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Persistence and detoxification of Pretilachlor and Fenclorim in rice (*Oryza sativa*)¹

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Abstract – The contents and persistence of pretilachlor (P) and of the safener fenclorim (F) in rice treated with P, F and their mixture were investigated in relation to the expression of glutathione-S-transferase (GST) activity in response to treatments. F failed to reduce the accumulation of P, however it was able to reduce its persistence in rice shoots. On the other hand, while P did not reduced F persistence, it determined a greater than two-fold F accumulation in plants. Therefore, a detoxification mechanism common to the two compounds but favourable to P was hypothesised. This mechanism consists in an activation of GST activity toward P as substrate [GST(P)] in response to all treatments. The activation levels were found to be proportional to the amount of persistent P and F, and to be additive following the P-F mixture treatment. The kinetic parameters V_{max} and K_m of GST(P) were increased and decreased, respectively, in rice shoots treated with P, F and particularly with P-F mixture. Therefore, a defence mechanism toward P, consisting in an increased expression of GST(P) in rice plant, both as the global amount of the enzyme in the protein “pull” and the induction of the enzyme isoforms more sensitive to P, in response to all treatments, especially the P-F mixture treatment, is confirmed. This finding is consistent with the observed effect of F on P persistence and detoxification in the rice shoots.

***Oryza sativa* / pretilachlor / fenclorim / persistence / glutathione-S-transferase**

Résumé – **Persistence et détoxification du pretilachlore et du fenclorim dans le riz (*Oryza sativa*)**. On a étudié la teneur et la persistance du pretilachlore (P) et du fenclorim (F), un antidote, dans le riz traité avec P, F et un mélange des deux par référence à l'activité de la glutathion-S-transférase (GST) en réponse aux traitements en question. F n'a pas réussi à réduire l'accumulation de P, mais a pu réduire sa persistance dans les pousses de riz. D'autre part, P n'a pas réduit la persistance de F, mais a déterminé une accumulation plus que double de F dans les plantes. Donc a-t-on soulevé l'hypothèse d'un mécanisme de détoxification commun aux deux composés, mais favorable à P. Ce mécanisme consiste en une activation de l'activité GST à l'égard de P comme substrat [GST(P)] en réponse à tous les traitements. On a relevé que les niveaux d'activation étaient proportionnels à la quantité de P et F persistants, et additifs dans le cas du traitement avec le mélange P-F. Les paramètres cinétiques V_{max} et K_m de [GST(P)] ont été respectivement augmentés et diminués dans les pousses de riz traitées avec P, F et particulièrement avec le mélange P-F. On a eu donc confirmation d'un mécanisme de défense à l'égard de P, consistant en une expression accrue de [GST(P)] dans la plante de riz, en ce qui concerne tant la quantité globale de l'enzyme dans le «pull» protéique que l'induction des isoformes enzymatiques plus sensibles à P, en réponse à tous les traitements et surtout au traitement à base de mélange P-F. Ce résultat concorde avec ce qui a été observé quant à l'effet de F sur la persistance et la détoxification de P dans les pousses de riz.

***Oryza sativa* / pretilachlore / fenclorim / persistence / glutathion-S-transférase**

1. INTRODUCTION

Pretilachlor [2-chloro-2',6'-diethyl-N-(2-propoxyethyl)-acetanilide = P] is an α -chloroacetanilide herbicide employed to control certain broadleaf weeds and annual grasses in transplanted rice fields. Since P failed to be satisfactorily

tolerated by water sown rice, the safener fenclorim (4,6-dichloro-2-phenylpyrimidine = F) was developed to avoid significant plant injury [2, 7, 14, 16, 22]. The development of F has facilitated the selective use of P in rice fields, and now P and F are generally co-formulated. Further, it has been reported that F enhances herbicide detoxification

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in rice plants [8, 15, 24]. The major detoxification pathway of all α -chloroacetanilide herbicides, P included, is their conjugation with reduced glutathione through the action of glutathione-S-transferase (E.C.2.5.1.18 = GST) enzymes which transform the parent molecule into an inactive derivative [4, 13, 18]. On the basis of experimental evidence it was supposed that F exerts a safening action in rice plants by inducing isoforms of GST enzymes [6, 24]. However, there is less experimental evidence as to the specificity and affinity of the F induced isoenzymes in rice toward P and the relationship between their activity and the rate of herbicide detoxification in plants.

The purpose of this study was to attain further information on the ability of F to induce of an enhanced expression of GST activity on the P substrate [GST(P)] and the role of this effect on the accumulation and persistence of P and F in rice plant.

2. MATERIALS AND METHODS

2.1. Chemicals

Standard pretilachlor (96.0%) and fenclorim (99.5%) were supplied by Lab. Ehrenstorfer-Schäfers (Augsburg, Germany). Reduced glutathione and brilliant blue G were obtained from Aldrich Chemie (Steinheim, Germany). Acetonitrile (HPLC grade), water (HPLC grade) were purchased from BDH (Poole, England). All other reagents employed were of ACS grade.

