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## Effects of DIMBOA on detoxification enzymes of the aphid *Rhopalosiphum padi* (Homoptera: aphididae)

S. Mukanganyama<sup>a,\*</sup>, C.C. Figueroa<sup>b</sup>, J.A. Hasler<sup>a</sup>, H.M. Niemeyer<sup>b</sup>

<sup>a</sup> University of Zimbabwe, Department of Biochemistry, P.O. Box MP 167, Mount Pleasant, Harare, Zimbabwe

<sup>b</sup> Universidad de Chile, Departamento de Ciencias Ecológicas, Facultad de Ciencias, Casilla 653, Santiago, Chile

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### Abstract

The presence of glutathione transferases and esterase activity was investigated in *Rhopalosiphum padi* and the effects of the cereal hydroxamic acid, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) on these detoxification enzymes was studied. Activity of glutathione *S*-transferases and general esterases was determined for adult aphids feeding on a natural diet lacking DIMBOA and on an artificial DIMBOA-containing diet for 48 hours. In vivo, DIMBOA in the diet inhibited the activities of esterases by 50–75% at all concentrations tested (0.5–4 mM). The activity of glutathione transferase was inhibited to a lesser extent (30%) at the higher concentrations of DIMBOA. In vitro, DIMBOA generally inhibited the activity of esterases with an  $IC_{50}$  of 33  $\mu$ M, and had a slight inhibitory effect on glutathione *S*-transferases. These effects of DIMBOA could make the aphids vulnerable to electrophilic agents and insecticides which may be metabolized via esterases and GSTs. In cereals, therefore, DIMBOA may act by interfering with esterase- or GST-mediated detoxification of xenobiotics by aphids.

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**Keywords:** Hydroxamic acids; DIMBOA; *Rhopalosiphum padi*; Glutathione *S*-transferases; Esterases

### 1. Introduction

Glutathione transferase (GST: E.C. 2.5.1.18) and esterases (E.C. 3.1.1.7) are important enzyme systems involved in the metabolism of a broad range of foreign and endogenous compounds in insects (Francis et al., 2001; Yu, 1996; Kanga et al., 1997; Conyers et al., 1998). Glutathione *S*-transferases are multifunctional detoxification enzymes that catalyse the conjugation of reduced glutathione (GSH) to electrophilic agents (Mannervik and Widersten, 1995). In insects, GST plays an essential role in herbivory through the detoxification of deterrent and toxic plant allelochemicals, and insecticides (Wadleigh and Yu, 1988; Leszczynski et al., 1994; Yu, 1992; 1996; Papadopoulos et al., 1999; Rufingier et al., 1999). Esterases constitute a widely distributed family of enzymes which hydrolyse carboxylester, amide, and thioester bonds in a variety of compounds. They are

of major importance in insects because of their role in the detoxification of organophosphorous insecticides and in the acquisition of pesticide resistance seen in many insect species (Georghiou and Taylor, 1977; Georghiou, 1986; Georghiou and Lagunes-Tejeda, 1991; Conyers et al., 1998).

Aphids are one of the main pests of cereal crops (Minks and Harrewijn, 1987; Leszczynski et al., 1994; Yu, 1992; 1996; Roditakis et al., 2000). The rising costs of pesticides, the increasing resistance of insects to them and their undesirable effects on the environment (Corey et al., 1993; Scott et al., 1998), have led to renewed efforts to identify and exploit host plant resistance to pests and diseases. Hydroxamic acids (Hx) present in cereals such as wheat, maize, and rye are plant secondary metabolites that can function as natural insecticides (Niemeyer and Pérez, 1995). They occur in the plant as glycosides, which are hydrolysed to the aglucone when the tissue is injured (Hofman and Hofmanova, 1969). The main aglucone isolated from wheat is DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) (Fig. 1). Resistance to aphids in cereals has been associated

\* Corresponding author. Tel.: +263-4-303211; fax: +263-4-308046.  
E-mail address: stan@xenobiot.icon.co.zw (S. Mukanganyama).

