia* shrubs (Araniti et al. 2016; Halligan 1973). Grassland vegetation occurs worldwide and is broadly defined to be dominated by herbaceous species, including grasses, legumes and other forbs, and at times woody species at rather low cover values (Allen et al. 2011). In addition to their unique biodiversity, many grasslands provide important services, such as production of livestock forage (Gibson 2009). Allelopathy may be relevant for a better understanding of the dynamics of these ecosystems, but also from an applied perspective, such as for weed management in pastures. Thus, by comprising studies conducted under different approaches, in ecosystems distributed around the world, studies in grasslands may offer a good prediction of general patterns in allelopathy research.

Here, we present a systematic review of allelopathy, in which quantitative data were used to evidence general and current trends in allelopathy research in grasslands, with a focus on used methods as well as underlying concepts. We included in the search all papers dealing with grassland ecosystems/species that followed clear criteria and evaluated 16

descriptive, methodological and conceptual aspects of each study. We separated our data into the last decade and the period before it, in order to be able to evaluate if suggestions that were given to improve allelopathy research have been actually considered.

2 Methods

We searched articles in Scopus (https://www.scopus.com), using the terms “allelopathic” or ”allelopathy” in combination with the terms “grassland” or “meadow” or “prairie” or “pasture” or “rangeland” or “savanna”. These terms had to appear in article title, abstract or keywords. The search resulted in 261 articles, which had been added to Scopus until July 1st, 2016. To be considered in our analyses, articles had to follow clear criteria: only direct or indirect interactions between plants were evaluated; the article was not a review or a book chapter; at least one phytotoxicity/allelopathy experiment or evaluation was conducted in the study. From our pool of 261, 152 articles were selected. Among these, 10 could not be obtained, and 12 were inaccessible due to the language (articles in English, Portuguese and Spanish were accessible to us). Besides the 130 obtained articles, we included more 13 articles that we previously knew, which evaluated allelopathy in grasslands (see Online Resource 1 for a reference list). These articles were not found in Scopus because one of the terms was not present in title, abstract or keywords, even though they were present in other parts of the text.

We classified the information of the 143 articles according to 16 aspects (Table 1). This included some descriptive information about the study region, the donor and recipient species, and the study approach (1-5). It also included aspects of the experimental design (6-14) and information about conclusions and the concept of allelopathy (15, 16).

Table 1. Data recorded in studies that evaluated allelopathy/phytotoxicity in grassland ecosystems, and established categories. The search was conducted in Scopus database and included 143 articles

Evaluated aspect	Categories
1 Study region	South America; North America; Oceania; Africa; Europe; Asia.
2 Donor species’ origin in relation to the study area	Native; exotic.
3 Donor species’ life form	Herbaceous monocotyledon; herbaceous dicotyledon; shrub (including subshrub); tree; fern.
4 Recipient species’ life form	Herbaceous monocotyledon; herbaceous dicotyledon; shrub (including subshrub); tree.

Evaluated aspect	Categories
5 Study approach	Ecological; applied to weed management; applied to herbicide development; applied to ecological restoration.
6 Study system	Laboratory; greenhouse (including glasshouse, shadehouse, screenhouse, common garden); field (natural or artificial).
7 Control	Included in laboratory assays; lack in laboratory assays; Included in field or greenhouse (including glasshouse, shadehouse, screenhouse, common garden) evaluations; lack in field or greenhouse evaluations.
8 Donor plant's material	Leaf; root; stem (including branches, bark, twigs); fruit; flower; shoot; whole plant; seed; litter; pure compound.
9 Allelochemical obtainment	Aqueous extract (plant tissues remaining in water for a given period); organic solvent extract (also including water + organic solvent); leachate (water passed by plant) or percolate; hot aqueous extract; plant tissue in/on/over substrate; donor species' soil; living donor species; essential oil; fog, rain or dew.
10 Effect type	Inhibitory; stimulatory; both inhibitory and stimulatory; no effects.
11 Affected parameter (inhibitory and/or stimulatory)	Germination; growth (length, biomass); physiologic (photosynthesis, stomatal conductance, wilting, chlorophyll content); morphologic (darkening, withering, abnormalities); plant community/population parameters (diversity, richness, composition, density, cover); survival; mycorrhizal interactions (nodulation, spore germination, colonization).
12 Selection of recipient species	Adequate to the study approach; not adequate (see text for details).
13 Substrate type	Filter paper (including other paper types); natural substrate (soil); artificial substrate (including sand, vermiculite, peat, perlite, pumice, mixed or not with natural soil); solid medium.
14 Chemical analysis	Characterization of main classes (e.g. terpenes, phenolics); characterization of chemical compounds; fractionation or isolation of compounds; no chemical analysis.
15 Main conclusion	Allelopathic; allelopathic potential; not allelopathic; low allelopathic potential; phytotoxic; herbicidal potential; others (another conclusion not related to allelopathy or no conclusion).
16 Consistency in the use of the term allelopathic	Yes; no.

In relation to the study region (1), when studies were not conducted in the field, but in a controlled environment, we considered the place to be where the donor species was collected, or the place where the greenhouse experiment was carried out. In many studies, not all information about the donor and recipient species was provided (2-4), and we obtained information from literature. In few cases we found that a species could present more than one life form, so we classified it according to the higher level (e.g. a plant that can be a shrub or a tree was classified as tree).

We categorized the study approach (5) according to information in the article abstract and introduction, based on the classification of Reigosa et al. (2013). The category "ecological" corresponded to allelopathy *sensu stricto*; "applied to weed management" corresponded to applied allelopathy; and "applied to herbicide development" was similar to the third approach proposed by the authors. In addition, for restoration studies, which present both ecological and applied components, we established the category "applied to ecological restoration".

Regarding the controls (7), studies were first categorized in laboratory and field/ greenhouse studies. Then, they were classified as included or lacking in each category. The lack of a control consisted in absence of a negative control (e.g. water in case of aqueous extract, or an organic solvent when it was used for extraction). For studies with leachates and aqueous extracts, it also consisted in the lack of pH and osmotic controls. In studies with plant material added to the substrate, absence of control was not using an artificial material or another plant material in/on the substrate. For studies with living donor plant, lack of control consisted in not establishing a control for competition. Recipient species was categorized as adequate or not (12) only when studies could be classified in one of the approaches according to aspect 5 (124 articles). For ecological studies and studies applied to ecological restoration, appropriate species would be native or exotic species that (potentially) co-occur with the donor species in natural ecosystems. For studies applied to weed management, and studies applied to herbicide development potential, appropriate species would be cultivated species and weeds that co-occur with them. However, the appropriate type of cultivated species can be specific in some cases. For example, in studies applied to weed management that aim at elucidating interactions in pastures for livestock production, species cultivated to feed livestock would be adequate, whereas species cultivated for food would be not.

In relation to substrate type (13), we did not consider studies conducted in the field, because researches did not deliberately choose the substrate used. As evaluations exclusively carried out in the field were performed only in ten papers, this reduced the number of studies evaluated here only slightly. Regarding the consistency in the use of the

term allelopathic (16), we first selected only studies that stated in conclusion that the species was phytotoxic or had allelopathic potential (i.e., allelopathy was not confirmed). Then, when the species/plant material was stated to be allelopathic in other parts of the text, we considered that it was not consistent.

Data was reported as the percentage of each category in relation to the number of analysed studies. Among the evaluated researches, the majority was performed, for example, with more than one donor and/or recipient species, or used more than one method. Hence, data was in many cases classified in more than one category, and the sum of categories reached higher values than 100%.

In order to evidence differences in old and current trends, we separated articles in two groups (except by descriptive data 1-5): studies conducted before 2006 (56 articles) and from 2006 to 2016 (87 articles). For these groups, analyses were performed and reported in the same manner that for the whole review.

3 Results and Discussion

3.1 Allelopathic interactions in grasslands: where and between which species?

We observed in our review that allelopathy studies in grasslands have been carried out in all the continents. Most studies were conducted in the Americas, with North and South America summing up 51.8% of them (Fig. 1a). The majority of studies in North America were carried out in United States (86%), whereas researches in South America were mostly conducted in Brazil (86%). In the other continents, studies did not predominate in a single country.

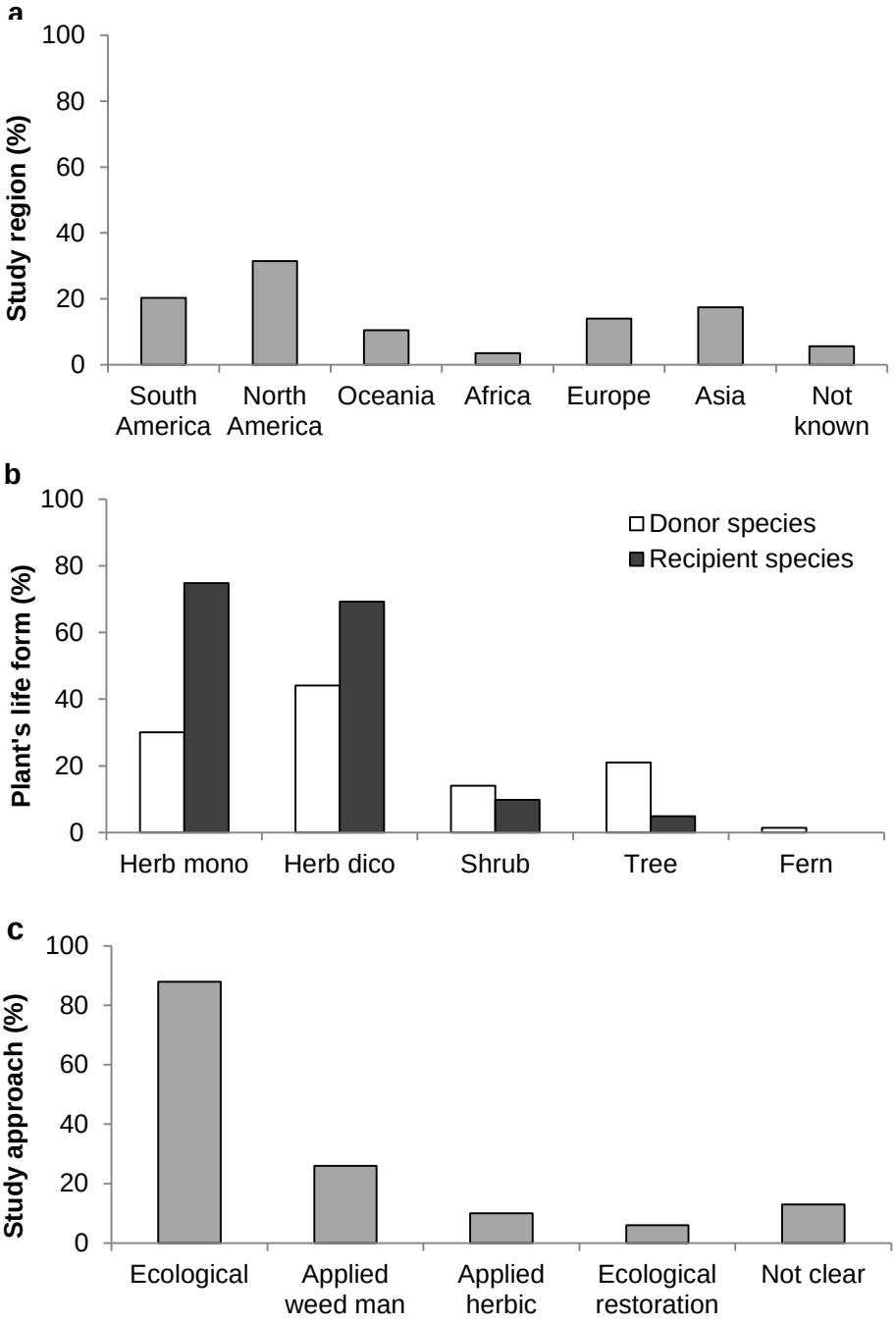


Figure 1. Descriptive characteristics of researches that tested phytotoxicity/ allelopathy in grassland ecosystems, including 143 articles obtained from Scopus database. a Study region, categorized by continent: America (separately presented in North and South America), Oceania, Africa, Europe, and Asia. b Donor and recipient species' life form, categorized in herbaceous monocotyledon (herb mono); herbaceous dicotyledon (herb dico); shrub (including subshrub); tree; and fern. c The used approach was classified in ecological; applied to weed management (applied weed man); applied to herbicide development (applied herbic); applied to ecological restoration; and not clear approach.

The evaluated grasslands are mainly comprised in tropical, subtropical and temperate zones, although studies in temperate grasslands were less common. Studies included

ecosystems that were defined, according to the authors, as coastal prairies (Bennett et al. 2011), tallgrass prairies (Rout et al. 2013), intermountain prairies (Metlen et al. 2013), grassy balds (Reinhart and Rinella 2011), alpine grasslands (Chou and Lee 1991), shrublands (Silva et al. 2015), steppes (Djurdjević et al. 2013), and savanna-type vegetation (Nyanumba and Cahill Jr 2012). Researches comprised both perennial (Wardle et al. 1994) and annual grasslands (Parker and Muller 1979). Ecosystems in diverse altitudes were assessed, from 200 m a.s.l. (Rout et al. 2013) to 3,500 m a.s.l. (Reinhart and Rinella 2011). Study areas also differed regarding precipitation, and included semiarid grasslands, with low annual rainfall (298 mm, Navarro-Cano et al. 2009), sites with a well-defined dry season (Gliessman and Muller 1978) and, on the other hand, areas with high annual precipitation distributed along the year (1,309 mm, Silva et al. 2015). Moreover, evaluated grasslands included moderate temperature areas (mean of 12.5 °C in the coolest month, and 23 °C in the hottest month, Tian et al. 2007), as well as cool sites (mean annual temperature of 1 °C, Zhang et al. 2015). They also comprised areas that reach high temperatures, with more extreme variation (mean monthly temperature between -1.8 °C and 21 °C, reaching maximum of 60 °C (Markó et al. 2011).

In the total, 213 donor species were evaluated in this review (Online Resource 2). In 56% of the studies, donor species were native to the study area, and in 44% they were exotic. Most of studies assessed effects of one or more herbaceous donor species. Herbaceous dycotyledons were the main evaluated group (44% of studies) (Fig. 1b), and half of them used species from the Asteraceae family. Many studies were also conducted with Poaceae species (30%). Regarding recipient plants, about 80% of studies used more than one species, and most of them were herbaceous.

3.2 Allelopathy studies: what for?

We observed that most studies followed the ecological approach (59.4%) (Fig. 1c). Ecological studies can be conducted in order to test hypotheses regarding allopatric and sympatric plant response to allelochemicals. These hypotheses include species-specific biochemical recognition – effects of sympatric chemicals; intraspecific biochemical recognition – autotoxicity; phytogeneric biochemical recognition – effects of allopatric and sympatric chemicals; and novel weapons hypothesis – effects of allopatric chemicals (Renne et al. 2014). Based in our data about native and exotic donor species, most of ecological studies aimed to tested species-specific biochemical recognition (51.7%) and the new weapons hypothesis (38.8%). Autotoxicity was assessed in 18.8%, and phytogeneric biochemical recognition in 5.8% of the studies.

Studies applied to weed management mainly investigated plant interactions in pastures for livestock production. Some studies evaluated which species are less sensitive to allelochemicals from weeds and should be used in invaded pastures (e.g. Wardle et al. 1993). Other studies tested which desired pasture species could be used to suppress weeds through allelopathy (e.g. Wardle et al. 1992b).

Among the studies applied to herbicide development potential, 88% used native donor species and 33% used exotic species. This indicates that native species have been viewed as the main possible sources of bioherbicides. We observed a small quantity of studies in this approach. This may be due to the criteria or keywords we used in our review, because for this type of study, information about the typical ecosystem of the donor species may be not so relevant to be stated. Thus, we can not affirm that having few studies in the bioherbicide development approach is a pattern for allelopathy studies with grassland species.

Most of studies applied to restoration of natural ecosystems evaluated which species were resistant to chemicals of exotic species and hence could thrive in invaded ecosystems (66% of these studies, e.g. Alford et al. 2009). The other studies investigated if native plant species, owing to their allelopathic potential, could be used to control invasive exotic species and otherwise to facilitate natives (Perry et al. 2009).

The uncertainty about the approach, found for more than 10% of studies, raises a concern: if the study approach is not clear, how can be appropriate methods be defined? A clear objective in an allelopathy study consists in the first step for obtaining clear answers.

3.3 Experimental design in allelopathy investigations

3.3.1 Study system and controls

Almost half of the studies were carried out exclusively in the laboratory, with an increased number in the last decade (Fig. 2a). The amount of researches that include field evaluations was similar or reduced in the past 10 years. Studies in three system types were reduced, with even fewer in the last decade. However, some species were evaluated in more than one study (19% of the donor species), and among these, the majority comprised different systems. We observed that most of laboratory studies did not use controls (68%) (Fig. 2b). The same was observed for the majority of field and greenhouse studies before 2006. Nevertheless, from 2006 to 2016 the proportion of studies that used controls in these systems substantially increased.

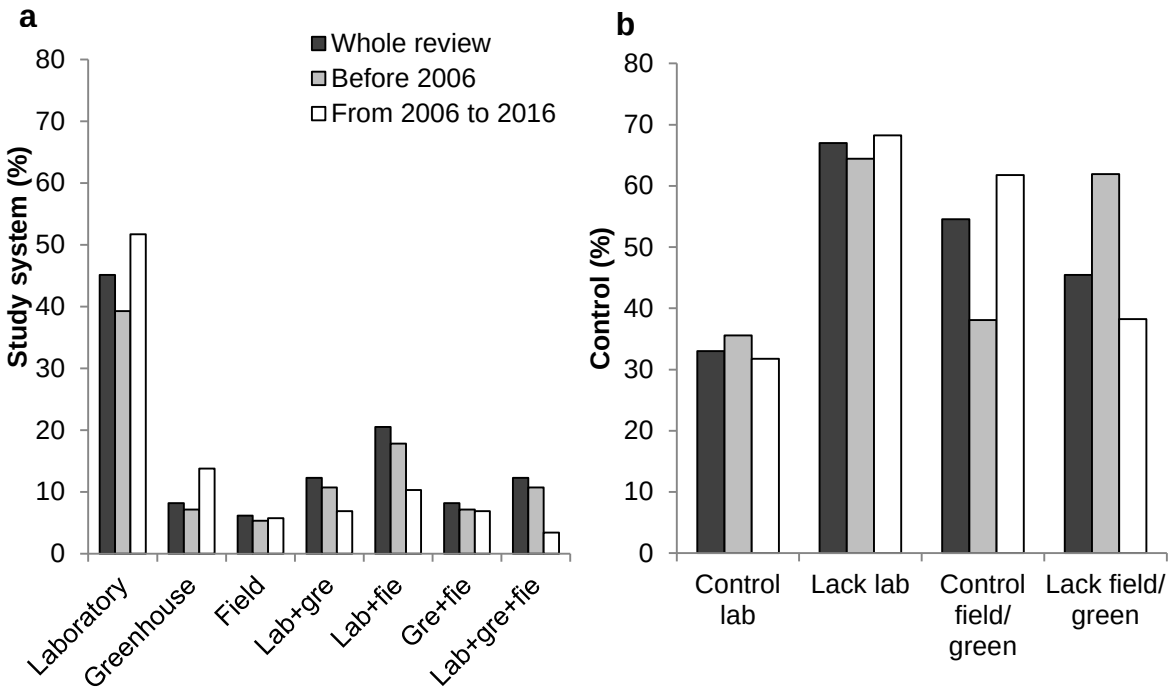


Figure 2. Study system and controls in allelopathy researches in grassland ecosystems, including 143 articles obtained from Scopus database (whole review); and the same articles divided in two groups: 56 articles published before 2006, and 87 articles published from 2006 to 2016. Data was presented as the percentage of each category in relation to the number of analysed studies in each group/whole review. a Study system was classified in laboratory; greenhouse; field; laboratory and greenhouse (Lab+gre); laboratory and field (Lab+fie); greenhouse and field (Gre+fie); laboratory, greenhouse and field (Lab+gre+fie). b Use of controls was classified as included in laboratory assays (control lab); lack in laboratory assays (lack lab); included in field or greenhouse evaluations (control field/green); lack in field or greenhouse evaluations (lack field/green).

Data obtained in our review show that many studies were restricted to laboratory assays, which became even more evident in recent years. Laboratory studies cannot be exclusively used to confirm that a donor species affects the dynamics of the local vegetation (Inderjit and Weston 2000). Nevertheless, studies that include field or greenhouse evaluations diminished in the past ten years. Although greenhouse or common garden studies cannot replace field assessments, they are more realistic systems than a growth room. In some cases, well conducted greenhouse studies can give good clues about the allelopathic potential or not of a species (e.g. Qin et al. 2007). We recognise that in some cases field evaluations can be time consuming and expensive; then, at least greenhouse/common garden experiments should be considered.

Field assessments are also mandatory to applied studies. In our review, some studies applied to weed management included evaluations in natural or artificial fields (e.g. Chou et al. 1987). However, researches applied to bioherbicide development were mostly restricted

to laboratory conditions, which has been highlighted as one of the limitations for bioherbicide development (Soltys et al. 2013). For these studies, laboratory experiments can already be time-consuming, because they may require elucidating allelochemical mode of action, isolating bioactive compounds, and even testing possible toxicological effects of allelochemicals (Soltys et al. 2013). Nevertheless, if the allelochemical is not effective in the field, is not worth performing extensive and exhausting laboratory assays.

In relation to the use of controls, usual negative controls were provided in laboratory assays (water, or organic solvent), and the results reflect mainly the lack of pH and/or osmolality controls for extracts and leachates. Extract pH and osmolality may change dramatically from water and affect recipient species (Chou and Young 1974; Wardle et al. 1992a). If it is uncertain that the inhibition was caused by allelochemicals, the species may not present the assumed allelopathic potential. In addition to a negative control, some studies applied to bioherbicide development used a positive control, which consisted in a commercial herbicide (e.g. Habermann et al. 2016; Imatomi et al. 2013). This type of control can give a good indication about the efficiency of the allelochemical.

In recent years, the implementation of controls in field/greenhouse studies increased a lot. For studies in these systems, control type is more variable than for laboratory assays. In cases of studies that use the living donor species, it is very relevant to control for competition. According to Inderjit and Nilsen (2003), an alternative is to use as control a species that is similar to the suspected allelopathic species, but otherwise with low to absent phytotoxicity (e.g. Del Moral et al. 1978; Silva et al. 2015). When plant material is added to soil, the control can be material from a non-phytotoxic species, or even a mix of several species similar to the donor, as used by Heděnec et al. (2014). An artificial material can also be added to soil, in order to control physical effects (e.g. Ruprecht et al. 2010). These examples of controls, among others, are simple and should be used in ecological studies - if confounding factors were not excluded, allelopathy can not be evidenced.

A control can be also established by neutralizing allelochemicals in soil. For this, activated carbon can be used, which binds organic molecules (Inderjit and Callaway 2003). If recipient species are less affected by the donor species in the presence of activated carbon, then allelopathy can be assumed. Some studies included in this review used activated carbon (9%). Nevertheless, the use of carbon may present restrictions: it is not completely certain all allelochemicals will be adsorbed, and pH and water retention may change (Inderjit and Callaway 2003). A possibility to certify adsorption is to characterize allelochemicals in soil before and after adding carbon; however, we do not know any study that did this. Furthermore, carbon may affect nutrient concentrations in soil, leading to increase in plant growth (Lau et al. 2008). To control this, recipient species should be exposed to soil without

allelochemicals, with and without activated carbon; if results did no differ, carbon can be used (e.g. Chen et al. 2012). Thus, the use of activated carbon can be interesting in allelopathy studies, but caution should be taken.

3.3.2 Plant material and allelochemical obtainment

Most studies analysed by us tested the phytotoxic/allelopathic effects of compounds present in plant leaves (53% plus 18% for shoot, which also includes leaves), followed by roots (28%) (Fig. 3a). In general, results were similar before and after 2006. In relation of allelochemical obtainment from plants, we observed that many studies performed an aqueous extraction (78%), followed by using plant tissues in/on/over substrate (52%), and the living donor species (46%) (Fig. 3b). In the last decade, the main changes include fewer studies that used plant tissue and living donor species.

Allelochemicals may be often released from leaves and roots in larger amounts than from ephemeral reproductive organs, owing to their quantity and constancy in plants, which probably motivated ecological studies and studies applied to weed management. Furthermore, leaves may be of interest to studies applied to bioherbicide development, because large amounts of plant material are usually needed for extraction of bioactive compounds.

Allelochemicals may be released from plants by water leaching, root exudation, volatilization and decomposition of plant residues (De Albuquerque et al. 2011). For each suspected mode of emission, some methods are more adequate. The larger use of aqueous extracts indicates that leaching has been the most suspected type of allelochemical release. Studies that follow the ecological approach, as well as the ones applied to weed management and to restoration of natural ecosystems, require that allelochemicals are obtained by conditions more similar to natural release as possible. In this sense, allelochemical extraction with organic solvents would be the most unrealistic procedure, and should be avoided (Inderjit and Dakshini 1995). Fortunately, in our review, most of studies in these approaches did not use organic solvents, or if they used, other methods were also employed. The use of water as solvent is thus preferred (since the water is not heated) (Inderjit and Dakshini 1995). However, according to Reigosa et al. (2013), laboratory studies should replicate the expected effects of rainfall or dew on the leaching of substances supposed to occur in the natural environment. Thus, leachates (e.g. Renne et al. 2014) or percolates (e.g. Bernhard-Reversat 1999) would be more adequate than an extract. Collection of fog, rain or dew would be even more appropriate, although this was only conducted in few studies (e.g. Batish et al. 2001) and not in the recent years.

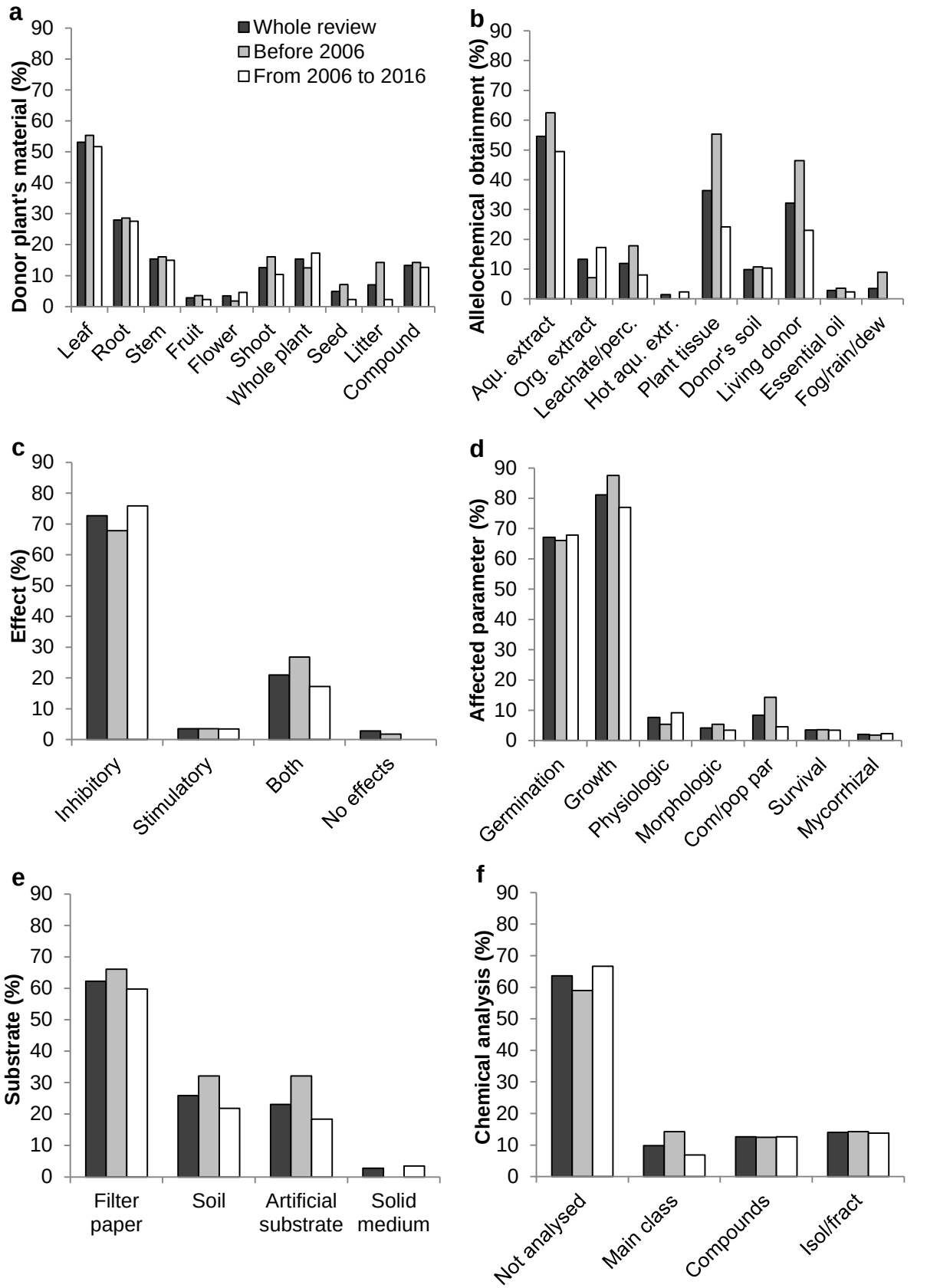


Figure 3. Experimental design in allelopathy researches in grassland ecosystems, including 143 articles obtained from Scopus database (whole review); and the same articles divided in two groups: 56 articles published before 2006, and 87 articles published from 2006 to 2016. Data was presented as the percentage of each category in relation to the number of analysed studies in each group/whole review. **a** Plant material was classified in leaf; root;

stem (including branches, bark, twigs); fruit; flower; shoot; whole plant; seed; litter; pure compound. **b** Allelochemical obtainment was classified in aqueous extract; organic solvent extract; leachate or percolate; hot aqueous extract (hot aqu. extr.); plant tissue in/on/over substrate; donor species' soil; living donor species; essential oil; fog, rain or dew. **c** Effect type was classified in inhibitory; stimulatory; both inhibitory and stimulatory; no effects. **d** Affected parameters were classified in germination; growth (length, biomass); physiologic (photosynthesis, stomatal conductance, wilting, chlorophyll content); morphologic (darkening, withering, abnormalities); plant community/population parameters (com/pop par, e.g. diversity, richness, composition); survival; mycorrhizal interactions (nodulation, spore germination, colonization). **e** Substrate type was classified in filter paper; soil; artificial substrate (including sand, vermiculite, peat, perlite, pumice, mixed or not with soil); solid medium. **f** Studies were classified in no chemical analysis (not analysed); characterization of main class (e.g. terpenes, phenolics); characterization of chemical compounds (compounds); and fractionation or isolation of compounds (isol/fract).

In many studies, the living donor plant was used, which mainly reflects field assessments that tested donor's effects on plant populations/communities (e.g. Ridenour and Callaway 2001). In other cases, studies with the living plant were conducted in greenhouse (e.g. Reinhart and Rinella 2011). This is an advantage for studies with grassland species, which are mostly herbaceous and can be grown quickly. When root exudation is suspected, using the living plant is the most appropriate choice, as the constancy and quantity of allelochemicals in exudates can be more precisely mimicked (e.g. Qin et al. 2007). By the same reasons, the use of living plant is the best method to comprehend the volatilization mechanism (e.g. Zhao-Jiang et al. 2011). Few studies in our review gave attention to volatilization, with some of them also evaluating volatile emission from tissues removed from plants (e.g. Silva et al. 2014), or in a less realistic manner, using essential oils (e.g. Komai and Tang 1989). We observed studies that evaluated exudation and volatilization from living species have been carried out only in the last years. This indicates recent improvement in methodologies for assessing these two modes of allelochemical emission. Allelochemical release during plant material decomposition was mostly evaluated by using plant tissues into or on the substrate. Some studies used fresh or dried material (e.g. Souza et al. 2006), whereas others used litter (e.g. Loydi et al. 2015), which would be a better approximation from the actual decomposition process. Few studies used litter, which may be related to the study system, as grasslands are mainly composed of a mixture of small herbaceous species. Hence, it is difficult to identify and collect litter from a single species, which should be easier in forests where litter quantity is also higher.

Most of studies applied to bioherbicide development used organic solvent extract (e.g. Imatomi et al. 2013). Some of them also used aqueous extracts and/or pure compounds (e.g. Novaes et al. 2013). This is very pertinent, as studies with this approach aim to maximize

obtainment of bioactive substances. Essential oils, which were used in one study (Souza Filho et al. 2009), are also adequate for bioherbicide potential researches.

3.3.3 Evaluated parameters

Studies included in our review mainly reported inhibitory effects of donor on recipient species (73%); few researches described only stimulatory effects or their absence (Fig. 3c). In recent years, more studies reported inhibitory effects, and fewer described both types. In relation to affected parameters, most of the studies reported effects on plant growth (81%) and on germination (67%) (Fig. 3d). Stimulatory effects were almost exclusively observed on growth and germination (55.8% and 38.2% of studies that showed stimuli, respectively).

The almost lack of stimulatory effects or absence of effects raises two possibilities: the first is that only “interesting” results have been published, i.e., inhibitory effects that indicate allelopathic potential. In many disciplines, there is an increasing tendency for not publishing negative or null results (Fanelli 2012). This is a conceivable explanation for data in our review, because stimulatory effects were almost only reported in cases that inhibitory effects were also described. The second possibility is well stated in the words of Harper (1994): “almost all species can, by appropriate digestion, extraction and concentration, be persuaded to yield a product that is toxic to one species or another”. This is also a likely explanation, as many studies in this review were conducted with extracts, and from our experience, extracts almost always present inhibitory effects at some concentration. Stimulatory effects, on the other hand, are more difficult to observe, and low concentrations are needed. Reigosa et al. (2013), in their review, also observed that few studies reported stimulatory effects. The authors noted the importance of screening for substances that promote germination or plant growth, which is particularly relevant for development of bioherbicides and plant growth regulators.

Plant allelochemicals can affect from individuals to ecosystems, from germination to senescence, and thus several parameters can be assessed. However, most of studies were designated to test effects on germination and plant growth. Regarding plant growth, effects in seedling length were mainly shown in laboratory assays (e.g. Silva et al. 2014), whereas effects in plant biomass were more commonly reported in field or greenhouse studies (e.g. Houx et al. 2008). Other morphological effects were scarcely reported, and include plant darkening and withering (Komai and Tang 1989), reduced length in metaxylem cells (Habermann et al. 2016), inversion of gravitropism and effects in secondary roots and root hairs (Oliveira and Campos 2006). In some field studies, effects on diversity, richness and plant cover of populations or communities were assessed (Silva et al. 2015). These

parameters were more scarcely reported in recent years, reflecting the reduction in field studies. Allelopathic interactions mediated by microorganisms were also assessed, such as effects in mycorrhizal nodulation (Wardle et al. 1994), colonization and spore germination (Bainard et al. 2009).

Physiologic effects other than germination were rarely assessed, revealing that few studies have investigated allelochemical mode of action. Reported parameters included wilting (Chou and Leu 1992), oxidative damage (Weir et al. 2006), and changes in chlorophyll content, photosynthesis and stomatal conductance (Zhao-Jiang et al. 2011). For studies applied to bioherbicide development, elucidating allelochemical mode of action is imperative (Soltys et al. 2013), but in our review, no physiologic parameter was assessed in these studies. Allelochemicals may act by many different mechanisms, even simultaneously (Gniazdowska and Bogatek 2005). Thus, investigations should be based in observed effects that indicate some phytotoxicity mechanism (e.g. plant withering indicates changes in chlorophyll content and photosynthesis). Excellent reviews about allelochemical mode of action were written by Weir et al. (2004) and Gniazdowska and Bogatek (2005), in which many studies that used diverse techniques were reported.

3.3.4 Selection of recipient species and substrate

In our review, 86% of studies used adequate species (see criteria in Methods section), while 29% did not. The proportion of studies that used inadequate species decreased from 37% before 2006 to 22% from 2006 to 2016. All studies applied to ecological restoration and to bioherbicide development potential used appropriate species. Recipient species were inadequate in 34% of applied to weed management studies, which mostly investigated interactions in pastures for livestock, and in 34% of ecological studies. In both cases, the most used inappropriate species was *Lactuca sativa* L. (lettuce). In relation to the substrate, most of studies used filter paper (67%) (Fig. 3e). In the past 10 years, fewer studies used soil as substrate.

Selection of appropriate recipient plants is fundamental and should be introduced into research design, if not at the beginning, at least prior to its completion (Macías et al. 2007). Some cultivated and weed species have been used as models for applied to bioherbicide development studies (e.g. *L. sativa, Allium cepa* L., *Lycopersicon esculentum* L.) (Macías et al. 2000), which is pertinent to standardize bioassays in this approach. In our review, studies with this approach used either cultivated or weed species as recipients, but not both of them. Including both type of species would be ideal, owing to the importance of identifying donor

species that produce substances capable of inhibiting weeds, but that do not inhibit - or even stimulate - cultivated species (Macías et al. 2000).

For ecological studies, it has long been highlighted that recipient species need to be representative of the natural conditions (Inderjit and Weston 2000), and thus model species are not appropriate for this approach. The main argument for the still current use of model species in ecological studies is that they show high, fast and homogeneous germination and are commercially available. We recognize that for allelopathy studies, species that occur in natural ecosystems are not always that easy to obtain, as large amounts of seeds are usually needed, and some of them present dormancy and low viability. However, the majority of studies in this review has shown that adequate grassland recipient species can be indeed acquired. Even if not native, many species can be better options than model species. As an example, in South Brazilian grasslands (Campos Sulinos region, Overbeck et al. 2007), Fabaceae species used for forage were introduced some decades ago (*Trifolium repens* L., *Medicago sativa* L., *Lotus corniculatus* L., among others). Nowadays, these species are considered naturalized - they occur in grasslands independent of human action forming stable populations (Schneider 2007). These plants are commercially available and present germination characteristics as good as standard species do. But, in contrast to the model recipients, they do grow in the studied system and co-occur with the species of interest.

The relevance of using natural soil in allelopathy studies instead of artificial substrates has been substantially highlighted (Inderjit and Callaway 2003; Inderjit and Weston 2000). However, a small number of studies have used soil, especially in recent years. As an example of misleading that can result from inadequate substrate use, a plant species that had presented great allelopathic potential in artificial potting substrate, recently showed lower allelopathic potential in natural soil (Parepa and Bossdorf 2016). Allelochemical activity can be increased or reduced by interacting with microorganisms, inorganic ions, nutrients and pH, sometimes by complex reactions involving many of these factors (Inderjit and Weiner 2001). Microorganisms, in special, have shown important roles in allelopathic interactions, mainly by reducing allelochemical activity (e.g. Ehlers 2011; Kaur et al. 2009), thus the soil should be not sterilized. Furthermore, the soil from the donor’s occurrence area/cultivated system should be used, as allelochemical persistence can vary according to soil type (e.g. for Andosols and Cambisols, Yamamoto 2009). Regarding studies applied to bioherbicide development, allelochemical time of residence in soil needs to be specially addressed (Soltys et al. 2013).

3.3.5 Chemical characterization

Most of the studies included in our review did not investigate which were the compounds involved in plant phytotoxicity/allelopathy (63%), with similar patterns before and after 2006 (Fig. 3f). Many types of allelochemicals have already been characterised, which can be classified in 14 classes according to their biosynthetic origin (e.g. coumarins, condensed tannins, terpenoids and sterols, flavonoids) (Rice 1984). A starting point for chemical characterization may thus be difficult to define. For allelochemicals dissolved in water (e.g. aqueous extracts), phenolics are usually the first ones to be investigated, owing to their recognised water-solubility (Inderjit and Dakshini 1995). In addition, some monoterpenes (as well as other compounds) also show solubility in water (Weidenhamer et al. 1993) and may be involved in phytotoxicity. However, characterization of one or few main classes of substances does not necessarily guarantee that these are the classes involved in phytotoxicity. In the case of phenolics, their relation with activity can be determined by removing them from solutions using adsorbents such as insoluble polyvinylpolypyrrolidone (PVPP) and polystyrene resins (Gray 1978), as reported in the studies of Macfarlane et al. (1982a, b). If extracts still showed bioactivity after phenolic removal, then other classes of substances should be investigated. In contrast, in studies about volatiles, such as essential oils, it is less difficult to assume which are the main classes of allelochemicals. These compounds are mostly mono- and sesquiterpenes, which can be characterized by gas chromatography (GC) coupled to mass spectrometry and by GC combined with flame ionization detector (e.g. El Ayeb-Zakhama et al. 2015). A more refined characterization can be performed by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (Zhu et al. 2005).

In addition, it is relevant to perform a more refined characterization. Bioactivity can be determined by synergistic or additive effects of many compounds (Einhellig 1996), or then mostly by one or few compounds (e.g. for (±)-catechin in *C. stoebe*, Inderjit et al. 2008). Characterization of allelochemicals is especially necessary in studies applied to bioherbicide development, which aim at identifying bioactive compounds in plants. In our review, half of the studies that followed this approach at least characterized chemical compounds. In some studies, an excellent method for understanding which compounds are related to phytotoxicity was reported: bioactivity-guided fractionation (e.g. Imatomi et al. 2013; Novaes et al. 2013). Here, fractions of extracts are tested for their bioactivity, and the active ones are purified. Then, pure active compounds are identified, isolated and tested again for their phytotoxicity. Hence, the compounds, or set of compounds responsible for activity are identified.

Chemical investigations are also relevant to comprehend the role of allelopathy in the environment. These analyses are pertinent, for example, to elucidate if exotic species

allelochemicals are new to an ecosystem and may be thus involved in allelopathic interactions (e.g. Inderjit et al. 2008). Also, when phytotoxic substances are identified, they can be added to soil, which can give substantial insights into allelopathy (Inderjit and Callaway 2003). Furthermore, strong evidences of allelopathy can be provided by determining presence and permanence of allelochemicals in the environment. Some examples include characterization in soil of terpenes (e.g. Del Moral and Muller 1970; He et al. 2014) and phenolics (e.g. Djurdjević et al. 2013). Also, there are methods that allow determining temporal and spatial chemical dynamics of the rhizosphere (Weidenhamer et al. 2014).

3.4 To be or not to be allelopathic - a question of terminology

Most authors were careful in their final conclusions if allelopathy occurred or not and mainly stated the species had allelopathic potential (45.5%) (Fig. 4). However, in 58% of studies that concluded allelopathic potential or phytotoxicity, the plant species/material was stated to be allelopathic in other parts of the text. The inconsistency in terminology was larger in the past 10 years (62%) than before it (52%). This may be possibly due to a conceptual matter, by considering the terms phytotoxic and allelopathic as synonyms, which is not the case. Terms should be appropriately used to make scientific communication clearer.

The concept of allelopathy has changed since it was first coined by Hans Molisch in 1937, from the combination of the two Greek terms “allelo” and “pathos”, meaning “mutual harm”, to describe biochemical interactions among plants or microorganisms (Rice 1984). Molisch’s concept, together with evidences that indirect and positive effects may also play a role in allelopathic interactions, led to the most widely used definition of allelopathy. This definition was given by Rice (1984) as any direct or indirect stimulatory or inhibitory effect of one plant (including microorganisms) on another through production of chemical compounds that escape into the environment. Later, the International Allelopathy Society (IAS 1996) proposed a definition that specifies types of organisms (plants, algae, bacteria and fungi), and also the systems where allelopathy may occur (natural and agricultural). However, allelopathic interactions may also occur in marine systems, among organisms such as phytoplankton, seaweeds, sponges and corals (Granéli and Pavia 2006), which makes it difficult to include type of organism in the concept. Recent studies have often summarized the term to explicit the goal of a specific research. For example, Inderjit and Callaway (2003) defined allelopathy as “the negative effect of one plant on another one through the release of chemical compounds into the environment”. In spite of variations, the concept of allelopathy

is always consistent in one aspect: allelopathy has been described as a phenomenon that occurs in the environment – and thus needs to be evidenced there.

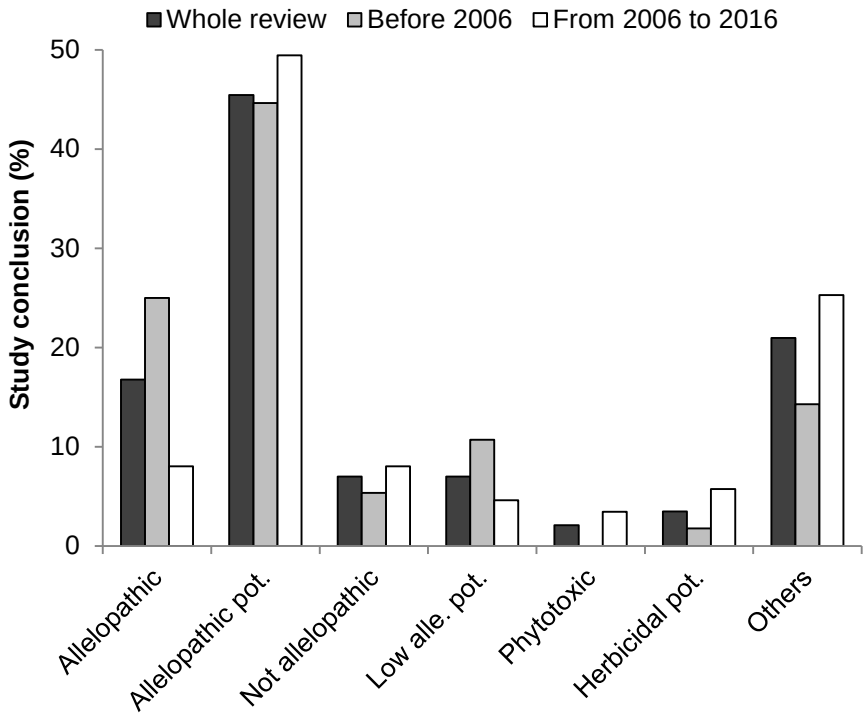


Figure 4. Main conclusion of allelopathy researches in grassland ecosystems, including 143 articles obtained from Scopus database (whole review); and the same articles divided in two groups: 56 articles published before 2006; and 87 articles published from 2006 to 2016. Data was presented as the percentage of each category in relation to the number of analysed studies in each group/whole review. Reported conclusions included if the donor species was considered allelopathic; with allelopathic potential (allelopathic pot.); not allelopathic; with low allelopathic potential (low alle. pot.); phytotoxic; with herbicidal potential (herbicidal pot.); and another conclusion not related to allelopathy, or no conclusion (others).

