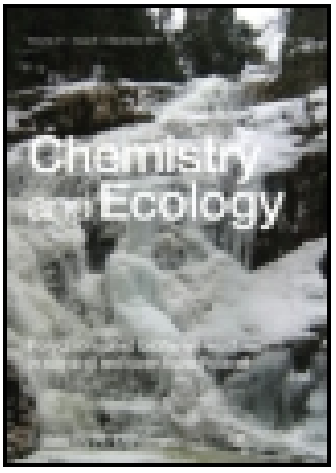

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Effects and identification of chemical compounds released from the invasive *Acacia dealbata* Link

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The invasion process of *Acacia dealbata* is partially mediated by releasing allelochemicals that affect native plants and soil microbes. However, non-volatile chemical compounds responsible for the allelopathic effect remain unknown, even though the allelopathic potential of this species has been studied under Europe conditions. We examined the allelopathic potential of aqueous extracts obtained from different plant materials of *A. dealbata*: litter from leaf, flowers, pods and seeds and fresh leaves and also litter extract's phytotoxicity evolution on germination and early seedling growth of *Lactuca sativa* L. Bioassays based on aqueous extracts and direct effect of plant materials showed that radicle length (RL), degree of root necrosis and germination percentage were the variables that gave the best prediction for assessing the inhibitory allelopathic effect. Chemical compounds present in all types of litter significantly reduced the hypocotyl and RL of *L. sativa*. GC–MS analysis reveals the presence predominantly of resorcinol, maculosin and moretenone in leaves; stigmasterol, D-alpha-tocopherol quinone, and lupanin in pods; and methyl *p*-anisate, *p*-anisyl alcohol, stigmasterol and anisal were identified in flowers. The inhibitory effect induced on *L. sativa* by the different plant parts supports the hypothesis that *A. dealbata* exerts its allelopathic potential throughout its phenological cycle.

Keywords: allelochemicals; allelopathy; biological invasions; phytotoxicity; secondary metabolites

1. Introduction

Several invasive plants have the capacity to spread rapidly. This fact has been well documented in different ecosystems worldwide.[1–3] Invasive species can produce alterations on plant composition of communities, reduce regeneration rates of native species and alter landscape structures, among other implications.[2,4,5] The genus *Acacia* (Fabaceae) is considered one of the most invasive taxa in the world, especially most of Australian species.[4,6] According to the global database of Australian acacia records, 386 species have been transferred outside Australia by humans, 71 species are naturalised and some are consider as weeds but 23 are unequivocally invasive.[7] The invasion success of *Acacia* species may be related to disturbance events and reproductive characteristics.[8] In fact, traits such as the ability to effectively reproduce in new locations are common to Australian acacias.[9] In addition, the status of *Acacia* invasions may

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be accelerated under the current global change scenario. Climatic models suggest that about a third of the world's land surface is climatically suitable for Australian acacias.[3]

Acacia dealbata Link (Subfamily: Mimosoideae) is native from Australia and it is considered as invasive species in Chile,[10] South Africa, Portugal, Spain, Italy and Madagascar.[3,8,11,12] *A. dealbata* was introduced for ornamental purposes in Chile and it is part of the 27 exotic Fabaceae species recognised in the country.[10,13] Currently, it is widely distributed and it is associated with riparian habitats, roads and human disturbance, covering about 100,000 hectares in the Biobío Region.[10] According to the novel weapons hypothesis,[14] the invasive success of *A. dealbata* in Europe is partially related to the release of allelopathic compounds that affects both plant species and soil microbes.[15–18] The allelopathic effect and competitive pressure exerted by this exotic in the new distribution range produce either inhibition or growth limitations of different 'new-neighbor' species, which in turn enhances the competitive capacity of *A. dealbata* against native species.[12,17,19–21]

Most of the compounds released by plants are considered secondary metabolites, synthesised as a result of secondary-metabolic pathways such as mevalonate, shikimate and malonate.[22] Secondary metabolites may have allelopathic effects depending on their phytotoxic action, bioactive concentration, persistence and fate in the environment in which they are released.[23] In nature, allelopathy can be a result of the joint action of several allelochemicals,[24] which can affect crucial physiological processes of native plants. Chemical compounds naturally released by *A. dealbata* have shown allelopathic effects on seed germination, seedling growth, net photosynthetic and respiration rates of agricultural and understorey plants and on functional diversity and structure of soil microbes in the invaded range.[15–18,25] However, in such studies the chemical compounds responsible for the biological activity were not identified. Many of these allelopathic studies use a model species; for example, *Lactuca sativa* L. (Asteraceae) due to rapid germination and allelopathic sensitivity.[15,26,27]

Most of the allelopathic studies of *A. dealbata* were conducted in Europe, while the allelopathic potential of *A. dealbata* in other invaded areas, such as South America, remains unknown. Our hypotheses to help explain the rapid invasion of *A. dealbata* are (a) natural concentrations of chemicals released by different plant materials of *A. dealbata* reduce germination and seedling growth of *L. sativa*, (b) direct contact with *A. dealbata* litter accentuates the phytotoxicity effect, (c) this inhibitory effect occurs throughout the phenological cycle of *A. dealbata* and (d) chemical compounds implicated in the phytotoxicity vary depending on the plant material.

This study aims (i) to evaluate the effect of aqueous extracts from *A. dealbata* using *L. sativa* as model species, (ii) to determine the phytochemical's release rate and variation in different plant parts and (iii) to identify the main chemical compounds present in different materials of *A. dealbata*. All these results are based on a study model and help to explain the successful invasion of this exotic species in its non-native range, especially in the Biobío region of Chile.

