Changes in antioxidants and kinetics of glutathione-S-transferase of maize in response to isoproturon treatment

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Abstract
Isoproturon at the recommended field dose (RFD) significantly reduced fresh and dry weights of shoots and roots as well as chlorophylls and carotenoids contents of 10-d-old maize seedlings during the following 20 d. The high the herbicide dose, the greater the reduction was. Meanwhile, ascorbate (AsA) and reduced glutathione (GSH) were increased in leaves for only the first few d. Similar increases in activities of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) were detected. Low doses caused general increases while high doses induced diminutions, however, CAT and APX activities were inhibited by all doses. Nevertheless, H2O2 was significantly accumulated throughout the experiment; the magnitude of accumulation increased with time and herbicide dose. On the contrary, there were significant inhibitions in activities of the glutathione S-transferase (GST) isoforms (GST(EDNB), GST(ALA), or GST(MET)) with no variation in GST(ATR), the inhibition was greater with increasing isoproturon doses. These findings suggest the occurrence of an oxidative stress in maize by isoproturon, a state that prolonged with increasing the herbicide dose and/or the treatment time. Moreover, Vmax of GST was lowered by isoproturon, however, Km unchanged indicating that the herbicide is a competitive inhibitor to GST.

Key words: Antioxidants, Isoproturon, Kinetics of GST, Maize, CAT, APX

Introduction
Isoproturon [arelon, 3-(4-isopropylphenyl)-1,1-dimethylurea] is a photosynthetic herbicide blocks the flow of electrons through PSII, and thus blocks the transfer of excitation energy from chlorophyll molecules to the PSI reaction center (Kleczkowski 1994). Excited chlorophyll molecules (singlet chlorophyll) spontaneously form triplet chlorophyll which reacts with O2 to form singlet oxygen (O2). Reactive oxygen species (ROS) typically result from the excitation of O2 to form 1O2 or from the transfer of one, two or three electrons to O2 to form, respectively, a superoxide radical (O2--), hydrogen peroxide (H2O2) or a hydroxyl radical (HO-). ROS are capable of unrestricted oxidation of various cellular components and can lead to the oxidative destruction of the cell (Mittler 2002, Hassan and Nemat Alla 2005). However, plants use ROS as second messengers in signal transduction cascades regulating diverse processes. ROS accumulation is crucial to plant development as well as defense (Foyer and Noctor 2005a, Pavet et al. 2005). To protect cellular membranes against the harmful ROS levels, plants developed defense antioxidants (Foyer et al. 2001, Aravind and Prasad 2005). Antioxidants are crucial for plant defense against oxidative stress. They act to detoxify ROS and induce enzymes active in ROS removal (Foyer et al. 2001, He and Hader 2002, Gomez et al. 2004). Major ROS-scavenging mechanisms of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione S-transferases (GSTs; EC 2.5.1.18) (Polidoros and Scandalios 1999, Mittler 2002, Hassan and Nemat Alla 2005). Therefore, this work was aimed to study the responses of some non-enzymatic and enzymatic antioxidants in maize to treatments with isoproturon. More attention was paid to the activity and kinetics of GST.
Materials and methods

