

# Effects of the Herbicide Pretilachlor and the Safener Fenclorim on Glutathione Content and Glutathione-Dependent Enzyme Activity of Rice\*

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The influence of individual or combined treatments of the chloroacetanilide herbicide pretilachlor and the safener fenclorim on glutathione content and the activity of glutathione reductase (GR, EC 1.6.4.2) and glutathione-S-transferase (GST, EC 2.5.1.18) of rice (*Oryza sativa* L., var. Lamont) was investigated in a time-course study including 0, 12, 24, 48, and 72 h periods after treatment. Pretilachlor applied alone at 2  $\mu$ M did not influence the levels of total or reduced glutathione of germinating rice seedlings. Fenclorim applied alone at 20  $\mu$ M increased significantly the levels of total and reduced glutathione of rice seedlings at 12, 24, and 72 h after treatment. The combination of pretilachlor and fenclorim increased the levels of total and reduced glutathione of rice at all time periods. GR activity extracted from pretilachlor-treated rice seedlings was significantly lower than that extracted from untreated rice seedlings. Pretreatment of rice seedlings with the safener fenclorim increased GR activity at 12 and 24 h, but not at 48 and 72 h after treatment. GR activity extracted from rice seedlings treated with the combination of pretilachlor and fenclorim was significantly higher than that of untreated controls at 12, 24, and 48 h after treatment. Pretreatment of rice seedlings with pretilachlor reduced the activity of GST which catalyzes the conjugation of this herbicide with reduced glutathione at time intervals greater than 24 h. Pretreatment with fenclorim did not have any effect on GST activity of rice seedlings. Pretreatment with the combination of pretilachlor and fenclorim increased GST activity of rice seedlings at the 48 and 72 h periods. These results suggest that a simultaneous application of pretilachlor and fenclorim is critical for the enhancement of glutathione and glutathione-related enzyme activity and the protection of rice from pretilachlor injury.

## Introduction

The recent development of the safener fenclorim by Ciba-Geigy Corporation has allowed the selective use of the chloroacetanilide herbicide pretilachlor in wet sown rice [1, 2].

**Abbreviations:** CDNB, 1-chloro-2,4-dinitrobenzene; CGA-133205, O-[1,3-dioxolan-2-yl-methyl]-2,2,2-trifluoro-4'-chloroacetophenoneoxime (now known as fluxofenim); Dichlormid, 2,2-dichloro-N,N-di-2-propenylacetamide; Fenclorim, 4,6-dichloro-2-phenyl-pyrimidine; Flurazole, phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione (glutathione disulfide); GST, glutathione-S-transferase; Pretilachlor, 2-chloro-2',6'-diethyl-N-(2-propoxyethyl)acetanilide; TLC, thin-layer chromatography.

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The exact mechanism of action of the safener fenclorim is not known. However, most of the currently available evidence indicates that this safener acts by enhancing the metabolic detoxification of pretilachlor *via* conjugation to reduced glutathione (GSH) [3–6]. This reaction can be either spontaneous (non enzymatic) or enzymatic mediated by glutathione S-transferase (GST, EC 2.5.1.18) [7]. The degree of protection provided by dichloroacetamide, oxime ether, and thiazole safeners on grass crops against injury from chloroacetanilide herbicides has been shown to correlate rather strongly with the ability of these safeners to enhance GST and/or the levels of reduced glutathione [7].

Glutathione reductase (GR, EC 1.6.4.2), a chloroplasmic enzyme which reduces oxidized glutathione (GSSG) to reduced glutathione (GSH), plays an important role in the protection of chloroplast membranes against peroxidative damage caused by toxic oxygen species [8]. Komives *et al.* [9] and Yenne and Hatzios [10] reported that the



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safeners dichlormid and fluxofenim (formerly known as CGA-133205) increased the activity of GR in corn and grain sorghum seedlings, respectively.

Therefore, the objectives of this research were to determine the influence of the herbicide pretilachlor and its respective safener fenclorim, applied individually or in combination, on the levels of glutathione and the activity of GR and GST enzymes of rice seedlings.

## Materials and Methods

### Chemicals

Analytical grade (95% pure) and radiolabeled ( $[^{14}\text{C}]$ ring labeled) pretilachlor as well as analytical grade (99% pure) fenclorim were obtained from Ciba-Geigy Corporation, Basel, Switzerland. All other chemicals and reagents used were obtained from various commercial sources.