2. Materials and methods

2.1. *Study area and plant material*

Plant material sampling was conducted in the Quillón Commune, approximately 67 km north of the city of Concepción, in the Biobío Region of Chile (36°50′58.81″S; 72°32′4.91″W), characterised by a Mediterranean climate. The natural predominant vegetation in this area is a forest dominated by *Quillaja saponaria* Mol., *Lithraea caustica* (Mol.) Hook.et Arn. and *Peumus boldus* Mol. with a rich understorey of herbs and leguminous shrubs, which are being invaded by *A. dealbata*. Plant material was collected under *A. dealbata* canopy after falling naturally in 2013: pods litters and seeds were collected in January, meanwhile leaves and flowers (glomerulus

globular inflorescence) litters were collected in May and August, respectively. Additionally, fresh leaves were also collected directly from trees in May. Subsequently, all plant samples were stored in plastic bags under refrigeration conditions ($\sim$8°C) until its use for bioassays. The model specie for all bioassays was *L. sativa* and its seeds were purchased from the Agroflora local market.

2.2. *Effect of extracts concentration on the germination and early growth*

The plant material naturally fallen was quantified per square meter using 25 random quadrats of 0.24 m^2. Predominant plant material inside the quadrats was collected, weighed and used to calculate a fall biomass rate. Averages were approximately: pods and leaf litter 32 g/m^2, flowers 48 g/m^2 and 32 g of fresh leaves were collected. In the study region, annual average rainfall is 827 L/m^2 [28] and the average daily rainfall was estimated to be about 2 L/m^2. According to this and to mimic natural conditions, different stock solutions by each plant material were established by soaking (72 h at 10°C) the estimated fallen plant material in a water volume similar to the amount of fallen rain in one day, always by square meter. On these cases, the concentrations were 16 g/L for pods litter, leaf litter and fresh leaves, and 24 g/L for flowers litter. As well, from these initial solutions several secondary stocks solutions were established in order to mimic different rain intensities (100%, 75%, 50%, 25% and 0%), distilled water was used as control in all cases. The pH values ranged from 6.5 to 7 in stock solutions, an optimal value to the germination and seedling growth of *L. sativa*.[29] It was measured with a TESTO portable pHmeter PH-206 (Lenzkirch, Germany).

Bioassays were performed separately for each plant part. Different secondary stock solution concentrations were considered as treatments and were replicated seven times (seven Petri dishes). Thirty seeds of *L. sativa* were uniformly distributed in Petri dishes (9 cm diameter) lined with a Whatman No. 1 paper disc soaked with 3 mL of either aqueous solution or distilled water. The dishes were sealed with Parafilm® to prevent evaporation and randomly placed in a growth chamber at 70–75% relative humidity, 12 h light/dark ($\sim 80\ \mu mol\ m^{-2}\ s^{-1}$) and at 20°C. After 8 days, the germinated seeds percentage (GP) was determined. RL and hypocotyl length (HL) of each seedling were measured. Additionally, radicle necrosis degree (RN) was assessed and classified into five categories as shown in Table 1.

2.3. *Effect of extract's evolution on the germination and early growth*

With previously prepared solutions once again bioassays were performed separately for each plant part. Aliquots from each secondary stock solution were taken at different days after its preparation (1, 2, 4, 8 and 16), days were considered as treatments. Also, distilled water was used as control and each treatment was seven times replicated. The pH values for all aliquots and distilled water were between 6.5 and 7. Bioassay conditions and the same previous variables were also evaluated in this experiment.

Table 1. Radical necrosis degree classification.

Necrosis degree	Description
0	Radicle without discoloration and with abundant root hairs
1	Radicle light brown and reduction of root hairs up to 50% of their length
2	Radicle brown and 5–10% necrosis. No root hairs observed
3	Radicle dark brown and *ca*. 50% necrosis. No root hairs observed
4	Radicle dark brown and more than 75% necrosis. No root hairs observed

2.4. *Direct effect of plant materials on the germination and early growth*

Since bioassays were performed in Petri dishes (63.6 cm^2), different plant materials were collected in an equivalent area. Samplings of leaves, flowers, pods and seeds were collected as described in the first experiment. Litter averages of leaves, pods and seeds for a Petri dish were 2, 3 and 4.3 g, respectively. Flowers were not uniformly distributed under the canopy; they ranged from 50 flowers (1 g) to 200 flowers (3.3 g). For this reason, four treatments with flowers were established: 50 flowers (F50), 100 flowers (F100), 150 flowers (F150) and 200 flowers (F200). In addition, since the *A. dealbata* canopy was predominantly covered by pods and leaf from January to July, and by pods and seeds from December to April, combined plant material treatments (seeds + leaves and leaves + pods) at natural proportions were also established.

Each different plant litter or combination was placed in a Petri dish, covered with a Whatman No. 1 paper disc and then watered with 20 mL (leaves, pods, leaves + pods and seeds + leaves), 15 mL (F50, F100, F150 and F200) or 8 mL (seeds) of distilled water. The volume of water was chosen based on previous experiments to ensure the minimum amount of water to allow germination. Petri dishes without any plant material, but lined with a Whatman No. 1 paper disc soaked with 3 mL of distilled water, were used as controls. Thirty seeds were also used in Petri dish and each treatment was replicated seven times. Also, the same previous variables were also evaluated. Growth conditions and *L. sativa* measurements were the same as in the first experiment. The pH was measured directly from Petri dishes at the beginning and end of the experiment by pH-indicator strips pH 0–14 (Acilit® MERCK, Darmstadt, Germany), and values were similar to those of previous experiments.