We did not find in literature, not even in the reviews that are available, a clear differentiation between the terms phytotoxic and allelopathic. As the concept of allelopathy includes effects that occur in the environment, we propose the use of the term “allelopathic species” only when both laboratory/greenhouse and field evaluations provide evidences that a species negatively or positively affects another by releasing chemical compounds into the environment (Fig. 5). If the study is not conclusive, we suggest always using the term “phytotoxic” (or “phytostimulatory” when effects are positive). Alternatively, a phytotoxic species can be affirmed to present “allelopathic potential”, which is more adequate (although not limited) to studies that employ more realistic methods, i.e., simulating field conditions. However, the term phytotoxicity/phytostimulation can be also used in field evaluations as it is a component of allelopathic interactions - when phytotoxicity/phytostimulation is involved in determining vegetation patterns, it implies in allelopathy. The same can be applied to studies

that deal with other recipient organisms than plants, for which the terms toxicity and stimulation can be used. For positive biochemical interactions, Nyanumba and Cahill Jr (2012) coined the term “allelarexis” based on the Greek roots “allelo” meaning “one another” and “arexis” meaning “help”, which may be also used for this type of allelopathic interaction.

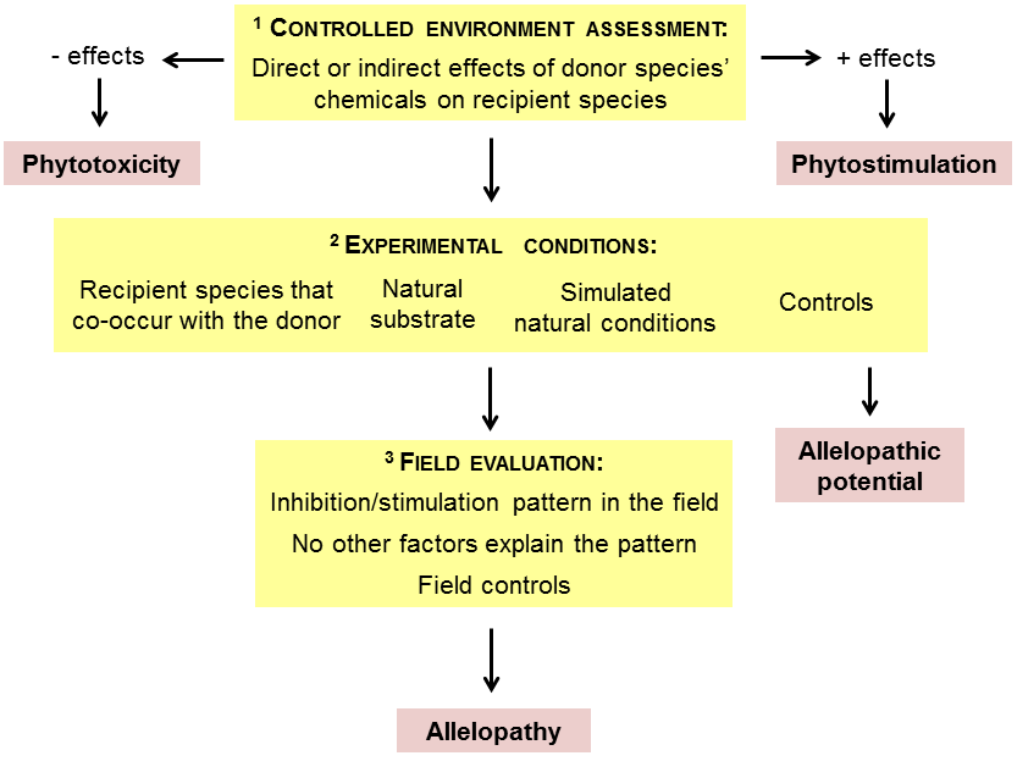


Figure 5. Concepts and terminology for allelopathy studies, according to evidences obtained in controlled conditions (laboratory/greenhouse) and in field. Some terms can be also used when a different combination of yellow boxes were comprised - phytotoxicity/phytostimulation (1; 1 and 2; 1 and 3; 1, 2 and 3), and allelopathic potential (1; 1 and 2; 1 and 3; 3) - although they fit better according to indication in the diagram. In these cases, content of the yellow boxes can be totally or partially achieved. Otherwise, allelopathy is only evidenced when all contents of all yellow boxes are obtained. For studies with other recipient organisms than plants, the terms phytotoxicity and phytostimulation should be adapted, or simply be mentioned as toxicity and stimulation. See supplementary material 3 for a Portuguese version.

In addition to clearly defined concepts, some criteria should be followed in order to minimize erroneous affirmations about allelopathy. Willis (1985) proposed detailed rules to evidence allelopathy. However, some of these rules are very difficult to follow, mostly because they include demonstrating the movement of chemicals from donor to recipient plant, and their uptake in bioactive concentration by a recipient species. This was successfully shown in one laboratory study (Macías et al. 2014), but demonstration in soil and/ or in field settings is still a challenge. Inderjit and Weiner (2001) discussed that acquiring such high level of proofs – not required to evoke any other plant interactions – may

not be essential. The authors argued that in numerous cases allelopathy may represent the simplest and most reasonable explanation for phenomena observed in the field, even though specific mechanisms were not totally elucidated. Hence, we propose that at least the following four guidelines, adapted from Willis (1985) are followed to evidence allelopathy (Fig. 5):

(1) a donor species must produce compounds that negatively or positively affect a recipient species, either directly or indirectly;

(2) effects must be observed in experimental conditions as similar to natural as possible, and include the use of natural substrate and recipient species that (potentially) co-occur with the donor (see Inderjit and Callaway 2003);

(3) an inhibition/stimulation pattern of one species by another must be shown in the field;

(4) the possibility that physical or other biotic factors explain the pattern must be ruled out, which must include the use of appropriate controls.

We are aware that it may be hard to decide, in some cases, when “experimental conditions are as similar to natural as possible”, and which are “appropriate controls”. This may depend on characteristics of each species and study system.

A small number of studies have provided substantial evidences that allelopathy explains (at least partly) field patterns, considering the points comprised in our guidelines. Examples include investigations about *Eucalyptus camaldulensis* (Del Moral and Muller 1970), *A. californica* (Halligan 1973), *Pteridium aquilinum* (Gliessman and Muller 1978), and *C. stoebe* (Inderjit et al. 2008; May and Baldwin 2011; Thorpe et al. 2009). These studies stand out by extensively observing the species’ behaviour and interactions. Knowing the study system is very important because together with allelopathy, other processes may be determining vegetation patterns (e.g. Halligan 1973). Thus, allelopathy must be viewed as only a part of a complex system, and its relevance needs to be detected among many variables.

In the approach applied to weed management, evidences suggest that some weeds inhibit desired forage species through allelopathy, which is the case of the weed *Carduus nutans* (Wardle et al. 1993, 1994). However, we did not find information if knowledge about allelopathy has been indeed considered in management of pastures, as well as in restoration of grasslands. Regarding the approach applied to herbicide development, some promising bioherbicides have been identified (Soltys et al. 2013). Furthermore, some natural herbicides that can be used in organic farming are currently in the market in the United States. They are mostly essential oil products, of species such as *Pinus* spp., *Citrus* spp., *Eugenia*

caryophyllus, *Cymbopogon* spp., and *Mentha piperita* (Dayan and Duke 2010). However, there are limitations for large use of these bioherbicides: they are often non-selective and their effects last for a short period. Thus, more research is still needed for applied used of allelochemicals in cultivated systems.

4 Conclusions

Allelopathy studies have evolved a lot in the last years with innovative approaches and methods, especially with robust chemical analysis, which can give substantial evidences of allelopathy – or at least phytotoxicity – and its mechanisms. We found in this review that the quality of research has increased in some aspects, such as the use of appropriate recipient species and controls for field and greenhouse studies. Nevertheless, some problems that led to critics about relevance of allelopathy, and which have been extensively highlighted, are still present. This includes the excess of laboratory studies that have used artificial substrates and unrealistic extraction procedures, and also the small number of field evaluations.

We still observe confusion about fundamental aspects, as for example, how to define an allelopathic species. In many cases a species only presents allelopathic potential, but is considered to be allelopathic, and studies have not advanced much in this regard. However, the results from our review also clearly show the potential to overcome this situation. For all of the critical issues in allelopathy studies, there are also positive examples that conduct experiments in natural conditions, or at least close approximations, and that observe interaction in the field. We already know that a large number of species is phytotoxic: it is time to know which of them are indeed allelopathic, or that present an applied potential.

A better knowledge on allelopathy and its mechanisms, in grasslands and in other systems, will lead to advances not only in science, but also in different applied fields. The potential for allelopathic species as new sources for bioherbicides is well-established. Recently, allelopathy has entered as an important issue into the ecological restoration debate. We hope and believe that in a near future, knowledge about allelopathy is more strongly used for solving these applied questions, so that allelopathy plays a significant role in conservation of biodiversity.

Acknowledgments

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the PhD scholarship granted to the first author.

References

Alford ÉR, Vivanco JM, Paschke MW (2009) The effects of flavonoid allelochemicals from knapweeds on legume-rhizobia candidates for restoration. Restor Ecol 17:506–514

Allen VG, Batello C, Berretta EJ et al. (2011) An international terminology for grazing lands and grazing animals. Grass Forage Sci 66:2–28

Araniti F, Gulli T, Marrelli M, Statti G, Gelsomino A, Abenavoli MA (2016) *Artemisia arborescens* L. leaf litter: phytotoxic activity and phytochemical characterization. Acta Physiol Plant 38:1-12

Aslam F, Khaliq A, Matloob A, Tanveer A, Hussain S, Zahir ZA (2017). Allelopathy in agro-ecosystems: a critical review of wheat allelopathy-concepts and implications. Chemoecology 27:1–24

Bainard LD, Brown PD, Upadhyaya MK (2009) Inhibitory effect of tall hedge mustard (*Sisymbrium loeselii*) allelochemicals on rangeland plants and arbuscular mycorrhizal fungi. Weed Sci 57:386–393

Batish DR, Singh HP, Kohli RK (2001) Vegetation exclusion under *Casuarina equisetifolia* L.: Does allelopathy play a role? Community Ecol 2:93–100

Belz RG (2007) Allelopathy in crop/weed interactions – an update. Pest Manag Sci 63:308–326

Bennett AE, Thomsen M, Strauss SY (2011) Multiple mechanisms enable invasive species to suppress native species. Am J Bot 98:1086–1094

Bernhard-Reversat F (1999) The leaching of Eucalyptus hybrids and *Acacia auriculiformis* leaf litter: laboratory experiments on early decomposition and ecological implications in congolese tree plantations. Appl Soil Ecol 12:251–261

Bhowmik PC, Inderjit (2003) Challenges and opportunities in implementing allelopathy for natural weed management. Crop Prot 22:661–671

Chen S, Xiao S, Callaway RM (2012) Light intensity alters the allelopathic effects of an exotic invader. Plant Ecol Divers 5:521–526

Chou CH (1999) Roles of allelopathy in plant biodiversity and sustainable agriculture. Crit Rev Plant Sci 18:609–636

Chou CH, Hwang SY, Peng CI et al. (1987) The selective allelopathic interaction of a pasture-forest intercropping in Taiwan. Plant Soil 98:31–41

Chou CH, Lee YY (1991) Allelopathic dominance of *Miscanthus transmorrisonensis* in an alpine grassland community in Taiwan. J Chem Ecol 17:2267–2281

Chou CH, Leu LL (1992) Allelopathic substances and interactions of *Delonix regia* (BOJ) RAF. J Chem Ecol 18:2285–2303

Chou CH, Young CC (1974). Effects of osmotic concentration and pH on plant growth. Taiwania 19:157-162

Dayan FE, Duke SO (2010) Natural products for weed management in organic farming in the USA. Outlooks Pest Manag 21:156–160

De Albuquerque MB, Santos RC, Lima LM et al. (2011) Allelopathy, an alternative tool to improve cropping systems. A review. Agron Sustain Dev 31:379–395

Del Moral R, Muller CH (1970) The allelopathic effects of *Eucalyptus camaldulensis*. Am Midl Nat 83:254–282

Del Moral R, Willis RJ, Ashton DH (1978) Suppression of coastal heath vegetation by *Eucalyptus baxteri*. Aust J Bot 26:203–219

Djurdjević L, Gajić G, Kostić O et al. (2013) Allelopathic effects of *Chrysopogon gryllus* L. in *Chrysopogonetum pannonicum* Stjep.-Ves. steppe community at Deliblato Sands (Serbia). Allelopathy J 32:133–148

Ehlers BK (2011) Soil microorganisms alleviate the allelochemical effects of a thyme monoterpene on the performance of an associated grass species. PLoS One 6:e26321

Einhellig FA (1996) Interactions involving allelopathy in cropping systems. Agron J 88:886–893

El Ayeb-Zakhama A, Sakka-Rouis L, Bergaoui A et al. (2015) Chemical composition and allelopathic potential of essential oils obtained from *Acacia cyanophylla* Lindl. cultivated in Tunisia. Chem Biodivers 12:615–626

Fanelli D (2012) Negative results are disappearing from most disciplines and countries. Scientometrics 90:891–904

Gibson (2009) Grasses and grassland ecology. Oxford University Press, New York

Gliessman SR, Muller CH (1978) The allelopathic mechanisms of dominance in bracken (*Pteridium aquilinum*). J Chem Ecol 4:337–362

Gniazdowska A, Bogatek R (2005) Allelopathic interactions between plants. Multi site action of allelochemicals. Acta Physiol Plant 27:395–407

Granéli E, Pavia H (2006) Allelopathy in marine ecosystems. In: Reigosa MJ, Pedrol N, González L (eds) Allelopathy: A physiological process with ecological implications. Springer, Netherlands, pp 415-431

Gray JC (1978) Absorption of polyphenols by polyvinylpyrrolidone and polystyrene resins. Phytochemistry 17:495–497

Habermann E, Pontes FC, Pereira VC et al. (2016) Phytotoxic potential of young leaves from *Blepharocalyx salicifolius* (Kunth) O. Berg (Myrtaceae). Brazilian J Biol 76:531–538

Halligan JP (1973) Bare areas associated with shrub stands in grassland: The case of *Artemisia californica*. Bioscience 23:429–432

Harper JL (1994) Population Biology of Plants. Academic Press, London

He H, Song Q, Wang Y, Yu S (2014) Phytotoxic effects of volatile organic compounds in soil water taken from a *Eucalyptus urophylla* plantation. Plant Soil 377:203–215

Heděnec P, Novotný D, Ustak S et al. (2014) Allelopathic effect of new introduced biofuel crops on the soil biota: A comparative study. Eur J Soil Biol 63:14–20

Houx JH, Garrett HE, McGraw RL (2008) Applications of black walnut husks can improve orchardgrass and red clover yields in silvopasture and alley cropping plantings. Agrofor Syst 73:181–187

IAS – International Allelopathy Society (1996) Constitution and Bylaw of IAS. IAS Newsletter, Cádiz-Spain

Imatomi M, Novaes P, Matos AP et al. (2013) Phytotoxic effect of bioactive compounds isolated from *Myrcia tomentosa* (Myrtaceae) leaves. Biochem Syst Ecol 46:29–35

Inderjit, Callaway RM (2003) Experimental designs for the study of allelopathy. Plant Soil 256:1–11

Inderjit, Dakshini KMM (1995) On laboratory bioassays in allelopathy. Bot Rev 61:28–44

Inderjit, Nilsen ET (2003) Bioassays and field studies for allelopathy in terrestrial plants: Progress and problems. Crit Rev Plant Sci 22:221–238

Inderjit, Pollock JL, Callaway RM, Holben W (2008) Phytotoxic effects of (+/-)-catechin in vitro, in soil, and in the field. PLoS One 3:e2536

Inderjit, Wardle DA, Karban R, Callaway RM (2011) The ecosystem and evolutionary contexts of allelopathy. Trends Ecol Evol 26:655–662

Inderjit, Weiner J (2001) Plant allelochemical interference or soil chemical ecology? Perspect Plant Ecol Evol Syst 4:3–12

Inderjit, Weston LA (2000) Are laboratory bioassays for allelopathy suitable for prediction of field responses? J Chem Ecol 26:2111–2118

Kaur H, Kaur R, Kaur S et al. (2009) Taking ecological function seriously: Soil microbial communities can obviate allelopathic effects of released metabolites. PLoS One 4:e4700

Komai K, Tang CS (1989) Chemical constituents and inhibitory activities of essential oils from *Cyperus brevifolius* and *C. kyllingia*. J Chem Ecol 15:2171–2176

Lau JA, Puliafico KP, Kopshever JA et al. (2008) Inference of allelopathy is complicated by effects of activated carbon on plant growth. New Phytol 178:412–423

Lipinska H, Harkot W (2007) Allelopathic activity of grassland species. Allelopathy J 19:3-36

Loydi A, Donath TW, Eckstein RL, Otte A (2015) Non-native species litter reduces germination and growth of resident forbs and grasses: allelopathic, osmotic or mechanical effects? Biol Invasions 17:581–595

Macfarlane MJ, Scott D, Jarvis P (1982a) Allelopathic effects of white clover 1. Germination and chemical bioassay. New Zeal J Agric Res 25:503–510

Macfarlane MJ, Scott D, Jarvis P (1982b) Allelopathic effects of white clover 2. Field investigations in tussock grasslands. New Zeal J Agric Res 25:511–518

Macías FA, Castellano D, Molinillo JMG (2000) Search for a standard phytotoxic bioassay for allelochemicals. Selection of standard target species. J Agric Food Chem 48:2512–2521

Macías FA, Molinillo JMG, Galindo JCG et al. (2001) The use of allelopathic studies in the search for natural herbicides. Journal of Crop Production 4:237-255

Macías FA, Molinillo JMG, Varela RM, Galindo JCG (2007) Allelopathy – a natural alternative for weed control. Pest Manag Sci 63:327–348

Macías FA, Oliveros-Bastidas A, Marín D et al. (2014) Evidence for an allelopathic interaction between rye and wild oats. J Agric Food Chem 62:9450−9457.

Markó G, Ónodi G, Kertész M, Altbäcker V (2011) Rabbit grazing as the major source of intercanopy heterogeneity in a juniper shrubland. Arid L Res Manag 25:176–193

May L, Baldwin LK (2011) Linking field based studies with greenhouse experiments: the impact of *Centaurea stoebe* (=*C. maculosa*) in British Columbia grasslands. Biol Invasions 13:919–931

Metlen KL, Aschehoug ET, Callaway RM (2013) Competitive outcomes between two exotic invaders are modified by direct and indirect effects of a native conifer. Oikos 122:632–640

Navarro-Cano JA, Barberá GG, Ruiz-Navarro A, Castillo VM (2009) Pine plantation bands limit seedling recruitment of a perennial grass under semiarid conditions. J Arid Environ 73:120–126

Novaes P, Imatomi M, Varela RM et al. (2013) Allelopathic potential of *Rapanea umbellata* leaf extracts. Chem Biodivers 10:1539–1548

Nyanumba SM, Cahill Jr JF (2012) Effect of aboveground litter on belowground plant interactions in a native Rough Fescue grassland. Basic Appl Ecol 13:615–622

Oliveira SCC, Campos ML (2006) Allelopathic effects of *Solanum palinacanthum* leaves on germination and seedling growth of *Sesamum indicum*. Allelopathy J 18:331–338

Overbeck G, Muller S, Fidelis A et al. (2007) Brazil's neglected biome: The South Brazilian Campos. Perspect Plant Ecol Evol Syst 9:101–116

Parepa M, Bossdorf O (2016) Testing for allelopathy in invasive plants: it all depends on the substrate! Biol Invasions 18:2975–2982

Parker VT, Muller CH (1979) Allelopathic dominance by a tree-associated herb in a California annual grassland. Oecologia 37:315–320

Perry LG, Cronin SA, Paschke MW (2009) Native cover crops suppress exotic annuals and favor native perennials in a greenhouse competition experiment. Plant Ecol 204:247–259

Qin B, Lau JA, Kopshever J et al. (2007) No evidence for root-mediated allelopathy in *Centaurea solstitialis*, a species in a commonly allelopathic genus. Biol Invasions 9:897–907

Reigosa M, Gomes AS, Ferreira AG, Borghetti F (2013) Allelopathic research in Brazil. Acta Bot Brasilica 27:629–646

Reinhart KO, Rinella M (2011) Comparing susceptibility of eastern and western US grasslands to competition and allelopathy from spotted knapweed [*Centaurea stoebe* L. subsp. micranthos (Gugler) Hayek]. Plant Ecol 212:821–828

Renne IJ, Sinn BT, Shook GW et al. (2014) Eavesdropping in plants: delayed germination via biochemical recognition. J Ecol 102:86–94

Rice EL (1984) Allelopathy, 2nd ed. Academic Publishers, New York

Ridenour WM, Callaway RM (2001) The relative importance of allelopathy in interference: the effects of an invasive weed on a native bunchgrass. Oecologia 126:444–450

Rout ME, Chrzanowski TH, Smith WK, Gough L (2013) Ecological impacts of the invasive grass *Sorghum halepense* on native tallgrass prairie. Biol Invasions 15:327–339

Ruprecht E, Józsa J, Ölvedi TB, Simon J (2010) Differential effects of several "litter" types on the germination of dry grassland species. J Veg Sci 21:1069–1081

Schneider AA (2007) A flora naturalizada no Estado do Rio Grande do Sul, Brasil: herbáceas subespontâneas. Biociências 15:257–268

Scognamiglio M, D'Abrosca B, Esposito A et al. (2013) Plant growth inhibitors: allelopathic role or phytotoxic effects? Focus on Mediterranean biomes. Phytochem Rev 12:803–830

Silva ER, Ferreira PMA, Overbeck GE, Soares GLG (2015) Does the phytotoxic shrub *Heterothalamus psiadioides* affect a plant community through allelopathy? Plant Ecol 216:87–97

Silva ER, Overbeck GE, Soares GLG (2014) Phytotoxicity of volatiles from fresh and dry leaves of two Asteraceae shrubs: Evaluation of seasonal effects. South African J Bot 93:14–18

Smith AE (1999) Allelopathy in the grassland ecosystems. In: Narwal SS (ed) Allelopathy update: Basic and applied aspects. Science Publishers, United States, pp 91-109

Soltys D, Krasuska U, Bogatek R, Gniazdowska A (2013) Allelochemicals as bioherbicides - present and perspectives. In: Price AJ, Kelton JA (eds) Herbicides - current research and case studies in use. InTech, Croatia, pp 517-542

Souza LS, Velini ED, Martins D, Rosolem CA (2006) Efeito alelopático de capim-braquiária (*Brachiaria decumbens*) sobre o crescimento inicial de sete espécies de plantas cultivadas. Planta Daninha 24:657–668

Souza Filho APS, Vasconcelos MAM, Zoghbi MGB, Cunha RL (2009) Efeitos potencialmente alelopáticos dos óleos essenciais de *Piper hispidinervium* C. DC. e *Pogostemon heyneanus* Benth sobre plantas daninhas. Acta Amaz 39:389–396

Thorpe AS, Thelen GC, Diaconu A, Callaway RM (2009) Root exudate is allelopathic in invaded community but not in native community: field evidence for the novel weapons hypothesis. J Ecol 97:641–645

Tian Y, Feng Y, Liu C (2007) Addition of activated charcoal to soil after clearing *Ageratina adenophora* stimulates growth of forbs and grasses in China. Trop Grasslands 41:285–291

Wardle DA, Nicholson KS, Ahmed M (1992a) Comparison of osmotic and allelopathic effects of grass leaf extracts on grass seed germination and radicle elongation. Plant Soil 140:315–319

Wardle DA, Nicholson KS, Ahmed M, Rahman A (1994) Interference effects of the invasive plant *Carduus nutans* L. against the nitrogen fixation ability of *Trifolium repens* L. Plant Soil 163:287–297

Wardle DA, Nicholson KS, Rahman A (1992b) Influence of pasture grass and legume swards on seedling emergence and growth of *Carduus nutans* L. Weed Res 32:119–128

Wardle DA, Nicholson KS, Rahman A (1993) Influence of plant age on the allelopathic potential of nodding thistle (*Carduus nutans* L.) against pasture grasses and legumes. Weed Res 33:69–78

Weidenhamer JD, Macías FA, Fischer NH, Williamson GB (1993) Just how insoluble are monoterpenes? J Chem Ecol 19:1799–1807

Weidenhamer JD, Mohney BK, Shihada N, Rupasinghe M (2014) Spatial and temporal dynamics of root exudation: How important is heterogeneity in allelopathic interactions? J Chem Ecol 40:940–952

Weir TL, Bais HP, Stull VJ et al. (2006) Oxalate contributes to the resistance of *Gaillardia grandiflora* and *Lupinus sericeus* to a phytotoxin produced by *Centaurea maculosa*. Planta 223:785–795

Weir TL, Park SW, Vivanco JM (2004) Biochemical and physiological mechanisms mediated by allelochemicals. Curr Opin Plant Biol 7:472–479

Willis RJ (1985) The historical bases of the concept of allelopathy. J Hist Biol 18:71–102

Yamamoto Y (2009) Movement of allelopathic compound coumarin from plant residue of sweet vernalgrass (*Anthoxanthum odoratum* L.) to soil. Grassl Sci 55:36–40

Zhang Y, Tang S, Liu K et al. (2015) The allelopathic effect of *Potentilla acaulis* on the changes of plant community in grassland, northern China. Ecol Res 30:41–47

Zhao-Jiang Z, Ru-Min Z, Pei-Jun G et al. (2011) Allelopathic effects of *Artemisia frigida* Willd. on growth of pasture grasses in Inner Mongolia, China. Biochem Syst Ecol 39:377–383

Zhu S, Lu X, Dong L et al. (2005) Quantitative determination of compounds in tobacco essential oils by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry. J Chromatogr A 1086:107–114

Supplementary material

Supplementary material 1. Reference list of analysed studies that were selected according to a systematic search in Scopus database. In the total, 143 articles that tested phytotoxicity/allelopathy in grassland ecosystems were included

Ahmed M, Wardle DA (1994) Allelopathic potential of vegetative and flowering ragwort (*Senecio jacobaea* L.) plants against associated pasture species. Plant Soil 164:61–68

Alford ÉR, Vivanco JM, Paschke MW (2009) The effects of flavonoid allelochemicals from knapweeds on legume-rhizobia candidates for restoration. Restor Ecol 17:506–514

Andrews TS, Jones CE, Whalley RDB (1997) Factors affecting the germination of Giant Parramatta grass. Aust J Exp Agric 37:439–446

Archibold OW, Brooks D, Delanoy L (1997) An investigation of the invasive shrub European buckthorn, *Rhamnus cathartica* L., near Saskatoon, Saskatchewan. Can. Field-Nat 11:617–621

Bai Y, Thompson DON, Broersma K (2000) Early establishment of Douglas-fir and ponderosa pine in grassland seedbeds. J Range Manag 53:511–517

Bainard LD, Brown PD, Upadhyaya MK (2009) Inhibitory effect of tall hedge mustard (*Sisymbrium loeselii*) allelochemicals on rangeland plants and arbuscular mycorrhizal fungi. Weed Sci 57:386–393

Bao G, Saikkonen K, Wang H et al. (2015) Does endophyte symbiosis resist allelopathic effects of an invasive plant in degraded grassland? Fungal Ecol 17:114–125

Batish DR, Singh HP, Kohli RK (2001) Vegetation exclusion under *Casuarina equisetifolia* L.: Does allelopathy play a role? Community Ecol 2:93–100

Belgeri A, Adkins S (2015) Allelopathic potential of invasive parthenium weed (*Parthenium hysterophorus* L.) seedlings on grassland species in Australia. Allelopathy J 36:1–14

Bennett AE, Thomsen M, Strauss SY (2011) Multiple mechanisms enable invasive species to suppress native species. Am J Bot 98:1086–1094

Bernhard-Reversat F (1999) The leaching of Eucalyptus hybrids and *Acacia auriculiformis* leaf litter: laboratory experiments on early decomposition and ecological implications in congolese tree plantations. Appl Soil Ecol 12:251–261

Bokhari UG (1978) Allelopathy among prairie grasses and its possible ecological significance. Ann Bot 42:127–136

Callaway RM, Nadkarni NM, Mahall BE (1991) Facilitation and interference of *Quercus douglasii* on understory productivity in Central California. Ecology 72:1484–1499

Carral E, Reigosa MJ, Carballeira A (1988) *Rumex obtusifolius* L: Release of allelochemical agents and their influence on small-scale spatial distribution of meadow species. J Chem Ecol 14:1763–1773

Chapla TE, Campos JB (2010) Allelopathic evidence in exotic guava (*Psidium guajava* L.). Brazilian Arch Biol Technol 53:1359–1362

Chen S, Xiao S, Callaway RM (2012) Light intensity alters the allelopathic effects of an exotic invader. Plant Ecol Divers 5:521–526

Chou CH, Chang SJ, Cheng CM et al. (1989) The selective allelopathic interaction of a pasture-forest intercropping in Taiwan II. Interaction between kikuyu grass and three hardwood plants. Plant Soil 116:207–215

Chou CH, Fu CY, Li SY, Wang YF (1998) Allelopathic potential of *Acacia confusa* and related species in Taiwan. J Chem Ecol 24:2131–2150

Chou CH, Hwang SY, Peng CI et al. (1987) The selective allelopathic interaction of a pasture-forest intercropping in Taiwan. Plant Soil 98:31–41

Chou CH, Lee YF (1991) Allelopathic dominance of *Miscanthus transmorrisonensis* in an alpine grassland community in Taiwan. J Chem Ecol 17:2267–2281

Chou CH, Leu LL (1992) Allelopathic substances and interactions of *Delonix regia* (BOJ) RAF. J Chem Ecol 18:2285–2303

Colvin WI, Gliessman SR (2011) Effects of fennel (*Foeniculum vulgare* L.) interference on germination of introduced and native plant species. Allelopathy J 28:41-52

Del Fabbro C, Güsewell S, Prati D (2014) Allelopathic effects of three plant invaders on germination of native species: a field study. Biol Invasions 16:1035–1042

Del Moral R, Muller CH (1970) The allelopathic effects of *Eucalyptus camaldulensis*. Am Midl Nat 83:254–282

Djurdjević L, Gajić G, Kostić O et al. (2013) Allelopathic effects of *Chrysopogon gryllus* L. in *Chrysopogonetum pannonicum* Stjep.-Ves. steppe community at Deliblato Sands (Serbia). Allelopathy J 32:133–148

El Ayeb-Zakhama A, Sakka-Rouis L, Bergaoui A et al. (2015) Chemical composition and allelopathic potential of essential oils obtained from *Acacia cyanophylla* Lindl. cultivated in Tunisia. Chem Biodivers 12:615–626

Ferreira NR, Medeiros RB, Soares GLG (2008) Potencial alelopático do capim-annoni-2 (*Eragrostis plana* Nees) na germinação de sementes de gramíneas perenes estivais. Rev Bras Sementes 30:43–50

Fonseca RR, Ortiz-Ramírez FA, Cavalcanti DN et al. (2012) Allelopathic potential of extracts the from marine macroalga *Plocamium brasiliense* and their effects on pasture weed. Brazilian J Pharmacogn 22:850–853

Furness NH, Adomas B, Dai Q et al. (2008) Allelopathic influence of houndstongue (*Cynoglossum officinale*) and its modification by UV-B radiation. Weed Technol 22:101-107

Gatti AB, Ferreira AG, Arduin M et al. (2010) Allelopathic effects of aqueous extracts of *Artistolochia esperanzae* O.Kuntze on development of *Sesamum indicum* L. seedlings. Acta Bot Brasilica 24:454–461

Ghebrehiwot HM, Aremu AO, Van Staden J (2014) Evaluation of the allelopathic potential of five South African mesic grassland species. Plant Growth Regul 72:155–162

Ghermandi L, Franzese J, Gonzalez SL et al. (2013) Disentangling *Fabiana imbricata* (Solanaceae) regeneration: The importance of disturbance and rainfall. J Arid Environ 97:9–13

Gliessman SR, Muller CH (1978) The allelopathic mechanisms of dominance in bracken (*Pteridium aquilinum*). J Chem Ecol 4:337–362

Goodall J, Witkowski ETF, Ammann S, Reinhardt C (2010) Does allelopathy explain the invasiveness of *Campuloclinium macrocephalum* (pompom weed) in the South African grassland biome? Biol Invasions 12:3497–3512

Grant DW, Peters DPC, Beck GK, Fraleigh HD (2003) Influence of an exotic species, *Acroptilon repens* (L.) DC. on seedling emergence and growth of native grasses. Plant Ecol 166:157–166

Grassi RF, Resende UM, Silva W et al. (2005) Estudo fitoquímico e avaliação alelopática de *Memora peregrina* – "ciganinha" – Bignoniaceae, uma espécie invasora de pastagens em Mato Grosso do Sul. Quim Nova 28:199–203

Greer MJ, Wilson GWT, Hickman KR, Wilson SM (2014) Experimental evidence that invasive grasses use allelopathic biochemicals as a potential mechanism for invasion: chemical warfare in nature. Plant Soil 385:165–179

Guo H, Cui H, Jin H et al. (2015) Potential allelochemicals in root zone soils of *Stellera chamaejasme* L. and variations at different geographical growing sites. Plant Growth Regul 77:335–342

Gyenes V, Béres I (2006) The allelopathic potential of common dandelion (*Taraxacum officinale* WEB.). J Plant Dis Prot 471–478

Habermann E, Pontes FC, Pereira VC et al. (2016) Phytotoxic potential of young leaves from *Blepharocalyx salicifolius* (Kunth) O. Berg (Myrtaceae). Brazilian J Biol 76:531–538

Hagan DL, Jose S, Lin C-H (2013) Allelopathic exudates of cogongrass (*Imperata cylindrica*): implications for the performance of native pine savanna plant species in the southeastern US. J Chem Ecol 39:312–322

Halligan JP (1973) Bare areas associated with shrub stands in grassland: The case of *Artemisia californica*. BioScience 23:429–432

Halvorson JJ, Belesky DP, West MS (2017) Inhibition of forage seed germination by leaf litter extracts of overstory hardwoods used in silvopastoral systems. Agrofor Syst 91:69–83

Harnden J, MacDougall AS, Sikes BA (2011) Field-based effects of allelopathy in invaded tallgrass prairie. Botany 89:227–234

Heděnec P, Novotný D, Ustak S et al. (2014) Allelopathic effect of new introduced biofuel crops on the soil biota: A comparative study. Eur J Soil Biol 63:14–20

Heisey RM, Delwiche CC (1985) Allelopathic effects of *Trichostema lanceolatum* (Labiatae) in the California annual grassland. J Ecol 73:729–742

Henn H, Petit D, Vernet P (1988) Interference between *Hieracium pilosella* and *Arrhenatherum elatius* in colliery spoils of north of France: Allelopathy or competition? Oecologia 76:268–272

Houx JH, Garrett HE, McGraw RL (2008) Applications of black walnut husks can improve orchardgrass and red clover yields in silvopasture and alley cropping plantings. Agrofor Syst 73:181–187

Hussain F, Ahmad B, Ilahi I (2010) Allelopathic effects of *Cenchrus ciliaris* L. and *Bothriochloa pertusa* (L.) A. Camus. Pakistan J Bot 42:3587–3604

Imatomi M, Novaes P, Matos AP et al. (2013) Phytotoxic effect of bioactive compounds isolated from *Myrcia tomentosa* (Myrtaceae) leaves. Biochem Syst Ecol 46:29–35

Inderjit, Pollock JL, Callaway RM, Holben W (2008) Phytotoxic effects of (+/-)-catechin in vitro, in soil, and in the field. PLoS One 3:e2536

Jankowska J, Ciepiela GA, Jankowski K et al. (2014) The allelopathic influence of *Taraxacum officinale* on the initial growth and development of *Festuca rubra* L. J Ecol Eng 15:38–44

Keay J, Rogers WE, Lankau R, Siemann E (2000) The role of allelopathy in the invasion of the chinese tallow tree (*Sapium sebiferum*). Texas J. Sci. 52:57–64

Khanh TD, Hong NH, Nhan DQ et al. (2006) Herbicidal activity of *Stylosanthes guianensis* and its phytotoxic components. J Agron Crop Sci 192:427–433

Komai K, Tang C-S (1989) Chemical constituents and inhibitory activities of essential oils from *Cyperus brevifolius* and *C. kyllingia*. J Chem Ecol 15:2171–2176

Kraus E, Voeten M, Lambers H (2002) Allelopathic and autotoxic interactions in selected populations of *Lolium perenne* grown in monoculture and mixed culture. Funct Plant Biol 29:1465–1473

Laterra P, Bazzalo ME (1999) Seed-to-seed allelopathic effects between two invaders of burned Pampa grasslands. Weed Res 39:297–308

Lipinska H, Harkot W (2005) Allelopathic effects of *Poa pratensis* on other grassland spp. Allelopathy J 16:251-260

Lipinska H, Lipinski W (2009) Initial growth of *Phleum pratense* under the influence of leaf water extracts from selected grass species and the same extracts improved with $MgSO_4$ $7H_2O$. J Elem 14:101–110

Lodhi MAK, Rice EL (1971) Allelopathic effects of *Celtis laevigata*. Bull. Torrey Bot. Club. 98:83-89

Loydi A, Donath TW, Eckstein RL, Otte A (2015) Non-native species litter reduces germination and growth of resident forbs and grasses: allelopathic, osmotic or mechanical effects? Biol Invasions 17:581–595

Macfarlane MJ, Scott D, Jarvis P (1982a) Allelopathic effects of white clover 1. Germination and chemical bioassay. New Zeal J Agric Res 25:503–510

Macfarlane MJ, Scott D, Jarvis P (1982b) Allelopathic effects of white clover 2. Field investigations in tussock grasslands. New Zeal J Agric Res 25:511–518

Makepeace W, Dobson AT, Scott D (1985) Interference phenomena due to mouse-ear and king devil hawkweed. New Zeal J Bot 23:79–90

Markó G, Ónodi G, Kertész M, Altbäcker V (2011) Rabbit grazing as the major source of intercanopy heterogeneity in a juniper shrubland. Arid L Res Manag 25:176–193

Martin LD, Smith AE (1994) Allelopathic potential of some warm-season grasses. Crop Prot 13:388–392

Martins D, Martins CC, Costa NV (2006) Potencial alelopático de soluções de solo cultivado com *Brachiaria brizantha*: efeitos sobre a germinação de gramíneas forrageiras e plantas daninhas de pastagens. Planta Daninha 24:61–70

May L, Baldwin LK (2011) Linking field based studies with greenhouse experiments: the impact of *Centaurea stoebe* (=*C. maculosa*) in British Columbia grasslands. Biol Invasions 13:919–931

Meier CL, Keyserling K, Bowman WD (2009) Fine root inputs to soil reduce growth of a neighbouring plant via distinct mechanisms dependent on root carbon chemistry. J Ecol 97:941–949

Metlen KL, Aschehoug ET, Callaway RM (2013) Competitive outcomes between two exotic invaders are modified by direct and indirect effects of a native conifer. Oikos 122:632–640

Mincheva T, Barni E, Siniscalco C (2016) From plant traits to invasion success: Impacts of the alien *Fallopia japonica* (Houtt.) Ronse Decraene on two native grassland species. Plant Biosyst 150:1348–1357

Moameri M, Khalaki MA, Tavili A (2011) Effects of *Haloxylon Aphyllum* (Minkw.) Lljin extracts on seeds germination and seedling growth of *Agropyron elongatum* (Host.) and *Agropyron desertorum* (Fisch.). Research Journal of Seed Science 4:40-50

Muller CH, Muller WH, Haines BL (1964) Volatile growth inhibitors produced by aromatic shrubs. Science 143:471–473

Nascimento MC, Alcantara SF, Haddad CRB, Martins FR (2007) Allelopathic potential of *Pouteria torta* (Mart.) Radlk., a species of the Brazilian cerrado. Allelopathy J 20:279–286

Nasir H, Iqbal Z, Hiradate S, Fujii Y (2005) Allelopathic potential of *Robinia pseudo-acacia* L. J Chem Ecol 31:2179–2192

Navarro-Cano JA, Barberá GG, Ruiz-Navarro A, Castillo VM (2009) Pine plantation bands limit seedling recruitment of a perennial grass under semiarid conditions. J Arid Environ 73:120–126

Novaes P, Imatomi M, Varela RM et al. (2013) Allelopathic potential of *Rapanea umbellata* leaf extracts. Chem Biodivers 10:1539–1548

Novaes P, Torres PB, Santos DYAC (2016) Biological activities of Annonaceae species extracts from Cerrado. Brazilian J Bot 39:131–137

Nyanumba SM, Cahill Jr JF (2012) Effect of aboveground litter on belowground plant interactions in a native Rough Fescue grassland. Basic Appl Ecol 13:615–622

Oliveira SCC, Campos ML (2006) Allelopathic effects of *Solanum palinacanthum* leaves on germination and seedling growth of *Sesamum indicum*. Allelopathy J 18:331–338

Paoletti MG, Rascio N, Moro I et al. (2012) Ethnobiology of *Munnozia pinnatipartita* (Compositae: Liabeae), an intriguing weed of dairy farms and its use as an herbicide to convert pastures into cloud forest in the Otonga reserve in highland Ecuador. J Sustain Agric 36:69–82

Parker VT, Muller CH (1979) Allelopathic dominance by a tree-associated herb in a California annual grassland. Oecologia 37:315–320

Rodrigues APDC, Laura VA, Pereira SR et al. (2012) Allelopathy of two brachiaria species in seeds of three species of stylosanthes. Cienc Rural 42:1758–1763

Perry LG, Cronin SA, Paschke MW (2009) Native cover crops suppress exotic annuals and favor native perennials in a greenhouse competition experiment. Plant Ecol 204:247–259

Perry LG, Johnson C, Alford ER et al. (2005) Screening of grassland plants for restoration after spotted knapweed invasion. Restor Ecol 13:725–735

Pinto GFS, Kolb RM (2016) Seasonality affects phytotoxic potential of five native species of Neotropical savanna. Botany 94:81–89

Pisula NL, Meiners SJ (2010) Allelopathic effects of goldenrod species on turnover in successional communities. Am Midl Nat 163:161–172

Prestidge RA, Thom ER, Marshall SL et al. (1992) Influence of *Acremonium lolii* infection in perennial ryegrass on germination, emergence, survival, and growth of white clover. New Zeal J Agric Res 35:225–234

Qin B, Lau JA, Kopshever J et al. (2007) No evidence for root-mediated allelopathy in *Centaurea solstitialis*, a species in a commonly allelopathic genus. Biol Invasions 9:897–907

Quddus MS, Bellairs SM, Wurm PAS (2014) *Acacia holosericea* (Fabaceae) litter has allelopathic and physical effects on mission grass (*Cenchrus pedicellatus* and *C. polystachios*) (Poaceae) seedling establishment. Aust J Bot 62:189–195

Reinhart KO, Rinella M (2011) Comparing susceptibility of eastern and western US grasslands to competition and allelopathy from spotted knapweed [*Centaurea stoebe* L. subsp. micranthos (Gugler) Hayek]. Plant Ecol 212:821–828

Renne IJ, Rios BG, Fehmi JS, Tracy BF (2004) Low allelopathic potential of an invasive forage grass on native grassland plants: a cause for encouragement? Basic Appl Ecol 5:261–269

Renne IJ, Sinn BT, Shook GW et al. (2014) Eavesdropping in plants: delayed germination via biochemical recognition. J Ecol 102:86–94

Ridenour WM, Callaway RM (2001) The relative importance of allelopathy in interference: the effects of an invasive weed on a native bunchgrass. Oecologia 126:444–450

Rodrigues IMC, Souza Filho APS, Ferreira FA, Demuner AJ (2010) Prospecção química de compostos produzidos por *Senna alata* com atividade alelopática. Planta Daninha 28:1–12

Rout ME, Chrzanowski TH, Smith WK, Gough L (2013) Ecological impacts of the invasive grass *Sorghum halepense* on native tallgrass prairie. Biol Invasions 15:327–339

Ruprecht E, Donath TW, Otte A, Eckstein RL (2008) Chemical effects of a dominant grass on seed germination of four familial pairs of dry grassland species. Seed Sci Res 18:239–248

Ruprecht E, Józsa J, Ölvedi TB, Simon J (2010) Differential effects of several “litter” types on the germination of dry grassland species. J Veg Sci 21:1069–1081

Safari H, Tavili A, Saberi M (2010) Allelopathic effects of *Thymus kotschyanus* on seed germination and initial growth of *Bromus tomentellus* and *Trifolium repens*. Front Agric China 4:475–480

San Emeterio L, Arroyo A, Canals RM (2004) Allelopathic potential of *Lolium rigidum* Gaud. on the early growth of three associated pasture species. Grass Forage Sci 59:107–112

