The antagonistic and synergistic effects of naphthalic anhydride and 1-aminobenzotriazole to imazethapyr toxicity in maize

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Abstract

Ten-day-old maize seedlings, grown from grains dressed with naphthalic anhydride (NA, 0.4% w/w by seed weight), were treated with imazethapyr (Imz), 1-aminobenzotriazole (ABT), or their combination and samples were collected after 5 days. Imz provoked significant reduction in growth parameters concomitant with inhibition in acetohydroxyacid synthase (AHAS) activity, protein, branched-chain amino acids, glutathione (GSH), and ascorbate (AsA). However, lipid peroxides and H_2O_2 were elevated while the activities of nitrate reductase/nitrite reductase (NR/NiR), glutamine synthetase/glutamate synthase (GS/GOGAT), and catalase/ascorbate peroxidase (CAT/APX) were inhibited. The application of NA alleviated these impacts and declined Imz residues while ABT led to high levels; nonetheless, NA became no longer effective when ABT was present. These findings indicate that Imz toxicity in maize is antagonized by NA and synergized by ABT. Such antagonism or synergism could be concluded to be attributed to enhanced detoxification or protraction of herbicide persistence, respectively. On the other hand, the changes in both AHAS activity and the branched-chain amino acids due to treatments were highly correlated. The slight changes in NR/NiR and the inhibition in GS/GOGAT reveal that protein drop is not due to ammonia demand but to decreased branched-chain amino acids. Moreover, Imz decreased Vmax of AHAS, NR, NiR, GS, GOGAT, CAT, and APX but unchanged their Km except the increased Km of only AHAS indicating that the inhibition is mixed for AHAS and noncompetitive for the others.

Keywords: acetohydroxyacid synthase; branched-chain amino acids; catalase/ascorbate peroxidase; glutamine synthetase/glutamate synthase; nitrate reductase/nitrite reductase

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Introduction

Imazethapyr {Imz, 2-[4,5-dihydro-4-methyl-4[1-methylethyl]-5-oxo-1-H-imidazol-2-yl]-5-ethyl-3-pyridine carboxylic acid} is an imazolinone herbicide that affects protein synthesis in plant through the inhibition of acetohydroxyacid synthase (AHAS); it is the first enzyme involved in valine, leucine, and isoleucine biosynthesis (Relton et al., 1986; Sondhia et al., 2015). In general, herbicides drastically influence all aspects of plant metabolism. The whole plant resistance to herbicides was attributed to less sensitivity of the target site and/or increased

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herbicide degradation (Nemat Alla et al., 2007; Huang et al., 2019). The inhibitory effect of herbicides on metabolism could result in retardation in many metabolic processes and lead to growth inhibitions. Most likely, several herbicides promote their toxic effects on photosynthetic organisms by affecting the production of reactive oxygen species (ROS) which disturb redox homeostasis and enhances membrane lipid peroxidation, protein oxidation, enzyme inhibition, DNA and RNA damage, biosynthesis of osmolytes, ion homeostasis, and gene expression (Nemat Alla et al., 2008a, b; Chakraborty et al., 2019).

Herbicide tolerance is a highly desirable trait in crops that can be explained by effectors of stress adaptation that mediate scavenging of ROS. To cope with harsh conditions, plants possess endogenous mechanisms to overcome stresses and scavenge ROS to protect cells from oxidative damage through efficient antioxidants (Nemat Alla and Hassan, 2006). GSH and AsA participate in ROS scavenging through AsA-GSH cycle (Nayyar and Gupta, 2006). However, these mechanisms are often not enough in the susceptible species; therefore, exogenous supporters are needed for the plant to withstand stress imposed by herbicides and to protect itself from toxicity by mitigating the herbicide effects (Jang et al., 2019) or by increasing its degradation via glucose conjugation and hydroxylation (Hatzios, 2000). Of these supporters are the safeners such as 1,8-naphthalic anhydride (NA) which are compounds that are utilized to protect crops from herbicidal injury by accelerating their detoxification and metabolism (Hatzios, 2000; Deng and Hatzios, 2003; Nemat Alla and Hassan, 2008) or antagonizing the herbicides at a common target site (Deng and Hatzios, 2003; Nemat Alla and Hassan, 2008). Nemat Alla and Hassan (2019) indicated that NA might cause mitigation of chlorimuron-ethyl toxicity to soybean by detoxifying the herbicides. Moreover, detoxification of several groups of herbicides involves the oxidative pathway by cytochrome P450 monooxygenases (Reichhart, 1995); however, the inhibitors of P450s such as 1-aminobenzotriazole (ABT) increase the phytotoxicity of most herbicides metabolized by P450 enzymes and can be used as a synergist of most herbicides metabolized by P450s (Moreland et al., 1993; Reichhart, 1995). Although the functions of NA and ABT are well elucidated, the effect of NA regarding the interaction with inhibitors of P450s such as ABT is scarce. So, the present work aims at investigating the toxicity of Imz to maize, the alleviation of Imz toxicity by NA, the kinetic of Imz inhibition to the examined enzymes, and if it is safe to use NA as a safener while ABT is used to synergize the herbicide for controlling weeds and grasses. For this purpose, maize seedlings grown from dressed grains were treated with Imz and/or ABT then tests were performed on the herbicide residues, protein, and branched-chain amino acids levels, activity of AHAS and protein-related enzymes (NR, NiR, GS, GOGAT) as well as the antioxidants either enzymatic (CAT and APX) or non-enzymatic (GSH and AsA), and oxidative stress indices (MDA and H_2O_2).

Materials and Methods

Plant material and growth conditions

A part of maize grains (*Zea mays* L.) was dressed with naphthalic anhydride (NA, 0.4% w/w by seed weight) and another part was left as control. Fifteen seeds were planted in plastic pots (40 × 20 × 10 cm) in quartz sand (pre-washed with hydrochloric acid) approximately 2 cm-depth and spaced 5 cm apart in 5 cm adjacent rows and placed in greenhouse at 30±5/20±5 °C, day/night temperature with a 14-h photoperiod at 450-500 µmol m^{-2} s^{-1} PPFD and watered as required. When seedlings were 10-day-old, the pots of each part were divided into 4 groups; one for control (Cont), one for treatment with imazethapyr (Imz), one for 1-aminobenzotriazole (ABT), and the fourth for combination of Imz+ABT (8 treatments). Imz and ABT were applied at rates equivalent to 35 and 225 g ai ha^{-1}, respectively. The quantities were mixed in a suitable amount of water and Tween-20 (0.25%) was added as a surfactant, then applied to pots twice with a mechanical sprayer, in one direction and crosswise. The shoots were harvested on th 5^{th} day after the herbicide treatment, washed with copious amounts of water, and used for the subsequent analyses, a part was weighed for recording fresh weights then

dried in an oven for dry weight and water content measurements. The 2nd leaf was collected for analysis, cut into small pieces, and homogenized in the respective extraction buffer. The experiment was repeated twice and samples were taken from both experiments for each analysis in triplicates (n=6).

Activity assay of acetohydroxyacid synthase (AHAS)

The enzyme was extracted in potassium phosphate (50 mM, pH 7.5) containing 1 mM from each of magnesium sulfate, pyruvate, and leucine and valine, 0.5 mM FAD, 10% ethandiol with 0.05% Triton-X-100 and polyvinyl polypyrolidone then centrifuged at 20000 ×g for 30 min and the supernatant fractioned with ammonium sulfate (Relton et al., 1986). The activity was assayed by measuring the production of acetolactate from pyruvate upon its decarboxylation to acetoin in sodium phosphate (100 mM, pH 7.0) containing 10 mM $MgCl_2$, 2.5 mM TPP, and 50 mM sodium pyruvate, and 10 µM FAD (Singh et al., 1988). The amount of the acetoin produced was quantified by adding 0.5% creatine and 5% 1-naphthol in 4 N NaOH, incubated at 60 °C for 15 min and at room temperature for another 15 min with frequent mixing then the absorbance change was recorded at 525 nm.

