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ORIGINAL PAPER

Herbicide tolerance in maize is related to increased levels of glutathione and glutathione-associated enzymes

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Abstract Treatment of 10 days old maize seedlings with metribuzin and pretilachlor near the recommended field-dose resulted in differential reductions in shoot fresh and dry weights during the following 16 days. Metribuzin showed great and consistent reductions, however, the reduction induced by pretilachlor, mostly nullified by the end of the experiment. Moreover, there were differential accumulations of lipid peroxides, carbonyl groups and H_2O_2 in maize leaves; metribuzin caused the greatest accumulation. Meanwhile, levels of thiol forms and reduced glutathione (GSH) were much more induced by pretilachlor than metribuzin; the contrary was true regarding oxidized glutathione (GSSG). The ratio of GSH/GSSG was highest following pretilachlor treatment and least by metribuzin. On the other hand, activities of glutathione-*S*-transferases (GSTs, EC 2.5.1.18), γ-glutamyl-cysteine synthetase (γ-GCS, EC 6.3.2.2), glutathione synthetase (GS, EC 6.3.2.3), glutathione peroxidase (GPX, EC 1.15.1.1) and glutathione reductase (GR, EC 1.6.4.2) were more enhanced in maize leaves by pretilachlor than metribuzin. These findings suggest the occurrence of an oxidative stress differentially induced in maize by the herbicides, a state that was most pronounced with metribuzin. Pretilachlor was concluded to be the least phytotoxic to maize, while metribuzin was the most, this differential tolerance seemed to be related to the induction of GSH and GSH-associated enzymes.

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Abbreviations

CDNB	1-Chloro-2,4-dinitrobenzene
DTNB	5,5-Dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothritol
γ-GCS	γ-Glutamylcysteine synthetase
GSH	Reduced glutathione
GPX	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSSG	Oxidized glutathione
GST	Glutathione-*S*-transferase
$GST_{(alachlor)}$	GST towards alachlor
$GST_{(atrazine)}$	GST towards atrazine
$GST_{(CDNB)}$	GST towards CDNB
$GST_{(metolachlor)}$	GST towards metolachlor
LSD	Least significant differences
MDA	Malonaldehyde
PVPP	Polyvinylpolypyrrolidone
ROS	Reactive oxygen species
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

Introduction

Metribuzin (4-amino-6-tert-butyl-4,5-dihydro-3-methyl-thio-1,2,4-triazin-5-1) is a photosynthetic inhibitor, blocks the energy transport and produces the oxidative stress agents (reactive oxygen species, ROS) through its interference with PSII (Kuzniak 2002; Hassan and Nemat Alla 2005; Nemat Alla and Hassan 2006, 2007). ROS react with

lipids, proteins, pigments and nucleic acids, and cause lipid peroxidation, membrane damage and inactivation of enzymes, thus affecting cell viability. However, pretilachlor [2-chloro-2,6-diethyl-*N*-(2-propoxyethyl) acetanilide] affects protein synthesis, cell division, mineral uptake and cell permeability. It disturbs gibberellic acid and carotenoids formation, which act as a buffer protecting chlorophyll from photodestruction, leading therefore to ROS generation (Kearney and Kaufman 1988; Nemat Alla et al. 2008). However, some plant species can tolerate herbicides through an efficient defense mechanism to detoxify herbicides and to scavenge ROS through a number of metabolites and enzymes (Edwards 1996; Kuzniak 2002; Anderson and Davis 2004; Misra et al. 2006; Nemat Alla and Hassan 2006). Thiol and GSH are important metabolites, which are regarded as detoxicants and antioxidants (Aravind and Prasad 2005). GSH participates in ROS scavenging through ascorbate–GSH cycle (Nayyar and Gupta 2006). GSH is synthesized from the combined action of γ-GCS and GS (Volohonsky et al. 2002; Gupta et al. 2005) and maintained in reduced state by GR. The enzymatic conjugation of herbicide with GSH is mediated by GSTs (Jablonkai and Hatzios 1993). GSTs are enhanced under certain conditions to increase the plant defense against several biotic and abiotic agents (Misra et al. 2006). Some isoforms of GST show dual activity and can also function as GSPX (Ye et al. 2000; Volohonsky et al. 2002). These enzymes, in addition to GSH might regulate the plant tolerance to herbicides. Therefore, the present work was aimed to relate the differential tolerance of maize to metribuzin and pretilachlor; both are used to control weeds in maize fields, with responses of oxidative stress indices, and the internal levels of thiol and GSH as well as activities of GSH-associated enzymes.

