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Comparison Study of Allelochemicals and Bispyribac-Sodium on the Germination and Growth Response of *Echinochloa crus-galli* L.

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Abstract

The phytotoxic effects of two allelochemicals (*trans*-cinnamic acid and syringaldehyde) at different concentrations (1000, 100, 10, and 1 µM) on seed germination, seedling growth, and physiological and biochemical changes of *Echinochloa crus-galli* L. were tested by comparison to a commercial herbicide 'Nominee' (that is, 100 g/L bispyribac-sodium). *trans*-Cinnamic acid and the herbicide inhibited seed germination completely at 100 µM, whereas for syringaldehyde, complete inhibition required 1000 µM. However, with 100 µM syringaldehyde, the seed germination of the test species was 53% of the control. Allelochemicals and the herbicide delayed seed germination and significantly affected the speed of germination index (*S*), speed of cumulative germination index (AS), and coefficient of germination rate (CRG). The roots were more affected when nutrients were not added to the growth bioassay. In general, with the increasing concentration of allelochemicals from 100 to 1000 µM, the inhibitory effects increased. Via microscopy analysis, we found leaf blade wilting and necrosis at concentrations above 100 µM in allelochemical-treated plants. Roots of *E. crus-galli* treated with 1000 µM allelochemicals had black points on root nodes but had no root hairs. The anatomy of roots treated with allelochemicals (1000 µM) showed contraction or reduction of root pith cells as well as fewer and larger vacuoles compared to the control. The allelochemicals also showed remarkable effects on seedling growth, SPAD index, chlorophyll content, and free proline content in a pot culture bioassay, indicating that *trans*-cinnamic acid and syringaldehyde are potent inhibitors of *E. crus-galli* growth and can be developed as herbicides for future weed management strategies.

Keywords Allelochemicals · Herbicide · *Echinochloa crus-galli* · Phytotoxicity · Germination indices · Growth inhibition · Weed management

Introduction

Weeds have been a serious problem since the beginning of crop cultivation. In fact, in agricultural lands, weeds cause large reductions in crop yield and quality, increase the time and costs involved in crop production, interfere with harvesting, and create problems in animal feeding (including poisoning) and livestock management, among other issues (Kraehmer and Baur 2013). Many strategies have been developed for controlling weeds, including hand or mechanical weeding, smothering with mulch, lethal wilting with high heat, burning, and the least expensive and most popular strategy, chemical attack with herbicides (weed killers). Unsurprisingly, weed management in current agriculture relies on herbicides because they are highly effective (Senseman 2007). However, the extensive use of herbicides to manage weeds has resulted in the emergence of herbicide resistance

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among target weeds as well as a host of health risks, particularly their ability to kill placental and umbilical cord cells. In addition, negative environmental and ecological effects occur from the use of herbicides. Therefore, in recent years, there has been considerable desire to reduce herbicide use and search for alternative ways to control weeds (Ackerman et al. 2014). The biochemicals, molecules or secondary metabolites released by a plant species or organism that influence the germination, physiology, growth, survival, and reproduction of other plant species or organisms are known as allelochemicals. Allelopathy, the study of biochemical plant–plant interactions based on biochemicals, molecules or secondary metabolites, including positive and negative effects of biological and agricultural systems (IAS 1996), is a promising option for weed management. Tens of thousands of secondary metabolites of plants have been identified, and some of these natural products show inhibitory activity on other plants (Macias et al. 2007). Thus, allelochemicals may help to overcome weed problems through the use of allelopathic crop varieties or use of natural (from plants or microbes) or synthetic-derivative phytotoxin plant growth inhibitors (Macias et al. 2000).

Considering both ecological and economic perspectives, natural products may provide clues to develop new herbicide chemicals through modifications that could be more active, selective, persistent or cost effective. Cinmethylin, a derivative of 1,4-cineole, a strong growth inhibitor, is a good example of a herbicide that was developed using this approach (Haig 2008). Another good example, leptospermone from *Callistemon citrinus*, which was initially found to be too weak to use as an herbicide, was transformed to be more effective via chemical synthesis into mesotrione (trade name Callisto), which is used as a commercial selective herbicide for maize (Bhowmik and Zhang 2003; Cornes 2005). Moreover, some allelochemical inhibitory activities are similar to herbicides, and their features allow them to be treated as bio-herbicides (Soltys et al. 2013). Therefore, there is a wide scope for use of plant-based herbicides in integrated management of weeds. Although naturally released allelochemicals have low bioactivity, less specificity and wide inconsistency compared to herbicides, they have different modes of action and have a short half-life as they are biodegradable. Hence, they perform better in agroecosystem, and the receiver may not easily adapt or develop resistance against them. Therefore, allelochemicals are considered to be environmentally and toxicologically safer than synthetic compounds (Bhowmik and Inderjit 2003).

Each year, natural product chemists isolate and identify hundreds of phenolics, alkaloids, terpenoids, polyacetylenes, fatty acids, and steroids from higher plants and microbes as allelochemicals (Inderjit et al. 2008). However, because a chemical can be extracted from a plant does not imply that it is released from the plant naturally, but rigorous studies are required to demonstrate allelopathy (Duke 2015). Regrettably, often, only a minute amount of a potential compound can be purified from a natural source, and it is even more difficult to prove the incidence of allelopathy. Therefore, a synthetic source is essential to sufficiently study the agent's mode of action as an allelochemical (Vyvyan 2002).

We previously investigated the Bangladesh indigenous rice 'Boterswar' as an allelopathic variety and reported four biologically active compounds along with syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) as an allelochemical (Masum et al. 2018). It is important to evaluate the degree of herbicidal or biological activities of these compounds for developing natural herbicides from this rice. As the isolated amount was too low to identify the mode of action, commercially available syringaldehyde was compared to another allelochemical, *trans*-cinnamic acid, and bispyribac-sodium (Nominee) to develop an understanding of rice allelopathy and the phytotoxicity of the allelochemicals. Seeds of *E. crus-galli* were used in a bioassay as *E. crus-galli* has superior biology and tremendous ecological adaptations and is one of the top 15 herbicide-resistant weeds in the world. Its proliferation seriously impacts rice production and can result in major losses in rice yield (Khanh et al. 2008). Thus, the present investigation was undertaken to evaluate the degree of the allelochemical interactions involved in this rice, their modes of action and biochemical or physiological changes of the receptor. This will allow us to develop new strategies in developing natural herbicides.

