American Journal of Plant Physiology

ISSN 1557-4539

Academic Journals Inc.

www.academicjournals.com

American Journal of Plant Physiology 2 (3): 195-205, 2007
ISSN 1557-4539
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Induction of Glutathione and Glutathione-associated Enzymes in Butachlor-tolerant Plant Species

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Abstract: The present study was conducted to ascertain the differential capability of wheat, maize and soybean to tolerate butachlor through checking levels of GSH and GSH-associated enzymes. The recommended field dose of butachlor differentially reduced shoot fresh and dry weights of ten-day-old wheat, maize and soybean seedlings during the following 16 days. The reduction was either slightly retracted and mostly nullified by the end of the experiment in wheat and maize or great and consistent in soybean. Meanwhile, there were elevations in protein-bound, non-protein, total thiol and reduced glutathione (GSH) in treated seedlings whereas oxidized glutathione (GSSG) was decreased, these changes were generally undetectable in soybean. On the other hand, activities of the isoforms of Glutathione-S-Transferases (GSTs, EC 2.5.1.18) to the substrates CDNB, atrazine, alachlor, metolachlor and butachlor as well as the activities of γ-glutamylcysteine synthetase (γ-GCS, EC 6.3.2.2), glutathione synthetase (GSS, EC 6.3.2.3), glutathione peroxidase (GSPX, EC 1.15.1.1) and glutathione reductase (GSR, EC 1.6.4.2) were greatly enhanced in wheat and maize by butachlor, however, the inductions in soybean were most likely negligible. The slight phytotoxic effect of butachlor to wheat and maize relative to soybean suggested that soybean could be considered as a more susceptible species than wheat and maize, both could tolerate the herbicide effects. The differential tolerance of plant species seemed to be in relationship with the varied induction of thiols, GSH and activities of GSH-associated enzymes.

Key words: Butachlor, GSH, GSH-associated enzymes, maize, soybean, tolerance, wheat

INTRODUCTION

Butachlor N-butoxymethyl-(2-chloro-2,6-diethylacetanilide is a chloroacetanilide herbicide used to control weeds in wheat, maize and soybean fields. It affects protein, pigments and gibberellic acid syntheses, cell division, mineral uptake and cell permeability (Kearney and Kaufman, 1988). However, some plant species can tolerate herbicide phytotoxicity through an efficient defense mechanism; i.e., detoxicants that convert herbicides into non-toxic compounds and antioxidants that scavenge Reactive Oxygen Species (ROS) (Edwards, 1996; Kuzniak, 2002; Anderson and Davis, 2004). These ROS are detoxified by sequential and simultaneous action of a number of enzymes including GST, GSPX and GSR and metabolites including GSH and thiols (Misra *et al*., 2006; Nemat Alla and Hassan, 2006). Moreover, detoxification of butachlor, as the other chloroacetanilide herbicides, proceeds mainly through the conjugation with GSH to be converted into a non-toxic compound (Kearney and Kaufman, 1988; Cobb and Kirkwood, 2000). Herbicide conjugation with GSH may take place non enzymatically or enzymatically mediated by GSTs (Jablonkai and Hatzios, 1993). Increasing thiol and GSH was

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regarded as limiting factor for herbicide detoxification (Aravind and Prasad, 2005). In addition, Jablonkai and Hatzios (1993) affirmed that resistance to herbicides depends on the GSTs-mediated conjugation of GSH. The GSTs are enhanced under certain conditions to increase the plant defense against several biotic and abiotic agents (Jablonkai and Hatzios, 1993; DeRidder *et al*., 2002; Misra *et al*., 2006). Therefore, the activity of GST might contribute to the physiological selectivity of plants to tolerate herbicides. Some isoforms of GST show dual activity and can also function as GSPX (Vontas *et al*., 2002). GSH is synthesized from the combined action of γ-GCS and GSS (Volohonsky *et al*., 2002; Gupta *et al*., 2005) and maintained in reduced state by GSR (Yoon *et al*., 2005). So, plants with high levels of GSH and GSH-associated enzymes are predicted to tolerate the phytotoxic effects of the GSH-conjugated herbicides. Therefore, the present study was aimed to ascertain the differential capability of wheat, maize and soybean to tolerate butachlor through investigating their internal levels of thiol and GSH as well as activities of GSH-associated enzymes.