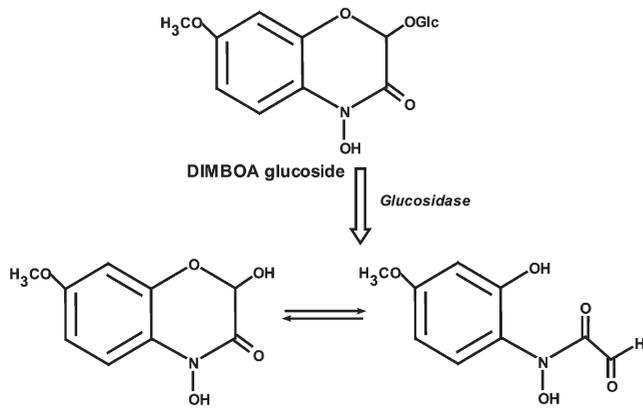


Fig. 1. The main hydroxamic acids in wheat. The scheme shows the biochemical reaction of the naturally present glucoside produced by plant cell rupture, and the tautomers of DIMBOA present in aqueous solutions.

with the concentrations of this secondary metabolite (Niemeyer and Pérez, 1995). Evidence has been presented for the accumulation (Niemeyer et al., 1989), excretion (Givovich et al., 1992) and enzymatic detoxification of Hx by aphids (Leszczynski et al., 1992). It is possible that glutathione transferases and esterases are part of insects' systems for biotransformation of secondary plant metabolites such as DIMBOA. In this paper, GST and general esterases are identified in the aphid *Rhopalosiphum padi* (L.) and the in vivo and in vitro effects of DIMBOA on these enzymes are investigated in the context of exploring a possible mechanism of toxicity of DIMBOA towards aphids.

## 2. Materials and methods

### 2.1. Chemicals and equipment

All reagents used were of analytical grade. 1-Chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), dithiothreitol (DTT), ethacrynic acid, hexylglutathione, bromosulphophthalein,  $\alpha$ -naphthyl acetate ( $\alpha$ -NA), fast blue B salt (FBB), bovine serum albumin (BSA), Bradford reagent, and sodium dodecylsulphate (SDS) were purchased from Sigma Chemical Co. (St. Louis, USA).  $\alpha$ -naphthol ( $\alpha$ -NO) was obtained from Merck Chemical Co. (Darmstadt, Germany). DIMBOA was isolated from etiolated maize shoots (*Zea mays* L. cv T55s) according to a published procedure (Woodward et al., 1978). Ultra-high-speed centrifugations were carried out in a Beckman L5-50 ultracentrifuge equipped with Ti-50 rotor and all spectrophotometric measurements were performed on a Shimadzu UV-240 recording spectrophotometer.

### 2.2. Insects

Aphids (*R. padi*) were collected from fields near Santiago, Chile, and multiclonal stock cultures were reared on oats (*Avena sativa* L. cv. Nahuén), a cereal lacking hydroxamic acids, under controlled conditions (20 °C and L:D 16:8).

### 2.3. Feeding of *R. padi* with artificial diets containing DIMBOA

One hundred and fifty wingless adult aphids were placed on a single jacket Parafilm "M" (American National Can™, Chicago, USA) sachet encompassing a 60-mm-diameter Petri dish. The sachet contained artificial diet composed of an aqueous solution of 30% sucrose, amino acids, vitamins and mineral salts at pH 5.5 (Auclair, 1965) and containing 0–4 mM DIMBOA. These concentrations of DIMBOA were chosen on the basis that: i) wheat leaf extracts may contain up to 8 mmol/kg fresh weight hydroxamic acids (Nicol et al., 1992), ii) they are comparable with concentrations determined in different plant compartments in wheat (Argandoña et al., 1987), iii) the concentration of hydroxamic acids in wheat phloem sap, which the aphid ingests, is between ca. 50 and 90% of leaf extract concentration, depending on the cultivar (Givovich et al., 1994) and iv) in related cereals such as maize (*Zea mays* L.), vacuolar sap of seedlings may contain up to 10 mM levels of benzoxaninones (Raveton et al., 1997). DIMBOA was prepared as solutions in dimethylsulfoxide (DMSO) and added to the artificial diet so that the final concentrations were 0.5, 1.0, 2.0, and 4.0 mM; DMSO was 0.1% in all cases.