Determination of amino acids

Amino acids were extracted with methanol and phase separated by chloroform and distilled water and eluted on Dowex 50-H^+ with NH_4OH (Rhodes et al., *19*87). The eluent was dried, re-dissolved in methanol/1 M sodium acetate/trimethylamine (TEA, 2:2:1) then derivatized to phenylisothiocyanate-derivatives (Bidlingmeyer et al., 1987) and 500 µl sodium phosphate (5 mM, pH 7.6) containing 5% acetonitrile were added then 6.0 µl were injected into Perkin-Elmer HPLC assembled from pumps (Series 410 LC), injector (Rheodyne Model 7125-075, 235), detector (diode array), a LC-18 DB column Supelcosil (25 cm x 4.6 mm, 5 µm particle size) protected with a Super guard pre-column (3 cm long), solution A (140 mM sodium acetate containing TEA, and adjusted to pH 6.4 with glacial acetic acid), solution B (acetonitrile: water, 60: 40), the flow rate (0.8 ml min^{-1}), the flow was gradient with respect to solution B (10% at start, 6 min to 12.5%, 32 min to 58%, 33 min step 100%, and 12 min wash for re-equilibration at 10% again).

Determination of Imz residues

Residues were extracted with 0.5 N NaOH and methanol then partitioned with dichloromethane (Sondhia et al., 2015). The lower layer was collected and evaporated then dissolved in methanol and cleaned up using a glass column packed with Celite and activated charcoal between anhydrous sodium sulfate at each end. The extract was eluted with methanol/water (60/40), the elute was collected and evaporated then dissolved in methanol and filtered through Pall Nylon 0.45-µm filter paper prior to HPLC analysis using Perkin-Elmer HPLC assembled from pumps (Series 410 LC), injector (Rheodyne Model 7125-075, 235), detector (diode array) with λmax of 250 nm for detection purposes, a Phenomenex C-18 (ODS) column (250×4.6 mm) and methanol:water (70:30) as a mobile phase at a flow rate of 0.9 ml min^{-1}.

Protein extraction and determination

Samples were homogenized with chilled acetone, filtered, and residues were spread on filter paper to dry for obtaining the acetone powders (Harborne, 1988). Aliquots of powders were mixed with Tris-HCl (0.05 M, pH 9.0) at 4 °C, centrifuged at 48200 ×g for 15 min at 4 °C then absorbance was read at 595 nm after reaction with Brilliant Blue G (Bradford, 1976).

Activity assay of nitrate reductase (NR) and nitrite reductase (NiR)

Extraction and assay of NR were carried out according to Nakagawa et al. (1985). Extraction was performed in potassium phosphate (250 mM, pH 8.0) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), 5% isopropyl alcohol, 1 mM β-mercaptoethanol, 1 mM EDTA, 5 mM potassium nitrate, 0.02 mM FAD, and 6.3% polyclar AT then centrifuged at 10000 ×g for 15 min. The activity was determined in potassium

phosphate (25 mM, pH 7.5) containing 5 mM potassium nitrate and 0.1 mM NADH, then the reaction was stopped by the diazo-coupling reagents [1% sulphanilamide in 3 N HCl and 0.02% N-(1-naphthyl)-ethylenediamine dihydrochloride] and absorbance was measured at 540 nm after 20 min.

NiR was extracted in Tris-HCl (50 mM, pH 7.9) containing 5 mM cysteine, 2 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, and 5% polyclar AT (Nagaoka et al., 1984). The assay was performed in potassium phosphate (33 mM, pH 7.5) containing 2 mM potassium nitrite, 1 mM methyl viologen, and 11.6 mM sodium dithionite (Wray and Filner, 1970). The reaction was started by the addition of 2 mg sodium dithionite in 0.29 M sodium bicarbonate, incubated in open tubes, then the reaction was stopped by vigorous shaking until the loss of the blue color, then the diazo-coupling reagents [1% sulphanilamide in 3 N HCl and 0.02% N-(1-naphthyl)-ethylenediamine dihydrochloride] were added and absorbance was measured at 540 nm after 20 min.

Activity assay of glutamine synthetase (GS) and glutamate synthase (GOGAT)

The extraction and assay of GS were carried out according to Lea et al. (1990). The extraction was performed in Tris-HCl (50 mM, pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol, 10 mM magnesium sulphate, 5 mM sodium glutamate and 10% ethandiol, and centrifuged at 15000 ×g for 15 min. The assay was performed in Tris-HCl (100 mM, pH 7.8) containing 50 mM glutamate, 5 mM hydroxylamine hydrochloride, 50 mM magnesium sulphate and 20 mM ATP, and incubated then the reaction was terminated by ferric chloride reagent (0.67 M ferric chloride, 0.37 M HCl, and 20% trichloroacetic acid, TCA). The amount of γ-glutamylhydroxamate released was determined by following the absorbance at 540 nm.

GOGAT was extracted in potassium phosphate (50 mM, pH 7.5) containing 100 mM potassium chloride, 5 mM EDTA, 12.5 mM β-mercaptoethanol, 1 mM PMSF, 2 mM 2-oxoglutarate, 20% ethandiol, and 0.05% Triton X-100, and centrifuged at 15000 ×g for 15 min (Marquez et al., *19*88). The assay was performed in potassium phosphate (100 mM, pH 7.5) containing 0.1 mM NADH, 10 mM glutamine and 10 mM 2-oxoglutarate (Hecht et al., 1988). The reaction was followed at 340 nm for measuring the oxidation of NADH for about 10 min at 30 °C.

Determination of lipid peroxides and H_2O_2

Lipid peroxides and H_2O_2 were extracted with chilled 0.1% TCA. Lipid peroxides were assayed as malondialdehyde by reaction with thiobarbituric acid in boiling water bath for 10 min, cooled and diluted with distilled water and the absorbance was read at 535 nm (Buege and Aust, 1972). H_2O_2 was assayed in phosphate buffer (375 mM, pH 6) containing 12.5 mM 3-dimethylaminobenzoic acid and 1.3 mM 3-methyl-2-benzothiazolinone hydrazine then the increase in absorbance at 590 nm was monitored for 3 min (Okuda et al., 1991).

Determination of glutathione (GSH) and ascorbic acid (AsA)

GSH was extracted in TCA (5%) containing EDTA (10 mM) and centrifuged at 12000 ×g for 15 min. GSH was assayed in phosphate buffer (100 mM, pH 6.8) containing 10 mM EDTA, 1 mM 1-chloro-2,4-dinitrobenzene and 1.0 U equine glutathione-S-transferase and incubated at 35 ℃ for 30 min. The absorbance at 340 nm was recorded before commencing the reaction and after the reaction had run to completion (Anderson and Gronwalds, 1991). AsA was extracted in 62.5 mM phosphoric acid and centrifuged at 12000 ×g for 20 min then eluted with 4.5 mM H_2SO_4 at a flow rate of 0.5 ml min^{-1}. AsA was determined in sodium molybdate (0.66%) containing 0.05 N H_2SO_4 and 0.025 mM sodium phosphate, then centrifuged at 4000 ×g for 5 min and absorbance was read at 660 nm (Ahn et al., 1999).