MATERIALS AND METHODS

Plant materials and growth conditions

This study was conducted in Botany Department, Faculty of Science at Damietta, Mansoura University, Egypt during the year 2006. Wheat (*Triticum aestivum* L. Giza, 168), maize (*Zea mays* V.S.C.129) and soybean (*Glycine max* cuttler 71) seeds were surface sterilized by immersing in 3% sodium hypochlorite solution for ten min, thoroughly washed, soaked for 8 h and germinated in sand/clay soil (1:1, v/v) in plastic pots (25 cm diameter×20 cm height). The pots were kept at 14 h photoperiod with 450-500 μmol m^{-2} sec^{-1} photosynthetic photon flux density, 75-80% relative humidity and 22/10°C day/night regime for wheat or 28/14°C for maize and soybean. When seedlings were ten-d-old, irrigation water was substituted with one-fourth strength Hoagland solution. At this stage, the pots were divided into two groups, one was left to serve as control and the other for butachlor treatment at the recommended field dose (3.0 L ha^{-1}). The herbicide quantity was calculated in relation to the surface area per pot and mixed in a suitable amount of water enough to spray the surface area of each pot twice; in one direction and crosswise. The herbicide was applied only once. Shoots of both species were collected just before herbicide application (used for zero time) and also after 4, 8, 12 and 16 days from treatments, rinsed with copious amounts of water and dried by plotting with paper towels.

Measurement of total, protein-bound and non-protein thiol contents

Plant samples were homogenized in 20 mM EDTA. The clear supernatants were separated by centrifugation at 12000 x g for 15 min. Total thiols were measured in 200 mM Tris-HCl (pH 8.2), 10 mM 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and absolute methanol (Sedlak and Lindsay, 1968). The absorbance was read at 412 nm. The quantity of thiol was calculated from the extinction coefficient E = 13, 100 mM^{-1} cm^{-1}. To determine non-protein thiols, the supernatant was mixed with Trichloroa Cetic Acid (TCA, 50% w/v) and centrifuged at 10,000xg for 15 min. The absorbance was read as above. The protein-bound thiols were calculated by subtracting the non-protein thiols from total thiols.

Determination of reduced and oxidized glutathione (GSH and GSSG)

Plant tissues were homogenized at 4°C in TCA (5%, w/v) and 10 mM EDTA (Anderson and Gronwald, 1991). The extracts were centrifuged at 12000 x g for 15 min. Reduced glutathione was assayed in 100 mM phosphate buffer, pH 6.8 containing 10 mM EDTA, 1 mM 1-Chloro-2,4-Dinitro Benzene (CDNB) and 1.0 U equine GST and incubated at 35°C for 30 min. The absorbance at 340 nm was recorded before commencing the reaction and after the reaction had run to completion. A control

assay without equine GST was performed to check the possible nonenzymatic reaction. For the assay of GSSG, 2-vinylpyridine was added to mask GSH. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 5 mM EDTA, 0.2 mM NADPH, 0.6 mM DTNB and 10 U GSR. The absorbance at 412 nm was immediately measured and again after 30 min of incubation at 35°C.

Assay of γ-Glutamyl-cysteine Synthetase (γ-GCS) and Glutathione Synthetase (GSS):

Plant samples were homogenized in 50 mM Tris-HCl (pH 7.5) containing 40 mM phenyl methyl sulfonyl fluoride and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The extracts were centrifuged at 15,000 x g for 20 min (Aravind and Prasad, 2005). The assay of γ-GCS was performed in 20 mM sodium glutamate, 20 mM L-amino butyrate, 40 mM Na_2-EDTA, 0.4% (w/v) BSA, 20 mM $MgCl_2$, 50 mM Na_2-ATP 20 mM Tris–HCl (pH 8.2) and the enzyme preparation (Nagalakshmi and Prasad, 2001). The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by TCA to estimate phosphate content at 660 nm. The assay of GSS was carried out in 100 mM Tris–HCl (pH 8.0) containing 50 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA, 10 mM ATP, 2.5 mM dithiothritol (DTT), 5 mM glycine and 5 mM γ-glutamylcysteine (Volohonsky *et al*., 2002). The reaction mixtures were incubated at 37°C and GSH was determined every 5 min up to 20 min.

Assay of Glutathione-S-Transferases (GSTs):

Plant tissues were homogenized in 100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 14 mM β-mercaptoethanol and 7.5% (w/v) PVPP. After centrifugation at 15,000 g for 15 min, ammonium sulfate was added to 80% saturation (Dixon *et al*., 1995). Routine assay of GST was performed towards the substrate chlorodinitrobenzene (CDNB) GST(CDNB) in 100 mM phosphate, pH 6.5 containing 5 mM GSH and 1 mM CDNB. After an incubation period for an h at 35°C, the reaction was stopped by HCl. The absorbance was measured at 340 nm. The enzyme activity was calculated by the extinction coefficient E = 9.6 mM^{-1} cm^{-1} (Askelof *et al*., 1975).

