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Effects of Chiral 3-Dichloroacetyl Oxazolidine on Glutathione S-Transferase and Antioxidant Enzymes Activity in Maize Treated with Acetochlor

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ABSTRACT

The objective of this paper was to investigate the protective effect of three potential herbicide safeners (3-dichloroacetyl oxazolidine and its two optical isomers) on detoxifying to chloroacetanilide herbicide acetochlor in maize. In this study, physiological and biochemical tests were conducted under laboratory condition in 2015. All safeners increased the expression levels of herbicide detoxifying enzymes, including glutathione S-transferases (GST), catalase (CAT) and peroxidase (POD) to reduce chloroacetanilide herbicide phytotoxicity in maize seedlings. Our results suggest that the R-isomer of R-29148 can induce glutathione (GSH) expression, GST activity, and affinity for the 1-chloro-2,4-dinitrobenzene (CDNB) substrate in maize, which can protect maize from injury by chloroacetanilide herbicide acetochlor. 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.

Keywords: Herbicide safener; Chiral 3-dichloroacetyl oxazolidine; Biological activity; GST activity; Acetochlor

1. Introduction

Acetochlor is a kind of selective herbicide before sprout. It is principally used for control of most annual grasses and certain broadleaf weed species of corn, cotton, cabbage, citrus, and peanut crops. Acetochlor is absorbed mainly by germinating plant shoots, and secondly by roots. It appears to inhibit geranylgeranyl pyrophosphate (GGPP) cyclisation enzyme synthesis in susceptible plants (Braswell et al 2016). However, studies showed that crops injury from acetochlor was greater in wet soil conditions within a two week period after application (Bouchonnet et al 2011; Hausman et al 2013). Corn injury from acetochlor was often associated with the phenomenon that leaves couldn't pull free from the whorl and form a "ladder" like plant. General symptoms included stunted plants with abnormally thick, short roots or twisted shoots and dark green leaves, also (Jursik et al 2011; Braswell et al 2016).

Herbicide safener was a widely used agrochemical with the unique ability to selectively protect crop plants from herbicide damage and

improve the selectivity of herbicide (Elmore et al 2016). Herbicide safeners are particularly effective in protecting monocot crops by increasing herbicide detoxification (Mhlanga & Chauhan 2016; Bartucca et al 2017). The current studies suggested herbicide safener appeared to induce a set of enzymes and improve herbicide metabolism (Stoilkova & Yonova 2010; Buono & Ioli 2011). It was found that the ability of safeners to protect maize from herbicide damage was related to the induction of glutathione-s-transferase activity (Fu et al 2011; Li et al 2017). Moreover, the researches had suggested that the detoxification ability of safener was involved in the level of glutathione conjugation in plant (Jo et al 2011; Ye et al 2016). However, the effects of chloroacetanilide safeners on the herbicide detoxification pathway were rarely reported. Safeners naphtbalic anhydride and dichlormid could increase crop tolerance to herbicide acetochlor by increasing the content of GSH and enhancing the activity of GST (Kraehmer et al 2014). It was also found that dichloromethyl-dioxolane safener protected maize by enhancing the activity of GST on catalyze glutathione conjugation in the metabolic detoxification of chloroacetanilide herbicide acetochlor (Rezaei et al 2013).

Studies indicated that some 3-dichloroacetyl-substituted oxazolidines with a chiral center often have different biological activities (Sriharsha & Shashikanth 2006; Zhao et al 2015). One of the most widely used dichloroacetamide safeners in maize was 3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine (R-29148). It could protect corn effectively by enhancing the expression of GST enzymes, which involved in herbicide detoxification (Li et al 2017). The activity of several antioxidative system enzymes, such as CAT and POD, was responsible for alleviating the oxidative stress generated by herbicides (Martins et al 2011; Rajasekar et al 2015; Sytykiewicz 2015). However, there is no clear mechanism for explaining the principle of action of the chiral safeners (Jablonkai 2013). Chiral R-29148 and 3-dichloroacetyl substituted oxazolidines were successfully synthesized in our previous research (Gao et al 2012). Therefore, this study was concerned with the possible mechanism of one chiral centers safeners to alleviate toxicity of acetochlor to maize. Enzyme activities of GSH, GST, POD, and CAT in maize, which treated with safener racemic R-29148 and its chiral isomers, were investigated. It was hypothesized that three potential herbicide safeners could effectively protect maize against herbicide injury.