### 2.4. Preparation of cytosol

After 48 hours of exposure to the diet, the living and obviously feeding aphids (proboscis inserted into diet sachet and antennae pointing backwards) were transferred to 1.5 ml Eppendorf tubes. Aphids on the sachets unable to walk after a gentle touch with a fine brush were considered dead and excluded from the study. Living aphids were sacrificed by exposure to –20 °C for 20 min. One hundred adults aphids from the sachet were homogenised using a plastic pestle in 300  $\mu$ l of 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.2 mM DTT, and 1 mM sodium azide. For each concentration of DIMBOA there were three replicates. All tissue homogenisation and centrifugation were performed at 4 °C. The homogenates were centrifuged at 10,000  $\times$  g for 15 min. The post-mitochondrial supernatant was centrifuged at 120,000  $\times$  g for 45 min to isolate the cytosol from organelles. Cytosols were also prepared from three groups of 100 adult aphids which were previously reared on oats. Cytosols were stored at –20 °C prior to analysis.

Protein concentration was determined by the method of Bradford (1976), using BSA as standard.

### 2.5. Determination of enzyme activities

The activities of GST and esterases were determined using end-point assays as described previously (Yan et al., 1995; Ortego et al., 1998). Reactions were linear up to 6 minutes, and therefore, all reactions were run for 3 minutes. Glutathione *S*-transferase activity was measured in 1 ml of reaction mixture containing 50  $\mu$ l of GSH (2 mM final concentration) and 3  $\mu$ g cytosolic protein as the enzyme source in 0.1 M phosphate buffer pH 6.5 containing 1 mM EDTA. The reaction tubes were pre-incubated at 30 °C for 2 min, then 50  $\mu$ l of CDNB in ethanol (1 mM final concentration) was added to start the reaction and the solution incubated for 3 min at 30 °C. Addition of 5% trichloroacetic acid ended the reaction (Yan et al., 1995). Absorbance at 340 nm was determined and activity expressed in  $\mu$ moles/min/mg protein using an extinction coefficient of 9.6 mM<sup>-1</sup> cm<sup>-1</sup> for *S*-(2,4-dinitrophenyl) glutathione (Mannervik and Widersten, 1995).

General esterase activity was determined using  $\alpha$ -NA as substrate based on the procedure of Gomori (1953), modified by Ortego et al. (1998). The reaction was initiated by the addition of 50  $\mu$ l of  $\alpha$ -NA in ethanol (0.25 mM final concentration) to 1 ml reaction mixture containing 1  $\mu$ g cytosolic protein as the enzyme source in 0.1 M phosphate buffer pH 7.0 supplemented with 150 mM NaCl and 5 mM MgCl<sub>2</sub>. The mixture was incubated at 30 °C for 30 min and the reaction ended by the addition of 250  $\mu$ l of an aqueous solution containing 1.6 mg/ml fast blue B salt and 60 mg/ml sodium dodecyl sulphate. The mixtures were then incubated for 1 h at room temperature for color development and the absorbance determined at 600 nm. The activity was expressed as nmoles of hydrolysed substrate/min/mg protein using  $\alpha$ -naphthol as the standard.

### 2.6. In vitro inhibition of enzyme activity and the effects of DIMBOA

The effects of increasing the concentration of standard GST inhibitors, or DIMBOA, on GST activity in cell supernatants was studied by comparing reaction rates in the presence and absence of these compounds. The enzyme was pre-incubated with the inhibitor which was dissolved in 95% ethanol or buffer for 2 minutes before the addition of the substrates GSH (2 mM) and CDNB (1 mM).

The inhibitors used were hexylglutathione, ethacrynic acid and bromosulphophthalein. DIMBOA was dissolved in 90% ethanol and was used in a concentration range of 0–100  $\mu$ M for the effects on GST and esterases activities. The activity in  $\mu$ moles/min/mg protein was

transformed into percentage activity with the 0  $\mu$ M concentration of inhibitor serving as the 100% activity value. The IC<sub>50</sub> values, i.e., the concentration of inhibitors required to reduce the activity by 50% (Mannervik and Danielson, 1988), were determined from non-linear regressions of the average percent activity against inhibitor concentration using the Graphpad Prism™ software package (GraphPad Software, Inc., California, USA).

