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Full Length Article

Inducing Glutathione S-Transferase Expression and Activity using an Herbicide Safener against Metolachlor in Maize

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Abstract

The effect of three potential herbicide safeners including N-Dichloroacetyl-2, 2 and 5-trimethyl-1and 3-oxazolidine (R-29148) and its two optical isomers on preventing maize (*Zea mays* L.) injury by herbicide metolachlor were determined. Treatment on maize with chiral safeners caused a progressive increase in glutathione-s-transferase activity in roots and reduced chloroacetanilide herbicide phytotoxicity in maize seedlings. After pretreating with the R-enantiomer *in vivo* and *in vitro* glutathione S-transferases activities in roots were increased by 107 and 17%, respectively. Compared with an untreated control the glutathione S-transferase kinetic parameter V_{max} of the maize after treatment with the R-enantiomer and racemic R-29148 increased by 60 and 18% respectively, while the K_M decreased by 24 and 7%. The R-enantiomer of R-29148 could induce glutathione expression glutathione S-transferase activity and affinity for the substrate 1-chloro-2 and 4-dinitrobenzene in maize, which meant the compound could protect maize against injury from chloroacetanilide herbicides. Catalase and peroxidase activities were higher when the plants responsed in the presence of a stressor agent like herbicides. We also observed that two antioxidant enzyme activities in maize were significantly changed due to the presence of the safener. © 2017 Friends Science Publishers

Keywords: Herbicide safener; Biological activity; GST activity; Metolachlor

Introduction

Metolachlor is a broad spectrum herbicide that is used to control certain broadleaf weeds in crops such as maize, soybean and sorghum etc. When absorbed into the target weed through roots and shoots metolachlor inhibited plant growth by suppressing synthesis of fatty acid, lipid, protein and gibberellin (Liu, 2010; Li *et al*., 2016). However, studies showed that crops injury from metolachlor were greater in wet soil conditions within a two week period after application (Bouchonnet *et al*., 2011; Christos *et al*., 2017). Maize injury from metolachlor is often associated with the leaves unable to pull free from the whorl and form a "ladder" like plant. General symptoms include stunted plants with abnormally thick roots, twisted shoots and dark green leaves (Vyn *et al*., 2006; Otto *et al*., 2012).

Herbicide safeners are widely used agrochemicals with the unique ability to protect crop plants from the injury caused by certain herbicides (Taylor *et al*., 2013). Safening is due to an increase in cereal plant tolerance to herbicides by the increasing detoxification rates. Therefore, herbicide safeners effectively improve herbicide selectivity (Stoilkova and Yonova, 2010; CorreiaL and Gomes, 2015; Matthew *et al*., 2015). The present studies show herbicide safeners induce a series of enzymes and the biosynthesis of cofactors associated with herbicide detoxication (Buono and Ioli, 2011; Bernasinskaa *et al*., 2013). Reports show that catalase (CAT) and peroxidase (POD) are involved in metabolizing the oxidative stress due to high herbicide doses and they can protect plants from the stress generated by herbicide (Rajasekar *et al*., 2015; Sytykiewicz, 2015). It is generally believed that safeners protected crops by enhancing the activity of glutathione-s-transferase (GST) to catalyze glutathione conjugation in the metabolic detoxification of herbicides (Fu *et al*., 2011; Bartucca *et al*., 2017). These observations suggest that safeners induce GST activity, which enhances GSH conjugation for protective activity against the herbicide (Ye and Xu, 2008; Ye *et al*., 2016; Bartucca *et al*., 2017). Nevertheless, the research on safeners for the protection of maize to detoxify chloroacetanilide herbicide is limited. The dichloromethyl-dioxolane safener MG-191 can protect maize against chloroacetanilide herbicide acetochlor injury by GSH conjugation. The protective effect is due to the ability of MG-191 to increase the levels of GST enzymes (Jablonkai and Hatzios, 1991; Assaha *et al*., 2015). The safener fenclorim protects rice against chloroacetanilide herbicide injury by enhancing the expression of detoxifying GST

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(Brazier *et al*., 2008). Dichloroketal safeners could reduce phytotoxicity of acetochlor to maize by enhancing the GSH content and GST activity in shoots and roots of maize (Kraehmer *et al*., 2014).

