Research Article

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Herbicidal properties of the commercial formulation of methyl cinnamate, a natural compound in the invasive silver wattle (*Acacia dealbata*)

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Abstract

Plants that release molecules affecting other plants are a source of potential bioherbicides. Silver wattle (*Acacia dealbata* Link), considered invasive worldwide, was found to be phytotoxic to various other plant species. Combining the search for alternative bioherbicides while reducing the spread of this invader by preventing seed formation is a good potential strategy to solve both agricultural and environmental problems. This study aimed to identify nonvolatile compounds from *A. dealbata* flowers and explore their phytotoxicity on the germination process and seedling and plant growth of lettuce (*Lactuca sativa* L.), wheat (*Triticum aestivum* L.), and rigid ryegrass (*Lolium rigidum* Gaudin). We identified methyl cinnamate and methyl anisate as potential phytotoxins in the extracts, but we used pure commercial molecules to conduct bioassays. Methyl cinnamate showed higher phytotoxicity than methyl anisate and was selected for further bioassays. Methyl cinnamate reduced guaiacol peroxidase activity by 57% and 85% in *L. rigidum* and lettuce, respectively, and α-amylase by 6% in *L. rigidum*. This compound also inhibited early stem and radicle growth of dicotyledonous lettuce (60% and 89%, respectively) and monocotyledonous *L. rigidum* (76% and 87%, respectively), both species having small seeds. However, wheat with a larger seed size was not affected by the phytotoxin. The results obtained indicate a potential bioherbicidal effect for methyl cinnamate, and its application might be useful in wheat crops infested by *L. rigidum*. We suggest that collecting *A. dealbata* flowers would prevent *Acacia* seed formation and thus play a role in invasive pest management, as well as serving as a source of potential herbicides to other species.

Introduction

Maximizing food production to feed an increasing human population relies heavily on the use of agrochemicals such as herbicides (Bhadoria 2011). Repeated and continuous use of herbicides has resulted in the evolution of herbicide-resistant weeds and environmental pollution (Bhadoria 2011; Dayan et al. 2009; Duke and Heap 2017; Green 2014; Rosculete et al. 2019). However, growers are still heavily reliant on herbicides for weed control (Duke et al. 2018; Green 2014), and there is increasing criticism of maintaining agricultural production in this way. Changes in agricultural practices are deemed necessary in order to develop more sustainable and integrated agronomic practices to help human populations and agroecosystems and to preserve natural resources (Storkey et al. 2019). Natural compounds or bioherbicides can be used as alternatives to synthetic herbicides (Macías et al. 2007; Storkey et al. 2019; Westwood et al. 2018). They may also provide a basis for developing new compounds for weed control (Dayan and Duke 2014; Dayan et al. 2012; Yan et al. 2018). Although natural molecules have some limitations in general weed control (Dayan et al. 2012), they are expected to be less toxic and more environmentally friendly than synthetic herbicides (Bhadoria 2011; Vurro et al. 2019).

According to the novel weapon hypothesis, invasive plants may partially outcompete native flora in ecosystems where they invade by releasing bioactive natural chemicals (Becerra et al. 2018; Callaway and Aschehoug 2000; van Kleunen et al. 2018). This phenomenon, commonly referred to as allelopathy (Einhellig 2018), also includes positive effects and makes invasive plants a source of allelochemicals or phytotoxins that could be used as bioherbicides

(Benchaa et al. 2018; Puig et al. 2018). Silver wattle (*Acacia dealbata* Link) is an invasive plant that seriously affects ecosystem functioning and services worldwide (Aguilera et al. 2015a; Lazzaro et al. 2014; Lorenzo et al. 2010b, 2012, 2013, 2017; Ngorima and Shackleton 2019). Allelopathy differentially contributes to the invasion process of *A. dealbata* (Aguilera et al. 2015a, 2015b, 2015c; Lorenzo et al. 2017). However, this invasive species has largely shown a potential allelopathic or phytotoxic effect on different physiological parameters of plants (Aguilera et al. 2015a, 2015b, 2015c; Lorenzo et al. 2008, 2010a, 2011, 2016, 2019; Reigosa and Carballeira 2017a) and soil microbes (Kamutando et al. 2019; Lorenzo et al. 2013).

Recently, Souza-Alonso et al. (2017) suggested using *A. dealbata* debris to control weeds in agriculture due to the phytotoxic effect of its plant material. Phytochemical composition of nonvolatile compounds has been reported for litter including leaves, flowers, and pods in Chile (Aguilera et al. 2015b) and for volatiles of fresh leaves and flowers and litter in Spain (Souza-Alonso et al. 2014). Leaf litter and fresh leaves seem to be the most phytotoxic parts of *A. dealbata* (Aguilera et al. 2015a, 2015b; Lorenzo et al. 2016), followed by flowers and pod litter (Aguilera et al. 2015a, 2015b). However, in Spain, fresh leaves directly left on soil or incorporated into soil were rarely toxic to weeds (Souza-Alonso et al. 2018), suggesting that natural compounds with potential herbicidal activity might be obtained from flowers or pods. Nevertheless, use of pods should be preferentially avoided, because seeds can accidentally propagate invasion. Decomposing flowers alone or combined with leaves significantly reduced the germination and radicle length of plants when incorporated into soil (Reigosa and Carballeira 2017b). Additionally, using flowers prevents seed formation and, hence, reduces dispersion of this invasive plant.

With the idea of finding potential uses for *A. dealbata* flowers to prevent spread by seeds while contributing to reducing reliance on synthetic herbicides, this study aimed to explore new phytotoxic activities for nonvolatile compounds identified in flowers of *A. dealbata* collected in the northwestern Iberian Peninsula. First, we evaluated the phytotoxicity of identified compounds on germination and seedling growth of lettuce (*Lactuca sativa* L.), a model species in phytotoxic bioassays. Then, we explored the potential herbicidal effect of the most active compound on the germination process, seedling growth, and biometric and biochemical parameters of well-established plants; these were lettuce; a widely consumed wheat crop (*Triticum aestivum* L.); and rigid ryegrass (*Lolium rigidum* Gaudin), a weed common in winter cereal and wheat crops (Cirujeda and Taberner 2009; Owen et al. 2015).