### 2.7. Statistical analyses

Differences between treatments were compared using the Student *t*-test or Tukey–Kramer multiple comparison test with the Graphpad INSTAT™ software.

## 3. Results

### 3.1. Presence of detoxification enzyme activities in aphids

The activities of the detoxification enzymes from *R. padi* fed on oats are shown in Table 1. The observed activities of GST and esterases were comparable with those of other insects (Leszczynski et al., 1994; Conyers et al., 1998).

### 3.2. In vivo effects of DIMBOA on GST and esterase activities

Ingestion by *R. padi* of a DIMBOA-containing diet for 48 hours generally had no effect on GST activity at the lower concentration of 0.5 mM but DIMBOA at 1–

Table 1  
Glutathione *S*-transferase and esterase activity in the aphid *Rhopalosiphum padi*

Enzyme	Substrate	Activity	
		<i>R. padi</i> <sup>c</sup>	Other insects
Glutathione <i>S</i> -transferase <sup>a</sup>	CDNB	1.55 ± 0.14	1.320 ± 0.230 <sup>d</sup>
			0.308 ± 0.002 <sup>e</sup>
			0.292 ± 0.010 <sup>f</sup>
			0.727 ± 0.041 <sup>g</sup>
Esterase <sup>b</sup>	$\alpha$ -NA	62.4 ± 2.2	26.86 ± 1.65 <sup>d</sup>

<sup>a</sup> values for GST activity are mean  $\mu$ mol/min/mg protein  $\pm$  SD for 3 different preparations of aphid cytosol

<sup>b</sup> values for esterases are mean nmol/min/mg protein  $\pm$  SD (n=3)

<sup>c</sup> this work

<sup>d</sup> values obtained for the saw-toothed grain beetle *Oryzaephilus surinamensis* (Conyers et al., 1998)

<sup>e</sup> values obtained for *R. padi* (Leszczynski et al., 1994)

<sup>f</sup> values obtained for *Sitobion avenae* (Leszczynski et al., 1994)

<sup>g</sup> values obtained for *Metopolophium dirhodum* (Leszczynski et al., 1994)

4 mM inhibited GST activity by about 30% (Fig. 2A). In contrast, significant differences in esterase activity were found between 0 and all concentrations of DIMBOA, and between 0.5 and 4, and 2 and 4 mM DIMBOA (Fig. 2B). Generally, DIMBOA in the diet led to between 50% and 75% decrease in esterase activity.

### 3.3. *In vitro* effects of inhibitors and of DIMBOA on aphid detoxification enzymes

The  $IC_{50}$  values determined for standard GST inhibitors, and DIMBOA, for GST from *R. padi* are shown in Table 2. Typical plots of percent GST activity against inhibitor concentrations from which an  $IC_{50}$  value can be calculated for ethacrynic acid, a standard inhibitor of GSTs and DIMBOA are shown in Fig. 3. Whilst ethacrynic acid, hexylglutathione and bromosulphophthalein were potent inhibitors of aphid GST activity, the  $IC_{50}$  for DIMBOA was not determinable up to a concentration

Table 2

$IC_{50}$  values of standard mammalian GST inhibitors and DIMBOA for aphid glutathione *S*-transferase using CDNB as electrophilic substrate

Compound	Highest concentration tested ( $\mu$ M)	$IC_{50}^a$ ( $\mu$ M)
Bromosulphophthalein	100	$16 \pm 0.6$
Ethacrynic acid	50	$9 \pm 0.4$
Hexylglutathione	100	$52 \pm 4.9$
DIMBOA	100	$> 100$

<sup>a</sup> Cytosolic fractions of whole-body homogenates were used as the enzyme source and  $IC_{50}$  values were obtained by analysis of data using non-linear regression of plots of average percent activity versus inhibitor concentration. Error bars represent the mean  $\pm$  SD, where  $n = 3$ , i.e., 3 batches each of 100 aphids.

