



Kinetics of inhibition of isoproturon to glutathione-associated enzymes in wheat

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Abstract The present study aimed at investigating the kinetic of inhibition of isoproturon to the GSH-associated enzymes [γ -glutamyl-cysteine synthetase (γ -GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPX)] in wheat. Isoproturon, applied to 10-day-old seedlings for the following 12 days, provoked significant reductions in shoot fresh and dry weights, protein, thiols and glutathione (GSH); however, oxidized glutathione (GSSG) was elevated while GSH/GSSG ratio was declined with concomitant significant inhibitions in the activities of γ -GCS, GS, GR, GST and GPX; the effect was time dependent. IC₅₀ and K_i values of isoproturon were lowest for GPX, highest for both GST and GR, and moderate for both γ -GCS and GS. The herbicide markedly decreased V_{max} of γ -GCS, GS and GPX but unchanged that of GST and GR; however, K_m of γ -GCS, GS, GST and GR increased but unchanged for GPX. The pattern of response of changing V_{max}, K_m, V_{max}/K_m, k_{cat} and k_{cat}/K_m for in vivo and in vitro tests of each enzyme seemed most likely similar. These results indicate that a malfunction to defense system was induced in wheat by isoproturon resulting in inhibitions in GSH-associated enzymes, the magnitude of inhibition was most pronounced in GPX followed by γ -GCS, GS, GST, and GR. These findings could conclude that isoproturon competitively inhibited GST and GR; however, the inhibition was noncompetitive for GPX but mixed for both γ -GCS and GS.

Keywords Glutathione · Glutathione-associated enzymes · Isoproturon · Kinetic · Wheat

Introduction

Isoproturon (N, N-dimethyl-N-4-isopropyl phenylurea) is widely used in wheat fields for the control of annual grasses and broadleaf weeds. It is active with photosynthesis particularly on chloroplast electron-transport system (Yin et al. 2008). Most likely, several herbicides, including isoproturon, promote their toxic effects on photosynthetic organisms by affecting the production of reactive oxygen species (ROS) (Nemat Alla et al. 2008a,b; Reis et al. 2011). The use of isoproturon induces oxidative stress in wheat even at very low concentrations (Yin et al. 2008). Isoproturon inhibits photosynthesis through blocking the electrons flow. The formation of ROS is due to the electron transfer to molecular oxygen at photosystem I and II, resulting in formation of superoxide radicals (O₂⁻) and, thus, subsequently hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH[·]). Moreover, ROS formation comes from the transfer of excitation energy from triplet chlorophyll to molecular oxygen, resulting in the formation of singlet oxygen (¹O₂) (Jahns and Holzwarth 2012). ROS lead to severe changes in the redox state of the plastoquinone pool and react with lipids, proteins, pigments and nucleic acids and cause lipid peroxidation, membrane damage and inactivation of enzymes, thus affecting cell viability (Badran et al. 2015; Chakraborty et al. 2019).

However, plants protect themselves from oxidative damage through an efficient defense mechanistic antioxidant system for ROS scavenging. Glutathione (GSH, γ Glucyl-Gly) and other thiol compounds are important metabolites as non-enzymatic antioxidants (Anderson and

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Davis 2004; Misra et al. 2006; Zushi and Matsuzoe 2009; Nemat Alla et al. 2011; Hasanuzzaman et al. 2017). Nayyar and Gupta (2006) indicated that GSH participates in the scavenging of ROS via the ascorbate-GSH cycle. The synthesis of GSH takes place through the catalysis of γ -glutamylcysteine synthetase (γ -GCS) and glutathione synthetase (GS) (Hasanuzzaman et al. 2017). Reduced GSH is maintained by glutathione reductase (GR) (Volohonsky et al. 2002; Gupta et al. 2005; Yamazaki et al. 2019). Glutathione-S-transferase (GST) mediates the conjugation of GSH with several herbicides; however, some isoforms of GST show dual activity and can also function as glutathione peroxidase (GPX) (Volohonsky et al. 2002). These enzymes, in addition to GSH might regulate plant tolerance to herbicides and so, they could depend on GSH availability. Some herbicides increase GSH and the associated enzymes for efficient detoxification; however, some others such as phenylurea herbicides do not but on the contrary act as inhibitors. Although the phytotoxicity of isoproturon is well documented through generating ROS and destroying defense system, nothing was detected about the kinetics of isoproturon inhibition to the GSH-associated enzymes. So, the aim of the present work is to investigate both the kinetics of inhibition and the inhibition type of isoproturon to the enzymes γ -GCS, GS, GR, GST and GPX in wheat. For this purpose, the inhibition constants (IC₅₀ and K_i) were calculated in response to isoproturon application, in addition to determining the kinetic parameters (V_{max}, K_m, k_{cat}, k_{cat}/K_m and V_{max}/K_m) of the GSH-associated enzymes through performing in vivo and in vitro tests.

Materials and methods

Plant materials and growth conditions

Grains of wheat (*Triticum aestivum*) were surface sterilized by immersing in 3% sodium hypochlorite solution for ten min, thoroughly washed, soaked for 8 h and 20 seeds were planted in quartz sand (pre-washed with hydrochloric acid), approximately 2 cm-depth in plastic pots (40 × 20 × 10 cm). The pots were kept at 24 ± 2/12 ± 2 °C, 10-h photoperiod and watered from overhead to the soil surface as required. The pots of 10-day-old seedlings were divided into two groups; one for isoproturon treatment at the recommended field dose (900 g ha⁻¹) and the other was left as control. On the 5th day and the 12th day from treatments, shoots were collected, rinsed with copious amounts of water and dried by plotting with paper towels and used for the subsequent analyses, a part was weighed for recording the fresh weights, then dried in the oven at 80 °C for 2 days for dry weight measurements.

Determination of protein content

Protein was extracted in Tris-HCl (80 mM, pH 7.4) (Laemmli 1970). After centrifugation at 14,000×g for 5 min, chilled trichloroacetic acid (TCA, 10%, w/v) in acetone was added to precipitate protein over night at 4 °C. Protein pellets were separated by centrifuged at 12,000×g for 15 min, washed with chilled acetone, allowed to dry in air and reconstituted in the buffer. Protein content was determined spectrophotometrically according to Bradford (1976). Aliquots were pipetted into test tubes, and the volume was adjusted to 100 μl with Tris-HCl (50 mM, pH 9) then 5 ml of Coomassie Brilliant Blue G-250 were added. The absorbance was measured at 595 nm after 2 min and before 1 h.

Determination of glutathione contents (GSH and GSSG)

An aliquot of tissue was homogenized in TCA (5%, w/v) containing EDTA (10 mM) at 4 °C and centrifuged at 12,000×g for 15 min (Anderson and Gronwald 1991). The assay of GSH was performed in phosphate buffer (100 mM, pH 6.8) containing EDTA (10 mM), 1-chloro-2,4-dinitrobenzene (1 mM) and equine GST (1 U), then the absorbance was recorded at 340 nm twice, firstly at the start of the reaction and secondly after its completion. For GSSG assay, 2-vinylpyridine was added to mask GSH. The assay was performed in potassium phosphate buffer (100 mM, pH 7.5) containing EDTA (5 mM), NADPH (0.2 mM), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.6 mM) and glutathione reductase (GR, 10 U), then absorbance was immediately read at 412 nm and again after 30 min of incubation at 35 °C.

