Chiral 3-dichloroacetyl Oxazolidines Induced the Activity of Enzymes in Maize for the Detoxified Herbicide

Fei Ye¹, Hai-Feng Cao¹, Li-Na Wei¹, Chen Wang¹, Ying Fu¹, Chun-Yan Li² and Shuang Gao¹*
¹College of Science, Northeast Agricultural University, Harbin, P.R. China
²College of Resources and Environment, Northeast Agricultural University, Harbin, China
*For correspondence: gaoshuang@neau.edu.cn; yefei@neau.edu.cn

Abstract

The expression of detoxifying enzyme glutathione S-transferase (GST) and antioxidant enzyme activities in maize seedlings were studied in response to three potential herbicide safeners (3-dichloroacetyl oxazolidine and its two optical isomers). The protective effect of chiral herbicide safeners on detoxifying to chloroacetanilide herbicides butachlor in maize was evaluated. All safeners increased the expression levels of herbicide detoxifying enzymes, including GST, catalase (CAT) and peroxidase (POD) to reduce chloroacetanilide herbicide phytotoxicity in maize seedlings. Our results indicate that the R-isomer of 3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine can induce glutathione (GSH) content, activity of GST, and the affinity for 1-chloro-2,4-dinitrobenezene (CDNB) substrate in maize, which can protect maize from injury by chloroacetanilide herbicide butachlor. The information of antioxidative enzymes activation was obtained to discuss the role of chiral safener in overcoming oxidative stress caused by the herbicide in maize. © 2018 Friends Science Publishers

Keywords: Chiral safener; Biological activity; Antioxidative enzyme activity; Protection mechanism; Butachlor

Introduction

Butachlor is a selective pre-emergent acetonilide herbicide. It is extensively used to control perennial grasses and a variety of broad leaf weeds of rice, corn, soybeans, and peanuts crops (Liu et al., 2011). Butachlor is absorbed mainly by germinating plant shoots, and secondly by roots. It appears to affect the formation of long chain fatty acids in susceptible plants (Cao et al., 2016). Previous studies have shown that butachlor harmed crops more often in conditions that soil containing relatively many water within two weeks of application (Hausman et al., 2013; Rasool et al., 2014). Butachlor is safe for soybean and peanut, so it applies to intercrop a corn field with soybean and peanut. The phytotoxic effects of butachlor on corn are often manifested as the inability of leaves to separate from the spiral. Affected by phytotoxicity, plant roots may become abnormally short, branches distort and the leaves turn dark green (Alfa et al., 2008).

Herbicide safeners are applied with herbicides to protect crops against their injury, without affecting weed control efficacy of the herbicides (Elmore et al., 2016). Herbicide safeners are particularly effective in protecting monocot crops by increasing herbicide detoxification (Mhlanga and Chauhan, 2016; Bartucca et al., 2017). The current studies suggested herbicide safener appeared to induce a set of enzymes and improve herbicide metabolism (Stoilkova and Yonova, 2010; Buono and Ioli, 2011). It was found that the ability of safeners to protect maize from herbicide damage was related to the induction of the activity of GST (Fu et al., 2011; Li et al., 2017). The main detoxification pathway represents the conjugation of chloroacetanilide and thiocarbamate sulfoxides herbicides with glutathione. (Jo et al., 2011; Ye et al., 2016). However, the effects of chloroacetanilide safeners on herbicide detoxification pathway were rarely reported. Safeners naphtallic anhydride and dichlormid could increase crop tolerance to herbicide butachlor by increasing the content of GSH and enhancing the activity of GST (Scarpone et al., 2006; Kraehmer et al., 2014). It was also found that dichloromethyl-dioxolane safener protected maize by enhancing the activity of GST on promote the conjugation of glutathione with chloroacetanilide herbicide butachlor in the metabolic detoxification (Rezaei et al., 2013).