Materials and methods

Plant materials and growth conditions

Grains of maize (*Zea mays* V.S.C.129) were surface sterilized by immersing in 3% sodium hypochlorite solution for 10 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} s^{-1} photosynthetic photon flux density, 75–80% relative humidity and 28/14°C day/night regime. When seedlings were 10 days old, irrigation water was substituted with one-fourth strength Hoagland solution. At this stage, the pots were divided into three groups; one was left to serve as control and one for each herbicide treatment at the recommended field dose used in maize fields (1.0 kg ha^{-1} and 1.6 L ha^{-1} for metribuzin and pretilachlor, respectively). 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 were collected just before herbicide application (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.

Determination of oxidative stress indices (lipid peroxidation, carbonyl groups and H_2O_2)

Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid- (TBA-) reacting substances as described by Buege and Aust (1972). Plant leaves were homogenized in 150 mM KCl and centrifuged at 7,000*g* for 15 min. One milliliter of the supernatant was incubated at 37°C for 2 h, after which, 1 ml of 0.6 M trichloroacetic acid (TCA) was added. After thorough mixing, the reaction mixture was centrifuged at 5,000*g* for 10 min. One milliliter of supernatant was taken with an equal volume of TBA and placed in a boiling water bath for 10 min, cooled and diluted with 1 ml distilled water. Spectrophotometric readings at 535 nm were calculated as nanomoles malonaldehyde (MDA).

Carbonyl groups were assayed using the dinitrophenyl hydrazine method (Levine et al. 1991). Proteins were extracted from leaf tissue with 100 mM potassium phosphate (pH 7.0), 0.1% Triton X-100 and 1 mM Na_2EDTA. After precipitation of the possible contaminating nucleic acids in the sample with 1% (w/v) streptomycin sulfate, an aliquot of the extract was reacted with 20 mM dinitrophenyl hydrazine (in 2 M HCl) for 1 h, with vigorous shaking. Proteins were then precipitated with 0.6 M TCA, washed with 1:1 (v/v) ethanol:ethyl acetate, solubilized in 6 M guanidine–HCl (pH 4.5) and the absorbance of the hydrazones (derivatized carbonyls) was measured at 370 nm.

H_2O_2 was extracted in 200 mM perchloric acid, centrifuged at 5,000*g* for 10 min and the supernatant was neutralized with 4 M KOH. After centrifugation at 3,000*g* for 5 min, 0.2 ml of the supernatant was loaded on 1 ml column of Dowex 1X8-100 anion exchange resin and eluted with 0.8 ml of distilled water. According to Okuda et al. (1991); the assay mixture contained 1 ml extract; 0.4 ml, 12.5 mM 3-dimethylaminobenzoic acid in 375 mM phosphate buffer (pH 6.5); 0.08 ml, 1.3 mM 3-methyl-2-benzothiazolinone hydrazone and 0.02 ml (0.25 units), horseradish peroxidase. The reaction was initiated by the addition of peroxidase and the increase in absorbance at 590 nm was monitored for 3 min.

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

Plant leaves were homogenized in 20 mM EDTA. The clear supernatants were separated by centrifugation at 12,000*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 $E = 13{,}100\ \text{mM}^{-1}\ \text{cm}^{-1}$. To determine non-protein thiols, the supernatant was mixed with TCA (50% w/v) and centrifuged at 10,000*g* 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 leaves were homogenized at 4°C in TCA (5%, w/v) and 10 mM EDTA (Anderson and Gronwald 1991). The extracts were centrifuged at 12,000*g* for 15 min. GSH was assayed in 100 mM phosphate buffer, pH 6.8, containing 10 mM EDTA, 1 mM 1-chloro-2,4-dinitrobenzene (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 non-enzymatic 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 GR. The absorbance at 412 nm was immediately measured and again after 30 min of incubation at 35°C.