2.2. Apparatus

A Perkin-Elmer Series 410 HPLC (Norwalk CT USA) was used. It was equipped with a 235 model diode array detector, with a C-18 column (4.6 mm i.d.; 25 cm length) and a pre-column (3 cm length) with the same characteristics.

A Varian Model Cary 210 double-beam grating spectrophotometer (Melbourne, Australia) was used.

2.3. Plant materials and growth conditions

Rice seeds (hybrid Pegaso) were used. Seeds were surface sterilized with two 3-min rinses in full strength commercial bleach (5.25% NaOCl) followed by a rinse in sterile distilled water. The seeds were germinated in sand quartz (pre-washed with hydrochloric acid) in plastic pots (40 × 20 × 10 cm) in a growth chamber in the dark at 25 °C and a relative humidity of 75%. Each pot contained 50 g of germinating seeds. Distilled water (50 ml) was applied daily to each pot. After 4 days the seedlings were submitted to day-night conditions (12 h of light, 5000 lux, and 12 h of darkness) at 25 °C. When the seedlings were 6 days old, one-fourth-strength Hoagland nutrient solution [10] was applied instead of distilled water and the pots were divided into four groups: one group was left as control, the second group was treated with P (5.0 mg·pot⁻¹), the third group was treated with F (2.5 mg·pot⁻¹) and the fourth group was treated with P-F mixture. Treatment concentrations corresponded to the suggested field dosage. To apply the required quantity of compounds, the rate per hectare was calculated according to the surface area per pot, and the

treatment solutions were applied utilising a mechanical sprayer in one direction and then crosswise. Seedlings were collected at 24, 48, 72, 96, 120, 144 h after treatment, rinsed with water and then washed with copious amounts of tap water to get rid of any adsorbed herbicide. Samples of shoots were dried by blotting with paper towels and utilised for the determination of P and F residues and for GST enzyme extraction.

2.4. Determination of P and F residues

The determination of P and F in rice shoots were performed according to the procedure of Vischetti et al. [23]. Samples of shoots (2 g) were powdered in liquid nitrogen and extracted and homogenized with a 20 ml solution of methanol-water (4:1 v/v) for 3 min. The mixture was then centrifuged for 15 min at 5000 rpm and filtered under vacuum. The liquid phase was added with water (20 ml), and partitioned with CHCl₃ (2 × 30 ml). The organic phase was evaporated to dryness, rinsed with 1 ml of water/acetonitrile (3:7 v/v) and submitted to HPLC analysis. The determinations were performed at 210 nm wavelength with a mobile phase (water/acetonitrile; 3:7 v/v) flow of 1 ml·min⁻¹. Under these conditions the retention time was 11.5 min for P and 13.0 min for F.

2.5. GST extraction

Shoot samples (2 g), obtained as described above, were collected and homogenized by sonication with acetone chilled at -30 °C (20 ml) for 1 min. The homogenate was filtered through a Büchner funnel and washed twice with chilled acetone. The residues were spread out on filter paper and allowed to dry at room temperature to obtain the acetone powders [9]. For the complete protein extraction, the powders were covered with buffer solution (20 ml of phosphate buffer, 0.1 M, pH 9.0) and allowed to stand for 10 min at 4 °C. The mixture was then centrifuged at 48 200 rpm for 20 min at 4 °C and the supernatant was used as the crude enzyme extract.

The total protein content in the enzyme extract was determined spectrophotometrically by reaction with brilliant blue G according to Bradford procedure [1].

2.6. GST assays

The GST(P) activity was evaluated from the rate of pretilachlor-GSH conjugation in the reaction mixtures prepared according to Scarponi et al. [19]. A suitable volume of enzyme extract was added to 3.5 ml of phosphate buffer (0.1 M, pH 7.4), which had been deaerated by bubbling N₂ through it, so as to have 2.5 mg of protein in the reaction mixture, and then in sequence: 10 μ mol of GSH and 160 nmol of P dissolved in the phosphate buffer. The final volume of the mixture was adjusted at 6.0 ml with the phosphate buffer. After 2 h of incubation at 35 °C the reaction was stopped by freezing in a dry-acetone bath and the reaction mixtures were then lyophilized. To check any herbicide losses by nonconjugating reactions, control tests were carried out by replacing GSH solution with 0.5 ml of the phosphate buffer, and the evaluation of nonenzymatic conjugation was performed by substituting the enzyme extract with equivalent