Certain 3-dichloroacetyl-substituted oxazolidines with a chiral center exhibit different biological activities (Sriharsha and Shashikanth, 2006; Zhao *et al*., 2012). The dichloroacetamide safener 3-(dichloroacetyl)-2,2 and 5-trimethyl-1 and 3-oxazolidine (R-29148) are widely used in for weed control in maize. Studies have shown that R-29148 can effectively protect maize by inducing GST activity to detoxify herbicides (Nelson and Penner, 2006; Kraehmer *et al*., 2014). However, few investigations have focused on chiral safener detoxification mechanisms (Jablonkai, 2013; Ye *et al*., 2016). In our previous studies, we successfully synthesized chiral R-29148 and 3-dichloroacetyl substituted oxazolidines (Gao *et al*., 2012). Thus, this study was conducted to explore possible mechanism of racemic R-29148 and its chiral isomers with one chiral centers to prevent maize from being harmed by chloroacetamide herbicides to validate the hypothesis that safeners could enhance detoxification. Our work was also aimed to ascertain that chiral safeners induce antioxidant enzymes activities to counteract oxidative stress produced by herbicide metolachlor. Therefore, this study was concerned with the possible mechanism of one chiral centers safeners to alleviate toxicity of metolachlor to maize. GSH, GST, POD, and CAT enzyme activities treated with safener racemic R-29148 and its chiral isomers were investigated and the results showed that expression of the mediating enzymes after treated with chiral safeners were increased. It was hypothesized that three potential herbicide safeners could effectively protect maize against herbicide injury.

Materials and Methods

Materials and Chemical Reagents

Seeds of maize (Dongnong 253) were used as the test crop for our experiments. Racemic R-29148, R-enantiomer, and S-enantiomer were synthesized in our laboratory, and their purity levels were greater than 99.0% (Table 1). Metolachlor emulsifiabale concentrate (720 g/L) was provided by Dongtai Agricultural Chemistry Co., Ltd (Shandong, China). Metolachlor standards were purchased from Aladdin Reagent Co., Ltd. 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB) and GSH were purchased from Sigma (Shanghai, China). Methanol was provided by Dikma Technologies Inc. Other chemical solvents were obtained from Aladdin Chemistry (Shanghai, China).

Plant Material and Growth Conditions

For chemical induction safeners were applied as a seed coating and maize seeds were soaked with safener solution for 12 h at 26.5°C before sowing. The non-treated control seeds were immersed in distilled water under the same conditions. After soaking, the seeds were germinated at 26.5°C for 24 h. Next, the seeds were directly sown in cups containing quartz sand prewashed with 10% (v/v) hydrochloric acid solution and sterilized in 5% (w/v) sodium hypochlorite solution. The metolachlor solution at 30 mg/L was added to quartz sand for a 60% water holding capacity. The control was treated with water. Seedlings were grown at 26.5°C under a 12 h photoperiod using artificial light (relative humidity 75%). Plant material was harvested 8 d after the treatment began. For completely randomized designs, each treatment was replicated thrice. Maize shoots and roots were collected after the treatments rinsed with water and dried through blotting. The shoots and roots length as well as fresh weight (FW) were determined. The maize growth index recovery rates were calculated to determine the optimal safener concentration. The growth index recovery rates were calculated using the following formula (Ercoli *et al*., 2004).

Table 1: Chemical names for the safeners used in our experiments

Safener	Chemical name
R-29148	3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine
R-enantiomer	(R)- 3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine
S-enantiomer	(S)- 3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine

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

In addition, the shoots and roots were frozen in liquid nitrogen and stored at -80°C for enzymatic assays (GSH, GST, POD, and CAT). The experiment was carried out with three replicates.

GSH Content Assay

To determine the GSH content, the maize tissue was homogenized in 5% (w/v) sulfosalicylic acid and the homogenates were centrifuged at 15 000 × g for 20 min at 4°C. The GSH content was determined according to the procedure of Gronwald *et al.* (1987). The maize roots and shoots were measured using spectrophotometry at 412 nm with the DTNB reagent and calculated through a comparison with the known concentration.