Material and Methods

Plant Material, Extraction Procedure, Isolation, and Identification of Chemical Compounds

Fresh flowers (7.80 kg) of *A. dealbata* were collected and extracted with 97% methanol as described in Lorenzo et al. (2016) to obtain a crude extract (535.36 g). About 50 g of this extract was re-dissolved in methanol–water (2:1 v/v) and sequentially extracted with *n*-hexane and ethyl acetate (800 ml each). Organic solvents were removed under reduced pressure, and the remaining solution was freeze-dried in flasks under high vacuum (0.1 to 0.01 mm Hg) with a −80 C cooling trap until no ice remained inside the flasks (LyoQuest −85 Plus model, Telstar, Tarrasa, Spain). This resulted in 2.92 g of hexane extract (FH). A portion of FH

(2.50 g) was subjected to column chromatography on silica gel using hexane/ethyl acetate mixtures of increasing polarity from 0% to 100% ethyl acetate. Several fractions were obtained after thin-layer chromatography analysis. One of them, fraction FH2 (828 mg) was obtained by elution with 5% and 10% ethyl acetate in hexane. FH2 was subjected to medium-pressure column chromatography on silica gel using a gradient mixture of ethyl acetate (5% to 30%) in hexane to obtain 11 fractions. From these, fractions FH2.5 (154 mg) and FH2.6 (65 mg) were subjected to high-performance liquid chromatography (column: XTerra MS C18, 5 microns, 150 by 4.6 mm; flow: 2.5 ml min^{-1}, [Agilent, Rozas de Madrid, Spain]; eluent: hexane/ethyl acetate 95:5, isocratic; detector: UV at 220 and 254 nm). Methyl cinnamate and methyl anisate were isolated with retention times of 10 and 12 min, respectively, and were identified by ^{1}H and ^{13}C nuclear magnetic resonance spectrum (methyl cinnamate: Supplementary Figures S1 and S2; methyl anisate: Supplementary Figures S3 and S4). These aromatic compounds were selected based on their chemical structures to further study their potential phytotoxic activity.

Commercial formulations of methyl anisate and methyl cinnamate were purchased (Merck Millipore, Darmstadt, Germany, 99.5%) and used to conduct bioassays. Commercial formulations guaranteed available quantities of chemicals and avoided interfering effects of potential unknown chemicals present in methyl anisate and methyl cinnamate extracts.

Preliminary Bioassay: Evaluation of the Phytotoxic Effects of Methyl Cinnamate and Methyl Anisate

The phytotoxic activities of methyl anisate and methyl cinnamate were explored using the sensitive model species lettuce ('Trocadero') to compare results of bioassays with these two compounds (Lorenzo et al. 2016). Methyl anisate or methyl cinnamate were dissolved in dimethyl sulfoxide (DMSO) (5 μl DMSO ml^{-1} MES buffer) and diluted with MES buffer (10 mM 2-[*N*-morpholino] ethanesulfonic acid and 1 M NaOH, pH 6.0) to obtain aqueous solutions with concentrations of 10, 50, 100, 500, and 1,000 μM according to the procedure of Macías et al. (2010). Control solutions received all diluted chemicals (i.e., 5 μl DMSO ml^{-1} of MES buffer) except methyl anisate and methyl cinnamate (0 μM).

Twelve seeds of lettuce were sown in petri dishes (3.7-cm diameter) lined with a sterile Whatman No. 2 paper and watered with 1.2 ml of each test solution. All treatments were replicated six times. Petri dishes were sealed with Parafilm® to prevent desiccation (Lorenzo et al. 2016) and maintained at 12/12 h (light/dark) and 20 C for 7 d, with their arrangement changed daily. Then, plates were frozen at −20 C to stop seedling growth (Lorenzo et al. 2016). After that, we determined the number of germinated seeds and measured the stem and radicle lengths (cm) of all seedlings in each plate using Image J v. 1.45 software (Rasband 1997–2014).

Phytotoxic Effect of Methyl Cinnamate on Germination and Initial Seedling Growth: Dose–Response Assay

The potential phytotoxicity of methyl cinnamate was evaluated in terms of inhibition in seed germination and seedling growth on lettuce; *L. rigidum*, a problematic weed in wheat that has evolved resistance to multiple herbicidal action modes (Broster and Pratley 2006; Broster et al. 2011; Chen et al. 2018; Cirujeda and Taberner 2009); and wheat ('Bastide') a widely cultivated winter cereal crop.

Twelve seeds each of lettuce, *L. rigidum*, and wheat were sown in petri dishes (3.7-, 6-, and 14-cm diameter, respectively) lined

with a sterile Whatman No. 2 paper and watered with 0.1 ml cm^{-2} of different solutions of methyl cinnamate. Methyl cinnamate was dissolved as described before and assayed at different concentrations (0, 250, 375, 500, 625, 750, 875, 1,000, and 1,250 μM). Plates were sealed with Parafilm®. Six replications of each treatment were kept at 12/12 h (light/dark) and 22 C for 7 d in the case of lettuce and for 9 d in the cases of *L. rigidum* and wheat. The number of germinated seeds and the stem and radicle lengths (cm) were recorded as described in the previous experiment.

Phytotoxic Effect of Methyl Cinnamate on Biochemical Parameters Related to the Germination Process

Seeds of lettuce (45), *L. rigidum* (35), and wheat (35) were sown in petri dishes (14 cm diameter) lined with a sterile Whatman No. 2 paper and moistened with 15 ml of different solutions of methyl cinnamate. Methyl cinnamate was dissolved as described earlier and assayed at concentrations of 0, 250, 500, 750, and 1,000 μM, with five replicates per treatment. Growth conditions were the same as in the previous experiments. At 4 d after sowing, germinated seeds were frozen in liquid nitrogen and preserved at −80 C.

General Extraction Procedure

Cotyledons (100 mg) were ground with liquid nitrogen using a mortar and pestle and extracted with 2 ml of extraction buffer (0.5% polyvinilpyrrolidone, 3 mM ethylenediaminetetraacetic acid [EDTA, disodium salt 2-hydrate], and 0.1 M potassium phosphate buffer, pH 7.5). The homogenate was centrifuged at 7,500 rpm for 15 min at 4 C (Horii et al. 2006). The supernatant was used as the crude protein extract to determine protein concentration and enzyme activities. Samples were kept at 0 to 4 C during the process.

Protein Concentration

Total protein concentration was determined according to Bradford's spectrophotometric assay (Bradford 1976) using 100 μl of supernatant mixed with 3 ml of Bradford reagent. Absorbance was measured at 595 nm after 5 min. Quantification of the protein content was determined by comparing absorbance values to a standard curve of bovine serum albumin. Protein concentration was expressed as mg g^{-1} fresh weight (FW).