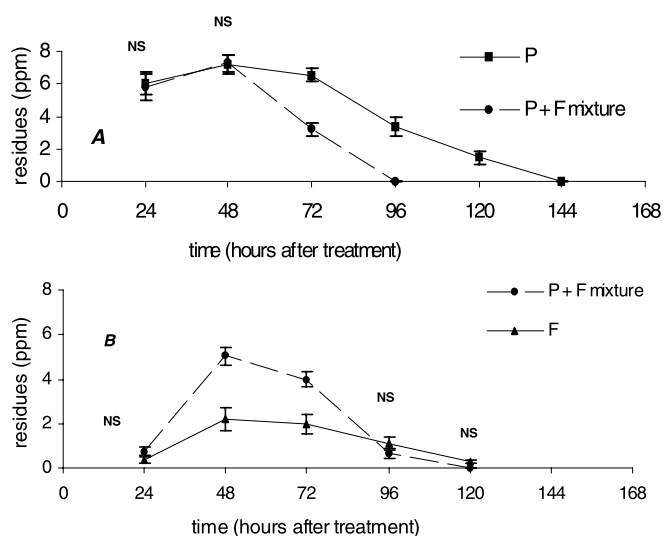


Figure 1. Residual amount of pretilachlor (A) and fenclorim (B) in treated rice shoots. Each value is the mean \pm SD of three determinations. NS, not significantly different from the respective sample treated with the P + F mixture ($P < 0.01$ on the basis of LSD test).

aliquot of phosphate buffer. The lyophilized material was extracted with 5 ml of methanol and centrifuged at 34 800 rpm for 10 min at 4 °C. The supernatant was utilized to determine the residual nonconjugated herbicide by the HPLC procedure as described above. The GST(P) activity was expressed as nmol of conjugated P per hour and protein unit.

The kinetic parameters of GST for P were determined using rice shoots collected 48 h after treatments with P, F and P-F mixture. In these tests enzyme activity was determined with the following concentrations (μM) of P: 1.0–2.0–3.0–4.0–5.0–10–20–30–40–50, so to obtain the V_{max} and K_m values by the linear regression analysis of $1/v$ versus $1/s$ of the linear transformation of Michaelis-Menten equation.

Each plant extraction, analytical determination and GST assay was run in triplicate. All data were statistically analyzed using the least significant difference (LSD) method.

3. RESULTS

Figures 1A, B report the amount and the persistence of P and F, added to rice shoots alone or in combination, during a 144-hour period after treatment. The data show a prevalence of P and F incorporation into plant tissues over their detoxification during the 48 hours initial period. Thereafter, degradation prevailed until negligible residue levels (below the 5 ppb detection limit). When P and F were applied alone, their maximum accumulation in the shoots was 7.2 and 2.2 ppm, respectively, and degradation time was 144 and 120 hours, respectively. Treatment with P-F mixture did not result in a different maximum accumulation of P while it reduced P persistence from 144 to 96 hours; on the other hand, while it did not modify the persistence of F, it significantly increased its maximum accumulation from 2.2 to 5.0 ppm.

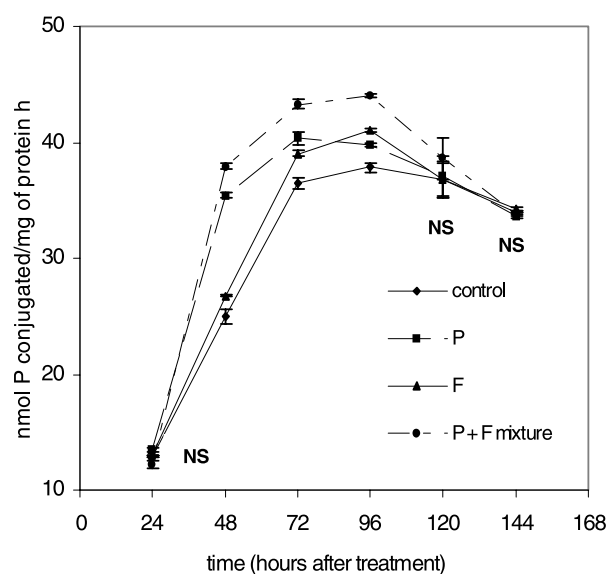


Figure 2. Activity of GST(P) in rice shoots. Each value is the mean \pm SD of three determinations. NS, not significantly different from the respective untreated control ($P < 0.01$ on the basis of LSD test).

Figure 2 illustrates the activities of GST(P) extracted from untreated shoots and from the shoots treated with P, F and their mixture, throughout the entire experiment period. Unlike the untreated shoots, significant increases in enzyme activity in all the treated shoots were found from 48 to 120 hours after treatment. P and F determined more or less the same increases in GST(P) activity (with the exception of P at 48 hours) and their mixture resulted in an almost additive effect. The increases ranged from 5.2%, 7.0% and 5.2% to 41.6%, 8.4% and 51.6% in the shoots treated with P, F and P-F mixture, respectively.