Santos S, Moraes MLL, Rezende MOO, Souza Filho APS (2011) Potencial alelopático e identificação de compostos secundários em extratos de calopogônio (*Calopogonium mucunoides*) utilizando eletroforese capilar. Eclética Química 36:51–68

Santos S, Moraes MLL, Souza Filho APS, Rezende MOO (2005) Allelopathic potential and systematic evaluation of organic extracts from *Canavalia ensiformis* leaves (Jack Beans). J Environ Sci Health 40:77-84

Scott D (1975) Allelopathic interactions of resident tussock grassland species on germination of oversown seed. New Zeal J Exp Agric 3:135–141

Shang ZH, Tang Y, Long RJ (2011) Allelopathic effect of *Aconitum pendulum* (Ranunculaceae) on seed germination and seedlings of five native grass species in the Tibetan Plateau. Nord J Bot 29:488–494

Shang ZH, Xu SG (2012) Allelopathic testing of *Pedicularis kansuensis* (Scrophulariaceae) on seed germination and seedling growth of two native grasses in the Tibetan plateau. Int J Exp Bot 81:75–79

Silva ER, Ferreira PMA, Overbeck GE, Soares GLG (2015) Does the phytotoxic shrub *Heterothalamus psiadioides* affect a plant community through allelopathy? Plant Ecol 216:87–97

Silva ER, Overbeck GE, Soares GLG (2014) Phytotoxicity of volatiles from fresh and dry leaves of two Asteraceae shrubs: Evaluation of seasonal effects. South African J Bot 93:14–18

Silva RMG, Santos VHM, Borges FM et al. (2012) Potencial alelopático e levantamento do banco natural de sementes sob a copa de *Copaifera langsdorffii* Desf. Biosci J 28:641–653

Smith AE (1990) Potential allelopathic influence of certain pasture weeds. Crop Prot 9:410-414

Snyman HA (2010) Allelopathic potential, seed ecology and germination of the encroacher shrub *Seriphium plumosum*. African J Range Forage Sci 27:29–37

Souza LS, Velini ED, Martins D, Rosolem CA (2006) Efeito alelopático de capim-braquiária (*Brachiaria decumbens*) sobre o crescimento inicial de sete espécies de plantas cultivadas. Planta Daninha 24:657–668

Souza Filho APS (2006) Interferência potencialmente alelopática do capim-gengibre (*Paspalum maritimum*) em áreas de pastagens cultivadas. Planta Daninha 24:451–456

Souza Filho APS, Rodrigues LRA, Rodrigues TJD (1997) Potencial alelopático de forrageiras tropicais: efeitos sobre invasoras de pastagens. Planta Daninha 15:53–60

Souza Filho APS, Vasconcelos MAM, Zoghbi MGB, Cunha RL (2009) Efeitos potencialmente alelopáticos dos óleos essenciais de *Piper hispidinervium* C. DC. e *Pogostemon heyneanus* Benth sobre plantas daninhas. Acta Amaz 39:389–396

Stowe LG (1979) Allelopathy and its influence on the distribution of plants in an Illinois old-field. J Ecol 67:1065–1085

Tan DKY, Daley AT, Wu H (2007) Allelopathic potential of lippia (*Phyla canescens*) on germinating seeds. Allelopath J 19:257–266

Taylor JE, Thomson JA (1998) Bracken litter as mulch: glasshouse evaluation of phytotoxicity. Aust J Exp Agric 38:161–169

Thorpe AS, Thelen GC, Diaconu A, Callaway RM (2009) Root exudate is allelopathic in invaded community but not in native community: field evidence for the novel weapons hypothesis. J Ecol 97:641–645

Tian Y, Feng Y, Liu C (2007) Addition of activated charcoal to soil after clearing *Ageratina adenophora* stimulates growth of forbs and grasses in China. Trop Grasslands 41:285–291

Tseng M, Kuo Y, Chen Y, Chou CH (2003) Allelopathic potential of *Macaranga tanarius* (L.) Muell.-Arg. J Chem Ecol 29:1269–1286

Tsubo M, Nishihara E, Nakamatsu K et al. (2012) Plant volatiles inhibit restoration of plant species communities in dry grassland. Basic Appl Ecol 13:76–84

Tucat G, Bentivegna D, Fernández O et al. (2013) Efecto fitotóxico de *Baccharis ulicina* sobre la germinación y crecimiento inicial de *Avena sativa*, *Lolium perenne* y *Raphanus sativus*. Rev la Fac Ciencias Agrar la Univ Nac Cuyo 45:63–77

Vázquez-de-Aldana BR, Romo M, García-Ciudad A et al. (2011) Infection with the fungal endophyte *Epichloë festucae* may alter the allelopathic potential of red fescue. Ann Appl Biol 159:281–290

Viard-Crétat F, Baptist F, Secher-Fromell H, Gallet C (2012) The allelopathic effects of *Festuca paniculata* depend on competition in subalpine grasslands. Plant Ecol 213:1963–1973

Viard-Crétat F, Gallet C, Lefebvre M, Lavorel S (2009) A leachate a day keeps the seedlings away: mowing and the inhibitory effects of *Festuca paniculata* in subalpine grasslands. Ann Bot 103:1271–1278

Viles AL, Reese RN (1996) Allelopathic potential of *Echinacea angustifolia* D.C. Environ Exp Bot 36:39–43

Wardle DA, Ahmed M, Nicholson KS (1991) Allelopathic influence of nodding thistle (*Carduus nutans* L.) seeds on germination and radicle growth of pasture plants. New Zeal J Agric Res 34:185–191

Wardle DA, Nicholson KS, Ahmed M (1992a) Comparison of osmotic and allelopathic effects of grass leaf extracts on grass seed germination and radicle elongation. Plant Soil 140:315–319

Wardle DA, Nicholson KS, Ahmed M, Rahman A (1994) Interference effects of the invasive plant *Carduus nutans* L. against the nitrogen fixation ability of *Trifolium repens* L. Plant Soil 163:287–297

Wardle DA, Nicholson KS, Rahman A (1992b) Influence of pasture grass and legume swards on seedling emergence and growth of *Carduus nutans* L. Weed Res 32:119–128

Wardle DA, Nicholson KS, Rahman A (1993) Influence of plant age on the allelopathic potential of nodding thistle (*Carduus nutans* L.) against pasture grasses and legumes. Weed Res 33:69–78

Wardle DA, Nicholson KS, Rahman A (1996) Use of a comparative approach to identify allelopathic potential and relationship between allelopathy bioassays and “competition” experiments for ten grassland and plant species. J Chem Ecol 22:933–948

Watanabe Y, Novaes P, Varela RM et al. (2014) Phytotoxic potential of *Onopordum acanthium* L. (Asteraceae). Chem Biodivers 11:1247–1255

Weir TL, Bais HP, Stull VJ et al. (2006) Oxalate contributes to the resistance of *Gaillardia grandiflora* and *Lupinus sericeus* to a phytotoxin produced by *Centaurea maculosa*. Planta 223:785–795

Wu GL, Ren GH, Shi ZH (2011) Phytotoxic effects of a dominant weed *Ligularia virgaurea* on seed germination of *Bromus inermis* in an alpine meadow community. Plant Ecol Evol 144:275–280

Yager LY, Smeins FE (1999) Ashe juniper (*Juniperus ashei*: Cupressaceae) canopy and litter effects on understory vegetation in a juniper-oak savanna. Southwest Nat 44:6-16

Yamamoto Y (1995) Allelopathic potential of *Anthoxanthum odoratum* for invading *Zoysia*-grassland in Japan. J Chem Ecol 21:1365–1373

Yan Z, Guo H, Yang J et al. (2014) Phytotoxic flavonoids from roots of *Stellera chamaejasm*e L. (Thymelaeaceae). Phytochemistry 106:61–68

Zaller JG (2006) Allelopathic effects of *Rumex obtusifolius* leaf extracts against native grassland species. J Plant Dis Prot 463–470

Zhang RM, Zuo ZJ, Gao PJ et al. (2012) Allelopathic effects of VOCs of *Artemisia frigida* Willd. on the regeneration of pasture grasses in Inner Mongolia. J Arid Environ 87:212–218

Zhang S, Liu J, Bao X, Niu K (2011) Seed-to-seed potential allelopathic effects between *Ligularia virgaurea* and native grass species of Tibetan alpine grasslands. Ecol Res 26:47–52

Zhang Y, Tang S, Liu K et al. (2015) The allelopathic effect of *Potentilla acaulis* on the changes of plant community in grassland, northern China. Ecol Res 30:41–47

Zhao-Jiang Z, Ru-Min Z, Pei-Jun G et al. (2011) Allelopathic effects of *Artemisia frigida* Willd. on growth of pasture grasses in Inner Mongolia, China. Biochem Syst Ecol 39:377–383

Supplementary material 2 Evaluated donor species in studies that tested phytotoxicity/allelopathy in grassland ecosystems, including 143 articles obtained from Scopus database. Species name was reported according to currently accepted name

Family	Donor species	Reference
Amaranthaceae	*Haloxylon ammodendron* (C.A.Mey.) Bunge ex Fenzl	Moameri et al. 2011
Annonaceae	*Annona coriacea* Mart.	Novaes et al. 2016
	Annona crassiflora Mart.	Novaes et al. 2016
	Duguetia furfuracea (A. St.-Hil.) Saff.	Novaes et al. 2016
	Xylopia aromatica (Lam.) Mart.	Novaes et al. 2016
Apiaceae	*Foeniculum vulgare* Mill.	Colvin and Gliessman 2011
	Heracleum mantegazzianum Somm. et Lev.	Loydi et al. 2015
Aristolochiaceae	*Aristolochia esperanzae* Kuntze	Gatti et al. 2010
Asteraceae	*Achillea millefolium* L.	Scott et al. 1974
	Acroptilon repens (L.) DC.	Grant et al. 2003; Alford et al. 2009
	Ageratina adenophora (Spreng.) R.M.King & H.Rob.	Tian et al. 2007
	Ambrosia artemisiifolia L.	Perry et al. 2009
	Antennaria microphylla Rydb.	Perry et al. 2009
	Artemisia adamsii Besser	Tsubo et al. 2012
	Artemisia californica Less.	Muler et al. 1964; Halligan 1973
	Artemisia frigida Willd.	Zhao-Jiang et al. 2011; Zhang et al. 2012
	Artemisia tridentata Nutt. ssp. Wyomingensis Beetle & Young	Bai et al. 2000
	Baccharis patens Baker	Silva et al. 2014; Silva et al. 2015
	Baccharis psiadioides (Less.) Joch.Müll.	Silva et al. 2014; Silva et al. 2015
	Baccharis ulicina Hook. & Arn.	Tucat et al. 2013
	Campuloclinium macrocephalum (Less.) DC	Goodall et al. 2010
	Carduus nutans L.	Wardle et al. 1991; Wardle et al. 1993; Wardle et al. 1994

Family	Donor species	Reference
Asteraceae	*Centaurea solstitialis* L.	Qin et al. 2007
	Centaurea stoebe L.	Ridenour and Callaway 2001; Perry et al. 2005; Weir et al. 2006; Inderjit et al. 2008; Alford et al. 2009; Thorpe et al. 2009; May and Baldwin 2011; Reinhart and Rinella 2011; Chen et al. 2012; Renne et al. 2014
	Echinacea angustifolia DC.	Viles and Reese 1996
	Erigeron annuus (L.) Desf.	Del Fabbro et al. 2014
	Eupatorium capillifolium (Lam.) Small ex Porter & Britton	Smith 1990
	Euthamia graminifolia (L.) Nutt.	Pisula and Meiners 2010
	Gaillardia megapotamica var. scabiosoides (Arn. ex DC.) Baker	Renne et al. 2014
	Gochnatia polymorpha (Less.) Cabrera	Pinto and Kolb 2016
	Helenium amarum (Raf.) H. Rock	Smith 1990
	Helianthus annuus L.	Perry et al. 2009
	Heliopsis helianthoides (L.) Sweet	Renne et al. 2014
	Hieracium pilosella L.	Scott et al. 1974
	Hieracium praealtum Vill. ex Gochnat	Scott et al. 1974
	Hypochaeris radicata L.	Scott et al. 1974
	Ligularia virgaurea (Maxim.) Mattf. ex Rehder & Kobuski	Wu et al. 2011; Zhang et al. 2011
	Munnozia pinnatipartita (Hieron.) H. Rob. & Brettell	Paoletti et al. 2012
	Onopordum acanthium L.	Watanabe et al. 2014
	Parthenium hysterophorus L.	Belgeri and Adkins 2015
	Parthenium integrifolium L.	Renne et al. 2014
	Pilosella officinarum Vaill.	Makepeace et al. 1985; Henn et al. 1988
	Pilosella praealta (Vill. ex Cochn.)	Makepeace et al. 1985
	Raoulia australis Hook.f. ex Raoul	Scott et al. 1974

Family	Donor species	Reference
Asteraceae	*Raoulia parkii* Buchanan	Scott et al. 1974
	Raoulia subsericea Hook.f.	Scott et al. 1974
	Ratibida pinnata (Vent.) Barnhart	Renne et al. 2014
	Senecio jacobaea L.	Ahmed and Wardle 1994
	Seriphium plumosum L.	Snyman 2010
	Solidago canadensis L.	Perry et al. 2009; Pisula and Meiners 2010
	Solidago gigantea Aiton	Pisula and Meiners 2010
	Solidago juncea Aiton	Pisula and Meiners 2010
	Solidago nemoralis Aiton	Pisula and Meiners 2010
	Solidago rugosa Mill.	Pisula and Meiners 2010
	Taraxacum officinale F.H. Wigg.	Scott et al. 1974; Gyenes and Béres 2006; Jankowska et al. 2014
	Thelesperma megapotamicum (Spreng.) Kuntze	Renne et al. 2014
Balsaminaceae	*Impatiens glandulifera* Royle	Loydi et al. 2015
Betulaceae	*Alnus formosana* (Burk.) Makino	Chou et al. 1989
Bignoniaceae	*Adenocalymma peregrinum* (Miers) L.G.Lohmann	Grassi et al. 2005
Boraginaceae	*Cynoglossum officinale* L.	Furness et al. 2008
	Pholistoma auritum (Lindl.) Lilja ex Lindbl.	Parker and Muller 1979
Brassicaceae	*Raphanus raphanistrum* L.	Smith 1990
	Sisymbrium loeselii L.	Bainard et al. 2009
Cannabaceae	*Celtis laevigata* Willd.	Lodhi and Rice 1971
Caryophyllaceae	*Scleranthus uniflorus* Will.	Scott et al. 1974
Casuarinaceae	*Casuarina equisetifolia* L.	Batish et al. 2001
Cupressaceae	*Cunninghamia lanceolata* (Lamb.) Hook.	Chou et al. 1987
	Juniperus ashei J.Buchholz	Yager and Smeins 1999
	Juniperus communis L.	Markó et al. 2011
Cyperaceae	*Kyllinga brevifolia* Rottb.	Komai and Tang 1989
	Rhynchospora colorata (L.) H.Pfeiff.	Komai and Tang 1989

Family	Donor species	Reference
Dennstaedtiaceae	*Pteridium aquilinum* (L.) Kuhn	Gliessman and Muller 1978
	Pteridium esculentum (G. Forst.) Cockayne	Taylor and Thomson 1998
Ericaceae	*Styphelia fraseri* F.Muell.	Scott et al. 1974
Euphorbiaceae	*Macaranga tanarius* (L.) Müll.Arg.	Tseng et al. 2003
	Sapium sebiferum (L.) Roxb.	Keay et al. 2000
Fabaceae	*Acacia aulacocarpa* Cunn. ex Benth.	Chou et al. 1998
	Acacia auriculiformis Cunn. ex Benth.	Chou et al. 1998; Bernhard-Reversat 1999
	Acacia cincinnata F.Muell.	Chou et al. 1998
	Acacia confusa Merr.	Chou et al. 1998
	Acacia crassicarpa Cunn. ex Benth.	Chou et al. 1998
	Acacia cyanophylla Lindl.	El Ayeb-Zakhama et al. 2015
	Acacia holosericea Cunn. ex Don	Quddus et al. 2014
	Acacia leptocarpa Cunn. ex Benth.	Chou et al. 1998
	Acacia mangium Willd.	Chou et al. 1998
	Acacia polystachya Cunn. ex Benth.	Chou et al. 1998
	Acacia tortilis (Forssk.) Hayne	Chou et al. 1998
	Calopogonium mucunoides Desv.	Souza Filho et al. 1997; Santos et al. 2011
	Canavalia ensiformis (L.)DC.	Santos et al. 2005
	Copaifera langsdorffii Desf	Silva et al. 2012
	Coronilla varia L.	Stowe 1979
	Dalea purpurea Vent.	Renne et al. 2014
	Delonix regia (Bojer ex Hook.) Raf.	Chou and Leu 1992
	Lotus tenuis Waldst. & Kit.	Laterra and Bazzalo 1999
	Lupinus polyphyllus Lindl.	Loydi et al. 2015
	Medicago sativa L.	Smith 1990; Wardle et al. 1992b; Wardle et al. 1996
	Quercus alba L.	Halvorson et al. 2016

Family	Donor species	Reference
Fabaceae	*Quercus douglasii* Hook. & Arn.	Callaway et al. 1991
	Robinia pseudoacacia L.	Nasir et al. 2005
	Senna alata (L.)Roxb.	Rodrigues et al. 2010
	Stylosanthes guianensi (Aubl.)Sw.	Souza Filho et al. 1997; Khanh et al. 2006
	Trifolium arvense L.	Scott et al. 1974
	Trifolium pratense L.	Wardle et al. 1992b; Wardle et al. 1996
	Trifolium repens L.	Scott et al. 1974; Macfarlane et al. 1982a; Macfarlane et al. 1982b; Wardle et al. 1992b; Wardle et al. 1996
	Trifolium subterraneum L.	Wardle et al. 1992b; Wardle et al. 1996
Hypericaceae	*Hypericum perfloratum* L.	Scott et al. 1974
Juglandaceae	*Juglans nigra* L.	Houx et al. 2008
Lamiaceae	*Monarda fistulosa* L.	Renne et al. 2014
	Pogostemon heyneanus Benth	Souza Filho et al. 2009
	Salvia apiana Jeps.	Muler et al. 1964
	Salvia leucophylla Greene	Muler et al. 1964
	Thymus kotschyanus Boiss. & Hohen.	Safari et al. 2010
	Trichostema lanceolatum Benth.	Heisey and Delwiche 1985
Lauraceae	*Cinnamomum camphora* (L.) J. Presl	Chou et al. 1989
Magnoliaceae	*Liriodendron tulipifera* L.	Halvorson et al. 2016
Malpighiaceae	*Byrsonima intermedia* A. Juss.	Pinto and Kolb 2016
Malvaceae	*Luehea candicans* Mart.	Pinto and Kolb 2016
Melastomataceae	*Miconia chamissois* Naudin	Pinto and Kolb 2016
Myrtaceae	*Blepharocalyx salicifolius* (Kunth) O. Berg	Habermann et al. 2016
	Eucalyptus camaldulensis Dehnh.	Del Moral and Muller 1970
	Myrcia tomentosa (Aubl.) DC.	Imatomi et al. 2013
	Psidium guajava L.	Chapla and Campos 2010

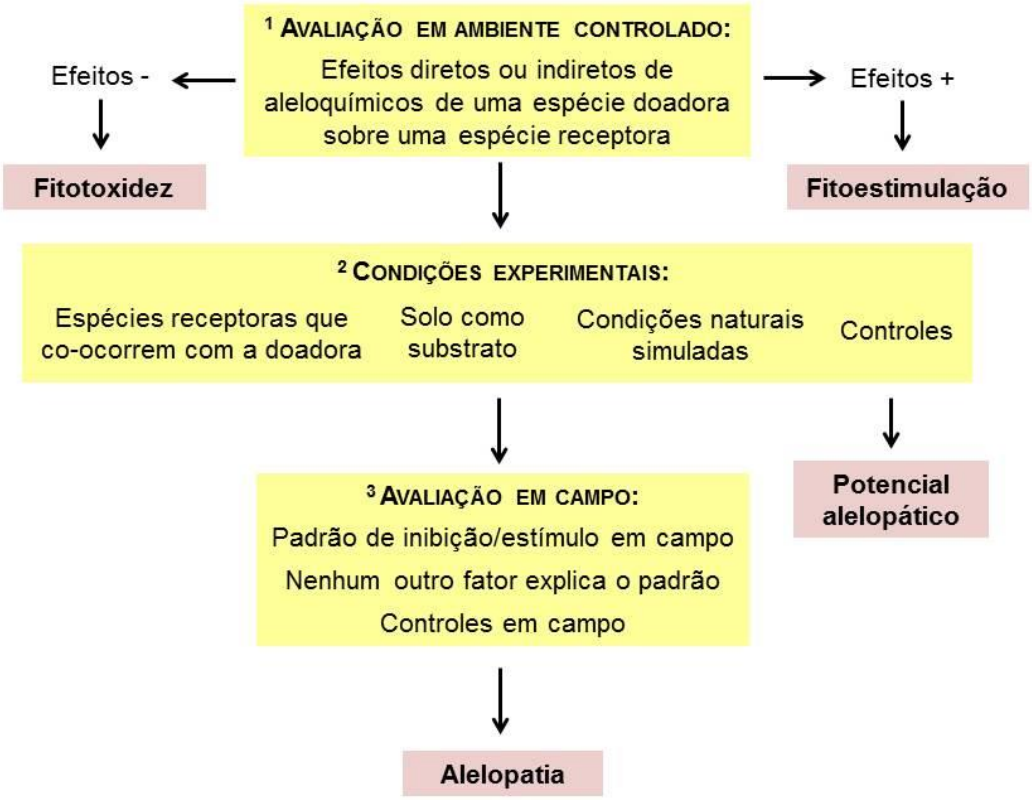
Family	Donor species	Reference
Orobanchaceae	*Pedicularis kansuensis* Maxim.	Shang and Shu 2012; Bao et al. 2015
Pinaceae	*Pinus halepensis* Mill.	Navarro-cano et al. 2009
	Pinus ponderosa Douglas ex C.Lawson	Bai et al. 2000; Metlen et al. 2013
	Pseudotsuga menziesii var. glauca (Beissn.) Franco	Bai et al. 2000
Piperaceae	*Piper hispidinervum* C. DC.	Souza Filho et al. 2009
Poaceae	*Agrostis capilaris* L.	Scott et al. 1974
	Andropogon gerardii Vitman	Greer et al. 2014; Renne et al. 2014
	Anthoxanthum odoratum L.	Scott et al. 1974; Yamamoto 1995
	Aristida niederleinii Mez	Renne et al. 2014
	Aristida junciformis Trin. & Rupr.	Ghebrehiwot et al. 2014
	Axonopus fissifolius (Raddi) Kuhlm.	Andrews et al. 1997
	Bothriochloa ischaemum (L.) Keng	Greer et al. 2014
	Bothriochloa pertusa (L.) A. Camus	Hussain et al. 2010
	Bouteloua gracilis (Kunth) Lag. ex Griffiths	Bokhari 1978
	Brachiaria brizantha (A.Rich.) Stapf	Souza Filho et al. 1997; Martins et al. 2006; Rodrigues et al. 2012
	Brachiaria decumbens Stapf	Souza Filho et al. 1997; Souza et al. 2006; Rodrigues et al. 2012
	Brachiaria humidicola (Rendle) Schweick.	Souza Filho et al. 1997
	Bromus catharticus Vahl	Wardle et al. 1992b; Wardle et al. 1996; Renne et al. 2014
	Bromus inermis Leyss.	Stowe 1979
	Cenchrus ciliaris L.	Hussain et al. 2010
	Chloris gayana Kunth	Andrews et al. 1997
	Chrysopogon gryllus (L.) Trin.	Djurdjevic et al. 2013
	Cynodon dactylon (L.) Pers.	Smith 1990
	Digitaria sanguinalis (L.) Scop.	Martin and Smith 1994

Family	Donor species	Reference
Poaceae	*Dactylis glomerata* L.	Stowe 1979; Wardle et al. 1992a; Wardle et al. 1992b; Wardle et al. 1996
	Echinochloa crus-galli (L.) P.Beauv.	Martin and Smith 1994
	Elymus repens (L.) Gould	Stowe 1979
	Elymus smithii (Rydb.) Gould	Bokhari 1978
	Eragrostis curvula (Schrad.) Nees	Ghebrehiwot et al. 2014
	Eragrostis plana Nees	Ferreira et al. 2008
	Festuca arundinacea Schreb.	Stowe 1979; Smith 1990; Wardle et al. 1992a; Wardle et al. 1992b; Wardle et al. 1996; Renne et al. 2004; Lipinska and Lipínski 2009
	Festuca campestris Rydb.	Bai et al. 2000
	Festuca novae-zelandiae (Hack.) Cockayne	Scott et al. 1974
	Festuca paniculata (L.) Schinz & Thell.	Viard-Crétat et al. 2009; Viard-Crétat et al. 2012
	Festuca rubra L.	Scott et al. 1974; Harnden et al. 2011; Vázquez-de-Aldana et al. 2011
	Hyparrhenia hirta (L.) Stapf	Ghebrehiwot et al. 2014
	Holcus lanatus L.	Wardle et al. 1992a; Wardle et al. 1992b; Wardle et al. 1996; Bennet et al. 2011
	Hordeum euclaston Steud.	Renne et al. 2014
	Imperata cylindrica (L.) P. Beauv.	Hagan et al. 2013
	Lolium multiflorum Lam.	Smith 1990; Lipinska and Lipinski 2009
	Lolium perenne L.	Prestidge et al. 1992; Wardle et al. 1992b; Wardle et al. 1996; Kraus et al. 2001; Lipinska and Lipinski 2009
	Lolium rigidum Gaudin	San Emeterio et al. 2004
	Megathyrsus maximus (Jacq.) B.K. Simon & S.W.L. Jacobs	Ghebrehiwot et al. 2014
	Miscanthus sinensis Andersson	Hedenec et al. 2014

Family	Donor species	Reference
Poaceae	*Miscanthus transmorrisonensis* Hayata	Chou and Lee 1991
	Paspalum maritimum Trin.	Souza Filho 2006
	Paspalum notatum Flueggé	Martin and Smith 1994
	Paspalum wettsteinii Hack.	Andrews et al. 1997
	Pennisetum clandestinum Hochst. ex Chiov.	Chou et al. 1987; Chou et al. 1989; Andrews et al. 1997
	Phalaris tuberosa L.	Wardle et al 1992a; Wardle et al. 1992b; Wardle et al. 1996
	Phleum pratense L.	Stowe 1979; Lipinska and Lipinski 2009
	Poa colensoi Hook.f.	Scott et al. 1974
	Poa palustris L.	Scott et al. 1974
	Poa pratensis L.	Lipinska and Wanda 2005; Lipinska and Lipinski 2009
	Rytidosperma setifolium (Hook.f.) Connor & Edgar	Scott et al. 1974
	Schizachyrium scoparium (Michx.) Nash	Keay et al. 2000
	Setaria faberi Herrm.	Martin and Smith 1994
	Setaria glauca (L.) P. Beauv.	Martin and Smith 1994
	Setaria sphacelata (Schumach.) Stapf & C.E.Hubb. ex Moss	Andrews et al. 1997
	Setaria viridis (L.) P.Beauv.	Martin and Smith 1994
	Sorghum halpense (L.) Pers.	Martin and Smith 1994; Rout et al. 2013
	Sporobulus indicus (L.) R.Br.	Andrews et al. 1997
	Stipa ichu (Ruiz & Pav) Kunth	Renne et al. 2014
	Stipa pulcherrima K.Koch	Ruprecht et al. 2008; Ruprecht et al. 2010
	Themeda triandra Forssk.	Ghebrehiwot et al. 2014
	Vulpia myuros (L.) C.C.Gmel.	Scott et al. 1974
Polygonaceae	*Fallopia japonica* (Houtt.) Ronse Decr.	Mincheva et al. 2016
	Fallopia sachalinensis (F. Schmidt) Ronse Decr.	Hedenec et al. 2014
	Muehlenbeckia axillaris (Hook. f.) Endl.	Scott et al. 1974

Family	Donor species	Reference
Polygonaceae	*Rumex acetosella* L.	Scott et al. 1974
	Rumex obtusifolius L.	Carral et al. 1988; Zaller 2010
	Rumex tianschanicus x *Rumex patientia*	Hedenec et al. 2014
Polytrichaceae	*Polytrichum juniperinum* Hedw.	Scott et al. 1974
Primulaceae	*Myrsine umbellata* Mart.	Novaes et al. 2013
Ranunculaceae	*Aconitum pendulum* N.Busch	Shang et al. 2011
Rhamnaceae	*Rhamnus canthartica* L.	Archibold et al. 1997
Rosaceae	*Acaena caesiglauca* Bergmans	Scott et al. 1974
	Acaena microphylla Hook.f.	Scott et al. 1974
	Geum rossii (R. Br.) Ser.	Meier et al. 2009
	Potentilla acaulis L.	Zhang et al. 2015
Sapindaceae	*Acer rubrum* L.	Halvorson et al. 2016
Sapotaceae	*Pouteria torta* (Mart.) Radlk.	Nascimento et al. 2007
Scrophulariaceae	*Verbascum thapsus* L.	Scott et al. 1974
Solanaceae	*Fabiana imbricata* Ruiz & Pav.	Ghermandi et al. 2013
	Solanum carolinense L.	Smith 1990
	Solanum lycocarpum A. St.-Hil.	Oliveira et al. 2004
	Solanum palinacanthum Dunal	Oliveira and Campos 2006
Thymelaeaceae	*Stellera chamaejasme* L.	Yan et al. 2014; Guo et al. 2015
Ulmaceae	*Zelkova serrata* (Thunb.) Makino	Chou et al. 1989
Verbenaceae	*Phyla canescens* (Kunth) Greene	Tan et al. 2007
Vochysiaceae	*Qualea cordata* (Mart.) Spreng.	Pinto and Kolb 2016
-	Not identified plant litter	Nyanumba and Cahill 2012

Supplementary material 3 Portuguese version of figure 5.



Conceitos e terminologia para estudos de alelopatia de acordo com evidências obtidas em condições controladas (laboratório, casa de vegetação) e em campo. Alguns termos podem ser utilizados quando uma diferente combinação de caixas amarelas for obtida – fitotoxidez/fitoestimulação (1; 1 e 2; 1 e 3; 1, 2 e 3) e potencial alelopático (1; 1 e 2; 1 e 3; 3) – embora se encaixem melhor de acordo com a indicação no diagrama. Nesses casos, o conteúdo das caixas amarelas pode ser totalmente ou parcialmente obtido. Por outro lado, a alelopatia (efeito alelopático) é apenas evidenciada quando todo o conteúdo de todas as caixas amarelas é obtido. Para estudos com outros organismos receptores além de plantas, os termos fitotoxidez e fitoestimulação devem ser adaptados, ou simplesmente ser mencionados como toxidez e estimulação (E.R. Silva et al. 2017).

CAPÍTULO II

Efeitos fitotóxicos do extrato e do óleo essencial das folhas da serapilheira de *Eucalyptus saligna* sobre espécies campestres

PHYTOTOXIC EFFECTS OF EXTRACT AND ESSENTIAL OIL OF *EUCALYPTUS SALIGNA* (MYRTACEAE) LEAF LITTER ON GRASSLAND SPECIES

Eliane Regina da Silva, Diana Carla Lazarotto, Joséli Schwambach,
Gerhard Ernst Overbeck, Geraldo Luiz Gonçalves Soares

Australian Journal of Botany, 65: 172–182, 2017. doi: 10.1071/BT16254

Resumo

Poucas plantas se estabelecem sob plantios de *Eucalyptus* na região dos campos do sul do Brasil, e a alelopatia pode estar envolvida em moldar esse padrão. Nós objetivamos avaliar a fitotoxidez do óleo essencial e do extrato aquoso das folhas da serapilheira de *Eucalyptus saligna* Sm. sobre espécies campestres. Nós testamos os efeitos do óleo puro e do extrato de *E. saligna* sobre a germinação, o crescimento de plântulas, os níveis de peróxido de hidrogênio (H_2O_2) e o vazamento de eletrólitos das membranas das plântulas de *Paspalum notatum* Flüggé, *Eragrostis plana* Ness (Poaceae), *Trifolium repens* L. e *Lotus corniculatus* L. (Fabaceae). O óleo essencial e o extrato aquoso de *E. saligna* afetaram todas as espécies receptoras, até mesmo nas menores quantidades/concentrações, inibindo a germinação e o crescimento inicial, e também aumentando os níveis de H_2O_2 e o vazamento de eletrólitos das membranas das plântulas. O óleo essencial consistiu principalmente em monoterpenos e apresentou α-pineno e 1,8-cineol como componentes majoritários. O extrato apresentou fenólicos, e níveis menores dessas substâncias no extrato foram associados à redução na fitotoxidez. Nós concluímos que as folhas da serapilheira de *E. saligna* possuem substâncias fitotóxicas que geram estresse oxidativo e levam a danos nas membranas, afetando sementes e o crescimento de plântulas. Além disso, nós relacionamos a fitotoxidez de *E. saligna* a monoterpenos que podem ser emitidos das folhas da serapilheira por volatilização, e a fenólicos que podem ser lixiviados pela chuva. O nosso estudo indica que *E. saligna* possui potencial alelopático sobre as espécies campestres testadas.

Palavras-chave: alelopatia; crescimento de plântulas; dano sobre membranas; estresse oxidativo; germinação; fenólicos; monoterpenos.

Abstract

Under *Eucalyptus* plantations in the South Brazilian grassland region, few plants establish, and allelopathy may be involved in shaping this pattern. We aimed to assess the phytotoxicity of essential oil and aqueous extract of *Eucalyptus saligna* Sm. leaf litter on grassland species. We tested the effects of *E. saligna* pure oil and extract on germination, seedling growth, hydrogen peroxide (H_2O_2) levels and electrolyte leakage of seedling membranes of *Paspalum notatum* Flüggé, *Eragrostis plana* Ness (Poaceae), *Trifolium repens* L. and *Lotus corniculatus* L. (Fabaceae). Essential oil and aqueous extract of *E. saligna* affected all recipient species, even at the lowest amounts/concentrations, inhibiting germination and initial growth, and also increasing H_2O_2 levels and electrolyte leakage of seedling membranes. Essential oil consisted mainly of monoterpenes and presented α-pinene and 1,8-cineole as the major compounds. The extract contained phenolics, and lower levels of these compounds in the extract were associated with decreased phytotoxicity. We conclude that *E. saligna* contains phytotoxic compounds in leaf litter that generate oxidative stress and lead to membrane damage, affecting seeds and seedling growth. In addition, we relate *E. saligna* phytotoxicity to monoterpenes that may be released from leaf litter by volatilisation, and phenolics that may be leached by rainfall. Our study indicates that *E. saligna* has allelopathic potential on the tested grassland species.

Keywords: allelopathy; germination; membrane damage; monoterpenes; oxidative stress; phenolics; seedling growth.

1 Introduction

Plants may suppress germination and development of other plants by producing and releasing secondary metabolites in the environment (Rice 1984; Andel 2006). The occurrence of this phenomenon – allelopathy – has been suspected to occur when vegetation cover and diversity are scarce under or near a particular species, as often observed under *Eucalyptus* stands (del Moral and Muller 1970; He *et al*. 2014). Allelopathy may be particularly relevant outside the natural range of the *Eucalyptus* species, where native species have not adapted to chemicals released by the species, as postulated by the novel weapons hypothesis (Renne *et al*. 2014). *Eucalyptus* allelochemicals are mainly present in leaves and consist of phenolics derivatives and terpenoids, which may be leached from foliage and litter by rainfall or dew (Muller *et al*. 1964; del Moral and Muller 1970).

Monoterpenes, which are found in essential oils, may be also released from *Eucalyptus* leaves by volatilisation (Muller 1965). Several studies have assessed phytotoxicity of *Eucalyptus* species' essential oils (e.g. Verdeguer *et al*. 2009; Kaur *et al*. 2011), leaf leachates or extracts (e.g. Bernhard-Reversat 1999; El-Rokiek and Eid 2009).

In the South American Pampa region, which includes areas of Southern Brazil, Uruguay and Argentina, large areas of natural grasslands have been replaced by *Eucalyptus* plantations (Overbeck *et al*. 2007), mainly for pulpwood production. Few grassland species persist under *Eucalyptus* plantations, but processes shaping this pattern have not been analysed in detail. In a grassland area in California coastal plain, the pattern of scarce vegetation under *Eucalyptus camaldulensis* Dehnh. could not be solely explained by abiotic factors, such as reduced light incidence (del Moral and Muller 1970). Rather, vegetation suppression was related to allelopathy due to phytotoxicity of compounds of *E. camaldulensis* leaves on grassland species (del Moral and Muller 1970). However, with the exception of the study by del Moral and Muller (1970), either crops (e.g. Mohamadi and Rajaie 2009), weeds (e.g. Verdeguer *et al*. 2009) or forest species (e.g. Fang *et al*. 2009; Chu *et al*. 2014) have been used as recipient plants in phytotoxicity studies about *Eucalyptus* species. Thus far, we lack data of allelopathic potential of *Eucalyptus* species in natural grassland systems.

Eucalyptus saligna Sm. is one of the most commonly cultivated *Eucalyptus* species in the Pampa region, but information about allelopathic potential of this species is scarce. Phytotoxic effects of the species have been only evaluated in one study, which showed *E. saligna* foliage extract affected germination and initial growth of crop species (Lisanework and Michelsen 1993). However, the potential for *E. saligna* to affect other plants by releasing allelochemicals is likely to be higher from leaf litter than from foliage because leaves remain in contact with water from rain for longer periods when accumulated on the soil surface. Also, in the case of the volatilisation mechanism, for which no study about *E. saligna* is available, only allelochemicals from litter have a chance to reach recipient plants. Even though several studies have evaluated the phytotoxicity of *Eucalyptus* spp. volatile oils, studies have used only essential oils extracted from foliage and not from leaf litter (e.g. Batish *et al*. 2007; Setia *et al*. 2007; Verdeguer *et al*. 2009; Kaur *et al*. 2011). Furthermore, in the study of Lisanework and Michelsen (1993), no chemical investigation was conducted to assess which compounds were involved in the extract effects. Phytotoxicity of *Eucalyptus* extracts has been mainly related to phenolics (Ziaebrahimi *et al*. 2007; Zhang and Fu 2010), but some studies have linked effects to both phenolics and terpenes (Padhy *et al*. 2000; El-Rokiek and Eid 2009).

Here, our aim was to evaluate the phytotoxicity of aqueous extract and essential oil of *E. saligna* leaf litter on grassland species. Specifically, we aimed to assess effects on

germination and seedling growth of species from the Poaceae (*Paspalum notatum* Flüggé and *Eragrostis plana* Ness) and Fabaceae (*Trifolium repens* L. and *Lotus corniculatus* L.), which are typical families of grasslands in the Pampa region. We also aimed to evaluate effects on H_2O_2 levels and electrolyte leakage from seedling membranes – physiological responses that indicate, respectively, oxidative stress and damage to membranes, which are possible mechanisms of *E. saligna* phytotoxicity. Additionally, we aimed to characterise *E. saligna* essential oil, to quantify phenolic content in the extract and to determine if phenolics contribute to the effects of the extract. We hypothesised that (1) leaf litter essential oil and extract affect germination and seedling growth of recipient species; (2) oil and extract damage membranes and increase H_2O_2 levels in seedlings; (3) *E. saligna* allelochemicals are not selective, and affect the four tested species; (4) extract effects are mainly related to phenolics.

2 Material and Methods

2.1 Essential oil extraction and aqueous extract preparation

Eucalyptus saligna Sm. essential oil and aqueous extract were prepared with leaf litter obtained in a plantation of the species located in the municipality of Eldorado do Sul, Rio Grande do Sul State, Brazil (30°11'02″S, 51°37'14″W). The site is located in the 'Campos Sulinos' region (Overbeck *et al*. 2007), where grassland is the vegetation type. The climate in the region is subtropical humid, without a dry season (Köppen's Cfa), and the soil type is Argissol (Streck *et al*. 2002). Mean annual temperature is 19.5 °C, and mean precipitation is 1309 mm $year^{-1}$ (Maluf 2000).

Essential oil was extracted from leaf litter collected in August 2014 by steam distillation. Steam distillation was conducted with 5 kg of dry leaves using an inox extractor with a steam flow rate of 3 L h^{-1} for 1 h, yielding 0.56% (w/v). Water in the *E. saligna* essential oil was eliminated with anhydrous sodium sulfate. The oil was then stored in an ultrafreezer at –80 °C until use.

Aqueous extract was prepared with leaf litter obtained from 10 collectors that were placed in the plantation (plastic mesh of 60 cm^2 surface area, 10.5 cm deep, 60 cm above the ground level). Leaves were collected monthly, from December 2014 to July 2015, and stored at –20 °C. In all experiments the extract was made with leaf litter in the same decomposition stage (1 month after falling from trees at most). This is important, as decomposition stage may influence quantity of allelochemicals in plant leaves (Bernhard-Reversat *et al*. 2003). Every time the extract was prepared, a mixed sample composed of

leaves collected in different months was used. This avoided possible differences due to changes in production of allelochemicals along the year (Silva *et al*. 2014). Aqueous extract (1 : 10 w/v) was prepared immediately before being used in each experiment, by static maceration: slightly chopped leaves were added to distilled water and remained for 72 h at room temperature (20 °C). Then, solid material was eliminated by percolation through qualitative filter paper. Crude extract (10%) was diluted in water to the concentrations of 7.5 and 5%.

2.2 Recipient species

Recipient species exposed to *E. saligna* allelochemicals were the Poaceae *Paspalum notatum* Flüggé and *Eragrostis plana* Ness, and the Fabaceae *Trifolium repens* L. and *Lotus corniculatus* L. These species are representative of two of the three richest families of South Brazilian grasslands (Overbeck *et al*. 2007). Species were chosen for their fast, high and homogeneous germination. *Paspalum notatum* is a native and very common species in the Pampa region; the three other species are naturalised exotic species, i.e. they occur in the grasslands independent of human action, forming stable populations (Schneider 2007). *Eragrostis plana* diaspores were collected at Estação Experimental Agronômica – UFRGS in Eldorado do Sul, Brazil (30°07'10″S, 51°41'06″W), and diaspores of the other species were obtained from commercial dealers.

2.3 Germination and early growth assays

In all experiments, groups were arranged in a completely random design with four replicates (Petri dishes) per group. In assays that evaluated leaf litter essential oil effects on germination, each replicate consisted of 40 diaspores of a recipient species sown in a Petri dish that contained filter paper moistened with 8 mL of distilled water. Then, pure essential oil was applied on a cotton piece, which was fixed in the inner face of the plate lid, in order to avoid direct contact between the oil and diaspores or water, and to allow volatilisation in the airspace within plates. Immediately after, plates were sealed with plastic film, to avoid loss of volatiles, and placed in a growth room (20 °C/16-h photoperiod and irradiance of 80.1 µmol m^{-2} s^{-1} provided by fluorescent lamps of 20 W). Oil amounts applied were 0 (control), 1, 10, 20, 30 and 50 µL. Germinated seeds (seeds that had root emerged) were counted until no more germination occurred, which took from eight to 18 days, depending on the recipient species. *Paspalum notatum* and *E. plana* seeds were counted every 24 h; *L. corniculatus* and *T. repens* seeds, which germinated faster than the two Poaceae, were counted every 12 h. Then, speed of accumulated germination (AS) was calculated, according to the formula:

AS = (N1/1 + N2/2+ … + Nn/n) (1) where N1, N2, N3, Nn are the cumulative number of seeds that germinate in counting 1, 2, 3, …, n (Anjum and Bajwa 2005). Germination rate was also calculated as the percentage of germinated seeds on the last day of the experiment.

In the experiment that tested essential oil effects on seedling growth, diaspores were sown in plates and placed at growth room until emergence of seedling shoot and root (144 h for *P. notatum*, 96 h for *E. plana* and 72 h for *T. repens* and *L. corniculatus*). Each replicate comprised twenty seedlings transferred to a plate and exposed to *E. saligna* essential oil (0, 1, 10, 20, 30 and 50 µL), in the same conditions of the germination assay. Plates were maintained in a growth room for 96 h, and then seedlings were photographed and measured using ImageJ 1.45s software (National Institutes of Health). Seedling root and shoot length were measured separately. For the Poaceae species, we considered the full shoot and the sum of length of all adventitious roots, and for the Fabaceae species the shoot except for the cotyledonary leaves and the primary root.

For the experiments that tested effects of *E. saligna* aqueous extract on seed germination and seedling growth, methods were similar to those described for assays with essential oil, but without addition of the oil. Extract (5, 7.5 and 10%) was added to moist filter paper in plates (8 mL). For the control group, we used distilled water.

2.4 Detection of H_2O_2 in seedlings

Effects of *E. saligna* leaf litter extract and essential oil on H_2O_2 accumulation in seedlings were assessed at the end of the early growth assays. Detection of H_2O_2 employed 3,3-diaminobenzidine (DAB), using a histochemical method adapted from work by Thordal-Christensen *et al*. (1997). Six seedlings per group were dipped in 1 mg mL^{-1} solution of DAB in water with adjusted pH (3.8) for 1 h 15 min, and then cleared in boiling ethanol. This treatment reveals a brownish coloration in seedlings due to a polymerisation reaction of DAB with H_2O_2. Accumulation of H_2O_2 was analysed in shoots of *P. notatum*, *T. repens* and *L. corniculatus*, using the full shoot, including cotyledonary leaves in the case of the Fabaceae species. The species *E. plana* showed dark stains (necrosis) in the shoot after exposure to *E. saligna* allelochemicals, and to avoid confounding effects, it was not included in this experiment.

