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Effect of safener fluxofenim on crop injury of chloroacetanilides and enzyme activity of glutathione S-transferase in grain sorghum seedlings

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Abstract : Effects of safener fluxofenim was investigated for crop injury of acetanilide's upland herbicides and for enzyme activity of glutathione S-transferase (GST) in grain sorghum. Bioassay with etiolated grain sorghum [*Sorghum bicolor* (L.) Moench. cv. 'G522DR'] seedlings grown in agar containing metolachlor or alachlor showed that they are strong inhibitors on root growth of grain sorghum (GI_{50} = 4.5 μM for metolachlor and 6.2 μM for alachlor). The safener fluxofenim applied by seed soaking protected growth of grain sorghum from crop injury of metolachlor or alachlor at the concentrations of 1 to 10 μM. There was a significant increase in glutathione-herbicide conjugates in root tissues of fluxofenim-treated seedlings. Activities of $GST_{\text{-metolachlor}}$ and $GST_{\text{-CDNB}}$ were increased by 82% and 70%, respectively, in the cytosolic fraction of roots with fluxofenim treatment. (Received December 26, 1997, accepted February 27, 1998)

Key words : fluxofenim, glutathione S-transferase, herbicide, safener.

Abbreviations used:

Alachlor, 2-chloro-N-(2,6-diethylphenyl)-N-(methoxy-methyl) acetamide; CDNB, 1-chloro-2,4-dinitrobenzene; fluxofenim, O-[1,3-dioxolan-2-yl-methyl]-2,2,2-trifluoro-4' -chloroacetophenone-oxime; CGA-133,205]; GSH, reduced glutathione; GST, glutathione S-transferase; Metolachlor, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1- methylethyl) acetamide

Introduction

Fluxofenim is an oxime ether safener developed and commercialized for sorghum against crop injury of metolachlor (Hatzios, 1989). Metolachlor and alachlor are the most popular chloroacetanilides and generally used in weed control for corn, sorghum, and soybean.

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Herbicide safeners can protect certain crops from herbicide injury. However, precise mechanisms of a safener has not been fully understood at the present time. A safenerinduced herbicide detoxification is currently recognized as a major mechanism of herbicide safeners. Safeners enhance the conjugation of chloroacetanilide and sulfoxidized carbamothioate herbicides with glutathione by elevating the levels of GSH or inducing the activity of specific GST (Lay and Casida1976; Hatzios, 1984; Fuerst and Gronwald, 1986; Gronwald, 1987, Hatzios and Wu, 1996). GST (EC 2.5.1.18) is a key metabolic enzyme catalyzing glutathione conjugation of several herbicides in plants (Lamoureux, 1989; Timmerman, 1989; Fuerst, 1993; Irzyk, 1993, Jepson, 1994).

GST have been mainly characterized in maize and showed that the efficacy of chemical safeners on maize against acetanilide herbicides depends on their ability to increase GST activity. The role of GSTs in selectivity of such herbicides as alachlor, atrazine,

fluorodifen and metolachlor has been determined in seedlings of maize (*Zea mays* L.) and the associated weed species *Abutilon theophrasti* Medic., *Digitaria sanguinalis* (L.) Scop. *Echinochloa crus-galli* (L.) P. Beauv., *Panicum miliaceum* L., *Setaria faberi* Herrm. and grain sorghum (Hatton, 1996) and showed to be detoxified by enhancing glutathione conjugation. Continuing advancements in molecular biology techniques will undoubtedly contribute towards the achievement of these goals in the near future. In this research we studied the safening effect of fluxofenim against crop injury caused by metolachlor or alachlor on etiolated sorghum seedlings and the fluxofenim-mediated GST induction in cytosolic fraction of sorghum seedlings.

Materials and Methods

Preparation of plant material

Seeds of grain sorghum (cv. G522DR) were submerged in water or safener solution and germinated in the dark with air bubbling for 72 hr at 25°C. After pre-germinated, they were seeded on 0.5% agar media with or without the herbicides in the dark condition for 4 days. The seedlings were then harvested to determine growth rate and GST activity.