### Plant material and chemical treatments

Rice (*Oryza sativa* L., cv. 'Lemont') seeds were screened in salted water (water containing NaCl as needed to raise its specific gravity to 1.06 at 20 °C), washed three times in tap water, and then immersed in water for 24 h. The imbibed seeds were germinated in the dark at 30 °C for 48 h. The germinated rice seedlings were then placed into 50 ml styrofoam cups containing filter paper and 15 ml of distilled water or the appropriate herbicide or safener solution, 60 seeds per cup. The cups were covered with parafilm and placed in a dark incubator at 30 °C. Chemical treatments included the herbicide pretilachlor at 2  $\mu\text{M}$ , the safener fenclorim at 20  $\mu\text{M}$  and combinations of pretilachlor plus fenclorim at 2  $\mu\text{M}$  and 20  $\mu\text{M}$ , respectively. At 0, 12, 24, 48, and 72 h after treatment, rice seedlings were harvested and had their shoots and roots separated from the seed tissue. Both parts (shoots and roots) were then frozen in liquid nitrogen and used for the determination of glutathione content or the activity of glutathione-related enzymes.

### Glutathione assay

For the determination of total and reduced glutathione content, a modification of the method of Tietz [11] was used. A complete description of the exact procedures has been given previously [10].

The content of reduced glutathione (GSH) in the crude extract was obtained by subtracting the content of oxidized glutathione (GSSG) from total glutathione. Glutathione concentration in the samples was calculated from standard curves using 0 to 2.0  $\mu\text{M}$  of GSH and GSSG. Data are expressed as  $\mu\text{mol/g}$  fresh weight of tissue.

### Glutathione reductase and GST extraction

Untreated and treated plant tissues (0.25 g) prepared as described above plus 0.13 g of polyvinylpolypyrrolidone were briefly ground with a mortar and pestle. Then 2.5 ml of 0.1 M K-phosphate buffer containing 0.5 mM EDTA (pH 7.5) was added and the slurry was ground again. This slurry was filtered through two layers of miracloth and then centrifuged at  $20,000 \times g$  for 20 min. The pellet was discarded and the supernatant was used as the crude enzyme extract for the following glutathione reductase (GR) and glutathione-S-transferase (GST) assays. Protein content was determined by the method of Bradford [12] which employs the Coomassie protein assay reagent.

### Glutathione reductase assay

GR was assayed according to the DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] procedure described by Smith *et al.* [8]. Formation of TNB (2-nitro-5-thiobenzoic acid) was monitored at 412 nm for 2 to 4 min, but the reaction was linear for at least 15 min. The rate of TNB formation is proportional to the amount of GR activity. The extinction coefficient for the TNB is  $11,500 \text{ moles cm}^{-1} \text{ min}^{-1}$  [10].

### Glutathione S-transferase assay

GST activity was assayed by the procedures described by Yenne and Hatzios [10]. Final concentrations of all reagents are given in parenthesis. The reaction mixture contained: 30  $\mu\text{l}$  of 0.1 M K-phosphate buffer (pH 6.5), 10  $\mu\text{l}$  of 60 mM reduced glutathione (10 mM) and 10  $\mu\text{l}$  of crude extract. The reaction was initiated by the addition of 10  $\mu\text{l}$  of 6 mM of uniformly ring-labeled  $[^{14}\text{C}]$ pretilachlor (1 mM; spec. act. 38.5 mCi/mg) in a final volume of 60  $\mu\text{l}$ . The reaction vessel was centrifuged in a microcentrifuge for 30 sec for thorough mixing and then incubated at 35 °C for 60 min. After incubation, the reaction was termi-

nated by adding 60  $\mu$ l of distilled water and 1 ml of dichloromethane. The reaction vessel was shaken vigorously and then microcentrifuged for 3 min. Then, 60  $\mu$ l of the aqueous phase was counted using liquid scintillation counting to determine the amount of GS-pretilachlor conjugate formed. TLC analysis of the aqueous phase has shown that the great majority of the radioactivity found in this fraction represents the GS-pretilachlor conjugate [4].

All experiments for glutathione determinations, GR, and GST were repeated in time and all assays were run in duplicate for each experiment.

## Results and Discussion

The results of the experiments conducted in this study are presented in Fig. 1 to 4 and in Table I.