Activity assay of catalase (CAT) and ascorbate peroxidase (APX)

CAT was extracted in phosphate buffer (50 mM, pH 7) containing EDTA (2 mM) and β-mercaptoethanol (5 mM) then centrifuged for 10 min at 12000 ×g at 4 ℃. CAT was assayed in

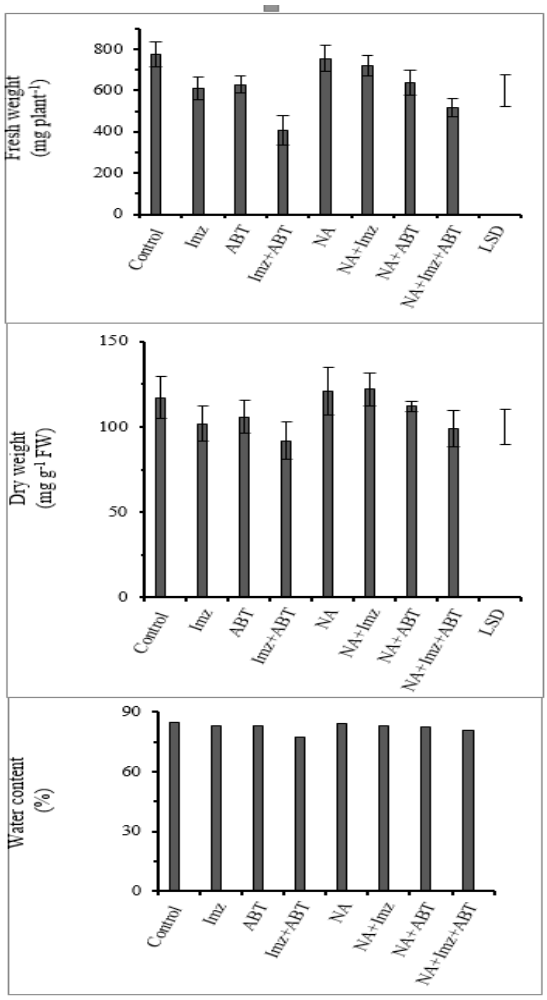

Fig. I. Changes in fresh weight, dry weight, and water content of maize shoots due to treatment with imazethapyr (Imz) and/or 1-aminobenzotriazole (ABT) at 35 and 225 g ai ha^{-1}, respectively; ten-day-old seedlings grown from undressed (Control) or dressed grains with naphthalic anhydride (NA, 0.4%) were subjected to the treatment and samples were collected 5 days after. Values are means ± SD, (n = 6 from two independent experiments). Data were subjected to ANOVA and LSD calculated at $P \leq 0.05$. s at the column top means significantly different from the untreated control.

phosphate buffer (50 mM, pH 7.5) containing 200 mM H_2O_2 by determining the consumption of H_2O_2 at 240 nm. APX was extracted in Tricine-KOH (0.1 M, pH 8) containing 1 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, and 0.28 mM PMSF. APX activity was determined in phosphate buffer (50 mM, pH 7.5) containing 40 mM Na ascorbate and 200 mM H_2O_2 at 270 nm (Nakano and Asada, 1981).

Determination of kinetic parameters of enzymes

Kinetic parameters (Vmax, Km, and Kcat) were determined by performing *in vitro* tests using enzyme preparations obtained from 10-day-old untreated seedlings. Imz was added to give a final concentration of 100 nM in the reaction mixtures. The velocities (v) of AHAS, NR, NiR, GS, GOGAT, CAT, and APX were assayed as functions of changing the concentrations of the respective substrate [S] (pyruvate, potassium nitrate, potassium nitrite, hydroxylamine, NADH, H_2O_2 and ascorbate, respectively) ranging from 15-400, 1-50, 0.5-15, 1-50, 2-75, 2-100, and 10-400 mM, respectively. v was plotted against [S] according to Michaelis-Menten saturation plot and equation {$v = Vmax\ [S]/(Km+[S])$}. Vmax and Km were calculated from Lineweaver-Burk plot and equation {$1/v= (1/[S])\ (Km/Vmax) + (1/Vmax)$} and further confirmed from Hanes plot and equation {$[S]/v=([S]/Vmax) +(Km/Vmax)$}. The catalytic rate (Kcat) was obtained from dividing Vmax by the concentration of the enzyme, then the catalytic efficiency (Kcat/Km) was calculated.

Statistical analysis

The experiment was repeated twice and designed as a complete randomized block consisting of 160 pots (8 set treatments) x (10 replications) x (2 repetitions). Each measurement was repeated until consistent results were obtained with at least six independent measurements (n=6). The values were represented as means ± SD. Samples were compared using one-way ANOVA and LSD was performed using SPSS.

Results

Ten-day-old maize seedlings, grown from grains dressed with naphthalic anhydride (NA, 0.4% w/w by seed weight), were treated with imazethapyr (Imz) and/or 1-aminobenzotriazole (ABT) then samples were collected 5 days after treatment. The results show that Imz significantly reduced shoot fresh and dry weights as compared to control, the magnitude of reduction augmented with the presence of ABT; however, ABT alone resulted in significant reduction too (Fig. I). On the

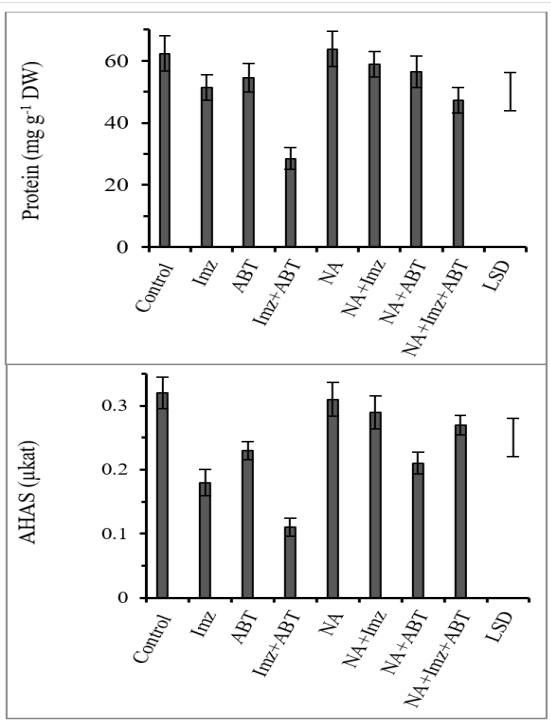

Fig. II. Changes in protein content and activity of acetohydroxyacid synthase (AHAS) of maize shoots due to treatment with imazethapyr (Imz) and/or 1-aminobenzotriazole (ABT) at 35 and 225 g ai ha^{-1}, respectively; ten-day-old seedlings grown from undressed (Control) or dressed grains with naphthalic anhydride (NA, 0.4%) were subjected to the treatment and samples were collected 5 after days. Values are means ± SD, (n = 6 from two independent experiments). Data were subjected to ANOVA and LSD calculated at P ≤ 0.05. s at the column top means significantly different from the untreated control.

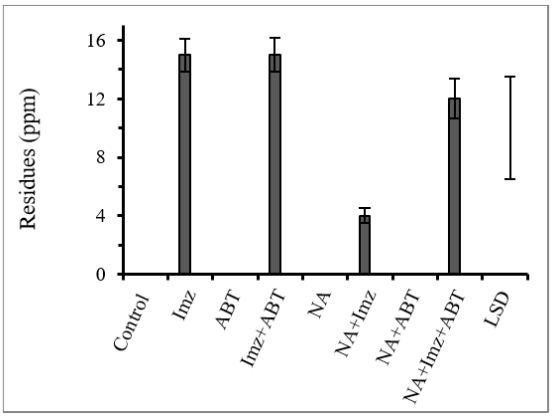

Fig. III. Residues of imazethapyr (Imz) in maize shoots due to treatment Imz and/or 1-aminobenzotriazole (ABT) at 35 and 225 g ai ha^{-1}, respectively. Ten-day-old seedlings grown from undressed (Control) or dressed grains with naphthalic anhydride (NA, 0.4%) were subjected to the treatment and samples were collected 5 days after. Values are means±SD, (n = 6 from two independent experiments). Data were subjected to ANOVA and LSD calculated at $P \leq 0.05$. s at the column top means significantly different from the untreated control.

contrary, NA treatment overcame the drastic effects of Imz on growth parameters; however, the presence of ABT was inconvenient. On the other hand, slight changes were detected in water content under all treatments; the loss of water content was highest when ABT was combined with Imz but least and seemingly negligible for seedlings derived from grains dressed with NA; nonetheless, each of ABT and Imz alone was moderately effective.