Plant materials and growth conditions

Maize grains (Zea mays L., hybrid 351) were germinated in sand/clay soil (1/1, v/v) in pots (25 cm diameter x 20 cm height). Pot, after being left in dark for 3 d, were transferred to a 14-h photoperiod with 450-500 μmol m-2 s-1 PPFD, 75-80% RH, and 28/14°C d/night regime. When seedlings were ten d old, pots were divided into two groups; one to serve as a control and the other for isoproturon treatment at the recommended field dose (RFD, 2.5 Kg ha-1), the herbicide was applied only once as foliar sprays. The rate per hectare was calculated according to the surface area per pot and the herbicide was mixed with a suitable amount of water. At harvest, shoots were collected just before herbicide application (used for zero time) and also after 2, 4, 8, 12, 16 and 20 d from treatment, washed with copious amounts of water and blotted dry with paper towels. Another trial was performed to check the dose effect of isoproturon. Isoproturon at 0.5, 1.0, 1.5 and 2.0 fold RFD was applied to a 14-h photoperiod with 450-500 μmol m-2 s-1 PPFD, 75-80% RH, and 28/14°C d/night regime. When seedlings were ten d old, pots were divided into two groups; one to serve as a control and the other for isoproturon treatment at the recommended field dose (RFD, 2.5 Kg ha-1), the herbicide was applied only once as foliar sprays. The rate per hectare was calculated according to the surface area per pot and the herbicide was mixed with a suitable amount of water. At harvest, shoots were collected just before herbicide application (used for zero time) and also after 2, 4, 8, 12, 16 and 20 d from treatment, washed with copious amounts of water and blotted dry with paper towels. Another trial was performed to check the dose effect of isoproturon. Isoproturon at 0.5, 1.0, 1.5 and 2.0 fold RFD was applied to ten-d-old seedlings. Shoots were harvested on the 8th d following treatment.

Determination of pigments

Chlorophyll a, chlorophyll b and carotenoids were determined in the fresh tissues at three different wavelengths of 452.5, 644, and 663 nm after extraction with 85% acetone following treatment. Isoproturon at 0.5, 1.0, 1.5 and 2.0 fold RFD was applied to a 14-h photoperiod with 450-500 μmol m-2 s-1 PPFD, 75-80% RH, and 28/14°C d/night regime. When seedlings were ten d old, pots were divided into two groups; one to serve as a control and the other for isoproturon treatment at the recommended field dose (RFD, 2.5 Kg ha-1), the herbicide was applied only once as foliar sprays. The rate per hectare was calculated according to the surface area per pot and the herbicide was mixed with a suitable amount of water. At harvest, shoots were collected just before herbicide application (used for zero time) and also after 2, 4, 8, 12, 16 and 20 d from treatment, washed with copious amounts of water and blotted dry with paper towels. Another trial was performed to check the dose effect of isoproturon. Isoproturon at 0.5, 1.0, 1.5 and 2.0 fold RFD was applied to ten-d-old seedlings. Shoots were harvested on the 8th d following treatment.

Determination of pigments

Chlorophyll a, chlorophyll b and carotenoids were determined in the fresh tissues at three different wavelengths of 452.5, 644, and 663 nm after extraction with 85% acetone according to the spectrophotometric method described by Metzner et al. (1965).

Determination of H2O2

H2O2 was extracted in 200 mM perchloric acid, centrifuged at 5000 xg for 10 min and the supernatant was neutralized with 4 M KOH. After centrifugation at 3000 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 8 mM H2O2, and 2 mM guaiacol. The absorption at 470 nm was recorded and the activity was calculated using the extinction coefficient of 26.6 mM-1 cm -1 (Ranieri et al. 1997). The reaction was initiated by the addition of peroxidase and the increase in absorbance at 590 nm was monitored for 3 min.

Determination of antioxidants, ascorbic acid (AsA) and reduced glutathione (GSH)

AsA was extracted in 62.5 mM metaphosphoric acid and centrifuged at 12000 g for 20 min at 4°C. Samples were filtered through a 0.5 μm FH-type Millipore filter. The filtrate was loaded onto an aminex HPX-87H ion exclusion column (300 x 7.8 mm, Bio-rad) connected to analytical HPLC system, and eluted with 4.5 mM H2SO4 at a flow rate of 0.5 ml min-1. The elution of AsA was detected at 245 nm (Ahn et al. 1999). GSH was extracted in 5% (w/v) trichloroacetic acid containing 10 mM EDTA and centrifuged at 12000 g for 15 min. GSH was assayed in 100 mM phosphate buffer, pH 6.8, 10 mM EDTA, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and the reaction was started by adding 1.0 U equine GST. The absorbance at 340 nm was recorded before commencing the reaction and after the reaction had run to completion (Anderson and Gronwalds 1991). A control assay without equine GST was performed to check the possible nonenzymatic reaction.