Table I reports the V_{max} and K_m parameters of GST(P) determined in the treated and untreated shoots 48 hours after treatment, which is the time of maximum P and F accumulation. In comparison to the untreated samples, the above kinetic parameters were significantly affected by the treatment to shoots. Compared to the untreated rice plants, V_{max} values were found to be increased by 33.3%, 33.3% and 41.6% and K_m decreased by 19.6%, 35.9% and 48.9% in response to treatment with P, F and P-F mixture, respectively.

4. DISCUSSION

Data concerning the accumulation and persistence of P and F in rice shoots (Figs. 1A, B) show that F did not interfere with P incorporation into rice shoots while it brought about a 33.3% reduction in P persistence. Conversely, P did not interfere with F persistence, however it determined more than twice the accumulation of F in the plant. The lower P persistence when added in mixture with F is not surprising since it is well known that F is able to protect rice from P injury by enhancing its detoxification [4, 8, 11, 22].

The greater accumulation of F when applied as P-F mixture rather than alone, suggests a similar mechanism of P and

Table I. K_m and V_{max} values of GST(P) extracted from untreated and treated rice shoots.

Treatments	V_{max} umol. of conjugated herbicide/min (g fw)	K_m mM	R^2
control	0.12a	0.92a	0.9963
pretilachlor	0.16b	0.74b	0.9861
fencloirim	0.16b	0.59c	0.9931
pretilachlor + fencloirim	0.17b	0.47d	0.9929

The data represent the means of triplicate determinations. Means within a same column followed by the same letter are not significantly different at 5% level using Duncan's multiple range test.

F detoxification occurs, favoring P because, unlike F, P did not accumulate in presence of F.

It is well known that the major detoxification pathway of α -chloroacetanilides in plants is their conjugation with glutathione via GST enzymes. This inactivation mechanism has been shown to be active in a large spectrum of tolerant crops, such as soybean, maize, sorghum, wheat, sugarcane, barley and rice for a number of α -chloroacetanilides, including pretilachlor [4, 12, 13, 17]. Such a mechanism has also been found to occur in rice with F as the GST substrate [5]. For this reason the GST(P) activity was checked in response to P, F and P-F mixture treatment during the period in which P and F residues were found to be present in rice shoots. Figure 2 shows that all treatments resulted in an inducing GST(P) activity and that this effect is proportional to the amounts of the persistent P during the time required for its detoxification in the plant. Such a trend suggests a direct effect of P and F on the activation of the enzyme of their detoxification. Induction of GST activity in response to P treatment to rice had already been observed by Deng et al. [6] and by Nagao et al. [15]. Similarly, GST induction was found in soybean treated with alachlor [17] or metolachlor [19], in vicia faba treated with propachlor [20] and in sorghum treated with herbicide antidotes [3].

Further information concerning the effect of P and F on the natural GST endowment in rice cv Pegaso can be drawn from the kinetic parameters – V_{max} and K_m – of GST(P) extracted from the treated and untreated shoots (Tab. I). The values of V_{max} (the rate when the enzyme is fully saturated by P substrate) were found to be significantly increased in response to all treatments. As V_{max} is proportional to enzyme concentration, it appears that P, F and especially the P-F mixture were able to strengthen the expression of the GST(P) in the protein pull of rice shoots.

The values of K_m (the P concentration required for 50% GST activity) were found to be significantly decreased by all treatments, especially by the P-F mixture. As the K_m parameter is inversely related to the substrate–enzyme affinity, it can be hypothesized that the treatments, particularly the P-F mixture, induced GST forms endowed with a greater affinity to the P substrate.

It has been ascertained that GST consists of many isoenzymes, therefore the increased GST(P) expression in the treated shoots could be the result of the induction of one or

more of these isoenzymes [21]. Deng et al. [6] demonstrated the existence of GST isoforms active on P and F substrates in rice and that the treatment with P-F mixture increased their activities greatly on the P substrate and to a far lesser extent on the F substrate [GST(F)]. The characteristics of the P and F induced GST isoenzymes and their affinity with P substrate would certainly deserve further investigation. However, a greater induction of the activity of GST(P) isoenzymes than that of the GST(F) isoenzymes by the P-F mixture, seems to be further confirmed by our data in that the shoots treated with the P-F mixture show a greater F accumulation and an unaffected P accumulation compared with the shoots treated with F or P alone.

In conclusion, the V_{max} increases and K_m decreases observed in all the treated rice shoots confirm the existence of a defence mechanism consisting in a greater expression of GST(P) activity, in terms of both the global amount of the enzyme in the protein pull of the plant and the induction of isoforms more sensitive to P in response to treatments with P, with F and particularly with P-F mixture. This finding is consistent with the role of the safener F on P persistence and detoxification and explains the greatly reduced persistence of P in the rice shoots when applied in mixture with F.

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