2.5. *Evolution of phytotoxicity in the pods litter*

Among all of the plant material, pods were the most persistent litter underneath the *A. dealbata*'s canopy. Pods fell between November and December and accumulated in the soil until next August. Along pods litter's decomposition process, samples of this material were collected in three different moments (December 2012, April 2013 and June 2013, respectively) which coincided with samples of 1, 4 and 7 months old after its fall. These moments were considered as treatments. All samples were collected in an area of 63.6 cm^2.

Pods were stored at 4°C in the dark until the experiment was established in June 2013. Petri dishes were filled with 3 g of pods litter, covered with a Whatman No. 1 paper disc and watered with 20 mL of distilled water. Control Petri dishes contained only 3 mL of distilled water and seeds. Equally, in this experiment 30 *L. sativa* seeds were used by Petri dish and each treatment was seven times replicated. As in the previous experiments, the same variables were measured.

2.6. *Extraction and identification of allelochemicals*

Fresh leaves (1600 g), flower litter (1920 g) and pods litter (177 g) were collected from or underneath 10 plants between December 2012 and July 2013. Extraction was carried with 96% of methanol for 10 days at 22°C. The resulting solutions were concentrated under reduced pressure with a rotatory evaporator (IKA HB10 digital, Staufen, Germany) to obtain the crude extracts, which were sequentially extracted with *n*-hexane, ethyl acetate (EtOAc) and distilled water. Extracts from hexane and EtOAc for each plant material were fractionated using a chromatography column packed with silica gel, eluted with *n*-hexane, EtOAc and methanol (MeOH) in order to increase polarity. Fractions obtained from each extract (approximately 10–15) were monitored

by thin layer chromatography in order to identify and mix similar fractions. Fractions were characterised by Gas Chromatography–Mass Spectrometry (GC–MS) (Agilent 7890A, California, USA), with an Agilent 5975C mass detector, using a HP5-MS type fused silica capillary column of 30 m, 0.25 mm inner diameter and 0.25 μm film thickness, under the following characteristics: temperature: 250°C; detector (mass): 280°C; furnace: initial 100°C for 5 min, increasing by 8°C/min up to 250°C and maintained for 15 min. The detector set in the scan mode ranged from 50 to 500 amu. The carrier gas flow (electronic degree helium) was at 1 mL/min. The compound characterisation was carried out by means of comparison with NIST® database.

2.7. *Statistical analyses*

Bioassays were established on the basis of a completely randomised experimental design.

The variables evaluated were RL, HL and RN. In all cases, the parametric statistics premises were evaluated, especially data normality and variances homogeneity by Kolmogorov–Smirnov and Levene test's. When premises were achieved, data were subjected to one-way ANOVA, using a significance level of 0.05. On samples with statistical significance, a Tukey's test was performed. When premises were not achieved, even with a data transformation consisted in Log $(n + 1)$ and root^4.[30,31] A non-parametric Kruskal–Wallis test was applied to the RN variable. All statistical analysis was made using STATISTICA 7.0 for Windows (Statsoft 2007).

3. Results

3.1. *Effect of extract's concentration on the germination and early growth*

L. sativa germination was never affected by extract's concentrations, reaching 100% in all treatments. The hypocotyl and radicle growth was inhibited in all treatments, which was observed by a reduction of HL and RL values; being highly significant ($p < .001$) for RL not only for the different concentrations but also for the plant parts (leaf, pods, flower litter's and fresh leaves) (Figure 1). The *L. sativa* radicle was reduced by up to 82% at 75 and 100% of the leaves litter extract (Figure 1(a)). The reduction of RL was associated with radicle necrosis (data not shown). On the other hand, radicles from control samples were white and had root hairs (degree 0, Table 1). Concentrations of 75% and 100% showed light brown radicles with fewer hairs in all plant materials (degree 1, Table 1). In leaf and pod litter extracts, radicles became light brown (degree 1, Table 1) from concentrations of 50% and 25%, respectively. HL was significantly ($p < .001$) affected by all of the extracts assayed (leaf litter; fresh leaves; flowers litter and pods litter) (Figure 1). However, a clear reduction of the hypocotyl was observed for the leaf litter extract at the highest concentrations (75% and 100%) (Figure 1(a)).

3.2. *Effect of extract's evolution on the germination and early growth*

Although germination was not affected by the aqueous extract time (values reached 100% in all treatments), HL and RL varied in a different significant way ($p < .001$) according to the period in which biomass was in contact with the aqueous solvent (Figure 2). HL is not a clear bioactivity indicator. Only leaf litter induced a constant decrease of this variable (Figure 2(a)), to the extent that contact time of biomass with the water elapsed. The rest of the extracts from the different plant parts maintained certain oscillations of HL, not always exceeded by the control (Figure 2(b)–2(d)). At the same time, leaf litter produced drastic decreases (from 3.5 cm to less than

