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Protective Responses Induced by 3-Dichloroacetyl Oxazolidine Safeners in Maize (*Zea mays*)

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Abstract

The protective effect of herbicide safener R-28725 and chiral 3-dichloroacetyl oxazolidine safeners in reducing the phytotoxicity of chlorsulfuron to maize were studied by physiological and biochemical tests. The results showed that R-28725 and compound A [(R)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine] could improve the GSH content, GST activity (*in vivo* and *in vitro*), ALS activity and GST affinity for the CDNB substrate in maize and the maize could be restored from the injury by chlorsulfuron. © 2015 Friends Science Publishers

Keywords: 3-dichloroacetyl oxazolidine; Biological activity; GST activity; GST affinity

Introduction

Sulfonylurea herbicides were discovered and commercialized by DuPont Crop Protection in 1980s, its mode of action was to inhibit acetolactate synthase (ALS), thereby block the biosynthesis of branched-chain amino acids. They have been developed worldwide in all major agronomic crops and worked on a broad range of grasses and broadleaf weeds. However, phytotoxic effects on non-target crop of sulfonylurea herbicide have been reported (Felsot *et al*., 1996; Geisel, 2007). Damage to crops and yield declining were observed because of the cultivation soil had been treated with chlorsulfuron in previous years (Amarjeet *et al.*, 2009; Wang *et al.*, 2010).

Herbicide safeners have been developed to reduce the phytotoxic effects of herbicide on crops (Bian *et al*., 2011). The herbicide safeners were a group of agrochemicals which could enhance herbicide tolerance of crops (Scarponi *et al.*, 2006). Safeners isoxadifen-ethyl and mefenpyr-diethyl could enhance sulfonylurea herbicides tolerance in cereal crops effectively by inducing cellular xenobiotic detoxification machinery (Behringer *et al.*, 2011). Safener 1,8-naphthalic anhydride (HN) could reduce phytotoxicity of chlorimuron-ethyl to maize by inducing the activity of ALS and glutathione-S-transferase (GSTs) (Guo, 2011). The protective effect of safener AD-67 in reducing the injury of monosulfuron were studied by bioassay and results showed that AD-67 could improve the glutathione (GSH) content in sorghum and maize treated with monosulfuron, and induce the conjugation of monosulfuron with GSH (Ye *et al.*, 2010a, b). Researchers generally believed that safeners increased GSH content and GST activity in plant greatly, and the potentially detoxification mechanism was GST catalyze the conjugation of GSH and herbicide by a nucleophilic substitution reaction to form inactive end product (Fu *et al*., 2011). It has previously been shown that 3-dichloroacetyl oxazolidine derivatives showed potential in protecting plants from injury by sulfonylurea, thiocarbamate, chloroacetanilide and imidazolinone herbicides (Davies and Caseley, 1999). R-28725 was a safener widely used for acetanilide herbicides and thiocarbamate herbicides. Some examined 3-dichloroacetyl substituted oxazolidines were chiral compounds and their biological activity were often related to their chirality (Kang *et al*., 2005; Sriharsha and Shashikanth, 2006). In previous studies, we synthesized some chiral 3-dichloroacetyl substituted oxazolidines successfully (Zhao *et al.*, 2011). Therefore, this study was concerned with understanding the mechanism by which R-28725 and 3-dichloroacetyl oxazolidine safeners with one chiral center protected maize from herbicide injury to test the hypothesis that safener enhance herbicide detoxification by elevating the activity of the mediating enzymes. The objective of this paper was to investigate the protective effects of R-28725 and chiral 3-dichloroacetyl oxazolidines for herbicide and the role of GSH, GST, ALS system in protective process by physiological and biochemical tests.

Materials and Methods

Materials and Chemical Reagents

The soils was Mollisols-cryolls clay loam type with a pH of 7.37. The available nutrient status field was medium in N (132 mg/kg), P (68.24 mg/kg), and K (189.63 mg/kg). Seed of maize (*Zea mays* L.) variety, “Dongnong253”, was used as test crop in the experiment. Chlorsulfuron wettable powder (20%) was provide by Liyang Chemistry Co. Ltd.,

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JiangSu province. R-28725, compound A, compound B and compound C were synthesized in our laboratory and their purity was over 99.0% (Table 1). Chlorsulfuron was obtained from Aladdin Reagent Co., Ltd. Methanol were purchased from Dikma Technologies Inc. Flavin adenine dinucleotide (FAD), GSH, 5,5'-dithiobis(2-nitrobenzoic)acid (DTNB), and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co. Ltd. Other chemical solvents were of the highest available purity and purchased from Aladdin Reagent Co., Ltd.