Moreover, a series of HPLC assays were performed to measure the activities towards the substrates atrazine, alachlor, metolachlor and butachlor herbicides, ($GST_{(atrazine)}$, $GST_{(alachlor)}$, $GST_{(metolachlor)}$ and $GST_{(butachlor)}$, respectively). A sample of about 2 mg of protein was incubated with 10 μmol of GSH and 160 nmol of the herbicide for 2 h at 35°C. The reaction was stopped in a dry ice-acetone bath and the mixture was then dried, extracted in methanol, and used for the HPLC determination of the residual non-conjugated herbicide (Scarponi *et al*., 1991). The following isocratic conditions were employed: mobile phase, water/acetonitrile (1/9, v/v); flow rate, 1 mL min^{-1} detection, 220 nm. Two control tests were carried out to check possible herbicide losses by non-conjugating reactions or the non-enzymatic GSH conjugation. The herbicides-GSH conjugation was calculated by subtracting the residues non-conjugated herbicides from the total quantity.

Assay of Glutathione Peroxidase (GSPX)

Plant tissues were homogenized in 100 mM Tris-HCl, pH 7.5, 1 mM EDTA and 2 mM DTT. The extracts were centrifuged at 15,000 x g for 20 min (Edwards, 1996). The reaction mixture constituted of 100 mM phosphate, pH 7.0, 2% (w/v) TritonX-100, 0.24 U GSR, 1 mM GSH, 0.15 mM NADPH, and 1 mM cumene hydroperoxide. After incubation at 30°C for 10 min, the rate of NADPH oxidation was measured by monitoring the absorbance at 340 nm for 3 min and calculated from the extinction coefficient E = 6.2 mM^{-1} cm^{-1} (Nagalakshmi and Prasad, 2001).

Assay of Glutathione Reductase (GSR)

Extraction was performed in 100 mM phosphate, pH 7.5 and 0.5 mM EDTA. The extracts were centrifuged at 15,000 x g for 20 min. The reaction mixture contained 100 mM phosphate, pH 7.5,

0.5 mM EDTA, 0.75 mM DTNB, 0.1 mM NADPH and 1 mM GSSG (Smith *et al*., 1988). The reaction mixture was incubated at 35°C meanwhile absorbance at 412 nm were being measured up to 5 min.

Protein was determined using the method of Bradford (1976). All values are means (±SD) of at least six determinations from two independent experiments. The full data were statistically analyzed using the least significant differences test at 5% level (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

This study was aimed to ascertain the differential tolerance of wheat, maize and soybean to butachlor. To achieve this purpose, growth parameters imposed as fresh and dry weight was determined. Moreover, level determinations of thiol forms, GSH and GSSG as well as activity assays of the enzymes GSTs, γ-GCS, GSS, GSPX and GSR were performed. Application of butachlor at the recommended field dose to wheat, maize and soybean resulted in differential significant decreases in shoot fresh weight below the control values, however, the reduction in wheat and maize reached maximally 13% and seemed to be leveled off mostly after the 4th day of treatment (Table 1). Nevertheless, fresh weight reduction of treated soybean was consistent and exceeded 53%. In the same pattern, the herbicide caused an inhibition in dry matter of wheat and maize only during the first 4 days from treatment. Thereafter, the reduction seemed to be recovered. Dry weight of soybean was significantly decreased by butachlor throughout the entire experimental period. Butachlor induced more than 46% reduction in soybean growth relative to 11% in maize and wheat.

The results, generally, showed differential reductions in fresh and dry weights following butachlor treatment. The reduction of wheat and maize growth was slight did not exceed 13% then retracted and even nullified by the end of the experiment. Nevertheless, growth reduction in soybean was greatest, reached more than 46%. As the other herbicides, α-chloroacetanilides cause reduction in growth of several plant species (Kearney and Kaufman, 1988; Farago *et al*., 1993; Nemat Alla and Younis, 1995; Cobb and Kirkwood, 2000; Hassan and Nemat Alla, 2005). The herbicide-induced growth reduction could result from alterations in certain metabolic processes (Farago *et al*., 1993; Chun Yan *et al*., 2000; Cobb and Kirkwood, 2000; Nemat Alla and Hassan, 2006). Butachlor, in the present study, provoked growth reduction either great in soybean or slight in wheat and maize. These results could generally conclude that soybean is susceptible to butachlor than wheat and maize, both species tolerated, to great extent, the effects of butachlor. As a whole, plant tolerance might result from some factors among which is the development of an efficient defense mechanism. This appeared the case in wheat and maize which seemed to overcome butachlor phytotoxicity.