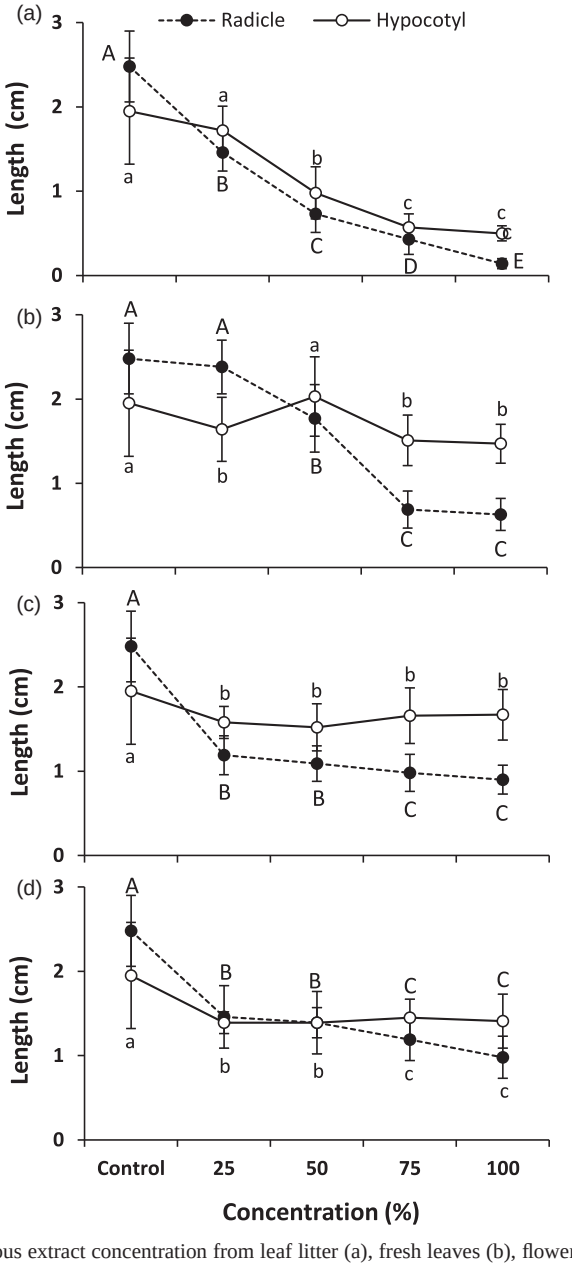

Figure 1. Effect of aqueous extract concentration from leaf litter (a), fresh leaves (b), flowers litter (c) and pods litter (d) of *A. dealbata* on the radicle and HL of *L. sativa* seedlings. Values shown are mean ± SD. Different letters denote significant differences between treatments after one-way ANOVA ($p < .05$) and Tukey's *post hoc* test. Capital letters represent the variation among RL while lowercase letters represent variation among HL.

1.0 cm) of the RL from 1 to 4 days, declining until day 16 (Figure 2(e)). In turn, fresh leaves, flowers litter and pods litter induced similar responses (Figure 2(f)–2(h)), but it is important to take into account that the biological activity remained relatively stable from the first day, when biomasses were in contact with distilled water. In all cases, HL and RL values of control samples significantly exceeded the rest of the treatments.