GST Extraction and Activity Assay

The GST extraction procedure was performed as described previously (Buono *et al*., 2011). To measure the GST activity 200 mg frozen maize seedling tissue was ground into powder under liquid nitrogen and homogenized in 1 mL of QB buffer (potassium phosphate buffer 100 mM pH 7.8 with EDTA 1 mM and polyvinylpyrrolidone at 5% w/v) at 4°C. The homogenate was centrifuged at 15 000 × g for 20 min at 4°C. The GST activity was measured using

spectrophotometry at 340 nm in accordance with Holt (Holt *et al.*, 1995). The final assay mixture consisted of 50 mM phosphate buffer (pH 6.5), 1 mM CDNB, 1 mM GSH, and 0.5 mM EDTA. The reaction began by adding the root extract. The reaction mixture was measured through spectrophotometry at 340 nm for 180 s (60 s intervals). The GST activity was expressed as the quantity of herbicide consumed by GSH catalyzed by GST per unit time per mg of enzyme (nmol s^{-1} mg^{-1} protein). The protein content was measured using the Bradford method and BSA as the standard (Bradford, 1976).

Moreover, High Performance Liquid Chromatography (HPLC) assays were performed to determine the GST activity towards the herbicide metolachlor as a substrate in accordance with Scarponi (2006). Metolachlor solution was added to glutathione and GST extract. The reaction mixture was incubated for 2 h. The reaction was stopped by adding 10 µL 3.6 M HCl and the mixture was extracted in methanol and injected into an HPLC. The GST activity was measured by comparing the initial and residual concentrations of metolachlor. The GST activity was expressed as the quantity of metolachlor consumed per minute per milligram of enzyme (nmol· min^{-1} ·mg^{-1} protein).

GST Kinetic Parameters Assay (CDNB)

Steady-state kinetic measurements for the GST enzyme were performed at 37°C in 50 mM phosphate buffer (pH 6.5) as described by Luciano and Elisa (2006). GST kinetic parameters were determined in the presence of 5 mM GSH. CDNB was used in the concentration range 1.0–32.0 mM and 25 µL of the enzymatic extract was added. The kinetic parameters constants V_{max} and K_M were determined using a linear regression analysis of 1/*V* vs. 1/*S* according to double reciprocal plots. The GST kinetic parameter activity was expressed as nanomoles per unit time per mg of enzyme used for the assay.

CAT Activity Assay

We determined the CAT activity using a modified method from Beers and Sizer (1952). The reaction mixtures (1.9 mL H_2O, 0.1 mL enzyme extract and 1 mL 0.3% (v/v) hydrogen peroxide) were measured by spectrophotometry at 240 nm through monitoring the decrease in H_2O_2 for 3 min. The catalase activity was expressed as µmol H_2O_2 min^{-1}·g^{-1} FW.

POD Enzyme Assay

The POD enzymatic activity was determined following the procedures described in Cakmak and Horst (1991) with certain modifications. The final assay mixture consisted of 1 mL 50 mM sodium phosphate buffer (pH 7.0) 2 mL 0.3% of hydrogen peroxide and 0.95 mL 0.2% guaiacol. The reaction was started by addition of 0.01 mL enzyme extract to reaction mixtures. Then, the POD enzymatic activity was measured through spectrophotometry at 470 nm for 5 min. The increase in absorbance was recorded upon adding H_2O_2. The peroxidase activity was expressed as mmol min^{-1} g^{-1} FW.

Statistical Analysis

The experiment was laid out in randomized complete block with three replications design to determine the protective effect of chiral safeners. The data were analyzed using SPSS version 16.0 software. The least significant difference was applied to assess differences between the treatments using the grouped mean and Duncan multiple range test at a 95% confidence level ($p = 0.05$).

Results

Maize Growth Level

In maize growth inhibition experiments, the metolachlor exhibited severe retardation in shoot and root growth. The treated maize growth index inhibition rate based on the plant height, fresh weight of shoot, root length, and root fresh weight decreased by 29, 31, 29 and 29%, respectively. The protective effects of three safeners (R-29148, R-enantiomer, and S-enantiomer) were tested using maize at different safener concentrations. The results show that three safeners significantly decreased the inhibition by the metolachlor herbicide and the maize seedling recovery rates upon treatment with the R-isomer and racemate were higher than with the S-enantiomer (Fig. 1). The maize growth indicator recovery rates ranged from 67 to 286% as shown in Fig. 1 when the racemic R-29148, R-enantiomer and S-enantiomer concentrations were 25, 5 and 50 mg·L^{-1}, respectively. Among the safeners, the R-enantiomer was highly protective against metolachlor herbicide injury in maize at a lower concentration.