Assays of antioxidant enzymes

All extraction steps were carried out at 4°C. Superoxide dismutase activity (SOD; EC 1.15.1.1) was extracted in 50 mM phosphate, pH 7.8, 0.1% (w/v) BSA, 5.5 mM AsA, and 8 mM β-mercaptoethanol. SOD was assayed in 50 mM phosphate, pH 7.8, 9.9 mM L-methionine, 0.057 mM NBT, 0.025% (w/v) Triton X-100, and 0.1 mM riboflavin by using the photochemical nitroblue tetrazolium (NBT) method in terms of SOD’s ability to inhibit reduction of NBT to form formazan by superoxide (Beyer and Fridovich 1987). The photoreduction of NBT (formation of purple formazan) was measured at 560 nm and an inhibition curve was made against different volumes of extract. One unit of SOD was defined as that caused inhibition of the photoreduction of NBT by 50% of control.

Catalase activity (CAT; EC 1.11.1.6) was extracted in 50 mM phosphate buffer, pH 7, and 1 mM dithiothreitol (DTT). CAT was evaluated spectrophotometrically by determining the consumption of H2O2 at 240 nm in 50 mM phosphate buffer, pH 7.5 and 200 mM H2O2 (Aebi 1984). One unit was defined as the decomposition of μmol H2O2 per min.

Ascobate peroxidase (APX; EC 1.11.1.7) was extracted in 0.1 M Tricine-KOH buffer, pH 8, 1 mM DTT, 10 mM MgCl2, 50 mM KCl, 1 mM EDTA, 0.1% (w/v) Triton X-100, and 0.28 mM phenylmethylsulfonyl flouride (PMSF). APX was evaluated at 270 nm in 50 mM phosphate buffer, pH 7.5 and 40 mM Na ascorbate and 200 mM H2O2 (Nakano and Asada 1981). One unit was defined as the decomposition of μmol H2O2 per min.

Guaiacol peroxidase (GPX; EC 1.11.1.7) was extracted in 220 mM Tris-HCl, pH 7.4, 250 mM sucrose, 50 mM KCl, 1 mM MgCl2, 160 mM β-mercaptoethanol, and 0.57 mM PMSF. GPX was assayed in 20 mM Na acetate, pH 5, 30 mM H2O2, and 2 mM guaiacol. The absorption at 470 nm was recorded and the activity was calculated using the extinction coefficient of 26.6 mM-1 cm -1 (Ranieri et al. 1997). The activity was converted to units using horseradish peroxidase standards.

Glutathione-S-transferase (GST; EC 2.5.1.18) was extracted in 100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 14 mM β-
Kinetic parameters of GST

To calculate the kinetic parameters of GST ($V_{\text{max}}$ and $K_m$), another assay was carried out using CDNB as a substrate for extracts obtained from leaves of treated and untreated maize on the 8th d following treatment with the different doses of isoproturon (0, 0.5, 1.0, 1.5 and 2.0 RFD). The reaction mixtures contained varied concentrations of CDNB up to 4 mM. The activity was determined using Lineweaver-Burk equation $[1/v = (1/[S]) (\frac{K_m}{V_{\text{max}}} + \frac{1}{V_{\text{max}}})]$ and further confirmed using Hanes equation, $[S]V = (S)(\frac{1}{V_{\text{max}}}) + (\frac{K_m}{V_{\text{max}}})$, the linear transformations of the Michaelis-Menten equation; $v = (V_{\text{max}})(S)/K_m + [S]$ (Engel 1984). Plotting $1/[S]$ against $1/v$, (Lineweaver-Burk plot), would give a straight line with the ordinate intercept $(1/V_{\text{max}})$, the abscissa intercept ($1/K_m$) and the slope ($-K_m/(V_{\text{max}})$). Further confirmation was carried by plotting $[S]$ against $[S]/v$ (Hanes plot) which should give a straight line, from with the ordinate intercept ($K_m/V_{\text{max}}$), the abscissa intercept ($K_m$) and the slope ($1/V_{\text{max}}$).