Extraction and assay of GSH-associated enzymes

Both γ-glutamyl-cysteine synthetase (γ-GCS, EC 6.3.2.2) and glutathione synthetase (GS, EC 6.3.2.3) were extracted from leaves 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*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. γ-GCS activity was expressed as μmol of phosphate liberated g^{-1} fresh weight min^{-1}. The assay of GS 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. GS activity was expressed as nmol of GSH liberated g^{-1} fresh weight min^{-1}.

For extraction of glutathione-*S*-transferases (GSTs, EC 2.5.1.18), plant leaves 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 CDNB [$GST_{(CDNB)}$] in 100 mM phosphate, pH 6.5, containing 5 mM GSH and 1 mM CDNB. After an incubation period for an hour 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 $E = 9.6\ \text{mM}^{-1}\ \text{cm}^{-1}$ (Askelof et al. 1975) and expressed as nmol CDNB conjugated g^{-1} fresh weight h^{-1}.

Moreover, a series of HPLC assays were performed to measure the activities towards the substrates alachlor, metolachlor and atrazine herbicides [$GST_{(ala)}$, $GST_{(met)}$ and $GST_{(atr)}$, 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} and 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. The enzyme activity was expressed as nmol herbicide conjugated g^{-1} fresh weight h^{-1}.

Glutathione peroxidase (GPX, EC 1.15.1.1) was extracted from leaves in 100 mM Tris–HCl (pH 7.5), 1 mM EDTA and 2 mM DTT. The extracts were centrifuged at 15,000*g* for 20 min (Edwards 1996). The reaction mixture constituted of 100 mM phosphate (pH 7.0), 2% (w/v) Triton X-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 $E = 6.2\ \text{mM}^{-1}\ \text{cm}^{-1}$ (Nagalakshmi and Prasad 2001). GPX activity was expressed as μmol NADPH oxidized g^{-1} fresh weight min^{-1}.

Glutathione reductase (GR, EC 1.6.4.2) was extracted in 100 mM phosphate (pH 7.5) and 0.5 mM EDTA. The extracts were centrifuged at 15,000*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 was being measured up to 5 min. GR activity was expressed as μmol TNB formed g^{-1} fresh weight min^{-1}.

Statistical analysis

All values are means (±SD) of at least six determinations from two independent experiments. The full data were first subjected to analysis of variance (ANOVA) followed thereafter by least significant differences (LSD) at 5% level (Snedecor and Cochran 1980).

Results

Fresh and dry weights

Application of metribuzin or pretilachlor near the recommended field dose to 10 days old maize seedlings resulted in differential significant decreases in shoot fresh and dry weights below the control values during the following 16 days (Fig. 1). The significant reduction of fresh and dry weights by metribuzin was consistent and continued up to the end of the experimental period. However, fresh weight reduction by pretilachlor seemed to be leveled off after 8 days of treatment, while dry weight reduction seemed to be restricted only to the first 4 days from treatment.

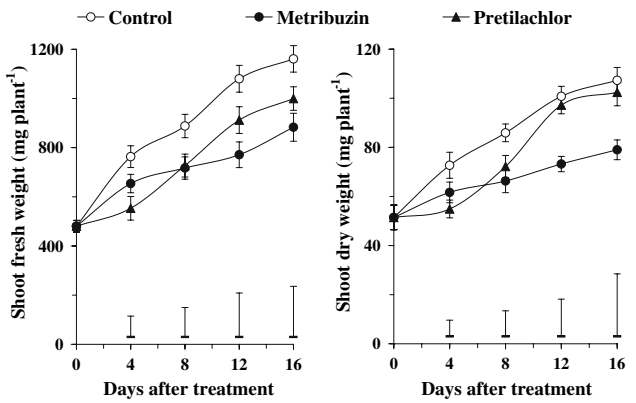

Fig. 1 Changes in shoot fresh and dry weights of 10 days old maize seedlings during the following 16 days from treatment with metribuzin and pretilachlor near the recommended field dose. Data are means (±SD) of at least six replications from two independent experiments. *Vertical bars* represent LSD at 5% level

Oxidative stress indices

Metribuzin application led to great accumulations of lipid peroxides, carbonyl groups and H_2O_2 in maize leaves throughout the whole experiment (Fig. 2). However, pretilachlor caused accumulations of carbonyl groups and H_2O_2 during the first 12 days, whereas lipid peroxides accumulation was detected up to the eighth day.

