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Disturbed activities of the detoxifying enzymes, acid and alkaline phosphatases, of *Galleria mellonella* L. (Lepidoptera: Pyralidae) by four plant growth regulators

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Abstract

The greater wax moth *Galleria mellonella* is a worldwide insect pest damaging wax combs and feeding on bee-hive products. The objective of the current study was to evaluate the impacts of the plant growth regulators (PGRs), *viz.*, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-benzyladenine (6-BA), on the activities of acid phosphatase (ACP) and alkaline phosphatase (ALP) in larvae and pupae of this pest. The 3rd instar larvae were force-fed on diet supplemented with LC_{50} values of these PGRs (0.24, 0.022, 0.16 and 0.085ppm, of IAA, IBA, 2,4-D and 6-BA, respectively). These larvae were continuously fed on the treated diet throughout the larval stage. Activities of acid phosphatase (ACP) and alkaline phosphatase (ALP) were determined in 5th and 7th instar larvae, as well as in pupae of three ages. The most important results could be summarized as follows. All PGRs predominantly induced the treated larvae to gain remarkably increasing activity of ACP, regardless the PGR or the larval instar. In contrast, the ACP activity in pupae of different ages was prevalently reduced by all PGRs. With regard to the disturbed ALP activity, IAA, IBA and 2,4-D exhibited considerably inducing effects on the enzyme activity in 5th and 7th instar larvae. On the contrary, 6-BA had remarkably reducing potency against ALP activity in the larvae. In addition, all PGRs exhibited predominant reducing effects on the enzyme activity throughout the pupal stage.

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Introduction

The greater wax moth or honeycomb moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) is widely distributed throughout the world, causing serious problems in temperate, tropical and subtropical beekeeping regions, where the warm temperature is favour for the rapid development of this insect (Spangler, 1989). It is the major destructive pest of wax combs because of their feeding and tunneling habits through the combs (Chandel *et al.*, 2003; Hanumanthaswamy *et al.*, 2013). Although the adults do not feed because they have atrophied mouth parts, the damage is cause by the larvae due to their voracious feeding leading to the destruction of honeycombs (Awasthi and Sharma, 2013; Ellis *et al.*, 2013; Kwadha *et al.*, 2017). On the other hand, *G. mellonella* larvae are used as a powerful model organism to test the ecotoxicological, immune and physiological effects of environmental pollutants (Altuntaş *et al.*, 2021) as well as to screen the immunotoxic effects of food preservative agents (Erbaş *et al.*, 2022). Also, *G. mellonella* is used as a good model for assessing the activity and toxicity of antimicrobial agents and for studying the immune response to pathogens (Piatek *et al.*, 2021).

The apiculture industry has traditionally relied on synthetic insecticides for the control of insect pests. Several fumigant insecticides, such as sulphur dioxide, acetic acid, formic acid, para dichloro benzene and phosphine have been used to control the infestation of *G. mellonella* on beeswax combs during storage. However, use of these chemicals is harmful to bee population and the bee products (Rajendran and Hajira Parveen, 2005; Kwadha *et al.*, 2017). In addition, the chemical pesticides are responsible for many problems of the environment and human health (Czeher *et al.*, 2008, Yadouleton *et al.*, 2010; Henry *et al.*, 2012; Hallmann *et al.*, 2014). Besides these deleterious effects, the developmental of resistance in insect pests to synthetic pesticides leads to annually economic losses of several billion dollars worldwide (Elzen and Hardee, 2003; Pereira *et al.*, 2006). In plants, pesticides lead to oxidative stress, inhibition of physiological and biochemical pathways, induce toxicity, impede photosynthesis and negatively affect yield of crops (Jan *et al.*, 2020).