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

An aliquot of tissue was homogenized in EDTA (20 mM) and centrifuged at 12,000×g for 15 min. Total thiols were measured in Tris-HCl (200 mM, pH 8.2) containing DTNB (10 mM) and absolute methanol and absorbance was read at 412 nm, then the extinction coefficient E = 13,100 mM⁻¹ cm⁻¹ was used to calculate thiol quantity (Sedlak and Lindsay 1968). For non-protein thiols determination, the supernatant was mixed with TCA (50% w/v) and centrifuged at 10,000×g for 15 min and absorbance was read at 412 nm. The difference between total thiols and the non-protein thiols gives the protein-bound thiols.

Activity assays of GSH-associated enzymes

According to Aravind and Prasad (2005), the extraction of γ -glutamyl-cysteine synthetase (γ -GCS) and glutathione synthetase (GS) was carried out in Tris–HCl (50 mM, pH 7.5) containing phenyl methyl sulfonyl fluoride (40 mM) and polyvinyl pyrrolidone (PVPP, 2%, w/v), then centrifuged at $15,000\times g$ for 20 min. γ -GCS activity was assayed by the method of Nagalakshmi and Prasad (2001) in Tris–HCl (20 mM, pH 8.2) containing sodium glutamate (20 mM), L-amino butyrate (20 mM), Na_2 -EDTA (40 mM), bovine serum albumin (0.4%, w/v), MgCl_2 (20 mM) and Na_2 -ATP (50 mM), then the absorbance was recorded at 660 nm. The assay of GS was carried out in Tris–HCl (100 mM, pH 8.0) containing KCl (50 mM), MgCl_2 (20 mM), EDTA (2 mM), ATP (10 mM), dithiothritol (DTT, 2.5 mM), glycine (5 mM) and γ -glutamyl-cysteine (5 mM), then GSH was determined every 5 min up to 20 min (Volohonsky et al. 2002).

GR was extracted in phosphate (100 mM, pH 7.5) containing EDTA (0.5 mM). The assay was performed in phosphate (100 mM, pH 7.5) containing EDTA (0.5 mM), DTNB (0.75 mM), NADPH (0.1 mM) and GSSG (1 mM), then absorbance at 412 nm was read up to 5 min (Smith et al. 1988).

The extraction of glutathione-S-transferase (GST) was performed in Tris–HCl (100 mM, pH 7.5) containing EDTA (2 mM), β -mercaptoethanol (14 mM) and PVPP (7.5%, w/v), then ammonium sulfate was added to 80% saturation (Dixon et al. 1995). The assay was performed in phosphate (100 mM, pH 6.5) containing GSH (5 mM) and 1-chloro-2,4-dinitrobenzene (1 mM) and absorbance was measured at 340 nm and the extinction coefficient $E = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was used for calculation of the enzyme activity (Askelof et al. 1975).

The extraction of glutathione peroxidase (GPX) took place in Tris–HCl (100 mM, pH 7.5) containing EDTA (1 mM) and DTT (2 mM) (Edwards 1996). The activity was assayed in phosphate (100 mM, pH 7.0) containing Triton X-100 (2%, w/v), GR (0.24 U), GSH (1 mM), NADPH (0.15 mM), and cumene hydroperoxide (1 mM). The absorbance at 340 nm was read for 3 min to measure the rate of NADPH oxidation and the extinction coefficient $E = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ was used for calculation of the enzyme activity (Nagalakshmi and Prasad 2001). Gradient polyacrylamide gel (7.5–15%, w/v) were used for SDS–PAGE. Coomassie brilliant blue R-250 was used for staining the proteins on the gels of the enzymes γ -GCS, GS, GR, GST and GPX according to Connell and Mullet 1986, Hou et al. 2003, Li et al. 2003, Ashida et al. 2005, Boisselier et al. 2011, respectively.

Determination of inhibition constants and kinetic parameters

The inhibition constants of isoproturon (IC_{50} and K_i) were determined from in vitro tests using enzyme preparations obtained from untreated shoots of 10-day-old wheat seedlings and isoproturon at different concentrations. The final concentration of isoproturon was 2, 4, 6, 8, 10 and 12 μM in reaction mixtures containing the enzyme extract. The velocity (v) of each enzyme was assayed as a function of isoproturon concentration using the appropriate [S] of γ -GCS, GS, GR, GST and GPX [amino butyrate, glutamylcysteine, 1-chloro-2,4-dinitrobenzene, cumene hydroperoxide and 5,5-dithiobis-(2-nitrobenzoic acid), respectively].

The determination of kinetic parameters was achieved by conducting in vivo tests using enzyme extracts obtained from wheat shoots of treated seedlings on the 5th day of treatment and in vitro tests using enzyme preparations obtained from shoots of untreated seedlings with the same age. Isoproturon was added (4 μM) to the reaction mixtures of the untreated samples. The velocities (v) of γ -GCS, GS, GR, GST and GPX were assayed as functions of their respective substrates [amino butyrate, glutamylcysteine, 1-chloro-2,4-dinitrobenzene, cumene hydroperoxide and 5,5-dithiobis-(2-nitrobenzoic acid), respectively]. The substrate concentrations [S] ranged, respectively from 2–100, 1–20, 0.1–1.0, 0.4–3.2 and 0.15–1.5 mM. v was plotted against [S] according to Michaelis–Menten saturation plot $\{v = V_{\text{max}} [S]/(K_m + [S])\}$. The data were fitted to Lineweaver–Burk plot $\{1/v = 1/V_{\text{max}} + (1/[S])(K_m/V_{\text{max}})\}$. A straight line is produced from plotting $1/v$ against $1/[S]$, its slope, ordinate intercept and abscissa intercept equal K_m / V_{max} , $1/V_{\text{max}}$ and $-1/K_m$, respectively.

The data were fitted also to Hanes plot $\{[S]/v = [S]/V_{\text{max}} + K_m/V_{\text{max}}\}$. A plot of $[S]/v$ against [S] gives a straight line with slope, ordinate intercept and abscissa intercept equal to $1/V_{\text{max}}$, K_m/V_{max} and $-K_m$, respectively. The catalyst rate (k_{cat}) was obtained from dividing V_{max} by the concentration of the enzyme, then the catalytic efficiency (V_{max}/K_m and k_{cat}/K_m) was determined. v was calculated with the presence and absence of isoproturon and used for the calculation of the % inhibition $[100 - (V_{\text{max}} - v)/100]$. Plotting isoproturon concentrations against $(V_{\text{max}} - v)/v$ for each enzyme gives straight lines (Yoshino and Murakami 2009). Values of both IC_{50} and K_i were obtained by data interpolation of % Inhibition and the equation $\text{IC}_{50} = K_i(1 + (S/K_m))$, respectively.

