INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
15–468/2016/18–4–698–702
DOI: 10.17957/IJAB/15.0153
http://www.fspublishers.org

Full Length Article

Identification and S-metolachlor-safening Effects of Compounds Extracted from *Ligusticum chuanxiong* on Rice

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Abstract

In this study, the effects of *Ligusticum chuanxiong* extract as herbicide safeners were determined on glutathione-*S*-transferase (GST) activity of rice crop. Two main extract compounds, Z-ligustilide and senkyunolide A, were identified by high performance liquid chromatography and liquid chromatography-mass spectrometry. In both bioassays conducted in agar and soil, the whole *L. chuanxiong* extract, as well as two active components individually were effective in safening the growth of rice seedlings against S-metolachlor toxicity. Z-Ligustilide was a better safener than senkyunolide A, and both compounds were more protective of shoots than roots. After herbicide-inhibited rice seedlings were treated with Z-ligustilide, GST activity significantly increased, suggesting the safening effect of this *L. chuanxiong* extract component involves GST. © 2016 Friends Science Publishers

Keywords: Herbicide safeners; Z-ligustilide; Glutathione-*S*-transferase; S-metolachlor

Introduction

Crop sensitivity to herbicides often causes severe reductions in crop production. Herbicide injury has affected crop growth on more than 50 million acres in China, reducing crop yields by 30–50%. Safeners play a crucial role in protecting crops from herbicide injury and can extend the application of some herbicides to more sensitive crops. Nearly 20 different safeners have been commercialized since the first safener, 1,8-naphthalic anhydride, was reported in 1969 (Kramer and Schirmer, 2007). Most aim to protect monocots from herbicide injury. For example, fenchlorazole-ethyl and mefenpyr-diethyl are used to safen aryloxyphenoxypropionates used on wheat and barley (Cummins *et al.*, 2009) and cloquintocet-mexyl can protect wheat from acetyl-CoA carboxylase inhibition (Brazier *et al.*, 2002; Hofer *et al.*, 2006). Benoxacor can be mixed with chloroacetamides to ensure corn safety (Davies and Caseley, 1999), while dichlormid and AD-67 mixed with acetochlor can achieve similar protection (Ekler *et al.*, 1993; Brazier *et al.*, 2002). Representative safeners protecting sorghum from chloroacetamide injury include fluxofenim, cyometrinil, oxabetrinil, and flurozole (Leif *et al.*, 1987; Bussler, 1996; Gronwald and Plaisance, 1998). Injury to rice caused by chloroacetamides can be alleviated by the safener fenclorim (Wu *et al.*, 1999; DeRidder *et al.*, 2002; Deng and Hatzios, 2002a), while isoxadifen-ethyl protects rice against sulfonylurea, daimuron, cumyluron and dimepiperate herbicide injury (Kramer and Schirmer, 2007). However, the abovementioned safeners are only able to safen one herbicide in a single crop. Bayer-developed isoxadifen-ethyl, on the other hand, takes herbicide safeners to a new level by safening multiple herbicides with various modes of action in multiple crops.

Chloroacetamides are a widely-used herbicide for controlling monocot weeds in Chinese rice fields. For example, pretilachlor is often applied in combination with fenclorim to prevent injury to rice. The chloroacetamide S-metolachlor is highly efficient in controlling monocot weeds, and rarely used in rice fields due to its toxicity to rice seedlings. In this study, we aimed to extend the application of S-metolachlor to rice by identifying a proper safener. Considering natural safeners are more environmentally friendly than synthetic ones, we screened botanical materials for compounds with the ability to safen S-metolachlor for application on rice. Of three plant species validated to protect rice from S-metolachlor injury in our previous study, *Ligusticum chuanxiong* (*L. chuanxiong*) was chosen for use in the current study. A *L. chuanxiong* extract was obtained by CO_2 supercritical fluid extraction (CO_2-SFE) and main active components were identified by high performance liquid chromatography (HPLC) and liquid chromatography-mass

To cite this paper: Li, J., W. Zheng, Y. Wang, H. Cai, C. Jin, X. Liu and L. Bai, 2016. Identification and S-metolachlor-safening effects of compounds extracted from *Ligusticum chuanxiong* on rice. *Int. J. Agric. Biol.*, 18: 698–702

spectrometry (LC-MS). Rice safening assays were conducted using the whole extract and two commercially available *L. chuanxiong* extract active compounds. In particular, potential effect(s) on glutathione-*S*-transferase (GST) activity was investigated with respect to safening against S-metolachlor injury of rice.