Dose-response to herbicides

Eight germinated seeds of grain sorghum were placed in 50-ml plastic centrifuge tubes containing 0.5% agar and metolachlor or alachlor at the concentrations of 0, 1, 10, and 100 μM. The tubes were kept in plastic boxes and placed in a dark incubator at 35℃ for 4 days. Root and shoot lengths of sorghum seedlings were then measured. Concentrations of metolachlor or alachlor causing 50% inhibition (GI_{50}) were determined from the dose-response relationship by probit analysis. All experiments were replicated with three times. Similar bioassay method was also used to evaluate the safening effect of fluxofenim on crop injury caused by metolachlor or alachlor.

Soluble GST extraction

Two grams of root tissues of etiolated sorghum seedlings were ground with liquid nitrogen in a mortar and pestle in 6 ml of 0.2 M tris-HCl (pH 7.8) buffer containing 25 mM mercaptoethanol, 1 mM EDTA and 10% glycerol(w/v). The homogenate was filtered through two layers of miracloth (Calbio-chem, La Jolla, CA) and then centrifuged at 4,000g for 30 min to get rid of debris. The supernatant was then re-centrifuged at 100,000g for 90 min.

After centrifuged, the supernatant (soluble fraction) was directly used to determine GST activity and protein content.

Assay of GST activity

CDNB-specific GST activity in soluble extracts of sorghum roots with or without fluxofenim treatment was determined according to Mannervik and Guthenberg (1981). The reaction mixture was consisted of 30 μl of the enzyme extract, 2 ml of 100 mM potassium phosphate buffer (pH 6.9), 0.9 ml of 3.3 mM GSH, and 100 μl of 30 mM CDNB. The reaction incubated at room temperature (25°C) was started by the addition of CDNB. The change of absorbance due to the formation of $GST_{\text{-CDNB}}$ conjugation was measured spectrophotometically at 340 nm. The enzyme activity was calculated using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ (Mannervik and Guthenberg, 1981).

$GST_{\text{-metolachlor}}$ activities in the soluble extracts of sorghum roots with or without fluxofenim treatment were determined by the method of Mozer et al. (1983) with slight modifications. The reaction mixture was 20 μl of soluble enzyme extract, 30 μl of 100 mM phosphate buffer (pH 6.9), and 10 μl of 60 mM GSH. The reaction incubated for 60 min at 30℃ was initiated by the addition of the radiolabeled substrate 10 μl of 100 mM [^{14}C]-metolachlor. The reaction was terminated by the addition of 60 μ l of 5% trichloroacetic acid.

The reaction mixture was then partitioned with 1 ml of dichloromethane to remove the unmetabolized herbicide. An aliquot of the aqueous phase was

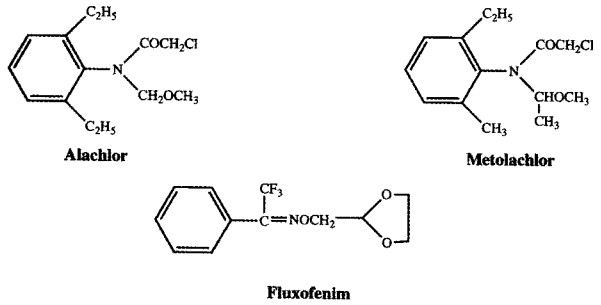

Fig. 1. Chemical structures of the herbicides and safener fluxofenim used in this study.

removed and the quantity of ^{14}C in the form of GST-metolachlor conjugates was determined by liquid scintillation counter. GST activity reported were calculated as described above. Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a protein standard. All experiments were repeated with three times.

Results

Effects of metolachlor and alachlor on grain sorghum growth

The herbicide concentration causing a 50% inhibition (GI_{50}) on root length of grain sorghum seedlings was 4.48 μM for metolachlor and 6.23 μM for alachlor (Fig. 2). On the other hand, the GI_{50} values of metolachlor and alachlor to sorghum shoot growth were 30.8 μM and 28.8 μM, respectively (Fig. 3). These results indicated that there was no great difference in growth response of grain sorghum between both herbicides and the roots were more sensitive to the herbicides than the shoots.

Effect of fluxofenim against crop injury of metolachlor or alachlor

Safening effect of fluxofenim was determined with seeds soaked in fluxofenim solution at the concentrations of 0.1, 1, and 10 μM for 48 hrs.