Statistical analysis

The design was completely randomized and repeated twice comprising 40 pots $[(2 \text{ isoproturon treatments}) \times (10$

Fig. 1 Influence of isoproturon application at the field dose to 10-day-old wheat seedlings on shoot fresh weight, dry weight and protein content during the following 12 days. White bars, Control; Black bars, Isoproturon. Values are means \pm SD ($n = 6$). Vertical bars represent LSD at $p \leq 0.05$; *significant difference from control

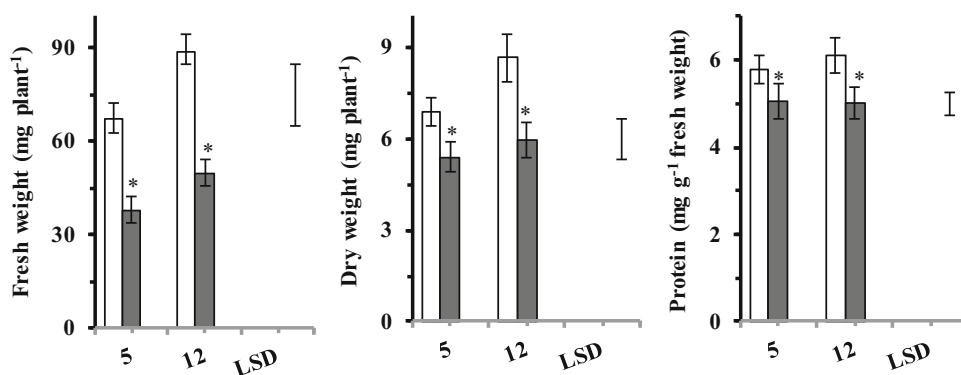
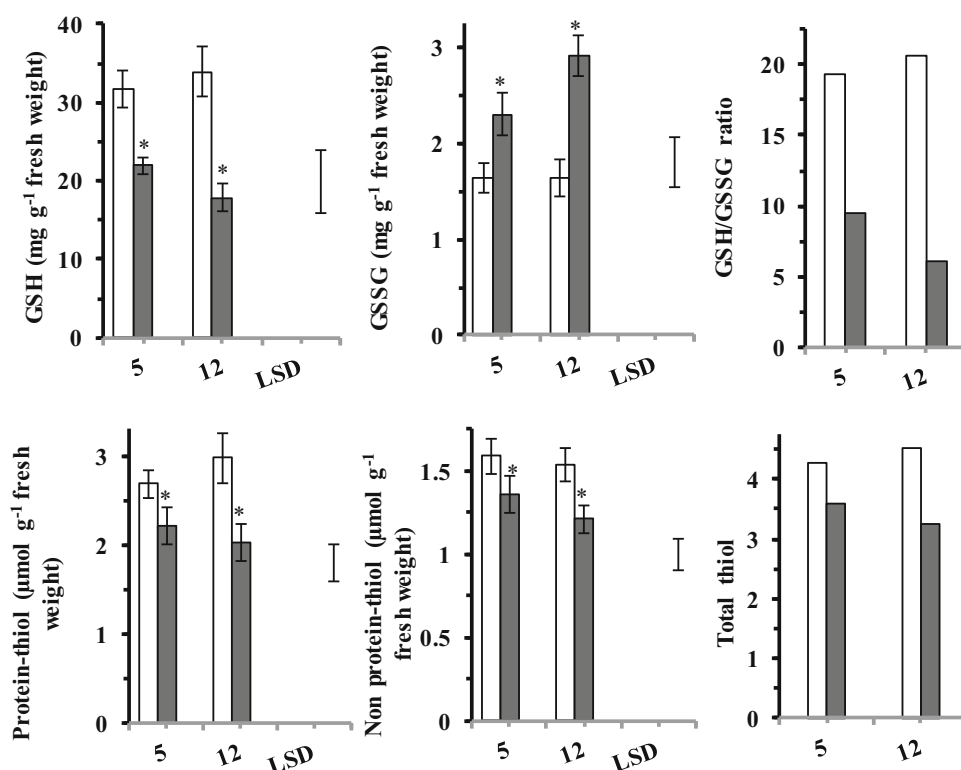


Fig. 2 Influence of isoproturon application at the field dose to 10-day-old wheat seedlings on reduced and oxidized glutathione (GSH and GSSG) and on thiol contents during the following 12 days. White bars, Control; Black bars, Isoproturon. Values are means \pm SD ($n = 6$). Vertical bars represent LSD at $p \leq 0.05$; *significant difference from control. GSH/GSSG ratio was obtained from dividing mean values while total thiol is just a sum of means



replications) \times (2 repetitions)]. Values are mean (\pm SD, $n = 6$). One-way ANOVA was used and LSD was calculated at $p \leq 0.05$.

Results

Isoproturon was applied at the recommended field dose to 10-day-old wheat seedlings. The shoot samples were harvested on the 5th day and the 12th day after treatment. Treatment with isoproturon provoked significant reductions in fresh and dry weights of wheat seedlings in relation to controls; the magnitude of reduction was higher for fresh than dry weight (Fig. 1). The effect of isoproturon was

higher at day 12 following treatment than at day 5. Meanwhile, the herbicide significantly decreased protein content; the decrease was most likely on the 5th day as similar as those detected on the 12th day following treatment.

Similar significant decreases were also detected in GSH; however, the oxidized form (GSSG) was highly elevated particularly on the 12th day following treatment (Fig. 2). Nonetheless, the ratio of GSH/GSSG was markedly dropped by isoproturon, the drop was time dependent. In addition, the contents of thiol forms were differentially decreased; the magnitude of decrease was higher in protein-bound than the non-protein form. Anyway, both forms were decreased more on the 12th day following treatment

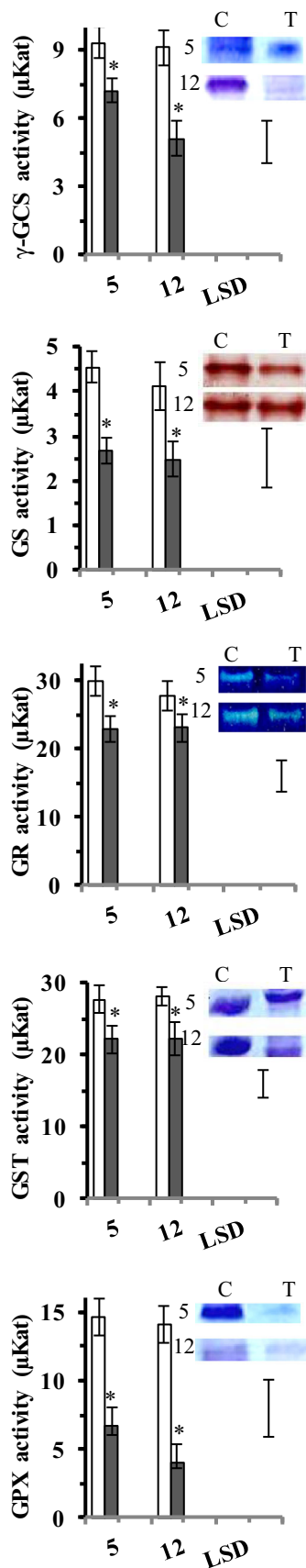


Fig. 3 Influence of isotroturon application at the field dose to 10-day-old wheat seedlings on the activities of γ -glutamyl-cysteine synthetase (γ -GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPX) during the following 12 days. White bars, Control; Black bars, Isotroturon. Values are means \pm SD (n = 6). Vertical bars represent LSD at $p \leq 0.05$; *significant difference from control. SDS-Page gels of the enzymes are shown on the 5th and the 12th day in control (C) and treated (T) samples