Materials and Methods

Materials and Equipment

Sun-dried *L. chuanxiong* was purchased from LBX Pharmacy (Changsha, China). Analytical grade S-metolachlor (97%) was purchased from Nutrichem Laboratory Co. Ltd. (Beijing, China). Z-ligustilide and senkyunolide A were purchased from Yaji Biotechnology Co. Ltd. (Shanghai, China). The Shimadzu HPLC system (Kyoto, Japan) included a SPD-20A Prominence UV/VIS detector, two LC-20AT pumps, a Shimadzu LC solution workstation, and a CNW C_{18} column (4.6 x 250 mm, 5 μm). The Agilent 1100 LC-MSD used was connected to a ZORBAX Extend C_{18} column (4.6 x 250 mm, 5 μm). The CO_2-SFE apparatus (HA231-50-06) included a CO_2-supplying steal bottle, pressure-control system (0-50 MPa), temperature controller (0-95ºC), separator and an extraction tank (Huaan Super Critical Extraction Co. Ltd., Nantong, China). Lastly, a Shimadzu UV mini-1240 spectrophotometer was also used.

L. chuanxiong Extract Preparation

Crushed *L. chuanxiong* (6 kg) was sieved (2 mm pore diameter), dehydrated in a freeze-dryer at -40ºC and subjected to CO_2-SFE as follows: CO_2 extraction was done in a 5 L extraction tank at 50ºC and 35 MPa, followed by separation from CO_2 in a separator at 55ºC and 8 MPa. The extract (0.2 kg) was stored at -20ºC until further analysis.

HPLC and LC-MS Extract Analysis

The LC-MS mobile phase consisted of 6:4 (v/v) 0.1% trifluoroacetic acid and acetonitrile, and the extract (10 μl injection volume) was eluted for 60 min at 1 $mL \cdot min^{-1}$. The extract was analyzed in positive ion mode using an APCI ion source at 120ºC, a nebulizer pressure of 35 psi, 350ºC desolvation and 8 $L \cdot min^{-1}$ dry gas (N_2) flow rate. The relative molecular masses of major components obtained by LC-MS were then matched with known *L. chuanxiong* compounds.

The identification of extract components was verified by HPLC using commercially available purified compounds. The extract was dissolved in ethyl acetate and subjected to HPLC (20 μL injection volume) at 35ºC and 254 nm. The mobile phase consisted of water and acetonitrile, and HPLC was completed in gradient elution mode at a flow rate of 1 $mL \cdot min^{-1}$ as follows: 60 to 52% water for 40 min, 52 to 60% water for 15 min and 60% water for 5 min.

S-Metolachlor Safening Bioassays of Rice Cultivated in Agar and Soil

Rice seeds *Zhuliangyou*-90: indica type) purchased from Nongfeng Seed Industry Co. Ltd. (Changsha, China) were germinated according to a previous study (Deng and Hatzios, 2002a). Uniformly-germinated seeds were grown in 0.2% (m/m) agar (150 mL) containing the appropriate chemical treatment in a 28ºC incubator without light for 5 d. The safening treatment included a mixture of S-metolachlor and *L. chuanxiong* extract or commercially available Z-ligustilide or senkyunolide A dissolved in 1 ml acetone and dispersed in the agar. Agar containing S-metolachlor dissolved in 1 ml acetone without safeners or extract was used as the herbicide treatment, while 1 mL acetone alone was used as control. Experiments were replicated three times. The shoots and roots of rice seedlings treated with a mixture of Z-ligustilide and S-metolachlor were harvested separately, frozen in liquid nitrogen and stored at -70ºC until use for the determination of GST activity.

