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The safener effect of chiral derivatives of 3-dichloroacetyl oxazolidine against haloxyfop-P-methyl-induced toxicity in maize

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Abstract

Herbicide safener, a diverse group of chemicals, is an important tool used to protect plants from herbicidal injury. With the aim of decreasing drift injury of haloxyfop-P-methyl to sensitive plants, the protective effect of four safeners (R-28725, 3-dichloroacetyl oxazolidine and its two optical isomers) was evaluated. Physiological and biochemical tests were conducted under laboratory conditions in Northeast Agricultural University, China, by using seed treatment with safener and soil treatment with haloxyfop-P-methyl, respectively. The maize seeds treated with these safeners were safe from haloxyfop-P-methyl treatment. A positive correlation between growth level and endogenous glutathione (GSH) content, glutathione S-transferases (GST) activity was observed in this research. Enhancement of GSH content, GST activity and affinity of GST to 1-chloro-2,4-dinitrobenzene (CDNB) in maize treated by R-28725 was maximum. However, the detoxification of herbicide was not accompanied by the increase of acetyl-CoA-carboxylase (ACC) activity in maize.

Key words: 3-dichloroacetyl oxazolidine, glutathione S-transferases activity, glutathione S-transferases affinity, haloxyfop-P-methyl.

Introduction

Haloxyfop-P-methyl [methyl(R)-2-[4-(3-chloro-5-trifluoromethyl-2-pyridyloxy)phenoxy] propionate], R-enantiomer of haloxyfop, is an aryloxyphenoxypropionic acid herbicide controlling annual and perennial weeds in dicotyledoneous crops (Bijanzadeh et al., 2010; Hammami et al., 2014). Haloxyfop-P-methyl controls weeds by competitive binding to acetyl-CoA-carboxylase (ACC), which is an important enzyme in the biosynthesis of fatty acids (Hijano et al., 2013). However, application of haloxyfop-P-methyl at recommended dose may cause injury to some soybean cultivars (Parsa et al., 2013). It also has been reported that strong inhibition of biosynthesis of fatty acids was observed in wheat and maize treated by haloxyfop (Banas et al., 2012).

Herbicide safeners, also referred to as herbicide antidotes, are a diverse group of chemicals that enhance the detoxification ability of crops to herbicide but without decrease in herbicidal activity (Kraehmer et al., 2014; Elmore et al., 2015). In the late 1940s, the phenomenon of herbicide safener was discovered serendipitously by Otto Hoffman. The products of safener were subsequently commercialized by agrochemical companies intensively in late 20th century (Jablonkai, 2013). Nevertheless intensive research has been conducted in recent years; the exact mechanism of safener is incompletely understood (Hull et al., 2008). Research on safeners previously focused on the structure-activity relationship of safener and the actions of safener on the detoxification process of herbicide in plants (Fu et al., 2012; Cataneo et al., 2013). It is generally believed that safeners enhance the expression of plant defense and detoxification genes such as glutathione S-transferases (GST), cytochrome P450, and endogenous glutathione (GSH) involved in exogenous compounds metabolism (Matola, Jablonkai, 2007; Del Buono, Ioli, 2011). It has been observed previously that GSH-mediated detoxification induced by safener was involved in the metabolic detoxification of herbicides (Riechers et al., 2010). However, to the best of our knowledge, no safener has been developed for haloxyfop-P-methyl so far.

R-28725 (3-dichloroacetyl-2,2-dimethyl-1,3-oxazolidine) has been proven effective in reducing herbicidal injury from ALS (acetolactate synthase)-inhibitor herbicides (Zhao et al., 2014). Its analogue, 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine, is a chiral molecule that has given rise to two optical isomers. With the aim of decreasing the herbicidal injury, bioactivity of optical isomers and racemate of 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine and R-28725 were accessed. In addition, this study was conducted to assess the protective ability of these compounds related to detoxification of herbicide, namely, GSH, GST and ACC activity.