At the same time, protein content was significantly decreased by Imz; the impact of the herbicide became virulent with the presence of ABT (Fig. II). On the other hand, dressing seeds with NA completely alleviated the decreases in protein induced by the herbicide; in fact, the application of NA antagonized the influence of Imz and rendered its effect to be non-significant. However, NA could not mitigate the herbicide effect when ABT was present. Indeed, ABT synergized the herbicide effect and even induced further decreases in protein content when present either alone or combined with the herbicide and/or with NA leading to significant decreases relative to control. It is apparent also that, treatment with Imz provoked inhibition in the activity of AHAS, the herbicide induced about 44% inhibitions in the enzyme activity relative to control (Fig. II). Further inhibitions were detected with the presence of ABT combined with Imz (66%). Dressing grains with NA counterbalanced the inhibition in AHAS activity by Imz; however, the presence of ABT retarded this recovery. On the other hand, ABT significantly inhibited AHAS activity even with the presence of NA.

Table 1
Changes in the endogenous levels of branched-chain amino acids in maize shoots due to the treatment with imazethapyr (Imz) and/or 1-aminobenzotriazole (ABT) at 35 and 225 g ai ha^{-1}, respectively; Ten-day-old seedlings grown from undressed (Control) or dressed grains with naphthalic anhydride (NA, 0.4%) were subjected to the treatment and samples were collected 5 after days.

	Val [a]	Leu	Ile
Control	76.4±6.44	87.6±8.01	111.3±9.82
Imz	36.5±5.12*	44.3±4.52*	59.8±4.79*
ABT	44.2±5.03*	49.9±4.16*	39.8±6.44*
Imz+ABT	28.8±3.11*	32.7±3.72*	41.2±3.52*
NA	74.8±6.87	88.9±7.49	115.2±10.71
NA+Imz	71.5±7.14	79.4±6.88	102.7±9.08
NA+ABT	40.9±3.88*	46.6±6.11*	52.7±7.81*
NA+Imz+ABT	39.6±3.32*	39.7±4.07*	49.7±4.28*
LSD [b]	16.38	21.92	27.18
r with AHAS [b]	0.95	0.95	0.83

[a] Values (nmol g^{-1} dry tissue) are means ± SE, (n=6 from two independent experiments). *, significantly different from control
[b] Data were subjected to ANOVA and LSD calculated at $P \leq 0.05$.
[b] r with AHAS, the correlations between % changes in AHAS activity and % changes in branched-chain amino acids

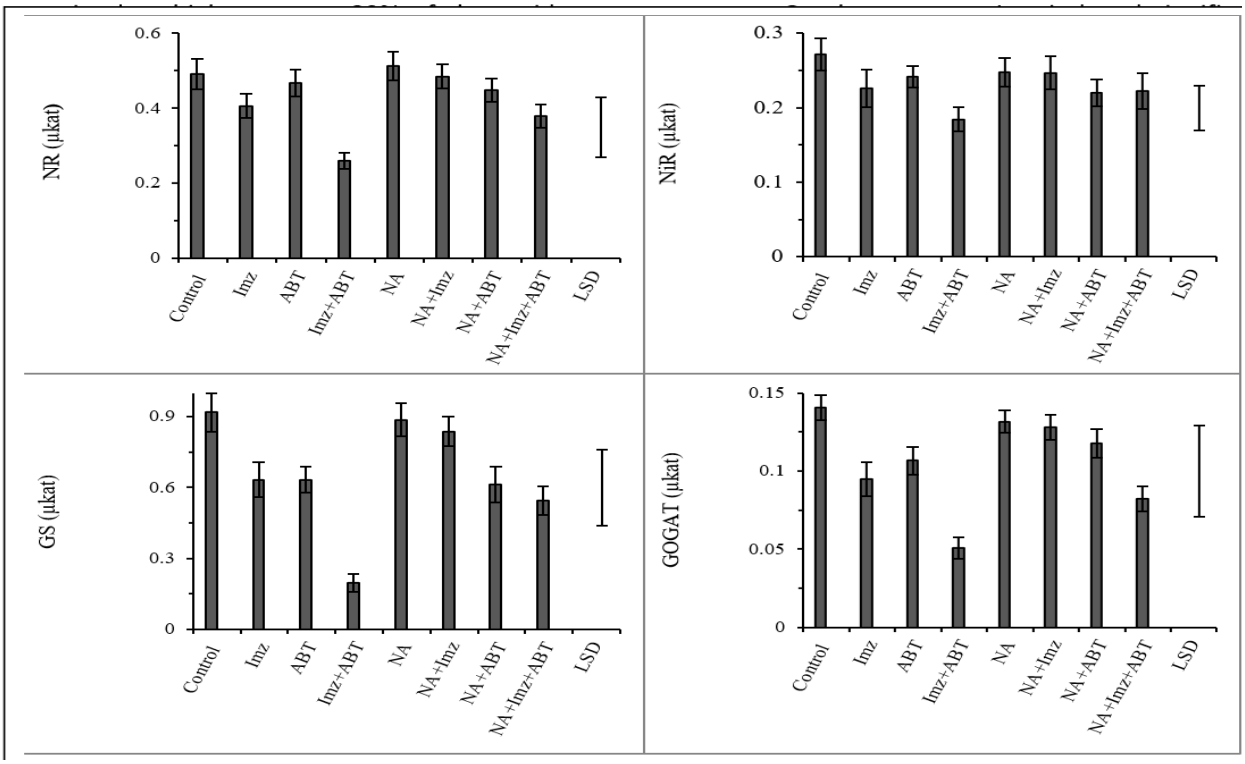

Fig. IV. Changes in activities of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT) of maize shoots due to treatment with imazethapyr (Imz) and/or 1-aminobenzotriazole (ABT) at 35 and 225 g ai ha^{-1}, respectively. Ten-day-old seedlings grown from undressed (Control) or dressed grains with naphthalic anhydride (NA, 0.4%) were subjected to the treatment and samples were collected 5 days after. Values are means±SD, (n = 6 from two independent experiments). Data were subjected to ANOVA and LSD calculated at *P ≤ 0.05*. s at the column top means significantly different from the untreated control.