Fluxofenim at the concentrations of 1 to 10 μM protected the growth of sorghum seedlings from metolachlor or alachlor injury. The shoot and root

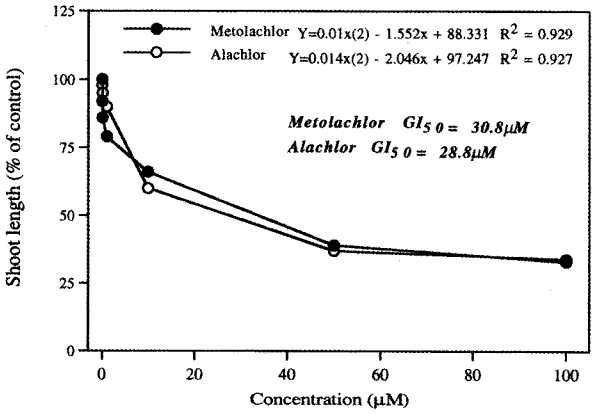

Fig. 2. Effect of metolachlor or alachlor on shoot growth of sorghum seedlings.

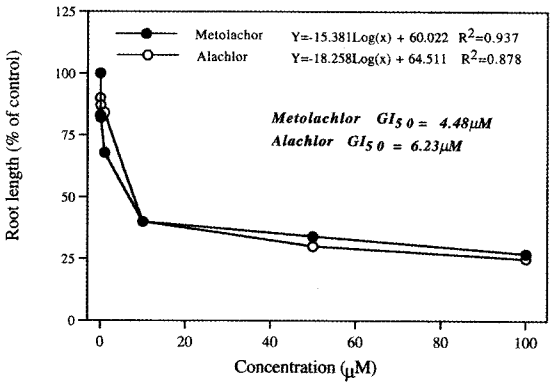

Fig. 3. Effect of metolachlor or root on shoot growth of sorghum seedlings.

lengths of grain sorghum seedlings treated with 1 μM fluxofenim and 10 μM metolachlor were about 25% and 20%, respectively, longer than those of the seedlings without fluxofenim and metolachlor (Fig. 4). The safening index (ratio of the seedling growth between treated with and without safener) of 1 μM fluxofenim and 20 or 40 μM metolachlor treatments were similar to that of 10 μM metolachlor treatments. A increased safening effect of 1 μM fluxofenim treatment against 5 μM alachlor was found (Fig. 5). The shoot length of grain sorghum seedlings treated with 1 μM of fluxofenim and 5 μM alachlor was almost same to that of untreated control. However, safening index between treated with and without 1 μM fluxofenim against 20 or 40 μM metolachlor treatments were similar to that of 10 or 20 μM alachlor.

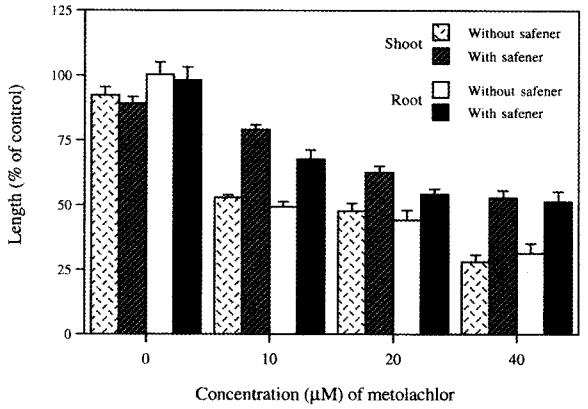

Fig. 4. Protection of sorghum seedlings against metolachlor injury with seed treatment of fluxofenim. Sorghum seedlings were grown in 0.5% agar treated with metolachlor. Safener was treated by seed soaking for 48 hrs in 1μM solution. Vertical bars represent SD of the means.