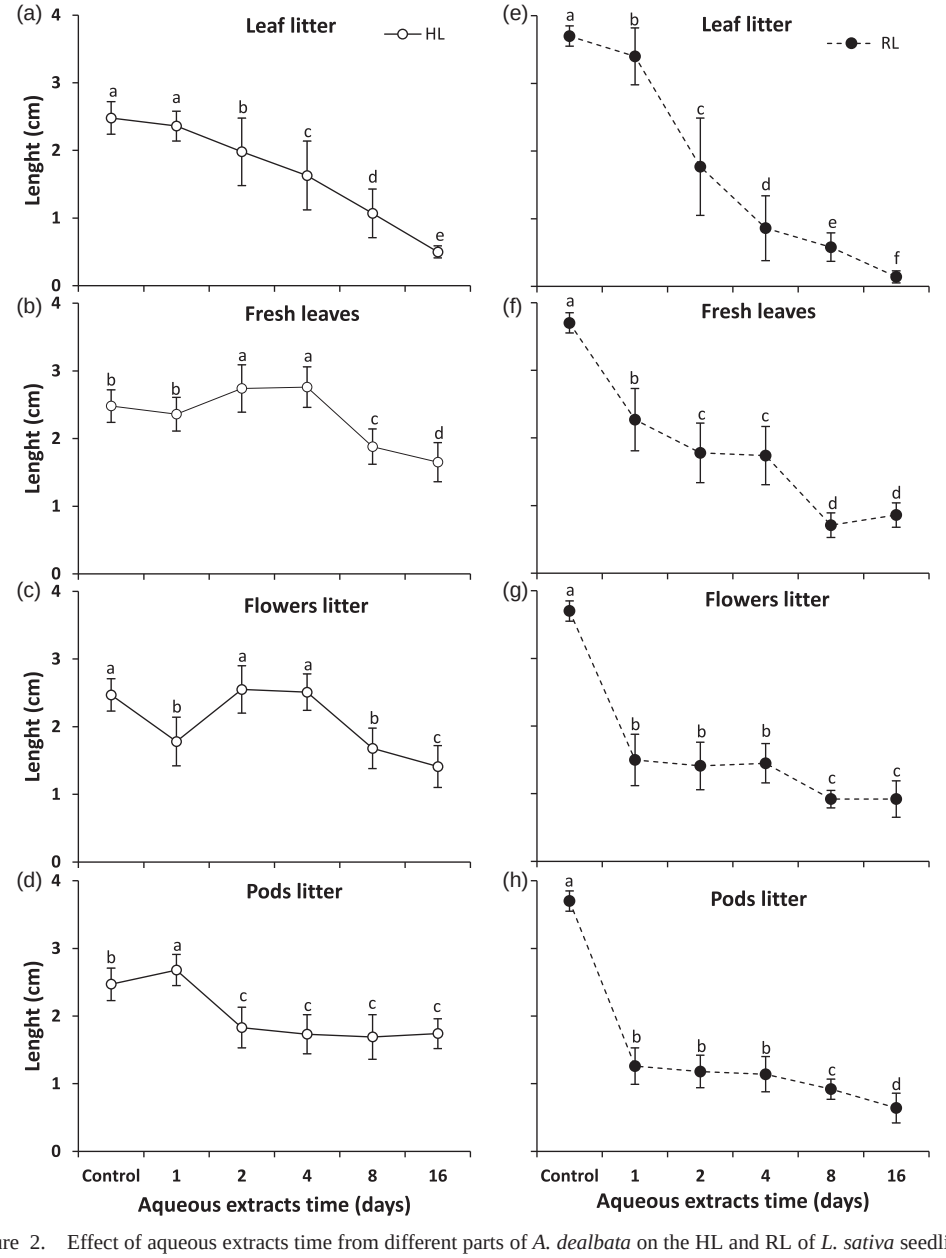

Figure 2. Effect of aqueous extracts time from different parts of *A. dealbata* on the HL and RL of *L. sativa* seedlings. Values shown are mean ± SD. Different letters denote significant differences between treatments after one-way ANOVA ($p < .05$) and Tukey's *post hoc* test.

3.3. *Direct effect of plant parts on the germination and early growth*

Different plant parts directly used in Petri dishes had contrasting effects on germination of *L. sativa*; for example, seed germination was totally inhibited by the leaf litter when it was alone or combined with pods (Figure 3). In contrast, the GP in other treatments including the control were 100%, similar to previous experiments. HL, RL and RN were also significantly affected by the biomass of different plant materials (Figure 3). HL values of seedlings grown in F100,

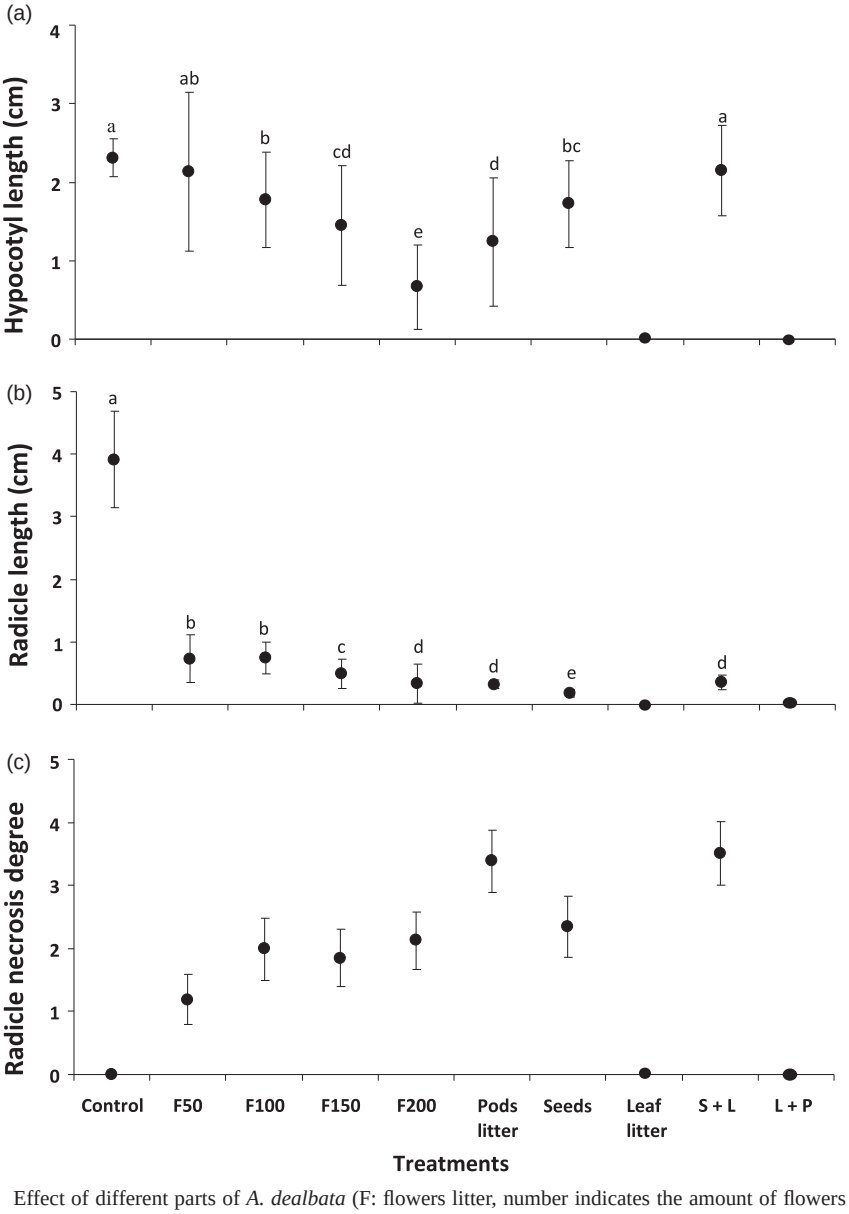

Figure 3. Effect of different parts of *A. dealbata* (F: flowers litter, number indicates the amount of flowers placed in Petri dishes; S + L: combination of seeds with leaf litter; L + P: combination of leaf litter with pods litter) on the HL (a), RL (b) and radicle necrosis degree (c) of *L. sativa* seedlings. Values shown are mean ± SD. Different letters denote significant differences between treatments after one-way ANOVA ($p < .05$) and Tukey's *post hoc* test. There were no germinated seeds for leaf litter and L + P treatments; in (c) nonparametric Kruskal–Wallis test was applied.