α-Amylase Activity

This was determined using the Jones and Varner starch–iodine procedure (Jones and Varner 1967). About 150 μl of the supernatant was diluted 1:5 (v/v) with distilled water, mixed with 1 ml of starch solution, and incubated for 10 min at 30 C. The reaction was stopped by addition of 1 ml of iodine reagent. Samples were diluted again 1:5 (ml, v/v) with distilled water, and absorbance was measured at 620 nm. One unit of α-amylase activity was defined as the amount of enzyme required to hydrolyze 1 mg starch min^{-1}, and the results were expressed as α-amylase activity mg^{-1} FW.

Guaiacol Peroxidase Activity (GPX)

GPX activity was determined using the guaiacol method previously described by Horii et al. (2006) and McCue et al. (2000). The reaction mixture contained the crude protein extract and buffer in a proportion of 1:5 (ml, v/v). Oxidation of guaiacol was assessed by monitoring absorbance at 470 nm over a period of 5 min. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 μmol guaiacol min^{-1} (U). GPX activity was expressed as U mg^{-1} FW.

Phytotoxic Effect of Methyl Cinnamate on Well-Established Plants

Five seeds of each species (lettuce, *L. rigidum*, and wheat) were sown in 100-ml pots (30.25 cm^2) filled with perlite (2- to 6-mm pore size). We did not use soil to avoid interference due to soil properties that could mask the actual effect of methyl cinnamate. Pots were irrigated with 10 ml of Hoagland solution (1:1) (pH 6.25 ± 1) once a week. Additional irrigation was done when necessary. Treatments were applied when seedlings reached 3 to 5 cm in height and were completely photosynthetically active. Plants were thinned to 1 plant per pot for lettuce and wheat; and 2 plants per pot for *L. rigidum*. Then, plants were watered with 10 ml of different concentrations of methyl cinnamate. This compound had previously been dissolved in DMSO (5 μl DMSO ml^{-1} solution) and diluted with Hoagland solution to achieve final concentrations of 10, 250, 500, 750, and 1,000 μM. Control pots received the same solutions without methyl cinnamate (0 μM). Application of methyl cinnamate was repeated after 14 d. Treatments were replicated 10 times. Plants were grown in a random arrangement at 14/10 h (light/dark) and 24/22 C (light/dark) for 21 d. After that, plants were harvested, and leaf area, fresh stem and root weights, and stem and root lengths were determined for each plant. Leaf area was recorded three times on 5 separate plants with a CI-202 Portable Laser Leaf Area Meter (CID Bio-Science, Vancouver, WA, USA) for lettuce and wheat and an LI-3000c model (Li-Cor Biosciences, Bad Homburg, Germany) for *L. rigidum*. Then, 5 plants per treatment were randomly selected and dried at 70 C to obtain stem and root dry weights (mg), and the other 5 plants were immediately processed to evaluate biochemical parameters.

Lipid Peroxidation in Stem and Leaves

Lipid peroxidation was indirectly determined by measuring the content of malondialdehyde (MDA), a by-product of lipid peroxidation (an indicator of membrane injury), using the thiobarbituric acid (TBA) method (Hodges et al. 1999) with slight modifications. Fresh whole stem and leaves (0.5 g) were extracted with 9 ml of 80% ethanol and centrifuged (4,400 rpm at 4 C for 30 min). Aliquots (0.75 ml) of supernatant were added to either 1.5 ml of 20% trichloroacetic acid (TCA) and 0.001% butylated hydroxytoluene or 1.5 ml of 20% TCA and 0.001% butylated hydroxytoluene plus 0.5% TBA. Samples were mixed vigorously, heated at 95 C for 25 min, quickly cooled in ice, and centrifuged at 4,000 rpm for 15 min. The absorbance of the supernatant was measured at 440, 532, and 600 nm. MDA equivalents were determined according to Hodges et al. (1999) and expressed as nmol g^{-1} FW.

Protein Concentration in Leaves

The extraction procedure for obtaining the crude extract from leaves and the quantification of total proteins (Bradford 1976) were determined as described in the previous protein concentration section.

Root Activity

This parameter was estimated using 2,3,5-triphenyltetrazolium chloride (TTC) (Onanuga et al. 2012), which is reduced to insoluble red-colored triphenyl formazan by living tissues (Ruf and Brunner 2003). Fresh roots (0.05 g) were chopped into 1-mm pieces and incubated with 1.2 ml of 0.4% TTC and 1.2 ml of 0.1 M sodium-potassium phosphate buffer for 3 h at 37 C.

Then, 0.75 ml of 95% ethanol was added and samples were incubated at 80 C for 15 min. The absorbance of the extract was measured at 410 nm. Triphenyl formazan content was expressed as A_{410} g^{-1} FW h^{-1}.

Crude Extract From Roots

Fresh roots (1 g) were powdered with liquid nitrogen using a mortar and pestle and extracted with 12 ml of extraction buffer (50 mM EPES-KOH, pH 7.8, containing 0.1 mM EDTA). The mixture was centrifuged at 6,150 rpm for 15 min at 4 C, and the supernatant was preserved at −20 C until use.

Protein Concentration in Roots

Protein concentration was determined according to Bradford's spectrophotometric assay (Bradford 1976) as indicated in the previous section using 100 μl of crude root extract.

Superoxide Dismutase Activity (SOD) in Roots

SOD activity was evaluated by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium as described by Beauchamp and Fridovich (1971) using 0.2 ml of crude root extract mixed with 2 ml of nitro blue tetrazolium solution. Samples were acclimated under 40-W fluorescent lamps for 3 min at room temperature. Reaction was started by adding 500 μl of 2 μM riboflavin. Then, samples were lit by 40-W fluorescent lamps for 10 min at room temperature. Absorbance was measured at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme that causes 50% inhibition of nitro blue tetrazolium, and the results were expressed as U mg^{-1} FW.