It is apparent from Fig. III that Imz was persisting after 5 days from treatment; the herbicide residues reached 15 ppb. This persistence continued with the presence of ABT without any reduction in the herbicide residues,

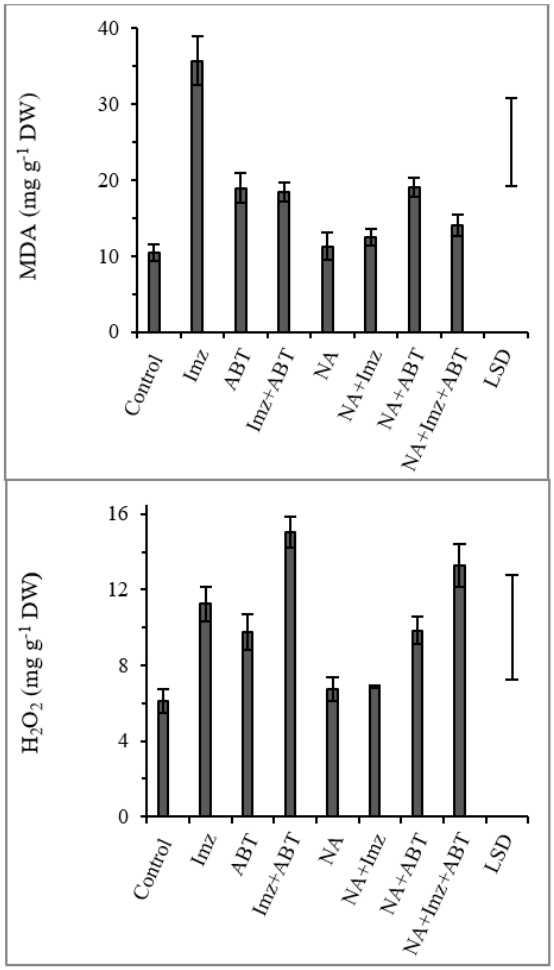

Fig. V. Changes in malondialdehyde (MDA) and H_2O_2 contents in maize shoots due to treatment with imazethapyr (Imz) and/or 1-aminobenzotriazole (ABT) at 35 and 225 g ai ha^{-1}, respectively; ten-day-old seedlings grown from undressed (Control) or dressed grains with naphthalic anhydride (NA, 0.4%) were subjected to the treatment and samples were collected 5 after days. Values are means ± SD, (n = 6 from two independent experiments). Data were subjected to ANOVA and LSD calculated at $P \leq 0.05$. s at the column top means significantly different from the untreated control.

the magnitude was very close to that detected in the herbicide-treated samples. However, a great reduction in the residues was induced in response to NA, the residues diminished to only 27% of that detected in the treated samples. Nonetheless, NA became less effective in decreasing the herbicide residues when ABT was present such that the herbicide persistence remained as high as up to 80% of the residues detected in the treated samples.

Meanwhile, Imz significantly decreased the levels of the branched chain amino acids

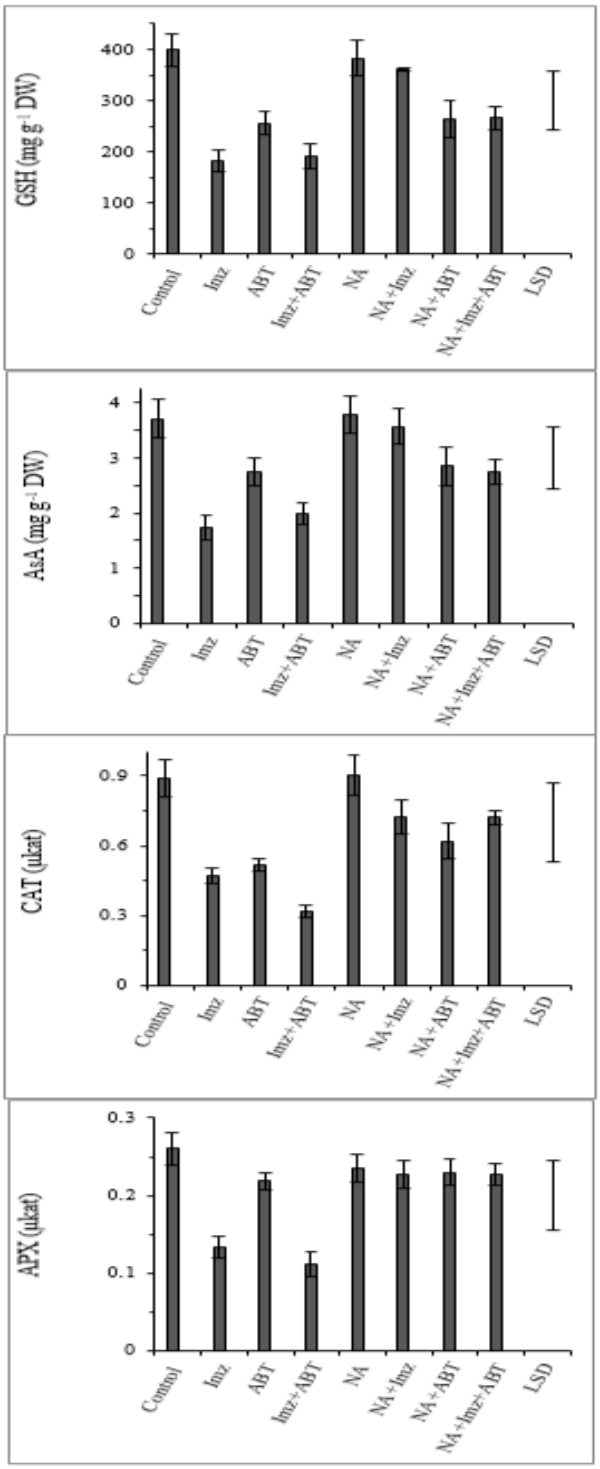

Fig. VI. Changes in contents of glutathione (GSH) ascorbic acid (AsA) and activities of catalase (CAT) and ascorbate peroxidase (APX) of maize shoots due to the treatment with imazethapyr (Imz) and/or 1-aminobenzotriazole (ABT) at 35 and 225 g ai ha^{-1}, respectively; ten-day-old seedlings grown from undressed (Control) or dressed grains with naphthalic anhydride (NA, 0.4%) were subjected to the treatment and samples were collected after 5 days. Values are means ± SD (n = 6 from two independent experiments). Data were subjected to ANOVA and LSD calculated at $P \leq 0.05$. s at the column top means significantly different from the untreated control.

Table 2
Changes in values of the kinetic parameters (Vmax and Km and their ratios), the catalytic rate (Kcat), and the catalytic efficiency (Kcat/ Km) of enzymes extracted from shoots of 10-day-old maize untreated seedlings. Imazethapyr (Imz) was added to the reaction mixtures to give a final concentration of 100 nM.

	Lineweaver-Burk					Hanes				
	Vmax [a]	Km	Vmax/Km	Kcat	Kcat/Km	Vmax	Km	Vmax/Km	Kcat	Kcat/Km
AHAS										
- [b]	0.36±0.042	11.14±1.06	0.032	0.103	0.009	0.37±0.03	13.41±1.03	0.028	0.106	0.008
+	0.11±0.016	22.12±1.52	0.005	0.031	0.001	0.11±0.01	25.01±1.34	0.004	0.032	0.001
NR										
-	0.51±0.071	1.47±0.12	0.346	0.145	0.099	0.52±0.06	1.38±0.14	0.375	0.148	0.107
+	0.43±0.037	1.46±0.16	0.293	0.122	0.084	0.45±0.03	1.66±0.15	0.271	0.128	0.077
NiR										
-	0.38±0.034	1.09±0.07	0.354	0.110	0.101	0.39±0.04	1.00±0.09	0.387	0.110	0.111
+	0.32±0.026	1.05±0.09	0.303	0.090	0.086	0.33±0.03	1.11±0.11	0.297	0.094	0.085
GS										
-	0.97±0.087	2.28±0.17	0.426	0.277	0.122	0.95±0.089	1.88±0.19	0.507	0.272	0.145
+	0.52±0.058	2.23±0.21	0.235	0.150	0.067	0.54±0.048	2.22±0.17	0.245	0.156	0.070
GOGAT										
-	0.15±0.014	2.07±0.18	0.074	0.044	0.021	0.15±0.013	1.32±0.18	0.113	0.043	0.032
+	0.11±0.012	2.02±0.16	0.055	0.032	0.016	0.11±0.009	1.92±0.13	0.059	0.032	0.017
CAT										
-	0.90±0.093	2.85±0.23	0.315	0.800	0.281	0.91±0.088	3.18±0.21	0.287	0.261	0.082
+	0.58±0.061	2.82±0.27	0.204	0.165	0.059	0.59±0.046	3.50±0.28	0.170	0.170	0.049
APX										
-	0.26±0.034	10.03±0.96	0.026	0.074	0.007	0.28±0.035	15.37±1.04	0.018	0.079	0.005
+	0.15±0.017	11.51±0.93	0.013	0.043	0.004	0.17±0.022	15.61±1.13	0.011	0.048	0.003

[a] Values of Vmax (µKat) and Km (mM substrate) are means ±S E, (n=6). Data were obtained from interpolation of the equations of Lineweaver-Burk and Hanes.
[b] -, without Imz; +, with Imz

(valine, leucine and isoleucine) (Table 1). The magnitude of the decrease was highest for isoleucine and lowest for valine, ranging from 46-52%. Similar significant decreases were detected when ABT was present regardless of its combination, alone induced 42-64% decreases but with Imz induced about 63% decreases. The application of NA as grain dressing mostly alleviated the decreases in amino acid levels induced by the herbicide.