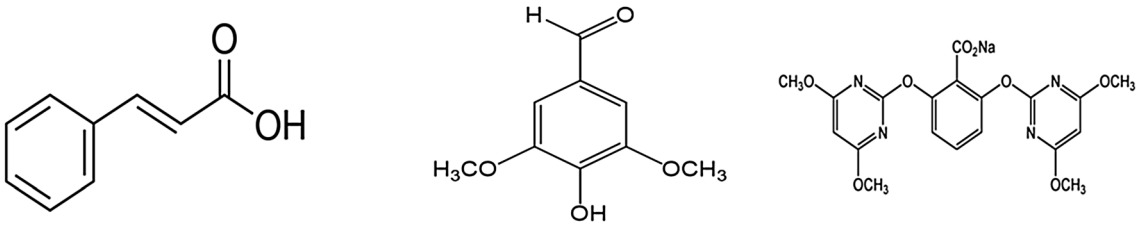
Materials and Methods

Plant Materials

According to Xuan et al. (2016), unfilled and immature seeds of *E. crus-galli* were screened by suspension in tap water. The remaining seeds were hermetically stored (− 20 °C) after air-drying. Stored seeds were sterilized with 1% sodium hypochlorite for 30 min and rinsed with distilled water before use. The germination percentage was randomly checked and was found to be above 80%.

General Procedure

The experimental procedure involved testing the phytotoxic effects of *trans*-cinnamic acid, MW 148.161 g/mol (Wako Pure Chemical Industries, Ltd. Osaka, Japan); syringaldehyde, MW 182.175 g/mol (Toronto Research Chemicals, Inc. Ontario, Canada); and bispyribac-sodium, MW 452.355 g/mol (Kumiai Chemical Industry Co. Ltd. Tokyo, Japan) (Fig. 1) on the seed germination and seedling growth of *E. crus-galli*.



trans-Cinnamic acid
Chemical formula: $C_9H_8O_2$
Molecular weight: 148.161

Syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde)
Chemical formula: $C_9H_{10}O_4$
Molecular weight: 182.175

Bispyribac-sodium
Chemical formula: $C_{19}H_{17}N_4NaO_8$
Molecular weight: 452.355

Fig. 1 Chemical structures of *trans*-cinnamic acid, syringaldehyde, and bispyribac-sodium (Nominee)

Chemical Solutions

To assess the effects of allelochemicals on *E. crus-galli*, stock solutions of test allelochemicals were prepared in a 1 M dimethyl sulfoxide solvent, from which different aqueous solutions of 1000, 100, 10, and 1 µM were prepared with the pH (6.0) adjusted by NaOH (Jose and Gillespie 1998). The herbicide solutions were prepared in distilled water at the same concentrations. These solutions were stored at 4 °C until use. In all experiments, distilled water along with a 1 M dimethyl sulfoxide solvent was used as the control (Reigosa and Pazos-Malvido 2007).

Germination Bioassay

Fifty seeds of *E. crus-galli* were placed on Whatman 2 MM paper in a Petri dish (9 cm), and 4 mL of a treatment solution or control was added. They were then placed in a thermostatically controlled incubator (total darkness at 25 °C) to germinate. Every 48 h, one milliliter of each solution was added per Petri dish. Germination was assessed (rupture of seed coats and the emergence of radicle, Mayer and Poljakoff-mayber 1963) every 12 h until no further seeds germinated. The total germinated seeds (%) were calculated from the cumulative germination data after 1 week (Weidenhamer et al. 1987). Treatments were replicated four times. The same data were then used to calculate and compare different indices. Four germination indices were selected because of their common use in germination studies and were calculated as proposed by Chiapusio et al. (1997).

Growth Bioassay

Glass beakers (500 mL volume, 12 cm depth, 9 cm diameter) containing 30 mL of 0.3% water agar without nutrients and containing 0.5 × Murashige and Skoog salts, 1 × Gamborg's B5 vitamins, 1% sucrose (w/v), and 2% Gelrite (w/v) adjusted to pH 6 were autoclaved (HMC EUROPE HG-50/HG-80, Tuessling, Germany). Ten uniformly pre-germinated seeds of *E. crus-galli* were placed and watered with 3 mL of solution or control treatments. The beaker was enclosed with parafilm and kept in the growth chamber with a daily light/dark cycle of 12/12 h, $3.56 \pm 0.16 \times 10^3$ lx fluorescent light intensity and temperature cycle of 25 °C/25 °C. An additional one milliliter of each solution was added every 48 h. The germination period of *E. crus-galli* seeds ranges from 5 to 12 days (Vengris et al. 1966). After finishing this stage, the embryo becomes a seedling and takes foods from the media by roots. Therefore, root and shoot growth were measured 6 days after starting the test without nutrients and 14 days after starting the test with nutrients (Reigosa and Pazos-Malvido 2007) to evaluate the degree of effects of trans-cinnamic acid, syringaldehyde and bispyribac-sodium.

Growth Chamber Culture Bioassay

After 36 h of soaking, uniformly pre-germinated seeds were placed in seedling trays (25-by-25-by-5 cm; two seeds per hole) filled with commercial potting mixture. All trays were placed in the glasshouse (12-h photoperiods; light intensity 21.5 ± MJ/m^2/day; 20–35 °C temperature and 70–80% relative humidity), and the seedlings were watered with tap water daily until use. After 12 DAS (days after sowing), uniform seedlings were transferred in conical flasks (200 mL, one seedling) containing Hoagland solution (250 mL; pH 5.5 and EC 1.2 ms/cm) and placed in a growth chamber under controlled conditions as previously described, and after 24 h, the allelochemicals, herbicide, and control were added as per each treatment. The conical flasks were kept for another 5 days (18 DAS) in the same environment as previously described, and at 24-h intervals, the solution level of the conical flasks was maintained by adding Hoagland nutrient solution. At the end of the experiment, the morphology of the leaves and roots of the bioassayed species as well as the root tip excised were observed under a microscope (Leica Microsystems LAS X). Thin sections (18 µm), cut with a

diamond knife on a Supernova microtome, were examined using a microscope (Leica Microsystems LAS X).