Data in Fig. 1 and 2 show that pretilachlor applied individually did not influence significantly the levels of total or reduced glutathione of germinating rice seedlings. Fenclorim applied alone increased significantly the levels of total glutathione of rice seedlings at all time periods after treatment, except the 48 h time period (Fig. 1). The effect of fenclorim, however, on the levels of reduced glutathione was not as pronounced (Fig. 2). These results are in agreement to those reported by Komives and Dutka [6] who also showed an enhancement of the glutathione levels of 'Starbonet' rice by the safener fenclorim. The levels of total or reduced glutathione reported in this study for 'Lamont' rice seedlings are much greater than those reported by Komives and Dutka [6] for 'Starbonet' rice. This observation emphasizes once again

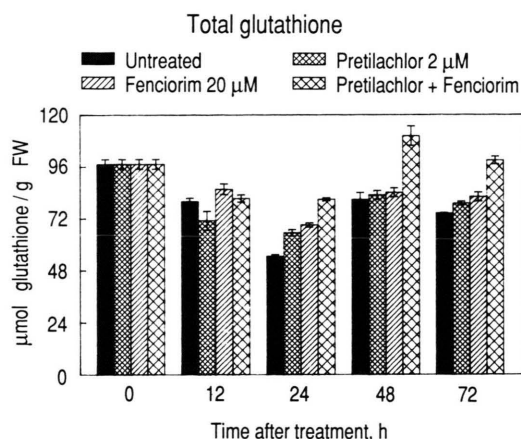


Fig. 1. Influence of individual or combined treatments with pretilachlor and fenclorim on the total glutathione content of rice seedlings. Bars on each histogram represent the standard error of each mean.

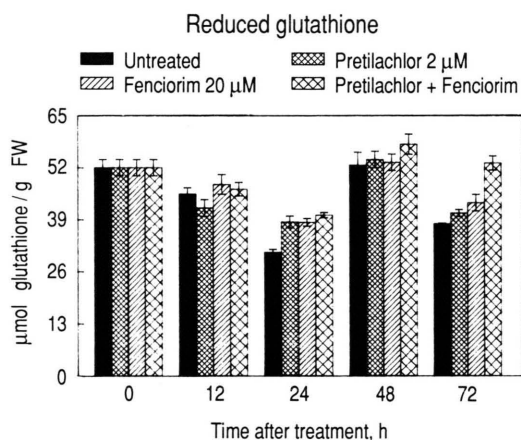


Fig. 2. Influence of individual or combined treatments with pretilachlor and fenclorim on the reduced glutathione content of rice seedlings. Bars on each histogram represent the standard error of each mean.

Table I. Effect of fenclorim concentration on *in vitro* activity of glutathione-S-transferase of rice seedlings.

Fenclorim concentration [ $\mu$ M]	Glutathione-S-transferase activity (nmol/mg protein $\cdot$ min) <sup>a</sup>	Percentage of control [%]
0	48.9 $\pm$ 0.1	100
1	47.0 $\pm$ 0.2	96
10	48.9 $\pm$ 1.2	100
20	51.1 $\pm$ 0.6	105
40	51.0 $\pm$ 1.7	104
80	52.0 $\pm$ 1.2	106
160	48.8 $\pm$ 1.4	100

<sup>a</sup> Mean values from three replications  $\pm$  SE of each mean.

the significance of potential varietal differences in studies of herbicide/safener interactions. The combination of pretilachlor and fenclorim increased the levels of total and reduced glutathione at all times after treatment (Fig. 1 and 2).

Fenclorim has been reported to conjugate with reduced glutathione in mammalian tissues [13] and in grass plants [Dr. B. Donzel, Ciba-Geigy, Basel, personal communication]. The formation of the GSH conjugate of fenclorim in treated rice seed-

lings reduces the amount of the available glutathione and may eliminate the feedback regulation exerted by high concentrations of GSH on its own biosynthesis. A similar mechanism has been reported by Breau *et al.* [14] for the safener flurazole.

The activity of glutathione reductase extracted from pretilachlor-treated rice seedlings was significantly lower than that from untreated tissues (Fig. 3). Pretreatment of rice seedlings with the safener fencloirum increased GR activity at 12 and 24 h, but not at 48 and 72 h after treatment. The activity of GR extracted from rice seedlings treated with the combination of pretilachlor and fencloirum was increased significantly (50 to 60% of control) at 12, 24, and 48 h after treatment (Fig. 3). The activity of GR extracted from 'Lamont' rice seedlings used in this study was much lower than that reported by Yenne and Hatzios [10] for 'Funk G-522-DR' grain sorghum seedlings, illustrating again interspecific differences in the levels of glutathione-related enzymes.