The results depicted in Fig. IV show that Imz caused slight decreases in the activities of NR and NiR. Also, significant inhibitions in GS and GOGAT activities were detected in treated samples relative to the untreated controls. The presence of ABT synergized the herbicide effect on the enzyme activities; its combination with the herbicide significantly inhibited the activities of all enzymes. However, NA antagonized the inhibitions in GS and GOGAT activities by the herbicide and resulted in close values of GS and GOGAT as well as NR and NiR to that of the control. Nonetheless, the recovery of the herbicide impacts due to the application of NA became less effective when ABT was present in combination.

On the contrary, Imz induced significant increases in MDA and H_2O_2 relative to control values (Fig. V). These increases remained significantly higher than control when ABT was present. Nonetheless, the application of NA counterbalanced the overproduction of MDA and H_2O_2 and rendered these levels to be close to control. However, NA became ineffective on reducing the accumulation of MDA and H_2O_2 when ABT was present either alone or in combination with the herbicide.

Concomitantly, the herbicide treatment significantly decreased the contents of GSH and AsA (Fig. VI). Most likely, the decreases still

remained lower than control by the presence of ABT either alone or when combined with the herbicide and/or NA. Dressing grains with NA completely overcame the herbicide-induced decreases in GSH and AsA; however, NA could not eliminate the herbicide effect when ABT was present. In the same pattern, Imz significantly inhibited CAT and APX activities as compared to control values; the magnitude of inhibition was higher in the activity of CAT than APX (Fig. VI). The application of NA greatly mitigated the inhibition in the enzyme activities and rendered the values to be close to those of the control. Nonetheless, ABT synergized the herbicide effects on CAT compared to APX activities and even augmented the inhibition.

The values of the kinetic parameters (Vmax, Km, and their ratios) of all tested enzymes (AHAS, NR, NiR, GS, GOGAT, CAT, and APX) as well as the catalytic rate (Kcat) and the catalytic efficiency (Kcat/ Km) were calculated using *in vitro* assays of v as functions of [S] from Lineweaver-Burk equation {1/v= (1/[S]) (Km/Vmax) +(1/Vmax)} then further confirmed by Hanes equation {[S]/v=([S]/Vmax) +(Km/Vmax)}and presented in Table 2. As a whole, there were very little variations among the calculations using Lineweaver-Burk or Hanes equations. The data showed that Imz lowered the magnitudes of Vmax of AHAS, NR, NiR, GS, GOGAT, CAT, and APX as compared to their respective untreated blank. On the other hand, the herbicides unchanged the Km values of NR, NiR, GS, GOGAT, CAT, and APX which remained close to their blanks; nonetheless, Km of only AHAS was increased. The decrease in Vmax, concomitant with whatever an increase or unchanged in Km, resulted in general decreases in the values of Vmax/Km for all enzymes. Moreover, Imz treatment led to depression in Kcat values of all enzymes with a subsequent decrease in Kcat/Km of these enzymes; the depression was most pronounced in AHAS followed by CAT but least for NR and NiR.

Discussion

Results showed that the treatment with Imz reduced the growth parameters of maize. The reduction might be due to the inhibitory effect of herbicide on plant metabolism which would lead to retardation in many metabolic processes and growth cessations. In general, herbicides drastically influence all aspects of plant metabolism. Several reports indicated reductions in plant growth following the application of several herbicides (Dvorak et al., 2002; Nemat Alla et al., 2007, 2008). In addition, Rutherford and Krieger-Liszkay (2001) hypothesized that the plant is killed by oxidative stress. So, the reduction in maize growth by Imz could result from alterations in metabolism and the production of oxidative stress. The most common metabolic processes related to Imz are the alterations in the branched-chain amino acids and protein synthesis due to the inhibition AHAS activity. Therefore, the reduction of maize growth caused by Imz was synchronized with inhibition of AHAS activity and decreases in the levels of valine, leucine, and isoleucine. These findings could conclude that the phytotoxicity of Imz is due to the inhibition of AHAS with a consequent block in the biosynthesis of the branched-chain amino acids, the influences that would lead to reduction in protein biosynthesis. On the other hand, the inhibition of AHAS activity was related to the amount of the herbicide residues detected which could disturb plant metabolism with a consequent reduction in growth. Generally, the susceptibility of crops to herbicides depends on their persistence and/or their detoxification; high persistence or low detoxification could result in high toxicity. The detected residues, in the present results were reduced by the application of NA concluding the prevalence of the detoxification with lowering the residues and resulting in less furiousness of herbicide toxicity.

The retraction of herbicide residues was synchronized with losses in the inhibition of AHAS activity and also in the contents of the branched-chain amino acids. The alleviation of NA for the herbicide effects was coincided with recovery in plant growth could result from its role as a safener in protection from herbicidal injury. Generally, safeners protect plants from herbicides by accelerating their detoxification or antagonizing them at a common target site leading to a reduction of herbicide amounts reaching its target site in an active form, thereby protecting plants from phytotoxic doses of herbicides (Deng and Hatzios, 2003; Nemat Alla and Hassan, 2008).

Metabolism and detoxification of several groups of herbicides involves the oxidative pathway by cytochrome P450 monooxygenases. ABT is a P450s inhibitor which increases the phytotoxicity of most herbicides metabolized by P450 enzymes (Reichhart, 1995) and can be used as synergists of most herbicides metabolized by P450s (Moreland et al., 1993). In fact, the presence of ABT synergized the impacts of Imz on maize in the present results. Therefore, NA treatment completely antagonized the phytotoxic effects of Imz on maize, whereas ABT synergized such effects. This might be due to the differential degradation and detoxification of Imz by maize either induction or hindrance by the presence of NA or ABT, respectively. Moreover, ABT kept the Imz residue too high, so the persistence of the herbicide would be high enough to inhibit the activity of AHAS and lower the levels of the branched chain amino acids causing a consequent drop in protein formation. In accordance, Nemat Alla and Hassan (2019) indicated that chlorimuron-ethyl induced an inhibition for AHAS and diminished the formation of branched-chain amino acids and protein, nonetheless, an external supplement of their mixture overcame the herbicide toxicity via compensating the drop in the endogenous branched-chain amino acids while.

The decrease in protein content was counterbalanced by the application of NA; in contradistinction, ABT resulted in a cumulative and severe inhibition. The decrease in protein content by Imz could arise from ammonia demand and/or inhibition in the branched-chain amino acids. Results declare drops in valine, leucine, and isoleucine contents due to AHAS inhibition. Nevertheless, ammonia availability was tested using NR-NiR system and ammonia assimilation into organic forms using the GS-GOGAT system. However, NR/NiR system was not greatly modified by the herbicide whereas GS-GOGAT system was highly inhibited; NA mostly alleviated the effects of Imz while ABT caused synergism to the herbicide effects.