Protein content was determined according to Bradford (1976). All values are means ($\pm$SD) of at least six replications from two independent experiments. The full data were statistically analyzed using the least significant difference (LSD) test at 5% level (Snedecor and Cochran 1980).

Results and Discussion

Fresh and dry weights of shoots and roots of 10-d-old maize seedlings were significantly reduced by treatment with isoproturon at the recommended field dose (RFD) (Figure 1). The herbicide significantly decreased fresh weight of shoots and roots up to the 16th d and dry weight up to the 12th d in shoots and roots, respectively. Linear fitting of the dose-dependent curve shows that 10% reduction of fresh weight was induced by 0.4 RFD and 0.2 RFD, respectively and of dry weight by 0.4 RFD and 0.5 RFD, respectively. 50% reductions were caused by 2.2 RFD in shoot and root fresh weight, or by 2.1 RFD and 1.9 RFD in dry weight of shoots and roots, respectively (table 1).

The reduction of growth by herbicides could result from alterations in certain metabolic processes (Cobb and Kirkwood 2000, Nemat Alla and Hassan 2006). Isoproturon is a photosynthetic inhibitor interferes with electron flow from PSII to PSI (Kleczkowski 1994). Such interference might result in possible photodestruction of excited chlorophyll.

In figure 2, chlorophyll a, chlorophyll a and carotenoids contents increased gradually with time, however, this rise was lower in isoproturon-treated than in control seedlings. Isoproturon induced significant reduction in pigments up to the 16th d in chlorophylls and 12th d in carotenoids. Curve fitting indicates that 10% reduction of chlorophyll a, chlorophyll a and carotenoids was induced by 0.4 RFD, 0.3 RFD and 0.3 RFD, respectively while 50% reductions were caused by 2.3 RFD, 1.9 RFD and 1.7 RFD (table 1).

Isoproturon, like other herbicides, is known to act by affecting stability of the D1 protein in the photosynthetic machinery. It interferes with the light-mediated part of the photosynthetic process by inhibiting PSII mediated electron transport, lead to inhibition of ATP and NADPH production (Nadasy et al. 2000). The general decreases in pigments by isoproturon are in conformity with its mode of action on electron flow causing therefore a destruction in chlorophyll. Further, the decrease in carotenoids confirms the photodestruction of pigments. Kenyon and Duke (1985) indicated that carotenoids are, in general, lost more rapidly than chlorophylls concluding that carotenoids are highly susceptible to such attack. Carotenoids act as chemical buffer to protect chlorophylls and chloroplasts from photooxidation by removing oxygen from the excited chlorophyll-oxygen complexes via a carotenoid-expoxide cycle. Rutherford and Krieger-Liszkay (2001) hypothesized that the plant is killed by light-induced oxidative stress initiated by damage caused by formation of singlet oxygen in the reaction center itself. Thus, triplet chlorophyll and singlet oxygen might be released causing oxidative stress and would induce lipid peroxidation.

Isoproturon resulted in significant increases in AsA and GSH contents only during the first 4 d of treatment (Figure 3). These levels retracted on the 8th d to reach control levels but significantly decreased from the 12th d onward. The dose-dependent curve shows increases in contents of both AsA and GSH by low doses of isoproturon whereas high doses led to great diminutions. Linear fitting of the dose-
Antioxidants and GST kinetics of maize

