3-Dichloroacetyl oxazolidine protect maize from imazethapyr herbicide injury

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ABSTRACT

Safeners are an important tool used to ensure the safe using of herbicide. The objective of this paper was to investigate the protective effect of four 3-dichloroacetyl oxazolidine safeners (3-dichloroacetyl-2,2-dimethyl-1,3-oxazolidine [R-28725], racemate of 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine, and its two chiral stereoisomers) in reducing the injury caused by imazethapyr. Physiological and biochemical tests were conducted under laboratory condition, by using seed treatment with safeners and soil treatment with imazethapyr, respectively. The interaction of two safeners (R-28725 and *R*-stereoisomer) and imazethapyr reduced the injury of maize significantly, and also increased glutathione content, activity of glutathione *S*-transferases, and activity of acetolactate synthase in maize. When induced by *R*-stereoisomer, the GSH content in root and in shoot increased 100.7% and 73.6%, respectively. When induced by R-28725, the GST activity *in vivo* increased threefold and the GST activity *in vitro* more than doubled. The kinetic parameter V_{max} of GST in the maize treated with R-28725 and *R*-stereoisomer increased by 102.2% and 81.9%, respectively, compared with the control. The results also showed that R-28725 and *R*-stereoisomer induced glutathione *S*-transferases affinity for the substrate of conjugation reaction significantly.

Key words: 3-Dichloroacetyl oxazolidine, biological activity, GST activity, GST affinity, *Zea mays*.

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INTRODUCTION

Imazethapyr [(*RS*)-5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid], an imidazolinone herbicide, was discovered in the 1980s. Imazethapyr controls weeds by inhibiting the activity of acetolactate synthase (ALS) which catalyzes the first step in the biosynthesis of valine, leucine and isoleucine (Maja and Branko, 2011). Imazethapyr has been used worldwide in weed control. As a consequence, phytotoxic effects to certain rotational crops were observed because of imazethapyr residue in cultivation soil and weed resistance to imazethapyr has developed into a more serious problem (Zhou et al., 2009; Pinto et al., 2009; Heap, 2014).

Herbicide safeners are a group of synthetic compounds that could protect selected plants against herbicide injury without decreasing herbicidal activity to target weeds (Kraehmer et al., 2014). In 1947, Hoffman realized that safener was a useful tool to protect crop from herbicide injury. Nearly 20 safeners has subsequently been developed and commercialized by famous agrochemical companies (Jablonkai, 2013). While the exact mechanism of safener is still not completely understood, it seems that safeners could increase crop tolerance by inducing the activity of detoxifying enzyme glutathione *S*-transferases (GST) and content of glutathione (GSH) involved in detoxification of exogenous compounds (Del Buono and Ioli, 2011; Matola and Jablonkai, 2007). GST are multifunctional enzymes which catalyze conjugation of GSH with herbicide molecules such as some sulfonylureas, diphenylethers, sulfonamides (Hatzios, 2001; Cummins et al., 2011). Therefore, a possible mechanism was safeners increased the metabolic regulation of herbicide to inactive metabolites in the safened plant (Liu et al., 2009). The speed and effect of detoxification was closely related with the level of activity of GST and content of GSH.

R-28725 (3-dichloroacetyl-2,2-dimethyl-1,3-oxazolidine), a 3-dichloroacetyl oxazolidine derivative, was proven effective in protecting maize (*Zea mays* L.) from injury by ALS-inhibitor (Davies and Caseley, 1999; Zhao et al., 2014). Racemate of 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine, an analogue of R-28725, has one chiral C atom bonded to four different groups. Chirality is one of the most important properties of molecule because there is always one stereoisomer of chiral molecule exist in living creatures. While one stereoisomer may has some good properties, the other one can be toxic. So, these two stereoisomers which have different configurations may differ in their detoxification ability due to their chirality. However, little work has been done on the different detoxification ability of

chiral safener. In order to reduce the amount of pesticides used and avoid the possible injury to plant caused by the unnecessary stereoisomers, it is of necessity to develop chiral safeners. In this study, two stereoisomers and the racemate of 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine and R-28725 have been evaluated for bioactivity as safener of imazethapyr. Hence, the purpose of this article was to examine the protective effect of R-28725 and chiral 3-dichloroacetyl oxazolidine safeners and further discover the important role of GSH, GST, and ALS in detoxification process through physiological and biochemical tests.