Table 1: Changes in shoot fresh and dry weight of ten-day old wheat, maize and soybean by the recommended field dose of butachlor during the following 16 days. Data are means (±SD) of at least six replications from two independent experiments[a]

	Wheat		Maize		Soybean	
Days after Treatment	Control	Treated	Control	Treated	Control	Treated
Fresh weight (mg plant^{-1})						
0	203±11.2		490±23.6		287±14.6	
4	288±17.4	237±13.6*	763±44.2	663±34.1*	468±22.3	314±32.1*
8	345±19.7	298±18.5	888±47.8	800±47.2	610±31.8	341±33.6*
12	380±27.9	348±26.4	1080±52.7	979±64.1	712±42.1	405±42.4*
16	432±21.6	400±19.7	1132±56.2	1039±46.8	873±41.9	410±37.9*
Dry Weight (mg plant^{-1})						
0	28.1±2.1		51.4±4.7		37.5±2.9	
4	38.3±3.2	34.6±2.7*	72.7±4.2	64.4±5.3*	51.7±3.7	42.1±3.1*
8	45.4±2.7	41.9±3.1	85.9±3.7	79.6±4.2	70.4±4.1	50.8±4.4*
12	53.9±2.4	51.0±2.1	100.8±4.3	94.2±4.4	83.2±3.9	49.4±4.3*
16	64.3±2.5	62.2±2.9	105.9±4.6	99.3±5.6	86.4±5.2	46.6±4.7*

[a]Values of treated samples with an (*) are significantly different at 5% level with respect to untreated control

Table 2: Changes in protein-bound, Non protein and total thiol of ten-day old wheat, maize and soybean by the recommended field dose of butachlor during the following 16 days. Data are means (±SD) of at least six replications from two independent experiments[a]

	Wheat		Maize		Soybean	
Days after Treatment	Control	Treated	Control	Treated	Control	Treated
Protein-bound thiol (μM g^{-1} shoot fresh weight)						
0	2.11±0.15		2.73±0.16		0.91±0.06	
4	2.14±0.14	3.09±0.17*	2.98±0.17	4.28±0.19*	0.97±0.05	1.09±0.07
8	2.04±0.16	3.29±0.15*	2.85±0.11	4.23±0.17*	1.03±0.09	1.10±0.09
12	1.98±0.14	2.87±0.09*	2.89±0.09	3.99±0.18*	0.91±0.07	0.99±0.06
16	2.09±0.16	2.56±0.13	2.96±0.18	3.33±0.15	0.87±0.05	0.89±0.07
Non protein thiol (μM g^{-1} shoot fresh weight)						
0	1.16±0.09		1.54±0.11		0.33±0.02	
4	1.25±0.04	2.04±0.13*	1.62±0.13	2.65±0.14*	0.39±0.03	0.38±0.04
8	1.23±0.05	1.89±0.12*	1.63±0.12	2.63±0.18*	0.35±0.03	0.42±0.03
12	1.17±0.07	1.71±0.13*	1.57±0.11	2.19±0.16*	0.35±0.04	0.36±0.04
16	1.25±0.09	1.72±0.11*	1.64±0.13	1.77±0.14	0.41±0.04	0.38±0.03
Total thiol (μM g^{-1} shoot fresh weight)						
0	3.26±0.21		4.24±0.27		1.24±0.09	
4	3.39±0.19	5.13±0.19*	4.60±0.22	6.88±0.35*	1.36±0.07	1.47±0.08
8	3.25±0.22	5.18±0.31*	4.48±0.19	6.86±0.31*	1.35±0.11	1.53±0.11
12	3.15±0.26	4.58±0.21*	4.47±0.34	6.18±0.24*	1.26±0.12	1.35±0.07
16	3.34±0.21	4.31±0.22*	4.56±0.28	5.11±0.21	1.28±0.06	1.27±0.04

[a]Values of treated samples with an (*) are significantly different at 5% level with respect to untreated control

Butachlor treatment resulted in high increases in protein-bound, non-protein and total thiol contents in shoots of wheat and maize seedlings (Table 2). Anyway, protein-bound thiol in both species was significantly induced by butachlor up to the 12th day of treatment. The increases in soybean were insignificant. However, the magnitude of increase seemed higher in maize than in wheat. Also, butachlor significantly increased non-protein thiol content either during the whole experiment in wheat or up to the 12th day in maize. The changes in soybean seemed undetectable. The pattern of response of total thiol was most likely similar to those of protein-bound and non-protein thiol forms. The induction of total thiol by butachlor was extended to the end of the experimental time in wheat and to the 12th day in maize with no significant induction in soybean. Any way, butachlor provoked inductions of all forms of thiol in wheat and maize particularly during the first 12 days from treatment. Moreover, there were great inductions of GSH in wheat and maize by butachlor during the whole experiment, the induction was slight in soybean restricted only to the first 4 days (Table 3). In spite of the consistent induction during the entire time, greater was the magnitude detected on the first few days relative to those detected during the following period. In contrast to GSH, GSSG contents were significantly decreased by butachlor during the first 8 days of treatment in wheat and maize and during the first 4 days in soybean. Thereafter, contents of GSSG reached nearly those of untreated controls.