Glasshouse Pot Culture Bioassay

A glasshouse pot (Wagner pot, 0.02 m^2) experiment was conducted to evaluate the effect of the allelochemicals and herbicide on *E. crus-galli*. Each pot was filled with 4 kg of gray soil (coarse sand 3.61%, fine sand 30.94%, silt 24.32%, clay 32.84%, apparent density 0.90 g/cm^3, pH 7.43, C 0.96%, N 0.12%, P 4.60 µ/g soil, K 42.89 µ/g soil, Ca 2604.15 µ/g soil, Mg 279.30 µ/g soil, S 2765.07 µ/g soil, Fe 0.16 µ/g soil, Na 102.36 µ/g soil, and Al 5.42 µ/g soil). *E. crus-galli* seedlings were raised as previously described, and at the three-leaf stage (12 DAS), one seedling per pot was transplanted. After 5 days (17 DAS), the pots were randomly divided into four groups as per the treatments and irrigation was stopped. Tween®20 (Polyoxyethylene Sorbitan Monolaurate) was mixed into the solutions and the control 0.01% to wet the leaves. The treatment solutions (250 mL) and 250 mL of control solutions were applied using a hand sprayer. Six days after the pot treatments, *E. crus-galli* seedlings (23-day-old) were harvested and their height (from the basal node to the end of leaf), tiller number, SPAD index, chlorophyll content and free proline (Pro) were measured.

Chlorophyll Content Determination

Based on the absorbance value, calculations were made using Arnon's (1949) equation, and the amount of chlorophyll (Chl) *a* and Chl *b* were estimated. Fully expanded leaves (0.5 g) were removed and then homogenized with an ice cold mortar and pestle using 80% acetone as the extraction buffer. The samples were then centrifuged at 0–4 °C using a rotor with a speed of 15,000 rpm for 10 min. The absorbance of the supernatant was measured at 480, 645, and 663 nm in a spectrophotometer-UV-1700 (Shimazdu, Japan).

The concentrations of chlorophyll *a* and chlorophyll *b* were calculated using the following equations:

$$\text{Chl } a = \frac{\left(12.7 \times A_{663} - 2.6 \times D_{645}\right) \times \text{Volume of 80\% acetone}}{1000 \times \text{Weight of leaf sample } (g)} \text{ mg/g fresh weight}$$

$$\text{Chl } b = \frac{\left(22.9 \times D_{645} - 4.68 \times D_{663}\right) \times \text{Volume of 80\% acetone}}{1000 \times \text{Weight of leaf sample (g)}} \text{ mg/g fresh weight}$$

Proline Determination

The proline (Pro) content was appraised according to the method of Bates et al. (1973). A 0.5 g sample from an upper fully expanded fresh leaf was homogenized in 5 mL of 3% sulfosalicylic acid, and the homogenate was filtered for use as an extract solution for extermination of the Pro content. Two milliliters of the filtrate was mixed with 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent, and the solution was heated at 100 °C for 1 h. After the solution cooled, 4 mL of toluene was added and it was then transferred to a separating funnel. Toluene-containing chromophores was separated and adjusted to the absorbance value at 520 nm with a spectrophotometer (UV-1700; Shimadzu Co., Ltd.). The concentration of Pro was estimated using a standard curve from the known concentration of Pro.

Statistical Analysis

Germination and growth bioassay experiments were repeated six times using a completely randomized design with four replications, and the data were compared with respect to the controls. Growth chamber and glasshouse pot culture bioassay experiments were repeated three times with three replications using a completely randomized design. All statistical comparisons were analyzed using Fisher's Protected Least Significant Difference test with the Type I error (0.05).

Results

In all bioassay experiments, the organic solvents used to dissolve the allelochemicals had no significant effects.

Effects of Allelochemicals on Germination at Each Exposure Time

The two allelochemicals and herbicide used, *trans*-cinnamic acid, syringaldehyde and Nominee, acted differently (Table 1), and the allelochemicals showed significant inhibitory effects on seed germination. At concentrations of 1000 and 100 µM, *trans*-cinnamic acid and the herbicide induced complete inhibition of germination, whereas syringaldehyde induced complete inhibition at 1000 µM. However, at 100 µM, syringaldehyde induced delayed germination. At lower concentrations, the allelochemicals had no significant effect on the germination of *E. crus-galli*.

Effects of the Allelochemicals on Germination Based on the Calculated Indices

The calculated indices are provided in Table 2. Although the four indices were calculated using the same data, they provided different results with low variability. Total germination, G_T, is a commonly used index that is affected by treatments at the highest concentrations. *trans*-Cinnamic

Table 1 Effects of different concentrations of *trans*-cinnamic acid, syringaldehyde and herbicide on the germination of barnyardgrass seeds every 12 h for 84 h

Treatment and concentration (μM)	Exposure time (h)				
	36	48	60	72	84
Control	4.33 ± 0.58	8.33 ± 1.53	13.67 ± 2.08	38.67 ± 3.06	40.00 ± 2.00
trans-Cinnamic acid					
1000	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
100	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
10	1.67 ± 0.58*	8.00 ± 1.00	10.33 ± 1.53*	28.67 ± 5.86*	29.67 ± 5.77*
1	3.00 ± 1.00*	8.33 ± 1.53	12.67 ± 2.52	38.33 ± 2.08	39.00 ± 3.00
Syringaldehyde					
1000	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
100	0.00 ± 0.00*	0.00 ± 0.00*	3.00 ± 1.00*	16.00 ± 2.00*	21.33 ± 3.06*
10	2.33 ± 0.58*	7.33 ± 1.53	13.00 ± 3.00	32.33 ± 2.52*	32.67 ± 2.52*
1	4.33 ± 0.58	6.33 ± 1.53*	12.67 ± 3.06	40.33 ± 2.89	41.00 ± 1.73
Herbicide					
1000	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
100	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
10	0.00 ± 0.00*	6.33 ± 1.53*	11.33 ± 0.58	29.33 ± 3.06*	29.33 ± 3.06*
1	3.67 ± 0.58	9.00 ± 2.00	15.67 ± 2.52	35.33 ± 3.51	35.33 ± 3.51*
$LSD_{5\%}$	0.76	1.90	2.91	4.38	4.33