Chemical-free soil was air-dried and then sifted (0.85 mm pore diameter). Uniformly germinated seeds were planted in 180 g of the sifted soil in plastic pots (13×8.5×5 cm) and grown in an incubator with a 16/8 h (day/night) photoperiod at 28/26.5ºC (day/night) and 7500 lx maximal illumination. For safening treatments, S-metolachlor mixed with the *L. chuanxiong* extract or commercially available Z-ligustilide or senkyunolide A was dissolved in 2 mL acetone and diluted to a final volume of 1 L with water. The nonionic chemical surfactant Tween-80 (0.25% [v/v]) was used to maximize dispersion of the mixture in water (Deridder and Goldsbrough, 2006). The herbicide treatment included S-metolachlor, 0.2% acetone, and 0.25% Tween-80, while the control treatment only contained 0.2% acetone and 0.25% Tween-80. Each solution (5 mL) was poured into the plastic pots 2 d after rice seedling growth; bioassays were conducted 8 d after treatment. The experiment was replicated three times.

Glutathione-*S*-transferase (GST) Activity

Shoots and roots frozen in liquid nitrogen were ground into a powder and then extracted with 0.2 $mol \cdot L^{-1}$ Tris-HCl buffer (pH 7.2) containing 5 $mmol \cdot L^{-1}$ 2-mercaptoethanol, 1 $mmol \cdot L^{-1}$ ethylene diamine tetraacetic acid, and 7.5% polyvinylpolypyrrolidone. After centrifugation of the extract at 20,000 *x g* for 30 min, the supernatant was harvested. Protamine sulfate at 10% (v/v) was then added to the supernatant to precipitate nucleic acids. Finally, an equal volume of ammonium sulfate was added to the supernatant to precipitate proteins, which were collected and suspended in 1 ml Tris-HCl (pH 7.2) as an enzyme solution **(**Deng and Hatzios, 2002a).

The enzyme reaction system included 25 μL enzyme solution, 225 μL of 3.3 $mmol \cdot L^{-1}$ glutathione and 3 mL of 0.1 $mol \cdot L^{-1}$ phosphate buffer (pH 7.4); 25 μL of 30 mmol L^{-1}

1-chloro-2,4-dinitrobenzene was used to activate the reaction, and changes in absorbance were measured within 2 min at 340 nm. A non-enzyme reaction control system was used to eliminate the impact of the reaction apart from enzymes. Bio-Rad protein concentration determination was completed using bovine serum albumin as a standard.

Statistical Analysis

Data were analyzed using SPSS (version 18.0.0) and differences in different treatments were determined by ANOVA. Means were separated using Least Significant Difference test at 5% level of significance.

Results

Identification of Main Extract Active Compounds

According to LC-MS, the relative molecular masses of four main extract components (1-4) were 194, 162, 192 and 190, respectively (Fig. 1). Compared to the relative molecular masses of known *L. chuanxiong* compounds, components 3 and 4 corresponded to Z-ligustilide and senkyunolide A, respectively, while components 1 and 2 were unmatched. HPLC analysis showed that retention times for components 3 and 4 were 33.8 and 50.7 min., respectively corresponding to pure Z-ligustilide and senkyunolide A, respectively (Fig. 2). The identity of components 1 and 2 remained unknown.

Safening Effect on Rice in Agar

S-Metolachlor evidently inhibited rice growth at a concentration of 0.20 mg·L^{-1} in agar. Shoot height and root length declined to 70% and 50% of control, respectively (Table 1 and 2). The herbicide-inhibitive effect was significantly reversed by the *L. chuanxiong* extract (Table 1) in a concentration-dependent manner from 3.33–13.33 mg·L^{-1}; higher extract concentrations (26.67–53.33 mg·L^{-1}) were less effective. Both commercially available Z-ligustilide and senkyunolide A were able to safen S-metolachlor applied to rice in a similar manner and concentration range (Table 2). Z-Ligustilide produced total reversion of S-metalochlor herbicide injury of shoots and roots at 4 mg·L^{-1}, but was phytotoxic to rice at concentrations ranging from 8–16 mg·L^{-1}. A similar trend was presented by senkyunolide A, though it was somewhat less protective than Z-ligustilide.