Pretreatment of rice seedlings with pretilachlor reduced significantly the activity of rice GST catalyzing the conjugation of this herbicide with GSH at 24, 48, and 72 h after treatment (Fig. 4). Pretreatment of rice seedlings with the safener fencloirum had no effect on GST activity of rice (Fig. 4). Similarly, fencloirum applied at a range of micromolar ( $\mu\text{M}$ ) concentrations did not affect the *in vi-*

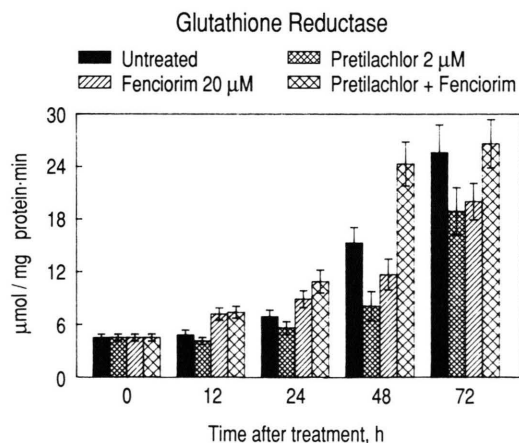


Fig. 3. Influence of individual or combined treatments with pretilachlor and fencloirum on glutathione reductase activity of rice seedlings. Bars on each histogram represent the standard error of each mean.

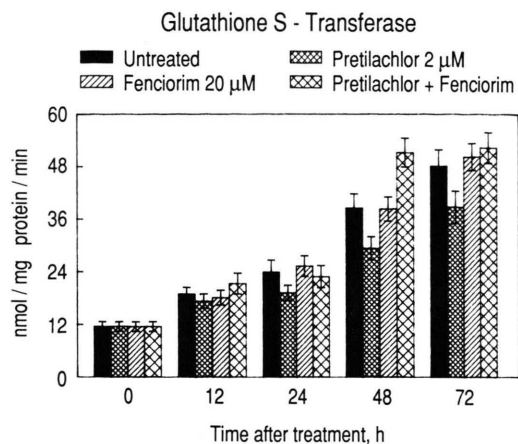


Fig. 4. Influence of individual or combined treatments with pretilachlor and fencloirum on glutathione-S-transferase activity of rice seedlings. Bars on each histogram represent the standard error of each mean.

*tro* activity of rice GST catalyzing the conjugation of pretilachlor with GSH (Table I). Pretreatment with the combination of pretilachlor and fencloirum increased GST activity of rice seedlings which utilizes pretilachlor as a substrate, but only at the 48 and 72 h periods (Fig. 4).

A 2- to 3-fold increase in GST activity of rice shoots and roots following pretreatment with the safener fencloirum at concentrations ranging from 0.5 to 10  $\mu\text{M}$ , has been reported recently by Komives and Dutka [6]. However, these investigators used CDNB (1-chloro-2,4-dinitrobenzene) as a substrate for determining GST activity and a different cultivar of rice than the one used in our studies. Setting aside the potential varietal differences in terms of GST activity in rice, it appears that GST activity using CDNB as a substrate [6] was about 10-fold higher than that found in our study which used radiolabeled pretilachlor as a substrate for GST activity. Such differences are not unexpected since it is now well accepted that GST isozymes with differential affinity for CDNB and chloroacetanilide herbicides are present in many grass plants such as corn and grain sorghum [15–18]. Dichlormid and other safeners of corn and grain sorghum have been shown to be selective in terms of their ability to enhance the activity of specific GST isozymes and to induce the *de novo* synthesis of some of these isozymes in corn and



grain sorghum [15–18]. Further research is definitely needed to elucidate the presence of GST isozymes in rice and the ability of the safener fenclorim in enhancing selectively the activity of such GST isozymes.

## Conclusion

Overall, these results suggest that the safener fenclorim may enhance the metabolic detoxication of pretilachlor *via* conjugation to glutathione by stimulating the direct or indirect synthesis of glutathione rather than the activity of the GST enzyme catalyzing this reaction. In addition, these results suggest that a simultaneous application of pretilachlor and fenclorim is critical for the enhancement of glutathione and glutathione-related en-

zyme activity and the protection of rice from pretilachlor injury.

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