The slight changes in NR and NiR activities with small depletion of the produced ammonia, due to the inhibited GS/GOGAT activities, might give rise to increased ammonia accumulation. So, the slight effects on NR-NiR as parameters of ammonia production coincided with great inhibition in GS-GOGAT as parameters of ammonia assimilation could indicate that there was no ammonia demand but only the losses in the branched-chain amino acids would alter the biosynthesis of protein. In addition to these alterations, the induction of oxidative stress could retard growth.

Most likely, Imz led to production of MDA and H_2O_2 concluding an induction of oxidative stress, the findings that are in conformity with other researches who stated that several herbicides promote their toxic effects on photosynthetic organisms by affecting the production of reactive oxygen species (ROS). They react with lipids, proteins, pigments, and nucleic acids and cause lipid peroxidation, membrane damage, and inactivation of enzymes, thus affecting cell viability (Nemat Alla et al., 2008a, b). The induced oxidative stress by Imz was overcome by NA; however, ABT supported the herbicide effects. These findings point to the antagonistic effect of NA on Imz whereas ABT induced synergism. To cope with these conditions, plants developed endogenous defense mechanisms for scavenging of ROS

Tolerance was associated with high levels of the antioxidants. AsA-GSH cycle, in which H_2O_2 is reduced to water, is catalyzed for the maintenance of GSH (Nayyar and Gupta, 2006). GSH and AsA participate in ROS scavenging through the reduction of H_2O_2 to water for the maintenance of GSH (Hassan and Nemat Alla, 2020). Nemat Alla and Hassan (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 the tolerance of plants to xenobiotics and environmental stresses. The antioxidant function of GSH is mediated by the sulfhydryl group of cysteine. Consequently, the drop in GSH by the herbicide might cause a deficiency in the defense system. The decrease in GSH level is not only due to the failure in reduction but also to an inhibition in its biosynthesis (Hassan and Nemat Alla, 2020). So, the decreases in GSH and AsA contents in response to Imz treatment were consistent with the elevation of MDA and H_2O_2

indicating the destruction of the antioxidant system. Therefore, an exogenous supporter is needed for the plant to withstand the herbicide stress by mitigating its impacts (Jang et al., 2019) or increasing its degradation (Hatzios, 2000). So, NA treatment supported maize in counterbalancing the effects of Imz; however, ABT synergized the herbicide effects. Moreover, DeRidder et al. (2002) concluded that safener-induced protection in cereals is associated with increased expression of herbicide detoxifying enzymes. Moreover, Nemat Alla and Hassan (2019) indicated that NA caused mitigation of chlorimuron-ethyl toxicity by detoxifying the herbicide.

The decrease in Imz persistence by NA synchronized with increases in GSH and AsA contents could support that the plant tends to tolerate the herbicides toxicity. In coincidence, similar effects were detected for CAT and APX activities, including inhibition by Imz and/or ABT and then recovery by NA. These results could conclude the suffering of maize under treatment with Imz and/or ABT; nonetheless, NA eliminated the Imz-induced stress. On the contrary, Abedini et al. (2017) found that vanadium increased the activity of guaiacol peroxidase in *Helianthus annuus* while the activities of CAT, glutathione reductase and ascorbate peroxidase decreased. Consequently, the relief of GSH, AsA, CAT and APX, in the present results, concomitant with the retraction in MDA and H_2O_2 could suggest that NA supported maize to overcome oxidative stress via declining the persistence of the herbicide concomitantly with saving AHAS and protein thereby reducing the herbicide toxicity. However, NA was no longer effective with the presence of ABT, suggesting that the synergism overlap the antagonism.

Taken together, these findings could consider Imz as a wholly inhibitor for the examined enzymes; the inhibitions might result from a decrease in the enzyme concentration and/or an interference with the structural integrity with probable changes in the kinetic parameters. Vmax and Km are the kinetic parameters which define the rate of an enzyme-catalyzed reaction as substrate concentration is varied. Vmax is regarded as a function of the enzyme concentration while Km reflects an enzyme's affinity for its substrate and can be related to isoenzyme distribution, enzyme-substrate affinity, etc. (Engel, 1984; Tovar-Mendez and Munoz-Clares, 2001). Imz induced a decrease in Vmax of AHAS concomitant with an increase in Km deducing that the herbicide effect was exerted on the enzyme concentration and the structural integrity too. Such finding tend to regard Imz as causing a mixed type of inhibition to AHAS. On the other hand, Vmax of NR, NiR, GS, GOGAT, CAT and APX were decreased by the herbicide which coincided with no change in Km values concluding that Imz exerted its effect only on the enzyme synthesis affecting only their concentration concluding that the herbicide could be regarded as a noncompetitive inhibitor to these enzymes. These findings support that Imz is an inhibitor for all enzymes at the enzymes concentration level and/or at the catalytic efficiency level. Moreover, the general decrease in Vmax of all enzymes would result in a consequent drop in kcat values while the decrease in Vmax concomitant with unchanging or increasing Km values would lead to decline the Vmax/Km ratios. Therefore, the decrease in kcat would drop the kcat/Km value which is a measure of how efficiently an enzyme converts a substrate into product. So, the decrease in Kcat of all enzymes by Imz could confirm the general inhibition induced by Imz while the drop in kcat/Km and Vmax/Km confirms the loss in their catalytic efficiency; the effect was highest for AHAS and lowest for NR and NiR.

Conclusion

Treatment of 10-day-old maize seedlings, grown from NA-dressed and undressed grains, with Imz significantly reduced growth parameters and inhibited AHAS activity with concomitant decreases in protein, branched-chain amino acids, GSH, and AsA as well as activities of GS, GOGAT, CAT, and APX but elevated lipid peroxides and H_2O_2. The application of NA or ABT antagonized or synergized the herbicide impacts, respectively; however, NA became no longer effective when ABT was present in combination. The changes in AHAS activity was highly correlated with the changes in the branched-chain amino acids concluding its regulatory role. The slight changes in NR and NiR activities concomitant with great

decrease in protein and inhibition in GS and GOGAT could conclude that the decrease in protein might not be attributable to ammonia demand but only to the loss in the branched-chain amino acids. These findings indicated that NA partially or completely alleviated the effects of Imz; however, ABT augmented them concluding that the differential antagonism or synergism of NA and ABT might be attributed to the enhanced detoxification or to the protraction of herbicide persistence, respectively. Moreover, the kinetic of Imz inhibition to the enzymes AHAS, NR, NiR, GS, GOGAT, CAT, and APX indicated decreases in Vmax values of all enzymes; however, Km was unchanged except for the increase in only AHAS. These findings indicate that Imz induced a mixed type of inhibition for AHAS and noncompetitive inhibition for NR, NiR, GS, GOGAT, CAT, and APX.

References

Abedini, M., F. Mohammadian, B. Daie-Hassani and **L. Zarandi-Miandoab.** 2017. 'Antioxidant responses of *Helianthus annuus* L. under vanadium stress'. *Iranian J Plant Physiol,* 8: 2239-2245.

Ahn, Y.O., S.Y. Kwon, H.S. Lee, I.H. Park and **S.S. Wak.** 1999. 'Biosynthesis and metabolism of vitamin C in suspension cultures *Scutellaria baicalensis'*. *J Biochem Mol Biol,* 32: 451-455.

Anderson, M.P. and **J.W. Gronwalds.** 1991. 'Atrazine resistance in velvetleaf (*Abutilon theophrasti*) biotype due to enhanced glutathione S-transferase activity'. *Plant Physiol,* 96: 107–109.

Bidlingmeyer, B.A., S.A. Cohen, T.L. Tarvin and **B. Frost.** 1987. 'A new, rapid, high sensitivity analysis of amino acids in food type samples'. *J Assoc Off Anal Chem,* 70: 241.

Bradford, M.M. 1976. 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding'. *Anal Biochem,* 72: 248-254.

Buege, J.A. and **S.D. Aust.** 1972. 'Microsomal lipid peroxidation'. *Methods Enzymol,* 52: 302-310.