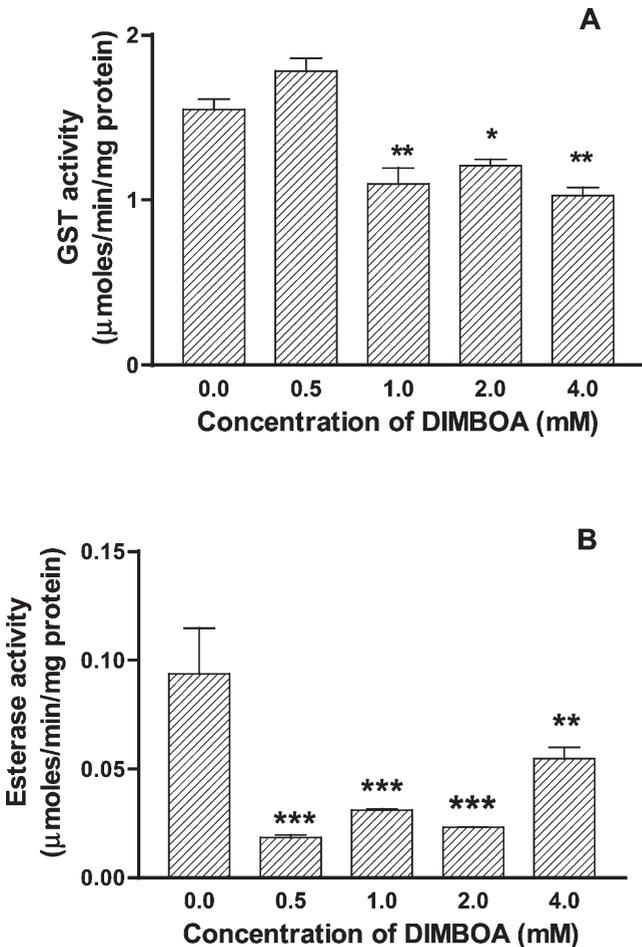


Fig. 2. The effects of DIMBOA on GST (A) and esterase (B) activity *in vivo*. DIMBOA was prepared as solutions in dimethylsulfoxide (DMSO) and added to the artificial aqueous diet so that the final concentrations were 0.5, 1.0, 2.0, and 4.0 mM; DMSO was 0.1% in all cases. One hundred wingless aphids were combined for each determination. Error bars represent the mean  $\pm$  SD, where  $n = 3$ , i.e., 3 batches each of 100 aphids. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$

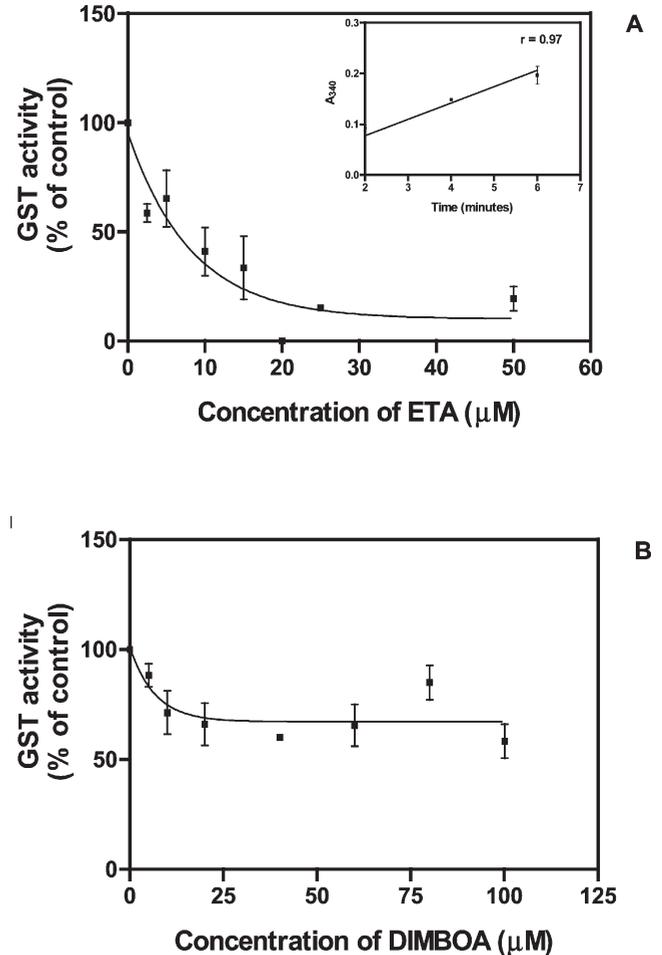


Fig. 3. The effects of DIMBOA on *R. padi* GST activity *in vitro*. A shows the effects of a standard GST inhibitor, ethacrynic acid, and B shows the effects of DIMBOA. Insert shows  $A_{340}$  against time for the CDNB reaction. This graph shows the linearity of the reaction between CDNB and GSH with time for aphid GST. Error bars represent the mean  $\pm$  SD, where  $n = 3$ , i.e., 3 batches each of 100 aphids. The determination of activity for each batch was done in quadruplicate