2. Material and Methods

The experiments were carried out with maize seeds Dongnong 253 (*Zea mays* L.). Racemic R-29148, R-isomer, and S-isomer were synthesized in our laboratory, and their purity levels were greater than 99.0% (Table 1). Acetochlor emulsifiabale concentrate (50%) was provided by Zhongshi Pharmaceutical Co., Ltd (Shandong, China). Acetolachlor standards were purchased from Aladdin Reagent Co., Ltd. And 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB) and GSH were purchased from Sigma (Shanghai, China). Methanol (99.9%) was provided by Dikma (Beijing, China).

Table 1- Chemical name of safeners

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

Safeners were applied as a seed coating, and maize seeds were soaked in safener solution (0, 1, 5, 10, 25, 50, 100 mg L^{-1}) for 12 h at 26.5 ℃ 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 ℃ for 24 h in a growth chamber with a 12/12 photoperiod. Next, the seeds were directly sown in paper-cups (8 cm × 12 cm) containing 150 mL quartz sand prewashed with 10% (v v^{-1}) hydrochloric acid solution and sterilized in 5%

(w v^{-1}) sodium hypochlorite solution, 6 seeds per cup. The treatments were performed by adding 60 mL of acetochlor solution (10 mg L^{-1}) to the sand quartz, each cup with water holding capacity at 60%. The control was treated with water. Seedlings were grown at 26.5 ℃ 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. After the treatments, maize shoots and roots were collected and rinsed with water, and then dried through blotting. We determined the shoot and root length as well as fresh weight (FW). The maize growth index recovery rates were calculated to determine the optimal safener concentration. The growth index recovery rates were calculated by Equation (1).

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

GSH level assay: GSH level was measured in accordance with Ismaiel & Papenbrock (2014). The maize tissue was homogenized in 5% (w v^{-1}) sulfosalicyclic acid and the homogenates were centrifuged at 15000 × g for 20 min at 4 °C. GSH levels in 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 enzyme extraction and assay *in vivo*: The extraction and assay of GST was performed as described by Buono & Ioli (2011). To measure the

$$\text{Recovery rate (\%)} = \frac{\text{Treated with compounds and acetochlor - Treated with acetochlor}}{\text{Contrast - Treated with acetochlor}} \quad (1)$$

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^{-1}) at 4 °C. The homogenate was centrifuged at 15000 × g for 20 min at 4 °C. 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). 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).

GST activity assay *in vitro*: To determine the GST activity *in vitro* against acetochlor in this study, HPLC assays were performed to determine the GST activity towards the herbicide acetochlor as substrate in accordance with Scarponi et al (2006). The GST enzyme extraction was added to GSH and an acetochlor standard solution. 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 with methanol and injected into an HPLC. The GST activity was measured by comparing the initial and residual concentrations of acetochlor. GST activity was expressed as the quantity of acetochlor consumed per minute per milligram of enzyme (nmol min^{-1} mg^{-1} protein).

Kinetic parameters of GST assay: 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 (Scarponi et al 2006). The GST activity was determined over a range of 1-chloro-2,4-dinitrobenzene (CDNB) concentration (1.0-32.0 mM) at a single GSH concentration of 5 mM.

POD enzyme extraction and assay: To investigate the effect of safener to target enzyme, POD activity was determined as described a modified method from Rajasekar et al (2015) 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 peroxidase activity was expressed as mmol min^{-1} g^{-1} FW.

CAT enzyme extraction and assay: CAT enzymatic activity was determined following the procedures described in Hemanth Kumar et al (2016). The reaction mixtures (1.9 mL H_2O, 0.1 mL enzyme extract and 1 mL 0.3% (v v^{-1}) 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.

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 ($P<0.05$). Data were expressed as mean±standard deviation (n= 3).

3. Results and Discussion

3.1. Growth index of maize

In inhibited growth experiments of the maize, the acetochlor showed a severe shoot and root growth retardation. The treated maize growth index inhibition rate based on the plant height, fresh weight of shoot, root length, and root fresh weight decreased by 42, 27, 36 and 24%, respectively. To determine a suitable treatment regime, a range of concentrations of safeners were tested for their ability to decrease the injury caused by acetochlor. The protective effects of three safeners at different concentrations were recorded for R-29148 at the concentration 25 mg L^{-1}, R-isomer at the concentration 5 mg L^{-1}, and S-isomer at the concentration 50 mg L^{-1}. The results showed that all the three safeners significantly decreased the inhibition by the acetochlor herbicide, and the order of protective ability of three chiral safeners was as follows: R-isomer > R-29148 > S-isomer. The maize growth indicator recovery rates ranged from 54 to 139% as shown in Figure 1, respectively.