Data Analyses

Data were analyzed separately for each species. We conducted general linear models (LMs) or generalized linear models (GLMs) to test for the concentration effect of methyl cinnamate or methyl anisate (only in the preliminary bioassay) on germination and stem and radicle length (preliminary, germination, and early growth bioassays); protein concentration, α-amylase activity, and GPX activity (germination process bioassays); and stem increment, root and stem biomass, foliar area, MDA concentration, protein concentration, TTC concentration, and SOD activity (well-established plant bioassays). The assumption of normality was assessed using the Shapiro-Wilk test. If response variables were normal, we conducted LMs, while GLMs with the appropriate error family and link function were used in the absence of data normality (Supplementary Tables S1–S4). We also checked for normality of each LM or GLM residual using the Shapiro-Wilk test. When a response variable was nonnormal and normality of GLM residuals could not be achieved through error family structure, we conducted a nonparametric Kruskal-Wallis (KW) test (Supplementary Table S4). Post hoc mean separations were conducted using Tukey's HSD by comparing the least-squares means obtained within each LM and GLM or by the Nemenyi test after a KW test. The LMs, GLMs, and KW tests were conducted using the STATS package, while post hoc comparisons were performed with the LSMEANS and MULTCOMP or STATS packages after LMs and GLMs or KW, respectively. All analyses were conducted in R v. 3.1.1 (R Development Core Team 2015). The level of significance was set at P ≤ 0.05 for all analyses.

Dose–response models plotting growth response against compound concentration were modeled by nonlinear regression curves to calculate IC_{50} and IC_{80} values (concentrations that cause 50% and 80% of inhibition, respectively). The most appropriate dose–response curve for each case was selected according to the best regression coefficient (R^2). Model adjustment and R^2 value (goodness of fit) were obtained by using scatter plots from the Excel program (Office 16). In *L. rigidum*, there were very few stem and radicle length records at 1,250 μM. Therefore, this concentration was removed from the IC_{50} and IC_{80} estimation for this species.

Results and Discussion

Preliminary Bioassay: Evaluation of the Phytotoxic Effects of Methyl Cinnamate and Methyl Anisate

Natural compounds are increasingly in demand to replace synthetic chemicals that cause environmental problems and human health concerns (Bhadoria 2011; Dayan et al. 2009; Katz and Baltz 2016). In the present study, we evaluated the potential herbicidal activity of the commercial formulations of methyl cinnamate and methyl anisate. We found these compounds in *A. dealbata* flowers (methyl cinnamate: Supplementary Figures S1 and S2; methyl anisate: Supplementary Figures S3 and S4), and their herbicidal activity has not been broadly explored in the literature. Methyl anisate was previously identified in flower litter of *A. dealbata* in the Chilean range (Aguilera et al. 2015b). However, methyl cinnamate was not found in *A. dealbata* plants from Chile (Aguilera et al. 2015b) or those from the Iberian Peninsula (Souza-Alonso et al. 2014). Methyl cinnamate showed a broadly antifungal activity (Lima et al. 2018; Prakash et al. 2015), inhibited bacterial growth (Malheiro et al. 2019), had a larvicidal effect (Fujiwara et al. 2017), and, furthermore, exibited some potential to inhibit plant growth (Fujiwara et al. 2017; Khanh et al. 2008). Essential oils from plants containing a large percentage of methyl cinnamte also showed high bioactivity (Mar et al. 2018; Noriega et al. 2018). In addition, methyl cinnamate is considered as an alternative to synthetic chemicals because it is safer (Fujiwara et al. 2017; Prakash et al. 2015). On the other hand, there is scarce literature reporting the phytotoxic effect of methyl anisate.

Our results demonstrated that the commercial formulations of methyl cinnamate and methyl anisate did not affect germination of lettuce (Supplementary Table S1). However, both compounds affected the stem and radicle length of lettuce (Figure 1; Supplementary Table S1). Dose–response curves showed that methyl cinnamate achieved the lowest IC_{50} and IC_{80} values of 844.57 and 1053.54 μM for stem length, respectively, and of 372.57 and 660.60 μM for radicle length, respectively (Figure 1). Methyl cinnamate stimulated the radicle length at the lowest concentration (10 μM), but reduced both stem (up to 75%) and radicle (up to 96%) growth of lettuce at the highest concentrations (500 and 1,000 μM) (Figure 1). However, methyl anisate showed a lower inhibitory effect on stem length (up to 15%) at 500 and 1,000 μM and on the radicle length at 1,000 μM (46%), although it also increased the radicle length at 10 and 50 μM (Figure 1). These results suggest that methyl cinnamate had higher potential phytotoxic activity than methyl anisate. Therefore, methyl cinnamate was selected to test for a putative bioherbicidal effect on different plant processes and parameters in subsequent bioassays.

The concentration of methyl cinnamate in the extract obtained from *A. dealbata* flowers was 16 mg kg^{-1} FW (i.e., 0.0016% w/w). This concentration is lower than that found for other natural compounds in studies conducted to find potential bioherbicides (Pardo-Muras et al. 2018; Takemura et al. 2013). Such a small

Table 1. Effect of the commercial formulation of methyl cinnamate on germination of lettuce, *Lolium rigidum*, and wheat.

Methyl cinnamate concentration	Lettuce	*L. rigidum*	Wheat
µM		%[a]	
0	91.67 ± 3.04	57.95 ± 9.43	50.09 ± 8.34
250	98.61 ± 1.39	58.01 ± 6.27	43.78 ± 6.83
375	90.15 ± 3.97	57.37 ± 6.39	38.71 ± 6.04
500	84.72 ± 4.52	61.65 ± 2.72	29.05 ± 6.00
625	94.84 ± 2.47	55.56 ± 10.02	45.82 ± 12.02
750	88.89 ± 4.12	59.19 ± 3.52	42.91 ± 4.34
875	91.67 ± 3.73	58.65 ± 7.60	34.64 ± 7.47
1,000	75.00 ± 9.13	46.23 ± 7.92	36.03 ± 6.28
1,250	60.68 ± 7.37	2.56 ± 1.62	20.69 ± 6.97
	(P < 0.01)*	(P < 0.001)*	

[a]Means ± SE are shown. $n = 6$. Asterisks (*) indicate statistical significance between concentrations and the control treatment according to Tukey's test after general or generalized linear models.

quantity of methyl cinnamate could compromise its potential role as a bioherbicide. However, a single individual of *A. dealbata* displays a massive production of flowers (Correia et al. 2014). Generally, *A. dealbata* forms dense invasive populations occupying large areas (Souza-Alonso et al. 2014) that provide large quantities of flowers, which could make it easier to obtain enough methyl cinnamate for practical application.