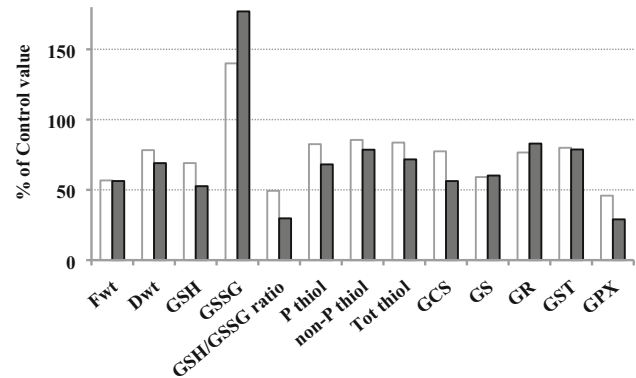


Fig. 4 Changes in the tested parameters relative to the respective control due to the influence of isotroturon at the field dose to 10-day-old wheat seedlings during the following 12 days. White bars, on the 5th day; Black bars, on the 12th day. Values represent percentages of the tested parameters mean values relative to their respective control means

than on the 5th day (Fig. 2). However, a great reduction was detected in total thiol contents.

On the other hand, isotroturon treatment significantly inhibited the activities of the GSH-associated enzymes [γ -glutamyl-cysteine synthetase (γ -GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPX)] (Fig. 3). The inhibition in the enzymes activities was greater on the 12th day than on the 5th day. The magnitude of inhibition was highest for GPX, however, the activities of both γ -GCS and GS were intermediate but lowest for both GST and GR. The activity gels of all enzymes show differential inhibition following isotroturon treatment. Any way, the band densities were lower in the treated samples than in their respective control; the effect was obvious for GPX followed by both γ -GCS and GS but seemed lowest for both GST and GR. The decrease in band densities in the treated samples augmented with the elapse of time.

Figure 4 shows that the percentage of change due to isotroturon treatment was higher for only GSSG than in the control. On the contrary, the other parameters were lower than the control values, the magnitude of decrease showed variations relative to their controls. The magnitude

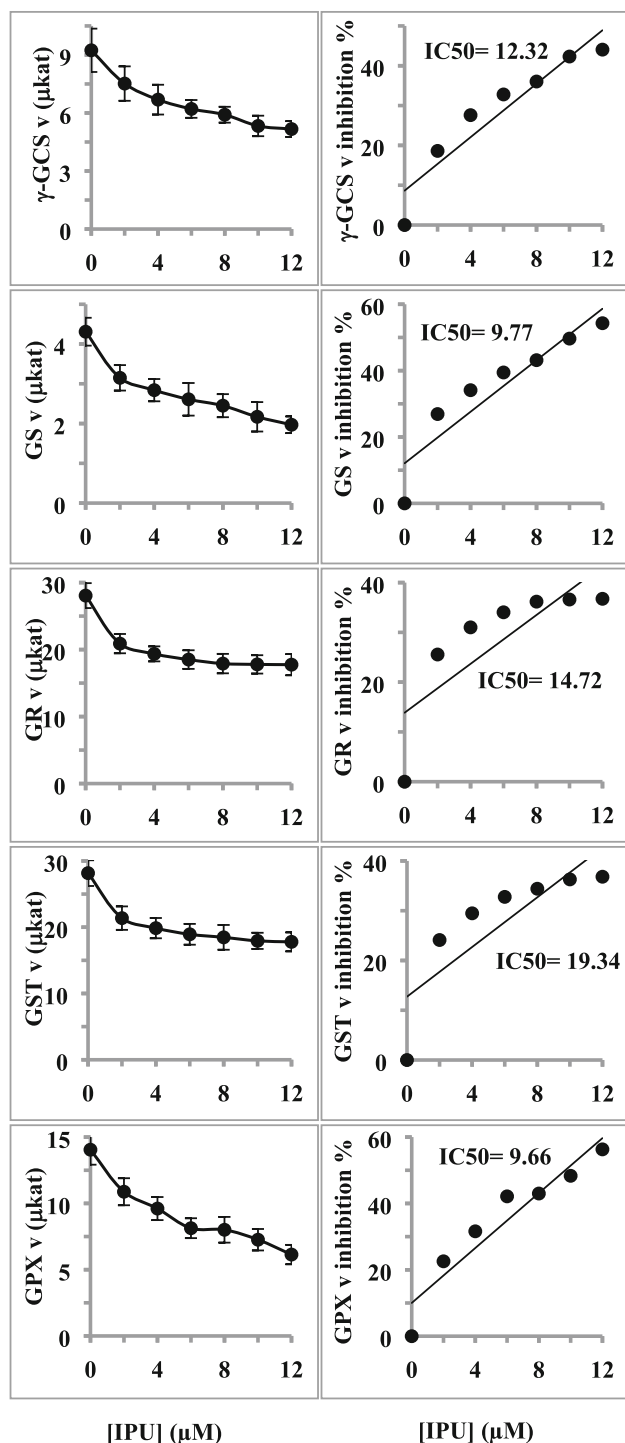


Fig. 5 In vitro tests of the velocities (v , μKat) and the % of inhibition (relative to blank) of the enzymes γ -glutamyl-cysteine synthetase (γ -GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPX) as functions of isoprotruron (IPU). The enzyme preparations were obtained from shoots of 15-day-old untreated wheat seedlings. Values of v are means \pm SD ($n = 6$). The straight lines of [IPU] versus the % of inhibition (relative to blank) were performed to calculate IC₅₀

Fig. 6 In vivo tests of the velocities (v , μKat) of the enzymes γ -glutamyl-cysteine synthetase (γ -GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPX) as function of the respective substrate concentration [S] according to Michaelis–Menten plot ([S] versus v), Lineweaver–Burk Plot ($1/[S]$ versus $1/v$), and Hanes Plot ([S] versus $[S]/v$). Samples were obtained from shoots of 15-day-old treated wheat seedlings. White circle, control; Black circle, treated. v and [S] values are means \pm SD ($n = 6$). Lineweaver–Burk and Hanes plots were performed using the final means of v and [S]

of decrease was highest for GSH/GSSG ratio and GPX activity; however, the least decreases were detected for GR and GST activities, and the non-protein thiol. Generally, the changes in the tested parameters at day 5 or at day 12 of treatment were most likely similar.

For measuring IC₅₀ of isoprotruron, in vitro tests using different concentrations of the herbicide were used with enzyme preparations obtained from shoots of untreated 15-day-old wheat seedlings. The enzyme velocity v was assayed as a function of isoprotruron concentration (Fig. 5). It is clear that v was gradually inhibited with increasing isoprotruron concentration up to 12 μM . The inhibition in v of GR and GST was steady with the presence of isoprotruron; it represented about 63% with 12 μM relative to v without the presence of the herbicide. However, the inhibition in v was most pronounced for GPX followed by GS and γ -GCS representing about 44, 46 and 56%, respectively with 12 μM isoprotruron relative to v without the presence of the herbicide.