*Significant differences between the control and treatment: $P < 0.05$, according to Fisher's LSD Test

acid and the herbicide completely inhibited germination at a concentration greater than 100 μM, whereas for syringaldehyde, 53% of the control germinated and demonstrated delayed germination. The herbicide proved to be the most deleterious and strongly inhibited the G_T of *E. crus-galli*. At very low concentrations, the allelochemicals had no significant effect on G_T. The allelochemicals and herbicide delayed *E. crus-galli* germination at concentrations of 1000, 100, and 10 μM and significantly affected the speed of the germination index (*S*) and the speed of the cumulative germination

Table 2 Effect of various concentrations of *trans*-cinnamic acid, syringaldehyde, and herbicide on germination indices

Treatment and concentration (μM)	Index			
	G_T	*S*	AS	CRG
Control	80.00 ± 4.00	15.74 ± 0.93	36.84 ± 2.66	16.75 ± 0.08
trans-Cinnamic acid				
1000	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
100	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
10	59.33 ± 11.55*	11.61 ± 2.01*	27.28 ± 3.83*	16.74 ± 0.16
1	78.00 ± 6.00	15.15 ± 1.11	35.15 ± 2.82	16.65 ± 0.21
Syringaldehyde				
1000	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
100	42.67 ± 6.11*	7.06 ± 1.03*	12.63 ± 1.93*	15.49 ± 0.05*
10	65.33 ± 5.03*	12.86 ± 0.50*	30.53 ± 0.46*	16.73 ± 0.27
1	82.00 ± 3.46	15.83 ± 0.46	36.28 ± 1.11	16.60 ± 0.16
Herbicide				
1000	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
100	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
10	58.67 ± 6.11*	11.17 ± 1.13*	25.86 ± 2.58*	16.48 ± 0.11*
1	70.67 ± 7.02*	14.33 ± 1.31	35.08 ± 3.26	16.93 ± 0.16
$LSD_{5\%}$	8.67	1.52	3.36	0.22

G_T total germination, *S* speed of germination, *AS* speed of accumulated germination, *CRG* coefficient of rate of germination

*Significant differences between the control and treatment: $P < 0.05$, according to Fisher's LSD Test

index (AS). In the case of the coefficient of germination rate (CRG), the allelochemicals at concentrations of 1000 and 100 µM controlled significantly, whereas the herbicide had a significant effect at concentrations greater than 10 µM. At the lowest concentration(1 µM), the two allelochemicals and herbicide had no significant effects on *S*, AS, and CRG.

Root and Shoot Elongation

Figures 2 and 3 present the inhibition effects of the evaluated allelochemicals and herbicide on root and shoot growth of *E. crus-galli* in the absence and presence of nutrients, respectively. All of the chemicals tested in the experiment demonstrated stronger inhibition effects on root growth than on shoot growth at higher concentrations (> 100 µM) in both with- and without-nutrients media, and the effects were more apparent when grown in a without-nutrients condition. At a lower concentration (1 µM), *trans*-cinnamic acid and the herbicide had no significant inhibition effect on seedling growth of *E. crus-galli*, whereas syringaldehyde showed a stimulatory effect on both root and shoot growth.

Morphological Attributes

The allelochemicals dose-dependently slowed or inhibited the growth of *E. crus-galli* seedlings. The bioassay species grown in a concentration of more than 100 µM allelochemicals were considerably smaller compared to the control plants and demonstrated leaf blade wilting and necrosis (Fig. 4) as well as inhibited root and root hair growth (Fig. 5). Visual differences in root systems were also observed. The allelochemicals inhibited the growth and quantity of roots. It was also observed that the toxic effect of *trans*-cinnamic acid and syringaldehyde was manifested as a dark brown discoloration on the root tip and black points on root nodes. On the other hand, in treatments with the herbicide at concentrations greater than 10 µM, lamina necrosis and a dark brown discoloration through the root pith and root tip were observed, but no root hair formation was observed. The lower concentration of allelochemicals either stimulated or did not affect the growth of the receiver species; however, herbicide at a 1 µM concentration showed leaf blade wilting. Microscopic images (Fig. 6) showed that root tip meristem cells treated with the allelochemicals at the highest concentration (1000 µM) demonstrated a significant contraction or reduction of root pith cells and fewer and larger vacuoles compared to the control root (Fig. 6a–c). The herbicide at the same concentration showed similar symptoms along with a black discoloration (Fig. 6d).

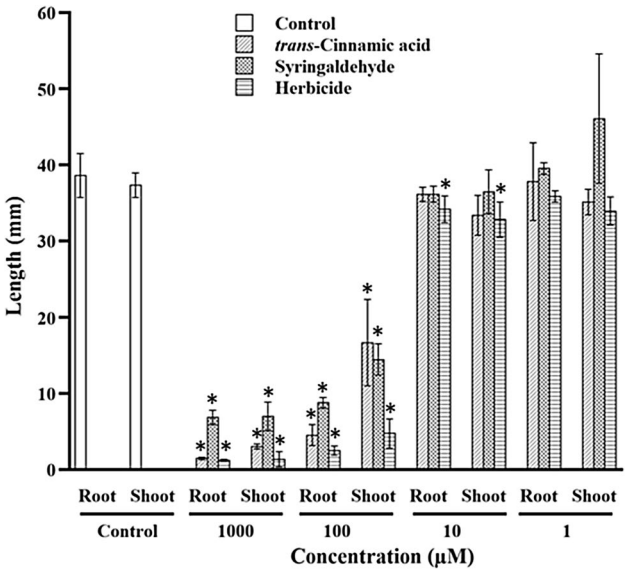


Fig. 2 Effect on root and shoot growth of *E. crus-galli* treated with *trans*-cinnamic acid, syringaldehyde, and herbicide at concentrations from 1000 to 1 µM under without nutrient condition. *Significant differences between the control and treatment: $P < 0.05$, according to Fisher's LSD Test