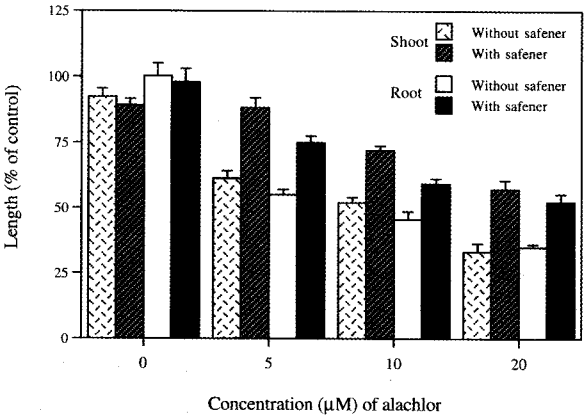

Fig. 5. Protection of sorghum seedlings against alachlor injury with seed treatment of fluxofenim. Sorghum seedlings were grown in 0.5% agar treated with alachlor. Safener was treated by seed soaking for 48 hrs in 1μ M solution. Vertical bars represent SD of the means.

GST activity

In most plant species tested, GST responsible for herbicide conjugation to GSH has been found to be soluble (Edwards and Owen, 1987). To show the relationship between the safening effect and induction of soluble GST activity by fluxofenim, the enzyme activity was assayed with plant roots treated with or without safener using metolachlor and CDNB as a substrate. The enzyme activity reached to the maximum level at 48 hrs after the treatment (data not shown). The ratio of GST activity between with and without safener, as measured with CDNB, indicated that the pre-treatment with 1 μM fluxofenim enhanced the extractable soluble GST activity from sorghum roots upto 70%.

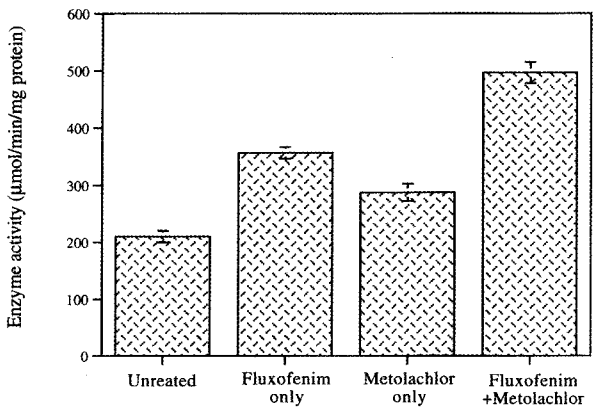

Fig. 6. GST_{-CDNB} activity in roots of sorghum seedlings.

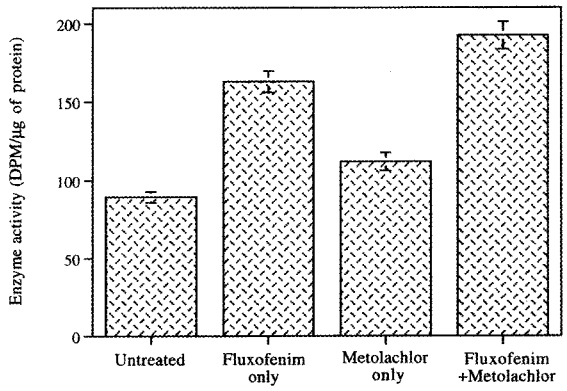

Fig. 7. $GST_{-metolachlor}$ activity in roots of sorghum seedlings.

The GST_{-CDNB} activity of sorghum roots treated with 10 μM metolachlor without fluxofenim also enhanced upto 37%. However, the treatment of 1 μM fluxofenim followed by 10 μM metolachlor resulted in 2.4 times increase in the extractable soluble GST_{-CDNB} activity of sorghum roots as compared with the untreated control (Fig. 6).

$GST_{-metolachlor}$ activities with treatments of fluxofenim only, metolachlor only, and 1 μM fluxofenim and metolachlor were also enhanced by 82, 25, and 120%, respectively, as compared with the untreated control (Fig. 7). The increase in GST_{-CDNB} and $GST_{-metolachlor}$ activity by the safener fluxofenim has also been observed with dichloroacetamide safeners such as dichlomid (Jepson et al., 1994) and benoxacor (Irzyk and Fuerst, 1993) in other grass crops(mainly corn). These results suggested that the fluxofenim-mediated increase in the GST activity is related to the safening action.