Plotting isoprotruron concentration versus the percentages of inhibition of v relative to blank for each enzyme gave straight lines from which IC₅₀ values were calculated. It is clear that IC₅₀ was highest for both GST and GR and lowest for GPX; however, moderate values were detected for both γ -GCS and GS. These results confirm the inhibitory effect of isoprotruron to these enzymes, the inhibition was greatest for GPX but lowest for both γ -GCS and GS; however, both GR and GST were moderately inhibited.

In vivo tests achieved v of enzyme extracts obtained from shoots of treated seedlings (on the 5th day from treatment) (Fig. 6). Plotting the changes of v for all enzymes against substrate concentration ([S]) gave the saturation curve according to Michaelis–Menten plot. These data were converted into linear equation using Lineweaver–Burk and Hanes equations gave plots with straight lines used to calculate V_{max} and K_{m} values and presented in Table 1. Isoprotruron lowered V_{max} of γ -GCS, GS and GPX; however, it did not change V_{max} of both GST and GR. There were very little variations among the calculations using Lineweaver–Burk or Hanes equations. On the other hand, increases in K_{m} values of γ -GCS, GS,

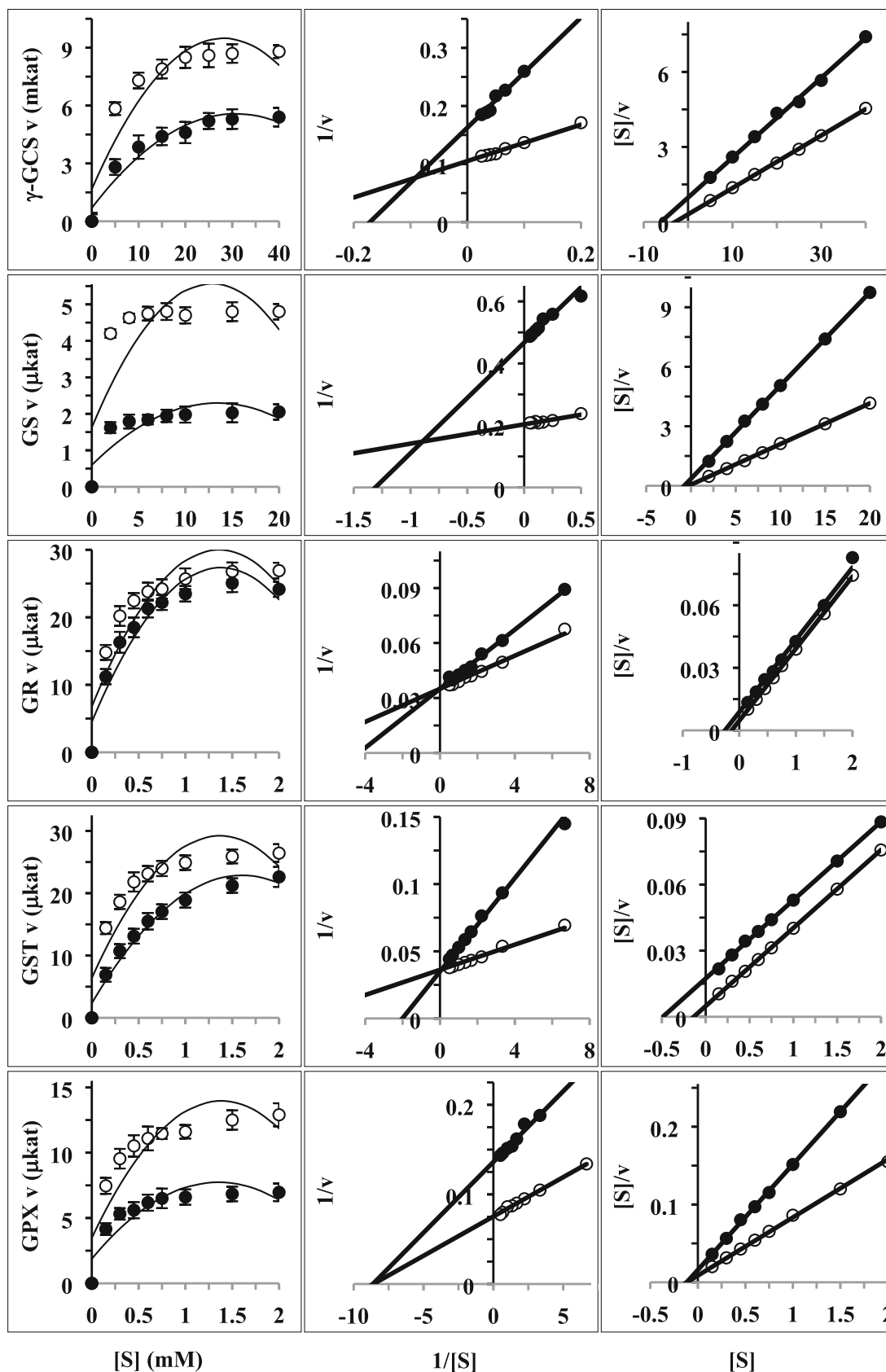


Table 1 Changes in kinetic parameters (V_{max} , K_m , k_{cat} , k_{cat}/K_m and V_{max}/K_m) of the glutathione-associated enzymes (γ -glutamyl-cysteine synthetase (γ -GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPX)) as functions of their substrates according to Lineweaver–Burk and Hanes equations