The DAB reaction has been used as a qualitative tool to characterise production of reactive oxygen species (ROS) in plants, by usually classifying coloration visible to naked eye from ‘not visible’ to ‘strong’. However, as the visibility of reaction increases with the quantity of H_2O_2 (Thordal-Christensen *et al*. 1997), a quantitative approach was used to more

precisely characterise differences in coloration. In some studies, H_2O_2 accumulation was characterised in DAB experiments by quantifying stained pixels in photographs with a commercial image processing software, which considers the area covered by dark spots in plants (Jin *et al*. 2008; Radville *et al*. 2011). This is an adequate method if H_2O_2 accumulation occurs in spots, but in our study some seedlings showed a general dark coloration that varied in intensity. Hence, we used a different method, as follows. First, seedlings were photographed with a digital camera (Leica DFC290 HD) connected to a stereo microscope (Leica S6D, ×6.3 magnification). This equipment was also connected to a light source (Leica L2), with the same light conditions for all groups of each recipient species, and to a computer. The same exposure pattern was set for all photographs, using Leica Application Suite software (LAS ver. 3.8) (exposure = 66.0, gain = 1.7, saturation = 1.3, gamma = 0.78). Then, coloration values of seedlings were determined from the digital images, using AxioVision software (Rel. 4.9., Zeiss). Each shoot was marked, and values of red, green and blue (RGB pattern) were determined for the total area and summed together. Hence, an average coloration value was obtained for each seedling, considering the stained area and colour intensity. Lower RGB values characterise darker coloration, and hence larger amounts of H_2O_2 can be assumed. Measurement of RGB values has been used to determine differences in coloration for other organisms, as birds (Vortman *et al*. 2011) and galls (Luz *et al*. 2015).

2.5 Electrolyte leakage of seedlings

Effects of *E. saligna* aqueous extract and essential oil on membrane integrity were evaluated by measuring the electrolyte leakage of seedling membranes. At the end of the early growth experiments, seedlings (10 g) from each plate were placed in tubes with distilled water (50 mL). Biomass of *E. plana* was not sufficient, and thus assays were only conducted with *P. notatum*, *T. repens* and *L. corniculatus*. Tubes were incubated in a growth room for 24 h and the initial electrical conductivity of the medium (EC1) was measured using a digital conductivity meter (Tecnal, TEC-4MP). Then, samples were frozen to release all electrolytes, defrost, left in the growth room for 24 h, and the final electrical conductivity (EC2) was measured. Relative electrolyte leakage (REL) was calculated according to the equation: REL(%) = (EC1/(EC1 + EC2)) × 10 (2).

2.6 Chemical characterisation of the essential oil

To assess leaf litter essential oil composition, chromatographic analysis was performed using a gas chromatograph coupled to a mass spectrometer detector (GC-MS) and a gas

chromatograph with a flame ionisation detector (GC-FID) (Hewlett Packard 6890). Analyses used two capillary columns HP-Innowax (GC-FID: 30 m × 320 µm × 0.50 µm; GC-MS: 30 m × 250 µm × 0.50 µ□m), (Hewlett Packard, Palo Alto, CA, USA), with an initial oven temperature of 40 °C (8 min) to 180 °C at 3 °C min^{-1}, 180–230 °C at 20 °C min^{-1}, 230 °C (20 min). Injector and interface temperatures were kept at 250°C for GC-FID and 280°C for GC-MS analyses. GC-FID used H_2 and GC-MS used He, with flow rate of 1.0 mL min^{-1}. Desorption occurred in split mode (1 : 10) and ionisation energy was 70 eV.

All of the components were tentatively identified by comparing experimental mass spectra with spectra stored in mass spectrometry databases (Wiley 275), and also with spectra registered in the literature (Adams 2001). Relative percentage of each component was directly obtained from chromatographic peak areas, assuming the sum of all eluted peaks totalled 100%.

2.7 Physico-chemical analyses of the extract

Changes in extract pH and osmolality may affect recipient species, and hence effects of extracts may be erroneously attributed to allelochemicals (Chou and Young 1974; Wardle *et al*. 1992). Osmolality differences may imply that seeds do not acquire water for germination. Therefore, effects of *E. saligna* extract pH and osmolality were assessed. Aqueous extract pH was measured with a pH meter (Sanxin PHS-3D), and osmolality was measured with a vapour pressure osmometer (Vapro 5520). Crude extract (10%) showed osmolality 12% higher than of water and pH 25% lower than of water. To control influence of these factors, the following groups were established: a pH control, consisting in a solution of distilled water and 1 M HCl adjusted to the same pH of crude extract; and an osmolality control, consisting in a solution of water and polyethylene glycol (PEG 400) in equal osmolality of crude extract. Effects of pH and osmolality controls were tested on germination, seedling growth, H_2O_2 levels and electrolyte leakage of seedlings, for all recipient species.

Total phenolic content of *E. saligna* aqueous extract (5, 7.5 and 10%) was determined using the Folin-Ciocalteu method, as described in work by Singleton and Rossi (1965). Briefly, extract (100 µL) was added to 7 mL of distilled water and 500 µL of Folin-Ciocalteu reagent. After 5 min, 1.5 mL of Na_2CO_3 solution (2 g Na_2CO_3:10 mL water) was added to the mixture and then water was added to 10 mL. Samples in triplicates were incubated in the dark at room temperature for 2 h, and the absorbance was read at 760 nm, using a spectrophotometer (Biospectro SP-220). Tannic acid was used as the phenolic standard to prepare a calibration curve in 10 concentrations ranging from 0.05 to 2.3 mg mL^{-1} (R^2 = 0.99). Total phenolic content was expressed as mg tannic acid equivalent mL^{-1} extract.

To test the influence of phenolic derivatives on phytotoxicity of *E. saligna* aqueous extract, assays were conducted exposing the recipient species to the crude extract (10%) and also to the extract with reduced concentration of phenolics. To reduce phenolic compounds of crude extract, insoluble polyvinylpolypyrrolidone (PVPP) was added to the extract (1 g per 100 mL). This mixture was stirred for 10 min. After PVPP precipitation (15 min), the supernatant was passed through filter paper to remove any remaining particulates. Total phenolic content of the crude extract before and after PVPP treatment was determined using the Folin-Ciocalteu method, as described above. PVPP was added to the extract until total phenolic content did not change anymore (three more times). Effects of the crude extract and of the extract with reduced concentration of phenolics (30% of total phenolics) were tested on germination, seedling growth, H_2O_2 levels and electrolyte leakage of seedling membranes of *P. notatum* and *L. corniculatus*. Effects were tested only on one Poaceae and one Fabaceae species, as all recipient species were similarly affected by *E. saligna* crude extract, i.e. almost all parameters were affected (see results section).

2.8 Statistical analysis

For all the experiments, the measured parameters for each recipient species (germination rate, speed of accumulated germination, shoot length, root length, electrolyte leakage and total RGB) were compared between groups by univariate analysis of variance with randomisation (PERMANOVA). When analysis of variance indicated significant differences between groups, contrast analyses were used for pairwise comparisons (Pillar and Orlóci 1996). Randomisation tests are not based on assumptions of normality and homogeneity of variances (Anderson 2001). Tests were conducted with 10,000 bootstrap iterations, used Euclidean distance as dissimilarity measure, and considered a significance level of $P \leq 0.05$. Analyses were performed with Multiv software (Pillar 2009).

3 Results

3.1 Inhibition of germination and seedling growth

Leaf litter essential oil and aqueous extract of *E. saligna* affected germination and growth parameters of all recipient species. In relation to germination assays, the oil significantly inhibited germination rate of the four species at 30 and 50 µL: at these amounts, no *L. corniculatus* seeds germinated (Fig. 1*a*). Furthermore, even the lowest amounts of essential oil affected germination rate of *E. plana* and *L. corniculatus*, except 1 µL for the

latter. All oil amounts reduced speed of accumulated germination of the Poaceae and Fabaceae species, except 1 µL for *P. notatum*, and 20, 30 and 50 µL caused more severe effects on all species (Fig. 1*c*). *Eucalyptus saligna* aqueous extract affected only germination rate of *P. notatum* (Fig. 1*b*), but did decrease the speed of accumulated germination of the four species, at most of concentrations and with greater reduction at 10% (Fig. 1*d*).

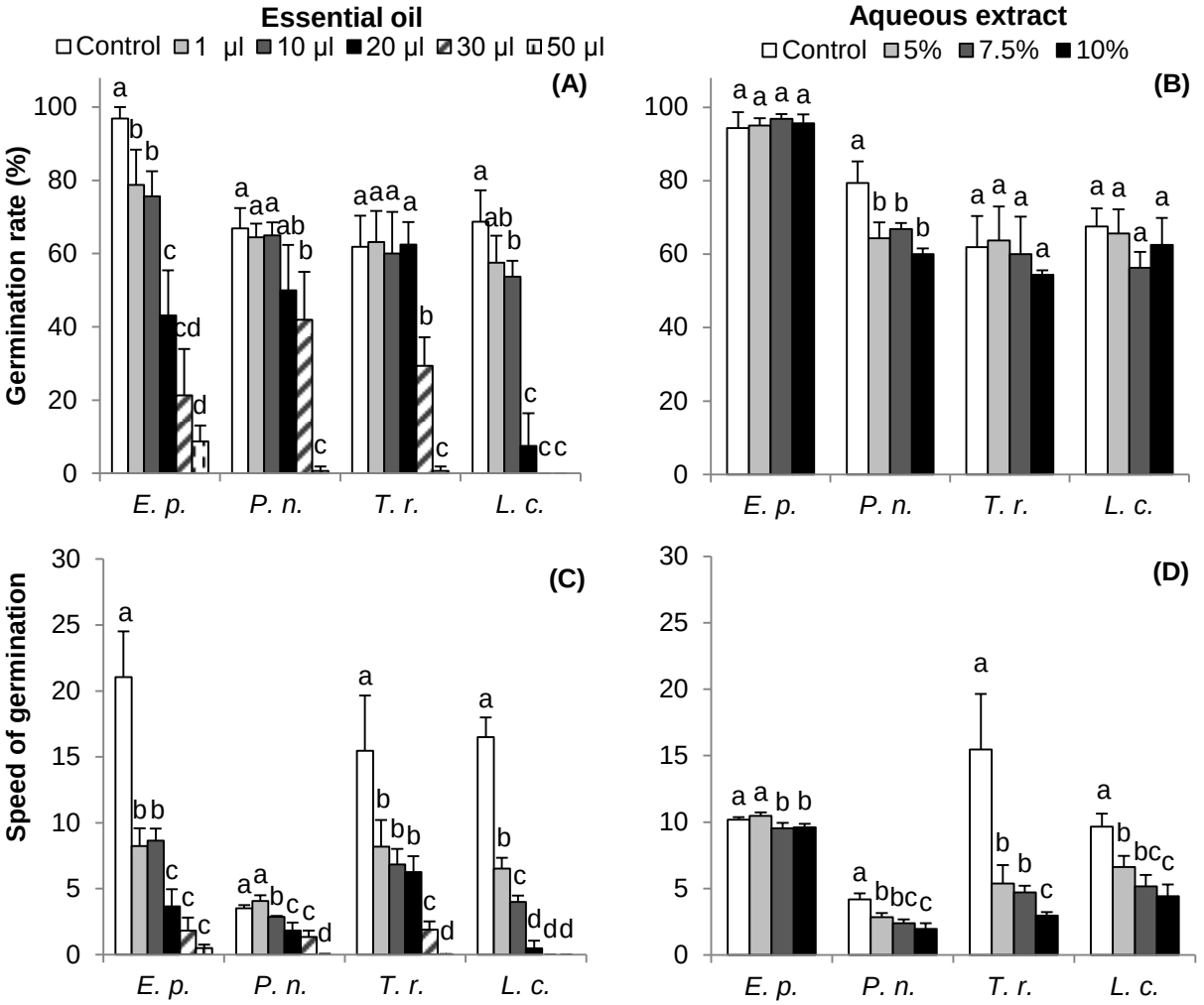


Figure 1. Effects of *Eucalyptus saligna* essential oil (1, 10, 20, 30 and 50 µL) and aqueous extract (5, 7.5 and 10%) on germination of *Eragrostis plana* (*E.p.*), *Paspalum notatum* (*P. n.*), *Trifolium repens* (*T. r.*) and *Lotus corniculatus* (*L. c*). Effects of oil and extract, respectively, on (*a*, *b*) germination rate; and (*c*, *d*) speed of accumulated germination. The data are presented as mean + s.d. (*n* = 4). Values with the same letter do not differ significantly, according to PERMANOVA at $P \leq 0.05$.

Eucalyptus saligna essential oil affected shoot growth of recipient species at concentrations from 10 to 50 µL (Fig. 2*a*). Amounts from 20 to 50 µL caused more severe inhibition in shoot growth of *L. corniculatus*, *E. plana* and *P. notatum*, and seedlings lost pigmentation at these amounts, except for *P. notatum*. Oil affected *T. repens* shoot growth only at 30 and 50 µL, and at these amounts seedlings were completely depigmented (white), with some pigmentation loss also at 10 and 20 µL. All oil amounts suppressed root growth,

except 1 µL for *L. corniculatus*, with stronger effects from 20 to 50 µL (Fig. 2*c*). The extract affected shoot growth of *P. notatum*, *T. repens* and *L. corniculatus* at the highest concentration (Fig. 2*b*). However, in the case of *T. repens*, we observed significant inhibition at 10% in relation to the two lowest concentrations of extract, and not to the control. Moreover, *E. plana* showed dark stains in shoot (necrosis) at all concentrations of extract, covering at most 10% of shoot area. *Eucalyptus saligna* extract inhibited root growth of the four species, at all concentrations (Fig. 2*d*).

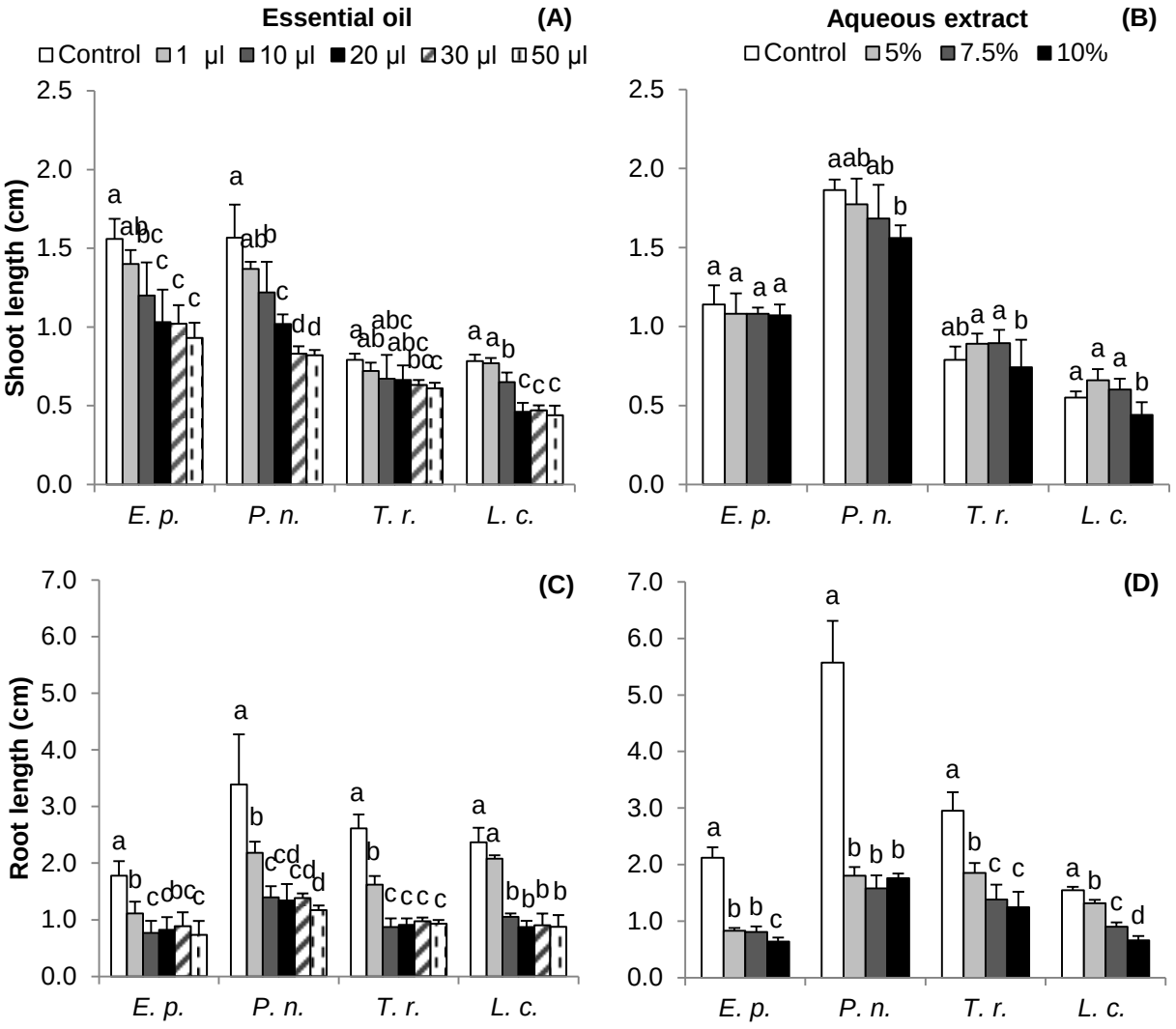


Figure 2. Effects of *Eucalyptus saligna* essential oil (1, 10, 20, 30 and 50 µL) and aqueous extract (5, 7.5 and 10%) on seedling growth of *Eragrostis plana* (*E.p.*), *Paspalum notatum* (*P. n.*), *Trifolium repens* (*T. r.*) and *Lotus corniculatus* (*L. c.*). Effects of oil and extract, respectively, on (*a*, *b*) shoot length; and (*c*, *d*) root length. The data are presented as mean + s.d. (*n* = 4). Values with the same letter do not differ significantly, according to PERMANOVA at $P \leq 0.05$.

3.2 Accumulation of H_2O_2 in seedlings

Eucalyptus saligna leaf litter essential oil and extract increased H_2O_2 levels in all recipient species at most of amounts and concentrations. Species exposed to all essential oil amounts showed significantly lower total RGB (red, green and blue values; lower RGB characterises darker coloration) than control, except *L. corniculatus* at 1 µL (Fig. 3*a*). At all concentrations of *E. saligna* aqueous extract, *T. repens* and *L. corniculatus* presented significantly lower total RGB, whereas *P. notatum* only showed lower values at 10% (Fig. 3*b*). We observed darker coloration (indicating greater H_2O_2 accumulation) in seedling shoots exposed to *E. saligna* oil and extract than in controls, with more evident differences in the highest oil amounts (Fig. 3*c*–*h*).

3.3 Increase in electrolyte leakage of seedlings

Eucalyptus saligna leaf litter essential oil and aqueous extract increased electrolyte leakage of seedling membranes. Electrolyte leakage of *P. notatum* and *T. repens* was increased by amounts of oil from 10 to 50 µL, and of *L. corniculatus* from 20 to 50 µL oil (Fig. 4*a*). Amounts of 30 and 50 µL caused a 4-fold increase in leakage of *P. notatum* and *L. corniculatus*, and an 8-fold increase in leakage of *T. repens*. Extract caused a significant increase in electrolyte leakage of *T. repens* at all concentrations, and of *Paspalum notatum* and *L. corniculatus* only at 10% (Fig. 4*b*). The highest concentration of extract caused a 2-fold increase in electrolyte leakage of *L. corniculatus*, with less remarkable differences in the other species.

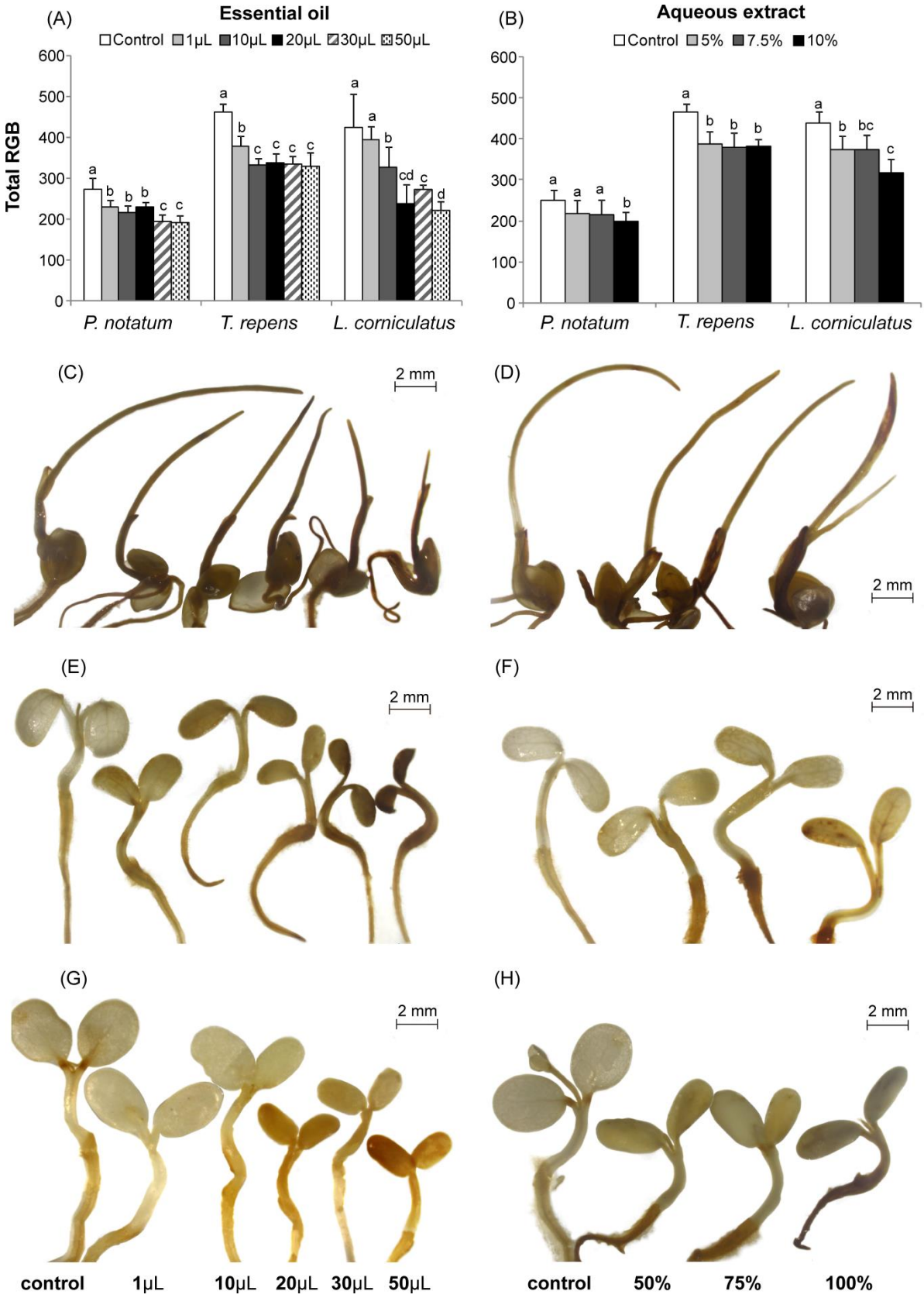


Figure 3. Effects of *Eucalyptus saligna* essential oil (1, 10, 20, 30 and 50 µL) and aqueous extract (5, 7.5 and 10%) on accumulation of H_2O_2 of *Paspalum notatum*, *Trifolium repens* and *Lotus corniculatus*. (*a*, *b*) Total RGB (sum of red, green and blue values) of seedling shoot after exposure to oil and extract, respectively; lower values characterise darker coloration – given by exposure to DAB reagent – and greater H_2O_2 accumulation. The data

are presented as mean + s.d. (*n* = 6). Values with the same letter do not differ significantly, according to PERMANOVA at *P* ≤ 0.05. Seedlings representing each evaluated treatment, which were exposed to oil and extract, respectively: (*c*, *d*) *P. notatum*; (*e*, *f*) *T. repens*; and (*g*, *h*) *L. corniculatus.*

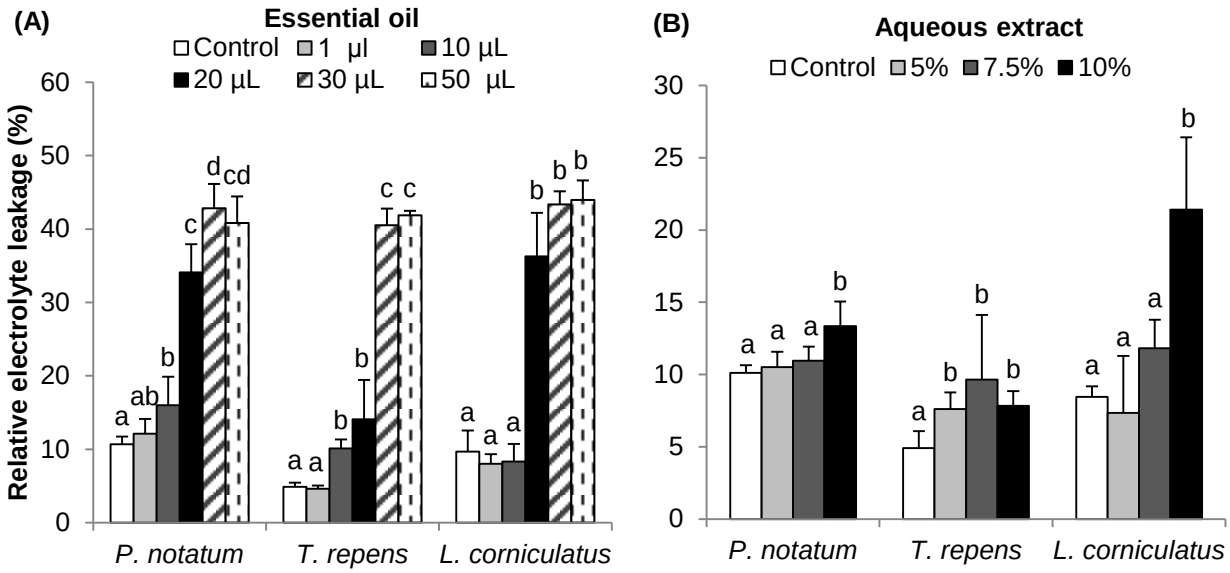


Figure 4. Effects of *Eucalyptus saligna* essential oil (1, 10, 20, 30 and 50 µL) and aqueous extract (5, 7.5 and 10%) on electrolyte leakage of seedling membranes of *Paspalum notatum*, *Trifolium repens* and *Lotus corniculatus*. (*a*) Effects of the oil. (*b*) Effects of the extract. The data are presented as mean + s.d. (*n* = 4). Values with the same letter do not differ significantly, according to PERMANOVA at *P* ≤ 0.05. Please note different scales of *y*-axis

3.4 Chemical characterisation of the essential oil

Essential oil of *E. saligna* was mainly composed of monoterpenes (Table 1). The most abundant fraction was monoterpene hydrocarbons (48.3%), followed by oxygenated monoterpenes (45.8%), sesquiterpene hydrocarbons (3.2%) and oxygenated sesquiterpenes (2.6%). The major compounds of the oil were the monoterpenes α-pinene (34.9%) and 1,8-cineole (28.5%). Other monoterpenes that were present in high quantities were limonene (6.5%) and isobornyl formate (5.6%).

Table 1. Chemical composition of *Eucalyptus saligna* essential oil.

Compound	Area (%)
Monoterpene hydrocarbons	
α-Pinene	34.95
α-Fenchene	0.68
Camphene	2.34
β-Pinene	0.27
Limonene	6.50
m-Cymenene	3.13
Terpinolene	0.39
Oxygenated monoterpenes	
1,8-Cineole (Eucalyptol)	28.46
n.i.	0.41
α-Campholenal	2.85
trans-Pinocamphone	0.54
n.i.	0.28
n.i.	0.38
Pinocarvone	1.01
n.i.	2.19
n.i.	0.33
Terpinen-4-ol	0.29
n.i.	0.98
cis-Pinocarveol	2.09
n.i.	0.38
Isobornyl formate	5.63
Sesquiterpene hydrocarbons	
α-Muurolene	0.29
Bicyclogermacrene	0.23
n.i.	0.29
δ-Cadinene	0.70
cis-Calamenene	0.70
n.i.	0.23
α-Calacorene	0.16
n.i.	0.40
n.i.	0.23
Oxygenated sesquiterpenes	
Ledol	0.48
n.i.	0.50
Spathulenol	1.67

Relative percentage of each component was directly obtained from chromatographic peak areas, considering the sum of all eluted peaks was 100%. Abbreviation: n.i.: not identified.

3.5 Physico-chemical analyses of the extract

Recipient species exposed to pH and osmolality controls did not differ from control in germination and growth parameters, H_2O_2 accumulation and electrolyte leakage of seedling

membranes (Table S1, available as supplementary material to this paper). Phenolic content in *E. saligna* extract showed a linear increase with concentration. At the concentrations of 5, 7.5 and 10%, total phenolic content was, respectively, 0.8, 1.2 and 1.6 mg tannic acid equivalents mL^{-1} extract. Crude extract (100% total phenolics) affected all evaluated parameters in *P. notatum* and *L. corniculatus* seeds and seedlings (as reported above), whereas extract after PVPP treatment (30% total phenolics) affected only a few parameters: speed of accumulated germination and root growth of *P. notatum*, and speed of accumulated germination and electrolyte leakage of *L. corniculatus* (Table 2). Effects of extract with 100% phenolics in root growth of *P. notatum* and electrolyte leakage of *L. corniculatus* were significantly stronger than in seedlings exposed to extract with 30% phenolics.

Table 2. Effects of *Eucalyptus saligna* aqueous extract and of the extract with reduced amount of phenolics (30% total phenolics) on seeds and seedlings of *Paspalum notatum* and *Lotus corniculatus*.

	Recipient species	Control	Extract with 30% phenolics	Extract with 100% phenolics
Germination rate (%)	*P. notatum*	51.88 ± 4.27a	28.75 ± 17.02ab	23.75 ± 5.20b
	L. corniculatus	61.25 ± 3.23a	55.00 ± 8.66a	60.63 ± 5.54a
Speed of accumulated germination	*P. notatum*	2.92 ± 0.23a	0.90 ± 0.51b	0.98 ± 0.23b
	L. corniculatus	6.46 ± 0.54a	4.28 ± 1.08b	4.36 ± 0.25b
Root length (cm)	*P. notatum*	2.73 ± 0.59a	1.40 ± 0.28b	1.22 ± 0.37c
	L. corniculatus	1.83 ± 0.53a	1.95 ± 0.51a	0.71 ± 0.17b
Shoot length (cm)	*P. notatum*	1.78 ± 0.28a	1.65 ± 0.21ab	1.56 ± 0.22b
	L. corniculatus	1.04 ± 0.18a	0.93 ± 0.16a	0.66 ± 0.14b
Total RGB	*P. notatum*	222.31 ± 4.97a	219.81 ± 10.9a	117.17 ± 21.06b
	L. corniculatus	389.11 ± 19.53a	359.76 ± 24.98a	277.12 ± 27.74b
Relative electrolyte leakage (%)	*P. notatum*	11.07 ± 1.18a	10.95 ± 1.62a	13.81 ± 0.61b
	L. corniculatus	3.25 ± 0.38a	5.31 ± 0.37b	6.99 ± 0.36c

Evaluated parameters were germination rate, speed of accumulated germination, seedling shoot and root length, electrolyte leakage of seedling membranes and H_2O_2 levels in seedlings (expressed as total red, green and blue (RGB) values). The data are presented as mean ± s.d. (n = 4, except for total RGB where n = 6). Values with the same letter do not differ significantly, according to PERMANOVA at $P \leq 0.05$.

4 Discussion

Essential oil and aqueous extract of *E. saligna* leaf litter affected germination, seedling growth, increased H_2O_2 levels and damaged membranes of all recipient species, which may be related to the action of monoterpenes in the oil and in considerable part to phenolics in the extract. The major components of essential oils often define quite well their biophysical and biological features (Bakkali *et al*. 2008). Essential oil was practically only constituted by monoterpenes, and among these, α-pinene and eucalyptol (1,8-cineol) were the major compounds. Previous studies have shown the phytotoxicity of α-pinene and eucalyptol, which inhibited germination and seedling growth and caused oxidative stress on recipient species (Zunino and Zygadlo 2004; Nishida *et al*. 2005; Singh *et al*. 2006). However, eucalyptol is more phytotoxic than many other monoterpenes, including α-pinene (Muller and Muller 1964; del Moral and Muller 1970; Nishida *et al*. 2005). Thus, eucalyptol is likely responsible for at least a considerable part of *E. saligna* oil phytotoxicity.

In the case of the aqueous extract, changes in pH and osmolality did not affect seeds and seedlings, and therefore, effects were indeed due to allelochemicals. Even though the importance of controlling these variables has long been highlighted (Wardle *et al*. 1998), many studies of *Eucalyptus* extracts have not established pH and osmolality controls (e.g. El-Rokiek and Eid 2009; Fang *et al*. 2009; Dejam *et al*. 2014), which are necessary to avoid biased inferences about phytotoxicity. Furthermore, we showed that the crude extract (100% phenolics) was more phytotoxic than the extract with a reduced concentration of phenolics (30%), which affected ~30% of the evaluated parameters. As phenolics could not be totally removed from the extract, we could not determine if these effects were due to either remaining phenolics and/or to terpenes. Nevertheless, our results indicate that even if terpenes are present in *E. saligna* extract, the most relevant role in extract phytotoxicity should be played by phenolics. We recognise that *E. saligna* extract still needs to be more specifically characterised. Despite this fact, we present herein substantial evidence of which chemical class is related to phytotoxicity of an extract. This has been rarely shown in allelopathy studies, in which extracts are usually investigated by one or few chemical classes and presence of identified substances is often related to activity, which may not always be true.

Leaf litter essential oil and extract reduced germination rate and speed of accumulated germination in most cases, but high amounts of oil caused the most severe effects. We did not assess by which mechanisms *E. saligna* allelochemicals affected germination. However, as we observed H_2O_2 accumulation in seedlings exposed to oil and extract, we assume that

this may also occur in seeds. Increased production and accumulation of reactive oxygen species (ROS) is one of the primary mechanisms by which allelochemicals affect germination, which can cause oxidative damage to membranes and seed reserves and lead to cell death (Oracz *et al*. 2007). Phytotoxins may also affect seeds by inhibiting or delaying reserve mobilisation, a process that occurs during early stages of germination, providing seeds with ATP (ATP) and carbon products required for the biosynthesis of seedlings (Gniazdowska and Bogatek 2005). Allelochemicals may interfere in reserve mobilisation by disrupting mitochondrial respiration, thus reducing glycolytic activity (Weir *et al*. 2004). These compounds may also change phytohormone levels, such as gibberellic acid and abscisic acid (e.g. Kamal and Bano 2008), which respectively stimulate and prevent aleurone cells to produce hydrolytic enzymes that participate in the breakdown of reserves (Jacobsen and Chandler 1987). Therefore, high amounts of essential oil may have inhibited reserve mobilisation or caused high ROS accumulation that led to cell death, causing the strong observed effects (absence of germination); the extract and low amounts of oil may have only delayed reserve mobilisation and led to some ROS accumulation, causing less severe effects (reduced speed of accumulated germination).

We observed that both essential oil and extract affected growth of seedling roots to a greater extent than shoots. Extract almost only inhibited root growth, and even though the oil inhibited both roots and shoot, the smallest amount (1 µL) only inhibited roots. Many studies have shown that seedling roots are more sensitive to phytotoxins than shoots (e.g. Batish *et al*. 2002; Turk and Tawaha 2003; Nishida *et al*. 2005; Zhang *et al*. 2014). Compounds present in essential oil may dissolve in lipophilic cell structures of recipient plants (Muller 1965), and therefore they can get in direct contact with roots and shoot. However, the surface of plant shoot is covered with a well-developed cuticle layer, whereas that of roots is not, and thus roots may have higher permeability to volatiles (Yoshimura *et al*. 2011). In the case of extract, the highest sensitiveness of roots may be additionally explained by the fact that roots were the first to interact with extract.

The main mechanism by which *E. saligna* allelochemicals affected seedling growth may be generation of ROS-induced oxidative stress. In our study, recipient species exposed to *E. saligna* essential oil and extract showed H_2O_2 accumulation in shoot tissues, and also an increase in electrolyte leakage, revealing damages on membranes. Uncontrolled production and accumulation of ROS is one of the primary mechanisms that initiate phytotoxic activity (Weir *et al*. 2004; Bakkali *et al*. 2008). One of the cellular targets of oxidative stress is lipid peroxidation, a free-radical chain process that leads to the deterioration of polyunsaturated fatty acids (PUFAs) present in membranes. This may cause generalised cellular disruption because of damage on membranes and consequently cell

death (Scandalios 1993). In addition, the highest amounts of oil caused even more severe effects on seedlings, as we observed a 4- to 8-fold increase in electrolyte leakage of seedlings and depigmentation of their shoot. Loss of pigmentation can also occur due to oxidative damage, which can lead to a decrease in chlorophyll content (Niu *et al*. 2013) and hence affect photosynthesis. Therefore, the strongest suppression in growth of seedlings exposed to *E. saligna* allelochemicals is likely related to reduction of photosynthesis added to severe membrane disruption, both mechanisms that can be primary triggered by oxidative stress.

The quantitative method we used to analyse differences in seedling coloration due to H_2O_2 accumulation – an adaptation in DAB experiment – showed satisfactory results. The method allowed us to detect increased H_2O_2 levels even when differences were not so evident. Moreover, the method allowed us considering different patterns of H_2O_2 accumulation in seedlings (stained area and darker coloration). However, differences in H_2O_2 quantity can be also assessed by other techniques, e.g. those involving absorbance measurements (Velikova *et al*. 2000). In our study, application of this kind of method was not possible, as it requires high amounts of plant biomass. We therefore suggest further studies be conducted, in which more seedling biomass can be obtained. These studies should use a quantitative method to determine H_2O_2 accumulation in addition to the method we used, to assess if statistical differences between groups are detected by both approaches in the same manner.

In this study we have shown that *E. saligna* produces phytotoxic compounds – both terpenes and phenolics – that may be released from leaf litter by volatilisation or leached by rainfall. Essential oil was extracted from leaf litter, and not from foliage as in most phytotoxicity studies. Hence, we reported effects of compounds that may volatilise from leaves that are on the soil and may get in contact with recipient species. *Eucalyptus saligna* oil affected grassland species in very small amounts, which increases the possibility of allelopathy. Furthermore, taking into account essential oil yield, 50 µL of oil, which was highly inhibitory on germination and seedling growth, is contained in ~9 g of *E. saligna* leaves; this quantity of leaves is equivalent to leaf litter that covers only ~2 cm^3 of soil in the plantations. We also showed the phytotoxicity of compounds extracted from *E. saligna* leaf litter by just placing leaves in water, thus avoiding unrealistic procedures such as grinding plant material or using organic solvents (Inderjit and Dakshini 1995). This revealed the allelopathic potential of compounds that may be leached by rainfall. However, we are aware that our study does not confirm allelopathy; it only indicates a plausible possibility. According to Blum *et al*. (1999), a donor species can be only considered allelopathic if allelochemicals accumulate in soil at phytotoxic levels, as shown for *E. camaldulensis* and *Eucalyptus urophylla* (del Moral

and Muller 1970; He *et al*. 2014), and if inhibition patterns in the field are not solely determined by other biotic and abiotic factors. In spite of the restrictions that a phytotoxicity study presents, our findings present insights that will be helpful in further field evaluations. As *E. saligna* allelochemicals affected some species from different families (Poaceae and Fabaceae) in a similar way, we can hypothesise that *E. saligna* allelochemicals present little selectivity on grassland species. Thus, if in a field investigation *E. saligna* suppressed Poaceae and Fabaceae species, or even species from other groups, in the same manner, *E. saligna* allelopathy is likely to play a relevant role in shaping vegetation patterns.

5 Conclusions

Eucalyptus saligna leaf litter contains phytotoxic compounds that inhibit germination and initial growth of the tested grassland species. We suggest that the main primary mechanism of *E. saligna* phytotoxicity may be generation of oxidative stress, as essential oil and aqueous extract increased H_2O_2 levels and damaged membranes of recipient species. In addition, we related phytotoxicity of oil to monoterpenes that may be released from *E. saligna* leaf litter by volatilisation, and of extract mainly to phenolic derivatives that may be leached by rainfall. However, field studies are required to test if phytotoxicity of *E. saligna* implies in allelopathic effects on grassland vegetation.

Acknowledgements

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the PhD scholarship granted to the first author, and CMPC Celulose Riograndense for financial support. We thank PhD Fabiana Agostini for help with chemical characterisation of the oil. Furthermore, the authors are thankful to the Laboratório de Extração de Óleos Essenciais at UCS for essential oil extraction, and to the following laboratories at UFRGS for the use of equipment: Centro de Ecologia, Laboratório de Anatomia Vegetal, Laboratório de Fisiologia Vegetal and Laboratório de Ecofisiologia Vegetal. We also thank the two anonymous referees and the editors for their suggestions that improved the manuscript.

References

Adams RP (2001) 'Identification of essential oil components by gas chromatography/ quadrupole mass spectroscopy.' (Allured Publishing Corporation: Carol Stream)

Andel JV (2006) Species interactions structuring plant communities. In 'Vegetation Ecology'. (Ed E Van der Maarel) pp. 238-264. (Blackwell Publishing: Oxford)

Anderson M (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 26, 32-46.

Anjum T, Bajwa R (2005) Importance of germination indices in interpretation of allelochemical effects. *International Journal of Agriculture and Biol*ogy 7, 417-419.

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils – A review. *Food and Chemical Toxicology* 46, 446-475.

Batish DR, Singh HP, Kohli RK, Saxena DB, Kaur S (2002) Allelopathic effects of parthenin against two weedy species, *Avena fatua* and *Bidens pilosa*. *Environmental and Experimental Botany* 47, 149-155.

Batish DR, Singh HP, Setia N, Kohli RK, Kaur S,Yadav SS (2007) Alternative control of littleseed canary grass using eucalypt oil. *Agronomy for Sustainable Development* 27, 171-177.

Bernhard-Reversat F (1999) The leaching of *Eucalyptus* hybrids and *Acacia auriculiformis* leaf litter: laboratory experiments on early decomposition and ecological implications in congolese tree plantations. *Applied Soil Ecology* 12, 251-261.

Bernhard-Reversat F, Main G, Holl K, Loumeto J, Ngao J (2003) Fast disappearance of the water-soluble phenolic fraction in eucalypt leaf litter during laboratory and field experiments. *Applied Soil Ecology* 23, 273-278.

Blum U, Shafer SR, Lehman ME (1999) Evidence for inhibitory allelopathic interactions involving phenolic acids in field soils: concepts vs. experimental model. *Critical Reviews in Plant Sciences* 18, 673-693.

Chou CH, Young CC (1974) Effects of osmotic concentration and pH on plant growth. *Taiwania* 19, 157-162.

Chu C, Mortimer PE, Wang H, Wang Y, Liu X, Yu S (2014) Allelopathic effects of *Eucalyptus* on native and introduced tree species. *Forest Ecology and Management* 323, 79-84.

Dejam M, Khaleghi SS, Ataollahi R (2014) Allelopathic effects of *Eucalyptus globulus* Labill. on seed germination and seedling growth of eggplant (*Solanum melongena* L.). *International Journal of Farming and Allied Sciences* 3, 81-86.

Del Moral R, Muller CH (1970) The allelopathic effects of *Eucalyptus camaldulensis. The American Midland Naturalist* 83, 254-282.

El-Rokiek KG, Eid RA (2009) Allelopathic effects of *Eucalyptus citriodora* on *Amaryllis* and associated grassy weed. *Planta Daninha* 27, 887-899.

Fang B, Yu S, Wang Y, Qiu X, Cai C, Liu S (2009) Allelopathic effects of *Eucalyptus urophylla* on ten tree species in south China. *Agroforestry Systems* 76, 401-408.

Gniazdowska A, Bogatek R (2005) Allelopathic interactions between plants. Multi site action of allelochemicals. *Acta Physiologiae Plantarum* 27, 395-407.

He H, Song Q, Wang Y, Yu S (2014) Phytotoxic effects of volatile organic compounds in soil water taken from a *Eucalyptus urophylla* plantation. *Plant and Soil* 377, 203-215.

Inderjit, Dakshini KMM (1995) On laboratory bioassays in allelopathy. *The Botanical Review* 61, 28-44.

Jacobsen JV, Chandler PM (1987) Gibberellin and abscisic acid in germinating cereals. In 'Plant hormones and their role in plant growth and development'. (Ed PJ Davies) pp. 164-193. (Martinus Nijhoff Publishers: Dordrecht)

Jin X, Yang X, Islam E, Liu D, Mahmood Q (2008) Effects of cadmium on ultrastructure and antioxidative defense system in hyperaccumulator and non-hyperaccumulator ecotypes of *Sedum alfredii* Hance. *Journal of Hazardous Materials* 156, 387-397.

Kamal J, Bano A (2008) Effects of sunflower (*Helianthus annuus* L.) extracts on wheat (*Triticum aestivum* L.) and physicochemical characteristics of soil. *African Journal of Biotechnology* 7, 4130-4135.

Kaur S, Singh HP, Batish DR, Kohli RK (2011) Chemical characterization and allelopathic potential of volatile oil of *Eucalyptus tereticornis* against *Amaranthus viridis*. *Journal of Plant Interactions* 6, 297-302.