Some reports indicated that 3-dichloroacetyl-substituted oxazolidines containing one chiral center usually have certain bioactivity (Sriharsha and Shashikanth, 2006; Zhao et al., 2015). Among the most widely used dichloracetamide safeners in maize, (R,S)-3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidine (R-29148) was particularly effective at protecting maize against herbicides. It could protect corn effectively by enhancing the expression of GST enzymes which involved in herbicide detoxification (Li et al., 2009). Activities of some antioxidative system enzymes including CAT and POD, are responsible for alleviating the oxidative stress caused by

To cite this paper: Ye, F., H.F. Cao, L.N. Wei, C. Wang, Y. Fu, C.Y. Li and S. Gao, 201x. Chiral 3-dichloroacetyl oxazolidines induced the activity of enzymes in maize for the detoxified herbicide. Int. J. Agric. Biol. 00: 000-000
herbicides, heavy metal and salt. Herbicide was found to cause oxidative stress in plants, which responded by an increase in the activity of antioxidative enzymes such as POD and CAT in the root tissues (Farombi et al., 2008; Martins et al., 2011; Bajpai and Srivastava, 2015; Obermeier et al., 2015). However, there is no report on the action principle of chiral safeners (Jablonkai, 2013). Chiral R-29148 and 3-dichloroacetyl substituted oxazolidines were successfully synthesized in previous report (Gao et al., 2012). Therefore, the present study aimed to ascertain the mechanism by which the raceme R-29148 and its chiral isomers protect maize from injury caused by butachlor and verify the supposition that detoxification of herbicide can be enhanced by safeners. The protective effects of chiral safener were examined on maize-seedling growth, GSH levels, GST, POD, and CAT enzyme activities, and to prove chiral safeners’ potential to promote herbicidal metabolism on the expression of detoxifying and antioxidative enzymes activity.

Materials and Methods

Materials and Chemical Reagents

Butachlor emulsifiable concentrate (60%) was purchased from Zhongshi Pharmaceutical Co., Ltd (Shandong, China). Butachlor standards were provided by Aladdin Chemistry (Shanghai, China). 5, 5'-dithiobis-(2-nitrobenzoic) acid (DTNB), 1-chloro-2, 4-dinitrobenzene (CDNB) and GSH were purchased from Sigma (Shanghai, China). Other chemical solvents were obtained from Aladdin Chemistry (Shanghai, China). Racemic R-29148, R-isomer, and S-isomer were synthesized in our laboratory, with the purity levels more than 99.0% (Table 1) (Fig. 1). The maize seeds, named ‘Dongnong 253’ (Zea mays L.), were germinated and raised in a growth chamber at the Pesticide Chemistry Laboratory, Northeast Agricultural University.

Plant Material and Growth Conditions

Before sowing, seeds were soaked in treatment solutions containing safener (0, 1, 5, 10, 25, 50, 100 mg·L⁻¹) at 26.5°C for 12 h. The controls were soaked in distilled water instead of safener solution. The seeds were germinated at 26.5°C for 24 h after soaking and then were sown in paper-cups (8 cm × 12 cm) directly. 6 seeds per cup. Each cup contained 150 mL quartz sand which was prewashed with solution and treated for sterilized. Quartz sand was sterilized by sodium hypochlorite solution (5%, w/v) after being washed with hydrochloric acid (10%, v/v). 60 mL butachlor solution (100 mg·L⁻¹) was added to the sand quartz, and water-holding capacity in each cup was controlled at 60%. Water was used for the treatment on the control. The seedlings were cultured in the growth chamber with a relative humidity of 75% at 26.5±1°C and with a 12 h light photoperiod.

Samples from maize shoot and root under each treatment were collected at 8 d, and liquid nitrogen was used to frozen samples immediately. Frozen samples were stored at −80°C. Frozen tissues were then grounded in liquid nitrogen until determination of enzyme activities (GSH, GST, POD, and CAT). Length and fresh weight (FW) of shoot and root were also determined. All experiments were performed in triplicate. The optimal safener concentrations were determined by calculating the recovery rate of maize growth index. Recovery rate of growth index was calculated by the formula followed:

\[ \text{Recovery rate} = \frac{\text{Growth index of maize treated by herbicide} - \text{Growth index of maize untreated}}{\text{Growth index of maize treated by herbicide}} \times 100\% \]

GSH Content Assay

GSH level was measured in accordance with Ismaiel and Papenbrock (2014). Homogenizing was carried out in sulfosalicyclic acid (5%, w/v). After being centrifuged at 15 000 × g for 20 min at 4°C, supernatant of the homogenates was immediately used for the GSH contents assays. GSH contents were determined spectrophotometrically at 412 nm with the DTNB reagent.

Extraction and Activity Determination of GST

Extraction and activity determination of GST was performed according with Buono and Ioli (2011). 200 mg frozen maize seedling tissue was used to measure the GST activity. After being ground into powder with the attendance of liquid nitrogen, the tissue was homogenized in 1 mL 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 final assay mixture consisted of 50 mM phosphate buffer (pH 6.5), 1 mM CDNB, 1 mM GSH, and 0.5 mM EDTA. The increase in absorbance at 340 nm was recorded after adding the DTNB reagent (5% w/v) and incubation at 4°C for 10 minutes. The increase in absorbance was measured spectrophotometrically at 412 nm and defined as the amount of herbicide reaction with GSH catalyzed by 1 mg GST in unit time (mmol·s⁻¹·mg⁻¹ protein).