Phytotoxic Effect of Methyl Cinnamate on Germination and Initial Seedling Growth: Dose–Response Assay

Methyl cinnamate inhibited germination of lettuce and *L. rigidum* at 1,250 µM (Table 1; Supplementary Table S2), whereas no significant effect was found for wheat (Table 1; Supplementary Table S2). Regarding seedling growth, methyl cinnamate reduced stem and radicle length of lettuce at 375 µM and higher concentrations and of *L. rigidum* at ≥875 µM (Figure 2; Supplementary Table S2), whereas the stem and radicle length of wheat were only inhibited at 1,000 µM (Figure 2; Supplementary Table S2). In lettuce, nonlinear dose–response curves showed that concentrations of methyl cinnamate that inhibited stem and radicle length by 50% (IC_{50}) were 1,121.68 and 176.29 µM, respectively (Figure 2). In *L. rigidum*, the IC_{50} values were established at 900.15 and 780.41 µM for stem and radicle length, respectively (Figure 2). In wheat, the IC_{50} values were 1,048.69 and 1,472.05 (out of range) µM for stem and radicle growth, respectively (Figure 2). The IC_{80} values obtained for stem and radicle length of each plant species are also shown in Figure 2. Our results partially agree with those obtained in previous studies. Methyl cinnamate was found to inhibit germination and growth of lettuce at 0.1% (Fujiwara et al. 2017) and slightly reduced germination and root length on *L. rigidum* at 640 nl cm^{-3} (Vasilakoglou et al. 2013), although the effect was dependent on concentration in both cases. However, low concentrations such as 100 ppm reduced radicle and shoot of radish (*Raphanus* sp.) (Khanh et al. 2008).

Phytotoxic Effect of Methyl Cinnamate on Biochemical Parameters during the Germination Process

Methyl cinnamate affected α-amylase and GPX activities in lettuce and GPX in *L. rigidum* and had a marginal effect on GPX in wheat during the germination process compared with the control treatment (Figure 3; Supplementary Table S3). In lettuce, α-amylase

was reduced at 750 and 1,000 µM, whereas GPX was stimulated at low concentrations (250 µM) and severely inhibited at high concentrations (750 to 1,000 µM) (Figure 3). In addition, methyl cinnamate caused a significant severe reduction in protein concentration when assayed at 250 and 500 µM (Figure 3). In *L. rigidum*, the GPX activity was inhibited at 750 to 1,000 µM (Figure 3). However, in wheat, methyl cinnamate only marginally stimulated the GPX activity at 250 µM (Figure 3). Our results demonstrated that methyl cinnamate did not seem to affect the number of total germinated seeds of the species assayed, except at 1,250 µM. However, lower concentrations such as 450 and 1,000 µM of this compound reduced α-amylase activity in lettuce and GPX activity in lettuce and *L. rigidum* during the germination process. α-Amylase activity hydrolyzes starch into sugars that are essential not only for embryonic development but also for maintaining the water potential during seed imbibition (Doria 2010; Taiz and Zeiger 2006). There is strong evidence that peroxides and radicals are abundantly produced within seeds during germination (Bailly 2004) and stressful conditions (Sharma et al. 2012). Therefore, GPX enzymes are expected to intensify their activity in seed germination as a result of the accumulation of reactive oxygen species produced by byproducts of mitochondrial respiration. This is in concordance with our results: the observed increased GPX activity at low methyl cinnamate concentrations protected the germination process. The decrease in α-amylase and GPX activities observed at the highest concentrations may result in reduced seed germination processes.

Phytotoxic Effect of Methyl Cinnamate on Well-Established Plants

Before the different concentrations of methyl cinnamate were applied, the initial stem length was recorded in each plant, and there were no significant differences for this parameter among plants assigned to each treatment within each species (Supplementary Table S4).

In lettuce, the increment in stem length was significantly and severely inhibited (from 76% to 96%) by methyl cinnamate at 500, 750, and 1,000 µM (Figure 4; Supplementary Table S4). The concentration of 1,000 µM also reduced stem and root biomass (Table 2; Supplementary Table S4). However, all tested concentrations of methyl cinnamate reduced both leaf area (Table 2; Supplementary Table S4) and root activity (TTC content) (Table 3; Supplementary Table S4). In addition, the content of MDA in stems was increased at 1,000 µM, whereas the concentration of total proteins in roots was reduced at the same concentration (Table 3; Supplementary Table S4). Methyl cinnamate did not affect root length, protein concentration in stems, or SOD in roots (Figure 4; Table 3; Supplementary Table S4).

In *L. rigidum*, methyl cinnamate reduced stem growth and root activity at 1,000 µM (Figure 4; Table 3; Supplementary Table S4) and concentration of root proteins at 250 and 1,000 µM (Table 3; Supplementary Table S4) and increased stem proteins at 500 µM (Table 3; Supplementary Table S4). There were no significant differences in the remaining parameters evaluated (Figure 4; Tables 2 and 3; Supplementary Table S4).