AsA and GSH are the most abundant low molecular weight non-enzymatic antioxidants in plant cells participating in ROS scavenging through the AsA-GSH cycle (Foyer et al. 2001, Barth et al. 2004). These antioxidants act to detoxify ROS through active enzymatic pathways. AsA and GSH are the major redox buffers of the plant cells, and they themselves are also signal-transducing molecules that can either signal independently or further transmit ROS signals (Barth et al. 2004, Pavet et al. 2005). They are thus intrinsic to redox homeostasis and redox-signaling events (Foyer and Noctor, 2005b). AsA maintains the membrane-bound antioxidant α-tocopherol in the reduced state, eliminates H₂O₂ through APX and acts as a cofactor in the xanthophyll cycle (Jimenez et al. 1997). AsA modulates growth through regulation of the cell cycle (Potters et al. 2004) and through regulation of elongation growth (Tokuna et al. 2005). In support, Pyon et al. (2004) stated that AsA is a powerful reducing agent in plants and plays an important role in scavenging free radicals in plants. However, plants with lower AsA content are more resistant to pathogen attack (Barth et al. 2004, Pavet et al. 2005). Barth et al. (2004) confirmed that low AsA is causing the deficient Arabidopsis mutant to enter at least some stage(s) of senescence prematurely with an accompanying increase in salicylic acid levels that results in a faster induction of defense responses. In confirmation, Pavet et al. (2005) concluded that AsA abundance modifies the threshold for activation of plant innate defense responses.

Moreover, GSH was suggested to be one of the limiting factors for tolerance to herbicides (Jiménez et al. 1997, Nemat Alla and Hassan 1998; Nemat Alla et al., 2007). GSH is an abundant plant metabolite that has many diverse and important functions including signal transduction (Gomez et al., 2004). It is a versatile antioxidant that can directly scavenge ROS and participate in the AsA-GSH cycle (Mittler 2002). Therefore, the inductions of AsA and GSH might increase plant defense to oxidative stress. In this context, Pyon et al. (2004) reported that AsA content was higher in the paraquat-resistant Erigeron Canadensis. Moreover, Nemat Alla et al. (2007) affirmed that the differential tolerance of plant species to butachlor was related to the induction of GSH and GSH-associated enzymes. So, the transient increase in GSH and AsA followed thereafter by consistent decreases might indicate a retraction in the antioxidative defense mechanism.

Figure 4 shows that isoproturon significantly increased H₂O₂ accumulation in maize during the entire experimental period as compared with control. The magnitude of accumulation increased with the elapse of time. However, there was no significant effect during the first 2 d of treatment. Moreover, all doses of isoproturon (0.5 to 2.0 fold RFD) caused substantial and consistent increases in the accumulation of H₂O₂ in maize ranged from 139% to 383%. In addition, curve fitting equations indicate that isoproturon at low doses (0.5 to 1.0 fold RFD) induced respectively 10% and 50% accumulation of H₂O₂ in maize (table 1).

H₂O₂ is an active signaling molecule and its accumulation leads to a variety of cellular responses that are dose dependent (Kurama et al. 2002). They confirmed that a high dosage of H₂O₂ results in hypersensitive cell death whereas a low dosage blocks cell cycle progression and functions as a developmental signal for the onset of secondary wall differentiation. Therefore, the accumulation of H₂O₂ confirms the existence of oxidative stress and appears to be a signaling intermediate in plant defense (Polidoros and Scandalios 1999, Hassan and Nemat Alla 2005, Nemat Alla and Hassan 2006). Murgia et al. (2004) stated that H₂O₂ can cause cell death to transgenic Arabidopsis thaliana. There was a negative interrelation between H₂O₂ and pigment content in treated seedlings, an observation that seems to convenient with isoproturon mode of action. Nevertheless, the accumulation of H₂O₂ in the isoproturon-treated maize might be due to an increased rate of production and/or decreased rate of degradation. The production of H₂O₂ is controlled by SOD while its degradation depends mainly on CAT, APX and GPX. Therefore, the great accumulation of H₂O₂ in the present study, would point to an increase in SOD activity and/or an inhibited activity of CAT, APX and GPX.