Materials and methods

Materials and chemical reagents. The tested soil was collected from Horticulture Station, Northeast Agricultural University, Harbin, China. It is classified as *Phaeozem* (*PH*) according to FAO (WRB, 2014), with a pH of 7.37. The seedlings of maize (*Zea mays* L.) cultivar 'Dongnong 253' were germinated and grown in a growth chamber at the Pesticide Chemistry Laboratory, Northeast Agricultural University during the period of 2012–2014. Haloxyfop-P-methyl (99.5%, powder) was obtained from Aladdin Chemistry (Shanghai, China). R-28725, 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine and its two optical isomers were synthesized in our laboratories (99.0%) (Table 1, Fig. 1).

Table 1. Chemical name of safeners used for the test

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

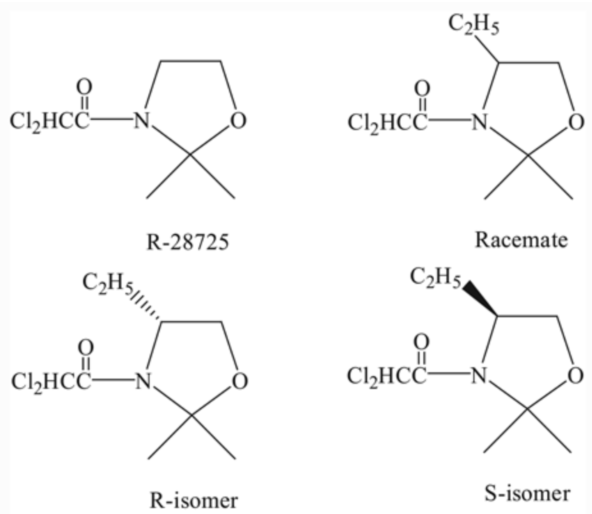

Figure 1. Structure of safeners used for the test

Growth conditions. Seeds of maize were soaked in solutions of safeners (0, 1, 5, 10, 25, 50 and 100 mg kg^{-1}) for 12 hours; the control was soaked in water. Then the seeds were germinated in dishes in a growth chamber for 24 hours. After that the seeds were sown in soil with a depth of 1cm in paper-cups (10 × 15 cm), 7 seeds per cup. The paper-cups contained soil added with haloxyfop-P-methyl (6 μg kg^{-1}) with a depth of 13 cm, the seedlings were incubated in a growth chamber with a photoperiod of 12h light and 12h dark, 26.5 ± 1°C temperature, 75% relative humidity. Each treatment was replicated three times.

The samples of maize plants were washed and collected 7 days after treatment. The recovery rate of growth index (plant height, root length, fresh weight of shoot, fresh weight of root) of maize was measured. Recovery rate of growth index was calculated according to the following formula:

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

where safener is R-28725, 3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine and its two optical isomers, respectively, and herbicide is haloxyfop-P-methyl. The recovery rate of growth index was calculated respectively and expressed as percent.

The shoot and root tissues of maize were stored in a refrigerator for biological activity assays.

Determination of endogenous glutathione (GSH) content. GSH content was measured by UV-visible spectrophotomer as described previously (Ismaiel, Papenbrock, 2014). To perform the determination, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was used as chromogenic agent, absorbance data collected at 412 nm, and GSH content were calculated by comparing with standard working curve.

Determination of glutathione S-transferases (*GST*) *activity.* The extraction and assay of GST 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 1-chloro-2,4-dinitrobenzene (CDNB) and expressed as amount of conjugate per minute per mg of protein (μmol min^{-1} mg^{-1} protein).