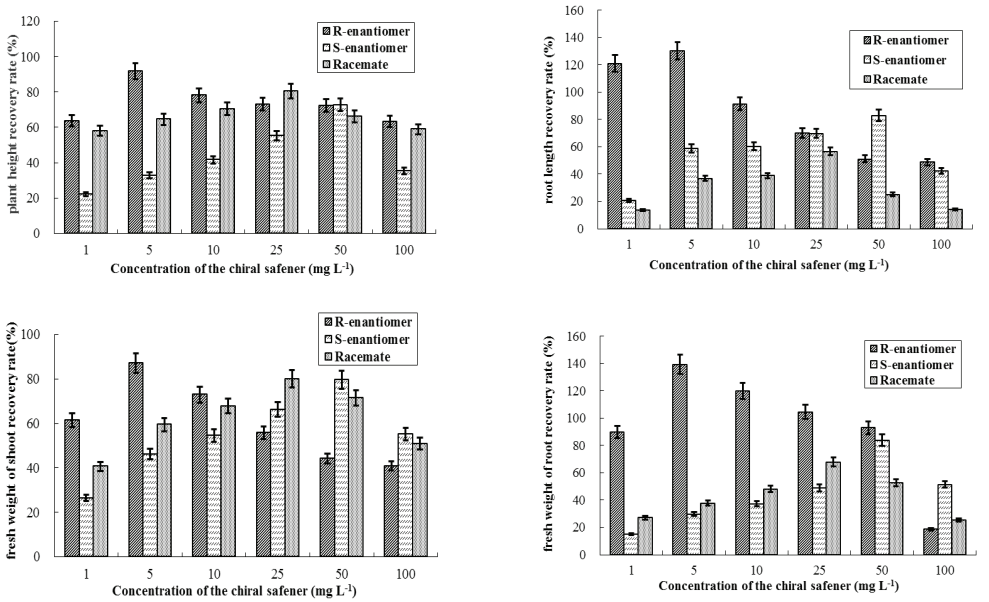

Figure 1- Recovery rate of growth indexes of maize affected by acetochlor and safeners

3.2. GSH level

In the maize root and shoot tissue, increases of GSH level after pretreatment with safeners were greater than with the acetochlor treatment (Table 2). Similarly, GSH level in the seedling tissue with the R-isomer-acetochlor treatment was greater than with the acetochlor treatment in maize. After pretreatment with the R-enantiomer the GSH contents markedly increased by 86 and 53% in the root and shoot respectively. Our results suggested that the enhanced GSH level in the maize seedlings may be related to the protective activity of herbicide safener.

Table 2- Effect of safeners and acetochlor on GSH level

Treatment	*GSH level in root ($\mu g\ g^{-1}$)*	*GSH level in shoot ($\mu g\ g^{-1}$)*
Control	4.043±0.331 d	10.044±0.171 d
Acetochlor	4.391±0.282 d	12.354±0.234 c
R-isomer+Acetochlor	8.192±0.149 a	18.868±0.178 a
S-isomer+Acetochlor	5.446±0.473 b	10.068±0.429 d
R-29148+Acetochlor	4.810±0.356 c	15.519±0.322 b

3.3. GST activity

The GST activity in vivo of maize seedling root treated by racemic R-29148 or R-isomer combined with acetochlor showed significantly increases (Table 3). The data indicated that the safener could enhance the GST activity, and the enhanced GST activity facilitated maize seedling survival at low acetochlor concentrations. When acetochlor was added as the substrate instead of CDNB, as expected, the in vitro activity of GST was also enhanced due to the chiral safeners (Table 3). The results of GST activity in maize indicated that different protect effects of safener in the maize root were due to the different levels of GST enzyme activity toward CDNB or acetochlor substrate. The R-isomer effectively promoted the GST activity among the three safeners.

3.4. Kinetic parameters of GST

The kinetic parameters tests of maize GST were carried out by using enzymatic extracts from maize roots (Table 4). The V_{max} of this process decreased, while the K_M increased under treatment with acetochlor. V_{max} increased by 60 and 18% after treated by R-isomer and racemic R-29148 compared with the untreated control, and the K_M decreased by 24 and 7%, respectively. The results in Table 4 showed that obvious influence of R-enantiomer to induction and dynamics of GST activity.