Plant Harvesting

Seeds of maize were soaked with safener solution for 12 h. The control was soaked with water. After soaking, the seeds were germinated for 24 h at 26.5°C. Then, the seeds were sown directly in cups containing soil treated with chlorsulfuron (0.2 μg/kg). The control was treated with water. Afterward, the cup was placed in growing chamber (relative humidity 75%, 12 h of light, 26.5°C). After 7 days, the growth of maize was tested to determine the optimum concentration of safener. Each treatment was replicated three times in a completely randomized design.

$$\text{Recovery rate (\%)} = \frac{\text{Treated with compounds and chlorsulfuron} - \text{Treated with chlorsulfuron}}{\text{Contrast} - \text{Treated with chlorsulfuron}}$$

The shoots and roots tissues of maize at the optimum concentration of safener were collected for biological activity assays (GSH、ALS、GST). The treatments were replicated thrice.

Determination of GSH Content

GSH content was assayed according to the procedure of Gronwald *et al.* (1987). GSH content in root and shoot of maize was analyzed spectrophotometrically with DTNB used as chromogenic agent. Absorbance at 412 nm was determined and GSH content was calculated by comparing with the known concentration.

Determination of GST Activity

GST extraction and GST enzyme assay *in vivo* were carried out according to the procedure of Jablonkai and Hatzios (1991). The GST activity was expressed in amount of conjugate constituted by GSH and CDNB catalyzed by GST per unit time per mg of enzyme (μmol·min^{-1}·mg^{-1} protein).

Assay of GST against chlorsulfuron *in vitro* was carried out by the Scarponi *et al.* (2006) method. Chlorsulfuron solution was added to glutathione and GST extract. The reaction mixture was cultivated 2 h and stopped. The residues of chlorsulfuron in this sample was determined by High Performance Liquid Chromatography (HPLC). The GST activity was measured by the differential value between the initial concentration of chlorsulfuron and the chlorsulfuron residue concentration after reaction. The concentration of chlorsulfuron lost in non-enzymatic reaction, measured by a blank sample in which the enzymatic extract was substituted by buffer solution, was reduced from the result. The GST activity was expressed as amount of herbicide consumed by GSH catalyzed by GST per unit time per mg of enzyme (nmol·min^{-1}·mg^{-1} protein).

Determination of Kinetic Parameters of GST (CDNB)

In order to understand the kinetic parameters V_{max} (maximal rate of reaction) and K_M (the substrate concentration which results in one-half the maximum velocity) of GST, the concentration of GSH in reaction was to remain constant (5 mM), the concentration of CDNB was distributed in 0.13-4.14 mM using 16 μL enzymatic extract. The kinetic constants V_{max} and K_M were measured by linear regression of a double reciprocal plot of GST activity (nmol·min^{-1}·mg^{-1} protein) and GSH concentration (Scarponi *et al.*, 2006).

Determination of ALS Activity

ALS enzyme extraction and assay were carried out as reported by Kobayashi *et al.* (1991). Acetolactate was measured spectrophotometrically (525 nm) and calculated by comparing with the known concentration. ALS activity was expressed as nmol·h^{-1}·mg^{-1} protein.

Determination of Protein Content

Protein content was measured according to the Bradford method with bovine serum albumin (BSA) as reference material (Nemat Alla and Hassan, 1998).

Statistical Analysis

The experiment was randomized complete block design with three replications. The test data treatment were carried out with Statistical Product and Service Solutions (SPSS 16.0) software. Grouped mean and Duncan multiple range test was measured to test for significant difference at 95% confidence level ($p = 0.05$).

Results

Effect of Safener on Maize Growth

To determine a suitable treatment regime, a range of concentrations of safener were tested for their ability to decrease the injury caused by chlorsulfuron. Chlorsulfuron could provoked a significant decrease on the growth of maize, but significant differences were observed after the introduction of safeners (R-28725, compound A, compound B and compound C) (Fig. 1). The highest recovery rate of the growth level of maize were recorded for R-28725 at the concentration 5 mg/kg, compound A at the concentration 10 mg/kg, compound B at the concentration 50 mg/kg, and compound C at the concentration 25 mg/kg. The growth level test showed that these compounds could defend maize

against herbicide injury partly. Therefore all subsequent testing was based on this observation.