To determine the GST activity *in vitro* (GST activity against haloxyfop-P-methyl in this study), the amount of haloxyfop-P-methyl was determined by high performance liquid chromatography (HPLC) as described previously (Scarponi et al., 2006; Li et al., 2012). GST enzyme was extracted from root of maize, and added with glutathione and haloxyfop-P-methyl solution. After cultivation for 2 hours, residues of haloxyfop-P-methyl in this mixture were determined by HPLC. The GST activity *in vitro* was expressed as amount of haloxyfop-P-methyl consumed per minute per mg 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 were determined by measuring GST activity over a range of CDNB concentrations (0.13–4.14 mM) and at a single GSH concentration of 5 mM.

Determination of acetyl-CoA-carboxylase (*ACC*) *activity.* To investigate the effect of safener on target enzyme, ACC activity was determined as described previously (Islam et al., 2014). ACC activity was expressed as amount of malonyl-CoA per min per mg of enzyme (nmol min^{-1} mg^{-1} protein).

Statistical analysis. Software *Statistical Product and Service Solutions* (*SPSS 16.0*) was used to determine statistical significance at 95% confidence level ($p < 0.05$). The data were expressed as mean ± standard deviation (n = 3).

Results

Growth index of maize. A significant reduction of growth index of maize was observed when haloxyfop-P-methyl was applied in soil. Haloxyfop-P-methyl applied at 6 µg kg^{-1} in soil caused 20.69, 27.62, 28.35 and 32.21 % injury to plant height, root length, fresh weight of shoot, and fresh weight of root of maize, respectively.

Dose response curves of safeners (R-28725, R-isomer, S-isomer and Racemate) were prepared to determine the appropriate concentration of safeners offering maximum protection. Figure 2 shows the recovery rate of growth index of maize treated with haloxyfop-P-methyl and four safeners. Significant differences were observed for recovery rate at different concentrations of safener in this study. The optimum concentration for R-28725, R-isomer, S-isomer and Racemate were 5, 25, 100 and 100 mg kg^{-1}, respectively. This observation clearly indicates that appropriate concentration of safener significantly decreases herbicidal injury from haloxyfop-