Table 1. Doses of isoproturon predicted to give 10% or 50% perturbation (inhibition and stimulation) in the content of tested parameters and enzyme activities in leaves of 10-d-old maize seedlings on day 8 following treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Inhibition</th>
<th>Stimulation</th>
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<tbody>
<tr>
<td></td>
<td>10%</td>
<td>50%</td>
</tr>
<tr>
<td>Shoot fresh weight</td>
<td>0.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Root fresh weight</td>
<td>0.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Shoot dry weight</td>
<td>0.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Root dry weight</td>
<td>0.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>0.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Ascorbic acid (AsA)</td>
<td>=</td>
<td>3.0</td>
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<tr>
<td>Reduced glutathione (GSH)</td>
<td>=</td>
<td>2.8</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>1.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>0.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Ascorbate peroxidase (APX)</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Guaiacol peroxidase (GPX)</td>
<td>1.1</td>
<td>3.2</td>
</tr>
<tr>
<td>GST towards CDNB</td>
<td>0.6</td>
<td>2.4</td>
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<tr>
<td>GST towards alachlor (GST (ALA))</td>
<td>0.5</td>
<td>2.1</td>
</tr>
<tr>
<td>GST towards metolachlor (GST (MET))</td>
<td>0.6</td>
<td>2.6</td>
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<tr>
<td>GST towards atrazine (GST (ATR))</td>
<td>0.6</td>
<td>2.2</td>
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</table>
SOD activity in maize was significantly enhanced by isoproturon up to the 12th d of treatment then became significantly lower than the control levels from the 16th d up to the end of the experiment (Figure 5). Similarly, isoproturon induced inductions in CAT activity only during the first 4 d of treatment, however the activity seemed to be comparable to control up to the 12th d and then significantly inhibited thereafter. On the other hand, APX activity was
inhibited by isoproturon up to the end of the experiment. Nevertheless, no significant changes of the enzyme activity from control level were observed during the first 4 d of treatment. On the contrary, GPX activity was significantly higher in treated samples than in control during the first 4 d of treatment, thereafter, the activity was inhibited up to the end of the experiment. The dose-dependent curve indicates enhancements in SOD and GPX activities of maize by low doses of isoproturon (0.5 and 1.0 fold RFD) while high doses (1.5 and 2.0 fold RFD) caused inhibitions. On the other hand, CAT and GPX activities were gradually decreased by all doses. 0.3 fold RFD increased SOD activity by 10% whereas 1.4 fold RFD and 3.6 fold RFD caused reductions by 10% and 50%, respectively (table 1). However, 50% inhibition in CAT and APX GPX, were by 2.9, 2.0 and 3.2 fold RFD, respectively.

SOD is a key enzyme in protecting cells against oxidative stress. It is responsible for the elimination of superoxide radicals generated in plant cells. Therefore, an increase in SOD activity might lead to great production of \( \text{H}_2\text{O}_2 \). Overproduction of SODs in plant chloroplasts was concluded to lead to protection against herbicides (Iannelli
et al. 1999). On the other hand, APX catalyzes the reduction of H$_2$O$_2$ by using AsA (Murgia et al. 2004). Moreover, CAT catalyzes the degradation of H$_2$O$_2$ preventing its accumulation (Polidoros and Scandalios 1999, Geoffroy et al. 2002, Hassan and Nemat Alla 2005). In confirmation, Jimenez et al. (1997) stated that CAT, APX and GPX are responsible for scavenging of H$_2$O$_2$. Thus the decreases in these activities could explain a drop in the detoxification of H$_2$O$_2$ with a subsequent increase in its accumulation.

Figure 3. Contents of ascorbic acid (A) and reduced glutathione (B) in leaves of 10-d-old maize seedlings after treatment with the recommended field dose of isoproturon during the following 20 d. (C and D), Dosage effect of isoproturon on the 8th d following treatment. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level.

Figure 4. Contents of H$_2$O$_2$ (A) in leaves of 10-d-old maize seedlings after treatment with the recommended field dose of isoproturon during the following 20 d. (B), Dosage effect of isoproturon on the 8th d following treatment. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level.
Figure 5. Activities of superoxide dismutase (A), catalase (B), ascorbate peroxidase (C) and guaiacol peroxidase (D) in leaves of 10-d-old maize seedlings after treatment with the recommended field dose of isoproturon during the following 20 d. (E, F, G and H). Dosage effect of isoproturon on the 8th d following treatment. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level.