To determine the activity of GST in vitro against butachlor, HPLC assays were carried out according with Scarpone et al. (2006). The GST enzyme extraction was mixed with GSH and butachlor standard solution. After being incubated for 2 h, 10 μL 3.6 M HCl was added into the reaction mixture to stop the reaction, and the mixture was added into methanol for extracting and then collected for HPLC analysis. By comparing the concentrations of butachlor before and after reaction, the GST activity was measured. GST activity was defined as the amount of butachlor reaction with in one minute with unit mass of GST (nmol·min⁻¹·mg⁻¹ protein).
GST Kinetic Parameters Assay (CDNB)

The procedure described by Buono et al. (2006) was followed for measuring kinetic parameters of GST with modification. According to double reciprocal plots, a linear regression analysis of 1/V vs. 1/S was used to determine the kinetic parameters $V_{\text{max}}$ and $K_M$. 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.

CAT Activity Assay

Decomposition rate of H$_2$O$_2$ was determined at 240 nm to evaluate the total activity of CAT according with Hemanth Kumar (2016). The reaction mixtures containing 1.9 mL H$_2$O, 0.1 mL enzyme extract and 1 mL hydrogen peroxide (0.3% ,v/v) were determined spectrophotometrically for 3 min. The CAT activity was determined as μmol H$_2$O$_2$ min$^{-1}$·g$^{-1}$ FW.

POD Enzyme Assay

To investigate the effect of safener to target, POD activity was determined based on a modified method from Obermeier et al. (2015). The mixture used for determination consisted of 1 mL 50 mM sodium phosphate buffer (pH 7.0), 2 mL 0.3% hydrogen peroxide and 0.95 mL 0.2% guaiacol. 0.01 mL enzyme extract was added to reaction mixtures. The mixture was determined spectrophotometrically at 470 nm for 5 min. The change in absorbance was recorded with the addition of H$_2$O$_2$. The peroxidase activity was determined as mmol·min$^{-1}$·g$^{-1}$ FW.

Statistical Analysis

SPSS statistic program (Version 16.0) software was used for data analysis. Differences between the treatments were evaluated by the least significance difference using the grouped mean and Duncan multiple range test.

Results

Growth Index of Maize

Growth index inhibition rate of maize treated by butachlor decreased significantly. Safener solutions with different concentration were used to test for the ability to decrease the injury, which was caused by butachlor, so as to obtain a suitable treatment regime. 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 protective effects of three safeners at different concentrations were assayed, and the results showed that the inhibition caused by butachlor could be significantly decreased after treated by all the three chiral safeners. The recovery rates of maize seedling treated by R-isomer and racemic R-29148 were higher than by S-isomer. The growth index recovery rates of maize were shown in Fig. 2.

GSH Content

The safener’s ability on detoxification was related to the conjugation level of glutathione in maize (Taylor et al., 2013). In this evaluation, the increasing of GSH level in root and shoot of maize which was treated by safeners were greater than those treated by butachlor (Table 2). Similarly, GSH level in the seedling tissue treated by the R-isomer-butachlor was greater than those treated by the butachlor. After treated by R-isomer, the GSH contents in the root and shoot increased observably. The results indicated that there was a relationship between the enhanced GSH level in the maize seedlings and the protective activity of herbicide safener. The increase of GSH induced by safener has also been reported previously (Jablonkái, 2013).

GST Activity

Safeners had the ability to motivate the activity of GST and toxicity could be effectively reduced by enzymecatalyzed conjugation of GSH with the herbicide. It was found that the activity of GST in the root of maize seedling which was treated by butachlor increased compared with the control. Treated by the safener and butachlor also caused increasing in the in vivo activity of GST (Table 3). The in vitro activity of GST was also increased attributed to the chiral safeners when butachlor was used as the substrate instead of CDNB (Table 3). The data indicated that difference in protecting effects of safener in the maize root might attribute to the different activity degree of GST on CDNB or butachlor substrate. Among the three safeners studied, R-isomer showed effectively induction on the activity of GST.