For these reasons, new environmentally safer alternative natural compounds are being encouraged (Hussein, 2005; Rehman *et al.*, 2009; Ilyas *et al.*, 2017). One alternative may be the use of plant growth regulators (PGRs) against pest species. PGRs are naturally occurring or synthetic compounds that have the potential to control pest insects through their chemosterilant activity (Becerikli Aksan *et al.*, 2022). Many researchers have focused on the effects of various PGRs on herbivores (Abdellaoui *et al.*, 2013, 2015). They showed that PGRs may have a significant impact on the development, survival and reproduction of herbivores (Kaur and Rup, 2002; Paulson *et al.*, 2005; Tsagkarakis *et al.*, 2012; Prado and Frank, 2013; Abo Elsoud *et al.*, 2021a,b; Nagaratna *et al.*, 2022). Exogenous applications of PGRs provide resistance to plants against pesticides by controlling production of reactive oxygen species, nutrient homeostasis, increase secondary metabolite production, and trigger antioxidant mechanisms (Jan *et al.*, 2020).

PGRs have been classified into different categories. Hopkins and Hüner (2004) classified the PGRs into six classes: Gibberellins (GAs), Auxins (Auxs), Ethylene (ET), Cytokinins (CTKs), Abscisic acid (ABA) and Brassinosteroids (BRs). Stamm *et al.* (2011) classified the PGRs into main nine classes: Auxs, GAs, CTKs, ET, ABA, Brassinosteroids (BRs), salicylic acid (SA), Jasmonates (JAs) and Strigolactones (SLs). Auxins (Auxs) were discovered as the first class of PGRs (Zhao, 2010). Aux is produced at the shoot apex in young leaves and actively moves down but not upwards into buds (Ljung *et al.*, 2001). Because of the wide usage of the indolic compounds as PGRs in the environment, non-target organisms, such as biological control agents could be negatively affected, many authors (Rup *et al.*, 2002; Uçkan *et al.*, 2011, 2014, 2015; Çelik *et al.*, 2017) reported that indolic compounds caused adverse effects on survival, developmental duration, adult longevity, reproductive potential, hemocytes

responses and haemolymph metabolites of various lepidopterous pest species. It is well known hitherto that CTKs have been produced in almost all higher plants as well as many mosses and prokaryotes (Salisbury and Ross, 1992). In addition, insects may produce CTKs, either directly or indirectly owing to their association with endosymbiotic bacteria (Giron and Glevarec, 2014; Zhang *et al*., 2016). However, CTK has been reported to affect the morphology, development and behavior of some insects (Rup *et al*., 1998).

In *G. mellonella*, activities of various detoxification enzymes, such as glutathione S-transferases, phosphatases, glutathione peroxidases, catalase, esterases, NADH dehydrogenase and superoxide dismutase, were disturbed after treatment with different PGRs, such as Gibberrilic acid (GA_3) (Altuntaş, 2015), Ethephon (ETF) (Altuntaş *et al*., 2016) and indole-3-acetic acid (IAA) (Özyılmaz *et al*., 2019). Also, some detoxification enzymes in other insects were disturbed after treatment with various PGRs, such as the mustard aphid *Lipaphis erysimi* (Rup *et al*., 2002, 2006), the fruit fly *Zaprionus paravittiger* (Sharma *et al*., 1997) and the melon fly *Bactrocera cucurbitae* (Kaur and Rup, 2003) after treatment with kinetin. Acid phosphatase (ACP, E.C.3.1.3.2) and Alkaline phosphatase (ALP, E.C.3.1.3.1) are hydrolyzing enzymes, which are responsible for the removal of phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids, in alkaline and acidic conditions, respectively (Janda and Benesova, 1991; Zibaee *et al*., 2011). In insects, ACP and ALP are responsible for cytolysis of tissues during the development and may act as hydrolases during the final stages of digestion, gonad maturation and the final stages of metamorphic molts (Cheug and Low, 1975; Tsumuki and kanehisa, 1984). In insects, also, ACP is responsible for synthesizing higher energy compounds (Hollander, 1971). ALP has the primary function to provide phosphate ions from mononucleotide and ribonucleo-proteins for a variety of metabolic processes (Etebari *et al*., 2005). It is an important synthesizing enzyme of tyrosine, the precursor of dopamine and octopamine, which are known to take part in the control of levels of juvenile hormone and 20-hydroxyecdysone (Rauschenbach *et al*., 2007). The objective of the present study was to evaluate the disturbing impacts of four PGRs, indole-3-acetic acid, indole-3-butyric acid, 2,4-Dichlorophenoxy acetic acid (Auxin compounds) and 6-benzyladenine (a cytokinin compound), on activities of acid and alkaline phosphatases in larvae and pupae of *G. mellonella*.