Under these conditions, where CAT, APX and GPX are diminished, the cell is not fully competent to remove $H_2O_2$ which would accumulate to toxic levels. Iannelli et al. (1999) correlated between the resistance of maize to paraquat and the increased activities of SOD and peroxidases. In addition, Pyon et al. (2004) reported that the activities of SOD, CAT and peroxidases were higher in paraquat-resistant *Erigeron canadensis* than in susceptible biotype. Geoffrey et al. (2002) found that CAT and APX of *Scenedesmus obliquus* were significantly stimulated by oxyfluorfen. Moreover, Nemat Alla and Hassan (2006) reported that the atrazine-induced oxidative stress was counterbalanced in tolerant but continued in susceptible maize lines. However, Stajner et al. (2003) found that paraquat, alachlor and metolachlor inhibited antioxidant enzyme activities (SOD, CAT and GPX) in lettuce, bean and pea seeds but lower concentrations of these herbicides increased activities of antioxidant enzymes in leaves. On the other hand, $H_2O_2$ content increased under severe drought conditions (Luna et al. 2005). Therefore, the decreases in activities of CAT and peroxidases indicate, if any, a shortage in $H_2O_2$ detoxification.
Figure 6. Activities of glutathione-S-transferase towards CDNB (A) or the herbicides alachlor (B), metolachlor (C) and atrazine (D) as substrates in leaves of 10-d-old maize seedlings after treatment with the recommended field dose of isoproturon during the following 20 d. (E, F, G and H), Dosage effect of isoproturon on the 8th d following treatment. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level.

In addition to these enzymes, GSTs are believed to play a role in antioxidant metabolism by mechanisms that probably aid in the reduction of secondary noxious products resulting from exposure to stress-induced ROS (Polidoros and Scandalios 1999, Hassan and Nemat Alla 2005, Nemat Alla and Hassan 2006). Moreover, GSTs catalyze the conjugation of electrophilic xenobiotics with GSH. Figure 6 represents the GST activities in maize towards the substrates CDNB, alachlor, metolachlor and atrazine (GST(CDNB), GST(ALA), GST(MET) and GST(ATR)). In general, isoproturon provoked significant inhibitions in activity of GST(CDNB), GST(ALA) and GST(MET) during the experimental period, however, no variations from control were detected during the first 4 d of treatment. On the other hand, the activity of GST(ATR) seemed to be non significantly affected by isoproturon allover the experimental period. Curve fitting indicates that activities of GSTs were generally decreased with increasing the isoproturon doses. GST(CDNB) and GST(MET) seemed to be more resistant to isoproturon followed by GST(ATR) whereas GST(ALA) appeared the most sensitive. 10% inhibition in activities of GST(CDNB), GST(ALA) and GST(MET) during the experimental period were mostly induced by 0.6 fold RFD while 50% inhibitions were induced respectively by 2.4, 2.1, 2.6 and 2.2 fold RFD (table 1).
The general inhibitions in GSTs activities by isoproturon concomitant with retraction in GSH contents suggest the exclusion of the GSH-mediated detoxification pathway of the herbicide and could support the negative effects of isoproturon on antioxidants. This observation might indicate an induced oxidative stress by the herbicide with a consequent suppression in the antioxidant defense systems. In this context, more susceptibility of soybean to butachlor was accompanied with lower induction of GSH and GST than the tolerant maize and wheat (Nemat Alla et al. 2007). In support, Geoffrey et al. (2002) confirmed that stimulated GST was related to protection of Scenedesmus obliquus against oxyfluorfen and diuron.

Table II. V_max and K_m of GST(CDNB) extracted from leaves of 10-d-old maize seedlings at the 8th d from treatment with different doses of isoproturon (0, 0.5, 1.0, 1.5, and 2.0 fold the recommended field dose, RFD) based on Lineweaver-Burk and Hanes equations.