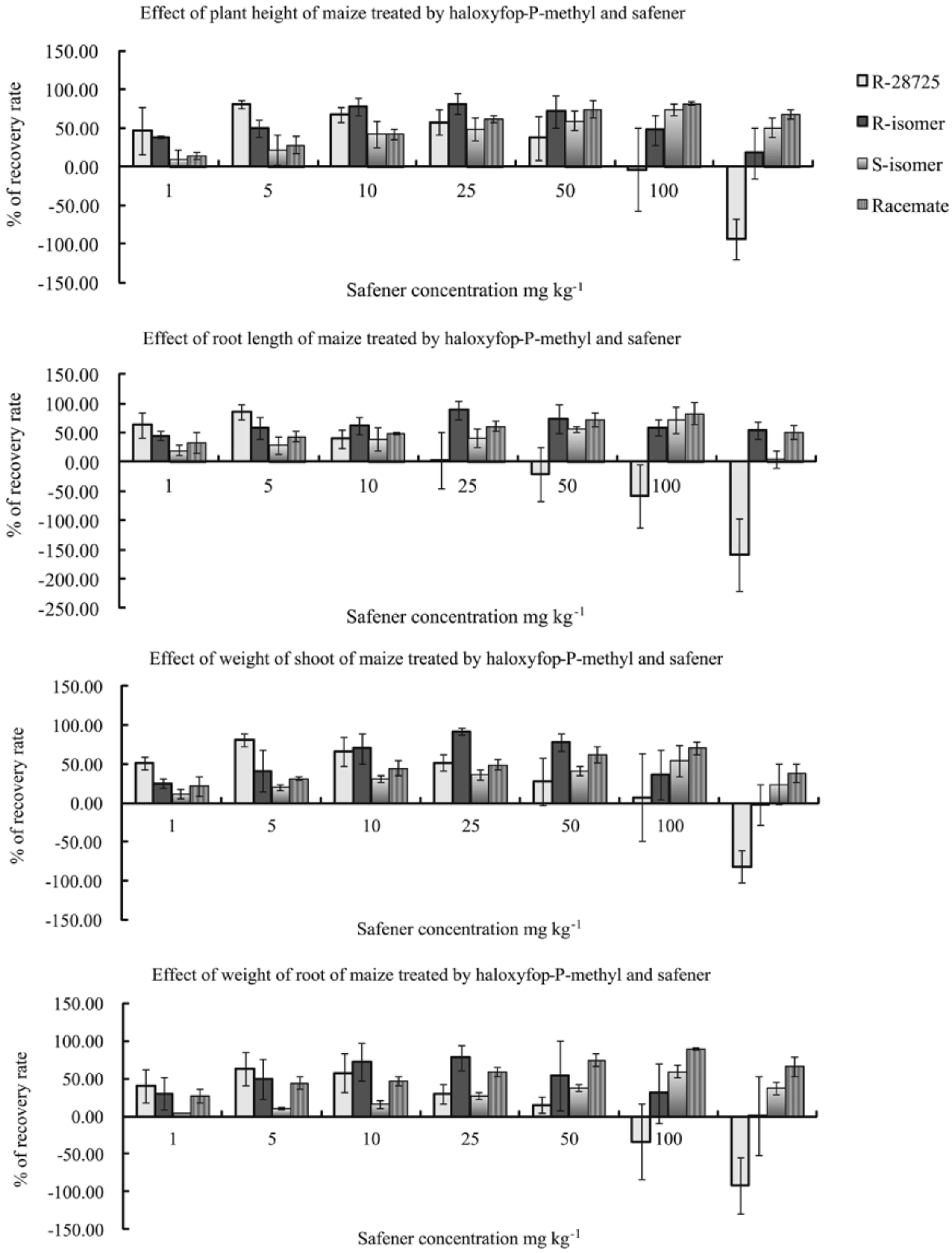

Figure 2. Recovery rate of growth level of maize treated with haloxyfop-P-methyl and safener

P-methyl. However, the increase in growth index of maize treated with R-28725 and haloxyfop-P-methyl started from 1 mg kg^{-1} and continued till 50 mg kg^{-1}, and thereafter it dropped rapidly. Optimum concentration for each safener was then applied to maize for subsequent testing.

Glutathione (GSH) content. It has been reported that safeners could increase the conjugation of xenobiotic compounds with GSH through elevating the level of GSH in plant (Requejo, Tena, 2012). Therefore, GSH content in plants treated with safener was an important index to access the protective ability of safener. In this evaluation, haloxyfop-P-methyl applied in soil at 6 μg kg^{-1} caused a slight increase in GSH content in maize (Table 2). As response to treatment with safener, the content of GSH in maize increased significantly. The increase of GSH induced by safener has also been reported previously (Jablonkai, 2013).

Table 2. Effect of safeners and haloxyfop-P-methyl on endogenous glutathione (GSH) content (μg g^{-1}) in maize

Treatment	In root	In shoot
Control	5.02 ± 0.229 d	11.45 ± 0.559 e
Haloxyfop-P-methyl	6.48 ± 0.462 c	15.03 ± 0.534 d
R-isomer + haloxyfop-P-methyl	10.47 ± 0.571 a	20.13 ± 1.064 a
S-isomer + haloxyfop-P-methyl	8.44 ± 0.483 b	16.84 ± 0.469 c
Racemate + haloxyfop-P-methyl	10.85 ± 0.168 a	18.33 ± 1.067 b
R-28725 + haloxyfop-P-methyl	10.88 ± 0.365 a	20.08 ± 0.807 a

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

Glutathione S-transferases (GST) activity. The role of GST is essential for detoxification ability of safener, depending on the action mechanism of safener. To access the functional role of GST in detoxification process, the effect of safener and haloxyfop-P-methyl on GST activity of maize was investigated in this study (Table 3). Combined application of haloxyfop-P-methyl and safener significantly increased the GST activity *in vivo* of maize. In a like manner, the GST activity *in vitro* of maize treated by R-isomer or R-28725 also showed a significant increase. These findings are in agreement with earlier reports (Benekos et al., 2010).