GSH Content

The GSH content of maize treated by different compounds exhibited significant differences (Table 2). The GSH content of root and shoot treated by chlorsulfuron was decreased to 57.3% and 50.6% of the control respectively. The maximum GSH content was found in treated by the combination of R-28725 and chlorsulfuron (99.0% in root and 98.6% in shoot), while treated by the combination of compound A and chlorsulfuron accumulated the second highest GSH content (67.9% in root and 89.9% in shoot).

GST Activity

The GST activity of maize *in vivo* treated by safener and chlorsulfuron were determined by the concentration of conjugate constituted by GSH and CDNB catalyzed by GST. Chlorsulfuron caused a small (5.1%) increase of GST activity as compared with the control. Treatment with the combination of safener and chlorsulfuron caused increase in GST activity. In particular, the GST activity of maize *in vivo* showed significantly increased in response to R-28725 treatment and compound A treatment (Table 3).

The GST activity *in vitro* against chlorsulfuron in maize was measured by using GST enzyme extracted from root of maize after treated by safener. The results showed that safeners increased GST activity against chlorsulfuron herbicide in some extent. The GST activity of maize against chlorsulfuron raised by 21.9% in response to R-28725 treatment and 7.2% in response to compound A treatment (Table 3).

The highest GST activity was found in maize treated by the combination of R-28725 and chlorsulfuron and significant increase of GST activity in maize was also observed in maize treated with the combination of compound A and chlorsulfuron.

Kinetics of GST

The kinetic parameters V_{max} and K_M of GST were researched to clarify the innate character of the inducement of GST and dynamics of GST induction caused by treatment of safener (Table 4). V_{max} decrease and K_M increase was noticed as a result of chlorsulfuron treatment. Otherwise, increase in V_{max} and decrease in K_M was observed in response to R-28725 and compound A treatment, which indicated a strong induction of GST caused by R-28725 and compound A.

ALS Activity

To verify the potential of compound to be used as safener of chlorsulfuron, ALS activity *in vivo* were assayed (Table 5). The results showed that chlorsulfuron caused a decrease of ALS activity by 37.3% compared with the control, combination of R-28725 and chlorsulfuron increased ALS activity by 17.2% compared with the control and compound A prevented and relieved the chlorsulfuron injury almost completely on the ALS activity while compound B and compound C just prevent the chlorsulfuron injury in some extent. This behavior suggests that R-28725 and compound A can enhance ALS activity significantly and protect maize from chlorsulfuron injury.

Discussion

Chlorsulfuron was detoxified via enzymatic conjugation with GSH catalyzed by GST (Ye and Xu, 2008). It was found that glutathione conjugation was responsible for herbicide resistance in plants (Del Buono and Ioli, 2011). The detoxification ability of safener was determined to some extent by the level of glutathione conjugation in the plant. R-28725 and compound A could protected maize from herbicide injury by improving content of GSH and GST activity, promoting glutathione conjugation of chlorsulfuron. Positive relationships occurred between content of GSH and GST activity in maize. These studies demonstrate that R-28725 and compound A decreases herbicide injury in maize by enhancing the activity of GST as well as GSH involved in processing the resulting conjugates which is in accordance with the results reported by Cummin *et al.* (2011). The results also showed that the enzyme affinity of GST for the CDNB substrate was decreased by chlorsulfuron treatment and increased by R-28725 and compound A treatment. Similar results were obtained when maize and wheat seeds were treated by cloquintocet-mexyl and fenchlorazole-ethyl (Scarponi *et al.*, 2006).

Chlorsulfuron was a inhibitors of ALS and inhibits ALS activity and thereby block the branched-chain amino acids biosynthesis (Fan *et al.*, 2003). So, ALS activity has close relationship to plant resistance to chlorsulfuron. The result suggested that the ALS activity of maize was improved by R-28725 and compound A. It was one of elementary pathways for herbicide detoxication in crops.

At last, the growth and physiological index of maize (GSH content, GST activity and ALS activity) was inhibited by chlorsulfuron and it can be recovered by R-28725 and compound A treatment significantly. As shown above, R-28725 and compound A induced GST activity in maize, and augmented chlorsulfuron detoxification through conjugation with GSH. This is one of elementary pathways for herbicide detoxication by safener in crops. Meanwhile, R-28725 and compound A can stimulate ALS activity and leads to detoxification of sulfonylurea herbicide chlorsulfuron.