Discussion

Chloroacetanilide herbicides, metolachlor and alachlor are strong inhibitor on root growth of grain sorghum. The herbicide concentrations required to inhibit 50% root growth were 4.5 μM for metolachlor and 6.2μ M for alachlor (Figs. 2 and 3). However, the inhibition was reduced with the treatment of safener fluxofenim treated by seed soaking to grain sorghum at 1 μM (Figs. 4 and 5). These results showed that fluxofenim has a safening effect on crop injury of acetanilide herbicides. However, the treatment of 1 μM fluxofrnim was not enough to protect completely sorghum seedling growth against 20, 40 μ M metolachlor and 10, 20 μM alachlor treatments. Also, the treatment of 0.1 μM fluxofenim did not show the safening effect on sorghum seedling growth against the concentrations of treatment and the inhibition of sorghum seedling growth occurred from the treatment of 10 μM fluxofenim (data not shown). These results showed that the safening effect occurred highly when the combination of safener and herbicide concentration is good.

A safener-induced herbicide detoxification is currently recognized as a major mechanism involved in the selectivity of herbicide with safeners. GSTs have been known to be a key metabolic enzyme catalyzing glutathione conjugation of several herbicides in plants (Lamoureux, 1989; Timmerman, 1989; Fuerst, 1993; Irzyk, 1993; Jepson, 1994). Other investigators have shown that GST activity in most mono-cotyledonous crops (e.g., corn and grain sorghum) is mainly associated with their roots (Lamoureux and Rusness, 1989; Gronwald, 1989; Jablonkai and Hatzios, 1993). Activities of $GST_{-metolachlor}$ and GST_{-CDNB} were increased upto 82% and 70%, respectively, in the root soluble fraction when applied with fluxofenim (Figs. 6 and 7). There was a significant increase in the formation of glutathione-herbicide conjugate in root tissues of fluxofenim-treated seedlings.

Plant GSTs have been mainly characterized in maize. The efficacy of chemical safeners as protectants of maize against crop injury of acetanilide herbicides depends on their ability to increase GST activity. Four isoforms of GST have been characterized in some detail in maize and two of four isoforms, GST II and GST IV, are induced by herbicide safeners (Jepson et al., 1994). GSTs induction by herbicide safeners are due to an increase of GST gene transcription (Shah et al., 1986; Jepson et al., 1994). Our separate experiment (unpublished) also showed that the fluxofenim-mediated induction of the mRNA expression coincided with a concomitant GST induction.

According to these results, we conclude that herbicide safener fluxofenim protects grain sorghum seedlings from crop injury of alachlor and metolachlor injury by enhancing GST activity.

Acknowledgements

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수수유묘에 있어서 fluxofenim의 약해경감효과와 glutathione S-transferase 효소활성

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요약 : 제초제 alachlor와 metolachlor가 처리된 수수의 생장에 대한 약해경감제 fluxofenim의 약해경감효과와 경감기작의 하나로 추정되는 GST 활성에 대하여 조사하였다. 제초제 metolachlor와 alachlor는 수수(품종;G522DR)의 유묘생장을 크게 억제 하였는데, 지상부 및 뿌리에 대한 50% 생장억제 농도가 각각 30.8, 28.8 μM과 4.48, 6.23 μM로 두 약제 모두 수수의 지상부에 대한 억제보다 뿌리에 대한 억제가 컸다. Fluxofenim을 종자에 처리하여 파종하고 metolachlor 또는 alachlor을 처리하면 수수의 유묘생장이 회복되어 fluxofenim처리에 의한 약해경감 효과가 크게 나타났다. 약해경감제 fluxofenim을 처리한 것과 처리하지 않은 수수 유묘로부터 추출한 GST의 활성을 비교한 결과, fluxofenim을 처리한 수수의 유묘로부터 추출한 GST의 활성이 CDNB를 기질로 사용하였을때 70% 증가되었고, [^{14}C]-metolachlor을 기질로 사용하였을 때에도 82% 증가되었다. 따라서 약해경감제 fluxofenim을 처리한 수수와 처리하지 않은 수수의 metolachlor 또는 alachlor에 대한 선택성의 차이는 fluxofenim 처리로 증가된 GST에 의한 metolachlor-glutathione 또는 alachlor-glutathione conjugation되기 때문인 것으로 생각된다.

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