Table 3. Effect of safeners and haloxyfop-P-methyl on activity of glutathione S-transferases (GST) in maize

Treatment	*in vivo* μmol min^{-1} mg^{-1} protein	Treatment	*in vitro* nmol min^{-1} mg^{-1} protein
Control	3.10 ± 0.215 e	Control	0.557 ± 0.0324 c
Haloxyfop-P-methyl	4.10 ± 0.229 d	Haloxyfop-P-methyl	0.207 ± 0.0316 d
R-isomer + haloxyfop-P-methyl	8.68 ± 0.558 b	R-isomer	1.809 ± 0.2505 a
S-isomer + haloxyfop-P-methyl	6.47 ± 0.529 c	S-isomer	0.681 ± 0.0551 bc
Racemate + haloxyfop-P-methyl	8.58 ± 0.325 b	Racemate	0.857 ± 0.0586 b
R-28725 + haloxyfop-P-methyl	9.76 ± 0.550 a	R-28725	2.015 ± 0.1585 a

Explanation under Table 2

Kinetic parameters of GST. To better understand the effect of safener on GST, the kinetic parameters of GST were determined for further research (Table 4). The maximum rate (V_{max}) and Michaelis constant (K_M) (the substrate concentration which results in one-half of the maximum velocity) of GST were determined by linear regression of a double reciprocal plot (Scarponi et al., 2006). V_{max} of GST for maize treated by R-28725 was significantly higher as compared to other safeners. At the same time, a significant decrease of K_M of GST for maize treated by R-28725 was observed, indicating the strong inducement of GST caused by R-28725. 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.

Table 4. Effect of safeners and haloxyfop-P-methyl on kinetic parameters of maize glutathione S-transferases (GST)

Treatment	V_{max} nmol min^{-1} mg^{-1} protein	K_M mmol L^{-1}
Control	0.790 ± 0.0300 e	1.950 ± 0.0557 b
Haloxyfop-P-methyl	0.623 ± 0.0732 f	3.627 ± 0.1069 a
R-isomer	1.437 ± 0.0231 b	1.320 ± 0.0100 d
S-isomer	0.913 ± 0.0666 d	1.673 ± 0.0569 c
Racemate	1.090 ± 0.0346 c	1.570 ± 0.0173 c
R-28725	1.597 ± 0.0907 a	1.167 ± 0.1443 d

Explanation under Table 2

Acetyl-CoA-carboxylase (ACC) activity. Safener protects crops by depressing the herbicide levels reaching the targeted site (Rushing et al., 2013). For that reason, ACC activity is critical for maize to decrease the injury caused by haloxyfop-P-methyl. Data obtained in this study showed that ACC activity of maize was significantly inhibited by haloxyfop-P-methyl (Table 5). However, it was noteworthy that no influence was observed in response to safener application.

Table 5. Effect of safeners and haloxyfop-P-methyl on acetyl-CoA-carboxylase (ACC) activity of maize

Treatment	ACC activity nmol min^{-1} mg^{-1} protein
Control	5.14 ± 0.561 a
Haloxyfop-P-methyl	2.35 ± 0.135 b
R-isomer + haloxyfop-P-methyl	2.67 ± 0.250 b
S-isomer + haloxyfop-P-methyl	2.56 ± 0.379 b
Racemate + haloxyfop-P-methyl	2.30 ± 0.261 b
R-28725 + haloxyfop-P-methyl	2.74 ± 0.314 b

Explanation under Table 2

Discussion

While herbicide contributes to improving crop yield, it can also pose a risk to the plants which are sensitive to them (Jursik et al., 2010). The use of herbicide at present has resulted in increased incidence of herbicidal injury (Grey et al., 2012; Cieslik et al., 2014). For that reason, it becomes essential to develop effective safeners. Even though, no safener has been developed to protect sensitive crops from haloxyfop-P-methyl. In order to develop safener for haloxyfop-P-methyl, the protective effects of four safeners were evaluated in our laboratory. The results conclusively demonstrated that the maize plants injured by haloxyfop-P-methyl were effectively protected by these safeners. The maize seeds that had been soaked in solution of safener were safe from haloxyfop-P-methyl treatment.