Materials and methods

The culturing of Galleria mellonella

A culture of susceptible strain of the greater wax worm *Galleria mellonella* L. (Lepidoptera: Pyralidae) was established in the Department of Zoology, Faculty of Science, Al-Azhar University, Cairo, Egypt, and maintained for several successive generations under controlled conditions (27±2°C, 65±5% R.H., photoperiod 14 h L and 10 h D). This culture was originally initiated by a sample of larvae obtained from Desert Research Center, Cairo, Egypt. Larvae were transferred into glass containers, tightly covered with muslin cloth. Different techniques for preparing the artificial diet had been described by some authors (Metwally *et al*., 2012; Nitin *et al*., 2012). In the present culture of *G. mellonella*, an artificial diet was formulated depending on the method of Bhatnagar and Bareth, (2004). The diet contained maize flour (400 g), wheat flour, wheat bran and milk powder, 200 g of each. Also, it was provided with glycerol (400g), bee honey (400g), yeast (100g). Larvae were allowed to continuously feed on this diet throughout the larval stage. However, improved manipulation of different developmental stages had been done according to Ghoneim *et al*. (2019).

The plant growth regulators and concentration preparation

Four plant growth regulators (PGRs) were tested against *G. mellonella*: Indole-3-Acetic Acid (IAA) is a synthetic auxin compound with chemical name: 2-(1H-indol-3-yl)ethanoic acid and molecular formula: $C_{10}H_9NO_2$. Indole-3-butyric acid (IBA) is a synthetic auxin compound with chemical name: 4-(1H-Indol-3-

yl) butanoic acid and molecular formula: $C_{12}H_{13}NO_2$. 2,4-Dichlorophenoxy acetic acid (2,4-D) is a synthetic auxin compound with molecular formula: $C_8H_6Cl_2O_3$. 6-Benzyladenine (6-BA) is a synthetic cytokinin with chemical name: 4-hydroxyphenethyl alcohol and molecular formula: $C_{12}H_{11}N_5$. These PGRs were purchased from Milipore Sigma, Burlington, MA 01803, USA Merk Ltd., Cairo, Egypt.

A series of six concentrations of each PGR was prepared by diluting the compound with distilled water in volumetric flasks, but IBA was exceptionally dissolved in acetone before diluting with distilled water, as follows: 100.0, 10.0, 1.0, 0.1, 0.01 and 0.001 ppm. On the basis of a toxicity bioassay, LC_{50} concentrations were determined as 0.24, 0.022, 0.16 & 0.085 ppm, for IAA, IBA, 2,4-D and 6-BA, respectively.

The larval treatment

For investigating the disturbed phosphatase activities, the 3rd instar larvae were force-fed on diets supplemented with LC_{50} value of each PGR, as follows. Ten grams of the previously described artificial diet were mixed with 2 ml of LC_{50} of each PGR before introduction to the newly moulted 3rd instar larvae, as a food. These larvae were continuously fed on the treated diet throughout the larval stage. Control larvae were provided with distilled water-treated diet. Ten replicates of treated and control larvae (one larva/replicate) were kept separately in suitable glass vials under controlled laboratory conditions (27±2°C, 65±5% R.H., photoperiod 14 h L and 10 h D).