MATERIALS AND METHODS

Materials and chemical reagents

The tested soil was Mollisols-cryolls clay loam type and collected from the Northeast Agricultural University Horticulture Station with a pH of 7.37. The seedlings of maize (*Zea mays* L.) 'Dongnong 253' were germinated and raised in a growth chamber at the Pesticide Chemistry Laboratory, Northeast Agricultural University. Imazethapyr (98.5%, wettable powder) was obtained from Aladdin Chemistry (Shanghai, China). R-28725, racemate of 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine and its two stereoisomers (*R*-enantiomer: (*R*)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine and *S*-enantiomer: (*S*)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine) were synthesized in our laboratories (99.0%) (Table 1).

Growth conditions

Seedlings of maize were soaking in solutions of safeners (0, 1, 5, 10, 25, 50, and 100 mg kg^{-1}) for 12 h, the control was soaking in water. Then, seeds were germinated in dishes in a growth chamber for 24 h. After seeds were sown in paper-cups (10 cm × 15 cm), seven seeds per cup, containing soil added with imazethapyr (0.5 mg kg^{-1}) with a depth of 13 cm, maize seeds were incubated in a growth chamber with a 12:12 h photoperiod, 26.5 ± 1 °C temperature, and 75% relative humidity. Each treatment was replicated three times.

The recovery rate of growth index (plant height, root length, fresh weight of shoot, fresh weight of root) of maize was measured 7 d after treatment and expressed as percentage:

$$\text{Recovery rate (\%)} = \frac{\text{Growth index of maize treated by safener and herbicide-Growth index of maize treated by herbicide}}{\text{Growth index of maize untreated-Growth index of maize treated by herbicide}}$$

where safeners were R-28725, racemate, and its two optically active enantiomers, and herbicide is imazethapyr.

All tissues of maize were washed and collected for subsequent biological activity assays (GSH, ALS, and GST).

Table 1. Safener chemical names used for test.

Safener	Chemical name
R-28725	3-dichloroacetyl-2,2-dimethyl-1,3-oxazolidine
R-stereoisomer	(*R*)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine
S-stereoisomer	(*S*)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine
Racemate	(*RS*)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine

Determination of GSH content, and GST activity

For determination of GSH content, 0.2 g tissue of maize (root or shoot) was homogenized in 5% sulfosalicylic acid and centrifuged at 15 000 g for 20 min. The supernatant was used for GSH content measurements by adding potassium phosphate buffer (pH = 8.0) and DTNB as the chromogenic agent. Absorbance was determined at 412 nm, whereas GSH content was calculated through comparison with a known concentration (Ismaiel and Papenbrock, 2014).

The extraction and GST assay were performed as described by Matola and Jablonkai (2007). The GST activity was obtained by measuring the amount of conjugate constituted from GSH and substrate CDNB and expressed as amount of conjugate per minute per milligram of protein (μmol min^{-1} mg^{-1} protein).

To determine the GST activity *in vitro* (GST activity against imazethapyr in this study), the amount of imazethapyr was determined by high performance liquid chromatography (HPLC) as described previously (Scarponi et al., 2006). GST enzyme was extracted from root of maize, and added with glutathione and imazethapyr solution. After 2 h cultivation, residues of imazethapyr in this mixture was measured through HPLC. The GST activity *in vitro* was expressed as amount of imazethapyr consumed per minute per milligram of enzyme (nmol min^{-1} mg^{-1} protein).

Determination of kinetic parameters of GST (CDNB)

The procedure described by Scarponi et al. (2006) was followed for measuring kinetic parameters of GST with modification. The kinetic parameters maximal rate of reaction (V_{max}) and the substrate concentration which results in one-half the maximum velocity (K_M) of GST were measured by linear regression of a double reciprocal plot. The kinetic parameters was determined by measuring GST activity over a range of CDNB concentration (0.13-4.14 mM) at a single GSH concentration of 5 mM.

Determination of ALS activity

To investigate the effect of safener to target enzyme, ALS activity was determined as described previously (Malkawi et al., 2003). ALS activity was expressed as amount of acetoin composed from acetolactate catalyzed by ALS per hour per milligram of enzyme (nmol h^{-1} mg^{-1} protein).