Lisanework N, Michelsen A (1993) Allelopathy in agroforestry systems: the effects of leaf extracts of *Cupressus lusitanica* and three *Eucalyptus* spp. on four Ethiopian crops. *Agroforestry Systems* 21, 63-74.

Luz FA, Gonçalves GL, Moreira GRP, Becker VO (2015) Description, molecular phylogeny, and natural history of a new kleptoparasitic species of gelechiid moth (Lepidoptera) associated with Melastomataceae galls in Brazil. *Journal of Natural History* 49, 1849-1875.

Maluf JRT (2000) A new climatic classification for the state of Rio Grande do Sul, Brazil. *Revista Brasileira de Agrometeorologia* 8, 141–150.

Mohamadi N, Rajaie P (2009) Effects of aqueous eucalyptus (*E. camadulensis* Labill) extracts on seed germination, seedling growth and physiological responses of *Phaseolus vulgaris* and *Sorghum bicolor*. *Research Journal of Biological Sciences* 4, 1292-1296.

Muller CH (1965) Inhibitory terpenes volatilized from *Salvia* shrubs. *Bulletin of the Torrey Botanical Club* 92, 38-45.

Muller WH, Muller CH (1964) Volatile growth inhibitors produced by *Salvia* species. *Bulletin of the Torrey Botanical Club* 91, 327-330.

Muller CH, Muller WH, Haines BL (1964) Volatile growth inhibitors produced by aromatic shrubs. *Science* 143, 471-473.

Nishida N, Satoshi T, Nagata N, Saito C, Sakai A (2005) Allelopathic effects of volatile monoterpenoids produced by *Salvia leucophylla*: inhibition of cell proliferation and DNA synthesis in the root apical meristem of *Brassica campestris* seedlings. *Journal of Chemical Ecology* 31, 1187-1203.

Niu Y, Wang Y, Li P, Zhang F, Liu H, Zheng G (2013) Drought stress induces oxidative stress and the antioxidant defense system in ascorbate-deficient vtc1 mutants of *Arabidopsis thaliana*. *Acta Physiologiae Plantarum* 35, 1189-1200.

Oracz K, Bailly C, Gniazdowska A, Côme D, Corbineau F, Bogatek R (2007) Induction of oxidative stress by sunflower phytotoxins in germinating mustard seeds. *Journal of Chemical Ecology* 33, 251-264.

Overbeck GE, Müller SC, Fidelis A, Pfadenhauer J, Pillar VD, Blanco CC, Boldrini II, Both R, Forneck ED (2007) Brazil's neglected biome: the South Brazilian Campos. *Perspectives in Plant Ecology, Evolution and Systematics* 9, 101-116.

Padhy PB, Patnaik PK, Tripathy AK (2000) Allelopathic potential of *Eucalyptus* leaf litter leachates on germination and seedling growth of fingermillet. *Allelopathy Journal* 7, 69-78.

Pillar VD (2009) 'MULTIV, software for multivariate exploratory analysis, randomization testing and bootstrap resampling, version Beta 2.6.8.' (Departamento de Ecologia, UFRGS: Porto Alegre) Available at http://ecoqua.ecologia.ufrgs.br [Verified 8 October 2013]

Pillar VD, Orlóci L (1996) On randomization testing in vegetation science: multifactor comparisons of relevé groups. *Journal of Vegetation Science* 7, 585-592.

Radville L, Chaves A, Preisser EL (2011) Variation in plant defense against invasive herbivores: evidence for a hypersensitive response in eastern hemlocks (*Tsuga canadensis*). *Journal of Chemical Ecology* 37, 592-597.

Renne IJ, Sinn BT, Shook GW, Sedlacko DM, Dull JR, Villarreal D, Hierro JL (2014) Eavesdropping in plants: delayed germination via biochemical recognition. *Journal of Ecology* 102, 86–94.

Rice EL (1984) 'Allelopathy.'(Academic Press: Orlando)

Scandalios JG (1993) Oxygen stress and superoxide dismutases. *Plant Physiology* 101, 7-12.

Schneider AA (2007) A flora naturalizada no Estado do Rio Grande do Sul, Brasil: herbáceas subespontâneas. *Biociências* 15, 257-268.

Setia N, Batish DR, Singh HP, Kohli RK (2007) Phytotoxicity of volatile oil from *Eucalyptus citriodora* against some weedy species. *Journal of Environmental Biology* 28, 63-66.

Silva ER, Overbeck GE, Soares GLG (2014) Phytotoxicity of volatiles from fresh and dry leaves of two Asteraceae shrubs: evaluation of seasonal effects. *South African Journal of Botany* 93, 14-18.

Singh HP, Batish DR, Kaur S, Arora K, Kohli RK (2006) α–Pinene inhibits growth and induces oxidative stress in roots. *Annals of Botany* 98, 1261-1269.

Singleton VL, Rossi Jr JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* 16, 144-158.

Streck EV, Kämpf N, Dalmolin RSD, Klamt E, Nascimento PC, Schneider P (2002) 'Solos do Rio Grande do Sul.' (EMATER/UFRGS: Porto Alegre)

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *The Plant Journal* 11, 1187-1194.

Turk MA, Tawaha AM (2003) Allelopathic effect of black mustard (*Brassica nigra* L.) on germination and growth of wild oat (*Avena fatua* L.). *Crop Protection* 22, 673-677.

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. *Plant Science* 151, 59-66.

Verdeguer M, Blázquez MA, Boira H (2009) Phytotoxic effects of *Lantana camara*, *Eucalyptus camaldulensis* and *Eriocephalus africanus* essential oils in weeds of Mediterranean summer crops. *Biochemical Systematics and Ecology* 37, 362-369.

Vortman Y, Lotem A, Dor R, Lovette IJ, Safran RJ (2011) The sexual signals of the East-Mediterranean barn swallow: a different swallow tale. *Behavioral Ecology* 22, 1344-1352.

Wardle DA, Nicholson KS, Ahmed M (1992) Comparison of osmotic and allelopathic effects of grass leaf extracts on grass seed germination and radicle elongation. *Plant and Soil* 140, 315-319.

Wardle DA, Nilsson M, Gallet C, Zackrisson O (1998) An ecosystem-level perspective of allelopathy. *Biological Reviews* 73, 305-319.

Weir TL, Park S, Vivanco JM (2004) Biochemical and physiological mechanisms mediated by allelochemicals. *Current Opinion in Plant Biology* 7, 472-479.

Yoshimura H, Sawai Y, Tamotsu S, Sakai A (2011) 1,8-Cineole inhibits both proliferation and elongation of BY-2 cultured tobacco cells. *Journal of Chemical Ecology* 37, 320-328.

Zhang C, Fu S (2010) Allelopathic effects of leaf litter and live roots exudates of *Eucalyptus* species on crops. *Allelopathy Journal* 26, 91-100.

Zhang J, An M, Wu H, Liu DL, Stanton R (2014) Phytotoxic activity and chemical composition of aqueous volatile fractions from *Eucalyptus* species. *Plos One* 9, e93189.

Ziaebrahimi L, Khavari-Nejad RA, Fahimi H, Nejadsatari T (2007) Effects of aqueous *Eucalyptus* extracts on seed germination, seedling growth and activities of peroxidase and polyphenoloxidase in three whet cultivar seedlings (*Triticum aestivum* L.). *Pakistan Journal of Biological Sciences* 10, 3415-3419.

Zunino MP, Zygadlo JA (2004) Effect of monoterpenes on lipid peroxidation in maize. *Planta* 219,303-309.

Supplementary material

Table S1. Effects of changes in pH and osmolality of *Eucalyptus saligna* aqueous extract on seeds and seedlings of *Eragrostis plana*, *Paspalum notatum*, *Trifolium repens* and *Lotus corniculatus.*

	Control	pH control	Osmolality control
Germination rate (%)			
E. plana	-	-	-
P. notatum	76.8 ± 5.9	79.4 ± 10.1	74.5 ± 14.7
T. repens	-	-	-
L. corniculatus	-	-	-
Speed of germination			
E. plana	10.2 ± 0.2	10.4 ± 0.8	9.6 ± 1.3
P. notatum	4.2 ± 0.5	4.2 ± 0.1	4.2 ± 0.4
T. repens	15.5 ± 4.2	13.8 ± 1.8	17.1 ± 1.6
L. corniculatus	9.6 ± 0.9	7.6 ± 0.8	9.2 ± 0.8
Root length (cm)			
E. plana	2.1 ± 0.2	1.9 ± 0.3	1.9 ± 0.4
P. notatum	5.6 ± 0.7	5.8 ± 0.2	5.3 ± 0.4
T. repens	2.9 ± 0.3	2.6 ± 0.3	2.9 ± 0.6
L. corniculatus	1.5 ± 0.1	1.7 ± 0.2	1.5 ± 0.1
Shoot length (cm)			
E. plana	-	-	-
P. notatum	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.2
T. repens	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.6
L. corniculatus	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.2
Total RGB			
P. notatum	250.3 ± 25.2	228.6 ± 26.9	289.3 ± 44.9
T. repens	466.0 ± 20.3	454.3 ± 35.6	504.7 ± 30.1
L. corniculatus	442.1 ± 31.9	418.2 ± 40.1	439.9 ± 52.5
Electrolyte leakage (relative %)			
P. notatum	10.1 ± 0.5	10.7 ± 1.1	10.0 ± 1.1
T. repens	4.9 ± 1.2	4.4 ± 0.6	5.3 ± 0.5
L corniculatus	8.4 ± 0.7	9.2 ± 1.6	10.2 ± 1.2

Control consisted in distilled water; pH control consisted in a solution of distilled water and 1 M HCl in the same pH of crude aqueous extract (10%); osmolality control consisted in a solution of water and polyethylene glycol in equal osmolality of crude extract. Evaluated parameters were germination rate, speed of germination, seedling shoot and root length, electrolyte leakage of seedling membranes and H_2O_2 levels in seedlings (expressed as total RGB - red, green and blue values). (-) Not evaluated parameter because no effect of the aqueous extract was observed; thus, establishing pH and osmolality controls was not necessary. The data are presented as mean ± standard deviation, with n = 4, except for total RGB, with n=6. Osmolality and pH control did not differ from control for any evaluated parameter in any recipient species, according to PERMANOVA, at $p \leq 0.05$.

“The natural habitat, even in a relatively simple community of the desert, is far too intricate a system of influences and factors, physical and biological, to hope that there may be found a single factor controlling the complicated life of a perennial species. An explanation, when it is arrived at, will be at least as intricate as the situation it seeks to describe.”

Cornelius H. Muller

CAPÍTULO III

Efeitos inibitórios da serapilheira de *Eucalyptus saligna* sobre espécies campestres: a alelopatia é um fator determinante?

INHIBITORY EFFECTS OF *EUCALYPTUS SALIGNA* LEAF LITTER ON GRASSLAND SPECIES: IS ALLELOPATHY A KEY FACTOR?

Eliane Regina da Silva, Luis Henrique Ruiz da Silveira, Gerhard Ernst Overbeck, Geraldo Luiz Gonçalves Soares

Manuscrito submetido (em revisão) para o periódico Plant Ecology and Diversity

Resumo

No sul do Brasil, grandes áreas de campos têm sido substituídas por plantios de *Eucalyptus*. A vegetação é escassa sob os plantios, o que pode estar associado à emissão de aleloquímicos. Nós objetivamos investigar os efeitos das folhas da serapilheira de *Eucalyptus saligna* sobre espécies campestres, e se esses efeitos estão relacionados à alelopatia. Em plantios de *E. saligna*, nós avaliamos os efeitos das folhas da serapilheira sobre o estabelecimento da comunidade vegetal e sobre espécies herbáceas que foram semeadas (*Paspalum notatum* e *Lotus corniculatus*). Nós estabelecemos grupos de parcelas, incluindo a adição de folhas da serapilheira de *E. saligna* em quantidades distintas, folhas artificiais, sombreamento, e ausência de serapilheira (controle). Nós também testamos a fitotoxidez do solo dos plantios de *E. saligna* sobre *P. notatum* e *L. corniculatus*. A riqueza de espécies, o índice de diversidade de Shannon, a altura, a cobertura e a biomassa de plantas foram menores em parcelas com folhas de *E. saligna* e folhas artificiais do que no grupo controle. *Paspalum notatum* e *L. corniculatus* apresentaram, em geral, menor biomassa e maior mortalidade nas parcelas com folhas de *E. saligna*, folhas artificiais e sombreamento. Os aleloquímicos não acumularam no solo em níveis fitotóxicos. Os resultados revelaram que as folhas da serapilheira de *E. saligna* inibiram a vegetação campestre, mas os efeitos foram principalmente físicos. Avaliações em campo com controles apropriados deveriam ser empregados de forma mais ampla para avaliar o real potencial alelopático de espécies de *Eucalyptus*.

Palavras-chave: avaliação em campo; Campos Sulinos; controles; efeitos físicos; plantio de árvores.

Abstract

In Southern Brazil, large areas of grasslands have been replaced by *Eucalyptus* plantations. Vegetation is scarce under plantations, which may be associated with releasing of allelochemicals. We aimed to investigate effects of *Eucalyptus saligna* leaf litter on grassland species and if these effects were related to allelopathy. In *E. saligna* plantations, we assessed effects of litter on establishment of the plant community and on seeded herbaceous species (*Paspalum notatum* and *Lotus corniculatus*). We established groups of plots, including addition of *E. saligna* leaf litter at different quantities, artificial leaves, shading, and absence of litter (control). We also tested phytotoxicity of soil from *E. saligna* plantations on *P. notatum* and *L. corniculatus*. Species richness, Shannon's diversity index, plant height, cover and biomass were lower in *E. saligna* leaves and artificial leaves plots than in control. *Paspalum notatum* and *L. corniculatus* generally showed lower biomass and higher mortality in plots with *E. saligna* leaves, artificial leaves and shading. Allelochemicals did not accumulate in soil at phytotoxic levels. Our results revealed that *E. saligna* leaf litter suppresses grassland vegetation, but effects are mainly physical. Field evaluations with appropriate controls should be more largely used to assess the actual allelopathic potential of *Eucalyptus* species.

Keywords: Campos Sulinos; controls; field evaluation; physical effects; tree plantation.

1 Introduction

In monoculture tree plantations, plant cover and diversity are often reduced when compared to native forests, and this is also true in *Eucalyptus* plantations (e.g. Loumeto and Huttel 1997; Proença et al. 2010; Souza et al. 2013). Many studies conducted in controlled settings have shown that *Eucalyptus* species produce chemical compounds that can inhibit germination and development of other plants (Batish et al. 2006; Fang et al. 2009; Ruwanza et al. 2015; Morsi and Abdelmigid 2016). Hence, plant suppression under *Eucalyptus* has been frequently attributed, among other factors, to allelopathic effects of *Eucalyptus* species (e.g. Omoro et al. 2010; Souza et al. 2013; Chu et al. 2014). Plant inhibition by allelopathy may be particularly relevant when *Eucalyptus* plantations are established outside their natural range, where native species have not adapted to *Eucalyptus* chemicals, as postulated by the novel weapon hypothesis (Callaway and Ridenour 2004).

In the South America Pampa region, which includes areas of Southern Brazil, Uruguay and Argentina, grasslands have been converted to other land uses, including *Eucalyptus* plantations (Overbeck et al. 2007; Vega et al. 2009). Few grassland species persist under these plantations. Although many studies about phytotoxicity of *Eucalyptus* species have been conducted worldwide, effects on grassland species have been scarcely tested (but see Saberi et al. 2013; Carvalho et al. 2015; Silva et al. 2017a). In fact, only one field study is available, and reported the allelopathic potential of *E. camaldulensis* on grassland vegetation in the California coastal plain (Del Moral and Muller 1970). In the South Brazilian grasslands, the 'Campos Sulinos' region (Overbeck et al. 2007), *Eucalyptus saligna* Sm. is one of the most cultivated species of the genus. In laboratory assays, the essential oil and the aqueous extract of *E. saligna* leaf litter showed high phytotoxicity on forb and graminoid species that occur in the Campos region (Silva et al. 2017a). Both oil and aqueous extract affected germination, seedling growth, generated oxidative stress and led to damages on seedling membranes (Silva et al. 2017a). Therefore, *E. saligna* has the potential to affect grassland species by releasing volatiles and water-soluble compounds from litter.

Eucalyptus allelochemicals may be produced and released from foliage (Kaur et al. 2011), leaf litter (Bernhard-Reversat 1999), or roots (Zhang et al. 2010). However, some studies have shown that *Eucalyptus* roots present lower allelopathic potential than leaves (May and Ash 1990; Zhang and Fu 2010; He et al. 2014). The potential of allelochemical release from leaf litter should be higher than from foliage, for both leaching and volatilisation mechanisms. For leaching, allelochemicals release from litter should be stronger because leaves remain in contact with water for longer periods when accumulated on the soil surface. For volatilisation, allelochemicals from litter have a much higher chance to reach recipient plants. For all types of release, actual occurrence of allelopathy is conditioned by allelochemical permanence in the soil at phytotoxic levels, as observed in plantations with *E. camaldulensis* (He et al. 2014).

Although *Eucalyptus* spp. are generally considered to be allelopathic, studies that have tested the relevance of allelopathy in field conditions are scarce for these species (e.g. Zhang and Fu 2009; Chu et al. 2014; Zhang et al. 2016). Moreover, even fewer studies have completely ruled out the relevance of other factors than allelopathy in shaping vegetation patterns under/near *Eucalyptus* plants (Del Moral et al. 1978). Plant inhibition under leaf litter may be related not only to chemical, but also to physical factors, such as mechanical impediment and shading (Downs and Cavers 2002; Quddus et al. 2014; Loydi et al. 2015). Effects of litter on vegetation may vary depending on many factors, such as ecosystem type, litter quantity and type, and recipient species (Xiong and Nilsson 1999; Loydi et al. 2013). In addition, different results about effects of litter may be observed

between greenhouse and field experiments (Rotundo and Aguiar 2005). Thus, in order to evidence allelopathy, effects of *E. saligna* on grassland species must be assessed in field studies, and the relevance of chemical effects over other factors needs to be demonstrated.

In this study, we aimed to evaluate effects of *E. saligna* leaf litter on grassland species, and, in case of effects, if they were related to allelopathy. We conducted experiments within *E. saligna* plantations and tested the following hypothesis. 1) Grassland species are suppressed by leaf litter under *E. saligna* plantations. 2) Effects of *E. saligna* leaf litter are both related to chemical compounds and to physical factors. 3) Inhibitory effects of *E. saligna* are observed on germination and plant growth, as reported in laboratory assays. 4) Allelochemicals accumulate in soil at phytotoxic levels, causing inhibition of grassland species even after leaf litter removal.

2 Material and Methods

2.1 Donor and recipient species

Many *Eucalyptus* species (Myrtaceae), which are native to Australia, have been planted in southern Brazil, mainly for pulpwood production. Most plantations are established in the Campos Sulinos region. *Eucalyptus saligna*, the donor species in our study, is currently one of the most widely planted species in the region, in monospecific cultures of seedling clones. Trees start losing their branches when they are three years old and a dense litter layer forms on the soil. Litter persists until trees are cut down, usually after seven to nine years, and forms a layer of 5 to 10 cm. Vegetation is scarce under *E. saligna* plantations. Patterns are similar in cultures of other *Eucalyptus* species that are planted in the region, including *E. saligna* hybrids (personal observation).

For experiment 1, recipient species were a natural plant community - all grassland species that spontaneously established in a *E. saligna* plantation. For experiment 2 and 3, recipient species were the grass *Paspalum notatum* Flüggé (Poaceae) and the forb *Lotus corniculatus* L. (Fabaceae). For both, phytotoxic effects of the essential oil and the aqueous extract of *E. saligna* leaf litter had been shown in laboratory experiments (Silva et al. 2017a). These species are representative of two of the three richest families of Campos Sulinos region. *Paspalum notatum* is a native and very common species in the region; *L. corniculatus* is a naturalised exotic species, i.e., it occurs in the grasslands independent of human action, forming stable populations (Schneider 2007); Fabaceae also presents many native species

in the region. Seeds of the recipient species were obtained from commercial dealers, and *L. corniculatus* seeds had been inoculated with *Rhizobium* sp..

2.2 Study areas

We conducted experiments in two different *E. saligna* plantations in the South Brazilian Campos Sulinos region. Climate in the region is subtropical humid, without a dry season (Köppen's Cfa). Experiment 1 was conducted in São Gabriel (30°53'S, 54°51'W, 180 m a.s.l.) – site I. The plantation area was surrounded by other *Eucalyptus* plantations, agricultural areas with soybean and wheat, and natural grassland areas. Mean annual temperature is 18.5 °C, and mean precipitation is 1,355 mm $year^{-1}$ (Maluf 2000). Planossol is the predominant soil type (Streck et al. 2002). Experiment 2 was carried out in Eldorado do Sul (30°18'S 51°62'W, 135 m a.s.l.) – site II. The site is surrounded by other *Eucalyptus* plantations and a small forest fragment. Mean annual temperature is 19.5 °C, mean precipitation is 1,309 mm $year^{-1}$ (Maluf 2000), and the soil type is Argissol (Streck et al. 2002). Composite samples of soil were collected at 10 cm depth in each site and physical-chemical analysis were conducted according to Embrapa (2011) (Table 1).

Table 1. Chemical and physical characteristics of soil in *Eucalyptus saligna* plantations in South Brazil, in the sites where experiment 1 (site I) and experiment 2 (site II) were carried out (mean values).

Soil parameter	Site I	Site II
pH (H_2O)	4.7	4.8
Soil organic matter content (%)	2.5	1.9
P (mg/dm^3)	3.5	1.7
K^+ (mg/dm^3)	64.8	54.0
Al^{3+} ($cmolc/dm^3$)	1.2	0.4
Ca^{2+} ($cmolc/dm^3$)	1.8	3.6
Mg^{2+} ($cmolc/dm^3$)	1.1	0.9
Cation exchange capacity ($cmolc/dm^3$)	11.5	9.6
Clay (%)	16.0	23.0
Sand (%)	60.5	62.0
Silt (%)	23.0	15.0

At both sites, trees were seven years old and no management had been employed in the last six years, i.e., no vegetation or litter under trees was removed. Trees presented

mean circumference at breast height of 23 cm, and were planted in rows at distance of 2 m x 3 m from each other. Litter layer in plantations was about 7 cm high.

2.3 Experimental design

2.3.1 Experiment 1 – Evaluation of effects of Eucalyptus saligna *leaf litter on the plant community*

Experiment 1 was designed to test effects of *E. saligna* leaf litter on establishment of the plant community and if these effects were related to chemical compounds. For this, we established four treatments in a eucalypt plantation at site I: 1) *E. saligna* leaf litter at high quantity, i.e. as found in eucalypt plantations (eucalypt leaves); 2) *E. saligna* leaf litter at half quantity (half eucalypt leaves); 3) artificial leaves at a quantity equivalent to that of eucalypt leaves; and 4) absence of leaves (control). All treatments were established in experimental plots of 1 m² (a sampling unit of 80 by 80 cm plus 10 cm² of buffer at all sides), in a randomised block design with six blocks, in January 2015. Blocks were established in two groups with distance of five km between them and of 200-500 m between plots in each group. In all experimental units, including control, we removed all plant litter and above-ground vegetation, by clipping at the ground level. To establish the quantity of the high and half litter treatment, we measured weight of eucalypt litter in ten plots of 1 m² within the plantation. The mean value was 720 g, which corresponded to a litter layer of several centimetres, covering soil totally. With half quantity of litter, some bare spots of soil persisted. A previous study about the effects of litter on grassland ecosystems (Loidy et al. 2013) had shown neutral to negative effects of high litter quantity (> 500 g/m²) on grassland vegetation, while lower amounts had neutral to positive effects. Artificial leaves were used to simulate physical effects (shading and mechanical impediment) of leaf litter, without the possibility of phytotoxic effects. We used pieces of ethylene vinyl acetate (EVA), cut to about 16 cm x 4 cm, 1.7 mm thick, with a similar weight of a *E. saligna* leaf from litter (0.3 g). Plots were surrounded by a plastic mesh (30 cm high) to avoid drifting of real or artificial litter by wind. We measured photosynthetic active radiation (PAR) over sampling units (at 10 cm high) and under litter with a quantum sensor (LI-COR LI-250A). Values varied considerably over units (from 50 to 1000 µmol m^{-2} s^{-1}). PAR was 0 µmol m^{-2} s^{-1} under eucalypt leaves and artificial leaves, and 1600 µmol m^{-2} s^{-1} outside the plantation.

Plant establishment was monitored at a three-month interval, until plant cover stopped increasing (in June 2016, after 17 months). Fallen leaves in control and artificial leaves plots were removed during the monitoring period. In each sampling unit, we identified all plant species and estimated their cover using the Londo's decimal scale (Londo 1976). We also

recorded vegetation height (mean of five measurements), and estimated total vegetation cover (using Londo's scale). After the last vegetation sampling, we clipped all live biomass at ground level and dried it at 60 ºC until constant weight. Along the experiment, animal(s) interfered in two blocks, removing part of the mesh and leaves, and possibly damaging some plants. Thus, these blocks were not considered in the experiment, remaining four blocks for plant evaluation.

2.3.2 Experiment 2 – Evaluation of effects of Eucalyptus saligna *leaf litter on* Paspalum notatum *and* Lotus corniculatus

Experiment 2 was designed to test effects of *E. saligna* leaf litter on seeded individuals of a grass (*P. notatum*) and a forb (*L. corniculatus*), and if these effects were related to chemical compounds. In May 2015, we established six blocks of sampling units per species at site II, with minimum distance of 100 m between blocks of each species. In the first part of the experiment, we tested if plant establishment was influenced by the quantity of *E. saligna* leaf litter. We removed all litter and vegetation, and determined three treatments of 1 m² experimental units (a sampling unit of 80 by 80 cm plus 10 cm² of buffer at all sides) per block: 1) eucalypt leaves; 2) half eucalypt leaves; and 3) absence of leaves (control). Sampling units and respective treatments were established similarly to experiment 1. Then, seeds of *L. corniculatus* and *P. notatum* were sown at a quantity of 1.8 and 2 g/m², respectively, following recommendations for seeding in pastures in the region. Seeds were sown, covered with a small amount of soil and watered. Five subplots made of wire (20 cm²) were randomly fixed in each sampling unit for plant counting. Seedling emergence was monitored in average at a 45-day interval during six months. Then, all plants were removed and counted. Aerial live biomass was dried at 60 ºC until constant weight.

The second part of the experiment started at the end of February 2016, and was conducted in a similar fashion as the first part. The following treatments were established within the same plots: 1) eucalypt leaves; 2) artificial leaves; 3) cover with shade cloth (shade); and 4) absence of litter (control). Here, we only used the high quantity of *E. saligna* litter, as the two quantities used in the first part of the experiment caused similar effects (see results). For the shade treatment, plots were covered with shade cloths at 30 cm from the ground level. PAR was measured over and under the shade cloth; under it, PAR was reduced by 84.8±9.6% (mean ± standard deviation). PAR values varied a lot over units (from 43 to 1700 µmol m^{-2} s^{-1} at midday). PAR was 0 µmol m^{-2} s^{-1} under eucalypt leaves and artificial leaves, and 1800 µmol m^{-2} s^{-1} outside the plantation.

Recipient species were sown similarly to the first part of the experiment and monitored after three weeks, and then at 45-day intervals. After six months, all plants were removed, counted and aerial live biomass was dried at 60 ºC until constant weight. To assess consistency of results, we repeated the second part of the experiment just after its final evaluation (at the end of August 2016). As in the first and second part of the experiment plant emergence did not change between three and six months, we conducted the final evaluation of the repetition after three months.

2.3.3 Experiment 3 – Laboratory phytotoxicity evaluation of soil from Eucalyptus saligna *plantation*

Experiment 3 was carried out in order to test if allelochemicals from *E. saligna* accumulated in soil at phytotoxic levels. At site II, we removed litter and collected soil at 10 cm depth. Activated charcoal was mixed in soil at 20 mL/L (Parepa and Bossdorf 2016), in order to remove allelochemicals possibly accumulated in soil. Pots of 57 cm of circumference and 10 cm of height were filled with 1 kg of soil or soil with activated charcoal, with four replicates per treatment. Seeds of *P. notatum* and *L. corniculatus* were sown, in separate pots for each species, with 30 seeds per replicate. Seeds were sown at a similar distance from each other, and a thin layer of soil was added to cover the seeds. Pots remained in a growth room (80 µmol m^{-2} s^{-1} provided by white LED light bulbs) and were watered when necessary to maintain soil humidity (in average at each three days). After a month, when plant emergence stopped increasing, all plants were removed from pots. Plants were counted and aerial biomass was dried at 60 ºC until constant weight.

2.3.4 Statistical analysis

For experiment 1, we submitted the matrix of species cover per plot to ordination analysis to detect vegetation patterns. We used principal coordinate analysis (PCoA), based on the Bray–Curtis dissimilarity measure. Results were submitted to bootstrap resampling (10,000 iterations) to verify stability of the ordination axes (Pillar 1999). To test effects of *E. saligna* leaf litter on establishment of the plant community (experiment 1), we evaluated differences between groups in plant richness, diversity, composition, biomass, cover and height. We calculated diversity and richness indexes based on the Rényi generalised entropy (Anand and Orlóci 1996). Renyi's entropy of α orders provides a profile of the most widely used diversity indices, as Shannon (α = 1) and Simpson (α = 2). For α = 0, the entropy value does not take into account the variation in the proportion of different species in a given

community, and corresponds to a richness index. The equitability effect is only stabilised when an α order much higher than Shannon's is used, such as 12 (Anand and Orlóci 1996). Thus, we used the α orders 0 (species richness index), 1, 2, and 12 (diversity indexes). To test for more specific effects, we also separated species cover in categories and summed values of each category per plot. These categories were graminoids (Cyperaceae and Poaceae), forbs (herbaceous non-graminoid) and bryophytes. We did not consider shrubs in this analysis, as the only recorded shrub species was present in only 6% of the plots. Differences in α orders, plant biomass, height and cover (total and per plant group) were compared between groups by ANOVA with randomisation. We tested differences in species composition between groups using MANOVA with randomisation.

In the first part of experiment 2, we tested differences in the final number of plants and in dry biomass between groups (eucalypt leaves, half eucalypt leaves and control). In the second part of the experiment, we evaluated differences in initial emergence of plants, final number of plants, mortality and dry biomass between groups (eucalypt leaves, shade, artificial leaves and control). Initial emergence was reported as plant number in the first counting (after three weeks), in which the higher plant number was obtained for the majority of blocks. Mortality was calculated as the difference between initial and final number of plants, and expressed as the percentage of reduction in the initial number. For experiment 3, we compared differences between groups (with and without activated charcoal) for number of plants and dry biomass. For experiments 2 and 3, differences between groups in evaluated parameters were compared by ANOVA with randomisation, separately for *P. notatum* and *L. corniculatus*.

Randomisation tests were conducted with Euclidean distance measure between sampling units, using 10,000 bootstrap iterations. Whenever the analyses indicated significant differences between groups, we performed contrast analyses for pairwise comparisons. Statistical tests were sum of squares between groups (Qb; Pillar and Orlóci 1996). All analyses were conducted in the software Multiv (Pillar 2009).

3 Results

3.1 Effects of *Eucalyptus saligna* leaf litter on the plant community

In experiment 1, we recorded a total of 38 (morpho-)species in the sampling units (Supplemental Table 1). Of these, 10 could only be identified to the genera/family level. The highest number of species occurred on Asteraceae, followed by Poaceae. Forbs represented

most of species (65.8%), followed by graminoids (26.4%). Shrubs and bryophytes were also recorded (2.6% and 5.3%, respectively).

Explanatory power of PCoA axes using species data of all sampling units was low (36% for axes 1 and 2 together, Figure 1). In spite of this, the exploratory ordination indicated a separation of control and half eucalypt leaves treatments from eucalypt leaves and artificial leaves treatments. Species that showed correlation with either one of the two first axes were arranged close to the first group; these species were ruderal forbs and grasses. Species composition in plots with eucalypt leaves, half eucalypt leaves, and artificial leaves differed from control (Qb = 2.9, *P* = 0.05).

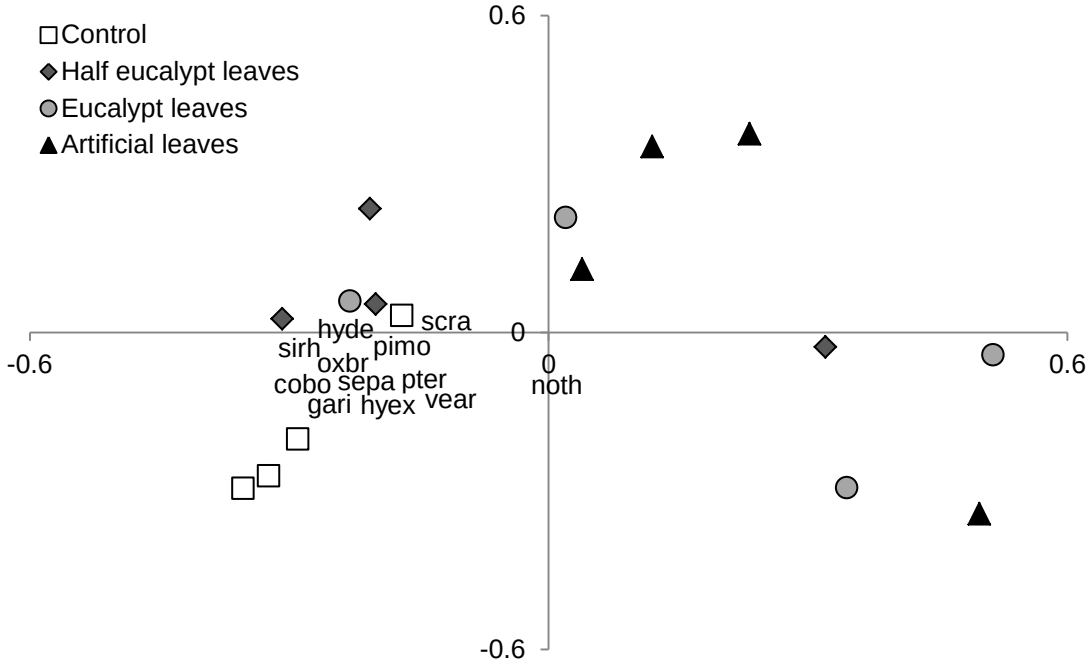


Figure 1. Ordination diagram by PCoA of the sampling units without leaves (control); with *Eucalyptus saligna* leaves at half quantity (Half eucalypt leaves); with *E. saligna* leaves at high quantity (Eucalypt leaves); and with artificial leaves at high quantity (Artificial leaves), in a *E. saligna* plantation. Bray–Curtis dissimilarity was used as resemblance measure. The ordination axes sum 36% of total variation (23 and 13% for axes 1 and 2, respectively). Variables correlated more than 0.2 were plotted in the diagram: cobo = *Conyza bonarie*nsis, gari = *Galium richardianum*, hyex = *Hydrocotyle exigua*, hyde = *Hypoxis decumbens*, noth = *Nothoscordum* sp., oxbr = *Oxalis brasiliensis*, pimo = *Piptochaetium montevidense*, pter = *Pterocaulon* sp., scra = *Scutellaria racemosa*, sepa = *Setaria parviflora*, sirh = *Sida rhombifolia*, vear = *Veronica arvensis*.

Species richness (α = 0), based on Rényi's entropy, was reduced in all groups in relation to control (Qb = 5.9; *P* < 0.001) (Figure 2). Shannon's diversity index (α = 1) was lower in plots with eucalypt leaves and artificial leaves than in control (Qb = 2.6; *P* = 0.01), but for the other diversity indexes, no significant differences were found.

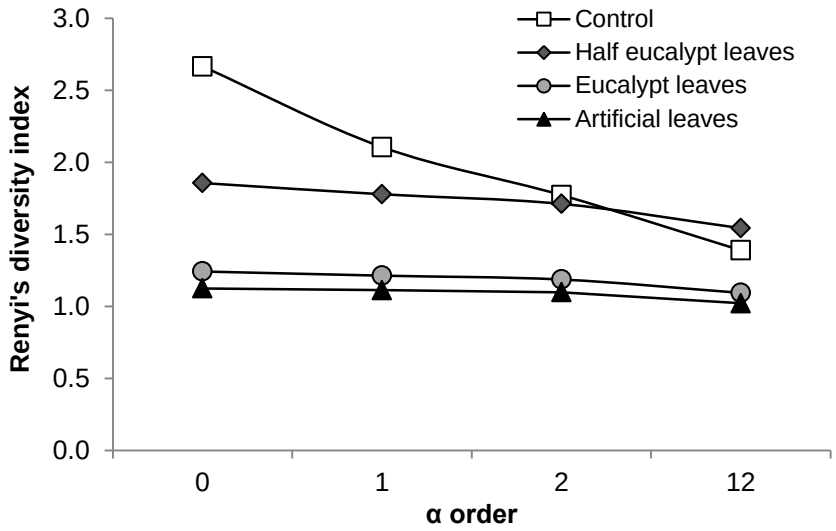


Figure 2. Diversity profiles of vegetation based on Renyi's entropy in plots without leaves (Control); with *Eucalyptus saligna* leaves at half quantity (Half eucalypt leaves); with *E. saligna* leaves at high quantity (Eucalypt leaves); and with artificial leaves at high quantity (Artificial leaves), in a *E. saligna* plantation. The α order 0 corresponds to species richness, α = 1 to Shannon's index, α = 2 to Simpson's index, and α = 12 is the order in which equitability effect should stabilise.

Plant height and biomass were lower in eucalypt leaves and artificial leaves treatments than in control (Figure 3A,B) (Qb = 82.3, *P* = 0.02; Qb = 911.6, *P* = 0.006, respectively). Plant cover was rather low in all sampling units, reaching maximum of 50% at a control plot. Total plant cover was lower in eucalypt leaves and artificial leaves treatments than in control (Qb = 2551.7, *P* = 0.001), which was also observed for plant cover separated in forbs and bryophytes (Figure 3C-E) (Qb = 928.2, *P* = 0.004; Qb = 40.5, *P* = 0.009, respectively). Graminoid cover was lower in artificial leaves plots than in control (Figure 3F) (Qb = 208.2, *P* = 0.036). Most of plants were etiolated in the sampling units.

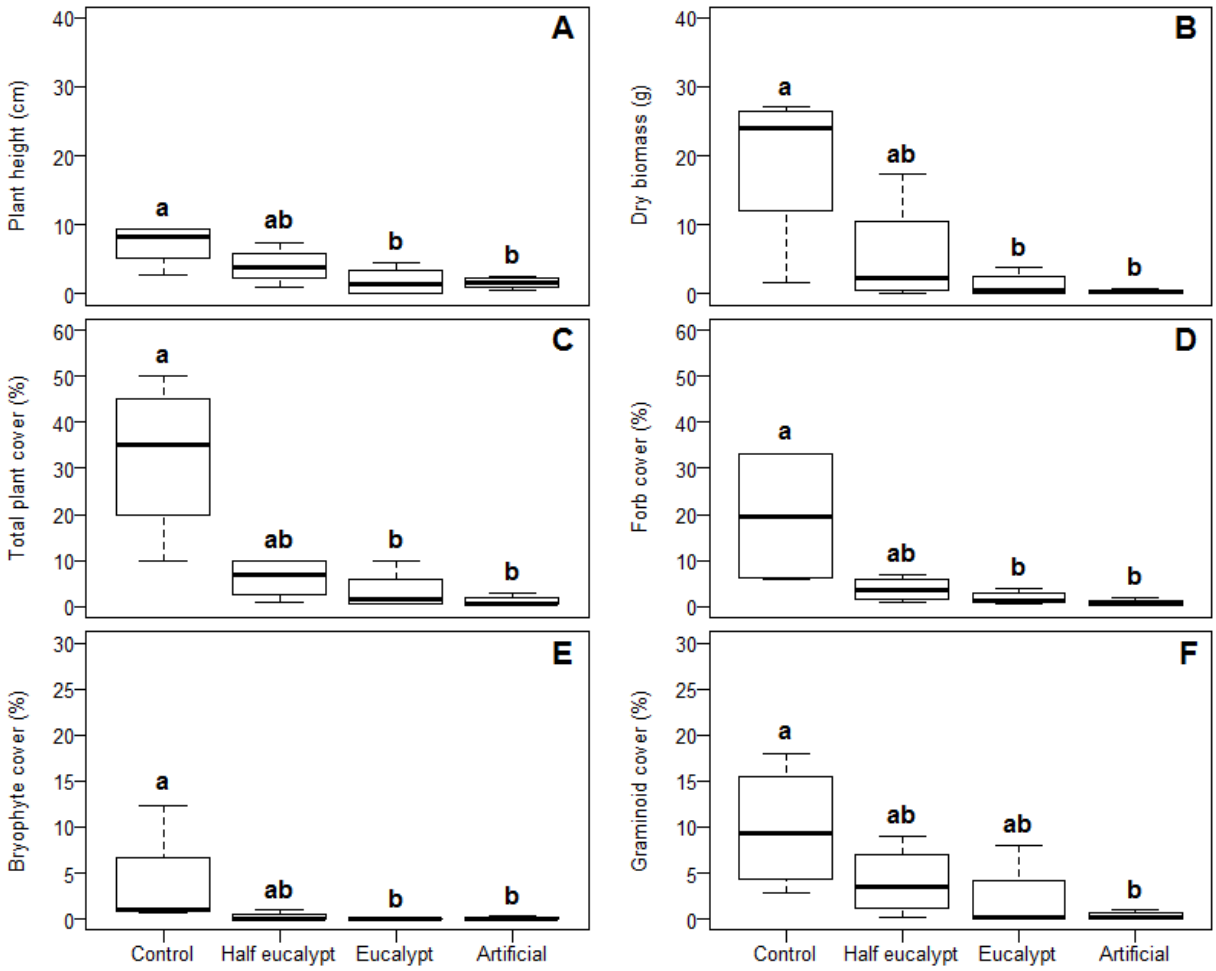


Figure 3. Vegetation parameters in a *Eucalyptus saligna* plantation in plots with absence of leaves (Control); with *E. saligna* leaves at half quantity (Half eucalypt); with *E. saligna* leaves at high quantity (Eucalypt); and with artificial leaves at high quantity (Artificial). Evaluated parameters were plant height (a); dry biomass (b); and plant cover, with total values and also separated in forb, bryophyte and graminoid (c-f). Groups sharing the same letter do not differ significantly according to ANOVA with randomisation, at $P \leq 0.05$ level, $n = 4$. Please note different scales of y-axis.

3.2 Effects of *Eucalyptus saligna* leaf litter on *Paspalum notatum* and *Lotus corniculatus*

In the first part of experiment 2, the number of *P. notatum* and *L. corniculatus* plants was lower in the treatments with eucalypt leaves (high and half quantity) than in control (Qb = 6434.1, $P < 0.001$; Qb = 2344.4, $P < 0.001$, respectively) (Figure 4). Dry biomass of *P. notatum* and *L. corniculatus* did not differ between groups (Qb = 7.18, $P = 0.2$; Qb = 9.7; $P = 0.3$, respectively).

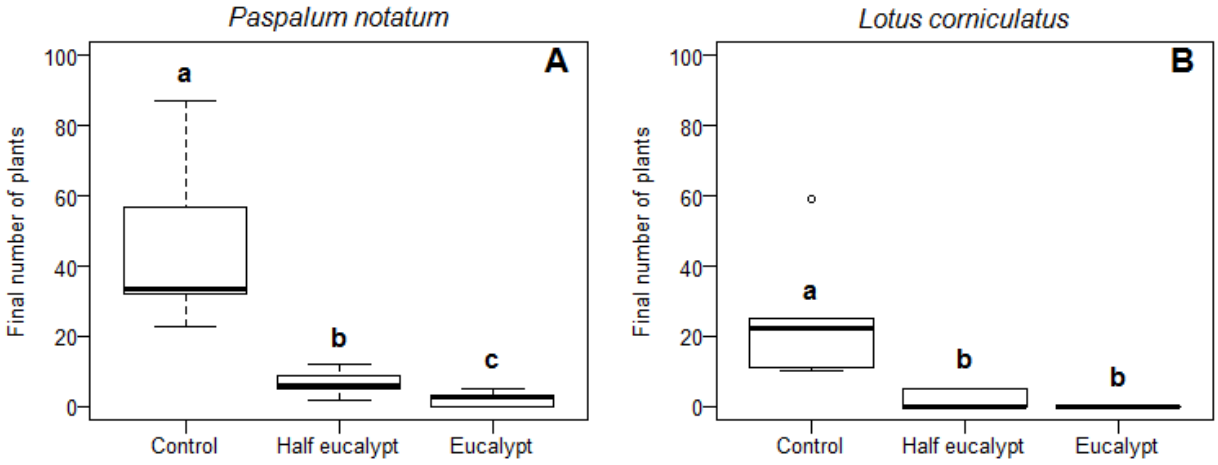


Figure 4. Final number of plants of *Paspalum notatum* (a) and *Lotus corniculatus* (b) in plots without leaves (Control); with *Eucalyptus saligna* leaves at half quantity (Half eucalypt); and with *E. saligna* leaves at high quantity (Eucalypt), in a *E. saligna* plantation. Groups sharing the same letter do not differ significantly when analysed by ANOVA with randomisation, at $P \leq 0.05$ level, $n = 6$.