Different forms of thiol have roles as detoxicants and antioxidants as efficient defense mechanism towards the herbicide itself or its consequences. Increasing thiol and GSH was regarded as limiting factor for herbicide detoxification (Aravind and Prasad, 2005). GSH was reported to be as an essential component of thiol pool and plays several roles in oxidative stress control and protection against xenobiotics and heavy metals (Mendoza-Cózatl and Moreno-Sánchez, 2006; Nemat Alla and Hassan, 2006). May *et al.* (1998) affirmed that GSH is an abundant and ubiquitous thiol with proposed roles in the storage and transport of reduced sulphur, the synthesis of proteins and nucleic acids and as a modulator of enzyme activity. They concluded that the level of GSH has also been shown to correlate with the adaptation of plants to extremes of temperature, in the tolerance of plants to xenobiotics and to biotic and abiotic environmental stresses. In fact, Farago *et al.* (1993) found that

Table 3: Changes in reduced glutathione (GSH) and oxidized glutathione (GSSG) of ten-day old wheat, maize and soybean by the recommended field dose of butachlor during the following 16 days. Data are means (±SD) of at least six replications from two independent experiments[a]

	Wheat		Maize		Soybean	
Days after Treatment	Control	Treated	Control	Treated	Control	Treated
GSH (mg^{-1} shoot fresh weight)						
0	9.8±1.1		14.1±1.3	4.4±0.3		
4	9.3±1.7	32.6±1.8*	14.7±0.7	31.2±1.9*	4.7±0.8	5.5±0.5*
8	8.4±0.8	29.9±2.1*	11.3±0.9	20.9±1.6*	5.0±0.7	4.6±0.7
12	8.4±0.6	23.3±1.7*	11.5±1.3	17.1±1.5*	3.9±0.5	3.6±0.4
16	7.9±0.9	20.4±1.5*	10.3±0.8	15.1±1.7*	3.8±0.4	4.1±0.3
GSSG (μg g^{-1} shoot fresh weight)						
0	36.1±4.8		107.1±9.1		20.4±1.9	
4	44.1±3.6	25.2±3.1*	119.7±8.1	68.6±5.4*	23.1±2.7	27.7±2.2*
8	48.5±3.8	28.4±2.5*	113.4±7.4	81.9±7.2*	23.9±2.1	22.8±1.7
12	45.9±3.2	52.5±4.3	119.1±9.3	130.2±8.1	21.5±1.5	23.1±1.4
16	48.9±3.6	50.4±3.6	119.3±7.5	123.9±9.3	20.5±1.8	21.5±2.4

[a]Values of treated samples with an (*) are significantly different at 5% level with respect to untreated control.

Table 4: Changes in γ-glutamyl cysteine synthetase (γ-GCS) and Glutathione synthetase (GSS) activities of Ten-day-old wheat, maize and soybean by the recommended field dose of butachlor during the following 16 day. Data are means (±SD) of at least six replications from two independent experiments[a]

	Wheat		Maize		Soybean	
Days after Treatment	Control	Treated	Control	Treated	Control	Treated
γ-GCS (μg phosphate liberated g^{-1} shoot fresh weight min^{-1})						
0	13.63±1.17		24.56±1.32		4.63±0.31	
4	13.85±1.23	21.64±0.96*	25.85±0.77	41.47±2.15*	4.11±0.25	4.96±0.36*
8	14.43±1.12	21.75±1.16*	27.33±1.68	39.55±1.88*	3.64±0.31	2.95±0.32
12	15.92±0.89	21.09±0.72*	30.04±1.15	35.85±2.41*	3.16±0.26	2.55±0.11
16	16.15±0.74	18.08±0.84	29.43±1.74	27.35±1.72	2.67±0.21	1.92±0.14
GSS (μg GSH released g^{-1} shoot fresh weight min^{-1})						
0	0.80±0.05		1.48±0.09		0.21±0.02	
4	0.76±0.03	1.10±0.06*	1.52±0.06	2.16±0.11*	0.29±0.03	0.37±0.02*
8	0.78±0.06	1.13±0.08*	1.61±0.07	2.17±0.09*	0.22±0.03	0.28±0.03
12	0.78±0.07	0.92±0.07*	1.42±0.08	2.09±0.11*	0.25±0.03	0.26±0.02
16	0.76±0.04	0.80±0.05	1.55±0.09	1.71±0.08	0.21±0.02	0.25±0.02