Chakraborty, K., S.K. Bishi, N. Goswami, A.L. Singh, D. Bhaduri and **P.V. Zala.** 2019. 'Salinity-induced changes in seed germination and the expression profile of antioxidant enzymes in peanut as early and late responses in emerging radicles'. *Acta Physiol Plant,* 41: 134-149.

Deng, F. and **K.K. Hatzios.** 2003. 'Characterization of cytochrome P450-mediated bensulfuron-methyl O-demethylation in rice'. *Pestic Biochem Physiol,* 74: 102-104.

DeRidder, B.P., D.P. Dixon, D.J. Beussman, R. Edwards and **P.B. Goldsbrough.** 2002. 'Induction of glutathione S-transferases in Arabidopsis by herbicide safeners'. *Plant Physiol,* 130: 1497-1505.

Dvorak, J. and **I. Remesova.** 2002.' Assessment of metribuzin effects on potatoes using a method of very rapid fluorescence induction'. *Rostlinna Vyroba,* 48: 107-117.

Engel, P.C. 1984. 'Enzyme Kinetics'. In *The chemistry of enzyme action.* Page, M.I. (Ed.). Elsevier, Amestrdam, New York, Oxford, pp: 73.

Harborne, J.B. 1988. 'Macromolecules'. In *Phytochemical Methods.* Harborne, J.B. (Ed.). Chapman and Hall, London, pp: 243-376.

Hassan, N.M. and **M.M. Nemat Alla.** 2020. 'Kinetics of inhibition of isoproturon to glutathione-associated enzymes in wheat'. *Physiol Mol Biol Plants,* In Press.

Hatzios, K.K. 2000. 'Herbicide safeners and synergists'. In *Metabolism of Agrochemicals in Plants*. Roberts, T. (Ed.) Wiley, Chichester, UK, pp: 259-294.

Hecht, U., R. Oelmüller, S. Schmidt and **H. Mohr.** 1988. 'Action of light, nitrate and ammonium on the levels of NADH- and ferridoxin-dependent glutamate synthases in the cotyledons of mustard seedlings'. *Planta,* 175: 130-138.

Huang, Z., B. Sui, C. Zhang and **H. Huang.** 2019. 'The basis of resistance mechanism to mesosulfuron-methyl in Tausch's goatgrass (*Aegilops tauschii* Coss.)'. *Pestic Biochem Physiol,* 155: 126-131.

Jang, S.J., S.S. Kim, H.Y. Bak, Y.B. Yun and **Y.I. Kuk.** 2019. 'Effects of organic acids on parquet activity in maize'. *South Afr J Bot,* 123: 286-292.

Lea, P.J., Robinson, S.A. and **Stewart, G.R.** 1990. 'The enzymology and metabolism of glutamine, glutamate and asparagine'. In *The*

biochemistry of plants. Miflin, B.J. and P.J. Lea (Eds.) Academic Press, NewYork, pp: 121-159.

Marquez, A.J., C. Avile, B.G. Forde and **R.M. Wallsgrove.** 1988. 'Ferredoxin-glutamate synthase from barley leaves: Rapid purification and partial characterization'. *Plant Physiol Biochem,* 26: 645-651.

Moreland, D.E., F.T. Corbin and **J.E. Mcfarland.** 1993. 'Oxidation of multiple substrates by corn shoot'. *Pestic Biochem Physiol,* 47: 206-214.

Nagaoka, S., M. Hirasawa, K. Fukushima and **G. Tamura.** 1984. 'Methyl viologen-linked nitrite reductase from bean roots'. *Agric Biol Chem,* 48: 1179-1188.

Nakagawa, H., Y. Yonimura, H. Yamamota, T. Sato, N. Ogura and **R. Sato.** 1985. 'Spinach nitrate reductase: Purification, molecular weight, and subunit composition'. *Plant Physiol,* 77: 124-128.

Nakano, Y. and **K. Asada.** 1981. 'Hydrogen peroxide is scavenged by ascorbate specific peroxide in spinach chloroplast'. *Plant Cell Physiol,* 22: 867-880.

Nayyar, H. and **D. Gupta.** 2006. 'Differential sensitivity of C3 and C4 plants to water deficit stress: Association with oxidative stress and antioxidants'. *Environ Exp Bot,* 58: 106-113.

Nemat Alla, M.M. and **N.M. Hassan.** 1998. 'Efficacy of exogenous GA3 and herbicide safeners in protection of *Zea mays* from metolachlor toxicity'. *Plant Physiol Biochem,* 36: 809-815.

Nemat Alla, M.M. and **N.M. Hassan.** 2019. 'Alleviation of chlorimuron-ethyl toxicity to soybean by branched-chain amino acids or naphthalic anhydride'. *Rendiconti Lincei,* 30: 759–766.

Nemat Alla, M.M. and **N.M. Hassan.** 2006. 'Changes of antioxidants levels in two maize lines following atrazine treatments'. *Plant Physiol Biochem,* 44: 202-210.

Nemat Alla, M.M., A.M. Badawi, N.M. Hassan, Z.M. El-Bastawisy and **E.G. Badran.** 2007. 'Induction of glutathione and glutathione-associated enzymes in butachlor-tolerant plant species'. *Am J Plant Physiol,* 2: 195-205.

Nemat Alla, M.M., A.M. Badawi, N.M. Hassan, Z.M. El-Bastawisy and **E.G. Badran.** 2008a. 'Effect of metribuzin, butachlor and chlorimuron-ethyl on amino acid and protein formation in wheat and maize seedlings'. *Plant Physiol Biochem,* 90: 8-18.

Nemat Alla, M.M., N.M. Hassan and **Z.M. El-Bastawisy.** 2008b. 'Changes in antioxidants and kinetics of glutathione-S-transferase of maize in response to isoproturon treatment'. *Plant Biosystems,* 142: 5-16.

Okuda, T., Y. Masuda, A. Yamanaka and **S. Sagisaka.** 1991.' Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat is caused by cold treatment'. *Plant Physiol,* 97: 1265-1267.

Reichhart, D.W. 1995. 'Herbicides metabolism and selectivity: role of cytochrome P450. Bright'. Crop Prot. Conf. - Weeds, 813-822.

Relton, J.M., R.M. Wallsgrove, J.P. Bourgin and **S.W. Bright.** 1986. 'Altered feedback sensitivity of acetohydroxyacid synthase from valine-resistant mutants of tobacco (*Nicotiana tabacum* L.)'. *Planta,* 169: 45-50.

Rhodes, D., A.L. Hogan, L. Deal, G.C. Jamieson and **P. Howorth.** 1987. 'Amino acid metabolism of *Lemna minor* L. II. Responses to chlorsulfuron'. *Plant Physiol,* 84: 775-780.

Rutherford, A.W. and **A. Krieger-Liszkay.** 2001. 'Herbicide-induced oxidative stress in photosystem'. *Opinion Trends Biochem Sci,* 26: 648 – 653.

Singh, B.K., M.A. Stidham and **D.L. Shaner.** 1988. 'Assay of acetohydroxyacid synthase'. *Anal Biochem,* 171: 173.

Sondhia, S., P.J. Khankhane, P.K. Singh and **A.R. Sharma.** 2015. 'Determination of imazethapyr residues in soil and grains after its application to soybeans'. *J Pestic Sci,* 40: 106–110.

Tovar-Mendez, A. and **R.A. Munoz-Clares.** 2001. 'Kinetics of phosphoenolpyruvate carboxylase from *Zea mays* leaves at high concentration of substrates'. *Biochem Biophys Acta,* 1546: 242-252.

Wray, J.L. and **P. Filner.** 1970.' Structural and functional relationships of enzyme activities induced by nitrate in barley'. *Biochem J,* 119: 715-725.