Thiol forms and GSH contents

Figure 3 shows high increases in protein-bound, non-protein and total thiol contents by herbicides in maize leaves. The magnitude of increase seemed to be higher by pretilachlor than metribuzin. Protein-bound thiol was induced by metribuzin only during the first 4 days of treatment, while pretilachlor induced significant increases during the first 12 days. Non-protein thiol was significantly increased by metribuzin only during the first 4 days, whereas pretilachlor resulted in significant increases up to the 12th day. Total thiol was significantly increased by metribuzin only during the first 4 days, however, the induction was extended by pretilachlor to the 12th day.

In Fig. 4, GSH in maize leaves was greatly induced by herbicides. The magnitude of induction was least with metribuzin and highest with pretilachlor. The significant induction by pretilachlor was continued during the whole experiment but the induction by metribuzin restricted only to the first 8 days. In contrast, metribuzin significantly increased GSSG contents; however, pretilachlor resulted in significant decreases. GSSG was significantly increased by metribuzin up to the end of the experiment and by pretilachlor during the first 8 days of treatment. The figure clearly indicated that the GSH/GSSG ratio was highly elevated following treatment with pretilachlor; however, these ratios seemed unchanged in metribuzin-treated seedlings.

GSH-associated enzymes

The activities of γ-GCS and GS in maize leaves were significantly increased by herbicide treatments (Fig. 5). The magnitude of increase was greatest with pretilachlor and least with metribuzin. γ-GCS activity was significantly induced by pretilachlor up to the 12th day and by metribuzin only during the first 4 days. The GS activity was also increased by pretilachlor up to the 12th day following treatment, whereas metribuzin induced significant enhancement up to the eighth day.

Figure 6 shows that herbicides differentially increased activity of GST towards the substrates CDNB

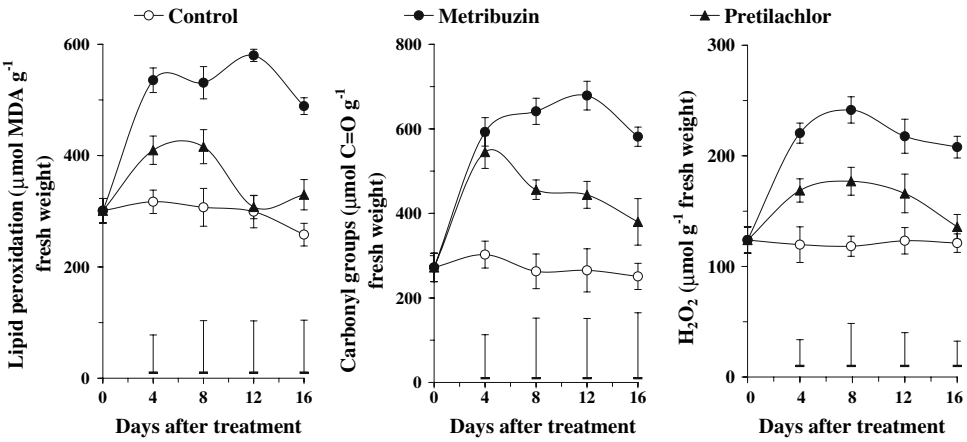

Fig. 2 Changes in lipid peroxides, carbonyl groups and H_2O_2 accumulation in leaves of 10 days old maize seedlings during the following 16 days from treatment with metribuzin and pretilachlor near the recommended field dose. Data are means (±SD) of at least six replications from two independent experiments. *Vertical bars* represent LSD at 5% level