Kinetic Parameters of GST

Enzymatic extracts from maize roots were used to determine the kinetic parameters of GST in maize (Table 4). After treated by butachlor, $V_{\text{max}}$ decreased while $K_M$ increased. Compared with the untreated control, $V_{\text{max}}$ increased after treated by R-isomer and racemic R-29148, and $K_M$ decreased by 23 and 8%, respectively. Table 4 showed the obvious influence of R-isomer to induction and dynamics of GST activity. Other safeners also induced the affinity of GST to substrate in the conjugated reaction. It was similar with the results of GSH content and GST activity.

POD and CAT Activity

It was reported that POD and CAT could protects crops from the stress caused by herbicide because their activities were connected with metabolizing the oxidative stress caused by high herbicide doses (Obermeier et al., 2015).
Table 1: Chemical name of test safeners

<table>
<thead>
<tr>
<th>Safener</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-29148</td>
<td>(R,S)-3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidine</td>
</tr>
<tr>
<td>R-isomer</td>
<td>(R)-3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidine</td>
</tr>
<tr>
<td>S-isomer</td>
<td>(S)-3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidine</td>
</tr>
</tbody>
</table>

Table 2: Effect of safeners and butachlor on GSH content in maize

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH content in root (μg·g⁻¹)</th>
<th>GSH content in shoot (μg·g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.319 ± 0.121 d</td>
<td>12.757 ± 0.092 e</td>
</tr>
<tr>
<td>Butachlor</td>
<td>5.092 ± 0.174 c</td>
<td>13.876 ± 0.166 d</td>
</tr>
<tr>
<td>R-isomer + Butachlor</td>
<td>7.862 ± 0.202 a</td>
<td>17.387 ± 0.353a</td>
</tr>
<tr>
<td>S-isomer + Butachlor</td>
<td>6.428 ± 0.216 b</td>
<td>16.340 ± 0.145c</td>
</tr>
<tr>
<td>R-29148 + Butachlor</td>
<td>6.775 ± 0.122 b</td>
<td>16.791 ± 0.184 b</td>
</tr>
</tbody>
</table>

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

Table 3: Effect of safeners and butachlor on activity of GST in maize

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GST activity in vivo (μmol·min⁻¹·mg⁻¹ protein)</th>
<th>GST activity in vitro (μmol·min⁻¹·mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.64 ± 0.92 a</td>
<td>75.36 ± 0.95 c</td>
</tr>
<tr>
<td>Butachlor</td>
<td>13.86 ± 0.24 a</td>
<td>96.71 ± 2.05 b</td>
</tr>
<tr>
<td>R-isomer + Butachlor</td>
<td>20.92 ± 1.61 a</td>
<td>75.63 ± 3.26 c</td>
</tr>
<tr>
<td>S-isomer + Butachlor</td>
<td>14.71 ± 0.50 b</td>
<td>88.69 ± 2.07 c</td>
</tr>
<tr>
<td>R-29148 + Butachlor</td>
<td>14.61 ± 0.88 b</td>
<td></td>
</tr>
</tbody>
</table>

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

Table 4: Effect of safeners and butachlor on kinetic parameters of GST in maize

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vₘₐₓ (μmol·min⁻¹·mg⁻¹ protein)</th>
<th>Kₘ (mmol·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.87 ± 0.30 c</td>
<td>0.52 ± 0.042 a</td>
</tr>
<tr>
<td>Butachlor</td>
<td>12.71 ± 0.44 d</td>
<td>0.51 ± 0.027 a</td>
</tr>
<tr>
<td>R-isomer</td>
<td>23.82 ± 0.23 a</td>
<td>0.39 ± 0.019 c</td>
</tr>
<tr>
<td>S-isomer</td>
<td>12.73 ± 0.67 d</td>
<td>0.50 ± 0.043 a</td>
</tr>
<tr>
<td>R-29148</td>
<td>17.54 ± 0.35 b</td>
<td>0.48 ± 0.037 b</td>
</tr>
</tbody>
</table>

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

Table 5: Effect of safeners and butachlor on POD and CAT activity of maize

<table>
<thead>
<tr>
<th>Treatment</th>
<th>POD Activity (nmol·min⁻¹·g⁻¹ FW)</th>
<th>CAT Activity (μmol·min⁻¹·g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1135 ± 2.28 e</td>
<td>2.09 ± 0.066 c</td>
</tr>
<tr>
<td>Butachlor</td>
<td>1574 ± 5.20 c</td>
<td>35.90 ± 1.65 c</td>
</tr>
<tr>
<td>R-isomer + Butachlor</td>
<td>1206 ± 3.11 d</td>
<td>20.60 ± 0.83 b</td>
</tr>
<tr>
<td>S-isomer + Butachlor</td>
<td>2039 ± 6.65 b</td>
<td>6.60 ± 0.21 c</td>
</tr>
<tr>
<td>R-29148 + Butachlor</td>
<td>2807 ± 4.06 a</td>
<td>12.70 ± 0.11 c</td>
</tr>
</tbody>
</table>