[a]Values of treated samples with an (*) are significantly different at 5% level with respect to untreated control

the decreased GSH levels in shoots of maize seedlings enhance the susceptibility of maize to metolachlor. Therefore, it could be predicted that the increased levels of thiols and GSH in wheat and maize could enhance the detoxification of butachlor in both species more than in soybean. In connection, the inductions of thiol and GSH by butachlor were slight in soybean but seemed inversely with wheat and maize growth reductions. These findings conclude, therefore, an important role of thiol and GSH to overcome the effects of butachlor probably through augmenting the detoxification rate. Nevertheless, these levels appeared insufficient in soybean to detoxify butachlor. Soybean, as the other leguminous species, contains GSH as homoglutathione (Hell, 1997). This could explain the lower level of GSH in soybean than in wheat and maize with a consequent deficiency in butachlor detoxification. On the contrary, GSSG was diminished in response to butachlor treatment greater in wheat and maize than in soybean suggesting its conversion into GSH to take a part in the detoxification mechanism. This transformation seemed negligible in soybean with least formation of GSH.

The increase in GSH level is not only due to GSSG reduction but also because of an increase in its biosynthesis rate. GSH biosynthesis takes place in two steps. First, L-cysteine is conjugated to L-glutamate by γ-GCS forming γ-glutamylcysteine (Volohonsky *et al*., 2002). In the second step, glycine is added by GSS (Gupta *et al*., 2005). Butachlor differentially increased the activities of γ-GCS and GSS in wheat, maize and soybean (Table 4). However, the magnitude of increase was

greater in maize than in wheat with very little rises in soybean for only 4 days. Nevertheless, the significant increase in γ-GCS activity by butachlor was being detected up to the 12th day in wheat and maize and only during the first 4 days in soybean. Similar to γ-GCS pattern, the activity of GSS was also stimulated by butachlor up to the 12th day following treatment in wheat and maize or during the first 4 days in soybean. Thereafter the activities of both enzymes reached those of the control.

Therefore, the increased γ-GCS and GSS activities concomitant with GSSG reduction could affirm the elevation of GSH levels, a state that was not obvious in soybean. Such elevation, if any, would facilitate the opportunity to conjugate with butachlor through the catalytic action of GSTs. These findings are related to Jablonkai and Hatzios (1993) who concluded that resistance to herbicides depends on the GSTs-mediated conjugation of GSH. Moreover, some reports concluded that the GSTs are enhanced under certain conditions to increase the plant defense against several biotic and abiotic agents (Jablonkai and Hatzios, 1993; DeRidder *et al*., 2002; Misra *et al*., 2006; Nemat Alla and Hassan, 2006). In Table 5, butachlor induced significant increases in GST activity towards CDNB as a substrate ($GST_{(CDNB)}$) in shoots of treated seedlings. The induction was consistent up to the end of the experiment in wheat and maize and to only 4 days in soybean. However, the magnitude of increase showed maximum values on the 8th day from treatment. Moreover, the activity of GST isoforms towards atrazine, alachlor, metolachlor and butachlor as substrates ($GST_{(atrazine)}$, $GST_{(alachlor)}$, $GST_{(metolachlor)}$ and $GST_{(butachlor)}$, respectively) were significantly increased in wheat and maize by

Table 5: Changes in activity of glutathione-S-transferase towards CDNB substrate (GST(CDNB), atrazine ($GST_{(atrazine)}$), alachlor ($GST_{(alachlor)}$), metolachlor ($GST_{(metolachlor)}$) and butachlor ($GST_{(butachlor)}$) of ten-day old wheat, maize and soybean by the recommended field dose of butachlor during the following 16 days. Data are means (±SD) of at least six replications from two independent experiments[a]