GSH Content

For safener-treated maize, the content of GSH in root and shoot tissue increased significantly and GSH contents were unaffected by the metolachlor herbicide compared with the non-treated control (Table 2). The rise of GSH contents in the maize root and shoot tissue after pretreatment with safeners were greater than those be treated with the metolachlor. Our results suggested that increase of GSH content in the maize seedlings may be related to the herbicide safener protective activity. Similarly, GSH content in the seedling tissue with safener-metolachlor treatments were greater than with the metolachlor treatment. After pretreatment with the R-enantiomer the GSH contents markedly increased by 64 and 27% in the root and shoot respectively.

GST Activity

The GST activity slightly increased in the metolachlor-

Table 2: Effect of safeners and metolachlor on shoot and root GSH content in maize seedlings

Treatment	GSH content of root ($\mu g \cdot g^{-1}$)	GSH content of shoot ($\mu g \cdot g^{-1}$)
Control	3.839±0.269e	13.433±0.138e
metolachlor	4.290±0.238d	14.347±0.135d
R-enantiomer + metolachlor	6.921±0.350a	18.319±0.210a
S-enantiomer + metolachlor	5.389±0.269c	16.769±0.318c
R-29148 + metolachlor	6.088±0.255b	17.468±0.312b

The data are the means from triplicate determinations ± standard deviation. Values that share the same letters show insignificant differences ($p \leq 0.05$)

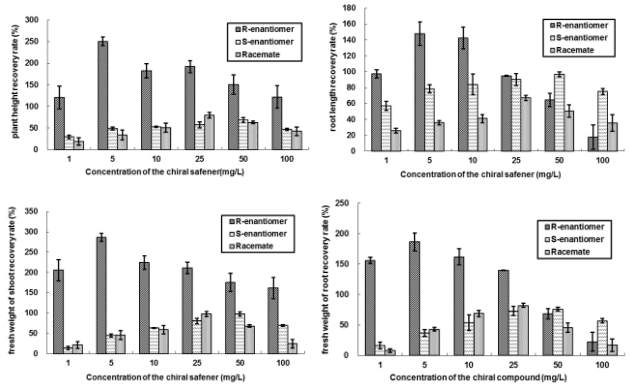

Fig. 1: Effect of safener concentration on maize growth indicator recovery rates

treated maize seedling root compared with the non-treated control. The safener and metolachlor treatment also increased the *in vivo* activity of GST. In treatments with the R-enantiomer, S-enantiomer and its racemate increased the GST (CDNB) activity by 107, 68, and 97% in the roots (Table 3). The data indicated that the safener enhanced the GST activity to facilitate maize seedling survival at low metolachlor concentrations. When metolachlor was added as the substrate instead of CDNB, as expected, it also enhanced the *in vitro* activity of GST due to the chiral safeners. However, we did not observe a clear effect on GST activity in vitro at different levels in response to various safener treatments (Table 3). According to the study of *in vivo* and *in vitro* GST activity, three safeners likely promote similar responses. It could be concluded that the different protect effects of three chiral safeners in the maize root were due to the different levels of GST enzyme activity toward CDNB or metolachlor substrate. The R-enantiomer effectively promoted the GST activity among the three safeners.

GST Kinetic Parameters

As shown in table 4, we examined the kinetic parameters constants V_{max} and K_M of maize GST using enzymatic extracts from maize roots. The V_{max} of this process decreased, while the K_M increased under treatment with metolachlor. The V_{max} increased by 60 and 18% after R-enantiomer and racemic R-29148 treatment compared with the untreated control, and the K_M decreased by 24 and 7% respectively. The results in Table 4 showed a clear effect from the R-enantiomer in the induction of and dynamics of GST activity.

CAT and POD Activity

After treated with the safener and metolachlor the antioxidant enzymes CAT and POD activities were also measured. CAT was involved in metabolizing the oxidative stress due to high herbicide doses and it protected plants from the stress generated by herbicide. In our experiments, CAT activity increased to 6.57 after the metolachlor treatment compared with the untreated control. The data in Table 5 showed that the CAT enzyme activity decreased to 4.89, 4.81 and 3.51 after three safeners treatment.

Compared with the control POD activity in the maize seedling roots exhibited a significant increase. In addition, an extreme increasing in POD activity was observed after treated with the S-enantiomer and racemic R-29148 compared with the metolachlor treatment alone. Upon treatment with the R-enantiomer POD enzyme activity decreased from 1870 to1560. Our results further suggested that the POD and CAT activity of maize were decreased by treated with the R-enantiomer. It also showed that chiral safeners induced a chain reaction that counteracts oxidative stress and arises in response to the herbicide activity.