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

The effect of safeners and butachlor on POD and CAT activity were determined to investigate the protective effectiveness of chiral safeners (Table 5). Compared with the control, a significant increase could be observed on POD activity in the roots of maize seedling which was treated by butachlor alone. After being treated by R-isomer, activity of POD decreased. The decreased activities of POD indicated that it might play essential roles in detoxifying herbicide under chiral safener treatment.

The activities of CAT could also protect crops and reduce the stress caused by herbicide by participating in metabolizing the oxidative stress. In this case, activity of CAT increased after being treated by butachlor compared with control. The decreasing on CAT activities after treatment with three safeners was shown in Table 5.

Discussion

Marked acceleration of glutathione conjugation responsible for herbicide resistance in plant had been well-documented (Ismaiel and Papenbrock, 2014). GST activity could be stimulated by safeners and enhance the conjugation of GSH with butachlor catalyzed by GST, which led to effectively detoxify (Scarponi et al., 2009; Jablonki, 2013). This meant that the detoxification ability of safener could be decided by the degree of conjugation of glutathione in maize to some extent. Overall, the R-isomer could enhance content of GSH and stimulate activity of GST to promote conjugation of glutathione with butachlor in the maize seedlings, and then protect maize from chloroacetanilide herbicide injury. Compared with those treated by butachlor,
Mechanism of Chiral Safener to Protect Maize from Herbicide Injury / Int. J. Agric. Biol., Vol. 00, No. 0, 201x

significant increasing could be observed on dynamics of GST activity toward CDNB in case treated by safener (Ye et al., 2016). Results indicated that the R-isomer altered the kinetic parameter $V_{\text{max}}$ and $K_M$ significantly. In this case, it was shown that different chiral safeners could induce GST to accelerate the conjugation of butachlor and GSH, which was responsible for the detoxification.

In our experiment, the antioxidative defense system consisting of POD and CAT was investigated. POD and CAT were involved in the mechanism of oxidative stress which was caused by herbicides. It could protect plants from the stress generated by herbicide overdoses. Increasing in POD and CAT activities in several plant species during herbicide exposure was reported (Bajpai and Srivastava, 2015; Sytykiewicz, 2015). Both herbicide and safener induced different trends of antioxidative enzymes activities and antioxidative stress response. In this study, further suggestion could be generated that the activities of POD and CAT in maize decreased after treated by R-isomer, which indicated resistance to oxidative stress in which the chiral safener played a certain role in maize. However, there was no significant change on POD activity after treated by racemic R-29148 and S-isomer. The activities of the two enzymes decreased after treated by R-isomer, which were in contrast to their response to herbicide. It could be concluded that R-isomer had the ability to reduce oxidative stress caused by butachlor in maize. This mechanism could be an important approach for the explanation of chiral safener detoxification in maize.

**Conclusion**

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 herbicide butachlor. The growth level of maize and GST activity were inhibited by butachlor significantly, which could be tempered by adding the R-isomer. The data indicated that the safener could enhance the GST activity, and the enhanced GST activity facilitated maize seedling survival at low butachlor concentrations. There are indications that the R-isomer can affect POD and CAT activity, which detoxified the plant from the effects of the...
butachlor. Moreover, further studies are still needed to determine the exact mechanism of chiral safener to protect maize from injury by chloroacetanilide herbicides.

Acknowledgements

This research was funded by the National Nature Science Foundation of China (No. 31572042), the Natural Science Foundation of Heilongjiang Province (No. ZD2017002, C2015013, C2015014), the Research Science Foundation in Technology Innovation of Harbin (No. 2017RAQXJ017), and the National Students’ Innovation and Entrepreneurship Training Program (No. 201710224002).

References


Ye, F., H.F. Cuo, Y. Fu, L.X. Zhao and S. Gao, 2016. The safener effect of chiral derivatives of 3-dichloroacetyl oxazolidine against haloxyfop-P-methyl-induced toxicity in maize. Zedmirbyste, 103: 29–34


(Rceived 28 November 2017; Accepted 28 December 2017)