	Wheat		Maize		Soybean	
Days after Treatment	Control	Treated	Control	Treated	Control	Treated
$GST_{(CDNB)}$ (nmol CDNB conjugated g^{-1} fresh weight h^{-1})						
0	15.15±1.16		12.77±1.11		4.15±0.39	
4	11.52±1.12	20.72±1.08*	14.18±1.05	34.02±2.98*	4.06±0.22	4.96±0.47
8	11.14±1.25	26.54±1.34*	12.89±1.27	36.29±3.27*	3.88±0.51	4.21±0.28
12	9.38±0.88	23.73±1.52*	14.53±1.44	29.34±3.11*	3.96±0.41	3.62±0.29
16	10.03±0.74	19.78±1.27*	11.43±1.36	27.43±2.07*	3.74±0.36	3.85±0.39
$GST_{(atrazine)}$ (nmol atrazine conjugated g^{-1} fresh weight h^{-1})						
0	2.17±0.24		4.37±0.42		1.445±0.113	
4	3.02±0.36	5.24±0.44*	3.87±0.27	6.99±0.51*	1.249±0.104	1.337±0.087
8	3.02±0.22	3.71±0.39	4.23±0.29	6.06±0.53*	1.342±0.122	1.356±0.117
12	3.38±0.22	3.82±0.31	4.91±0.44	6.02±0.36	1.243±0.095	1.375±0.107
16	3.24±0.31	3.84±0.21	4.94±0.35	5.45±0.42	1.386±0.088	1.278±0.121
$GST_{(alachlor)}$ (nmol alachlor conjugated g^{-1} fresh weight h^{-1})						
0	3.57±0.22		5.33±0.43		2.341±0.118	
4	4.45±0.28	8.82±0.49*	5.72±0.21	11.38±0.65*	2.154±0.147	2.331±0.311
8	4.81±0.41	9.84±0.61*	6.02±0.48	14.76±0.78*	2.332±0.169	2.345±0.226
12	5.32±0.33	10.17±0.62*	6.55±0.39	13.59±1.04*	2.475±0.088	2.312±0.181
16	6.33±0.47	8.22±0.53	6.57±0.41	12.14±0.95*	2.147±0.178	2.254±0.163
$GST_{(metolachlor)}$ (nmol metolachl or conjugated g^{-1} fresh weight h^{-1})						
0	3.54±0.23		6.34±0.22		2.113±0.177	
4	3.77±0.31	8.46±0.56*	6.85±0.51	12.18±1.02*	2.542±0.195	2.672±0.207
8	4.23±0.42	9.96±0.45*	6.13±0.41	11.72±0.92*	2.671±0.205	2.757±0.147
12	5.32±0.21	11.04±0.89*	6.24±0.42	9.12±0.88*	2.847±0.109	2.446±0.159
16	4.81±0.51	8.26±0.87*	6.62±0.36	9.67±0.57*	2.472±0.115	2.411±0.108
$GST_{(butachlor)}$ (nmol butachlor conjugated g^{-1} fresh weight h^{-1})						
0	3.05±0.21		4.51±0.41		1.381±0.112	
4	2.89±0.32	8.52±0.51*	4.72±0.44	8.53±0.62*	1.444±0.095	1.634±0.125
8	3.33±0.25	8.46±0.44*	4.88±0.32	9.47±0.38*	1.723±0.080	1.652±0.104
12	3.54±0.19	7.63±0.34*	4.35±0.30	8.86±0.47*	1.676±0.117	1.426±0.084
16	3.24±0.21	7.28±0.52*	4.23±0.25	7.64±0.51*	1.615±0.122	1.531±0.122

[a]Values of treated samples with an (*) are significantly different at 5% level with respect to untreated control

Table 6: Changes in activity of glutathione peroxidase (GSPX) and Glutathione Reductase (GSR) of ten-d-old Wheat, maize and soybean by the recommended field dose of butachlor during the following 16 days. Data are means (±SD) of at least six replications from two independent experiments[a]

	Wheat		Maize		Soybean	
Days After Treatment	Control	Treated	Control	Treated	Control	Treated
GSPX (μM NADPH oxidized g^{-1} shoot fresh weight min^{-1})						
0	25.74±1.55		46.44±3.11		6.79±0.55	
4	26.17±2.14	36.73±2.66*	48.73±5.87	75.29±3.69*	8.59±0.42	9.74±0.57*
8	25.33±1.36	38.03±2.05*	48.54±3.24	73.67±5.28*	6.58±0.39	7.14±0.21
12	26.35±1.12	39.83±3.33*	49.67±3.84	72.03±6.02*	5.17±0.51	6.12±0.32
16	26.89±1.91	30.51±3.21	48.33±4.17	51.67±3.11	5.84±0.26	6.03±3.14
GSR (mg TNB formed g^{-1} shoot fresh weight min^{-1})						
0	14.75±1.03		26.59±3.27		3.69±0.38	
4	13.81±0.95	19.71±1.22*	26.38±2.58	37.42±3.26*	3.84±0.25	4.51±0.21*
8	14.52±0.87	20.78±1.58*	24.31±1.98	39.85±2.58*	3.91±0.47	4.15±0.39
12	14.62±1.27	18.53±2.14*	27.37±2.07	32.47±2.07	4.52±0.12	4.27±0.31
16	14.99±1.54	15.71±1.07	27.72±1.82	29.61±2.49	4.11±0.29	3.74±0.27

[a]Values of treated samples with an (*) are significantly different at 5% level with respect to untreated control

butachlor but seemed unchanged in soybean. The activity of $GST_{(atrazine)}$ appeared the least inducible by butachlor; the induction was restricted only to the first 4 or 8 days in wheat or maize shoots, respectively. $GST_{(alachlor)}$ activity was consistently enhanced by butachlor in wheat up to the 12th day and in maize throughout the entire experimental period. For metolachlor as a substrate, butachlor showed also a great induction in $GST_{(metolachlor)}$ activity consistently up to the end of the experiment in both species. Additionally, butachlor highly induced the activity of $GST_{(butachlor)}$ in wheat and in maize throughout the entire experiment too.