Discussion

The conjugation of glutathione with herbicides was generally considered to be one of the major detoxification mechanisms of plants (Taylor *et al.*, 2013). Safeners could stimulate GST activity and effectively detoxify by enzyme-catalyzed conjugation of GSH with the metolachlor herbicide (Scarponi *et al.*, 2006). The detoxification ability of safener was determined to the degree of glutathione conjugation in maize to a certain extent (Zhao *et al.*, 2012). Our results showed enhancements of GST activity against metolachlor in response to chiral safener treatments. Overall the R-enantiomer could protect maize from chloroacetanilide herbicide injury by enhancing GSH content and stimulating GST activity to promote glutathione conjugation with metolachlor in the maize seedlings. Compared with treated by metolachlor, dynamics of GST activity toward CDNB increased significantly in each safener treatment (Al-Ayedh *et al.*, 2016; Ye *et al.*, 2016). The data showed that the R-enantiomer significantly altered the kinetic parameter V_{max} and K_M.

POD and CAT activities were involved in metabolizing the oxidative stress due to high herbicide doses and protects plants from the stress generated by herbicide (Rajasekar *et al.*, 2015). Reports showed that POD and CAT were involved in herbicide tolerance and two antioxidant enzymes activity increased during herbicide exposure (Hemanth Kumar *et al.*,

Table 3: Safener-mediated increase in GST activity toward metolachlor in maize seedling roots

Treatment	GST activity *in vivo* (nmol s^{-1} mg^{-1} protein)	Treatment	GST activity *in vitro* (nmol min^{-1} mg^{-1} protein)
Control	21.40±1.58d	Control	48.20±1.72 c
metolachlor	29.81±1.78c	metolachlor	—
R-enantiomer + metolachlor	44.23±2.21a	R-enantiomer	56.51±1.44a
S-enantiomer + metolachlor	35.94±2.58b	S-enantiomer	50.70±0.52b
R-29148 + metolachlor	42.32±2.78a	R-29148	54.11±1.22ab

The data are the means of triplicate determinations ± standard deviation. Values that share the same letter exhibit insignificant differences (p>0.05)

2016; Bakir *et al*., 2017). Our result showed that the POD and CAT activity of maize were decreased by treated with the R-enantiomer. However, it should be noted that there was no decrease of POD activity in response to racemic R-29148 and S-enantiomer application, which indicated that R-enantiomer played a certain role in maize to resist oxidative stress generated by herbicide metolachlor. This mechanism could be an important pathway for chiral safener detoxification in maize.

Conclusion

Based on a few available reports, we investigated the changes of GST, CAT and POD activity in maize after treated with racemic R-29148 and its chiral isomers as the safener. The maize growth level and physiological index were significantly inhibited by metolachlor. Three safeners were found effective to protect maize against metolachlor injury. Moreover, the R-enantiomer induced GST activity and promoted glutathione conjugation with metolachlor in root of maize seedlings. Further information on the chiral safener role in antioxidative enzymes activation was obtained from CAT and POD activity to overcome oxidative stress caused by the herbicide. The R-enantiomer exhibited high safening activity against metolachlor injury in maize and its activity exceeded that of the S-enantiomer and the racemic compound. This mechanism is an essential way for herbicide detoxification by chiral safeners in maize. The findings indicated the importance of the stereochemistry in the protective effectiveness.

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Table 4: Kinetic parameters for GST activity induced by safeners in maize seedling roots

Treatment	V_{max} (nmol min^{-1} mg^{-1}protein)	K_M (mM)
Control	14.87±0.030bc	0.51±0.042b
metolachlor	8.21±0.044d	0.78±0.027a
R-enantiomer	23.82±0.023a	0.39±0.019c
S-enantiomer	12.73±0.067c	0.50±0.043b
R-29148	17.54±0.035b	0.48±0.037b

The data are the means of triplicate determinations ± standard deviation. Values that share the same letter exhibit insignificant differences (p>0.05)

Table 5: Effects of safeners on CAT and POD activity in maize seedling roots

Treatment	CAT activity (U/g F.W.)	POD activity (U/g F.W.)
Control	2.21±0.02e	1171±1.08e
metolachlor	6.57±0.03a	1807±1.02a
R-enantiomer	4.89±0.02b	1560±1.04d
S-enantiomer	4.81±0.03c	1974±0.20b
R-29148	3.51±0.01d	1967±1.00c

The data are the means of triplicate determinations ± standard deviation. Values that share the same letter exhibit insignificant differences (p≤0.05)

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