F150, F200, pods litter and seeds were significantly lower than control ($p < .001$), showing the lowest values in the F200 treatment (Figure 3(a)). All tested plant parts significantly reduced the RL ($p < .001$), with values around zero in all cases (Figure 3(b)). Moreover, the seedling radicles were significantly damaged in all treatments in comparison to the control ($p < .001$) (Figure 3(c)). Seedlings grown in the pods litter and in a mixture of seed and leaf litter treatments showed the highest necrosis degree, with at least 50% of the radicle area damaged and without root hairs (Figure 3(c), degree 3 in Table 1).

Table 2. Chemical composition of leaf, flowers and pods of *A. dealbata* determined by GC–MS.

Plant parts	Compounds	Molecular formula	MW (g/mol)	RT (min)	RA
Leaf litter	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	14.509	4.89
	9,12-Octadecadienoic acid (Z,Z)-methyl ester	$C_{19}H_{34}O_2$	294	16.144	2.41
	9,12,15-Octadecatrienal	$C_{18}H_{30}O$	262	16.206	10.22
	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	292	16.211	12.33
	Moretenone	$C_{30}H_{48}O$	424	29.468	2.18
	Lupenone	$C_{30}H_{48}O$	425	30.314	8.26
	Resorcinol	$C_6H_6O_2$	110	6.773	46.76
	Benzophenone	$C_{13}H_{10}O$	182	11.448	61.35
	Maculosin	$C_{14}H_{16}N_2O_3$	260	14.608	25.51
Flowers litter	1-Dodecene	$C_{12}H_{24}$	168	5.445	17.41
	Anisal	$C_8H_8O_2$	136	6.586	28.60
	2-Tetradecene, (E)-	$C_{14}H_{28}$	196	8.356	22.92
	3-Hexadecene, (Z)-	$C_{16}H_{32}$	224	10.940	9.41
	1-Octadecene	$C_{18}H_{36}$	252	13.191	5.91
	Stigmasterol	$C_{29}H_{48}O$	412	29.354	7.70
	Methyl *p*-anisate	$C_9H_{10}O_3$	166	8.195	19.05
	Palmitic acid	$C_{17}H_{34}O_2$	270	14.541	20.86
	9,12-Octadecadienoic acid, methylester	$C_{18}H_{32}O_2$	280	16.165	9.02
	Methyl linolenate	$C_{19}H_{32}O_2$	292	16.232	19.60
	Methyl stearate	$C_{19}H_{38}O_2$	298	16.434	5.48
	Methyl *p*-hydroxycinnamate	$C_{10}H_{10}O_3$	178	12.896	70.59
	p-Anysil alcohol	$C_8H_{10}O_2$	138	6.893	32.56
Pods litter	Lupanine	$C_{15}H_{24}N_2O$	248	17.731	2.27
	D-alpha-tocopherol quinone	$C_{26}H_{44}O_3$	404	26.293	10.66
	Methyl hexadecanoate	$C_{17}H_{34}O_2$	270	14.644	9.66
	Octinoxate	$C_{18}H_{26}O_3$	290	18.328	6.82
	2-Heptadecanone	$C_{17}H_{34}O$	254	14.421	6.81
	Stigmasterol	$C_{29}H_{48}O$	412	29.619	12.82

Notes: MW, molecular weight from GC–MS data; RT, Retention times; RA, Relative peak area (peak area relative to total peak area per fraction).

3.4. *Evolution of phytotoxicity in pods litter*

The pods litter collected at different times did not affect the germination of *L. sativa*. However, the HL and RL were significantly ($p < .001$) reduced by this plant material (Figure 4(a) and 4(b)). Although this effect was not constant during the collection time, the highest length reduction was found at one-month-old pods for the hypocotyl and one- and four-month-old pods for the radicle (Figure 4(a) and 4(b)). Significant differences ($p < .001$) in necrosis symptoms were found for the radicle of seedlings grown in the pods litter accumulated at one and four months under *A. dealbata*'s canopy (Figure 4(c)). Both one- and four-month-old pods litter showed dark-brown radicles with a damaged area of 50% and without root hairs (Figure 4(c), degree 3 in Table 1).

3.5. *Identified chemical compounds*

Different chemical compounds were isolated depending on the plant parts of *A. dealbata* (Table 2). The highest number of identified chemicals was found in the flowers litter, followed by leaf litter and pods litter. The most abundant compounds in flowers were anisal, methyl *p*-hydroxycinnamate and *p*-anysil alcohol. Aromatic compounds such as resorcinol, benzophenone and the non-protein amino acid macusolin were the most abundant chemicals in leaf litter. The pods litter contained stigmasterol, D-alpha-tocopherol quinone compounds and others in lower quantities.