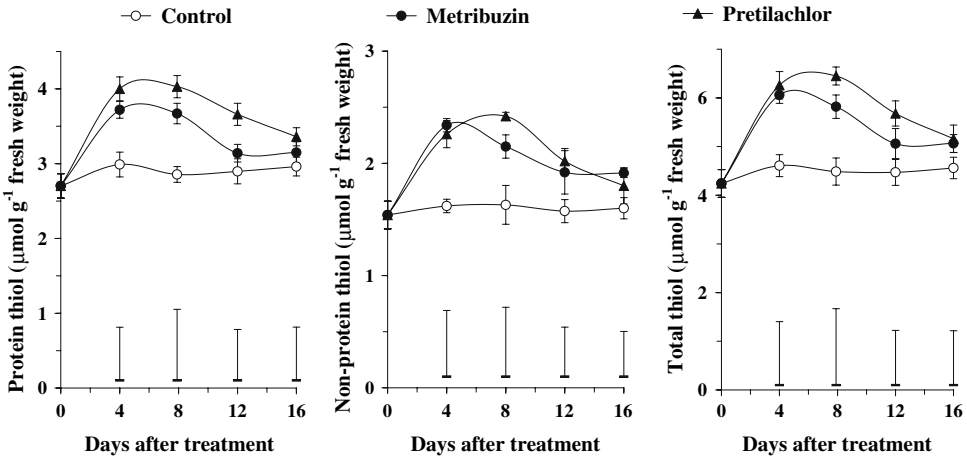

Fig. 3 Changes in protein-bound, non-protein and total thiol in leaves of 10 days old maize seedlings during the following 16 days from treatment with metribuzin and pretilachlor near the recommended field dose. Data are means (±SD) of at least six replications from two independent experiments. *Vertical bars* represent LSD at 5% level

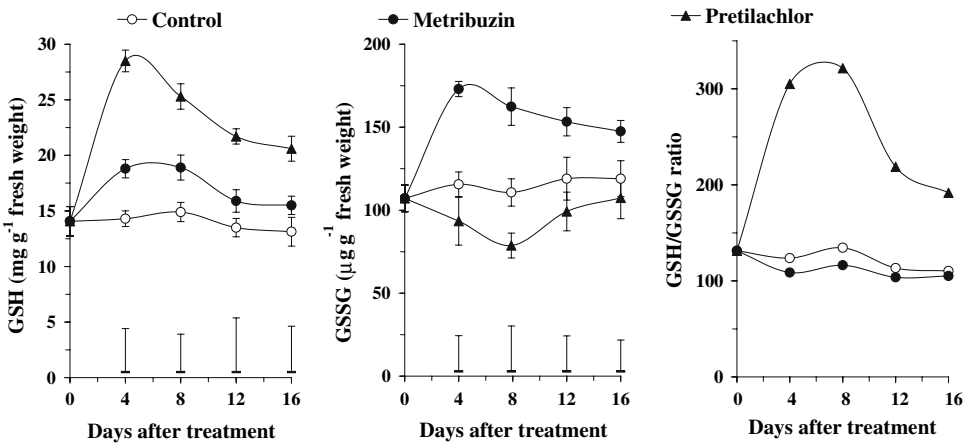

Fig. 4 Changes in reduced glutathione (GSH), oxidized glutathione (GSSG) and their ratios in leaves of 10 days old maize seedlings during the following 16 days from treatment with metribuzin and pretilachlor near the recommended field dose. Data are means (±SD) of at least six replications from two independent experiments. *Vertical bars* represent LSD at 5% level

[$GST_{(CDNB)}$], alachlor [$GST_{(ala)}$], metolachlor [$GST_{(met)}$] and atrazine [$GST_{(atr)}$]. $GST_{(CDNB)}$ was increased by all herbicides up to the eighth day. Thereafter, the increase by pretilachlor extended up to the end of the experiment and by metribuzin to the 12th day. A similar consistent increase in $GST_{(ala)}$ was induced by pretilachlor throughout the experimental period, however, metribuzin induced significant increases up to 12 days. Again, pretilachlor consistently induced $GST_{(met)}$ up to the end of the experiment. Also metribuzin resulted in significant increases during the first 8 days. On the other hand, $GST_{(atr)}$ was mostly induced by metribuzin throughout the entire experimental period, nevertheless, slight increases were induced by pretilachlor only during the first 8 days.