For evaluation of the enhancement of detoxification of maize, induced by safeners, the GSH content, GST activity, and ACC activity of maize treated by haloxyfop-P-methyl and safener were investigated. The results indicate that these safeners caused enhancement of GSH content, GST activity and affinity of GST enzyme to substrate. These results are in agreement with previous studies, according to which safeners significantly change the affinity of GST to substrate of conjugation reaction (Scarponi et al., 2006). It has also been observed that high GST activity may contribute to the tolerance of rice to glyphosate and chlorsulfuron (Hu, 2014). A positive correlation between growth level and GSH content, GST activity has been observed in this research. Enhancement of GSH content, GST activity and affinity of GST to CDNB in maize treated by R-28725 was maximum. It was observed that the order of protective ability of safeners was as follows: R-28725 > R-isomer > Racemate > S-isomer. Based on the results obtained in this study, it seems that these safeners induce the conjugation of herbicide with GSH catalyzed by GST to some extent. Consistent with this notion, it has been shown that some major crops are able to conjugate herbicides rapidly by different pathways (Cummins et al., 2011). However, it should be noted that there is no increase of ACC activity in response to safener application. Another assumption made above is that the detoxification of herbicide was not always accompanied by the release of target enzyme in plants.

Conclusions

1. Four safeners – R-28725, 3-dichloroacetyl oxazolidine and its two optical isomers – present protective ability against injury caused to maize by haloxyfop-P-methyl.

2. There are indications that the inducement of endogenous glutathione (GSH) content and glutathione S-transferases (GST) activity were not accompanied by the release of acetyl-CoA-carboxylase (ACC) activity in maize treated with haloxyfop-P-methyl and safener. This might suggest that the detoxification process may not always be accompanied by the induction of target enzyme.

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Apsauginis 3-dichloroacetilo oksazolidino chiralinių junginių poveikis kukurūzams nuo pažeidimo haloksifopo-P-metilu

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Santrauka

Herbicidų apsauginės medžiagos, sudarančios atskirą įvairių chemikalų grupę, yra svarbi priemonė, naudojama augalus apsaugoti nuo pažeidimo herbicidais. Siekiant sumažinti haloksifopo-P-metilo pernašos žalą jautriems augalams, tirtas poveikis keturių apsauginių medžiagų – R-28725, 3-dichloroacetilo oksazolidino ir jo dviejų optinių izomerų. Fiziologiniai ir biocheminiai tyrimai buvo atlikti laboratorinėmis sąlygomis Šiaurės Rytų žemės ūkio universitete. Tyrimo metu sėklos buvo apdorotos apsaugine medžiaga, o dirvožemis – haloksifopo-P-metilu. Sėklos, apdorotos apsauginėmis medžiagomis, buvo saugios nuo haloksifopo-P-metilo. Tyrimo metu nustatyta teigiama koreliacija tarp augimo lygio ir endogeninio glutationo (GSH) kiekio bei glutationo S-transferazės (GST) veiklos. GSH kiekio bei GST aktyvumo padidėjimas ir GST panašumas į 1-chloro-2,4-dinitrobenzeną (CDNB) kukurūzuose, apdorotuose R-28725, buvo maksimalūs. Tačiau dėl herbicido detoksikacijos acetil-CoA-carboksilazės (ACC) aktyvumo padidėjimas kukurūzuose neįvyko.

Reikšminiai žodžiai: glutationo S-transferazės aktyvumas, glutationo S-transferazės panašumas, haloksifop-P-metilas, 3-dichloroacetilo oksazolidinas.

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