Statistical analysis

Statistical Product and Service Solutions software (SPSS 16.0, IBM Corporation, Armonk, New York, USA) was used to determine statistical significance at 95% confidence level ($p = 0.05$). Data were expressed as mean ± standard deviation ($n = 3$).

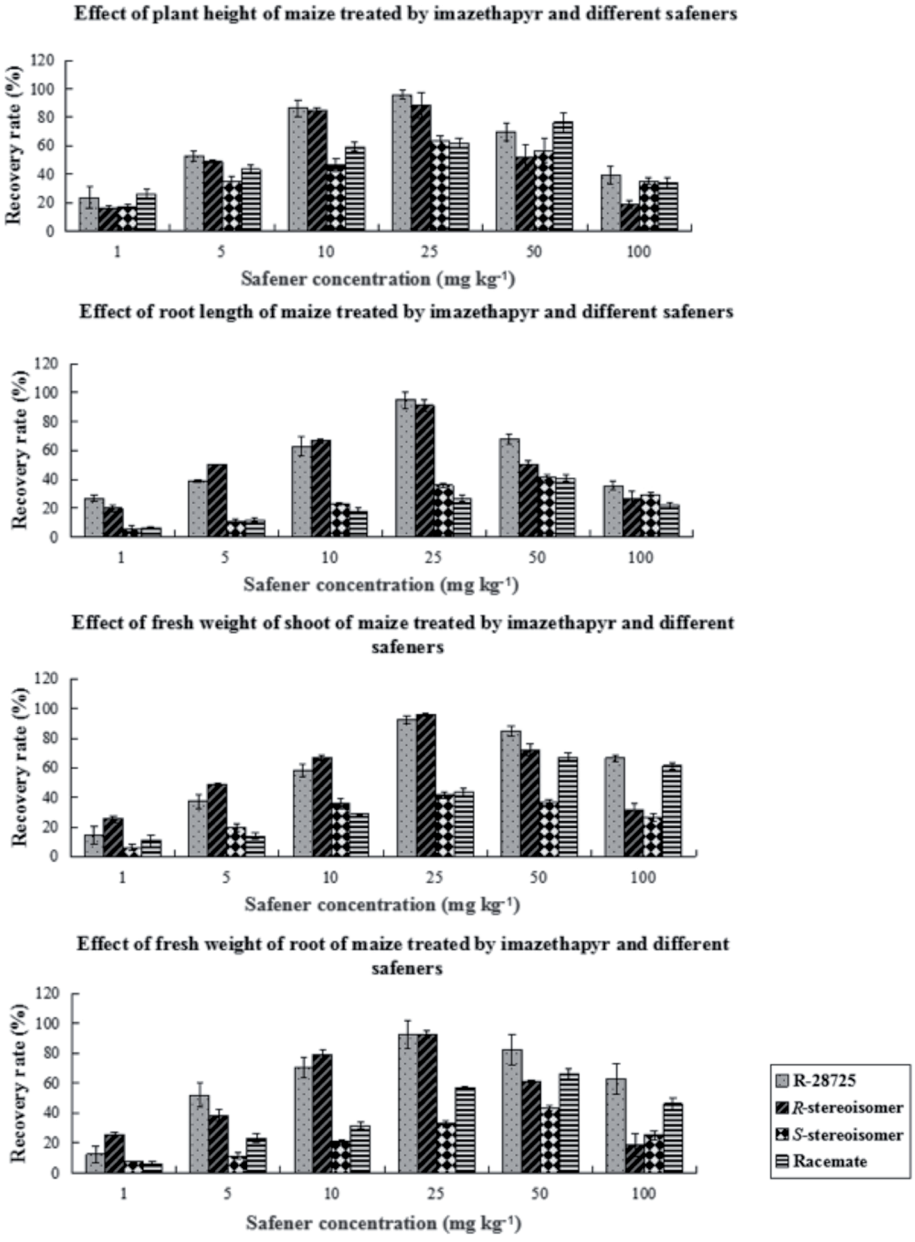
RESULTS AND DISCUSSION

Growth level of maize

The imazethapyr applied to maize at 0.5 mg kg^{-1} in soil reduced plant height 31.61%, root length 31.90%, fresh weight of shoot 35.72%, and fresh weight of root 33.54% compared with the control, respectively.