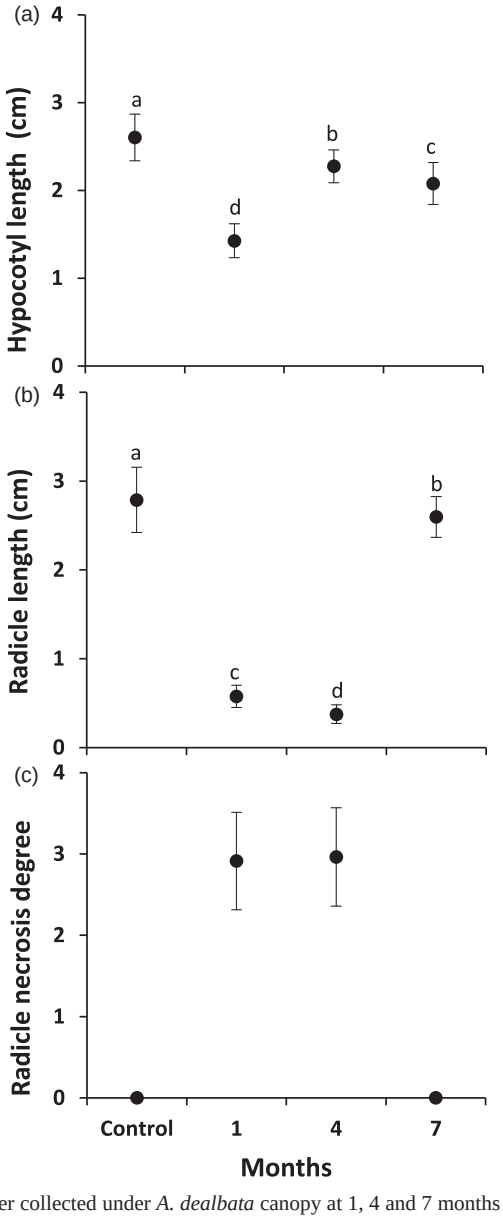

Figure 4. Effects of pods litter collected under *A. dealbata* canopy at 1, 4 and 7 months on HL (a), RL (b) and radicle necrosis degree (c) of *L. sativa* seedlings. Values shown are mean ± SD. Different letters denote significant differences between treatments after one-way ANOVA ($p < .05$) and Tukey's *post hoc* test; in (c) non-parametric Kruskal–Wallis test was applied.

4. Discussion

Although field studies are necessary to fully determine the role of allelopathy,[4] improved bioassays with aqueous extracts representing natural concentrations of released allelochemicals are still useful.[18,32–34] Present work is the first to explore the allelopathy potential of *A. dealbata*, based on the average rainfall in the study area and the amount of equivalent litter naturally deposited under canopy. This approach makes the experiment close to natural conditions. In our case, naturally based bioassays performed from a relevant ecological data of rainfall and plant

litter showed significant inhibitory effects. In fact, the annual average rainfall on this region is 827 L/m^2,[28] which corresponds to the highest concentration tested (100%). However, seedling growth was even affected at the lowest concentration (25%), which is equivalent to uncommon periods of rainfall. Moreover, the annual rainfall average in the area has been decreasing in the last decades.[35] Therefore, extracts tested in the present study could be less concentrated than leachates occurring under natural conditions, representing a plausible approach for evaluating potential allelopathic activity of *A. dealbata* and suggesting the real presence of allelopathic interactions in Chilean fields.

In the present study, aqueous extracts prepared with natural quantities of different *A. dealbata* plant parts did not affect the germination of *L. sativa* but had a negative effect on seedling growth. The allelopathic effect on HL depended on the extract concentration, plant parts and the time in which plant parts were kept in water. In particular, the radicle was severely reduced and damaged by low concentrations of short-term extracts released by all plant parts, the extract of leaf litter being the most phytotoxic solution. Moreover, plant materials directly used showed the same effects on the growth of *L. sativa* seedlings, with the leaf litter alone or combined with pods litter also inhibited seed germination. These results differ from those obtained in northwestern Europe by Lorenzo et al.,[16] who found that the radicle of *L. sativa* was stimulated by natural leachates from aerial parts of *A. dealbata* collected at pods and inflorescence formation stages and inhibited by natural leachates at the open flower phase. In the same way, inflorescence and open flower leachates of *A. dealbata* collected in the European range also stimulated the seedling growth and respiration rate of *Dactylis glomerata* L., a native species to this area.[16,17] Therefore, we suggest that initial growth of the test species *L. sativa* was at least partially dependent on allelochemicals released from *A. dealbata*. However, further tests with native species from the study system are required. In fact, preliminary results obtained in bioassays assessing the phytotoxic effect of *A. dealbata* extracts also collected in Chile showed a strong inhibitory effect on seedling growth of *Q. saponaria*, *Helenium aromaticum* and *Rhodophiala maculata* native species to South America (Aguilera, unpublished). Moreover, a recent study conducted in Northwest Spain showed that volatile organic compounds released from fresh flowers of *A. dealbata* inhibited germination and seedling growth of *Trifolium subterraneum*, *Lolium multiflorum* and *Medicago sativa* native to this region.[36] Other studies carried out in Northwest Spain and Italy have shown that *A. dealbata* has negative impacts on both native understorey plants and soil microbes,[11,12,20] although allelopathy was not directly implicated in these studies.