The preparation of tissue samples

Some healthy treated and control larvae of the two later instars (5th and 7th instars) were weighed and then homogenized in a saline solution (one larva/1 ml saline solution 0.7 %) using a fine electric homogenizer for 2 min. With regard to pupae, the phosphatase activities had been determined in successfully developed pupae, of the treated and control groups, at the ages: 3-day old pupae (early-aged pupae), 6-day old pupae (mid-aged pupae) and 9-day old pupae (late-aged pupae). The pupal homogenates were prepared using the same procedure used for larvae. All larval and pupal homogenates were centrifuged at 4000 r.p.m. for 15 min. The supernatant was used directly or frozen until use. Ten replicates were used and homogenates of two individuals were never mixed.

The determination of phosphatase activities

The activities of phosphatases were determined in homogenates of the whole body of larvae and pupae, using the following procedure. Acid phosphatase activity (IU/L) was determined according to the method of Tietz (1986) using the research kits purchased from Biodiagnostics Company (Dokki, Giza, Egypt). The enzyme activity was measured at wave length 405 nm by spectrophotometer. Alkaline phosphatase activity (IU/L) was determined according to the method of Klein *et al.* (1960) using the research kits purchased from Biodiagnostics Company (Dokki, Giza, Egypt). The enzyme activity was measured at wave length 550 nm by spectrophotometer.

Statistical analysis of data

Data obtained were analyzed by the Student's *t*-distribution, and refined by Bessel correction (Moroney, 1956) for the test significance of difference between means using GraphPadInStat© v. 3.01 (1998).

Results

Effects of PGRs on the acid phosphatase activity

After force-feeding of 3rd instar larvae of *G. mellonella* on diets supplemented with LC_{50} concentrations of Indole-3-Acetic Acid (IAA), Indole-3-butyric acid (IBA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-Benzyladenine (6-BA), data of disturbed activity of acid phosphatase (ACP) in homogenates of 5th instar larvae and 7th instar larvae were arranged in Table (1). Depending on these data, ACP level slightly elevated in control larvae with the instar (1.3±0.2 & 1.8±0.1 IU/L in 5th instar and 7th instar larvae, respectively). As obviously shown in the same table, all PGRs predominantly enhanced the treated larvae to gain

remarkably elevated activity of ACP, regardless the compound or the larval instar. Moreover, the strongest inducing potency was displayed by IBA, as determined in 5th instar larvae (1161.5% increasing ACP activity) while the least inducing potency was exhibited by IAA, as determined in 7th instar larvae (594.3% increasing ACP activity).

In the pupal stage, data of Table (2) revealed that the ACP activity gradually elevated with the age of control pupae (59.8±4.11, 70.9±1.33 &74.8±0.6 IU/L in 5th instar and 7th instar larvae, respectively). In contrast to the treated larvae, ACP activity in pupae was prevalently suppressed by all tested PGRs, regardless the pupal age. IBA exerted the most potent reducing action on ACP activity throughout the pupal stage (75.0, 72.4 & 68.5% reduction of ACP activity, in early-, mid- and late-aged pupae, respectively). On the other hand, 2,4-D exerted the least reducing action on the enzyme activity throughout the pupal stage (8.3, 1.6 & 1.4% reduction of the enzyme activity, in early-, mid- and late-aged pupae, respectively). In general, the strongest suppressing effect on the enzyme activity was exhibited by IBA, as determined in the early-aged pupae (75.0% ACP declination) while the least suppressing effect on the enzyme activity was exhibited by 2,4-D, as estimated in the late-aged pupae (1.4% ACP declination).

Table 1. Acid phosphatase activity (IU/L) in the homogenized whole body of *G. mellonella* larvae after force-feeding of 3rd instar larvae on diet mixed with LC_{50} concentrations of plant growth regulator (PGR).

PGR		Larval instar	
		5th instar	7th instar
Indole-3-Acetic Acid	Mean±SD	10.1±0.3 d	12.5±0.3 d
	Change (%)	+676.9	+594
Indole-3-butyric acid	Mean±SD	16.4±1.17 d	18.3±0.9 d
	Change (%)	+1161.5	+916.6
2,4-Dichlorophenoxy acetic acid	Mean±SD	11.2±0.6 d	17.3±0.7 d
	Change (%)	+761.2	+861.1
6-Benzyladenine	Mean±SD	10.8±0.5 d	15.3±1.2 d
	Change (%)	+730.7	+750.2
Control	Mean±SD	1.3±0.2	1.8±0.1

(d): very highly significantly different (p<0.001).