<table>
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<tr>
<th></th>
<th>Lineweaver-Burk equation</th>
<th>Hanes equation</th>
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<tbody>
<tr>
<td></td>
<td>V_max</td>
<td>K_m</td>
</tr>
<tr>
<td>Control</td>
<td>294 ± 28</td>
<td>0.44 ± 0.05</td>
</tr>
<tr>
<td>0.5 RFD</td>
<td>233 ± 26*</td>
<td>0.42 ± 0.06</td>
</tr>
<tr>
<td>1.0 RFD</td>
<td>208 ± 24*</td>
<td>0.44 ± 0.04</td>
</tr>
<tr>
<td>1.5 RFD</td>
<td>189 ± 19*</td>
<td>0.43 ± 0.05</td>
</tr>
<tr>
<td>2.0 RFD</td>
<td>141 ± 18*</td>
<td>0.45 ± 0.06</td>
</tr>
</tbody>
</table>

Data are means ± SE. (n = 6)
Data followed by (*) are significantly different from control at 1 %. V_max is expressed as nmol CDNB conjugated mg⁻¹ protein min⁻¹. K_m is expressed as mM CDNB.

In general, the inhibition of an enzyme could result not only from a decrease in its concentration but also from the interference with the structural integrity, which can be related to isoenzyme distribution and/or enzyme-substrate affinity. Activity of GST extracted from leaves of maize treated with different doses of isoproturon was examined on the 8th d as a function of CDNB concentration according to Michaelis-Menten plot (Figure 7). The velocity of GST was generally lowered by isoproturon, the decrease augmented with increasing the herbicide dose. Thus, isoproturon could be considered as an inhibitor for GST; with probable changes in the kinetic parameters K_m and V_max. Lineweaver-Burk plot clearly indicates that increasing isoproturon dose increased the ordinate intercept (1/V_max). There were little changes in the abscissa intercept (-1/K_m) and increases in slop values. These observations confirm drops in V_max of GST by the herbicide with a possible change in K_m. To support these findings, a plot of [S]/v against [S] (Hanes plot) confirmed an increase in the slop (1/V_max) in mixtures incubated with isoproturon with somewhat unclear changes in abscissa intercept (-1/K_m). These results confirmed decreases in V_max values; more decreases were observed as isoproturon dose increased.

Therefore, V_max and K_m values were calculated and obtained by data interpolation from equations presented in Figure 7 (Lineweaver-Burk plot and Hanes plot) (Table 2). There were progressive decreases in V_max of GST as isoproturon dose increased. On the contrary, K_m values seemed unchanged. Since V_max is a function of the enzyme concentration, a decrease in its value consequently suggests an inhibition in the enzyme synthesis. On the other hand, the unaffected K_m values confirm no interference of the herbicide with the structural integrity. Therefore, the consistence of K_m in the present results, concomitant with
decreases in the $V_{\text{max}}$ of GST could, thereupon, deduce that isoproturon altered only the synthesis and concentration of GST. So, isoproturon could be concluded to cause competitive inhibition to GST.

In conclusion, treatment of maize with isoproturon reduced growth parameters and pigment contents. Meanwhile, the herbicide temporarily increased the antioxidants, AsA and GSH at the start, however, these levels retracted thereafter. This behaviour was also detected, to some extent, for activities of SOD, CAT, APX and GPX. Whether low doses increased or decreased these parameters, high doses led to significant drops. Meanwhile, there were significant accumulation of H$_2$O$_2$ throughout the experimental period. Moreover, activities of GST isoforms (GST$_{\text{CTDHB}}$, GST$_{\text{ALT}}$, and GST$_{\text{MET}}$) were generally inhibited by isoproturon with no significant changes in GST$_{\text{ATR}}$. These observations confirm the existence of oxidative stress induced in maize by isoproturon with a consequent suppression in the protective mechanism by antioxidant defense system as time of treatment is prolonged and/or the herbicide dose is increased. In addition, the herbicide lowered $V_{\text{max}}$ values of GST with no variations in $K_{\text{m}}$ confirming that isoproturon is a competitive inhibitor to GST.

References