Table 4- Effect of safeners and acetochlor to kinetic parameters of GST

Treatment	*V_{max} (nmol min^{-1} mg^{-1} protein)*	*K_m (mmol L^{-1})*
Control	14.87±0.030 c	0.51±0.042 b
Acetochlor	6.69±0.044 e	0.56±0.027 a
R-isomer	23.82±0.023 a	0.39±0.019 d
S-isomer	12.73±0.067 d	0.50±0.043 b
R-29148	17.54±0.035 b	0.48±0.037 c

3.5. POD and CAT activity

The activities of POD and CAT were involved in metabolizing the oxidative stress due to high herbicide doses and protecting plants from the stress generated by herbicide. The effect of

Table 3- Effect of safeners and acetochlor on GST

Treatment	*GST activity in vivo (nmol s^{-1} mg^{-1} protein)*	*Treatment*	*GST activity in vitro (nmol min^{-1} mg^{-1} protein)*
Control	7.67±0.32 d	Control	68.17±2.65 c
Acetochlor	8.39±0.44 c	Acetochlor	-
R-isomer+Acetochlor	20.68±0.65 a	R-isomer	117.88±5.11 a
S-isomer+Acetochlor	8.17±0.38 c	S-isomer	34.60±2.39 d
R-29148+Acetochlor	11.68±0.49 b	R-29148	99.05±4.07 b

safeners and acetochlor on POD and CAT activity were determined to investigate the protective effectiveness of chiral safeners (Table 5). Compared with the control, POD activity in the maize seedling roots exhibited a significant increase. In addition, an extreme decrease in POD activity was observed after treatment with the S-isomer and racemic R-29148 compared with the acetochlor treatment alone. Upon treatment with the R-isomer, POD enzyme activity decreased from 2388 to 1834.

CAT was involved in metabolizing the oxidative stress due to high herbicide doses and then protecting plants from the stress generated by herbicide. In this case, CAT activity increased to 9.00 after the acetochlor treatment compared with the untreated control. The data in Table 5 showed that the CAT activity decreased to 7.60, 2.30 and 4.60 after treatment with three safeners, respectively.

Table 5- Effect of safeners and acetochlor on CAT and POD activity

Treatment	*CAT Activity (μmol min^{-1} g^{-1} FW)*	*POD Activity (mmol min^{-1} g^{-1} FW)*
Control	2.09±0.03 d	1135±3.25 e
Acetochlor	9.00±0.05 a	2388±4.87 b
R-isomer+Acetochlor	7.60±0.03 b	1834±1.62 d
S-isomer+Acetochlor	2.30±0.01 d	1988±2.21 c
R-29148+Acetochlor	2.09±0.03 d	1135±3.25 e

Marked acceleration of glutathione conjugation responsible for herbicide resistance in plant had been well-documented (Ismaiel & Papenbrock 2014). Safeners can stimulate GST activity and effectively detoxify by enzyme-catalyzed conjugation of GSH with the acetochlor herbicide (Scarponi et al 2006; Jablonkai 2013). This meant that the detoxification ability of safener could be decided by the degree of glutathione conjugation in maize to a certain extent. Overall, the R-isomer could protect maize from chloroacetanilide herbicide injury with enhanced GSH content and stimulated GST activity to promote glutathione conjugation with acetochlor in the maize seedlings. Compared with treated by acetochlor, dynamics of GST activity toward CDNB in safener-treatment increased significantly (Ye et al 2016). The data showed that the R-isomer significantly altered the kinetic parameter V_{max} and K_M.

Reports show that POD and CAT were involved in herbicide tolerance and two antioxidant enzymes activity increase during herbicide exposure (Rajasekar et al 2015; Sytykiewicz 2015). Our results further suggested that the activities of POD and CAT in maize were decreased by treated with the R-isomer, which indicates resistance to oxidative stress in which the chiral safener played a certain role in maize. This mechanism could be an important pathway for chiral safener detoxification in maize.

4. Conclusions

Based on data obtained in this study, it can be concluded that the effects of racemic R-29148 and its chiral isomers on growth and enzymes activity of maize could protect maize against injury from chloroacetanilide herbicides acetochlor. We investigated the changes of GST, CAT and POD activity after treatment with a safener. The maize growth level and GST activity were significantly inhibited by acetochlor, which could be tempered by adding the R-isomer. The results also suggested that the R-isomer can affect POD and CAT activity, which detoxified the plant from the effects of the acetochlor. Moreover, further studies are still needed to determine the exact mechanism of chiral safener to protect maize from injury by chloroacetanilide herbicide.

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