In the second part of the experiment, initial emergence of *P. notatum* and *L. corniculatus* seedlings did not differ between groups (Qb = 1241, $P = 0.4$; Qb = 10232, $P = 0.4$, respectively). These seedlings were etiolated and whitish in all treatments, but mainly in eucalypt leaves, artificial leaves and shade. The final number of *P. notatum* and *L. corniculatus* was lower in plots with eucalypt leaves and artificial leaves than in control (Qb = 19893, $P = < 0.001$; Qb = 673.2, $P = 0.01$, respectively) (Figure 5A,B). Mortality of *P. notatum* and *L. corniculatus* was higher in both leaves and shade treatments when compared to control (Qb = 27154, $P < 0.001$, Qb = 3116, $P = 0.001$, respectively) (Figure 5C,D). Regarding dry biomass, the control treatment differed from all others for *P. notatum* (Qb = 0.45, $P < 0.001$; Qb = 0.03, $P = 0.009$), with higher biomass, while only artificial leaves differed from control for *L. corniculatus* (Figure 5E,F).

Results were mostly similar in the repetition of the second part of the experiment. Exceptions were the shade treatment that did not show higher mortality than control for the two species, and eucalypt leaves and shade treatments that showed lower biomass of *L. corniculatus* than control.

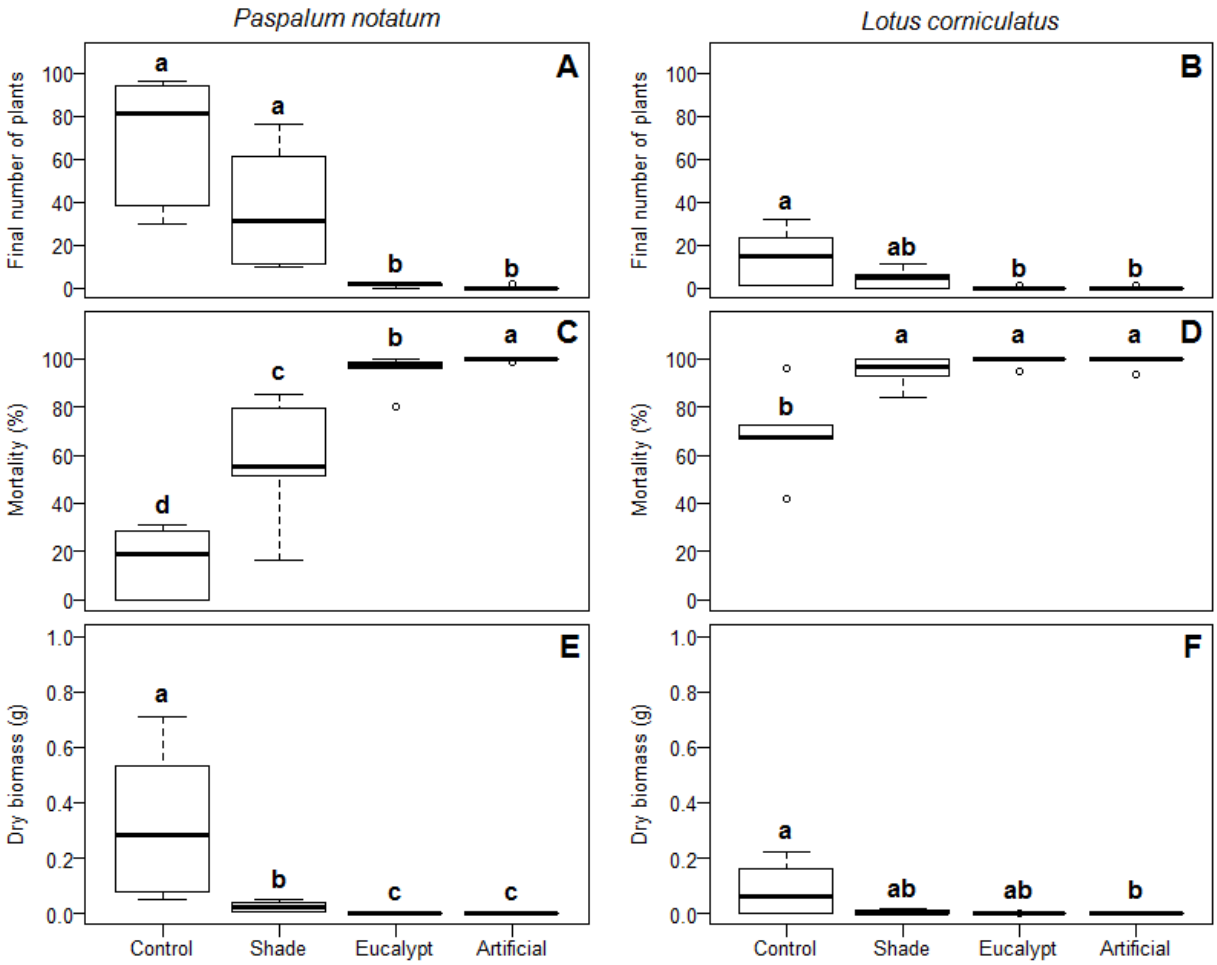


Figure 5. Effects on *Paspalum notatum* and *Lotus corniculatus* plants in plots without leaves (Control); with light incidence reduced with shade cloth (Shade); with *Eucalyptus saligna* leaves (Eucalypt); and with artificial leaves (Artificial), in a *E. saligna* plantation. Effects on *P. notatum* and *L. corniculatus* final number of plants (a, b), mortality (c, d), and dry biomass (e, f), respectively. Groups sharing the same letter do not differ significantly according to ANOVA with randomisation, at $P \leq 0.05$ level, $n = 6$. Please note different scales of y-axis.

3.3 Phytotoxicity evaluation of soil from *Eucalyptus saligna* plantation

The number of *P. notatum* plants did not differ between treatments with and without activated charcoal (15.5 ± 2.4 and 12.7 ± 1.7, respectively, mean ± s.d.; Qb = 15.1, $P = 0.17$). The same was observed for *L. corniculatus* (14.0 ± 2.9 and 13.5 ± 2.9 with and without charcoal, respectively; Qb = 0.5, $P = 0.9$). Dry biomass of *P. notatum* and *L. corniculatus* also did not differ between groups (Qb = 5.8, $P = 0.1$; Qb = 9.0, $P = 0.2$, respectively). Seedlings did not show morphological changes, and none of them died during the experiment.

4 Discussion

We observed that *E. saligna* leaf litter suppresses grassland vegetation, but effects do not seem to be related to allelochemicals. In sampling units with *E. saligna* leaves, establishment of the plant community was lower than in plots without leaves (control). We also observed that herbaceous species that were sown (*P. notatum* and *L. corniculatus*) germinated under *E. saligna* leaves, but survival was low, resulting in fewer plants and lower biomass than in control. However, effects of artificial leaves on vegetation were similar to effects of *E. saligna* leaves. In addition, in plots with half quantity of leaves, which had bare soil at some spots, effects on vegetation were less severe than in plots with high quantity of leaves. These results, altogether, indicate that physical effects of litter are associated with the inhibition pattern. Most of *P. notatum* and *L. corniculatus* seedlings became whitish, etiolated and died in plots with eucalypt leaves, artificial leaves and shade. Also, in shade treatment, in which light incidence was largely reduced, most of the evaluated parameters were affected. Thus, shading of leaf litter is likely the main physical factor driving plant suppression.

Eucalyptus saligna leaf litter affected graminoid and forb species, which comprise the most characteristic plant groups of grassland ecosystems affected by *Eucalyptus* plantations. In South Brazilian grasslands, graminoids represent most of plant biomass, whereas forbs account for most of species richness (e.g. Overbeck and Pfadenhauer 2007); woody components are less representative. In the plant community in our experimental plots, eucalypt leaves and artificial leaves inhibited forbs, artificial litter significantly affected graminoids, and shrubs were scarce. Leaf litter also suppressed a graminoid and a forb that were sown, with stronger effects on the graminoid. In *Eucalyptus* plantations, herbaceous species richness and density are generally lower than in grasslands (Loumeto and Huttel 1997; Souza et al. 2013). Nevertheless, in cases where plantations are surrounded by a forest matrix, many woody species manage to colonise the area and an understory establishes (Senbeta et al. 2002; Yirdaw and Luukkanen 2003; Alem and Woldemariam 2009). In fact, in site II that is close to a forest fragment, vegetation cover was far denser than in site I, with many herb, shrub and tree species (personal observation). Although we cannot generalise that *E. saligna* leaf litter inhibits all grassland species, our experiments indicate that the plant groups that predominate and characterise grasslands were affected. Negative effects of the medium quantity of litter (half leaves treatment) contrast other findings from field studies in grasslands (Loydi et al. 2013), indicating that indeed the grasslands studied by us are highly affected by eucalypt leaf litter.

In the *E. saligna* plantations, the plant community established slowly and scarcely even in the absence of leaf litter, with 50% of plant cover at most. In these plots, the most representative species were ruderals. Many factors can be associated with plant suppression in *Eucalyptus* plantations, such as competition by soil water (Jobbágy and Jackson 2004; Nosetto et al. 2005); changes in physical, chemical or biological properties of soil (Berthrong et al. 2009, 2012); and few seeds in the soil seed bank (Wang et al. 2009) or from seed rain. In site I, soil humidity was not a limiting factor, or bryophytes would not have established (we observed bryophytes in the plots even during summer). Soil was totally covered with herbaceous species at the edge of plantations, which shows that physical-chemical properties of soil were not restrictive. This also indicates that there are sources of diaspores nearby, although we do not have data about the soil seed bank to affirm it. Altogether, these findings point out that shading by *E. saligna* trees, which limits establishment of light-demanding herbaceous plants (Pillar et al. 2002), including the dominant C4 grasses, is an important factor limiting plant establishment in our experimental plots. Shading was partial under plantations, with a large variation in light incidence per plot, but the mean irradiance was obviously lower than outside plantation. Also, many plants of the grassland community were etiolated in the sampling units. Therefore, besides physical effects of leaf litter, shade of trees may have largely contributed to plant suppression.

The low plant cover under *E. saligna* trees also indicates the possibility of soil phytotoxicity. To test this hypothesis, activated charcoal was added to soil from plantations, in order to bind allelochemicals and reduce their effects on plant species. Plants germinated and grew similarly in soil with or without activated charcoal, indicating that allelochemicals did not remain in soil at phytotoxic levels. Nevertheless, it is possible that allelochemicals were released in soil, but degraded before or along the experiment. In fact, essential oil components may be degraded in soil in just one or two days (Isman 2000). If this occurred, residual effects of allelochemicals from leaf litter could be indeed ruled out in control plots. Otherwise, allelochemicals may be released from roots exudates (Zhang et al. 2010) or from foliage (Kaur et al. 2011). In these cases, if allelochemical release was frequent, effects could possibly occur even with quick degradation. Because our pot experiment does not allow mimicking constant flow of allelochemicals from these tissues, the possibility of such effects cannot be dismissed. Although physical effects of leaf litter and shade of trees seems to explain the major part of plant inhibition under *E. saligna*, minor effects of allelochemicals cannot be ruled out, and should be further studied in detail.

The field experiments conducted herein did not corroborate the results of laboratory assays. In the laboratory, the essential oil and aqueous extract of *E. saligna* leaf litter caused severe effects on germination and growth of graminoid and forb species (Silva et al. 2017a).

However, germination of *P. notatum* and *L. corniculatus* was not inhibited in the field, and allelopathic effects, if they occurred, only played a minor role on plant growth. In many cases, the allelopathic potential of *Eucalyptus* spp. has been assessed by some methods that do not resemble natural conditions, such as in the study about *E. saligna* (Silva et al. 2017a). These methods include the use of artificial substrates instead of natural soil (e.g. Batish et al. 2006; Fang et al. 2009; Saberi et al. 2013). Nevertheless, the use of soil in allelopathy studies is imperative (Inderjit and Weston 2000), because physical, chemical and biological soil factors can mediate phytotoxic activity (Inderjit and Weiner 2001), or even neutralise it (e.g. Kaur et al. 2009). As an example, microorganisms were shown to alleviate phytotoxicity of a *Eucalyptus* extract (Lu et al. 2017). Also, in some studies, aqueous extracts were used (e.g. Fang et al. 2009; Morsi and Abdelmigid 2016), which may not represent natural rates of allelochemical emission. Hence, the allelopathic potential of *Eucalyptus* species may have been largely overestimated in previous studies conducted in controlled settings.

Evidencing allelopathy requires, among other issues, that inhibition patterns are shown in the field, and that these patterns are not primarily explained by other biotic and abiotic factors (Blum 2014; Silva et al. 2017b). In many cases, inert materials were successfully used as controls, and allowed evidencing that physical effects of leaf litter were more relevant than chemical effects (e.g. Barritt and Facelli 2001; Downs and Cavers 2002; Quddus et al. 2014). However, few allelopathy studies have employed this type of control in the field, which is necessary because results obtained in a common garden/greenhouse may differ from field results (e.g. Ruprecht et al. 2010). In our study, we evidenced the importance of physical factors of *E. saligna* leaf litter by employing an artificial material as control in an experiment conducted in plantations, thus accounting for effects of litter in a real situation. Previous studies about the allelopathic potential of *Eucalyptus* species rarely included a field assessment (but see Blaise et al. 1997; Zhang and Fu 2009; Chu et al. 2014; Zhang et al. 2016). In addition, almost all field evaluations with *Eucalyptus* species have been conducted without a field control, which makes it difficult to connect results with those from laboratory experiments. An exception is the study of Del Moral et al. (1978), which used as control a species that was similar to the suspected allelopathic *Eucalyptus* species, but otherwise non-phytotoxic. Appropriate controls for field evaluations are in most cases quite simple and inexpensive, and should be introduced in the experimental design of allelopathy investigations. Only with field controls conclusive evidences about the role of allelopathy in shaping or not vegetation patterns can be obtained.

Our results may have implications for conservation and restoration of grasslands in areas that have or had *Eucalyptus* plantations. With reduced leaf litter (half quantity of eucalypt leaves), and diminished shading (by using a shade cloth), inhibitory effects on plant

establishment were not as strong as those of the high quantity of litter. If distance between trees was larger, litter layer and shade of trees would diminish, probably allowing more plants to establish. This seems reasonable as *P. notatum* grew vigorously inside a *Eucalyptus* plantation in Australia – where distance between trees was twice greater than in the area of our study – with even greater plant biomass than in full sun (Wilson et al. 1990). Also, density of understory species was higher in some *E. saligna* plantations with lower tree density and lower crown cover than in other plantations and a native forest (Senbeta et al. 2002). Moreover, our experiment with activated charcoal evidenced that allelochemicals did not accumulate in soil at phytotoxic levels. Thus, allelopathy should not be a restriction for restoration of grasslands in areas previously occupied by *E. saligna* plantations. This is also in line with findings by Torchelsen et al. (in press), who showed that after long-term use of original grassland sites for *Eucalyptus* plantations, grassland vegetation – albeit with difference to natural grassland without plantation history – can develop.

We are aware that we cannot extrapolate our results about *E. saligna* to other species of the genus that are planted in the Campos Sulinos region. However, other *Eucalyptus* species (e.g. *E. dunni*, *E. urophylla*, *E. grandis*) have been cultivated in Campos similarly to *E. saligna*, regarding time of cultivation, distance between trees, and management practices. Hence, similar amounts of litter accumulate on soil under plantations, and our results indicate that plant inhibition was largely determined by physical effects of litter and shade of trees. Therefore, even if a *Eucalyptus* species show allelopathic effects on grassland plants, these effects should not be highly relevant for shaping vegetation patterns. Nevertheless, further studies should investigate if allelochemicals of other *Eucalyptus* species accumulate and persist in soil in areas previously occupied by plantations.

5 Conclusions

We evidenced that *E. saligna* leaf litter suppresses the establishment of grassland species, but inhibitory effects were not related to allelopathy. Instead, inhibition of the plant community and of a graminoid and a forb species was mainly associated with physical effects of litter, which was evidenced by the control with artificial leaves in field experiments. Furthermore, additional plant inhibition in plantations seemed mostly related to shade of trees. Allelopathic potential of *Eucalyptus* species may have been overestimated and overgeneralised by the lack of connection between laboratory experiments and field assessments. Appropriate controls should be employed in field evaluations to better understand the role of allelopathy in shaping plant communities.

Acknowledgements

We thank CMPC Celulose Riograndense for financial support and Elias Frank de Araújo for providing information about the areas and for logistic support. We also thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the PhD scholarship granted to ER Silva. GE Overbeck receives a CNPq grant (310022/2015-0). We thank the anonymous referees for their helpful suggestions to the manuscript.

References

Alem S, Woldemariam T. 2009. A comparative assessment on regeneration status of indigenous woody plants in *Eucalyptus grandis* plantation and adjacent natural forest. Journal of Forest Research 20:31–36.

Anand M, Orlóci L. 1996. Complexity in plant communities: the notion and quantification. Journal of Theoretical Biology 179:179–186.

Barritt AR, Facelli JM. 2001. Effects of *Casuarina pauper* litter and grove soil on emergence and growth of understorey species in arid lands of South Australia. Journal of Arid Environments 49:569–579.

Batish DR, Singh HP, Setia N, Kaur S, Kohli RK. 2006. Chemical composition and inhibitory activity of essential oil from decaying leaves of *Eucalyptus citriodora*. Zeitschrift für Naturforschung C 61:52–56.

Bernhard-Reversat F. 1999. The leaching of *Eucalyptus* hybrids and *Acacia auriculiformis* leaf litter: laboratory experiments on early decomposition and ecological implications in congolese tree plantations. Applied Soil Ecology 12:251–261.

Berthrong ST, Piñeiro G, Jobbágy EG, Jackson RB. 2012. Soil C and N changes with afforestation of grasslands across gradients of precipitation and plantation age. Ecological Applications 22:76–86.

Berthrong ST, Schadt CW, Piñeiro G, Jackson RB. 2009. Afforestation alters the composition of functional genes in soil and biogeochemical processes in South American Grasslands. Applied and Environmental Microbiology 75:6240–6248.

Blaise D, Tyagi PC, Khola OPS, Ahlawat SP. 1997. Effects of *Eucalyptus* on wheat, maize and cowpea. Allelopathy Journal 4:341–344.

Blum U. 2014. Plant-plant allelopathic interactions II: laboratory bioassays for water-soluble compounds with an emphasis on phenolic acids. Switzerland: Springer.

Callaway RM, Ridenour WM. 2004. Novel weapons: invasive success and the evolution of increased competitive ability. Frontiers in Ecology and the Environment 2:436–443.

Carvalho FP, Melo CAD, Machado MS, Dias DCFS, Alvarenga EM. 2015. The allelopathic effect of *Eucalyptus* leaf extract on grass forage seed. Planta Daninha 33:193–201.

Chu C, Mortimer PE, Wang H, Wang Y, Liu X, Yu S. 2014. Allelopathic effects of *Eucalyptus* on native and introduced tree species. Forest Ecology and Management 323:79–84.

Del Moral R, Muller CH. 1970. The allelopathic effects of *Eucalyptus camaldulensis*. The American Midland Naturalist 83:254–282.

Del Moral R, Willis RJ, Ashton DH. 1978. Suppression of coastal heath vegetation by *Eucalyptus baxteri*. Australian Journal of Botany 26:203–219.

Downs MP, Cavers PB. 2002. Physical and chemical factors associated with the reduction or delay of seed germination and seedling emergence of bull thistle, *Cirsium vulgare* (Savi) Ten. under leaf litter. Écoscience 9:518–525.

Embrapa - Empresa Brasileira de Pesquisa Agropecuária. 2011. Manual de métodos de análise de solos. Rio de Janeiro (Brasil): Embrapa Solos.

Fang B, Yu S, Wang Y, Qiu X, Cai C, Liu S. 2009. Allelopathic effects of *Eucalyptus urophylla* on ten tree species in south China. Agroforestry Systems 76:401–408.

He H, Song Q, Wang Y, Yu S. 2014. Phytotoxic effects of volatile organic compounds in soil water taken from a *Eucalyptus urophylla* plantation. Plant and Soil 377:203–215.

Inderjit, Weiner J. 2001. Plant allelochemical interference or soil chemical ecology? Perspectives in Plant Ecology, Evolution and Systematics 4:3–12.

Inderjit, Weston LA. 2000. Are laboratory bioassays for allelopathy suitable for prediction of field responses? Journal of Chemical Ecology 26:2111–2118.

Isman MB. 2000. Plant essential oils for pest and disease management. Crop Protection 19:603-608.

Jobbágy EG, Jackson RB. 2004. Groundwater use and salinization with grassland afforestration. Global Change Biology 10:1299–1312.

Kaur H, Kaur R, Kaur S, Baldwin IT, Inderjit. 2009. Taking ecological function seriously: Soil microbial communities can obviate allelopathic effects of released metabolites. PLoS One 4:e4700.

Kaur S, Singh HP, Batish DR, Kohli RK. 2011. Chemical characterization and allelopathic potential of volatile oil of *Eucalyptus tereticornis* against *Amaranthus viridis*. Journal of Plant Interactions 6:297–302.

Londo G. 1976. The decimal scale for releves of permanent quadrats. Vegetatio 33:61–64.

Loumeto JJ, Huttel C. 1997. Understory vegetation in fast-growing tree plantations on savanna soils in Congo. Forest Ecology and Management 99:65–81.

Loydi A, Donath TW, Eckstein RL, Otte A. 2015. Non-native species litter reduces germination and growth of resident forbs and grasses: allelopathic, osmotic or mechanical effects? Biological Invasions 17:581-595.

Loydi A, Eckstein RL, Otte A, Donath TW. 2013. Effects of litter on seedling establishment in natural and semi-natural grasslands: a meta-analysis. Journal of Ecology 101:454-464.

Lu F, Zheng L, Chen Y, Li D, Zeng R, Li H. 2017. Soil microorganisms alleviate the allelopathic effect of *Eucalyptus grandis* x *E. urophylla* leachates on *Brassica chinensis*. Journal of Forest Research 28:1203-1207.

Maluf JRT. 2000. A new climatic classification for the State of Rio Grande do Sul, Brazil. Revista Brasileira de Agrometeorologia 8:141–150.

May FE, Ash JE. 1990. An assessment of the allelopathic potential of *Eucalyptus*. Australian Journal of Botany 38:245–254.

Morsi MM, Abdelmigid HM. 2016. Allelopathic activity of *Eucalyptus globulus* leaf aqueous extract on *Hordeum vulgare* growth and cytogenetic behaviour. Australian Journal of Crop Science 10:1551–1556.

Nosetto MD, Jobbágy EG, Paruelo JM. 2005. Land-use change and water losses: The case of grassland afforestation across a soil textural gradient in central Argentina. Global Change Biology 11:1101–1117.

Omoro LM, Pellikka PKE, Rogers PC. 2010. Tree species diversity, richness, and similarity between exotic and indigenous forests in the cloud forests of Eastern Arc Mountains, Taita Hills, Kenya. Journal of Forest Research 21:255–264.

Overbeck GE, Muller SC, Fidelis A, Pfadenhauer J, Pillar VD, Blanco CC, Boldrini II, Both R, Forneck ED. 2007. Brazil's neglected biome: The South Brazilian Campos. Perspectives in Plant Ecology, Evolution and Systematics 9:101–116.

Overbeck GE, Pfadenhauer J. 2007. Adaptive strategies in burned subtropical grassland in southern Brazil. Flora 202:27–49.

Parepa M, Bossdorf O. 2016. Testing for allelopathy in invasive plants: it all depends on the substrate! Biological Invasions 18:2975–2982.

Pillar VD. 1999. The bootstrapped ordination re-examined. Journal of Vegetation Science 10:895–902.

Pillar VD. 2009. MULTIV, software for multivariate exploratory analysis, randomization testing and bootstrap resampling. Version Beta 2.6.8. Departamento de Ecologia, UFRGS, Porto Alegre, Brazil. Available online at http://ecoqua.ecologia.ufrgs.br (accessed 8 October 2016).

Pillar VD, Boldrini II, Lange O. 2002. Padrões de distribuição espacial de comunidades campestres sob plantio de eucalipto. Pesquisa Agropecuária Brasileira 37:753–761.

Pillar VD, Orlóci L. 1996. On randomization testing in vegetation science: multifactor comparisons of relevé groups. Journal of Vegetation Science 7:585–592.

Proença VM, Pereira HM, Guilherme J, Vicente L. 2010. Plant and bird diversity in natural forests and in native and exotic plantations in NW Portugal. Acta Oecologica 36:219–226.

Quddus MS, Bellairs SM, Wurm PAS. 2014. *Acacia holosericea* (Fabaceae) litter has allelopathic and physical effects on mission grass (*Cenchrus pedicellatus* and *C. polystachios*) (Poaceae) seedling establishment. Australian Journal of Botany 62:189–195.

Rotundo JL, Aguiar MR. 2005. Litter effects on plant regeneration in arid lands: a complex balance between seed retention, seed longevity and soil-seed contact. Journal of Ecology 93:829–838.

Ruprecht E, Enyedi MZ, Eckstein RL, Donath TW. 2010. Restorative removal of plant litter and vegetation 40 years after abandonment enhances re-emergence of steppe grassland vegetation. Biological Conservation 143:449–456.

Ruwanza S, Gaertner M, Esler KJ, Richardson DM. 2015. Allelopathic effects of invasive *Eucalyptus camaldulensis* on germination and early growth of four native species in the Western Cape, South Africa. Southern Forests: a Journal of Forest Science 77:91–105.

Saberi M, Davari A, Tarnian F, Shahreki M, Shahreki E. 2013. Allelopathic effects of *Eucalyptus camaldulensis* on seed germination and initial growth of four range species. Annals of Biological Research 4:152–159.

Schneider AA. 2007. A flora naturalizada no Estado do Rio Grande do Sul, Brasil: herbáceas subespontâneas. Biociências 15:257–268.

Senbeta F, Teketay D, Näslund B. 2002. Native woody species regeneration in exotic tree plantations at Munessa-Shashemene Forest, southern Ethiopia. New Forests 24:131–145.

Silva ER, Lazarotto DC, Schwambach J, Overbeck GE, Soares GLG. 2017a. Phytotoxic effects of extract and essential oil of *Eucalyptus saligna* (Myrtaceae) leaf litter on grassland species. Australian Journal of Botany 65:172–182.

Silva ER, Overbeck GE, Soares GLG. 2017b. Something old, something new in allelopathy review: what grassland ecosystems tell us. Chemoecology 27:217-231.

Souza AF, Ramos NP, Pizo MA, Hübel I, Crossetti LO. 2013. Afforestation effects on vegetation structure and diversity of grasslands in southern Brazil: The first years. Journal for Nature Conservation 21:56–62.

Streck EV, Kampf N, Dalmolin RSD, Klamt E, Nascimento PC, Schneider P. 2002. Solos do Rio Grande do Sul. Porto Alegre (Brazil): EMATER/UFRGS.

Torchelsen FP, Cadenazzi M, Overbeck GE (in press). Do subtropical grasslands recover spontaneously after afforestation? Journal of Plant Ecology.

Vega E, Baldi G, Jobbágy EG, Paruelo J. 2009. Land use change patterns in the Río de la Plata grasslands: The influence of phytogeographic and political boundaries. Agriculture, Ecosystems & Environment 134:287–292.

Wang J, Ren H, Yang L, Li D, Guo Q. 2009. Soil seed banks in four 22-year-old plantations in South China: Implications for restoration. Forest Ecology and Management 258:2000–2006.

Wilson JR, Hill K, Cameron DM, Shelton HM. 1990. The growth of *Paspalum notatum* under the shade of a *Eucalyptus grandis* plantation canopy or in full sun. Tropical Grasslands 24:24–28.

Xiong S, Nilsson C. 1999. The effects of plant litter on vegetation: a meta-analysis. Journal of Ecology 87:984–994.

Yirdaw E, Luukkanen O. 2003. Indigenous woody species diversity in *Eucalyptus globulus* Labill. ssp. *globulus* plantations in the Ethiopian highlands. Biodiversity and Conservation 12:567–582.

Zhang C, Fu S. 2009. Allelopathic effects of eucalyptus and the establishment of mixed stands of eucalyptus and native species. Forest Ecology and Management 258:1391–1396.

Zhang C, Fu S. 2010. Allelopathic effects of leaf litter and live roots exudates of *Eucalyptus* species on crops. Allelopathy Journal 26:91–100.

Zhang C, Li X, Chen Y, Zhao J, Wan S, LinY, Fu S. 2016. Effects of *Eucalyptus* litter and roots on the establishment of native tree species in *Eucalyptus* plantations in South China. Forest Ecology and Management 375:76–83.

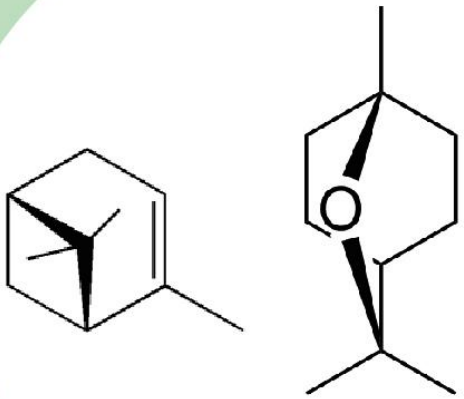
Zhang D, Zhang J, Yang W, Wu F. 2010. Potential allelopathic effect of *Eucalyptus grandis* across a range of plantation ages. Ecological Research 25:13–23.

Supplemental data

Table 1. Plant (morpho-)species cover in a *Eucalyptus saligna* plantation in plots with absence of leaves (Control); with *E. saligna* leaves at half quantity (Half eucalypt); with *E. saligna* leaves at high quantity (Eucalypt); and with artificial leaves at high quantity (Artificial). Results are expressed as mean cover per group (n = 4).

Family	Species	Control	Half eucalypt	Eucalypt	Artificial
Asteraceae	*Aspilia montevidensis* (Spreng.) Kuntze	-	0.05	-	-
	Baccharis dracunculifolia DC.	0.25	-	-	-
	Conyza bonariensis (L.) Cronquist	0.78	0.25	-	-
	Gamochaeta americana (Mill.) Wedd.	0.10	-	-	-
	Noticastrum decumbens (Baker) Cuatrec.	0.25	-	-	-
	Senecio selloi (Spreng.) DC.	-	0.50	-	-
	Pterocaulon sp. Elliott	0.35	-	-	-
	Asteraceae 1	-	-	0.13	-
Amaranthaceae	*Pfaffia tuberosa* (Spreng.) Hicken	1.00	-	0.05	0.06
Amaryllidaceae	*Nothoscordum montevidense* Beauverd	-	0.18	1.00	-
	Nothoscordum sp. Kunth	-	0.25	0.06	-
Araliaceae	*Hydrocotyle exigua* Malme	7.90	-	0.06	0.13
Convolvulaceae	*Dichondra sericea* Sw.	0.55	0.05	-	0.05
Cyperaceae	*Fimbristylis* sp. Vahl	0.43	0.05	0.18	0.19
	Kyllinga odorata Vahl	0.25	-	-	-
	Kyllinga vaginata Lam.	0.25	-	-	-
	Rhynchosphora sp. Vahl	1.13	1.05	0.50	-
Hypoxidaceae	*Hypoxis decumbens* L.	-	0.05	-	-
Lamiaceae	*Scutellaria racemosa* Pers.	0.20	0.25	-	-
Lythraceae	*Cuphea carthagenensis* (Jacq.) J.F.Macbr	2.50	-	-	-
Malvaceae	*Sida rhombifolia* L.	1.28	0.73	0.06	0.13
Orchidaceae	Orchidaceae 1	-	1.00	-	0.08

Family	Species	Control	Half eucalypt	Eucalypt	Artificial
Orchidaceae	Orchidaceae 2	0.35	-	-	-
Oxalidaceae	*Oxalis brasiliensis* G. Lodd	0.78	0.25	0.06	0.05
	Oxalis perdicaria (Molina) Bertero	0.18	-	-	-
Plantaginaceae	*Veronica arvensis* L.	0.50	-	-	-
Poaceae	*Dichanthelium sabulorum* (Lam.) Gould & C.A. Clark	-	0.50	0.13	-
	Echinochloa colona (L.) Link	1.53	0.85	1.05	0.25
	Paspalum umbrosum Trin.	1.00	-	-	-
	Piptochaetium montevidense (Spreng.) Parodi	0.25	-	-	-
	Setaria parviflora (Poir.) M.Kerguelen	-	0.50	0.50	-
	Sorghastrum sp. Nash	6.68	1.10	-	0.25
Ricciaceae	*Riccia* sp. L.	0.10	-	-	-
Rubiaceae	*Galium richardianum* (Gillies ex Hook. & Arn.) Endl. ex Walp.	0.78	-	-	-
Solanaceae	*Solanum americanum* Mill.	0.25	-	-	-
Verbenaceae	*Glandularia selloi* (Spreng.) Tronc.	-	0.25	0.13	-
	Verbena sp. L.	0.25	-	-	-
Undefined	Bryophyte	3.68	0.25	-	0.08



"Si conocemos la forma en la que las plantas hacen posibles sus relaciones inter e intraespecíficas en un determinado ecosistema, podremos imitar ciertos procesos y pensar en posibles aplicaciones como herbicidas, antibióticos, fungicidas e inseticidas naturales."

Grupo de Alelopatía de Cádiz

CAPÍTULO IV

Potencial do óleo essencial das folhas da serapilheira de *Eucalyptus saligna* como herbicida natural

BIOHERBICIDE POTENTIAL OF *EUCALYPTUS SALIGNA* LEAF LITTER ESSENTIAL OIL

Eliane Regina da Silva, José Manuel Igartuburu, Gerhard Ernst Overbeck, Geraldo Luiz Gonçalves Soares, Francisco Antonio Macías

Resumo

Óleos essenciais fitotóxicos podem ser potencialmente empregados como herbicidas naturais no manejo de plantas daninhas. Nós objetivamos avaliar os efeitos fitotóxicos do óleo essencial das folhas da serapilheira de *E. saligna*, e investigar os componentes relacionados à fitotoxidez. Os efeitos do óleo essencial e seus componentes majoritários foram testados por volatilização sobre a germinação e o crescimento de *Eragrostis plana*. As substâncias voláteis nas placas foram monitoradas durante o período experimental por microextração em fase sólida, e o óleo essencial e os voláteis foram caracterizados por cromatografia gasosa associada à espectrometria de massas. A fitotoxidez do óleo essencial, suas frações e componentes majoritários diluídos em solução aquosa foi avaliada. As espécies receptoras foram *Lactuca sativa* (espécie-padrão), *Amaranthus viridis* e *E. plana* (daninhas), e *Paspalum notatum* (forrageira). O óleo essencial consistiu em uma mistura de monoterpenos hidrocarbonetos (35,6%), monoterpenos oxigenados (52,5%), sesquiterpenos hidrocarbonetos (3,7%) e sesquiterpenos oxigenados (5,5%). No ensaio de volatilização, o óleo essencial foi mais fitotóxico, seguido dos majoritários eucaliptol e α-pineno. A proporção de monoterpenos oxigenados aumentou ao longo do período experimental, e a de monoterpenos hidrocarbonetos diminuiu. O óleo diluído em solução aquosa foi mais fitotóxico sobre *A. viridis* e *L. sativa*, seguido por *E. plana*, e não afetou *P. notatum*. *Amaranthus viridis* foi inibida por todos os tratamentos, principalmente o óleo essencial, α-pineno e uma das frações, enquanto *E. plana* foi mais afetada pelas frações, sem efeitos para os componentes majoritários. Os resultados revelaram os efeitos fitotóxicos espécie-específicos do óleo essencial de *E. saligna*, indicando seu uso potencial para controlar *A. viridis* em cultivos de poáceas e *E. plana* em pastagens. Os efeitos fitotóxicos de um óleo essencial ou de seus componentes não devem ser generalizados, uma vez que os efeitos podem diferir de acordo com a espécie receptora e a metodologia empregada.

Palavras-chave: componentes majoritários; fitotoxidez; frações; plantas daninhas; solução aquosa; voláteis.

ABSTRACT

Phytotoxic essential oils can be potentially employed as natural herbicides for weed management. We aimed to evaluate the phytotoxic effects of *Eucalyptus saligna* leaf litter essential oil, and to investigate which compounds were related to phytotoxicity. Effects of the essential oil and its major components were tested by volatilization on germination and growth of *Eragrostis plana*. Compounds in the airspace were monitored along the experimental period by solid-phase microextraction (SPME), and the essential oil and volatilizing compounds were characterized by gas-cromatography couple to mass-spectrometry (GC-MS). Phytotoxicity of the essential oil, its fractions and major compounds diluted in aqueous solution was assessed. Recipient species were *Lactuca sativa* (standard), *Amaranthus viridis* and *E. plana* (weeds), and *Paspalum notatum* (forage). The essential oil was a mixture of monoterpene hydrocarbons (35.6%), oxygenated monoterpenes (52.5%), sesquiterpene hydrocarbons (3.7%) and oxygenated sesquiterpenes (5.5%). In the volatilization experiment, the essential oil was more phytotoxic, followed by the major compounds eucalyptol and α-pinene. The proportion of oxygenated monoterpenes increased along the experimental period, and of monoterpene hydrocarbons diminished. The essential oil diluted in aqueous solution was more phytotoxic on *A. viridis* and *L. sativa*, followed by *E. plana*, and caused no effects on *P. notatum*. *Amarathus viridis* was inhibited by all treatments, mainly the essential oil, α-pinene and one of the fractions, whereas *E. plana* was more affected by the oil fractions, with no effects of the major compounds. Results revealed the species-specific phytotoxic effects of *E. saligna* essential oil, indicating its potential use for controlling *A. viridis* in crops of Poaceae species, and *E. plana* in pastures. Phytotoxic effects of an essential oil or its components should not be generalized, as effects may change according to recipient species and employed methodology.

Keywords: aqueous solution; fractions; major compounds; phytotoxicity; weeds; volatiles.

1 Introduction

Weeds have been a major problem for agriculture, causing greater reduction in crop yields than any other agricultural pest (Dayan et al. 2009). Weed control mostly relies in the use of huge amounts of synthetic herbicides, accounting for about 40% of all pesticides used world over (Stokstad 2013). However, the excessive use of synthetic pesticides has resulted in emergence of resistant biotypes (Heckel 2012), toxicological implications to human health

(Gilden et al. 2010), long-term persistence in the environment (Weber et al. 2010) and biodiversity loss (Geiger et al. 2010). In the last years, popularity and demand for organic foods has grown, which has resulted in the conversion of millions of hectares from conventional to organic agriculture (Willer and Lernoud 2017). Neither synthetic herbicides nor transgenic crops are accepted by organizations that approve products for use in organic farming. This leaves very few commercial options, with organic farmers relying on labor intensive and high cost methods, such as cover crops, mulches and hand labor (Dayan and Duke 2010). In this sense, natural products, either in pure form or as crude extracts or essential oils, can be used as alternatives for controlling weeds both in organic and in conventional farming (Dayan et al. 2009).

Essential oils of several species have been viewed as potential herbicides, and some of them have already been commercialized and successfully launched in organic agriculture (Dayan et al. 2009; Soltys et al. 2013). These plant products present very little or no mammalian toxicity, and are non-persistent in fresh water and in soil (Isman 2000; Isman and Machial 2006). Because of these characteristics, essential oils can be considered eco-friendly herbicides (bioherbicides). Essential oils of several *Eucalyptus* species have shown phytotoxicity on crops and weeds (Batish et al. 2008; Zhang et al. 2010; Ghnaya et al. 2013; Ootani et al. 2017), indicating their bioherbicide potential. In particular, a *Eucalyptus* oil was even demonstrated to effectively control weeds under field conditions (Batish et al. 2004, 2007). In addition, *Eucalyptus* essential oils have presented repellent/biocide activity on a number of harmful arthropods and nematodes (Batish et al. 2008; Zhang et al. 2010), indicating their wide potential for use in pest management.

Many *Eucalyptus* species have been cultivated for pulpwood production worldwide, such as *Eucalyptus saligna* Sm., one of the most planted species in South Brazil. Insecticidal and repellent activity was demonstrated for *E. saligna* essential oil (Mossi et al. 2011; Bett et al. 2016). Moreover, volatiles emitted from *E. saligna* essential oil affected germination, seedling growth, generated oxidative stress and led to damages on seedling membranes of recipient species (Silva et al. 2017). A volatilization method may be an interesting approach, for example, to explore potential weed suppressive effects of volatiles present in plant material that could be used as mulch (e.g. Petersen et al. 2001). On the other hand, the potential applicability of essential oils as herbicides depends on observing phytotoxic effects when these substances are diluted in water. In spite of the presumed insolubility of essential oil components in water, these compounds may present aqueous solubility at different levels and be phytotoxic in concentrations below their aqueous solubility (Weidenhamer et al. 1993).

Elucidating which compounds are related to activity is a necessary step in phytotoxicity investigations. Biological activity of volatiles is usually associated with compounds reported in essential oils. However, specific quantity of compounds volatilizing in the airspace may be different (e.g. Kumar et al. 2012). In addition, very often the major compounds of an essential oil fail to explain its whole activity, and effects are thus attributed to synergism of many compounds (e.g. Zhang et al. 2012). However, not all compounds are necessarily related to observed effects, with some of them possibly acting antagonistically (e.g. Savelev et al. 2003). In this sense, a deeper chemical investigation, as for example, with sets of compounds (fractions), may reveal which compounds are indeed related to bioactivity. This is relevant in order to comprehend if a better strategy for weed control consists in using a whole essential oil or only one or few compounds. This knowledge would also allow searching and selecting more bioactive plant chemotypes.

In this study, we aimed to evaluate the phytotoxic effects of *E. saligna* leaf litter essential oil. We also aimed to assess which compounds or set of compounds were related to the phytotoxicity of *E. saligna* oil. For this, we tested the phytotoxic effects of the essential oil and of its major compounds by volatilization, and monitored changes in chemical composition of volatiles with time. Moreover, we assessed the effects of the essential oil, its fractions and major compounds in aqueous solution on weeds, standard and desirable plant species.

2 Material and Methods

2.1 Collection of *Eucalyptus saligna* leaf litter and extraction of the essential oil

Eucalyptus saligna leaf litter was obtained in a plantation of the species located in the municipality of Eldorado do Sul, Rio Grande do Sul State, Brazil (30°11’02”S, 51°37’14”W). The site is located in the ‘Campos Sulinos’ region (Overbeck et al. 2007), where grassland is the vegetation type. Essential oil was extracted from leaf litter collected in December 2015 by steam distillation. Steam distillation was conducted with 5 kg of dry leaves using an inox extractor with a steam flow rate of 3 L/h for 1 h. Water in *E. saligna* essential oil was eliminated with anhydrous sodium sulfate. The oil, as well as some leaves, were then stored at -20 °C until use.

2.2 Chemical analysis

Compounds of *E. saligna* essential oil were analyzed by a gas chromatograph coupled to a mass spectrometer detector (GC-MS - Bruker Scion 436, USA). A capillary column SPB-5 (30 m x 250 µm x 0,25 µm, Bruker, USA) was used, with an initial oven temperature of 40 °C (10 min), raised by 3 °C/min to 180 °C and by 10 °C/min to 300 °C (5 min). Injector and detector temperatures were kept at 250 °C. Helium flow rate was 1 mL/min and desorption occurred in split mode (1:100). Linear temperature programmed retention indexes (LTPRIs) were determined from the retention data of an n-alkane solution (C7–C25), along with the retention data of volatile compounds from *E. saligna* samples. All components were tentatively identified through comparison of their LTPRI with those registered in the literature databases (Arithmetic Index, Adams 2007). Experimental mass spectra were also compared with spectra stored in mass spectrometry databases (NIST). Relative percentage of each component was obtained directly from chromatographic peak areas, assuming that the sum of all eluted peaks was 100%.

In previous experiments that tested the phytotoxicity of *E. saligna* essential oil (Silva et al. 2017), the oil was obtained from leaf litter collected in the same plantation area, in August 2014. In that study, the essential oil was characterized with different column and temperature conditions, and many compounds could not be identified. Thus, in order to determine possible differences in chemical composition of the essential oils, the sample of 2014 was characterized under the same optimized conditions described above.

Volatile compounds directly emitted from *E. saligna* leaf litter were determined by solid-phase microextraction (SPME). Macerated leaf litter (3 g) was added to a 25 mL sealed tube, where it remained by 24 h. The syringe needle of the SPME device was inserted into the tube and the PDMS fiber (100 µm, Supelco, USA) was exposed to the headspace during 10 min at room temperature (22 °C). The needle containing the SPME fiber was withdrawn and introduced into the port of a gas chromatograph at 250 °C. The SPME device was held in the port for 2 min to allow complete desorption of compounds. Chemical characterization was performed under the same conditions described for essential oil analysis.

2.3 Phytotoxicity of *Eucalyptus saligna* essential oil by volatilization

Effects of *E. saligna* essential oil and its major compounds were assessed on germination and seedling growth of a recipient species. In a previous study, the essential oil showed similar phytotoxic effects on the Poaceae *Eragrostis plana* Ness and *Paspalum notatum* Flüggé, and the Fabaceae *Trifolium repens* L. and *Lotus corniculatus* L., by

volatilization (Silva et al. 2017). Thus, only effects on *E. plana* were assessed herein. The major compounds of the essential oil used in the experiments were α-pinene and eucalyptol (1,8-cineole). A mixture of eucalyptol and α-pinene was also used, in the same proportion between these compounds found in the essential oil (54.2% and 45.8%, respectively). Eucalyptol and α-pinene were obtained from commercial source (Sigma-Aldrich).

Groups were arranged in a completely random design with four replicates (Petri dishes) per group. Each replicate consisted of 20 diaspores of *E. plana* sown in a 5 cm Petri dish that contained filter paper moistened with 1 mL of a buffer solution (distilled water with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH adjusted to 6.0) with 0.5% dimethylsulfoxide (DMSO). Then, the treatment (pure essential oil or compound) was applied on a cotton piece that was fixed in the inner face of the plate lid. This allowed volatilisation in the airspace within plates without direct contact between the treatment and diaspores or water. Immediately after, plates were sealed with Parafilm®, to avoid loss of volatiles, and placed in a growth chamber (25 °C/16-h photoperiod). Applied amounts of essential oil/compounds were 0 (control), 1, 5, 10, 20, and 30 µL. After seven days, the total of germinated seeds (root ≥ 2 mm), root and shoot length were measured using Fitomed® software. Germination rate was calculated as the percentage of germinated seeds.