of 100  $\mu\text{M}$ , although at that concentration GST activity was reduced by 30% (Fig. 3 b).

In contrast, DIMBOA in vitro inhibited the activity of general esterases with an  $\text{IC}_{50}$  of  $33 \pm 4 \mu\text{M}$  (Fig. 4).

#### 4. Discussion

The role of insect detoxification enzymes in the metabolism of insecticides, allelochemicals and other xenobiotics is well established (Wadleigh and Yu, 1988; Conyers et al., 1998; Ortego et al., 1998; Lee, 1991). Data presented here show that GST and esterases are present in *R. padi* at concentrations comparable with those in other insects, and that DIMBOA altered the activity of GST and esterases both in vitro and in vivo. Esterases appear much more sensitive to the inhibitory effect of DIMBOA than are GSTs. For esterases, the feeding deterrent effect of DIMBOA at 4 mM may cause reduced ingestion of the allelochemical (Argandoña et al., 1983), and hence an apparent decrease of the inhibitory effect of DIMBOA (Fig. 2B).

Glutathione *S*-transferase activity for *R. padi* was three times higher than that obtained for the same species by Leszczynski et al. (1994). The reason for the difference could be linked to the fact that in the present study, aphids were fed on oats, whilst those in the study of Leszczynski et al. (1994) were fed on wheat. Wheat con-

tains hydroxamic acids (Niemeyer et al., 1992) which, as shown herein, decrease the activity of GST in vivo.

DIMBOA decreases *R. padi* GST activity in vitro and in vivo by 33% and 30%, respectively. This may be due to reaction of DIMBOA with essential residues on the enzyme related to GSH binding. Most GSTs have a cysteine residue near the active site, and modification of this residue alters the binding of GSH as has been shown for human GST (Hayes and Pulford, 1995; Ricci et al., 1996; Stella et al., 1998). Interestingly, the aldehyde group of the open chain aldol of DIMBOA does react with hydroxyl groups, as for example, internally to form the predominant cyclic tautomer of DIMBOA (Fig. 1). It is also known that 4-hydroxylakenals can react with GSH in a GST catalysed reaction (Mannervik and Widersten, 1995). It is possible DIMBOA can react with GSH as it does with other thiols (Niemeyer et al., 1982) to form a conjugate. Further experiments would be required to determine the residues that constitute the active site of aphid GST and to test if DIMBOA could act as a substrate. Alteration of the activity of GST with respect to GSH is also caused by chemicals that compete for thiol groups (Mulder and Ouwerkerk-Mahadevan, 1997). It has been reported that DIMBOA reacts with thiols through the hydroxamic nitrogen atom and the aldehyde group of the open chain aldol with which it equilibrates in solution (Niemeyer et al., 1982; Pérez and Niemeyer, 1985); analogous reactions with GSH or GST may be responsible for the effects observed. The observations reported here contrast with other results showing increases in both the activity and expression of the enzyme after the treatment of insects such as beetles and aphids with some insecticides (Papadopoulos et al., 1999; Rufingier et al., 1999). Yan et al., (1995) reported that dietary DIMBOA stimulated glutathione *S*-transferase activity in the Asian corn borer *Ostrinia furnacalis* (Guenée). Apparently, the effect of the insecticide or plant allelochemical (increase/decrease) on the GST enzyme system in insects depends on the species of insect and on the physicochemical properties of the xenobiotic.