In order to investigate the protective effect of these safeners, R-28725, *R*-stereoisomer, *S*-stereoisomer, and racemate were used to test their protective ability at different concentrations. Growth level of maize were increased by safener application: the recovery rate of maize ranged from 5.53% to 95.98%. The recovery rate of maize treated by R-28725, *R*-stereoisomer were significantly higher than other safeners. At the same time, difference of recovery rate of maize treated by different concentration of safeners were observed, and safeners at too high or too low concentration caused decrease of protective effects. The optimum working concentration for R-28725, *R*-stereoisomer, *S*-stereoisomer, racemate were 25, 25, 25-50, and 50 mg kg^{-1}, respectively (Figure 1). The optimum recovery rates for four safeners were then applied to physiological and biochemical tests and all subsequent testing were based on this observation.

Figure 1. Recovery rate of growth level of maize treated by imazethapyr and different safeners.



GSH content

The content of GSH in maize is essential for detoxication of herbicide, depending on the mechanism of safeners. The results indicated that GSH content of maize treated by imazethapyr was elevated. Treated by the combination of safeners and imazethapyr accumulated high GSH content and specific combination of safener and imazethapyr were beneficial (Table 2). The GSH content of maize treated by R-28725 or *R*-stereoisomer combined with imazethapyr was significantly increased compared with the maize treated by imazethapyr.

GST activity

The interaction between seed treatment with safeners and the application of imazethapyr in soil were investigated. GST activity of maize treated by safeners and imazethapyr were tested to check its important role in detoxification process of imazethapyr. The GST activity of maize showed significant increases in response to *R*-stereoisomer and R-28725, while weak increases were observed in response to *S*-stereoisomer and racemate. In particular, the application of R-28725 and imazethapyr increased GST activity *in vivo* by 144.20% and the application of *R*-stereoisomer and imazethapyr increased GST activity *in vitro* by 40.22% (Table 3). These findings are in agreement with an earlier report (Benekos et al., 2010).

To confirm these results, a measurement of kinetic parameters of GST were conducted to clarify the innate character of the inducement and dynamics of GST.

Kinetic parameters of GST

In order to obtain more information about the effect of safeners and imazethapyr to GST, the kinetic parameters of GST were investigated. The V_{max} and K_M values are shown in Table 4; V_{max} values of GST for maize treated by R-28725 and *R*-stereoisomer were significantly increased, indicating the strong inducement of GST caused by safeners. K_M values of GST were notably decreased by R-28725 and *R*-stereoisomer treatment, indicating that R-28725 and *R*-stereoisomer increased the affinity of GST to substrate of GSH conjugated reaction. This increased GST affinity is an advantage to the process of detoxication.

ALS activity

As imazethapyr was a strong ALS inhibitor, we attempted to study the effect of safeners and imazethapyr on the ALS activity and investigate the protective effectiveness of safeners (Table 5).

The results showed that ALS activity was significantly inhibited by imazethapyr compared with the untreated control. Treatment of maize with safeners alone did not induce a significant change in the ALS activity compared with the untreated control. In contrast to other investigated safeners, R-28725 and *R*-stereoisomer almost reversed the

Table 2. Effect of safeners and imazethapyr on glutathione (GSH) content of maize.

Treatment	GSH content in root	GSH content in shoot
	μg g^{-1}	
Control	5.238 ± 0.1940d	11.703 ± 0.1390d
Imazethapyr	6.749 ± 0.1657c	14.354 ± 0.1940c
R-Stereoisomer+imazethapyr	10.515 ± 0.1571a	20.312 ± 0.2721a
S-Stereoisomer+imazethapyr	8.111 ± 0.1619b	14.962 ± 0.2767c
Racemate+imazethapyr	9.806 ± 0.2902ab	17.623 ± 0.2626b
R-28725+imazethapyr	10.183 ± 0.1940a	19.971 ± 0.2875a

Mean ± standard deviation of three replicates. Values with similar letter in the same volume are not significantly different ($p > 0.05$).

Table 4. Effect of safeners and imazethapyr on kinetic parameters of maize glutathione *S*-transferases (GST).