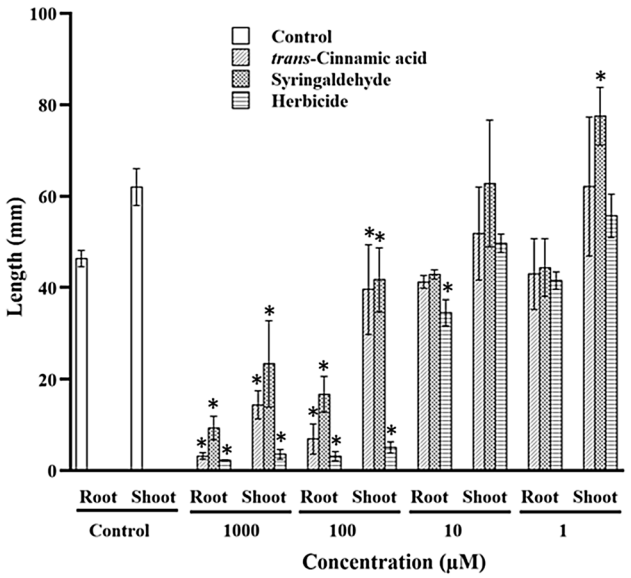


Fig. 3 Effect on root and shoot growth of *E. crus-galli* treated with *trans*-cinnamic acid, syringaldehyde, and herbicide at concentrations from 1000 to 1 µM under with nutrients condition. *Significant differences between the control and treatment: $P < 0.05$, according to Fisher's LSD Test

Effect on the Seedling Growth Parameters in Pot Culture Bioassay

The response of the growth parameters of *E. crus-galli* was significantly affected by different concentrations of the allelochemicals and herbicide (Fig. 7). The strongest inhibitory effects were found for the herbicide (> 10 µM

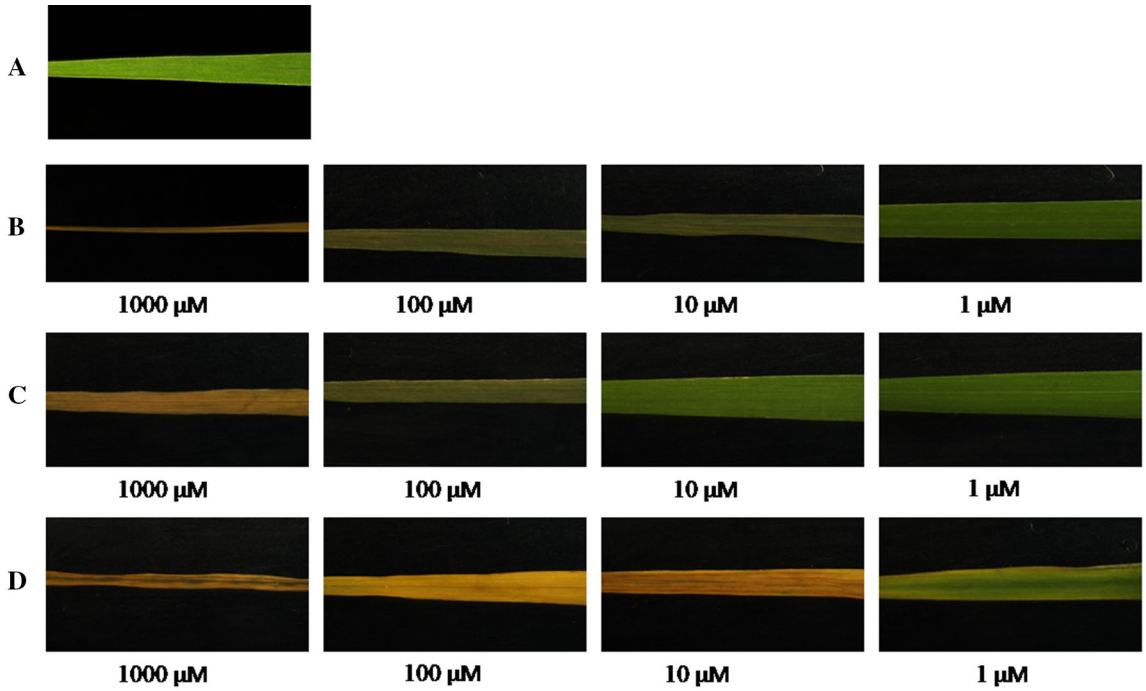


Fig. 4 Effect of control (**a**), *trans*-cinnamic acid (**b**), syringaldehyde (**c**), and herbicide (**d**) at concentrations of from 1000 to1 µM on leaf growth of *E. crus-galli* in a growth chamber bioassay

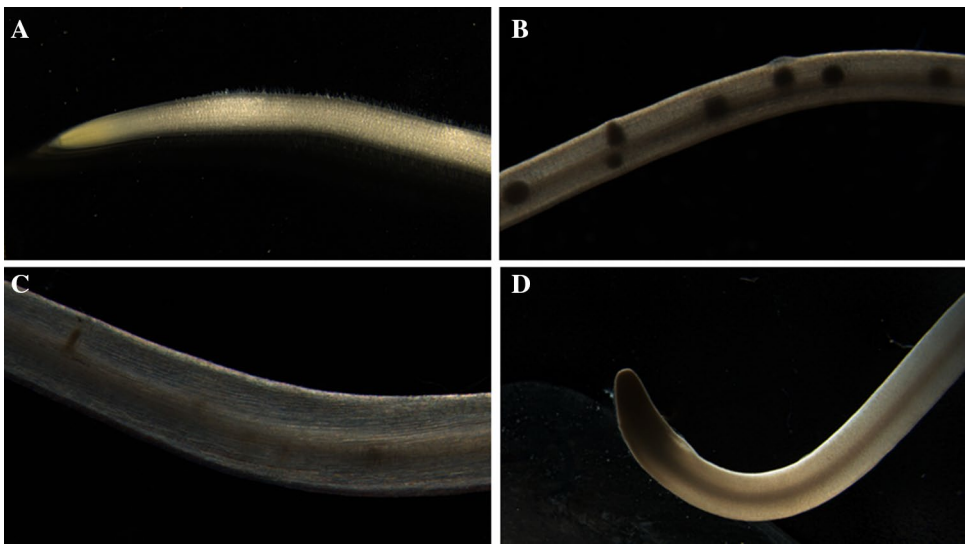


Fig. 5 Effect of control (**a**), *trans*-cinnamic acid (**b**), syringaldehyde (**c**), and herbicide (**d**) at a concentration of 1000 µM on root growth of *E. crus-galli* in a growth chamber bioassay