Pretilachlor induced significant increases in the GPX activity up to the 12th day of treatment, whereas metribuzin resulted in significant inhibition up to the eighth day (Fig. 7). Similarly, GR activity was significantly stimulated

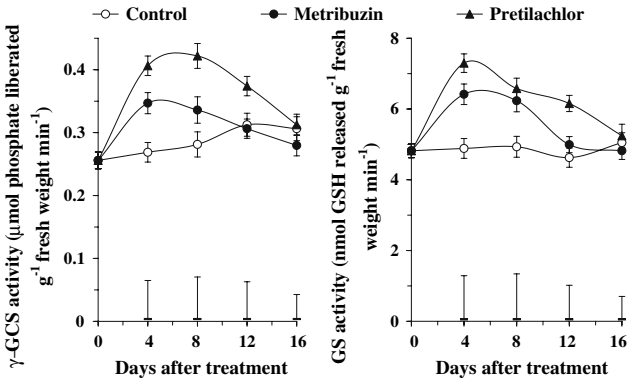

Fig. 5 Changes in γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS) activities in leaves of 10 days old maize seedlings during the following 16 days from treatment with metribuzin and pretilachlor near the recommended field dose. Data are means (±SD) of at least six replications from two independent experiments. *Vertical bars* represent LSD at 5% level

by pretilachlor during the first 8 days. In contrast, metribuzin significantly inhibited GR activity during the first 4 days.

Discussion

The results, generally, showed a great reduction in fresh and dry weights of maize shoots by metribuzin and pretilachlor, the reduction was most pronounced with metribuzin. Growth reduction of several plant species was reported following the application of triazine and triazinone herbicides (Dvorak and Remesova 2002; Nemat Alla et al. 2008) and also of α-chloroacetanilides (Hassan and Nemat Alla 2005; Nemat Alla et al. 2008). 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; Nemat Alla et al. 2008). Therefore, the greatest reduction of growth by metribuzin revealed relative susceptibility of maize to this herbicide. Such susceptibility seemed to be in relation to oxidative stress indices.

Levels of TBA-reacting substances, carbonyl groups and H_2O_2 increased following herbicide treatments, particularly metribuzin. These increases could give an index of oxidative stress. MDA, a decomposition product of polyunsaturated fatty acids hydroperoxides, has been utilized very often as a suitable biomarker for lipid peroxidation, which is an effect of oxidative damage (Dewir et al. 2006). Increases in TBA-reacting substances were observed in many plant species due to several factors (Sandalio et al. 2001). Fluometuron and atrazine significantly increased contents of lipid peroxides, carbonyl groups and H_2O_2 in broad bean and maize (Hassan and Nemat Alla 2005). Similar increases in H_2O_2 were induced

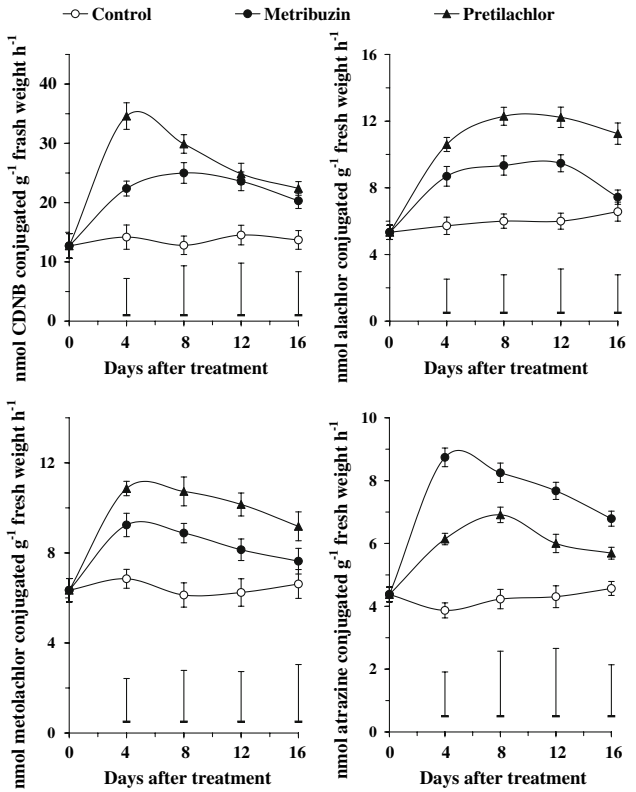

Fig. 6 Changes in activities of glutathione-*S*-transferase (GST) towards CDNB [$GST_{(CDNB)}$], alachlor [$GST_{(ala)}$], metolachlor [$GST_{(met)}$] and atrazine [$GST_{(atr)}$] in leaves of 10 days old maize seedlings during the following 16 days from treatment with metribuzin and pretilachlor near the recommended field dose. Data are means (±SD) of at least six replications from two independent experiments. *Vertical bars* represent LSD at 5% level