The enhancement of GSTs against several agents could conclude that the enzyme activities might contribute to the physiological selectivity of plants to tolerate herbicides. The present results revealed great increases in GST activities by butachlor in wheat and maize but little in soybean. So, more increases in GSH levels in wheat and maize concomitant with more activities of GSTs in response to butachlor could suggest fast and easy conjugation and subsequently detoxification of the herbicide. Nevertheless, these increases seemed not enough in soybean to achieve detoxification of butachlor. Thus, the recovery of wheat and maize from butachlor appeared to relate with the enhanced GSH, thiol and activities of GST, γ-GCS and GSS. On the contrary, soybean susceptibility to butachlor might result from inefficient detoxification system. These observation are supported with Stephensen *et al.* (2002) who reported that GSH and GSH-related enzymes are important components in the defenses against stress in aerobic organisms. On the other hand, some isoforms of GST showed dual activity and can also function as GSPX (Volohonsky *et al.*, 2002; Vontas *et al.*, 2002). However, Ye *et al.* (2000) reported that two distinctly different types of GSPX activity exist in plants; those types having only GSPX activity, and those having dual GST/ GSPX activities. In addition, Aravind and Prasad (2005) concluded that GSPX is a part of the arsenal of the protective enzymes which respond to stress.

Treatment with butachlor resulted in significant increases in the activities of GSPX and GSR in wheat and maize shoots (Table 6). Maize usually contained higher enzyme levels than wheat. The significant increase of the enzyme activities were detected up to the 12th day in wheat and maize and for only 4 days in soybean. Similar significant enhancements were also observed in GSR by butachlor during the first 12 days in wheat, the 8th day in maize and the 4th day in soybean. Therefore, the enhanced GST and GSPX activities, in the present study, accompanied with an increase in GSH could accelerate the capability of wheat and maize to detoxify butachlor. Supporting this conclusion, Anderson and Davis (2004) stated that GST, GSPX and GSR are enzymes that utilize GSH to play an important role in plant defense mechanism. In this respect, Gehin *et al.* (2006) found that the glyphosate-caused depletion of GSH was accompanied with GSPX disorders. GSR is responsible for

maintaining a high reduction state of the glutathione pool. GSR plays an essential role in a cell's defense against ROS and xenobiotics by sustaining the reduced status of GSH (Yoon *et al*., 2005). In support to the present study, Zabalza *et al*. (2006) concluded that the enhancement of the GSH content detected in imazethapyr-treated pea roots can be related to the increase of GSR activity. Nevertheless, Aravind and Pasad (2005) reported that the maintenance of GSH pool is not only because of GSR but also γ-GCS and GSS have a great role. Consequently, GSH could be much more produced in wheat and maize than in soybean by GSR together with γ-GCS and GSS. With the presence of active GSTs and GSPX, GSH could proceed efficient conjugation with butachlor causing its detoxification. Nevertheless, a shortage of butachlor detoxification in soybean might result from less induction of GSH and GSH-associated enzymes.

CONCLUSIONS

In conclusion, the present results indicated differential state of stress induced by butachlor, great in soybean allover the experimental time but slight in wheat and maize seedlings only during the first few days recovered thereafter. These findings conclude that soybean is susceptible to butachlor while wheat and maize exert some tolerance. This tolerance appeared to relate with the induction of detoxicants and detoxicative enzymes. Indeed, there were rises in levels of thiol and GSH as well as in activities of GSH-associated enzymes (γ-GCS, GSS, GSTs, GSPX and GSR). These elevations seemed negligible in soybean. The activity rises of these enzymes might be related to molecular basis. Further studies are required to evaluate the molecular basis and gene level of these enzymes in response to butachlor tolerance. Anyway, GSH was increased on the expense of GSSG reduction in addition to an active GSR, γ-GCS and GSS. With the presence of a sufficient concentration of GSH in wheat and maize, both GSTs and GSPX would be activated to catalyze butachlor-GSH conjugation with a consequent detoxification of the herbicide. In contrast, insufficient GSH level and less effective enzyme activities could not assist soybean to detoxify butachlor with a subsequent appearance of phytotoxicity. These findings conclude that soybean could be considered to be susceptible to butachlor but wheat and maize as tolerant species. The differential tolerance of plant species seemed to be in relationship with the varied inductions of thiol, GSH and GSH-associated enzymes.

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