Effects of PGRs on the alkaline phosphatase activity

After force-feeding of 3rd instar larvae of *G. mellonella* on diets supplemented with LC_{50} concentrations of IAA, IBA, 2,4-D and 6-BA, data of disturbed activity of alkaline phosphatase (ALP) in homogenates of 5th instar larvae and 7th instar larvae were arranged in Table (3). Depending on these data, ALP activity slightly elevated in control larvae with the instar (50.6±1.2 & 55.2±1.5 IU/L in 5th instar and 7th instar larvae, respectively). With regard to the disturbing effects of the tested PGRs on ALP activity, data of the same table clearly revealed considerably inducing effects of IAA, IBA and 2,4-D on ALP activity in both larval instars. The strongest enhancing effect was exhibited by 2,4-D, as estimated in 5th instar larvae (99.2% increasing ALP activity) while the least enhancing effect was exhibited by IAA, as estimated in 5th instar larvae (10.6% increasing ALP activity).

On the contrary, 6-BA had remarkably reducing potency against ALP activity in both larval instars (61.0 & 57.2% reduction of the enzyme activity in 5th instar and 7th instar larvae, respectively).

Data of ALP activity in the successfully developed pupae of three ages were distributed in Table (4). On the basis of these data, ALP activity gradually increased in the control pupal stage (196.0±6.5, 225.0±5.0 & 272.3±22.5 IU.L, in early-, mod- and late-aged pupae, respectively). Data of this table

revealed, also, predominant suppressing effects of all compounds on the enzyme activity. Moreover, IAA exerted the strongest suppressing action on ALP activity throughout the pupal stage (98.7, 97.9 & 96.7% reduction of ALP activity in early-, mid- and late-aged pupae, respectively) while IBA exerted the least suppressing action on the enzyme activity throughout the pupal stage (63.3, 65.2 & 69.6% reduction of ALP activity in early-, mid- and late-aged pupae, respectively). In general, the most potent reducing effect on the enzyme activity in the pupal stage was exhibited by IBA (98.7% reduction of ALP activity, as determined in the early-aged pupae) while the least reducing effect was exhibited by IAA (63.3% reduction of ALP activity, as determined in the early-aged pupae).

Table 2. Acid phosphatase activity (IU/L) in the homogenized whole body of *G. mellonella* pupae after force-feeding of 3rd instar larvae on diet mixed with LC_{50} concentrations of plant growth regulator (PGRs).

PGR		Pupal age		
		Early-aged pupae	Mid-aged pupae	Late-aged pupae
Indole-3-Acetic Acid	Mean±SD	29.4±0.8 d	31.7±0.7 d	35.0±1.1 d
	Change (%)	-50.8	-55.2	-53.2
Indole-3-butyric acid	Mean±SD	14.9±1.3 d	19.5±1.2 d	23.5±0.9d
	Change (%)	-75.0	-72.4	-68.5
2,4-Dichlorophenoxy acetic acid	Mean±SD	54.8±3.66 a	69.7±0.5 a	73.7±0.6 a
	Change (%)	-8.3	-1.6	-1.4
6-Benzyladenine	Mean±SD	41.1±2.5 c	44.8±1.8 d	51.0±1.8 a
	Change (%)	-31.2	-36.8	-31.8
Control	Mean±SD	59.8±4.11	70.9±1.33	74.8±0.6

Mean±SD followed with (a): insignificantly different ($p>0.05$). (c): highly significantly different ($p<0.01$), (d): see footnote of table (1).