concentration) on plant height, number of total tillers and SPAD index of *E. crus-galli*, whereas both allelochemicals only showed significant inhibitory effects compared to the control at 1000 µM. At the lower concentration (1 µM), in the case of the allelochemicals, there was no effect or a stimulatory effect was observed.

Effect on Chlorophyll Content

Seedlings grown in the presence of the allelochemicals showed chlorosis when exposed to more than 100 µM, whereas the herbicide negatively affected the Chl content upon exposure to a concentration above 10 µM (Fig. 8). At the lower concentration (1 µM), syringaldehyde slightly enhanced the Chl content. At the 1000 µM concentration, Chl *a* declined by 56, 49, and 69% compared to the control using

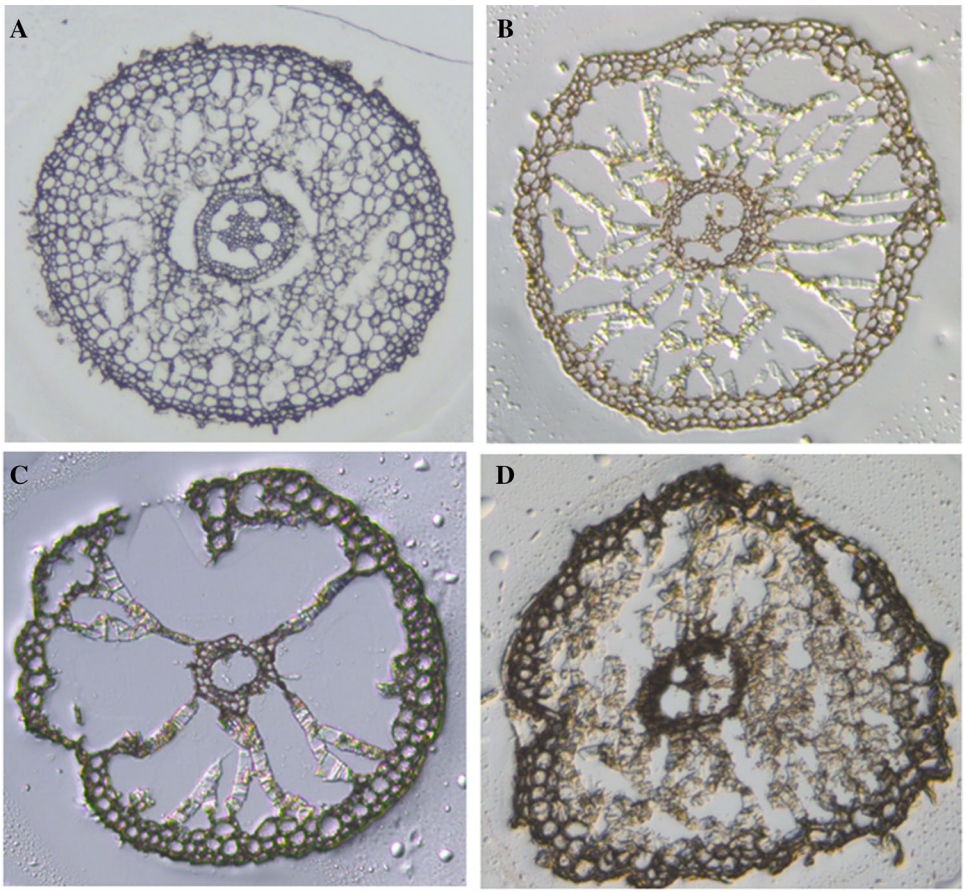


Fig. 6 Effect of control (**a**), *trans*-cinnamic acid (**b**), syringaldehyde (**c**), and herbicide (**d**) at a concentration of 1000 µM on the root anatomy (18 µm) of *E. crus-galli* in a growth chamber bioassay

trans-cinnamic acid, syringaldehyde, and herbicide, respectively, whereas the Chl *b* content decreased at a concentration of more than 10 µM for the herbicide treatments only.

Effect on Pro Content

The effects of the allelochemicals and herbicide on the changes in the Pro content are shown in Fig. 9. Application of the allelochemicals at concentrations of more than 100 µM ameliorated the Pro content compared to the control, and all of the herbicide treatments showed considerable variation compared to control plants.

Discussion

A set of biochemical, physiological and morphological changes take place in a well-defined sequence during the seed germination process (Bentsinka and Koornneef 2008), which can be interrupted by a biochemical reaction or an excess or deficiency of a compound. Therefore, germination bioassays are effective tools to evaluate the effect of any exogenously applied compound from a natural or artificial source (Hoagland and Williams 2003). Germination of *E. crus-galli* was inhibited by test solutions in a dose-dependent approach. In general, increasing concentrations resulted in a greater reduction of the germination percentage and also influenced the average germination time. The germination pattern (speed and synchrony) was also modified by allelochemical activity. Seed germination inhibited or delayed by allelochemicals has been reported in many plant studies (Gniazdowska and Bogatek 2005; Santana et al. 2006; Reigosa and Pazos-Malvido 2007; Hussain et al. 2008; Grisi et al. 2015; Oliveira et al. 2016). Seedling growth of *E. crus-galli* was affected to a great extent by the allelochemicals compared to seed germination, and the sensitivity of the root was more susceptible compared to that of the shoot because the permeability of the allelochemicals in root tissue is greater than in shoot tissue and the root absorbs the allelochemicals first (Nishida et al. 2005). A similar pattern of growth and development inhibition was also reported by Escudero et al. (2000). The without-nutrients condition inhibitory effects on seedling growth were very apparent, and the with-nutrients condition required a higher concentration to inhibit growth. Reigosa and Pazos-Malvido (2007) also found similar results and explained that this result may be due to the synergic effect of nutrient limitation with phytotoxicity. Belz and Hurle (2004) also observed