DIMBOA decreased esterase activity both in vivo and in vitro, suggesting that the nucleophilic residues of the esterases involved in catalysis may interact with the hydroxamic acid. The activity of membrane-bound acetylcholinesterases from insect and mammalian sources are suppressed by DIMBOA through the reaction of the hydroxamic acid with the serine moiety at the active centre (Cuevas and Niemeyer, 1993). A similar mechanism for the decrease in activity of cytosolic esterases reported here may be postulated. Since esterases are involved in the metabolism of insecticides such as organophosphates (Conyers et al., 1998; Rufingier et al., 1999), the decreased activity of esterases caused by DIMBOA could lead to increased susceptibility of aphids to these insecticides containing an ester bond. This possibility is

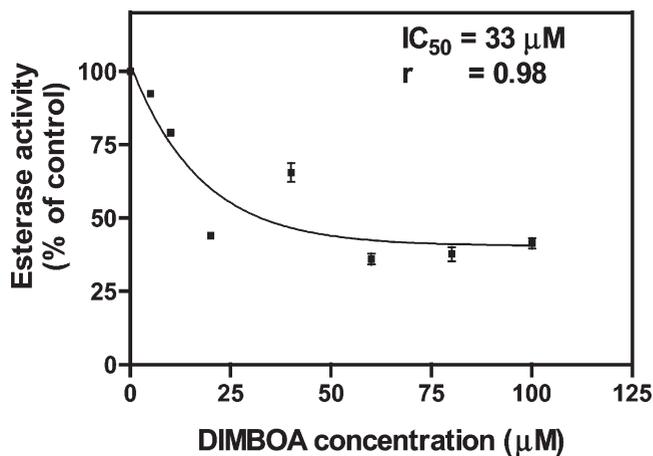


Fig. 4. Effects of DIMBOA on *R. padi* esterase activity in vitro. General esterase activity was assessed using the substrate  $\alpha$ -naphthyl acetate. The incubation mixture consisted of 50  $\mu\text{l}$   $\alpha$ -naphthyl acetate in 95% ethanol, with a final concentration 0.25 mM, 50  $\mu\text{l}$  enzyme (1  $\mu\text{g}$ ) in 0.1 M phosphate buffer pH 7.0 containing NaCl, 5 mM  $\text{MgCl}_2$  and DIMBOA (0–100  $\mu\text{M}$ ). Error bars represent the mean  $\pm$  SD, where  $n = 3$ , i.e., 3 batches each of 100 aphids. The determination of activity for each batch was done in quadruplicate. The  $\text{IC}_{50}$  values, i.e., the concentration of inhibitors required to reduce the activity by 50% (Mannervik and Danielson, 1988), were determined from non-linear regressions of the average percent activity against inhibitor concentration using the Graphpad Prism™ software package (GraphPad Software, Inc., California, USA).

supported by observation of increased susceptibility to the pesticide deltamethrin, reported for *Sitobion avenae* nymphs reared on several wheat cultivars with different DIMBOA levels (Nicol et al., 1993). The observation that there were decreased inhibitory effects for esterase activity between 0.5 and 1 mM DIMBOA and 2.0 and 4 mM DIMBOA, is puzzling. Decreased inhibitory effects at higher concentrations of DIMBOA suggest that, in *R. padi*, enhanced detoxification/binding by esterases could serve as a mechanism of resistance to DIMBOA as DIMBOA is known to inactivate acetylcholinesterases in *R. padi* (Cuevas and Niemeyer, 1993). The decreased inhibition in esterase activity after exposure to an insecticide is known for other insecticides (Kanga et al., 1997). Another explanation may be that for esterases at 4 mM, the feeding deterrent effect of DIMBOA cause reduced ingestion of the allelochemical (Argandoña et al., 1983), and hence an apparent decrease of the inhibitory effect of DIMBOA (Fig. 2B).

The resistance of Hx-containing cereals to aphids may be due to the inhibitory effect of hydroxamic acids on aphid detoxification enzymes, which decreases the capacity of the aphid to detoxify the allelochemicals. This in turn may increase aphid vulnerability to electrophilic agents and other insecticidal compounds. In conclusion, the present study demonstrates the presence of GST and esterases in *R. padi*, and that DIMBOA modifies the levels of activity of these detoxification enzymes both in vitro and in vivo. Thus, DIMBOA may act on aphids by interfering with both esterase and GST-mediated detoxification reactions.

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