In wheat, methyl cinnamate stimulated stem growth at 250 µM (Figure 4; Supplementary Table S4) and reduced root SOD activity at 500 and 1,000 µM (Table 3; Supplementary Table S4). However, this chemical compound did not alter any other parameter measured (Figure 4; Tables 2 and 3; Supplementary Table S4).
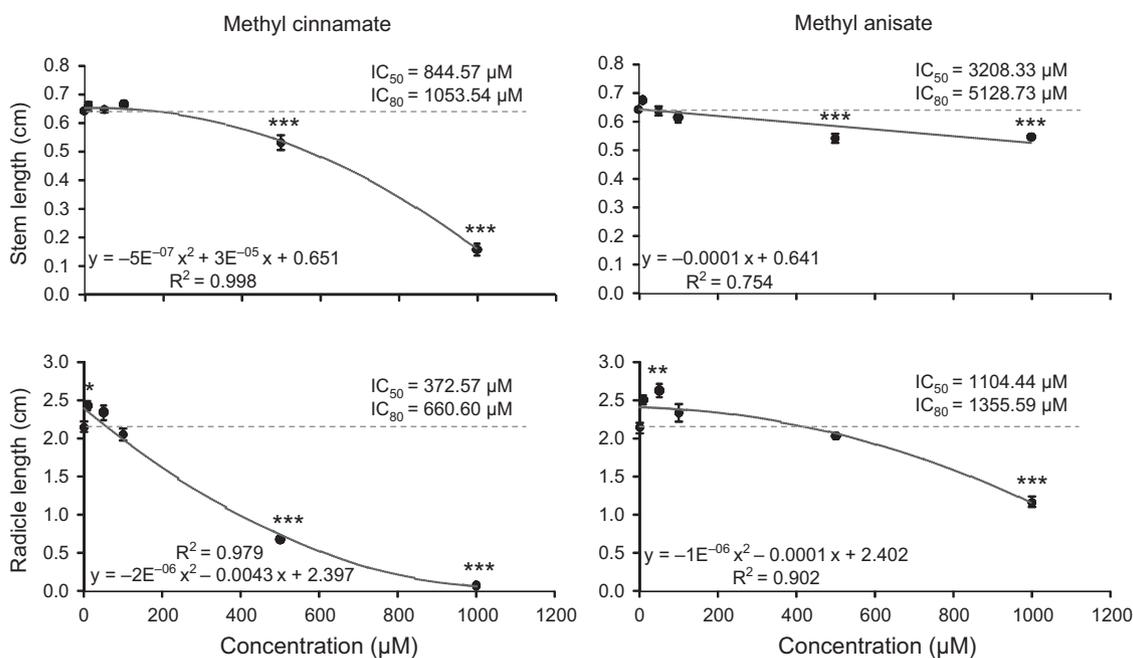
Figure 1. Preliminary bioassay. Percentage values with respect to the control for the stem and radicle lengths of lettuce seedlings in response to application of the commercial formulations of methyl cinnamate and methyl anisate. On the y axis, dashed lines indicate control values. Bars are means ± SE; $n = 6$. Asterisks indicate statistical significance between concentrations and the control treatment according to Tukey's test after general or generalized linear models: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.
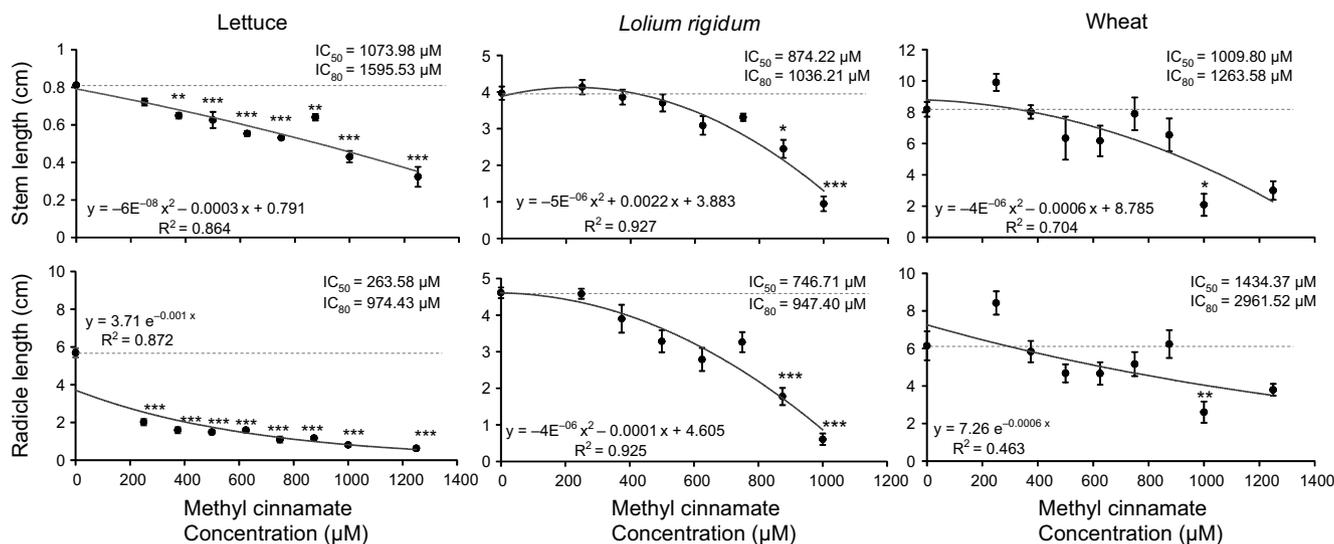
Figure 2. Effects on initial seedling growth. Nonlinear dose–response curves for the stem and radicle lengths of lettuce, *Lolium rigidum*, and wheat seedlings in response to application of the commercial formulation of methyl cinnamate. On the y axis, dashed lines indicate control values. The y axis shows a different scale for each species. In *L. rigidum*, stem and radicle length records at 1,250 μM were very low. Therefore, this concentration was removed from the IC_{50} and IC_{80} estimation for this species. Bars are means ± SE; $n = 6$. Asterisks indicate statistical significance between concentrations and the control treatment according to Tukey's test after general or generalized linear models: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. IC_{50} and IC_{80} indicate the concentration values of methyl cinnamate that cause 50% and 80% of inhibition, respectively.

Comparing the Phytotoxic Effect of Methyl Cinnamate between Seedlings and Well-Established Plants

The phytotoxic effect of methyl cinnamate is more likely to be seen on 0- to 9-d-old seedlings (preliminary bioassay, dose–response assay, and germination process assay) than on older plants (well-established plant bioassay). At the seedling stage, growth was reduced in *L. rigidum* and severely inhibited in lettuce. This effect was supported by the IC_{50} values, which indicated that 50% of seedlings of these two species were negatively affected by