Treatment	V_{max}	K_m
	nmol min^{-1} mg^{-1} protein	mmol L^{-1}
Control	0.790 ± 0.030e	1.950 ± 0.056b
Imazethapyr	0.417 ± 0.031f	3.987 ± 0.151a
R-stereoisomer	1.437 ± 0.023b	1.320 ± 0.010d
S-stereoisomer	0.913 ± 0.067d	1.673 ± 0.057c
Racemate	1.090 ± 0.035c	1.570 ± 0.017c
R-28725	1.597 ± 0.091a	1.167 ± 0.144d

Mean ± standard deviation of three replicates. Values with similar letter in the same volume are not significantly different ($p > 0.05$).

Table 5. Effect of safeners and imazethapyr on acetolactate synthase (ALS) activity of maize.

Treatment	ALS Activity
	nmol h^{-1} mg^{-1} protein
Control	0.093 ± 0.0022a
Imazethapyr	0.039 ± 0.0015d
R-stereoisomer	0.081 ± 0.0025a
S-stereoisomer	0.092 ± 0.0017a
Racemate	0.078 ± 0.0032a
R-28725	0.094 ± 0.0051a
R-stereoisomer + imazethapyr	0.075 ± 0.0031b
S-stereoisomer + imazethapyr	0.048 ± 0.0041d
Racemate + imazethapyr	0.062 ± 0.0007c
R-28725 + imazethapyr	0.085 ± 0.0028a

Mean ± standard deviation of three replicates. Values with similar letter in the same volume are not significantly different ($p > 0.05$).

Table 3. Effect of safeners and imazethapyr on glutathione *S*-transferases (GST) activity of maize.

Treatment	GST activity *in vivo* μmol min^{-1} mg^{-1} protein	Treatment	GST activity *in vitro* nmol min^{-1} mg^{-1} protein
Control	2.95 ± 0.136f	Control	2.36 ± 1.514e
Imazethapyr	4.05 ± 0.278e	Imazethapyr	4.60 ± 0.300c
R-Stereoisomer + imazethapyr	8.31 ± 0.269b	*R*-Stereoisomer	6.45 ± 0.601a
S-Stereoisomer + imazethapyr	6.30 ± 0.159d	*S*-Stereoisomer	4.30 ± 0.169d
Racemate + imazethapyr	7.49 ± 0.184c	Racemate	4.94 ± 0.498c
R-28725+ imazethapyr	9.89 ± 0.484a	R-28725	6.32 ± 0.236b

Mean ± standard deviation of three replicates. Values with similar letter in the same volume are not significantly different ($p > 0.05$).

injury caused by imazethapyr. These data could indicate that R-28725 and *R*-stereoisomer can elevate the ALS activity of maize inhibited by imazethapyr significantly.

Imazethapyr, an extensively used herbicide with two enantiomers, causes phytotoxic effects to rotational crops and weed resistance (Yi et al., 2007). In order to extend the use of imazethapyr, it is necessary to develop effective safeners. It has been reported that imazaquin can be detoxified by safener BAS-145138, but there is no report about safeners of imazethapyr with high activity (Loniovereror, 1997). Aiming to develop safener of imazethapyr, the protective effects of four safeners were studied in our laboratory, showing that the maize injured by imazethapyr were effectively protected by R-28725 or *R*-stereoisomer. The seed treatment with safeners R-28725 or *R*-stereoisomer provided complete protection against imazethapyr.

Another aim of our study was to investigate the mechanism of safeners. The results in this study showed that the GSH content, GST activity *in vivo*, and ALS activity were increased by safeners treatment under laboratory condition, which is in agreement with previous studies (Jablonkai, 2013). Without imazethapyr application, seed treatment with these safeners also promoted the increase of GST activity *in vitro* and affinity of GST enzyme. Data obtained at this study are corroborate by Scarponi et al. (2006), who reported five safeners significantly changed the kinetic parameters of maize GST. This might suggest that these safeners induce GST activity significantly, increase conjugation of imazethapyr with GSH to some extent, and result in release of target enzyme ALS in maize, finally.

CONCLUSIONS

According to data obtained in this study, it can be concluded that seed treatment with R-28725 or *R*-stereoisomer present protective ability to injury of maize caused by imazethapyr. This study is the first one on the protective effect of chiral 3-dichloroacetyl oxazolidine and their interaction with imazethapyr. The further studies will focus on the development of structural optimization of *R*-stereoisomer to improve the protective effects.

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