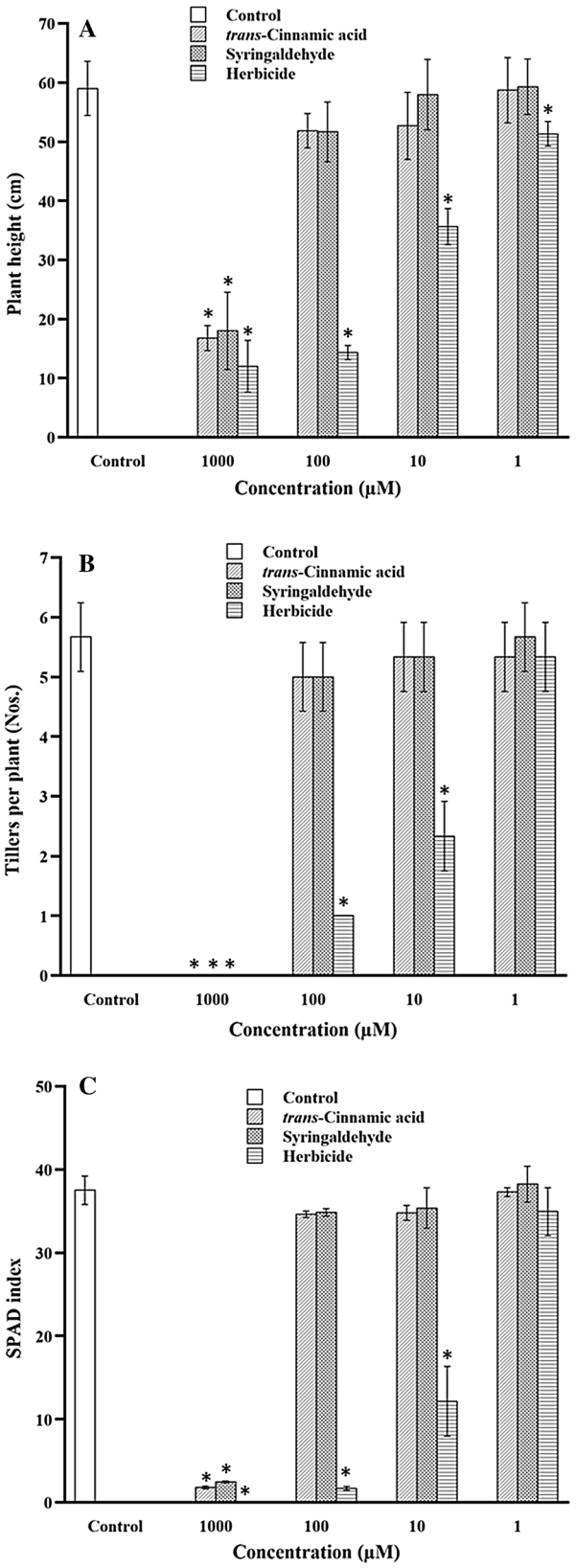


Fig. 7 Effect on plant height (**a**), tillers number plant (**b**), and SPAD index (**c**) of *E. crus-galli* treated with *trans*-cinnamic acid, syringaldehyde, and herbicide at concentrations from 1000 to 1 µM in a pot culture bioassay. *Significant differences between the control and treatment: $P < 0.05$, according to Fisher's LSD Test

that nutrient limitation increased the inhibition activities of allelochemicals. The phytotoxic effect of allelochemicals on the bioassay species was evident by lamina wilting, chlorosis, and necrosis. *trans*-Cinnamic acid and syringaldehyde may inhibit Chl biosynthesis, thereby causing retardation of the growth of the weed. This finding was in agreement with the findings of Sanchez-Moreiras and Reigosa (2005), who reported inhibitory effects of BOA on *Lactuca sativa* L plants. In this study, the allelochemicals slowed or stopped the growth of *E. crus-galli* seedling roots and suppressed the growth of root hairs dose-dependently. The root anatomy study demonstrated that there was contraction or reduction of root pith cells as well as fewer and larger vacuoles of root meristem. Similarly, widened and shortened root cells, damaged cell walls, an increase in both the size and number of vacuoles, cell autophagy, disorganization of organelles, reduced intercellular communication, and inhibited formation of root hairs by allelochemicals have been found in many plant studies (Liu and Lovett 1993; Kaur et al. 2005; Grana et al. 2013). It is often proposed that allelochemicals reduce cell division in the apical meristem (Sanchez-Moreiras et al. 2008) and strongly inhibit mitosis and/or disrupt organelle structure, for example, of the nuclei and mitochondria (Gniazdowska and Bogatek 2005). The reduction of *E. crus-galli* height and biomass in the greenhouse experiment indicated the inhibition potentiality of the allelochemicals as these compounds directly affect many physiological and biochemical reactions and therefore influence growth (Weir et al. 2004; Gniazdowska and Bogatek 2005; Lara-Nunez et al. 2006). Uddin et al. (2012) also observed burning and growth inhibition at 2–3 days after treatment with sorgoleone in sensitive species (*Rumex japonicas* Houttuyn, *Galium spurium* L. and *Aeschynomene indica* L.). Among the physiological effects caused by the allelochemicals, disturbance of photosynthesis is frequently observed (Gniazdowska and Bogatek 2005). Chlorophylls are the base component of pigment protein complexes, which are essential for photosynthesis. Any changes in the chlorophyll content are expected to bring about changes in photosynthesis (Reigosa et al. 2006). Because plant dry matter production depends on the Chl content (Buttery and Buzzell 1977), any diminution of the leaf Chl content would limit net photosynthesis and thus reduce total plant growth. Therefore, precise determination of Chl *a* and Chl *b* can provide a scientific basis for the plant growth state as they play a significant role in the plant growth process and are the key points of implementing accurate agriculture (Dong et al. 2008). In allelochemical-treated