Safening Effect on Rice in Soil

S-Metolachlor severely inhibited the growth of rice in soil (30% of control) at a concentration of 10 mg·L^{-1} (Table 3 and 4). Safening treatments with *L. chuanxiong* extract (400 mg·L^{-1}) and commercially available Z-ligustilide or senkyunolide A (both 120 mg·L^{-1}) reversed herbicide-inhibited rice growth to 96, 95 and 75% of control levels, respectively. However, their application at higher concentrations was less effective, as with agar-grown rice.

Table 1: *L. chuanxiong* extract safening of S-metolachlor applied to rice grown in agar

Treatments (mg·L^{-1}/mg·L^{-1})	Ratio in shoots [extract/control (%)]	Ratio in roots [extract/control (%)]
A(0.20)	71.83±2.14 a	51.60±1.89 a
B/A (3.33/0.20)	83.05±1.90 b	78.34±4.29 d
B/A (6.67/0.20)	92.09±2.30 c	89.51±1.53 e
B/A (13.33/0.20)	100.45±1.01 d	93.49±2.23 e
B/A (26.67/0.20)	86.83±1.91 b	67.69±3.18 c
B/A (53.33/0.20)	83.25±2.97 b	60.83±3.00 b

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). A: S-metolachlor; B: extract

Table 2: Z-Ligustilide and senkyunolide A safening of S-metolachlor applied to rice grown in agar

Treatments (mg·L^{-1}/mg·L^{-1})	Ratio in shoots [active compounds/control (%)]	Ratio in roots [active compounds/control (%)]
A(0.20)	71.83±2.14 a	53.94±1.40 a
B/A (1/0.20)	84.63±2.11 c	79.35±2.90 d
B/A (2/0.20)	96.73±3.70 d	84.45±3.97 d
B/A (4/ 0.20)	101.15±0.51 e	94.84±2.65 e
B/A (8/0.20)	87.25±0.79 c	67.34±2.17 c
B/A (16/0.20)	83.59±0.88 c	59.52±2.40 b
C/A (1/0.20)	79.23±1.29 b	62.39±0.97 b
C/A (2/0.20)	84.92±4.50 c	70.52±0.78 c
C/A (4 /0.20)	84.34±1.19 c	81.12±1.13 d
C/A (8/0.20)	89.28±1.91 c	83.60±2.24 d
C/A (16/0.20)	88.16±1.69 c	81.36±6.13 d

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). A: S-metolachlor; B: Z-ligustilide; C: senkyunolide A

Table 3: *L. chuanxiong* extract safening of S-metolachlor applied to rice grown in soil

Treatments (mg·L^{-1}/ mg·L^{-1})	Ratio in shoots [extract/control (%)]
A (10)	30.32±2.85 a
B/A (50/10)	56.76±2.27 b
B/A (100/10)	69.13±3.86 c
B/A (200/10)	88.52±3.42 e
B/A (400/10)	95.84±3.33 f
B/A (800/10)	77.99±2.40 d

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). A: S-metolachlor; B: extract

Table 4: Z-Ligustilide and senkyunolide A safening of S-metolachlor applied to rice grown in soil

Treatments (mg·L^{-1}/ mg·L^{-1})	Ratio in shoots [active compounds/control (%)]
A (10)	29.55±1.95 a
B/A (15/10)	63.11±5.51 cd
B/A (30/10)	70.33±5.81 de
B/A (60/10)	85.52±2.48 f
B/A (120/10)	95.43±2.68 g
B/A (240/10)	85.06±3.17 f
C/A (15/10)	46.02±5.03 b
C/A (30/10)	59.97±7.97 c
C/A (60/10)	66.99±3.38 cde
C/A (120/10)	75.19±4.71 e
C/A (240/10)	74.52±4.14 e