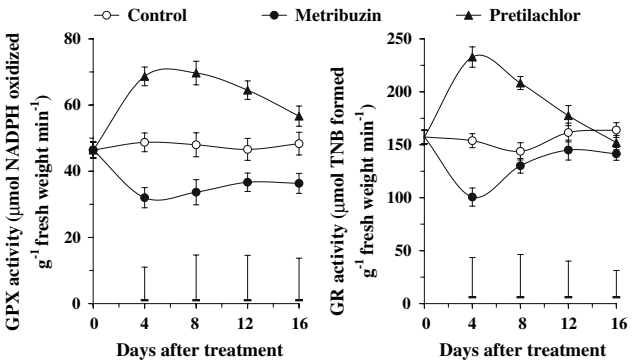

Fig. 7 Changes in activity of glutathione peroxidase (GPX) and glutathione reductase (GR) in leaves of 10 days old maize seedlings during the following 16 days from treatment with metribuzin and pretilachlor near the recommended field dose. Data are means (±SD) of at least six replications from two independent experiments. *Vertical bars* represent LSD at 5% level

in maize by isoproturon (Nemat Alla and Hassan 2007) and by butolachlor (Nemat Alla et al. 2007).

The accumulation of H_2O_2 could result from an increase in its formation through the dismutation of O_2^- by

superoxide dismutase and/or a decrease in its degradation by catalase and peroxidases (Polidoros and Scandalios 1999; Mittler 2002; Hassan and Nemat Alla 2005; Nemat Alla and Hassan 2006, 2007). In this context, Iannelli et al. (1999) reported that overproduction of superoxide dismutase in plant chloroplasts led to protection against herbicides. Moreover, Nemat Alla and Hassan (2007) indicated that isoproturon increased H_2O_2 and superoxide dismutase activity in maize but diminished catalase and peroxidases indicating that cells under these conditions are not fully competent to remove H_2O_2. In confirmation, Pyon et al. (2004) reported that the activities of superoxide dismutase, catalase and peroxidases were higher in paraquat-resistant *Erigeron canadensis* than in susceptible biotype. On the other hand, Reinheckel et al. (1998) reported that the functionality of proteins can be affected by ROS, either by oxidation of amino acid side chains or secondary reactions with aldehydic products of lipid peroxidation. Both primary and secondary reactions can introduce carbonyl groups into proteins. The great accumulation of H_2O_2 leads to a variety of cellular responses that are dose dependent (Nemat Alla and Hassan 2007). They confirmed that a high dosage of H_2O_2 results in hypersensitive cell death, whereas a low-dosage blocks cell cycle progression and functions. The accumulation of H_2O_2 might result from deterioration of the ascorbate–(AsA–)GSH cycle in which H_2O_2 is reduced to water (Aravind and Passad 2005) catalyzed by GSH-associated enzymes operating for the maintenance of GSH levels (Nagalakshmi and Prasad 2001). GSH acts to detoxify ROS through active enzymatic pathways.

Thiol-pool functions as a stress indicator and plays several roles in oxidative stress control and protection against xenobiotics and heavy metals (Mendoza-Cózatl and Moreno-Sánchez 2006). GSH is regarded as a key component of antioxidant defenses in most aerobic organisms and moreover, to be limiting for tolerance to herbicides. Farago et al. (1993) found that the decreased GSH levels in maize shoots enhance the susceptibility to metolachlor. 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. The antioxidant function of GSH is mediated by the sulfhydryl group of cysteine that upon oxidation forms GSSG. The extended drop in GSH by metribuzin might indicate a deficiency in the detoxification of the herbicide and also of ROS. On the other hand, the conjugation of GSH with pretilachlor might be considered as one of their major mechanisms of detoxification accompanied with high detoxification of ROS. In this context, Nemat Alla et al. (2007) concluded that plant tolerance to butachlor was related to GSH induction. The present findings conclude, therefore, an augmented detoxification rate of pretilachlor. In confirmation, Nemat Alla and Hassan (2007) concluded that the isoproturon-induced oxidative stress was accompanied with depletion of GSH. On the contrary, GSSG was diminished in response to pretilachlor treatment suggesting its conversion into GSH. In contrast, the slight increase in GSH by metribuzin accompanied with rises in GSSG suggests a failure of GSSG reduction into GSH. This interrelation and the utilization of GSH for detoxification of both herbicide and ROS are controlled by GSH-associated enzymes.