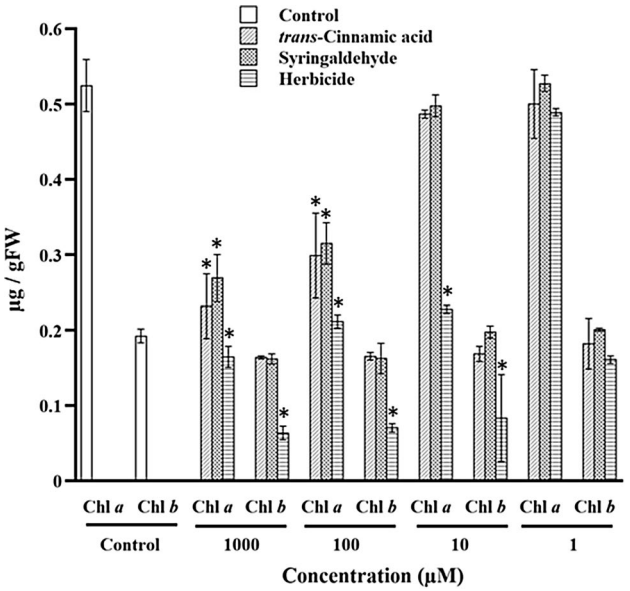


Fig. 8 Effect of *trans*-cinnamic acid, syringaldehyde, and herbicide at concentrations from 1000 to 1 µM on the chlorophylls *a* and *b* contents of *E. crus-galli* in a pot culture bioassay. *Significant differences between the control and treatment: $P < 0.05$, according to Fisher's LSD Test

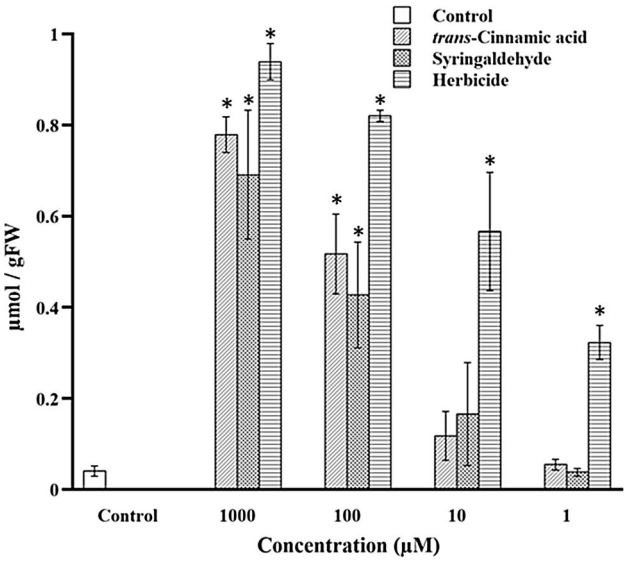


Fig. 9 Effect of *trans*-cinnamic acid, syringaldehyde, and herbicide at concentrations from 1000 to 1 µM on the free proline content of *E. crus-galli* in a pot culture bioassay. *Significant differences between the control and treatment: $P < 0.05$, according to Fisher's LSD Test

plants allelochemicals may act in three ways: inhibit Chl synthesis, stimulate Chl degradation, or both (Zhou and Yu 2006). The Chl content of *E. crus-galli* was dependent and dramatically affected by the allelochemical concentration. In our study, we found that lower concentrations of *trans*-cinnamic acid and syringaldehyde stimulated the Chl content, whereas higher concentrations produced inverse effects. Baziramakenga et al. (1994) also reported that high concentrations of allelochemicals (benzoic acid and *trans*-cinnamic acid) caused a reduction in the leaf Chl content of soybean, whereas a lower concentration promoted it. Meazza et al. (2002) found that allelochemicals reduce the key enzyme of the receiver for plastoquinone synthesis of *p*-hydroxyphenylpyruvate dioxygenase (HPPD). Inhibition of this enzyme interrupts the biosynthesis of carotenoids and results in foliar bleaching. Phytotoxic effects of allelochemicals are termed 'allelochemical stress' (Lara-Nunez et al. 2006). Allelochemicals can induce accumulation and increase synthesis of compatible osmolytes as stress proteins, such as Pro (Duran-Serantes et al. 2002). Pro accumulation could be due to de novo synthesis, decreased degradation or both (Lattanzio et al. 2009). As our results were well correlated with the results of growth and photosynthesis inhibition, we proposed to use Pro as a stress indicator to measure the effects produced by the allelochemicals and observed that Pro increased with the increasing concentration of allelochemicals, which may mitigate the deleterious effect of stress in *E. crus-galli* seedlings. This could be due to the generation of specific proteins in response to the oxidative damage caused by allelochemical stress (Mishra et al. 2006; Araniti et al. 2017). Djanaguiraman et al. (2005) reported that allelochemicals from *Eucalyptus* sp. leaves have an increased Pro content in receivers. Thapar and Singh (2006) also noted an induction of the Pro content in the leaves of *Parthenium hysterophorus* treated by leachate leaves of *Cassia tora*. Similar findings were also reported by Reigosa et al. (2001) and Duran-Serantes et al. (2002).

The overall observation of the germination and growth reduction in the test weed species at high concentrations of allelochemicals was inconsistent with the control treatments and provides support for the hypothesis that there is the allelochemicals cause a chemical interference, and in most cases, the results demonstrate the concentration-dependent phytotoxicity concept. Therefore, these studies may provide an understanding of allelochemical interactions and may help to distinguish the mechanisms involved in plant interference. In general, allelochemicals are less active than commercial herbicides, but they can be naturally released in crop fields through the development of allelopathic varieties of crops for weed management. Our results also confirm the phytotoxicity of syringaldehyde. However, the suppressive ability of syringaldehyde should be tested in other weeds as well.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to declare.

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