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). A: S-metolachlor; B: Z-ligustilide; C: senkyunolide A

Effect of Z-ligustilide on GST Activity in Rice

Results showed that there was no significant difference in

Table 5: Glutathione-*S*-transferase (GST) activity in crude preparations from treated or untreated rice seedlings

Treatments	Roots		Shoots	
($mg \cdot L^{-1}$/ $mg \cdot L^{-1}$)	GST activities (nmol min^{-1} mg^{-1})	Ratio (Z-ligustilide/control)	GST activity (nmol $min^{-1} \cdot mg^{-1}$)	Ratio (Z-ligustilide/control)
Control	135±80 a	-	81±50 a	-
A (0.20)	199±40 a	1.5	113±10 a	1.4
B/A (1/0.20)	465±31 b	3.4	187±19 b	2.3
B/A (2/0.20)	746±26 c	5.5	249±24 c	3.1
B/A (4/0.20)	1384±45 d	10.3	363±10 d	4.5
B/A (8/0.20)	1448±94 d	10.7	365±22 d	4.5
B/A (16/0.20)	1469±13 d	10.9	377±34 d	4.7

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). A: S-metolachlor; B: Z-ligustilide

GST activity in rice shoots and roots between the herbicide treatment and control (Table 5). When herbicide-inhibition of shoot and root growth was reversed by Z-ligustilide, GST activity in both shoots and roots was significantly enhanced compared to control. Z-Ligustilide applied at concentrations ranging from 1-4 $mg \cdot L^{-1}$ led to a gradual increase in GST activity in rice shoots (five-fold) and roots (11-fold) relative to control. GST activity in both shoots and roots was maintained at its highest level when Z-ligustilide was applied at concentrations greater than 4 $mg \cdot L^{-1}$.

Discussion

Except for extensive medical studies of *L. chuanxiong* as a traditional Chinese medicine, many studies have focused on its agricultural applications as insecticides and fungicides. To our knowledge, this is the first report about the safening effect of the *L. chuanxiong* extract on rice treated with the herbicide S-metolachor. Using LC-MS, the relative molecular masses of both volatile and nonvolatile active compounds extracted from *L. chuanxiong* were determined. Two components were identified and confirmed by HPLC to be Z-ligustilide and senkyunolide A. Previous studies have reported application of these two compounds as insecticides (Miyazawa *et al.*, 2004), fungicides (Meepagala *et al.*, 2005; Sim and Shin, 2008) and medicinally to treat cardiovascular and cerebrovascular diseases and a menstrual disorder (Chu *et al.*, 2011).

Few safeners have been developed for chloroacetamide application to rice, and none have proven effective against injury caused by S-metolachlor. In the present study, Z-ligustilide was found to be the most protective *L. chuanxiong* extract component by almost completely reversing S-metolachlor-inhibition of rice growth, demonstrating its potential as a safener for this chloroacetamide. As opposed to chemically synthesized safeners, application of botanically-derived Z-ligustilide to rice fields is more environment-friendly and avoids production of hazardous compounds from its chemical synthesis. Considering Z-ligustilide and senkyunolide A readily isomerize to other chemical compounds at room temperature (Cui *et al.*, 2006), it is critical to stabilize their structures long enough to be effective. On the other hand, Z-ligustilide could produce even more effective safening compounds by modification of its functional group, which may represent alternative route for developing new chloroacetamide safeners.