methyl cinnamate, and IC_{50} values were within the assayed range. However, the effect of methyl cinnamate on older plants was only evident for lettuce, with *L. rigidum* and wheat being only marginally affected. Parameters such as lipid peroxidation (MDA) and SOD are related to oxidative stress (e.g., Weir et al. 2004; Yadav and Singh 2013). In our study, methyl cinnamate did not affect MDA and barely influenced SOD in stem and root biomass of well-developed plants. This may indicate that well-established plants treated with this phytotoxin are not very stressed. The fact
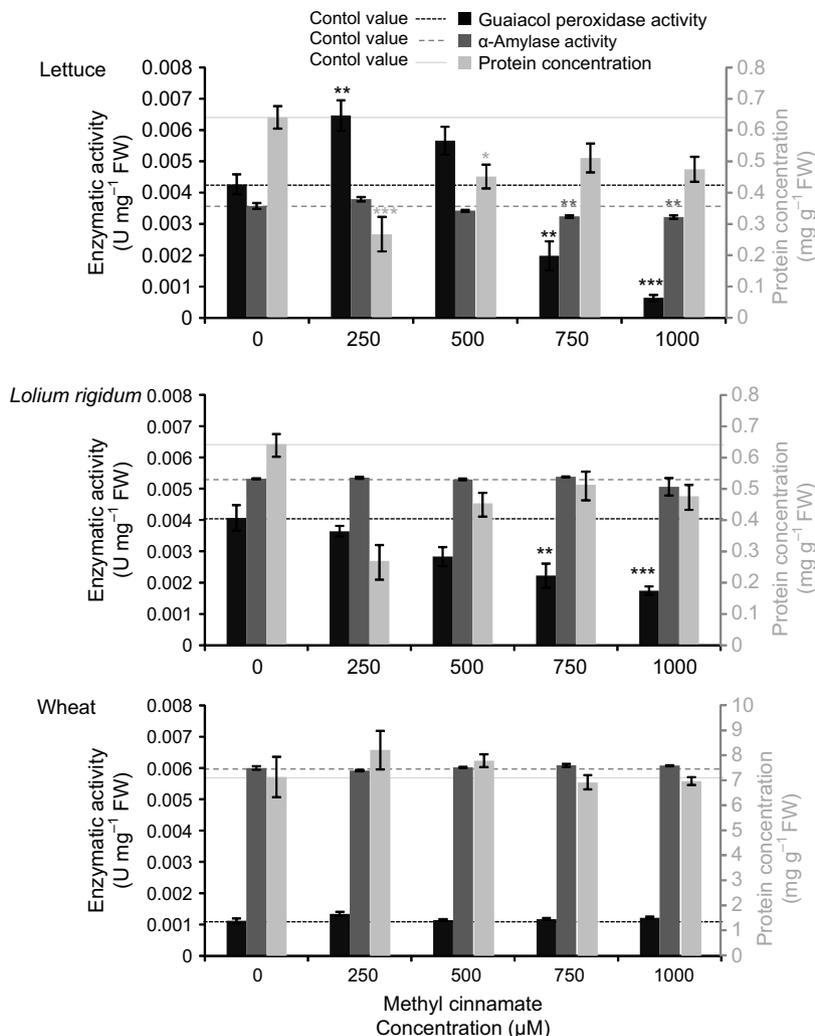
Figure 3. Effects on parameters related to the germination process. Mean ± SE values for guaiacol peroxidase activity, α-amylase activity, and protein concentration in lettuce, *Lolium rigidum*, and wheat seeds in response to the application of the commercial formulation of methyl cinnamate. The *y* axis on the right shows a different scale for each species. *n* = 5. Asterisks indicate statistical significance between concentrations and the control treatment according to Tukey's test after general or generalized linear models or to Nemenyi's test after Kruskal-Wallis analyses: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. FW, fresh weight.
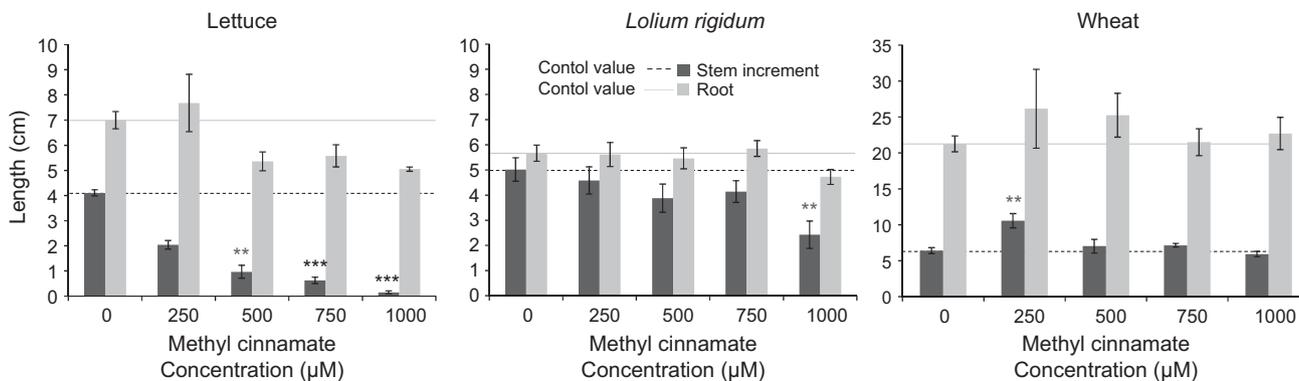
Figure 4. Effects on well-established plants. Mean ± SE values for the stem increment and root length of well-established lettuce, *Lolium rigidum*, and wheat plants in response to application of the commercial formulation of methyl cinnamate. The *y* axis shows a different scale for each species. *n* = 5. Asterisks indicate statistical significance between concentrations and the control treatment according to Tukey's test after general or generalized linear models or to Nemenyi's test after Kruskal-Wallis analyses: **, P ≤ 0.01; ***, P ≤ 0.001.

Table 2. Effects of the commercial formulation of methyl cinnamate on stem biomass, root biomass, and foliar area of well-established lettuce, *Lolium rigidum*, and wheat plants.

Species	Methyl cinnamate concentration	Stem biomass	Root biomass	Foliar area
	µM	————————mg[a]————————		cm^2 per leaf[a]
Lettuce	0	38.92 ± 4.84	4.36 ± 0.45	28.52 ± 2.72
	250	33.16 ± 2.91	3.46 ± 0.56	17.33 ± 1.81 (P = 0.005)*
	500	28.72 ± 3.34	2.82 ± 0.41	8.93 ± 2.89 (P < 0.001)*
	750	25.66 ± 4.80	2.66 ± 0.45	8.92 ± 1.75 (P < 0.001)*
	1,000	19.40 ± 2.74 (P = 0.011)*	2.08 ± 0.14 (P = 0.005)*	3.43 ± 0.71 (P = 0.008)*
Lolium rigidum	0	32.90 ± 5.75	7.10 ± 0.84	2.26 ± 0.30
	250	31.00 ± 4.00	6.60 ± 0.73	2.03 ± 0.30
	500	34.98 ± 6.82	7.66 ± 0.99	2.41 ± 0.34
	750	27.90 ± 5.35	7.24 ± 1.30	1.85 ± 0.40
	1,000	19.90 ± 3.21	4.82 ± 0.55	1.73 ± 0.34
Wheat	0	106.92 ± 12.98	26.13 ± 2.48	23.72 ± 2.39
	250	128.38 ± 37.45	31.56 ± 7.21	30.80 ± 8.14
	500	120.82 ± 10.28	30.36 ± 3.36	26.27 ± 1.62
	750	123.98 ± 12.53	27.08 ± 2.42	23.26 ± 1.93
	1,000	115.78 ± 4.09	27.72 ± 2.55	18.12 ± 0.87

[a]Means ± SE are shown. n = 5. Asterisks (*) indicate statistical significance between concentrations and the control treatment according to Tukey's test after general or generalized linear models.