In order to determine possible changes in chemical profile of volatiles along the experiment, volatiles in plates were obtained daily by SPME and analysed by GC-MS. Two replicates were prepared in the same manner described for the experiment with *E. plana* diaspores, and 30 µL of essential oil was added. The syringe needle of the SPME device was inserted into the plate and the fiber was exposed to the headspace by 10 min. The needle containing the SPME fiber was withdrawn and introduced into the port of a gas chromatograph at 250 °C, and held in the port by 2 min. Chemical characterization was performed under the same conditions described above. This procedure was made after the essential oil was added to plates, and then at a daily interval, for seven days. Plates were maintained in a growth chamber (25 °C), and sealed after each SPME procedure.

2.4. Coleoptile bioassay

The coleoptile assay consists in an initial bioactivity evaluation, and was used as a first step to assess effects of *E. saligna* essential oil diluted in aqueous solution. *Triticum aestivum* L. (wheat) diaspores were sown in Petri dishes that contained filter paper moistened with distilled water and maintained in a growth chamber at 25 °C in complete darkness. After 96 hours, four-millimeter-long coleoptile fragments were cut, immediately below the first two millimeters of the coleoptile apex, using a Van der Weij guillotine. The

essential oil was diluted in a buffer solution (distilled water with citrate and phosphate, pH adjusted to 5.6, supplemented with 2% sucrose) with 0.5% DMSO, at concentrations of 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL. For comparison of bioactivity, *E. saligna* essential oil obtained in a previous year (2014) was also used, at the same concentrations. Controls were the buffer solution with DMSO (negative control), and the buffer solution with DMSO containing the herbicide Logran® (positive control) at the same concentrations used for the essential oil. Five coleoptiles were incubated in test tubes containing 2 mL of the treatments, with three replicates per group. All procedures were conducted under a green safelight.

Tubes were incubated with constant stirring (6 rpm) in an SC2 Stuart Scientific culture rotator in the dark at 25 °C for 24 hours. After this period, the coleoptiles were photographed and measured using PhotoMed® software. The percentage of inhibition of coleoptile growth was calculated using the following formulae: ((((il-rl)-(il-cl))/(il-cl)) x 100), in which il = initial length of coleoptile (4 mm), rl = mean length of coleoptile in the replicate, cl = mean length of coleoptile in the negative control group.

2.5 Phytotoxicity of the essential oil in aqueous solution

In this experiment, effects of *E. saligna* essential oil was assessed on germination and seedling growth of the recipient species *Lactuca sativa* L. (Asteraceae), *Amaranthus viridis* L. (Amaranthaceae), *E. plana*, and *P. notatum*. *Lactuca sativa* (lettuce) is standard target species (Macías et al. 2000), a model for phytotoxicity evaluations. *Amaranthus viridis* (slender amaranth, caruru) is common weed in tropical and subtropical regions, and this exotic species has spread all over Brazil in crop plantations and in pastures. *Eragrostis plana* (South African lovegrass, capim-annoni-2) is a highly invasive exotic species in South Brazilian grasslands, causing problems for livestock production due to its low forage quality. This species is also invasive in other regions of the world, with high potential for expansion, mainly in southern South America (Barbosa et al. 2013). *Paspalum notatum* (pensacola bahiagrass, grama-forquilha), on the other hand, is a native species in South Brazilian grasslands and a desirable species in pastures for cattle production. This is the most adapted perennial grass to climate and soil in South Brazil, and one of the most cultivated forage species in the country.

The experiment was conducted in a random design with four repetitions per group, and methods were similar to the volatilization experiment. However, the essential oil was not added to the bottom of the plate, but diluted in the buffer aqueous solution with DMSO. For *L. sativa* and *A. viridis*, concentrations used were 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL. The grasses (*E. plana* and *P. notatum*) showed less sensitivity to the essential oil, which was

observed in a pilot assay; hence, the concentrations of 1.0, 0.8, 0.6, 0.4 and 0.2 mg/mL were used. Plates were sealed and maintained in a growth room for six days for *L. sativa* and *A. viridis*, seven days for *E. plana* and 14 days for *P. notatum*. Controls were the same that for the volatilization experiment, as well as growth room conditions, except by *L. sativa* that was maintained in darkness. At the end of the experiments, total of germinated seeds, root and shoot length were measured.

2.6 Phytotoxicity of fractions and major compounds of the essential oil in aqueous solution

Fractions of *E. saligna* essential oil were obtained by preparative thin-layer chromatography (TLC). Normal phase 200 mm x 200 mm plates (silica gel 60 F_{254}, Merck) were used for preparative TLC. The essential oil diluted in hexane (1:1, v/v) was applied to each plate (100 µL per plate), using an automated sample applicator. The mobile phase consisted of hexane: ethyl acetate (9:1, v/v), which was applied to plates twice. Compounds were separated into six bands, which were detected under UV light (254 and 365 nm). After drying plates at room temperature, bands were scraped off the plates and extracted with diethyl ether. Silica was removed and the solvent was evaporated, remaining the essential oil fractions. Fractions were characterized by GC-MS, as described above.

Effects of the six fractions and of the major compounds of *E. saligna* essential oil were tested on germination and growth of the weeds *A. viridis* and *E. plana*. Major compounds used were the same that for the volatilization experiment: α-pinene, eucalyptol and a mixture of eucalyptol (54.2%) and α-pinene (45.8%). Fractions and compounds were diluted in the buffer solution with DMSO at concentrations of 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL for *A. viridis* and 1.0, 0.6 and 0.2 mg/mL for *E. plana*. Experiments were conducted under the same conditions described before.

2.7 Statistical analyses

Differences between the measured parameters (coleoptile length, germination rate, root length and shoot length) were compared between groups by analysis of variance (ANOVA), for each species. When differences were significant, ANOVA was followed by Tukey's test. In cases that data did not follow normality and/or homogeneity of variances (tested by Shapiro-Wilk and Lavene's test, respectively), analysis of variance with randomisation (PERMANOVA) was performed. Randomisation tests are not based on assumptions of normality and homogeneity of variances (Anderson 2001). When

PERMANOVA indicated significant differences between groups, contrast analyses were used for pairwise comparisons (Pillar and Orlóci 1996). PERMANOVA was conducted with 10,000 bootstrap iterations and used Euclidean distance as dissimilarity measure. For all analyses, a significance level of $P \leq 0.05$ was considered. Results are presented as percentage of control. Positive values represent stimulation, and negative values indicate inhibition of germination or growth.

3 Results

3.1 Chemical characterization of essential oil and volatiles emitted from leaf litter of *Eucalyptus saligna*

Eucalyptus saligna leaf litter essential oil was mainly constituted by oxygenated monoterpenes (52.5%), followed by monoterpene hydrocarbons (35.6%) (Table 1). Oxygenated sesquiterpenes and sesquiterpene hydrocarbons were also found (5.5% and 3.7%, respectively). The major compounds of the oil were the monoterpenes eucalyptol (32.5%) and α-pinene (27.5%). The essential oil obtained in a previous year showed a very similar chemical composition, both qualitatively and quantitatively (Supplementary Table 1).

The chemical composition of volatiles emitted from *E. saligna* leaf litter was similar to the essential oil (Table 1). However, 24% of the peaks were not found in the leaves and some quantitative differences were observed. The most abundant compounds were monoterpene hydrocarbons (65.1%), followed by oxygenated monoterpenes (30%), sesquiterpene hydrocarbons (3.3%) and oxygenated sesquiterpenes (1.1%). α-Pinene was the major compound, found in a higher amount than in the essential oil (60.6%), followed by eucalyptol (29%).

Table 1. Chemical composition of *Eucalyptus saligna* leaf litter essential oil and of volatiles obtained from *E. saligna* leaf litter by SPME, analysed by GC-MS.

Compound	LTPR^{llit}	Essential oil		Leaf litter		Group
		LTPRI	Area (%)	LTPRI	Area (%)	
α-Pinene	932	934	27.49	936	60.58	MH
Camphene	946	946	2.45	949	0.29	MH
β-Pinene	974	972	0.35	976	0.21	MH
α-Phellandrene	1002	1001	0.10	1004	0.07	MH
n.i.		1014	0.13	1016	0.04	
p-Cymenene	1020	1025	4.65	1025	3.87	MH
1,8-Cineole (eucalyptol)	1026	1031	32.51	1032	29.01	OM
cis-β-Ocimene	1032	1038	0.01	-	-	MH
γ-Terpinene	1054	1056	0.11	1059	0.04	MH
n.i.		1068	0.28	1071	0.06	
Terpinolene	1086	1082	0.49	1085	0.04	MH
n.i.		1087	0.64	1089	<0.01	
Isopentyl isovalerate	1102	1106	0.12	1108	0.06	OM
trans-Thujone	1112	1113	0.03	-	-	OM
exo-Fenchol	1118	1116	1.80	1118	0.03	OM
α-Campholenal	1122	1123	2.79	1125	0.13	OM
trans-Pinocarveol	1135	1137	3.86	1138	0.13	OM
Camphor	1141	1141	0.13	-	-	OM
Nerol oxide	1154	1150	0.07	-	-	OM
trans-Pinocamphone	1158	1155	0.30	1157	0.02	OM
Pinocarvone	1160	1157	1.34	1159	0.14	OM
endo-Borneol	1165	1169	3.98	1172	0.04	OM
Terpinen-4-ol	1174	1178	0.04	1179	0.03	OM
trans-Isocarveol	1187	1186	0.18	-	-	OM
α-Terpineol	1186	1193	4.71	1194	0.34	OM
n.i.		1194	0.10	-	-	
trans-Carveol	1215	1218	0.28	-	-	OM
Isobornyl formate	1235	1223	0.24	1225	0.01	OM
n.i.		1227	0.12	-	-	
n.i.		1234	0.19	1236	0.01	
Carvone	1239	1239	0.07	1242	0.01	OM
Geranial	1264	1266	0.04	-	-	OM
trans-Pinocarvyl acetate	1298	1291	0.04	-	-	OM
Thymol	1289	1300	<0.01	-	-	OM
n.i.		-	-	1331	0.10	
α-Cubebene	1345	1344	0.03	1347	0.07	SH
Isoledene	1374	1367	0.03	1370	0.07	SH
α-Copaene	1374	1371	0.26	1375	0.39	SH
β-Bourbonene	1387	1379	0.06	1382	0.03	SH
β-elemene	1389	1379	0.03	1388	0.02	SH

Compound	LTPR^{lit}	Essential oil		Leaf litter		Group
		LTPRI	Area (%)	LTPRI	Area (%)	
cis-Jasmone*	1392	1388	0.05	-	-	NI
α-Gurjunene	1409	1402	0.13	1405	0.32	SH
trans-Caryophyllene	1417	1414	0.41	1417	0.62	SH
β-copaene	1430	1425	0.02	1428	0.02	SH
Aromadendrene	1439	1433	0.11	1437	0.19	SH
n.i.		1445	0.18	1447	0.05	
α-Humulene	1452	1450	0.16	1453	0.17	SH
Alloaromadendrene	1458	1455	0.67	1458	0.73	SH
γ-Gurjunene	1475	1467	0.11	-	-	SH
α-Curcumene	1479	1477	0.06	-	-	SH
n.i.		1483	0.29	1486	0.14	
Viridiflorene	1496	1486	0.26	1490	0.14	SH
n.i.		1490	0.17	1494	0.15	
α-Muurolene	1500	1494	0.14	1498	0.05	SH
δ-Cadinene	1522	1514	0.30	1518	0.21	SH
trans-Calamenene	1521	1517	0.72	1520	0.25	SH
n.i.		1530	0.07	-	-	
α-Calacorene	1544	1536	0.12	1540	0.03	SH
n.i.		1544	0.07	1547	0.01	
n.i.		1566	0.34	1568	0.06	
Spathulenol	1577	1573	3.13	1576	0.46	OS
Caryophyllene oxide	1582	1575	0.03	1579	0.12	OS
Globulol	1590	1585	0.76	1585	0.13	OS
Ledol	1602	1593	0.94	1594	0.16	OS
1-epi-Cubenol	1627	1624	0.45	1626	0.13	OS
Cubenol	1645	1638	0.16	1642	0.02	OS
Cadalene	1675	1667	0.08	-	-	SH
Total per group:						
Monoterpene hydrocarbons			35.66		65.13	
Oxygenated monoterpenes			52.55		29.96	
Sesquiterpene hydrocarbons			3.70		3.31	
Oxygenated sesquiterpenes			5.46		1.15	
Non isoprenoid			0.05		0.00	
Not identified			2.58		0.46	

LTPRI^{lit}: Linear Temperature Programmed Retention Indexes, tabulated; LTPRI: Linear Temperature Programmed Retention Indexes, calculated; area %: relative percentage of each component, which was directly obtained from chromatographic peak areas, considering that the sum of all eluted peaks was 100%; n.i.: not identified; -: not found in the sample. Compounds were grouped by class: Monoterpene hydrocarbon (MH); Oxygenated monoterpene (OM); Sesquiterpene hydrocarbon (SH); Oxygenated sesquiterpene (OS); Non isoprenoid (NI).

3.2 Phytotoxicity of *Eucalyptus saligna* essential oil by volatilization

Along the seven-day period of the experiment, almost all compounds of *E. saligna* essential oil volatilized in plates, and quantitative changes occurred (Fig. 1). Just after the essential oil was added to plates (time 0), monoterpene hydrocarbons and oxygenated monoterpenes predominated, in comparable amounts. At that moment, α-pinene and eucalyptol accounted for almost the total area of compounds in the airspace (49.8 and 41.2%, respectively) (See Supplementary Table 2 for a detailed chemical characterization). Substantial changes in chemical composition occurred from 48h, and then it remained similar to the end of the experiment. From 0h to 168h, monoterpene hydrocarbons diminished (from 54.8 to 3.9%), mainly α-pinene (2.7%), whereas oxygenated monoterpenes relatively increased (from 44.2 to 91.3%), especially eucalyptol (60.8%), *trans*-pinocarveol (from 0.4 to 8.2%), *endo*-borneol (from 0.08 to 5.95) and α-terpineol (from 0.17 to 6.34%).

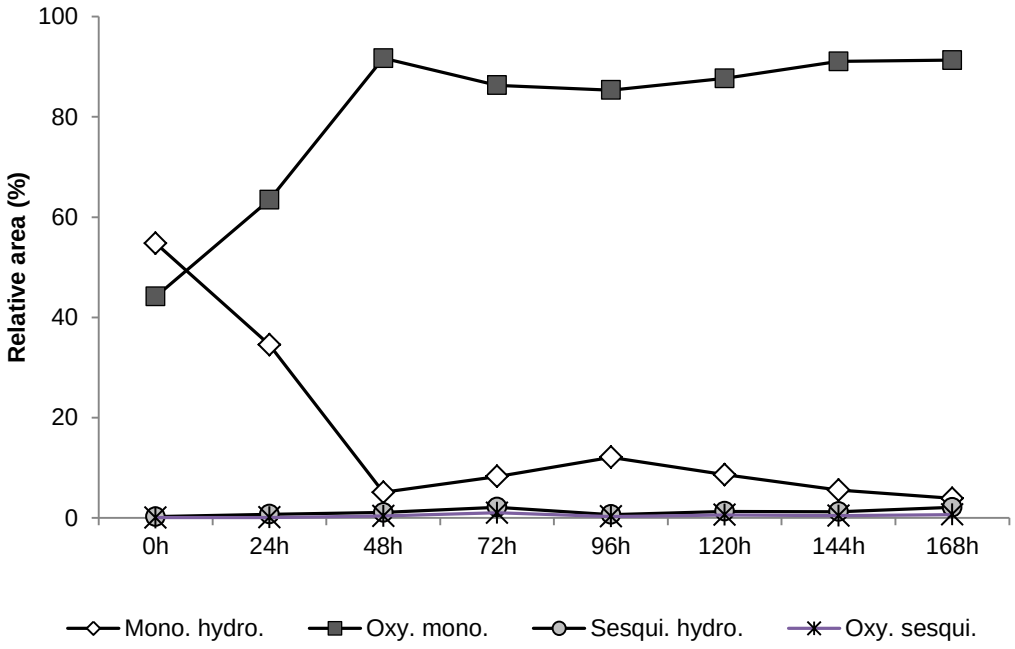


Figure 1. Relative area of volatiles emitted from *Eucalyptus saligna* leaf litter essential oil (30 µL) along the period that lasted the phytotoxicity experiment. Volatiles were obtained into closed plates by SPME and analysed by GC-MS. Compounds were grouped by class: monoterpene hydrocarbons (Mono. hydro.); oxygenated monoterpenes (Oxy. mono.); sesquiterpene hydrocarbons (Sesqui. hydro.); and oxygenated sesquiterpenes (Oxy. sesqui.).

The essential oil significantly inhibited germination, root and shoot growth of *E. plana* at all tested amounts, with total inhibition of these parameters at 20 and 30 µL (Fig. 2). In relation to the major compounds, eucalyptol and eucalyptol+α-pinene inhibited germination of

E. plana from 10 to 30 µL and from 20 to 30 µL, respectively, with no effect of α-pinene on germination. Eucalyptol significantly inhibited root growth from 5 to 30 µL, whereas the other treatments caused negative effects only at 20 and 30 µL. All treatments inhibited shoot growth of *E. plana*, at all tested amounts, although percentage of inhibition was lower in treatment with α-pinene.

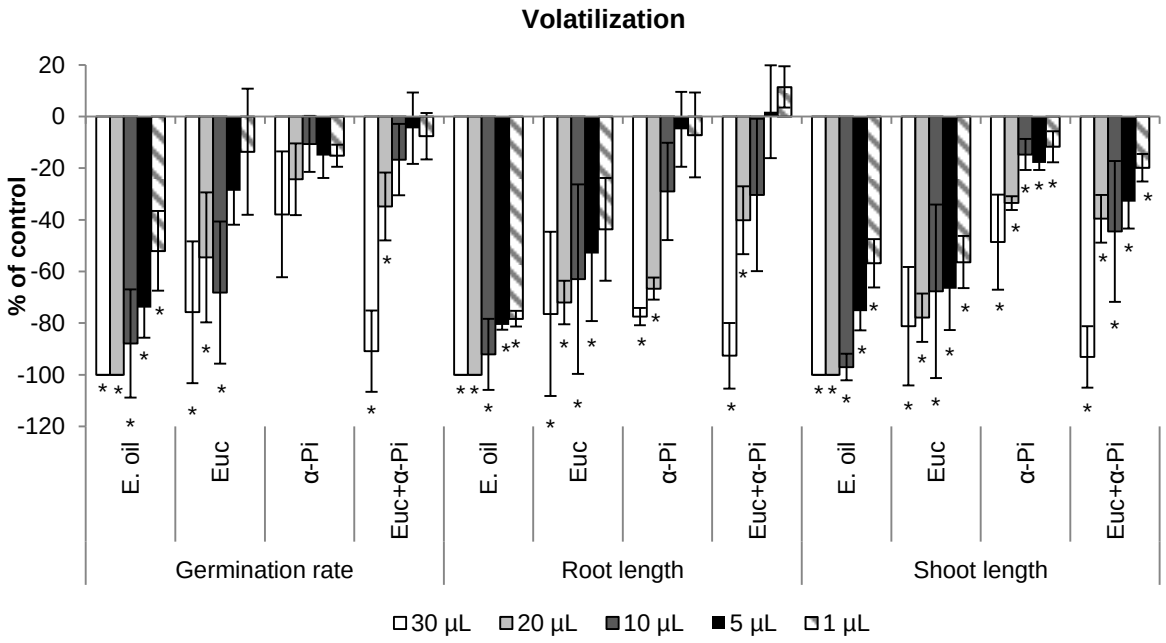


Figure 2. Effects of *Eucalyptus saligna* essential oil and its major compounds (1, 5, 10, 20, and 30 µL) on germination, root and shoot growth of *Eragrostis plana*. The major compounds were eucalyptol, α-pinene, and eucalyptol+α-pinene in the same proportion found in the essential oil (54.2%+45.8%). Data are presented as mean ± standard deviation of percentage of control (n = 4). (*) Significant difference from control according to ANOVA or PERMANOVA, at $P \leq 0.05$.

3.3 Coleoptile bioassay

Eucalyptus saligna essential oil negatively affected growth of *T. aestivum* coleoptile, from 0.4 to 0.1 mg/mL (Fig. 3). The essential oil obtained from *E. saligna* leaf litter in a previous year caused similar effects on coleoptile growth, but inhibition was only significant at 0.4 and 0.2 mg/mL. Effects of the herbicide Logran, the positive control, were observed at all tested amounts.

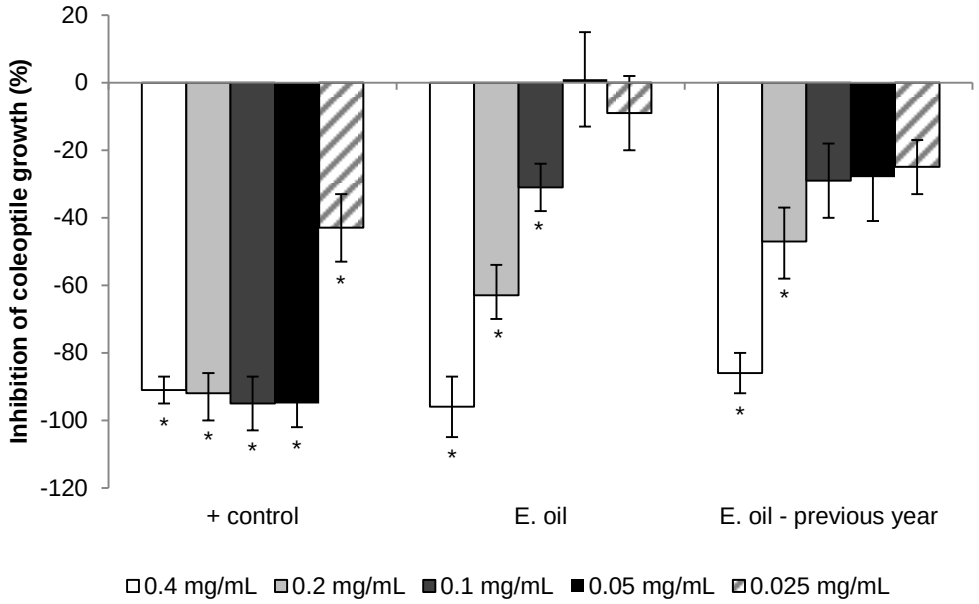


Figure 3. Effects of *Eucalyptus saligna* leaf litter essential oil on growth of *Triticum aestivum* coleoptile. Essential oil (E. oil) was diluted in a buffer solution of water and DMSO, at concentrations of 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL. Effects of the essential oil collected in a previous year and used in another phytotoxicity study were also assessed (E. oil – previous year). The herbicide Logran was used as a positive control (+ control). Data are presented as mean ± standard deviation of inhibition of growth (n = 3). (*) Significant difference from control according to ANOVA followed by Tukey's test, at $P \leq 0.05$.

3.4 Phytotoxicity of the essential oil in aqueous solution

The essential oil inhibited germination and growth of most recipient species (Fig. 4). *Eucalyptus saligna* oil negatively affected germination of the standard species *L. sativa* from 0.4 to 0.05 mg/mL, and root and shoot growth until 0.1 mg/mL (Fig. 4 A). The oil inhibited germination and growth of the weed *A. viridis* until 0.025 mg/mL and 0.05 mg/mL, respectively, with total inhibition of these parameters at 0.4 and 0.2 mg/mL (Fig. 4 B). In general, effects of *E. saligna* oil on *L. sativa* and *A. viridis* were comparable to effects of the herbicide that was used as positive control. However, the essential oil caused stronger effects on germination of these species. Regarding the Poaceae species, the essential oil only inhibited shoot growth of *E. plana* from 1.0 to 0.6 mg/mL (Fig. 4 C). The oil did not affect *P. notatum*, the desirable species, but the herbicide affected its germination and growth at all concentrations (Fig. 4 D).

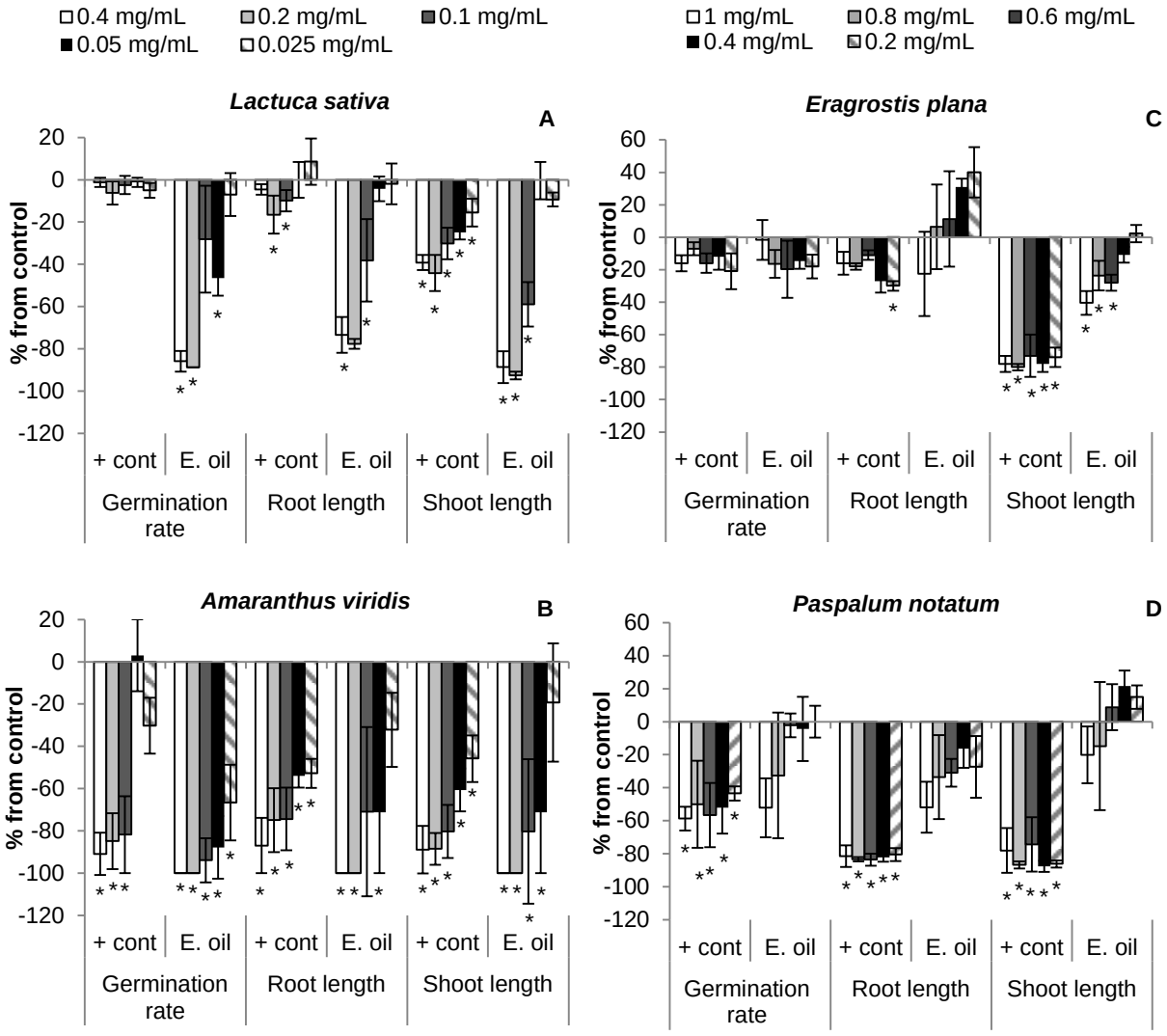


Figure 4. Effects of *Eucalyptus saligna* essential oil on germination and growth of *Lactuca sativa* (A), *Amaranthus viridis* (B), *Eragrostis plana* (C) and *Paspalum notatum* (D). The essential oil (E. oil) was diluted in a buffer solution of water and DMSO, at concentrations of 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL for *L. sativa* and *A. viridis* and at 1.0, 0.8, 0.6, 0.4 and 0.2 mg/mL for *E. plana* and *P. notatum*. The herbicide Logran was used as a positive control (+ cont). Data are presented as mean ± standard deviation of percentage of control (n = 4). (*) Significant difference from control according to ANOVA followed by Tukey's test or PERMANOVA followed by contrast analysis, at $P \leq 0.05$. Please note different scale of y-axis.

3.5 Phytotoxicity of fractions and major compounds of the essential oil in aqueous solution

In the total, six fractions were obtained from *E. saligna* essential oil, with qualitative and quantitative differences in their chemical composition (Table 2). Major compounds of all fractions were oxygenated monoterpenes, except by fraction 1 that also presented some sesquiterpene hydrocarbons in high amounts. Fraction 1 showed the highest amount of compounds (27 peaks), with alloaromadendrene (16%), *trans*-calamenene (16%) and

eucalyptol (13%) as major compounds. Fraction 2, 3 and 4 had eucalyptol as the most abundant compound (93%, 49% and 34%, respectively). However, fraction 3 also presented high amounts of pinocarvone (26%) and α-campholenal (15%), and fraction 4 had carvone (10%) and camphor (10%). Fraction 5 was mainly represented by *exo*-fenchol (72%), and fraction 6 presented α-terpineol (46%) and *endo*-borneol (36%) as major compounds.

Effects of fractions of *E. saligna* essential oil and its major compounds varied between recipient species (Fig. 5). Regarding *A. viridis*, the major compounds affected germination from 0.4 to 0.05 mg/mL (Fig. 5 A). All fractions inhibited *A. viridis* germination at 0.4 and 0.2 mg/mL, with effects of fractions 5 and 6 until 0.05 mg/mL. α–Pinene inhibited *A. viridis* growth until 0.1 mg/mL, whereas eucalyptol+α–pinene affected root and shoot growth until 0.4 and 0.2%, respectively (Fig. 5 B, C). Eucalyptol did not affect significantly *A. viridis* growth. All fractions inhibited root and shoot growth at 0.4 mg/mL, with effects of fraction 6 until 0.05 mg/mL.

The major compounds of the essential oil did not inhibit *E. plana* germination and growth (Fig. 5 D, E, F). Most of fractions inhibited germination at 1.0 mg/mL, with fraction 6 totally suppressing germination and causing effects also at 0.6 mg/mL. All fractions negatively affected *E. plana* root and shoot growth at 1 mg/mL, with effects of fractions 3, 4 and 6 also at 0.6 mg/mL.

Table 2. Chemical composition of fractions obtained from *Eucalyptus saligna* essential oil by preparative thin-layer chromatography, analysed by GC-MS.

Compound	Area (%)						LTPRI[lit]	LTPRI						Group
	F1	F2	F3	F4	F5	F6		F1	F2	F3	F4	F5	F6	
α-Pinene	2.03	0.56	1.09	6.75	1.22	0.09	932	932	933	933	933	933	933	MH
p-Cymenene	0.65	0.12		0.90	0.17		1020	1023	1024		1024	1023		MH
Limonene	0.54			1.07	0.23	0.04	1024	1028			1028	1028	1028	MH
1,8-Cineole (eucalyptol)	**13.26**	**93.10**	**48.77**	**34.45**	3.92	0.55	1026	1029	1031	1030	1030	1030	1030	OM
n.i.			1.11	10.48						1070	1070			
Isopentyl isovalerate		0.36					1102		1108					OM
trans-Thujone		0.12					1112		1115					OM
exo-Fenchol				1.01	**71.98**	0.10	1118				1116	1117	1117	OM
α-Campholenal	0.34	0.44	**15.56**	1.84			1122	1124	1124	1124	1124			OM
trans-Pinocarveol					0.44	7.06	1135					1137	1137	OM
Camphor				**10.25**			1141				1142			OM
Nerol oxide		0.19					1154		1152					OM
Pinocarvone			**26.11**	1.57			1160			1159	1158			OM
endo-Borneol						**36.12**	1165						1171	OM
n.i.			1.82	1.82						1172	1172			
Terpinen-4-ol					4.01		1174					1178		OM
trans-Isocarveol						1.77	1187						1187	OM
α-Terpineol						**46.45**	1186						1195	OM
trans-Carveol						2.30	1215						1218	OM
Isobornyl formate		1.55					1235		1225					OM
n.i.					1.79							1226		

Compound	Area (%)						LTPRI[lit]	LTPRI						Group
	F1	F2	F3	F4	F5	F6		F1	F2	F3	F4	F5	F6	
n.i.		1.87							1236					
Carvone				**10.33**			1239				1241			OM
n.i.				4.66							1246			
n.i.					5.56							1251		
trans-Pinocarvyl acetate		0.38					1298		1293					OM
Thymol					1.16		1289					1300		OM
n.i.	1.32							1309						
α-Copaene	4.24						1374	1374						SH
β-Bourbonene	0.95						1387	1382						SH
α-Gurjunene	1.01						1409	1405						SH
trans-Caryophyllene	0.62						1417	1417						SH
Aromadendrene	4.22						1439	1436						SH
n.i.	1.74			5.99				1448			1448			
n.i.	2.03							1451						
Alloaromadendrene	**16.24**						1458	1458						SH
n.i.	1.19							1464						
γ-Gurjunene	1.14						1475	1471						SH
n.i.	1.80							1475						
α-Curcumene	0.61						1479	1480						SH
n.i.	3.24	1.32						1487	1486					
α-Muurolene	2.57						1500	1498						SH
δ-Cadinene	4.73						1522	1518						SH

Compound	Area (%)						LTPRI[lit]	LTPRI						Group
	F1	F2	F3	F4	F5	F6		F1	F2	F3	F4	F5	F6	
trans-Calamenene	**15.97**						1521	1520						SH
α-Calacorene	2.76						1544	1539						SH
n.i.					1.91							1561		
n.i.			2.77							1568				
Spathulenol						0.22	1577						1575	OS
Caryophyllene oxide	10.06		1.31	2.13			1582	1578		1578	1578			OS
n.i.				6.75							1587			
Globulol						4.74	1590						1584	OS
n.i.	2.23							1606						
1-epi-Cubenol					7.61		1627					1627		OS
Cubenol						0.56	1645						1646	OS
Cadelene	1.68						1675	1671						SH
n.i.			1.47							1741				
Total per group:														
MH	3.21	0.68	1.09	8.72	1.61	0.13								
OM	13.60	96.13	90.45	59.46	81.51	94.35								
SH	56.75	0.00	0.00	0.00	0.00	0.00								
OS	10.06	0.00	1.31	2.13	7.61	5.52								
Not identified	16.38	3.19	7.16	29.70	9.27	0.00								

Area %: relative percentage of each component, which was directly obtained from chromatographic peak areas, considering that the sum of all eluted peaks was 100%; LTPRI[lit]: Linear Temperature Programmed Retention Indexes, tabulated; LTPRI: Linear Temperature Programmed Retention Indexes, calculated; F1 to F6: the six fractions obtained from the essential oil; n.i.: not identified. Compounds were grouped by class: Monoterpene hydrocarbon (MH); Oxygenated monoterpene (OM); Sesquiterpene hydrocarbon (SH); Oxygenated sesquiterpene (OS).

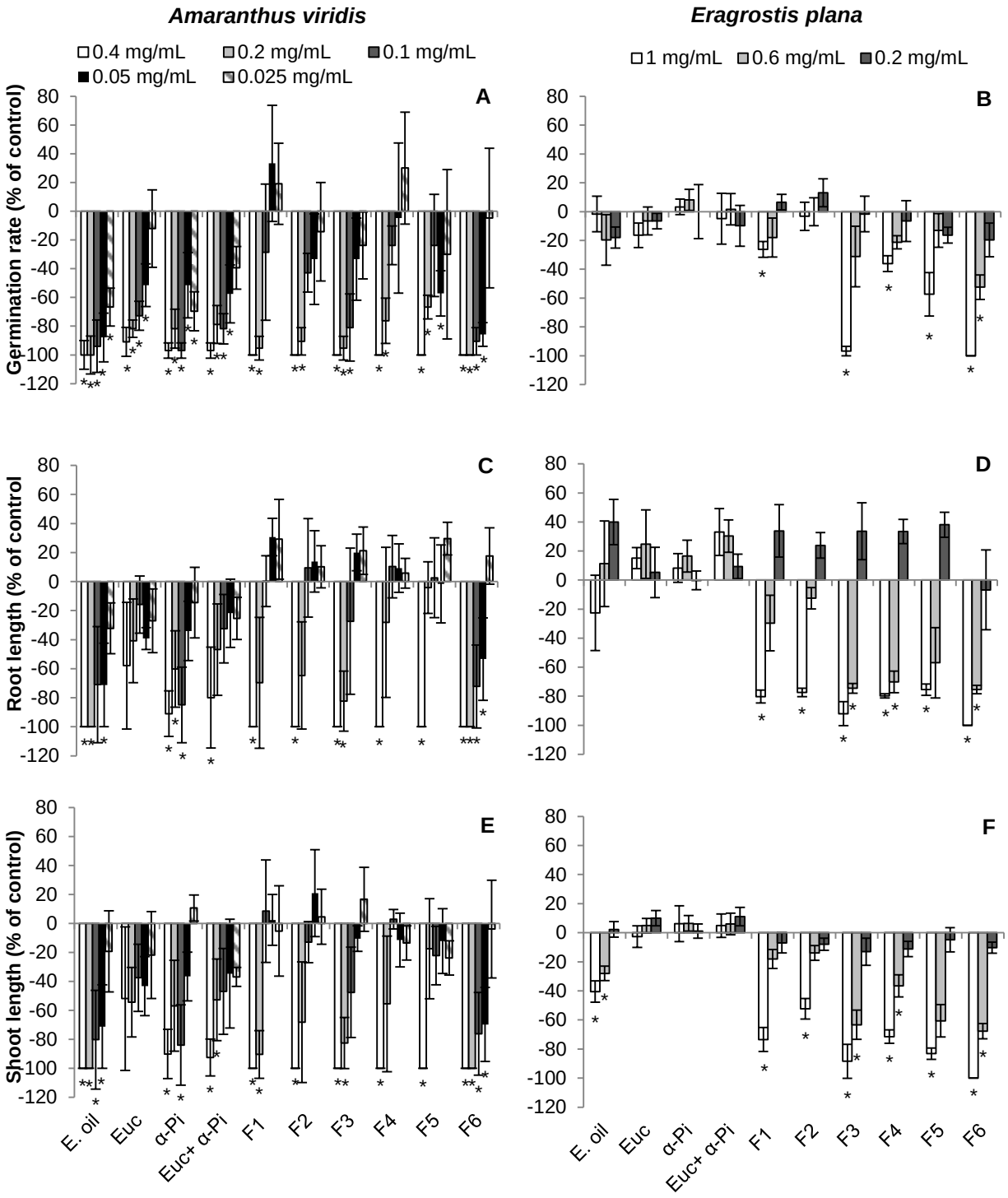


Figure 5. Effects of fractions obtained from *Eucalyptus saligna* essential oil and of the major compounds of the oil on recipient species. Effects were assessed on germination, root and shoot growth of *Amaranthus viridis* (A, C, and E, respectively) and *Eragrostis plana* (B, Dand F, respectively). Six fractions (F1 to F6) were obtained by preparative thin-layer chromatography. The major compounds were eucalyptol (euc), α-pinene (α-pi), and eucalyptol+α-pinene (euc+ α-pi) in the same proportion found in the essential oil (54.2%+45.8%). Fractions and compounds were diluted in a buffer solution of water and DMSO, at concentrations of 0.4, 0.2, 0.1, 0.05 and 0.025 mg/mL for *A. viridis*, and at 1.0, 0.6, and 0.2 mg/mL for *E. plana*. Results for the essential oil (E. oil) are shown for comparison. Data are presented as mean ± standard deviation of percentage of control (n = 4). (*) Significant difference from control according to ANOVA followed by Tukey's test or PERMANOVA followed by contrast analysis, at $P \leq 0.05$.

4 Discussion

Eucalyptus saligna essential oil showed phytotoxicity, but effects varied a lot, both in relation to method and recipient species. We observed differences between chemical compounds that volatilize from *E. saligna* leaf litter and from its essential oil. The major change was the higher amount of α-pinene in leaves than in the oil. This compound, by its high volatility, may have had a quantitative loss in steam distillation of the essential oil. Some minor compounds of the essential oil were not found in leaf litter, which may be due to the low quantity of leaves used in the SPME procedure. Another possibility is that the different compounds in the essential oil have resulted from break down and rearrangements of other compounds during the distillation (e.g. Cornwell et al. 2000). Moreover, the essential oils obtained in different years showed a very similar chemical composition. This is interesting in the view of the potential applied use of *E. saligna* essential oil, and allows comparing results obtained herein with a previous study about *E. saligna* phytotoxicity (Silva et al. 2017). In addition, when the essential oil was added to plates, the chemical composition of volatiles changed with time, with oxygenated monoterpenes predominating. It is possible that the more volatile compounds (i.e., monoterpene hydrocarbons) escaped from plates, or then were diluted in water. Thus, chemical compounds that volatilized in plates differed from the ones found in a typical essential oil characterization and from compounds that in fact volatilized from leaf litter. This may be one of the reasons why in many cases phytotoxicity observed in laboratory experiments does not imply in relevant allelopathic effects in the field, as we observed in our previous study in *E. saligna* plantations (Silva et al., submitted). Chemical differences also imply that suppressive effects observed for an essential oil may not be the same when leaves are used as mulch.

We observed that the essential oil was more phytotoxic than its major compounds by volatilization. α-Pinene was less phytotoxic than eucalyptol, which was also observed in other studies that used this method (Muller and Muller 1964; del Moral and Muller 1970; Nishida et al. 2005). Same level of phytotoxicity was observed for another *Eucalyptus* species with a similar chemical composition on *A. viridis*, with the oil showing stronger effects, followed by eucalyptol and α-pinene (Kaur et al. 2011). Eucalyptol was almost as phytotoxic as *E. saligna* essential oil, and this was the major compound volatilizing in plates during the experiment (about 60%). In general, the major components reflect the biological features of the essential oils from which they were isolated (Bakkali et al. 2008). However, the activity of the main compounds may be modulated by other minor molecules that together define cell penetration, lipophilic or hydrophilic attraction and fixation on cell walls and membranes, and cellular distribution (Bakkali et al. 2008). Therefore, eucalyptol should be responsible for

great part of the essential oil phytotoxicity by the volatilization method, with other compounds acting additively or synergistically.

The essential oil diluted in aqueous solution showed variable phytotoxicity on recipient species. Effects of *E. saligna* essential oils obtained in different years on *T. aestivum* coleoptile were comparable, reflecting their similar chemical composition. The essential oil was phytotoxic in low concentrations on *L. sativa,* a species recognized by its sensitiveness to allelochemicals. Strong effects were similarly observed on the weed *A. viridis*, which was also sensitive to *E. citridora* essential oil (Batish et al. 2004; Setia et al. 2007). *Eucalyptus saligna* essential oil was even more effective on this weed than the commercial herbicide. In relation to the Poaceae species, the essential oil negatively affected *E. plana* shoot growth, but only at higher concentrations than *A. viridis* and *L. sativa*, whereas caused no effects on the desirable species *P. notatum*. Therefore, effects of the oil were stronger on the eudicotyledons than on the monocotyledons tested herein. Nevertheless, we would need to have assessed effects on a higher number of species and families in order to affirm that the specificity is indeed due to plant group. Studies about *Eucalyptus* phytotoxicity have not evaluated differential effects between monocots and eudicots, or between plant families, and in few cases that species of both groups were tested, effects were not specific (Batish et al. 2004, 2006). In addition, our previous study that used the volatilization method revealed similar effects of *E. saligna* oil on both Poaceae and Fabaceae species (Silva et al. 2017), indicating that specific effects only occur when the oil is diluted in water. Hence, we highlight that phytotoxic effects of an essential oil or its compounds can not be generalized to any species or plant groups, and that effects may also depend on the employed methodology.

Phytotoxic effects of major compounds of the essential oil diluted in aqueous solution differed from effects by volatilization and also between recipient species. The major compounds caused no effects on *E. plana*, the same species that by volatilization was affected for all compounds, mainly eucalyptol. We even exposed *E. plana* to higher concentrations of the treatments (2 and 3 mg/mL), and while increased effects of the oil were observed, no phytotoxicity was detected for the major compounds (data not shown). Regarding *A. viridis*, all compounds were phytotoxic, but stronger effects were observed for the essential oil, followed by α-pinene. Essential oil fractions also revealed contradictory results; hence, we were not able to totally elucidate which compounds or set of compounds were responsible for *E. saligna* phytotoxicity. All fractions were as phytotoxic as *E. saligna* oil on *A. viridis* at the highest tested concentrations. At lower concentrations, only fraction 6 showed effects comparable to the oil, although major compounds in this fraction (α-terpineol and endo-borneol) are not the main compounds of *E. saligna* essential oil. All fractions strongly inhibited *E. plana*, especially fraction 6. The least phytotoxic fractions on *E. plana*

were the ones that had eucalyptol, or eucalyptol and some sesquiterpene hydrocarbons as major compounds (F1 and F2). Thus, *A. viridis* is affected by practically any combination of mono and/or sesquiterpenes diluted in water, whereas a more specific combination of compounds is required for inhibiting *E. plana*. The essential oil may present at the same time compounds that act synergistically on *E. plana*, since major compounds alone were not phytotoxic, but also compounds that act antagonistically, because fractions that lack some essential oil compounds showed stronger effects than the oil. The specific amount of each compound, and not only its identity, may also play an important role in effects of fractions, as reported by (Zhang et al. 2012). In that study, the least active *Eucalyptus* essential oils were the ones that presented moderate amounts of eucalyptol, and small variations in amounts of other compounds. In fact, *E. saligna* essential oils obtained in different years showed a slight difference in phytotoxicity level, reflecting very small qualitative and quantitative differences in their components. Thus, a careful quality control in chemical composition may be required for applied use of *E. saligna,* as well as other essential oils.