Enzyme	Treatment	V_{max}^a	K_m^a	k_{cat}^b	k_{cat}/K_m	V_{max}/K_m
In vivo	Lineweaver–Burk					
γ -GCS	Control	9.51 ± 0.747	2.98 ± 0.283	2.72	0.91	3.19
	Treated	6.15 ± 0.549*	5.83 ± 0.554*	1.76	0.30	1.06
GS	Control	4.92 ± 0.397	0.31 ± 0.033	1.41	4.59	16.08
	Treated	2.14 ± 0.258*	0.76 ± 0.066*	0.61	0.80	2.80
GR	Control	28.57 ± 2.39	0.13 ± 0.016	8.16	63.49	222.22
	Treated	28.57 ± 2.677	0.23 ± 0.022*	8.16	35.27	123.46
GST	Control	27.62 ± 2.147	0.13 ± 0.017	7.89	60.79	212.77
	Treated	28.66 ± 2.982	0.50 ± 0.036*	8.79	36.42	153.67
GPX	Treated	7.34 ± 0.779*	0.12 ± 0.011	2.10	18.08	63.29
	Hanes					
γ -GCS	Control	9.51 ± 0.844	2.88 ± 0.278	2.72	0.94	3.30
	Treated	6.27 ± 0.811*	6.10 ± 0.556*	1.79	0.29	1.03
GS	Control	4.88 ± 0.647	0.26 ± 0.033	1.39	5.40	18.90
	Treated	2.13 ± 0.303*	0.76 ± 0.064*	0.61	0.80	2.81
GR	Control	28.90 ± 2.912	0.13 ± 0.012	8.26	62.11	217.39
	Treated	28.82 ± 2.847	0.25 ± 0.024*	8.23	32.84	114.94
GST	Control	28.25 ± 3.345	0.14 ± 0.013	8.07	59.52	208.33
	Treated	28.09 ± 2.784	0.49 ± 0.038*	8.03	16.42	57.47
GPX	Control	13.39 ± 1.211	0.12 ± 0.014	3.82	33.22	116.28
	Treated	7.38 ± 0.813*	0.12 ± 0.013	2.11	17.97	62.89
In vitro	Lineweaver–Burk					
γ -GCS	Blank	9.43 ± 0.887	2.69 ± 0.217	2.69	1.18	4.12
	IPU	6.30 ± 0.721*	5.11 ± 0.678*	1.80	0.35	1.23
GS	Blank	4.67 ± 0.495	0.33 ± 0.038	1.34	4.05	14.18
	IPU	2.20 ± 0.227*	0.67 ± 0.054*	0.63	0.63	3.30
GR	Blank	28.33 ± 3.116	0.13 ± 0.018	8.09	62.11	217.39
	IPU	28.01 ± 2.921	0.29 ± 0.019*	8.00	27.74	97.09
GST	Blank	28.57 ± 2.873	0.11 ± 0.014	8.16	73.26	256.41
	IPU	27.93 ± 2.487	0.38 ± 0.032*	7.98	20.86	72.99
GPX	Blank	14.22 ± 1.547	0.10 ± 0.011	4.06	42.64	149.25
	IPU	8.37 ± 0.889*	0.10 ± 0.008	2.39	24.84	86.96
γ -GCS	Hanes					
	Blank	9.54 ± 0.965	2.41 ± 0.231	2.73	1.13	3.97
IPU	6.31 ± 0.686*	5.09 ± 0.477*	1.80	0.35	1.24	

Table 1 continued

Enzyme	Treatment	Vmax ^a	Km ^a	kcat ^b	kcat/Km	Vmax/Km
GS	Blank	4.68 ± 0.487	0.33 ± 0.041	1.34	4.05	14.18
	IPU	2.21 ± 0.255*	0.67 ± 0.088*	0.63	0.94	3.30
GR	Blank	28.09 ± 2.144	0.12 ± 0.019	8.03	64.94	227.27
	IPU	28.01 ± 2.776	0.29 ± 0.018*	8.00	27.21	95.24
GST	Blank	29.07 ± 3.008	0.11 ± 0.008	8.30	73.26	256.41
	IPU	28.09 ± 2.014	0.38 ± 0.029*	8.03	20.86	72.99
GPX	Blank	14.22 ± 1.621	0.10 ± 0.012	4.06	42.64	149.25
	IPU	8.37 ± 0.798*	0.10 ± 0.016	2.39	24.84	86.96

For in vivo test, shoots of 15-day-old treated wheat seedlings (5 days after treatment with the recommended field dose) were used. For in vitro test, shoots of 15-day-old untreated wheat seedlings were used with the addition of isotoproturon (IPU) to give a final concentration of 4 μM to reaction mixtures

^aVmax (μKat ± SD) and Km (mM substrate ± SD) values were obtained by data interpolation from equations presented in Lineweaver–Burk and Hanes plots (Figs. 6 and 7), (n = 6).

^bSignificant difference from control at $p \leq 0.05$

^ckcat was obtained from dividing mean values of Vmax by the concentration of the enzyme

GST and GR were detected in response to isotoproturon whereas Km of GPX remained unchanged. Also, the catalyst rate constant (kcat) of γ-GCS, GS and GPX were retracted in response to isotoproturon application whereas kcat of GST and GR was unchanged. Isoproturon declined the values of the catalytic efficiency (kcat/Km and Vmax/Km) of all enzymes.

v of enzyme extracts obtained from shoots of untreated 15-day-old seedlings was determined with the presence of isotoproturon in the reaction mixtures (in vitro tests) (Fig. 7). Plotting v against [S] followed also the saturation curve of Michaelis–Menten plot. Using both Lineweaver–Burk and Hanes equations, Vmax and Km were calculated from the obtained straight lines (Table 1). Similar to in vivo tests, isotoproturon decreased Vmax of γ-GCS, GS and GPX but unaffected that of both GST and GR, the calculations using both Lineweaver–Burk and Hanes equations were very close. Nonetheless, isotoproturon increased Km of γ-GCS, GS, GST and GR but unchanged that of GPX. The values of kcat for γ-GCS, GS and GPX were decreased by isotoproturon; however, kcat values for GST and GR were unchanged. Isoproturon generally declined kcat/Km and Vmax/Km.

However, the straight lines obtained from plotting isotoproturon concentration versus (Vmax – v)/v for each enzyme was performed to calculate Ki values (Fig. 8). Isoproturon concentration against (Vmax – v)/v for each enzyme gave straight lines according to Yoshino and Murakami (2009) and satisfied the equation $\{(V_{max}-v)/v\} = (1 + [I]/K_i)(K_m/[S])$ for competitive inhibition, the equation $\{(V_{max}-v)/v\} = (K_m/[S]) + (K_m/[S] + 1)([I]/K_i)$ for noncompetitive inhibition or the equation $\{(V_{max}-v)/v\} = (K_m/[S]) + (K_m/[S])(1/K_i + [I]/K_i[I])$ for the mixed type inhibition. It is obvious that the values of Ki were highest for both GST and GR and lowest for GPX; however, these values were moderate for both γ-GCS and GS. These results further confirm that the greatest inhibition of isotoproturon was induced in GPX; however, the inhibition was lowest for both GR and GST but moderate for both γ-GCS and GS.

Discussion

The results depicted herein indicate that isotoproturon provoked significant reduction in wheat growth as indicated from the decreases in shoots fresh and dry weights. Such reduction could result from general alterations in certain metabolic processes. Protein content was significantly decreased by isotoproturon treatment pointing to malfunction in plant metabolism. On the other hand, some processes using protein would suffer from retractions in their functions due to supply shortage. In general, several reports