The different phytotoxic effects found in both non-native ranges (South America and Europe) to *A. dealbata* could be related to a potential evolutionary process evolved by cohabiting native plants with allelochemicals naturally released by the invasive species.[4,37] In Southwestern Europe, native species have been exposed and hence have had the opportunity to evolve a potential resistance to *A. dealbata* chemistry for two centuries. However, the time elapsed since *A. dealbata* was introduced in South America is shorter, around a century and half.[13]

Regardless of the invasion time and observed effects, results obtained in the present study and by Lorenzo et al. seem to indicate an allelopathic interference by *A. dealbata* in its non-native ranges.[15–18] These allelopathic interactions were initially associated with allelochemicals released by flowers.[16–18,38] Nevertheless, our results indicated that the potential allelopathic activity of this exotic species was greatly related to flowers litter but also to leaf, seeds and pods litter. Leaf and pods litter was largely found underneath the *A. dealbata* canopy (Aguilera, personal observation), and their lixiviation may have contributed to a persistent allelopathic phenomenon along the phenological cycle.

All dropped plant parts had a consistently negative effect on radicle development. Interestingly, this effect was observed immediately after applying the treatments, indicating a rapid bioactivity of allelochemicals dissolved in water. However, the plant parts naturally deposited under the canopy of *A. dealbata* provoked stronger inhibition than fresh leaves. The higher

phytotoxic activity of leaf litter may be related to cell degradation and tissue decomposition, which could be accelerating the liberation of chemical compounds into the water.[5]

The observed inhibitory effect on *L. sativa* may be mediated by chemical compounds such as resorcinol (phenol), maculosin (cyclodipeptide), moretenone (triterpene), stigmasterol (steroid), lupanine (quinolizidine alkaloid), anisal (benzaldehyde), methyl *p*-anisate (phenylpropanoid), *p*-anysil alcohol (phenylpropanoid), D-alpha-tocopherol quinone (quinone) and fatty acids found in leaves, flowers and pods. To our knowledge, this is the first study to identify non-volatile organic compounds being possibly responsible for the phytotoxic activity of *A. dealbata*. Allelopathic activity of phenolic compounds has been well studied.[39–41] Previous studies have shown that phenolics altered cell membranes by disrupting permeability, ions flow and hydraulic activity at the root level, resulting in cascading effects that caused severe damage to the stomatal function and the photosynthesis and respiration rates.[42] In addition, phenolic compounds affected the germination of seeds inhibiting the mobilisation process of reserve substances.[5] Other secondary metabolites produced cellular damage in the apical meristems of roots. Abnormal cell division of plants with aberrant formations and less root hairs were characteristic of allelochemical-treated roots.[43] Changes in auxin content in treated roots could have played a role in the absence of root hairs,[44] as root hairs formation is sensitive to hormonal and environmental factors [45] and the local presence of auxin and expansions are required for the growth and development of root hairs.

In particular, root exudates of certain rice varieties containing resorcinol had an inhibitory effect on the germination and growth of weeds that usually coexist with these rice areas [46,47]; and the magnitude of the negative effect was correlated to the amount of this phenol.[48] The allelopathic effect of other phytotoxins found in the present study and belonging to other chemical groups is less known. For example, maculosin has shown biological activity in eukaryotes and prokaryotes,[49] and in the cell suspension of plants [50]; moretone was related to the negative effect on cell division,[5] and stigmasterol could have had a high cytotoxic capacity.[51] Other chemicals showed a more ambiguous phytotoxic activity. In this way, lupanine, an alkaloid present in epidermic cells which contains nitrogen, can be easily linked with amino acids and proteins greatly responsible for phytotoxic effects,[52,53] or with the ubiquitous fatty acids, which can induce allelopathic activity.[54,55] Additionally, the global allelopathic potential can not only be determined by individual activities, but also by the synergistic action of chemicals since they were not isolated in the treatments tested.[24]

Phytotoxic effects similar to those induced by *A. dealbata* on *L. sativa* have been reported by the action of *Hypericum myrianthum* and *Hypericum polyanthemum*.[56] In that case, the RL was significantly affected showing necrosis. Also in both species phenolic compounds were identified as the main components and they could be responsible for the inhibition of the germination and growth of *L. sativa*.[56] Likewise, aqueous extracts of leaves of *Cecropia pachystachya*, *Peltophorum dubium*, *Psychotria leiocarpa*, *Sapium glandulatum* and *Sorocea bonplandii* produced toxic effects on seedling growth and root reduction and debility of *L. sativa*.[57] The same reducing pattern of shoots and root length by allelochemical effects on *L. sativa* from several donor species is repeated in almost every allelopathy work.[58–62] In addition to phenolic compounds, many of the phytotoxic effects on *L. sativa* have been attributed mainly to sesquiterpene lactones,[63] benzoxazolin-2 (3H)-one (BOA) [64] and hydroxycinnamic acids.[60] As discussed above, *A. dealbata* releases other compounds, which also exert a strong phytotoxic effect on the early growth of *L. sativa*.

5. Conclusion

In this study *A. dealbata* released allelopathic compounds in the aqueous phase, which suggest that it could occur in natural conditions during rainfall periods. These aqueous allelopathic

compounds affected *L. sativa* seedling growth, suggesting a likely growth reduction of South American native plant species. This phenomenon was more remarkable due to the direct contact of plant material from *A. dealbata* with the model species. The inhibitory effect induced by different plant parts confers to this invader an allelopathic potential throughout its phenological cycle. The allelopathic activity can be greatly determined by leaf, flowers and pods litter chemicals such as resorcinol, maculosin, moretenone, stigmasterol, D-alpha-tocopherol quinone lupanine, anisal, methyl *p*-anisate and *p*-anysil alcohol which could be acting synergistically under natural conditions. Finally, these results confirm that allelopathy can be a biological phenomenon that facilitates invasion of *A. dealbata* in its non-native ranges.

Disclosure statement

No potential conflict of interest was reported by the authors.

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