Table 3. Effects of the commercial formulation of methyl cinnamate on malondialdehyde concentration (MDA) and protein concentration in stems and on superoxide dismutase activity (SOD), triphenyltetrazolium chloride (TTC), and proteins in roots of well-established lettuce, *Lolium rigidum*, and wheat plants.[a]

Species	Methyl cinnamate Concentration	Stem[b]		Root[b]		
		MDA	Proteins	SOD	TTC	Proteins
	—µM—	—nmol g^{-1} FW—	—mg g^{-1} FW—	—U mg^{-1} FW—	—A g^{-1} FW h^{-1}—	—mg g^{-1} FW—
Lettuce	0	73.00 ± 1.47	0.49 ± 0.12	0.023 ± 0.005	3.66 ± 0.23	1.19 ± 0.08
	250	127.60 ± 36.24	0.29 ± 0.09	0.042 ± 0.010	1.88 ± 0.19 (P < 0.001)*	0.81 ± 0.16
	500	88.76 ± 9.78	0.24 ± 0.09	0.043 ± 0.006	1.66 ± 0.09 (P < 0.001)*	0.89 ± 0.26
	750	115.16 ± 9.48	0.38 ± 0.11	0.038 ± 0.006	1.36 ± 0.13 (P < 0.001)*	0.61 ± 0.23
	1,000	138.50 ± 20.92	0.34 ± 0.06	0.018 ± 0.004	1.05 ± 0.11 (P < 0.001)*	0.26 ± 0.07 (P = 0.011)*
Lolium rigidum	0	21.94 ± 2.80	0.69 ± 0.09	0.077 ± 0.005	0.51 ± 0.11	4.12 ± 0.07
	250	31.85 ± 5.56	1.03 ± 0.13	0.087 ± 0.007	0.21 ± 0.05	3.58 ± 0.10 (P = 0.005)*
	500	21.35 ± 2.04	1.08 ± 0.05 (P = 0.05)*	0.082 ± 0.006	0.30 ± 0.05	3.79 ± 0.10
	750	25.97 ± 1.65	1.01 ± 0.04	0.077 ± 0.007	0.29 ± 0.06	4.08 ± 0.11
	1,000	29.41 ± 3.50	0.77 ± 0.11	0.058 ± 0.003	0.04 ± 0.00 (P = 0.002)*	3.72 ± 0.09 (P = 0. 05)*
Wheat	0	82.47 ± 16.35	2.71 ± 0.94	0.072 ± 0.006	7.69 ± 1.31	2.81 ± 0.35
	250	53.01 ± 6.55	2.97 ± 0.95	0.066 ± 0.003	6.51 ± 0.78	3.34 ± 0.37
	500	50.12 ± 4.52	5.43 ± 0.62	0.048 ± 0.005 (P = 0.012)*	6.08 ± 0.69	3.59 ± 0.09
	750	73.92 ± 17.73	2.35 ± 0.74	0.073 ± 0.004	6.76 ± 1.65	3.67 ± 0.06
	1,000	61.16 ± 4.62	3.86 ± 0.40	0.043 ± 0.004 (P = 0.002)*	5.12 ± 1.29	3.76 ± 0.20

[a]A, absorbance; FW, fresh weight.
[b]Means ± SE are shown. n = 5. Asterisks (*) indicate statistical significance between concentrations and the control treatment according to Tukey's test after general or generalized linear models or to Nemenyi's test after Kruskal-Wallis analyses.

that the germination-related enzymes and seedling stage were more affected by methyl cinnamate than older plants may suggest a potential PRE herbicidal effect instead of POST activity. So, as an example, although the highest concentrations assayed (750 and 1,000 µM) did not affect the total number of germinated seeds, these concentrations highly reduced germination-related enzymes (guaiacol peroxidase activity and α-amylase activity in lettuce; guaiacol peroxidase activity in *L. rigidum*). This may result in an anomalous germination process that leads to inviable seedlings.

Although the effect of methyl cinnamate on well-established plants can be considered marginal in terms of plant biomass, root activity evaluated by the reducing capacity of TTC was affected in two of the three assayed species (lettuce and *L. rigidum*). This parameter is related to aerobic respiration in roots, which is fundamental to proper functioning in sugar regulation, mineral absorption, and water uptake in plants (Onanuga et al. 2012;

Wang et al. 2006). In our study, we cannot conclude whether the effect on root activity of *L. rigidum* finally resulted in depletion of plant growth as observed for lettuce. However, this effect deserves further evaluation, because *L. rigidum* has largely evolved resistance to several herbicides (Broster and Pratley 2006; Broster et al. 2011; Chen et al. 2018; Cirujeda and Taberner 2009).

Phytotoxic Effect of Methyl Cinnamate and the Type of Target Species

Previous studies found that methyl cinnamate inhibited germination or growth of both monocotyledonous and dicotyledonous with small seed size such as radish (dicot) (Khanh et al. 2008), *L. rigidum* (monocot) (Vasilakoglou et al. 2013), and lettuce (dicot) (Fujiwara et al. 2017), although the effect may not be compared between species due to different concentrations used. However,

this phytotoxin did not affect the germination of chick pea (*Cicer arietinum* L.) (Ramirez et al. 2018), a dicot with large seed size. In our study, lettuce was the most affected by methyl cinnamate at all evaluated stages, followed by *L. rigidum*, which was affected during the germination process and initial growth at higher concentrations. Wheat (monocot with medium-sized seed), however, was negligibly affected by this compound. This may suggest a potential herbicidal effect of methyl cinnamate on both monocot and dicot with small seeds, but it is ineffective for species with large ones. The concentrations of methyl cinnamate that had a negative effect on *L. rigidum* did not reduce wheat growth. This suggests that methyl cinnamate could potentially be used as a selective herbicide for *L. rigidum* control in wheat. However, further studies on the germination process and early growth are required to evaluate the herbicide potential of methyl cinnamate under field conditions.

In conclusion, methyl cinnamate was more phytotoxic than methyl anisate. Our results showed that the application of methyl cinnamate might be more effective during the germination process and early growth of both monocot and dicot species with small seeds. Methyl cinnamate could potentially be used as a selective herbicide for *L. rigidum* control in wheat. However, further studies are required to assess the effectivity of methyl cinnamate under field conditions. The inhibitory effect of methyl cinnamate on seeds and seedlings may indicate a putative herbicidal effect of this compound and a potential use for *A. dealbata* flowers, contributing to the management of this invasive species.

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