Table 1: Safener chemical names used for test

Safener	Chemical name
R-28725	3-dichloroacetyl-2,2-dimethyl-1,3-oxazolidine
Compound A	(R)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine
Compound B	(S)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine
Compound C	(RS)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine

Table 2: Effect of safener to GSH content in maize root and shoot

Treatment	GSH content of root ($\mu g \cdot g^{-1}$)	GSH content of shoot ($\mu g \cdot g^{-1}$)
Control	10.533±0.291 a	18.452±0.391 a
Chlorsulfuron	6.030±0.308 d	9.345±0.266 d
R-28725 + Chlorsulfuron	10.423±0.172 a	18.194±0.437 a
Compound A + Chlorsulfuron	7.154±0.230 bc	16.583±0.359 b
Compound B + Chlorsulfuron	6.924±0.277 c	13.986±0.253 c
Compound C + Chlorsulfuron	7.412±0.181 b	14.152±0.484 c

Table 3: Effect of safener to GST activity in maize root

Treatment	GST activity (*in vivo*) ($\mu mol\ min^{-1} \cdot mg^{-1}$ protein)	GST activity (*in vitro*) ($nmol\ min^{-1}\ mg^{-1}$ protein)
Control	3.15±0.18 d	0.629±0.004 d
Chlorsulfuron	3.31±0.10 d	-----
R-28725 + Chlorsulfuron	9.19±0.56 a	0.767±0.007 a
Compound A + Chlorsulfuron	7.29±0.34 b	0.674±0.012 b
Compound B + Chlorsulfuron	3.45±0.05 d	0.573±0.006 e
Compound C + Chlorsulfuron	4.59±0.04 c	0.645±0.006c

Table 4: Effect of safener to kinetic parameters of GST activity in maize root

Treatment	V_{max} ($nmol\ min^{-1}\ mg^{-1}$ protein)	K_M (mM)
Control	0.790±0.030 e	1.950±0.056 a
Chlorsulfuron	0.470±0.044 f	2.120±0.139 a
R-28725	1.597±0.091 a	1.167±0.144 d
Compound A	1.437±0.023 b	1.320±0.010 cd
Compound B	0.913±0.067 d	1.673±0.057 b
Compound C	1.090±0.035 c	1.570±0.017 bc

Table 5: Effect of safener to ALS activity in maize shoot

Treatment	ALS activity ($nmol\ h^{-1}\ mg^{-1}$ protein)
Control	107.1±3.9 b
Chlorsulfuron	67.1±3.6 d
R-28725 + Chlorsulfuron	125.5±9.7 a
Compound A + Chlorsulfuron	104.3±5.0 b
Compound B + Chlorsulfuron	68.6±6.2 d
Compound C + Chlorsulfuron	82.9±5.5 c

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05). The values correspond to averages of three replicates

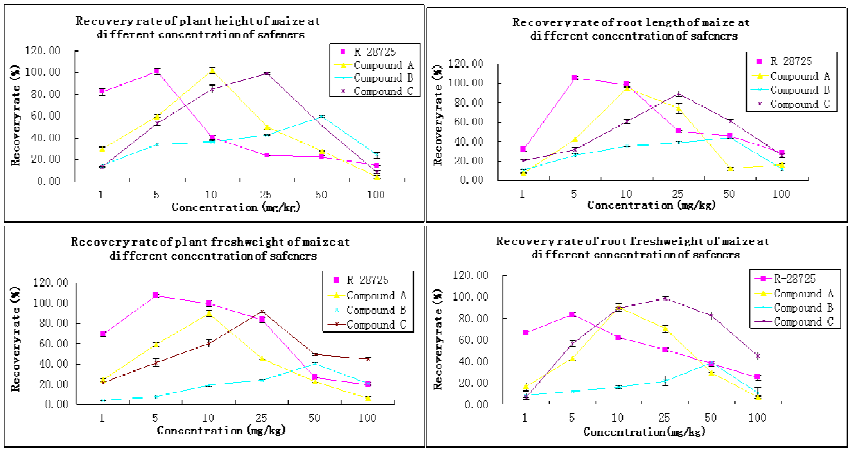

Fig. 1: Recovery rate of growth level of maize at different concentration of safener

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