The increase in GSH level is not only due to GSSG reduction but also because of an increase in its biosynthesis. 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 GS (Gupta et al. 2005). Therefore, the increased γ-GCS and GSS activities by pretilachlor concomitant with GSSG reduction could explain the elevation of GSH levels. On the other hand, resistance to herbicides depends on GSH conjugation through the catalytic action of GSTs. 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; Misra et al. 2006; Nemat Alla and Hassan 2006). This conclusion could explain the increased tolerance of maize to pretilachlor. Nemat Alla et al. (2007) confirmed that the differential tolerance of wheat, maize and soybean to butachlor was related to the differential induction of GSH and GSH-associated enzymes. The antioxidant metabolism has been shown to be important in determining the ability of plants to survive in hyperhydric stress and the up regulation of these enzymes would help to reduce the build up of ROS (Dewir et al. 2006). Thus, the recovery of maize from pretilachlor appeared to be in relation with the increased GSH/GSSG ratio and activities of GST, γ-GCS and GS.

On the other hand, some isoforms of GST showed dual activity and can also function as GPX (Volohonsky et al. 2002). However, Ye et al. (2000) reported that two distinctly different types of GPX activity exist in plants; those types having only GPX activity, and those having dual GST/GPX activities. In addition, Aravind and Prasad (2005) concluded that GPX is a part of the arsenal of the protective enzymes, which respond to stress. Therefore, the enhanced GST and GPX activities by pretilachlor, in the present study, accompanied with an increase in GSH could accelerate the capability of maize to detoxify this herbicide. In support, Anderson and Davis (2004) stated that GST, GSPX and GSR are enzymes that utilize GSH to play

an important role in plant defense mechanism. Gehin et al. (2006) found that the glyphosate-caused depletion of GSH was accompanied with GSPX disorders. In addition, Pyon et al. (2004) indicated that one of the paraquat-resistant mechanisms in *Erigeron canadensis* might be related to detoxicative enzymes and GSH content. Moreover, Nemat Alla and Hassan (2007) affirmed that high doses of isoproturon induced oxidative stress and caused inhibitions in activities of γ-GCS, GS, GPX, GST and GR. Aravind and Pasad (2005) reported that the maintenance of GSH pool is not only because of GR but also γ-GCS and GS have a great role. GR sustains the reduced status of GSH. In support to the present study, Zabalza et al. (2007) concluded that the enhancement of the GSH content detected in imazethapyr-treated pea roots can be related to the increase of GSR activity. Consequently, the production of GSH in pretilachlor-treated maize by GR together with γ-GCS and GS could facilitate GSTs and GPX to precede efficient conjugation and subsequently detoxification of this herbicide.

In conclusion, the present results indicated a differential state of stress induced by metribuzin or pretilachlor in maize seedlings. Growth, oxidative stress indices, thiol, GSH and GSH-associated enzymes were differentially responded to the herbicides. Maize seedlings tolerated the effects of pretilachlor rather than metribuzin. Actually, pretilachlor was the least depressive to growth, the least inductive to oxidative stress indices, and the most inductive of AsA, thiol, GSH and GSH-associated enzymes (γ-GCS, GS, GST, GPX and GR), however, the contrary was obvious for metribuzin. GSH is increased on the expense of GSSG reduction by GR in addition to a synthesis by γ-GCS and GS. Sufficient concentration of GSH could facilitate both GSTs and GPX to achieve the conjugation and consequently detoxification of pretilachlor. Manipulation of endogenous expression of GSH-associated enzymes through genetic means might elevate the defense ability of these plant species, thus, further studies are required to evaluate the gene level of these enzymes in response to herbicide tolerance. The present findings suggest the occurrence of a differentially induced-oxidative stress in maize by herbicides, a state that was most pronounced with metribuzin. Pretilachlor was concluded to be the least phytotoxic to maize, while metribuzin was the most. This differential tolerance seemed to be related to the induction of GSH and GSH-associated enzymes.

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