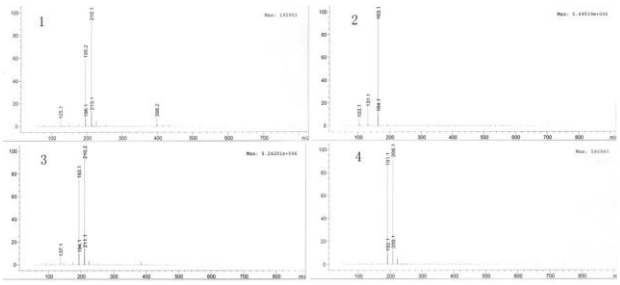

Fig. 1: Mass spectra of components 1-4

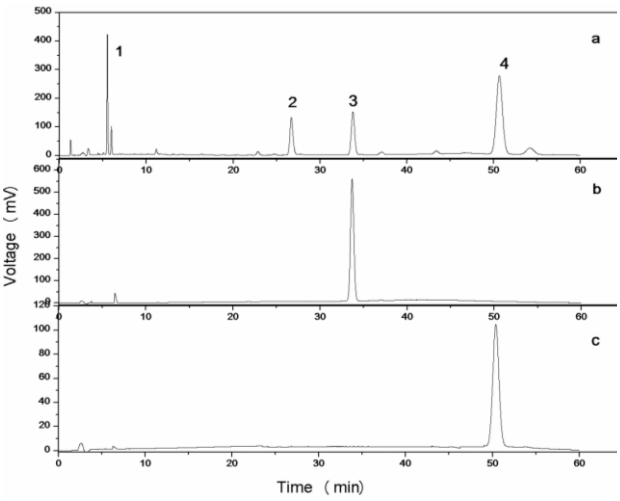

Fig. 2: HPLC chromatogram. (a): extract, (b): senkyunolide A, (c): Z-ligustilide

Recent studies on the mode of action of herbicide safeners have made it clear that safeners enhance degradation of herbicides to keep crops safe. In the degradation model, most hydrophobic herbicides are first transformed into hydrophilic compounds through reactions catalyzed by protective enzymes (e.g., oxidation by cytochrome P450 or conjugation by GST). Hydrophilic compounds can then be transported into the vacuole by ATP-binding cassette transporters or multidrug resistance-associated proteins and catabolized prior to their expulsion from the vacuole (Kolukisaoglu *et al.*, 2002; Schröder and Collins, 2011). For

example, fenclorim enhances the metabolism of pretilachlor by increasing GST activity in rice (Deng and Hatzios, 2002a). In fact, the GST isozyme OsGSTII-III plays an important role in the degradation of pretilachlor (Deng and Hatzios, 2002b). The accelerated metabolism of metazachlor in wheat by the safener BAS145-138 also involves an increase in GST activity (Riechers *et al.*, 2010). Other studies have shown that safeners including fencorim, benoxacor and fluxofenim induce expression of GST in *arabidopsis* (DeRidder *et al.*, 2002; Deridder and Goldsbrough, 2006). In present study, GST activity in rice shoots and roots was evidently increased by Z-ligustilide when applied with S-metolachlor. However, it should be noted that when used at high concentrations, Z-ligustilide can be phytotoxic to plant cells (Ho *et al.*, 2009).

An added consideration is detoxification mediated by other enzymes. For example, the safener naphthalic anhydride has been shown to induce cytochrome P450 activation and enhance conversion of bensulfuron-methyl to 4-hydroxy-bensulfuron-methyl in rice (Deng and Hatzios, 2003). Liu (2010) also reported greater induction of cytochrome P450 activity by naphthalic anhydride than by fenclorim in rice and maize. Moreover, induction of *O*-glucosyltransferase activity by safener cloquintocet mexyl has been shown to be pertinent to the metabolism of xenobiotics with a hydroxyl functional group (Brazier *et al.*, 2002). Edwards *et al.* (2011) suggested that metabolites with a hydroxyl functional group could be further *O*-glucosylated by *O*-glucosyltransferase. Therefore, future studies are needed to investigate whether enzymes other than GST are activated in rice by Z-ligustilide to detoxify S-metolachlor.

Conclusion

Z-ligustilide extracted from *L. chuanxiong* detoxifies the herbicide S-metolachlor when applied to rice through an increase in GST activity, suggesting its use as a potential herbicide safener.

Acknowledgements

This research was funded by national science research projects of China (No.201303031 and 31501661), Hunan Leading Academic Discipline Project (No. 090403), and Open-End Fund of the Inovative College Plantform of Hunan (No. 13K116 and 13K115).

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(Received 29 April 2015; Accepted 25 January 2016)