Our results indicate that *E. saligna* leaf litter essential oil could be potentially considered for controlling *A. viridis* in crop plantations, and *E. plana* in pastures. Application of the essential oil as a natural herbicide seems even more promising for *A. viridis*, because the essential oil was more phytotoxic in its pure form, and at low concentrations. Moreover, the oil caused substantially stronger effects on this weed than on Poaceae species - effects on germination and growth of *A. viridis* were detected at a concentration 12 times lower than a concentration that only affected shoot growth of *E. plana* and that caused no effects on another Poaceae species. This specificity indicates a potential for controlling *A. viridis* in plantations of some Poaceae crop (e.g. maize). In the case of *E. plana*, we evidenced a potential for using the essential oil to control this weed that invades South Brazilian grasslands, where *P. notatum* – the species that was not affected by the oil - is one of the most common native species. However, due to the diversity found in native pastures, effects of the essential oil should be still tested in a higher number of grassland species. In addition, strong inhibition of *E. plana* germination and growth were only observed for fractions of *E. saligna* oil, or by the oil at high concentrations. Nevertheless, the essential oil fractions can not be obtained by the same method used herein in a commercial scale. Therefore, further studies should evaluate different combinations of commercialized terpenes present in high amounts in the most active fractions, in order to determine if a set of compounds can be effective and specific on *E. plana*.

In this study, we observed species-specific phytotoxic effects of *E. saligna* oil, when one of the main limitations for applying essential oils as herbicides is that they are often non-selective (Dayan and Duke 2010). Moreover, a large amount of plant material is needed

for essential oil production, which can be restrictive in case of wild species, or high-cost plantations. In this sense, essential oils of many *Eucalyptus* species have been commercialized worldwide, which may not be difficult for *E. saligna* that has been largely planted for pulpwood production. However, effectiveness of *E. saligna* essential oil still needs to be tested in more realistic conditions, such as applying the essential oil on species planted in soil, and which are in more developed stages. Also, evaluating the efficacy of the essential oil in the field is required, as conducted in studies with *E. citridora* (Batish et al. 2004, 2007). In those studies, the essential oil was diluted in water and in a surfactant and sprayed on weeds, which were successfully controlled in greenhouse experiments and in field conditions. Even mortality was observed at some concentrations of *E. citridora* oil, which in many cases is not achieved with essential oils applications, resulting in regrowth of weeds (Dayan and Duke 2010). Essential oils of many species have been commercialized as herbicides (Dayan et al. 2009; Soltys et al. 2013); *E. saligna*, as well as other *Eucalyptus* species, should be viewed as potential new products for use in weed management.

5 Conclusions

In this study, we showed the phytotoxic effects of *E. saligna* leaf litter essential oil. We detected different levels of phytotoxicity between species, which indicates a potential use of the oil for controlling *A. viridis* in crops of Poaceae species, and *E. plana* in pastures. We recommend that future studies evaluate effects of *E. saligna* essential oil on these weeds in field conditions, in order to determine the actual potential of the oil as a natural herbicide. In addition, we observed that phytotoxicity of *E. saligna* essential oil is not exclusively related to its major compounds. However, effects of the essential oil and of its components varied considerably between species and employed method. This may be related to species-specific additive, synergistic and antagonistic effects of compounds, reflected by quantitative and qualitative changes in chemical composition. Therefore, the bioactivity of an essential oil or its components should not be generalized in cases that effects are tested with only one method and on one or few recipient species.

Acknowledgments

The authors thank Coordenação de Aperfeiçoamento de Nível Superior (CAPES) for the sandwich PhD scholarship granted to ER Silva. The authors also thank Instituto de Investigación en Biomoléculas (INBIO) at Universidad de Cádiz for equipment's use and staff's support in the experiments, and thank Laboratório de Extração de Óleos Essenciais at Universidade de Caxias do Sul and Matias Kohler for essential oil extraction.

References

Adams RP (2007) Identification of essential oil components by gas chromatography/ mass spectroscopy. 4 ed. Allured Publishing Corporation, Carol Stream

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32–46

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils - A review. Food Chem Toxicol 46:446–475

Batish DR, Setia N, Singh HP, Kohli RK (2004) Phytotoxicity of lemon-scented eucalypt oil and its potential use as a bioherbicide. Crop Prot 23:1209–1214

Batish DR, Singh HP, Kohli RK, Kaur S (2008) *Eucalyptus* essential oil as a natural pesticide. For Ecol Manage 256:2166–2174

Batish DR, Singh HP, Setia N, et al (2006) Chemical composition and phytotoxicity of volatile essential oil from intact and fallen leaves of *Eucalyptus citriodora*. Z Naturforsch C 61:465–471

Batish DR, Singh HP, Setia N, et al (2007) Alternative control of littleseed canary grass using eucalypt oil. Agron Sustain Dev 27:171–177

Barbosa FG, Pillar VD, Palmer A, Melo AS (2013) Predicting the current distribution and potential spread of the exotic grass *Eragrostis plana* Nees in South America and identifying a bioclimatic niche shift during invasion. Austral Ecology 38:260–267

Bett PK, Deng AL, Ogendo JO, et al (2016) Chemical composition of *Cupressus lusitanica* and *Eucalyptus saligna* leaf essential oils and bioactivity against major insect pests of stored food grains. Ind Crops Prod 82:51–62

Cornwell CP, Reddy N, Leach DN, Grant Wyllie S (2000) Hydrolysis of hedycaryol: The origin of the eudesmols in the Myrtaceae. Flavour Fragr J 15:421–431

Dayan FE, Cantrell CL, Duke SO (2009) Natural products in crop protection. Bioorganic Med Chem 17:4022–4034

Dayan FE, Duke SO (2010) Natural products for weed management in organic farming in the USA. Outlooks Pest Manag 21:156–160

del Moral R, Muller CH (1970) The allelopathic effects of *Eucalyptus camaldulensis*. Am Midl Nat 83:254–282

Ghnaya A Ben, Hanana M, Amri I, et al (2013) Chemical composition of *Eucalyptus erythrocorys* essential oils and evaluation of their herbicidal and antifungal activities. J Pest Sci 86:571–577

Gilden RC, Huffling K, Sattler B (2010) Pesticides and health risks. J Obstet Gynecol Neonatal Nurs 39:103–110

Heckel DG (2012) Insecticide resistance after Silent Spring. Science 337:1612–1614

Isman MB (2000) Plant essential oils for pest and disease management. Crop Prot 19:603–608

Isman MB, Machial CM (2006) Pesticides based on plant essential oils: from traditional practice to commercialization. In: Rai M, Carpinella MC (eds) Advances in Phytomedicine 3: Naturally Occurring Bioactive Compounds. Elsevier, pp. 29-44

Kaur S, Singh HP, Batish DR, Kohli RK (2011) Chemical characterization and allelopathic potential of volatile oil of *Eucalyptus tereticornis* against *Amaranthus viridis*. J Plant Interact 6:297–302

Kumar P, Mishra S, Malik A, Satya S (2012) Compositional analysis and insecticidal activity of *Eucalyptus globulus* (family: Myrtaceae) essential oil against housefly (*Musca domestica*). Acta Trop 122:212–218

Macías FA, Castellano D, Molinillo JMG (2000) Search for a standard phytotoxic bioassay for allelochemicals. Selection of standard target species. J Agric Food Chem 48:2512–2521

Mossi AJ, Astolfi V, Kubiak G, et al (2011) Insecticidal and repellency activity of essential oil of *Eucalyptus* sp. against *Sitophilus zeamais* Motschulsky (Coleoptera, Curculionidae). J Sci Food Agric 91:273–277

Muller WH, Muller CH (1964) Volatile growth inhibitors produced by *Salvia* species. Bull Torrey Bot Club 91:327–330

Nishida N, Tamotsu S, Nagata N, et al (2005) Allelopathic effects of volatile monoterpenoids produced by *Salvia leucophylla*: inhibition of cell proliferation and DNA synthesis in the root apical meristem of *Brassica campestris* seedlings. J Chem Ecol 31:1187–1203

Ootani MA, dos Reis MR, Cangussu ASR, et al (2017) Phytotoxic effects of essential oils in controlling weed species *Digitaria horizontalis* and *Cenchrus echinatus*. Biocatal Agric Biotechnol 12:59–65

Overbeck GE, Muller SC, Fidelis A, et al (2007) Brazil's neglected biome: The South Brazilian Campos. Perspect Plant Ecol Evol Syst 9:101–116

Petersen J, Belz R, Walker F, Hurle K (2001) Weed suppression by release of isothiocyanates from turnip-rape mulch. Agron J 93:37–43

Pillar VP, Orlóci L (1996) On randomization testing in vegetation science: multifactor comparisons of relevé groups. J Veg Sci 7:585–592

Savelev S, Okello E, Perry NSL, et al (2003) Synergistic and antagonistic interactions of anticholinesterase terpenoids in *Salvia lavandulaefolia* essential oil. Pharmacol Biochem Behav 75:661–668

Setia N, Batish DR, Singh HP, Kohli RK (2007) Phytotoxicity of volatile oil from *Eucalyptus citriodora* against some weedy species. J Environ Biol 28:63–66

Silva ER, Lazarotto DC, Schwambach J, et al (2017) Phytotoxic effects of extract and essential oil of *Eucalyptus saligna* (Myrtaceae) leaf litter on grassland species. Aust J Bot 65:172–182

Soltys D, Krasuska U, Bogatek R, Gniazdowska A (2013) Allelochemicals as bioherbicides - present and perspectives. In: Price AJ, Kelton JA (eds) Herbicides - current research and case studies in use. InTech, Croatia, pp 517–542

Stokstad E (2013) Pesticide Planet. Science 341:730–731

Weber J, Halsall CJ, Muir D, et al (2010) Endosulfan, a global pesticide: A review of its fate in the environment and occurrence in the Arctic. Sci Total Environ 408:2966–2984

Weidenhamer JD, Macías FA, Fischer NH, Williamson GB (1993) Just how insoluble are monoterpenes? J Chem Ecol 19:1799–1807

Willer H, Lernoud J (2017) The world of organic agriculture: Statistics and emerging trends 2017. Available at: https://shop.fibl.org/CHen/mwdownloads/download/link/id/785/?ref=1

Zhang J, An M, Wu H, et al (2010) Chemistry and bioactivity of *Eucalyptus* essential oils. Allelopath J 25:313–330

Zhang J, An M, Wu H, et al (2012) Chemical composition of essential oils of four *Eucalyptus* species and their phytotoxicity on silverleaf nightshade (*Solanum elaeagnifolium* Cav.) in Australia. Plant Growth Regul 68:231–237

Supplementary Table 1. Chemical composition of *Eucalyptus saligna* essential oil obtained from leaf litter collected in a previous year (2014), analysed by GC-MS.

Compound	LTPRI^{lit}	LTPRI	Area (%)	Group
α-Pinene	932	934	29.39	MH
Camphene	946	946	3.36	MH
β-Pinene	974	972	0.35	MH
α-Phellandrene	1002	1001	0.11	MH
n.i.		1013	0.12	
p-Cymenene	1020	1023	3.39	MH
1,8-Cineole (eucalyptol)	1026	1031	30.44	OM
cis-β-Ocimene	1032	1037	0.01	MH
γ-Terpinene	1054	1056	0.12	MH
n.i.		1068	0.19	
Terpinolene	1086	1082	0.67	MH
n.i.		1086	0.68	
Linalool*	1095	1099	0.32	OM
n.i.*		1103	0.09	
Isopentyl isovalerate	1102	1106	0.10	OM
trans-Thujone	1112	1113	0.03	OM
exo-Fenchol	1118	1115	1.69	OM
α-Campholenal	1122	1123	3.13	OM
trans-Pinocarveol	1135	1136	2.57	OM
Camphor	1141	1140	0.28	OM
Nerol oxide	1154	1150	0.02	OM
trans-Pinocamphone	1158	1155	0.42	OM
Pinocarvone	1160	1157	1.07	OM
endo-Borneol	1165	1169	4.16	OM
Terpinen-4-ol	1174	1179	0.15	OM
trans-Isocarveol	1187	1189	0.07	OM
α-Terpineol	1186	1193	3.89	OM
n.i.		1214	0.10	
trans-Carveol	1215	1218	0.06	OM
Isobornyl formate	1235	1223	0.24	OM
n.i.		1234	0.11	
n.i.		1235	0.20	
Carvone	1239	1239	0.06	OM
Geranial	1264	1265	0.31	OM
trans-Pinocarvyl acetate	1298	1291	0.04	OM
Thymol	1289	1302	0.02	OM
Geranyl acetate*	1379	1377	0.01	OM
α-Cubebene	1345	1343	0.08	SH
Isoledene	1374	1367	0.09	SH
α-Copaene	1374	1371	0.63	SH
β-Bourbonene	1387	1379	0.09	SH
β-elemene	1389	1385	0.06	SH

Compound	LTPRI^{lit}	LTPRI	Area (%)	Group
cis-Jasmone	1392	-	-	NI
n.i.		1388	0.04	
α-Gurjunene	1409	1402	0.35	SH
trans-Caryophyllene	1408	1414	0.76	SH
β-copaene	1430	1425	0.04	SH
Aromadendrene	1439	1433	0.28	SH
n.i.		1445	0.34	
α-Humulene	1452	1450	0.36	SH
Alloaromadendrene	1458	1455	1.26	SH
γ-Gurjunene	1475	1467	0.15	SH
α-Curcumene	1479	-	-	SH
n.i.		1470	0.13	
n.i.		1483	0.36	
Viridiflorene	1496	1486	0.45	SH
n.i.		1490	0.34	
α-Muurolene	1500	1494	0.33	SH
δ-Cadinene	1522	1514	0.54	SH
trans-Calamenene	1521	1517	0.97	SH
n.i.		1530	0.06	
α-Calacorene	1544	1536	0.12	SH
n.i.		1544	0.09	
n.i.		1566	0.29	
Spathulenol	1577	1573	1.68	OS
Caryophyllene oxide	1582	1576	0.58	OS
Globulol	1590	1586	0.37	OS
Ledol	1602	1594	0.92	OS
1-epi-Cubenol	1627	1625	0.12	OS
Cubenol	1645	1637	0.07	OS
Cadalene	1675	1666	0.08	SH
Total per group:				
Monoterpene hydrocarbons			37.40	
Oxygenated monoterpenes			49.09	
Sesquiterpene hydrocarbons			6.65	
Oxygenated sesquiterpenes			3.74	
Not identified			3.12	

LTPRI^{lit}: Linear Temperature Programmed Retention Indexes, tabulated; LTPRI: Linear Temperature Programmed Retention Indexes, calculated; area %: relative percentage of each component, which was directly obtained from chromatographic peak areas, considering that the sum of all eluted peaks was 100%; n.i.: not identified; *not found in the essential oil sample of 2015 (Table 1); -: only found in the sample of 2015. Compounds were grouped by class: Monoterpene hydrocarbon (MH); Oxygenated monoterpene (OM); Sesquiterpene hydrocarbon (SH); Oxygenated sesquiterpene (OS).

Supplementary Table 2. Chemical composition of volatiles emitted from *Eucalyptus saligna* essential oil along the period that lasted the phytotoxicity experiment by volatilization. Volatiles were obtained in closed plates by SPME and analysed by GC-MS. Data are expressed as the relative percentage of each component, which was directly obtained from chromatographic peak areas, considering that the sum of all eluted peaks was 100%.

Compound	0h	24h	48h	72h	96h	120h	144h	168h	Group
α-Pinene	49.78	31.26	3.68	5.49	9.69	7.57	4.17	2.76	MH
Camphene	3.06	1.66	0.22	0.31	0.53	0.38	0.25	0.17	MH
β-Pinene	0.39	0.31	0.06	0.07	0.09	0.07	0.08	0.04	MH
α-Phellandrene	0.11	0.12	0.03	0.04	0.04	<0.01	<0.01	<0.01	MH
p-Cymenene	0.69	0.51	0.63	1.81	1.31	0.30	0.79	0.72	MH
1,8-Cineole (eucalyptol)	41.18	51.36	68.28	58.49	68.94	61.10	65.10	60.87	OM
γ-Terpinene	0.13	0.12	0.05	0.09	0.06	0.05	0.04	0.02	MH
n.i.	0.18	0.36	0.60	0.61	0.52	0.54	0.57	0.54	
Terpinolene	0.60	0.55	0.42	0.46	0.38	0.23	0.20	0.17	MH
n.i.	0.46	0.55	0.60	0.69	0.66	0.50	0.50	0.41	
Isopentyl isovalerate	0.08	0.08	0.13	0.19	0.17	0.18	0.14	0.15	OM
trans-Thujone	0.02	0.02	<0.01	0.02	0.02	0.02	0.01	0.02	OM
exo-Fenchol	0.19	1.22	2.47	1.19	1.58	2.81	3.20	3.54	OM
α-Campholenal	1.31	2.22	2.86	3.37	2.57	2.16	1.96	1.90	OM
trans-Pinocarveol	0.39	2.84	6.13	6.82	4.18	6.84	7.07	8.20	OM
Camphor	0.03	0.06	0.14	0.62	0.11	0.15	0.16	0.20	OM
Nerol oxide	0.03	0.12*	0.17*	0.31*	0.18*	0.21*	0.18*	0.31*	OM
trans-Pinocamphone	0.06	0.17	0.11	0.31	0.16	0.17	0.23	0.26	OM
Pinocarvone	0.53	1.32	2.52	2.58	1.88	2.72	2.43	2.83	OM
endo-Borneol	0.08	1.84	3.91	3.70	1.77	5.19	4.81	5.95	OM
Terpinen-4-ol	0.02	0.11	0.17	0.39	0.18	0.28	0.17	0.24	OM
n.i.	0.01	0.04	0.05	0.10	0.06	0.12	0.15	0.26	
α-Terpineol	0.17	1.87	4.47	7.59	3.31	5.38	5.24	6.34	OM
n.i.	0.03	0.06	0.10	0.18	0.09	0.17	0.10	0.15	
Isobornyl formate	0.06	0.14	0.24	0.48	0.22	0.34	0.24	0.33	OM
n.i.	0.06	0.11	0.17	0.32	0.16	0.27	0.21	0.33	
Carvone	0.01	0.04	0.06	0.14	0.05	0.07	0.07	0.09	OM
trans-Pinocarvyl acetate	0.01	0.02	0.04	0.09	0.03	0.06	0.05	0.08	OM
α-Cubebene	<0.01	0.01	0.01	0.05	0.02	0.03	0.03	0.04	SH
Isoledene	<0.01	0.01	0.01	0.03	0.01	0.02	0.02	0.03	SH
α-Copaene	0.03	0.09	0.14	0.28	0.10	0.20	0.19	0.31	SH
β-Bourbonene	0.01	0.02	0.02	0.05	0.02	0.03	0.03	0.05	SH
β-elemene	<0.01	0.01	0.01	0.07	0.02	0.01	0.01	0.02	SH
α-Gurjunene	0.01	0.03	0.05	0.09	0.03	0.06	0.06	0.09	SH
trans-Caryophyllene	0.04	0.12	0.18	0.33	0.12	0.24	0.22	0.38	SH
β-copaene	<0.01	<0.01	<0.01	0.01	<0.01	0.01	0.01	0.01	SH
Aromadendrene	0.02	0.03	0.05	0.10	0.03	0.06	0.06	0.11	SH
n.i.	0.01	0.04	0.06	0.13	0.04	0.08	0.07	0.11	

Compound	0h	24h	48h	72h	96h	120h	144h	168h	Group
α-Humulene	0.02	0.05	0.07	0.14	0.05	0.09	0.08	0.14	SH
Alloaromadendrene	0.06	0.16	0.24	0.46	0.15	0.32	0.29	0.51	SH
n.i.	<0.01	0.04	0.08	0.14	0.04	0.08	0.08	0.14	
Viridiflorene	0.01	0.04	0.06	0.09	0.02	0.04	0.04	0.07	SH
n.i.	0.01	0.02	0.03	0.05	0.02	0.03	0.03	0.04	
α-Muurolene	0.01	0.01	0.03	0.05	0.01	0.03	0.02	0.04	SH
δ-Cadinene	0.02	0.03	0.06	0.11	0.02	0.04	0.04	0.07	SH
trans-Calamenene	0.03	0.08	0.14	0.25	0.05	0.13	0.11	0.17	SH
n.i.	<0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	
α-Calacorene	0.01	0.01	0.03	0.02	0.01	0.03	0.02	0.04	SH
n.i.	<0.01	0.01	0.04	0.07	0.02	0.04	0.03	0.05	
Spathulenol	0.02	0.05	0.24	0.62	0.17	0.37	0.25	0.40	OS
Globulol	<0.01	0.01	0.03	0.12	0.04	0.07	0.05	0.08	OS
Ledol	0.01	0.02	0.07	0.18	0.04	0.11	0.07	0.11	OS
1-epi-Cubenol	<0.01	0.01	0.02	0.06	0.03	0.04	0.03	0.07	OS
Total per group:									
MH	54.78	34.53	5.09	8.27	12.10	8.60	5.53	3.90	
OM	44.16	63.42	91.70	86.29	85.34	87.66	91.06	91.30	
SH	0.26	0.72	1.09	2.13	0.66	1.31	1.24	2.08	
OS	0.04	0.09	0.38	1.00	0.28	0.60	0.42	0.67	
Not identified	0.77	1.24	1.75	2.31	1.62	1.82	1.75	2.04	

*not identified in the sample. Compounds were grouped by class: Monoterpene hydrocarbon (MH); Oxygenated monoterpene (OM); Sesquiterpene hydrocarbon (SH); Oxygenated sesquiterpene (OS).

3 CONSIDERAÇÕES FINAIS

Os resultados desta tese de doutorado indicam que a alelopatia possui um potencial maior como alternativa no manejo de plantas daninhas do que como um fator determinante no estabelecimento e desenvolvimento da vegetação campestre. No primeiro capítulo, a revisão sistemática revelou que a ocorrência de alelopatia na natureza tem sido evidenciada para poucas espécies campestres. Isso se deve principalmente ao fato de muitos estudos serem realizados em condições pouco realistas, tais como certos procedimentos de extração, a falta do uso de solo como substrato e a escassez de avaliações em campo. Apesar de muitas recomendações na literatura, esse cenário não tem melhorado consideravelmente nos últimos anos. Em muitos casos, não há um objetivo claro nos estudos de alelopatia, os quais incluem alguns aspectos metodológicos que seriam mais adequados para a abordagem ecológica e outros para a abordagem aplicada ao manejo de plantas daninhas. Isso dificulta a evolução do conhecimento em pelo menos uma das direções. Por outro lado, novos métodos e ferramentas analíticas surgiram recentemente, além de uma nova abordagem, considerando a alelopatia na restauração de ecossistemas naturais. Contudo, essa aplicação depende da ciência básica, do conhecimento sobre uma espécie ser de fato alelopática.

Os herbicidas naturais já são uma realidade, embora sua aplicação em uma escala ampla ainda seja limitada, especialmente pelo custo elevado e pelos efeitos durarem pouco tempo. O uso potencial de muitos produtos naturais como novos bioherbicidas é restrito também pelo mesmo problema dos estudos ecológicos: a falta de avaliações em campo. Acredito que isso resulte principalmente do fato que as investigações em laboratório, em geral, apresentam menor custo e a sua execução costuma ser mais rápida. Em laboratório, dificilmente as hipóteses não são confirmadas, enquanto a campo, um longo tempo de estudo e de investimento pode não levar a respostas conclusivas ou à viabilidade de aplicação. Entretanto, é preciso arriscar e investir mais na pesquisa sobre alelopatia para que essa área se mova a passos mais largos.

O segundo capítulo da tese compreendeu um estudo de fitotoxidez feito em laboratório, o qual pode servir de base tanto para pesquisas ecológicas quanto aplicadas. Nesse trabalho, os efeitos fitotóxicos do óleo essencial e do extrato aquoso das folhas da serapilheira de *E. saligna* foram evidenciados sobre a germinação e o crescimento de plântulas. No entanto, para servir como uma base mais sólida para o terceiro capítulo, esse estudo deveria ter sido feito em condições mais realistas, incluindo solo, e não utilizando extrato e óleo essencial. Uma indicação de que esses procedimentos de extração não foram

representativos do que ocorre em campo é o fato de que no quarto capítulo foi observado que a composição química dos voláteis emitidos das folhas da serapilheira difere da do óleo essencial. Talvez, os ensaios poderiam ter empregado percolação, na qual a lixiviação seria mais bem simulada, e voláteis emitidos diretamente de folhas. Dessa forma, recomendo que para estudos ecológicos já se incluam procedimentos mais realistas desde os primeiros bioensaios. Para pesquisas aplicadas ao manejo de plantas daninhas, experimentos como os conduzidos no segundo capítulo são adequados, pois se busca maximizar a obtenção de substâncias bioativas. Além disso, nesse trabalho a fitotoxidez foi relacionada primariamente a estresse oxidativo, o qual levou a danos nas membranas. Com isso, foi dado um passo inicial para a compreensão de qual seria o possível modo de ação dos aleloquímicos de *E. saligna*, o que é fundamental para o desenvolvimento de bioherbicidas.

No terceiro capítulo, foi evidenciado que é muito difícil para espécies campestres se estabelecerem sob a serapilheira de *E. saligna*. Entretanto, os resultados indicaram que os efeitos da serapilheira são principalmente físicos, como observado também para *Casuarina equisetifolia*, invasora de restingas no Brasil (Zimmermann et al. 2017). Além disso, o sombreamento das árvores provavelmente consistiu em outro fator limitante no estabelecimento da vegetação. Não foi possível ter certeza que não houve a ação de aleloquímicos de *E. saligna*, mas se houve, os efeitos foram muito fracos para serem detectados. Em minha dissertação de mestrado, o mesmo foi observado para *B. psiadioides* (Silva et al. 2015). Nesses casos, foi possível chegar a conclusões em pouco tempo porque não foram encontradas evidências de alelopatia. Porém, se fosse o contrário, muito mais tempo (e investimento) seria necessário, como observado para alguns casos em que ocorrência desse fenômeno foi bem documentada. Um exemplo é o caso dos arbustos aromáticos *Artemisia californica* e *Salvia* spp. no Chaparral da Califórnia, o qual compreendeu muitos anos de estudo (Muller et al. 1964, Muller & Del Moral 1971, Halligan 1973). Esses trabalhos incluíram experimentos nas comunidades em longo prazo, avaliando todas as possíveis explicações alternativas à alelopatia, como a influência de animais e de competição. Mesmo assim, a relevância da alelopatia nesse sistema ainda é questionada. Outro caso é o de *Centaurea stoebe*, invasora em campos na América do Norte, o qual requereu esforço de vários pesquisadores ao longo de quase uma década para que a relevância da alelopatia fosse conclusivamente demonstrada (Bais et al. 2003, Inderjit et al. 2008, Thorpe et al. 2009, May & Baldwin 2011).

É importante destacar que os estudos que evidenciaram efeitos alelopáticos até agora foram realizados no hemisfério norte, em ecossistemas que em geral apresentavam uma riqueza menor de espécies do que, por exemplo, o Pampa. Em alguns casos, como o dos arbustos aromáticos, de *Pteridium aquilinum* (L.) Kuhn (Gilessman & Muller 1978) e de

Eucalyptus camaldulensis Dehnh. (Del Moral & Muller 1970), a baixa diversidade de plantas permitiu que as interações entre as principais espécies da comunidade pudessem ser testadas. Pode ser que a alelopatia atue com força maior sobre algumas espécies apenas, mas quantas e quais espécies seriam necessárias para de fato representar efeitos sobre a vegetação campestre? Além disso, a dificuldade em obter sementes de espécies nativas, como mencionado na seção de métodos, consiste em mais um empecilho. Como foi destacado na introdução, espécies do gênero *Eucalyptus* teriam grande chance de serem alelopáticas, comparado a outras espécies que ocorrem no Pampa, mas não foram detectados efeitos para *E. saligna*. Com isso, é questionável se em sistemas tão diversos como os campos (ou outros ecossistemas no Brasil), e com tantos fatores bióticos e abióticos agindo simultaneamente, será possível detectar a relevância da alelopatia para alguma espécie vegetal.

Os efeitos observados sobre o estabelecimento e desenvolvimento da vegetação campestre foram muito severos nos plantios, sendo que o estabelecimento foi baixo e levou muito tempo, devendo ter resultado principalmente do banco de sementes e não de rebrote. Nos últimos anos, grandes áreas de vegetação campestre têm sido substituídas por plantios de eucalipto o que, juntamente com outras mudanças no uso do solo na região, poderá levar a grandes perdas na diversidade de espécies vegetais. Visando a conservação da vegetação campestre, uma forma de mitigar esses efeitos negativos sobre a vegetação em monoculturas de eucalipto poderia ser realizar o plantio com um espaçamento maior entre as árvores. Isso resultaria em menor sombreamento das árvores e menor quantidade de folhas da serapilheira, implicando em maior estabelecimento das espécies campestres, como mencionado no capítulo III. Inevitavelmente, menor quantidade de árvores plantadas implicaria em menos lucro. Para contornar essa situação, uma opção economicamente rentável seria implantar um sistema silvipastoril, permitindo pastejo por gado nos plantios. Esse tipo de manejo tem sido bem-sucedido na região do Cerrado, em plantios de eucalipto onde o espaçamento entre as árvores é maior (10 m x 4 m, enquanto nas áreas de estudo se utiliza 3 m x 2 m) (Dube et al. 2002).

O quarto capítulo foi direcionado para a abordagem aplicada ao manejo de plantas daninhas. Nesse trabalho, análises químicas mais robustas foram incluídas e foi demonstrado que há variação na fitotoxidez do óleo essencial de *E. saligna* dependendo do método utilizado. O óleo essencial diluído em água - que seria a forma de aplicá-lo como herbicida - apresentou especificidade sobre as espécies receptoras, algo diferente do que havia sido observado pelo método de volatilização no segundo capítulo. Dessa forma, o método de volatilização de óleo essencial parece ser algo que não aproxima de nenhuma das abordagens em estudos de alelopatia. Como discutido anteriormente, investigações em

laboratório em condições controladas não são muito elucidativas para estudos ecológicos. Porém, para aplicação como herbicidas naturais, as pesquisas em laboratório podem revelar um potencial de fato (ex. Batish et al. 2007). Como os tecidos vegetais já passaram por extração, não deverá haver grandes mudanças em campo, a menos que a intenção seja no uso como pré-emergente (efeitos sobre germinação são mais complicados pelo fato de que os aleloquímicos podem se degradar rapidamente no solo). No caso de óleos essenciais, esses normalmente são aplicados como pós-emergentes sobre ervas daninhas, promovendo a remoção das ceras da cutícula e provocando a desidratação e morte dos tecidos (Dayan & Duke 2010). O que deve mudar a campo, principalmente, é a concentração necessária para afetar as plantas, bem como o tempo de duração do efeito, caso não cause a mortalidade dessas. Além disso, seria interessante investigar se o óleo essencial das folhas da copa apresenta composição química e fitotoxidez similar ao das folhas da serapilheira, uma vez que a comercialização do óleo essencial das folhas da copa seria mais viável. Embora ainda haja muitas coisas que poderiam ser elucidadas sobre o óleo essencial em laboratório, o passo seguinte no estudo sobre *E. saligna* já deveria ser testar sua efetividade como herbicida em campo.

O potencial bioherbicida de uma espécie que é exótica nos campos do sul do Brasil foi investigado, mas nessa região há uma diversidade muito grande de espécies nativas que podem apresentar substâncias com esse potencial. Embora certas substâncias bioativas possam ser sintetizadas, uma alternativa mais simples poderia ser a utilização de óleos essenciais ou extratos brutos. A maior dificuldade talvez seja a produção desses produtos, já que uma grande quantidade de material vegetal é necessária. Além disso, no quarto capítulo foi evidenciado que pequenas mudanças na composição química podem implicar em diferentes níveis de fitotoxidez sobre certas espécies. O controle de qualidade do óleo essencial de *E. saligna* não seria tão difícil, uma vez que os plantios consistem em clones cultivados em condições similares em áreas extensas. No entanto, para a maioria das espécies nativas campestres não há sequer a comercialização de sementes, e a coleta de plantas em diferentes ambientes poderia implicar em diferenças na composição química. Ainda assim, acredito que no Brasil a pesquisa sobre alelopatia na abordagem aplicada deverá trazer resultados mais promissores do que na abordagem ecológica.

Durante o desenvolvimento desta tese, foi possível perceber que o desenvolvimento da área de alelopatia não depende tanto do surgimento de novos métodos ou de recomendações sobre como os mesmos devem ser empregados. O maior desafio parece ser disseminar o conhecimento que já existe há muito tempo e que continua a ser gerado. Mas quais seriam as soluções? Certamente não é apenas a publicação de artigos de revisão em periódicos de alto impacto, pois como foi observado no capítulo I, isso não

implicou em mudanças consideráveis. Talvez seja mais construtivo divulgar as informações em um âmbito local, em cada país ou região. Um bom exemplo é o artigo “Allelopathy in Brazil” (Reigosa et al. 2013), publicado no periódico Acta Botanica Brasilica, o qual apresenta o estado da arte sobre a pesquisa de alelopatia no país e discute questões importantes. Esse artigo provavelmente foi e será encontrado pela maioria dos pesquisadores que trabalham ou que irão começar a trabalhar com alelopatia no Brasil. A disseminação de conhecimento pode também ser feita em cursos e eventos regionais ou nacionais, ou até mesmo na revisão cuidadosa de manuscritos em periódicos. Um passo importante foi dado recentemente quando criamos o Grupo de Alelopatia do Brasil (Alelo) em uma rede social, com a finalidade de divulgar as pesquisas que estão sendo produzidas no país e de possibilitar colaborações e encontros.

Finalmente, a maior consideração que quero deixar nesta tese de doutorado está na conclusão do capítulo I: “Um maior conhecimento sobre alelopatia e os seus mecanismos, em campos e em outros sistemas, levará a avanços não apenas na ciência, mas também em diferentes áreas aplicadas. (...) Eu espero e acredito que em um futuro próximo, o conhecimento sobre alelopatia seja utilizado mais fortemente para resolver questões aplicadas, de forma que a alelopatia desempenhe um papel importante na conservação da biodiversidade.”.

REFERÊNCIAS

Adams RP (2001) Identification of essential oil components by gas chromatography/ quadrupole mass spectroscopy. 2 ed. Allured Publishing Corporation, Carol Stream

Adams RP (2007) Identification of essential oil components by gas chromatography/ mass spectroscopy. 4 ed. Allured Publishing Corporation, Carol Stream

Anand M, Orlóci L (1996) Complexity in plant communities: the notion and quantification. J Theor Biol 179:179–186

Anderson M (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32-46

Anjum T, Bajwa R (2005) Importance of germination indices in interpretation of allelochemical effects. Int J Agric Biol 7:417-419

Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM (2003) Allelopathy and exotic plant invasion: from molecules and genes to species interactions. Science 301:1377-1380

Barbosa FG, Pillar VD, Palmer A, Melo AS (2013) Predicting the current distribution and potential spread of the exotic grass *Eragrostis plana* Nees in South America and identifying a bioclimatic niche shift during invasion. Austral Ecol 38:260–267

Batish DR, Singh HP, Setia N, et al (2007) Alternative control of littleseed canary grass using eucalypt oil. Agron Sustain Dev 27:171–177

Bernhard-Reversat F, Main G, Holl K, Loumeto J, Ngao J (2003) Fast disappearance of the water-soluble phenolic fraction in eucalypt leaf litter during laboratory and field experiments. Appl Soil Ecol 23:273-278

Callaway RM, Ridenour WM (2004) Novel weapons: invasive success and the evolution of increased competitive ability. Front Ecol Environ 2:436–443

Chou CH, Young CC (1974) Effects of osmotic concentration and pH on plant growth. Taiwania 19:157-162

Chu C, Mortimer PE, Wang H, Wang Y, Liu X, Yu S (2014) Allelopathic effects of *Eucalyptus* on native and introduced tree species. For Ecol Manag 323:79–84

Dayan FE, Cantrell CL, Duke SO (2009) Natural products in crop protection. Bioorganic Med Chem 17:4022–4034

Dayan FE, Duke SO (2010) Natural products for weed management in organic farming in the USA. Outlooks Pest Manag 21:156–160

De Albuquerque MB, Santos RC, Lima LM et al (2011) Allelopathy, an alternative tool to improve cropping systems. A review. Agron Sustain Dev 31:379–395

Del Moral R, Muller CH (1970) The allelopathic effects of *Eucalyptus camaldulensis*. Am Midl Nat 83:254–282

Del Moral R, Willis RJ, Ashton DH (1978) Suppression of coastal heath vegetation by *Eucalyptus baxteri*. Aust J Bot 26:203–219

Dube F, Couto L, Silva ML et al (2002) A simulation model for evaluating technical and economic aspects of an industrial eucalyptus-based agroforestry system in Minas Gerais, Brazil. Agrofor Syst 55:73–80

Fontaneli RS, Santos HP, Fontaneli RS (2012) Forrageiras para integração lavoura-pecuária-floresta na região sul-brasileira. 2. ed. Embrapa, Brasília

Gliessman SR, Muller CH (1978) The allelopathic mechanisms of dominance in bracken (*Pteridium aquilinum*). J Chem Ecol 4:337–362

Gonçalves CEP (2014) Alelopatia de Carqueja (*Baccharis trimera* Less) e ação de fungos em capim-annoni (*Eragrostis plana* Ness). Dissertação de Mestrado em Agrobiologia, UFSM, 88 p.

Granéli E, Pavia H (2006) Allelopathy in marine ecosystems. In Reigosa MJ, Pedrol N, González L (eds) Allelopathy: a physiological process with ecological implications. Springer, Netherlands, pp 415–431

Halligan JP (1973) Bare areas associated with shrub stands in grassland: The case of *Artemisia californica*. BioScience 23:429–432

Harper JL (1994) Population Biology of Plants. Academic Press, London

He H, Song Q, Wang Y, Yu S (2014) Phytotoxic effects of volatile organic compounds in soil water taken from a *Eucalyptus urophylla* plantation. Plant Soil 377:203–215

IAS-International Allelopathy Society (1996) Constitution and Bylaw of IAS. IAS Newsletter, Cádiz-Spain

Inderjit, Callaway RM (2003) Experimental designs for the study of allelopathy. Plant Soil 256:1–11

Inderjit, Dakshini KMM (1995) On laboratory bioassays in allelopathy. Bot Rev 61:28–44

Inderjit, Nilsen ET (2003) Bioassays and field studies for allelopathy in terrestrial plants: progress and problems. Crit Rev Plant Sci 22:221–238

Inderjit, Pollock JL, Callaway RM, Holben W (2008) Phytotoxic effects of (+/-)-catechin in vitro, in soil, and in the field. PLoS One 3:e2536

Inderjit, Weiner J (2001) Plant allelochemical interference or soil chemical ecology? Perspect Plant Ecol Evol Syst 4:3–12

Inderjit, Weston LA (2000) Are laboratory bioassays for allelopathy suitable for prediction of field responses? J Chem Ecol 26:2111–2118

Isman MB (2000) Plant essential oils for pest and disease management. Crop Prot 19:603–608

Jatoba LJ, Varela RM, Molinillo JMG et al (2016) Allelopathy of bracken fern (*Pteridium arachnoideum*): new evidence from green fronds, litter, and soil. PLoS ONE 11:e0161670.

Jensen K, Gutekunst K (2003) Effects of litter on establishment of grassland plant species: the role of seed size and successional status. Basic Appl Ecol 4:579–587

Lau JA, Puliafico KP, Kopshever JA et al (2008) Inference of allelopathy is complicated by effects of activated carbon on plant growth. New Phytol 178:412–423.

Lazarotto DC, Pawlowski A, Silva ER, Schwambach J, Soares GLG (2014) Phytotoxic effects of *Heterothalamus psiadioides* (Asteraceae) essential oil on adventitious rooting. Acta Physiol Plant 36:3163–3171

Lisanework N, Michelsen A (1993) Allelopathy in agroforestry systems: the effects of leaf extracts of *Cupressus lusitanica* and three *Eucalyptus* spp. on four Ethiopian crops. Agrofor Syst 21:63–74

Londo G (1976) The decimal scale for releves of permanent quadrats. Vegetatio 33:61–64

Macías FA, Castellano D, Molinillo JMG (2000) Search for a standard phytotoxic bioassay for allelochemicals. Selection of standard target species. J Agric Food Chem 48:2512–2521

May L, Baldwin LK (2011) Linking field based studies with greenhouse experiments: the impact of *Centaurea stoebe* (=*C. maculosa*) in British Columbia grasslands. Biol Invasions 13:919–931

Muller CH, Muller WH, Haines BL (1964) Volatile growth inhibitors produced by aromatic shrubs. Science 143:471–473

Muller CH, Del Moral R (1971) Role of animals in suppression of herbs by shrubs. Science 173:462-463

Omoro LM, Pellikka PKE, Rogers PC (2010) Tree species diversity, richness, and similarity between exotic and indigenous forests in the cloud forests of Eastern Arc Mountains, Taita Hills, Kenya. J For Res 21:255–264

Ootani MA, Reis MR, Cangussu ASR, et al (2017) Phytotoxic effects of essential oils in controlling weed species *Digitaria horizontalis* and *Cenchrus echinatus*. Biocatal Agric Biotechnol 12:59–65

Overbeck GE, Muller SC, Fidelis A, et al. (2007) Brazil's neglected biome: The South Brazilian Campos. Perspect Plant Ecol Evol Syst 9:101–116

Padovan A, Keszei A, Külheim C, Foley WJ (2014) The evolution of foliar terpene diversity in Myrtaceae. Phytochem Rev 13:695-716

Parepa M, Bossdorf O (2016) Testing for allelopathy in invasive plants: it all depends on the substrate! Biol Invasions 18:2975–2982

Pillar VD (1999) The bootstrapped ordination re-examined. J Veg Sci 10:895–902

Pillar VD (2009) MULTIV, software for multivariate exploratory analysis, randomization testing and bootstrap resampling. Version Beta 2.6.8 Departamento de Ecologia, UFRGS, Porto Alegre, Brazil (URL: http://ecoqua.ecologia.ufrgs.br). Accessed 8 Oct 2013

Pillar VD (2013) How accurate and powerful are randomization tests in multivariate analysis of variance? Community Ecol 14:153-163

Pillar VD, Orlóci L (1996) On randomization testing in vegetation science: multifactor comparisons of relevé groups. J Veg Sci 7:585-592

Reigosa M, Gomes AS, Ferreira AG, Borghetti F (2013) Allelopathic research in Brazil. Acta Bot Brasilica 27:629–646

Rice EL (1984) Allelopathy. 2 ed. Academic Publishers, New York

Rotundo JL, Aguiar MR (2005) Litter effects on plant regeneration in arid lands: a complex balance between seed retention, seed longevity and soil-seed contact. J Ecol 93:829–838

Schneider AA (2007) A flora naturalizada no Estado do Rio Grande do Sul, Brasil: herbáceas subespontâneas. Biociências 15:257-268

Silva ER (2014). Efeito de duas espécies arbustivas sobre a comunidade vegetal: a alelopatia é um fator determinante? Dissertação de Mestrado em Ecologia, UFRGS, 57 p.

Silva ER, Overbeck GE, Soares GLG (2014) Phytotoxicity of volatiles from fresh and dry leaves of two Asteraceae shrubs: evaluation of seasonal effects. S Afr J Bot 93:14-18

Silva ER, Ferreira PMA, Overbeck GE, Soares GLG (2015) Does the phytotoxic shrub *Heterothalamus psiadioides* affect a plant community through allelopathy? Plant Ecol 216:87–97

Singleton VL, Rossi Jr JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enology Vitic 16:144-158

Soltys D, Krasuska U, Bogatek R, Gniazdowska A (2013) Allelochemicals as bioherbicides - present and perspectives. In: Price AJ, Kelton JA (eds) Herbicides - current research and case studies in use. InTech, Croatia, pp 517–542

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 11:1187-1194.

Thorpe AS, Thelen GC, Diaconu A, Callaway RM (2009) Root exudate is allelopathic in invaded community but not in native community: field evidence for the novel weapons hypothesis. J Ecol 97:641–645

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. Plant Sci 151:59-66

Wardle DA, Nicholson KS, Ahmed M (1992) Comparison of osmotic and allelopathic effects of grass leaf extracts on grass seed germination and radicle elongation. Plant Soil 140: 315-319

Waterman PG, Mole S (1994) Analysis of phenolic plant metabolites. 1 ed. Blacwell Scientific Publications, Oxford

Weston LA (2005) History and current trends in the use of allelopathy for weed management. HortTechnology 15:529-534

Worthington M, Reberg-Horton C (2013) Breeding cereal crops for enhanced weed suppression: optimizing allelopathy and competitive ability. J Chem Ecol 39:213–231

Zhang C, Fu S (2010) Allelopathic effects of leaf litter and live roots exudates of *Eucalyptus* species on crops. Allelopathy J 26:91–100

Zimmermann TG, Andrade ACS, Richardson DM (2017) Abiotic barriers limit tree invasion but do not hamper native shrub recruitment in invaded stands. Biol Invasions 19:109–129