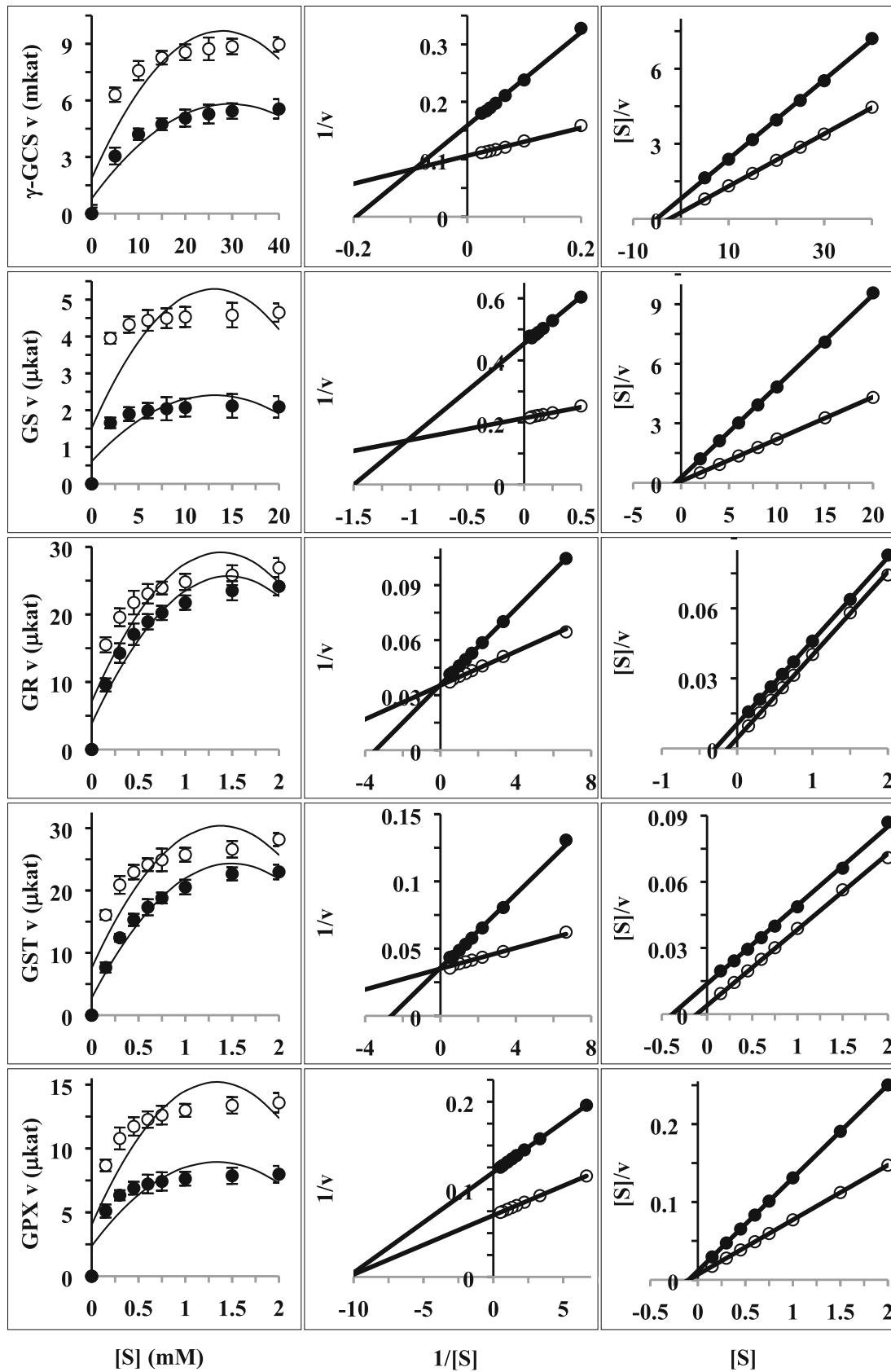


Fig. 7 In vitro tests of the velocities (v , μKat) of the enzymes γ -glutamyl-cysteine synthetase (γ -GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPX) as function of the respective substrate concentration $[S]$ according to Michaelis–Menten plot ($[S]$ versus v), Lineweaver–Burk Plot ($1/[S]$ versus $1/v$), and Hanes Plot ($[S]$ versus $[S]/v$). Isoproturon (IPU) was added to give a final concentration of $4 \mu\text{M}$ to reaction mixtures containing enzyme preparations obtained from shoots of 15-day-old untreated wheat seedlings. White circle, Blank; Black circle, IPU. v and $[S]$ values are means \pm SD ($n = 6$). Lineweaver–Burk and Hanes plots were performed using the final means of v and $[S]$

indicated reductions in plant growth following the application of several herbicides (Hassan and Nemat Alla 2005; Nemat Alla et al. 2007, 2008a; Yin et al. 2008; Reis et al. 2011; Jahns and Holzwarth 2012). Rutherford and Krieger-Liszky (2001) hypothesized that the plant is killed by oxidative stress. Isoproturon most likely affects the generation of reactive oxygen species (ROS), with a consequence of severe changes in the redox state (Nemat Alla et al. 2008b; Reis et al. 2011). It has been indicated that ROS cause lipid peroxidation, inactivation of enzymes and membrane damage, and affects cell viability (Badran et al. 2015; Chakraborty et al. 2019).

So, oxidative stress resulted from isoproturon might cause the reduction of wheat growth in the present study. However, plants have a system for scavenging of ROS to protect cells from oxidative damage; the most efficient non-enzymatic antioxidants are glutathione (GSH) and other thiol compounds (Nemat Alla et al. 2008b; Zushi and Matsuzoe 2009). So, the decreases in thiol pool and GSH by isoproturon, in the present results, might suggest the production of a state of oxidative stress. This stress might lead to destruction in the defense system that is based particularly on GSH biosynthesis. Thiol compounds play several roles in the control of oxidative stress and moreover in the protection against xenobiotics (Mendoza-Cózatl and Moreno-Sánchez 2006). In most aerobic organisms, GSH is considered as a key component of antioxidant defenses. Moreover, it could be regarded as limiting for herbicide tolerance. GSH has role as a modulator of enzymic activities and also in nucleic acids and proteins synthesis as well as in the transport and storage of reduced Sulphur and in plant adaptation to xenobiotics tolerance (Nemat Alla and Hassan 1998).

So, GSH acts to detoxify ROS through active enzymatic pathways. GSH-associated enzymes catalyze the reduction of H_2O_2 to water in the ascorbate-GSH cycle for the maintenance of GSH (Nagalakshmi and Prasad 2001; Aravind and Prasad 2005; Kong et al. 2017). GSH is oxidized to GSSG, so the decreased GSH level by isoproturon could lead to deficiency in the antioxidant system. On the other hand, the depletion of GSH concomitant with rises in

GSSG could suggest the failure in the reduction of GSSG to GSH through GR catalytic action. The decrease in GSH level is not only due to the failure in reduction of GSSG to GSH but also to an inhibition in its biosynthesis. GSH biosynthesis takes place in two steps; first, L-cysteine is conjugated to L-glutamate by γ -GCS forming γ -glutamyl-cysteine and in the second step, glycine is added by GS (Gupta et al. 2005; Hasanuzzaman et al. 2017). Hasanuzzaman et al. (2017) indicated that GSH is considered as a strong non-enzymatic antioxidant that regulates multiple metabolic functions and prevents the oxidative denaturation of proteins under stress conditions, and helps in the chelating of xenobiotics. Thus, the inhibited activities of GR, γ -GCS and GS due to isoproturon, in the present results, could explain the decreased levels of GSH particularly with the elevation of GSSG.

On the other hand, some reports concluded that GST increases the plant defense against several biotic and abiotic agents (Misra et al. 2006). However, some GST isoforms can also function as GPX (Volohonsky et al. 2002). Aravind and Prasad (2005) concluded that GPX is a part of the arsenal of the protective enzymes which respond to stress. Anderson and Davis (2004) concluded that GSH is utilized by GST, GPX, and GR to play roles in defense mechanisms of plants. Gehin et al. (2006) found that the glyphosate-caused depletion of GSH was accompanied with GPX disorders. Moreover, Aravind and Prasad (2005) stated that not only GR maintained GSH pool but also γ -GCS and GS have a great role. So, that activities of GST and GPX depend on the availability of GSH, which are maintained by GR and the depression of GSH/GSSG ratio in wheat by isoproturon was related with GSH depletion and/or GSSG rises in addition to the inhibited activities of GST, GR, γ -GCS, and GS. This suggestion could confirm the imposed stress status in wheat by isoproturon, concluding, therefore, that it could be considered as an inhibitor for the GSH-associated enzymes (γ -GCS, GS, GR, GST and GPX). In confirmation to these findings; the activity gels showed variable decreases in bands of all enzymes in samples treated with isoproturon. The treated samples had bands with less density relative to control. The decrease in the enzyme band densities was time-dependent. This could prove that the herbicide might affect the enzyme quantity following treatment. The herbicide impact was highest for GPX but lowest for both GST and GR.

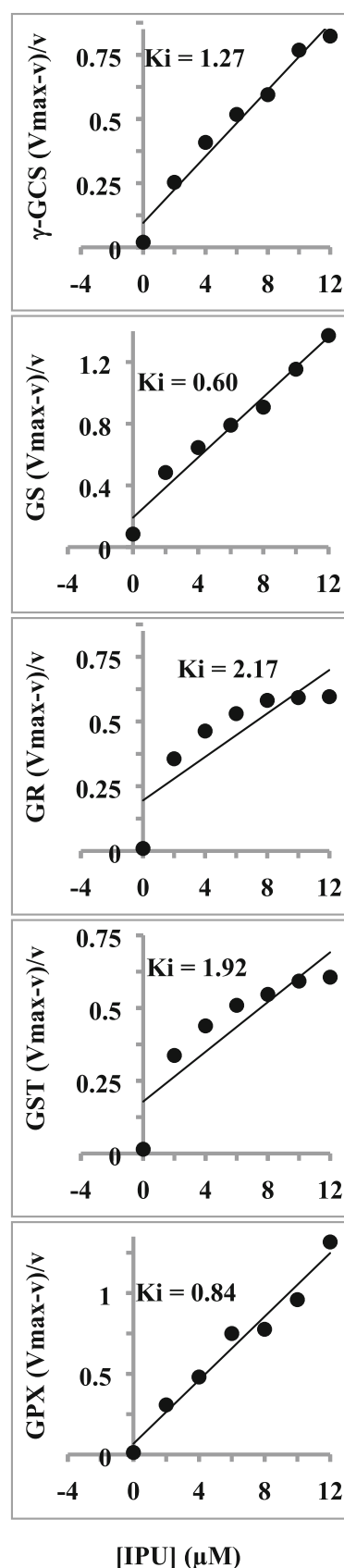
The changes in the enzyme kinetic parameters values (V_{max} and K_m) by isoproturon could result in variations of enzyme inhibition. These parameters are useful as descriptors of enzymes because they define the enzyme-catalyzed reaction rate. K_m represents the enzyme's affinity for its substrate while V_{max} is considered as enzyme concentration function (Engel 1984; Tovar-Mendez and Munoz-Clares 2001). The increases in K_m values

Fig. 8 In vitro tests of the velocities (v , μKat) {relative to V_{max} , $(V_{\text{max}}-v)/v$ } of the enzymes γ -glutamyl-cysteine synthetase (γ -GCS), glutathione synthetase (GS), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPX) as functions of isoproturon (IPU). The enzyme preparations were obtained from shoots of 15-day-old untreated wheat seedlings

of GST and GR by isoproturon while V_{max} remained unchanged could deduce that the herbicide effect was exerted on only the structural integrity of both enzymes. So, isoproturon could be regarded as a competitive inhibitor for both GST and GR enzymes. On the contrary, the decrease in V_{max} of GPX by the herbicide with unchanging K_{m} could deduce that the effect of isoproturon was exerted on only the enzyme synthesis with an effect only on its concentration concluding that GPX is non-competitively inhibited by isoproturon. Nevertheless, V_{max} values of both γ -GCS and GS were decreased by isoproturon concomitantly with increasing K_{m} values confirming an induced mixed type inhibition for γ -GCS and GS enzyme.

On the other hand, isoproturon depressed k_{cat} for γ -GCS, GS and GPX; nonetheless, both $k_{\text{cat}}/K_{\text{m}}$ and $V_{\text{max}}/K_{\text{m}}$ for all enzymes were highly declined. The decrease in K_{cat} of γ -GCS, GS and GPX could confirm their great inhibition by isoproturon. On the contrary, k_{cat} values of GST and GR remained unchanged indicating less inhibition following isoproturon application. However, the consequent drop in $k_{\text{cat}}/K_{\text{m}}$ and $V_{\text{max}}/K_{\text{m}}$ for all enzymes confirms the loss in their catalytic efficiency. So, these findings support that isoproturon affected all enzymes at the concentration level and/or at the catalytic efficiency level leading to greatest inhibition to GPX followed by γ -GCS, GS and finally by GST and GR.

In confirmation with this conclusion, IC_{50} of isoproturon was $9.66 \mu\text{M}$ for GPX; however, this value was raised to 19.34 , 14.72 , 12.32 and $9.77 \mu\text{M}$ for GST, GR, γ -GCS and GS, respectively. These results indicate that isoproturon inhibited GPX highly followed by GS, γ -GCS, GR and finally GST. Moreover, the magnitude of K_{i} values were 2.17 , 1.92 , 1.27 , 0.84 and $0.60 \mu\text{M}$ for GR, GST, γ -GCS, GPX and GS, respectively confirming that GPX and GS were the most inhibited enzymes by isoproturon; nonetheless, the least inhibited ones were GR and GST. The trends of the inhibition of these enzymes by isoproturon seemed alike on the basis of both IC_{50} and K_{i} , despite the slight differences, concluding that the inhibition was higher for GPX and GS followed by γ -GCS then both GR and GST.



Conclusion

Isoproturon significantly declined the wheat growth, protein, GSH and GSH/GSSG ratio, thiols compounds; however, it elevated GSSG. Meanwhile, isoproturon differentially inhibited the activities of the GSH-associated enzymes (γ -GCS, GS, GR, GST and GPX); the effect was time dependent. The inhibition constant were lowest for GPX, highest for both GST and GR, and moderate for γ -GCS and GS concluding that the inhibition was greatest for GPX but least for both GST and GR. Isoproturon provoked decreases in Vmax of GPX but unchanged Km; however, it increased Km of GST and GR but unchanged their Vmax but inhibited Vmax of both γ -GCS and GS concomitant with increasing their Km values were increased. There were no great differences among the in vivo and in vitro tests. These results indicate that isoproturon resulted in malfunction to defense system of wheat and induced inhibitions in GSH-associated enzymes, the inhibition was most pronounced in GPX followed by γ -GCS, GS, GST, and GR. These findings could conclude that isoproturon competitively inhibited GST and GR but noncompetitively inhibited GPX; however, the inhibition of both γ -GCS and GS was mixed type inhibition.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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