Discussion

The detoxifying enzymes are generally involved in the enzymatic defense against foreign compounds and play a crucial role for decreasing toxicity of toxic material and maintaining the normal physiological functions in the body (Visetson and Milne, 2001; Mukanganyama *et al.*, 2003; Li and Liu, 2007). In insects, some authors (Weirich *et al.*, 2002; Srinivas *et al.*, 2004; Altuntaş *et al.*, 2016) reported that the detoxification can be achieved by different enzymes, such as esterases, phosphatases, glutathione S-transferase, glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, *etc.*, and they have been reported as protectants against the oxidative stresses. In other words, some of the detoxifying enzymes are involved in the metabolism of xenobiotics or can detoxify the xenobiotics into non-toxic compounds and/or into a form more suitable for rapid elimination from the body (Kaur and Rup, 2003; Rup *et al.*, 2006 Panini *et al.*, 2016; Özyılmaz *et al.*, 2019). The activities of acid phosphatase (ACP) and alkaline phosphatase (ALP) have been disturbed by different botanicals (Diamantino *et al.*, 2001; de Almeida *et al.*, 2014; Ottaviani, 2014; Waheeb, 2020). The activity of some detoxification enzymes involved in the metabolism of xenobiotics can be increased (catalase, esterase, NADH dehydrogenase, glutathione peroxidase, superoxide dismutase and acid phosphatase) or decreased (alkaline phosphatase, esterase, ATPase and O-demethylase) with the influence of the plant growth regulator, kinetin, in the aphid *Lipaphis erysimi* (Rup *et al.*, 2002, 2006) and *B. cucurbitae* (Kaur and Rup, 2003b). However, changes of ACP and ALP activities, after treatment with some botanicals, indicate changes of the physiological balance in the insect (For review, see Senthil-Nathan, 2013).

Table 3. Alkaline phosphatase activity (IU/L) in the homogenized whole body of *G. mellonella* larvae after force-feeding of 3rd instar larvae on diet mixed with LC_{50} concentrations of plant growth regulator (PGRs).

PGR		Larval instar	
		5th instar	7th instar
Indole-3-Acetic Acid	Mean±SD	56.0±2.0 b	69.2±1.8 d
	Change (%)	+10.6	+25.3
Indole-3-butyric acid	Mean±SD	60.9±1.6 d	64.7±1.3 c
	Change (%)	+20.3	+17.0
2,4-Dichlorophenoxy acetic acid	Mean±SD	100.8±1.8 d	108.5±2.5 d
	Change (%)	+99.2	+96.5
6-Benzyladenine	Mean±SD	19.7±2.0 d	23.6±1.1 d
	Change (%)	-61.0	-57.2
Control	Mean±SD	50.6±1.2	55.2±1.5

(d): see footnotes of table (1). (c): see footnotes of table (2).

Disturbed ACP activity in G. mellonella by plant growth regulators (PGRs)

In insects, the detoxification enzyme, ACP, is generally demonstrated as the enzymatic defense against foreign compounds and play a significant role in maintaining their normal physiological functions (Li and Liu, 2007). It plays an important role in the detoxification process of toxic compounds entering the body (Zheng, *et al.*, 2007).

After continuously force-feeding of 3rd instar larvae of *G. mellonella* on diets supplemented with the PGRs, *viz.*, Indole-3-Acetic Acid (IAA), Indole-3-butyric acid (IBA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-Benzyladenine (6-BA), in the present study, activity of ACP in 5th and 7th instar larvae was disturbed, since all PGRs exhibited predominantly inductive effects on the treated larvae to gain remarkably increasing enzyme activity. These results were in agreement with some reported results of increasing ACP activity in different insects after treatment with some PGRs or certain plant-derived compounds. For example, coumarin (Cn) and Neemix (an azadirachtin formulation) caused significant increase of the ACP activity in 4th instar larvae of the Egyptian cotton leafworm *Spodoptera littoralis* (Gaaboub *et al.*, 2012). A significant elevated level of ACP was measured in larvae and pupae of the mosquito *Aedes aegypti* by exposure to Neemazal (a neem formulation) (Koodalingam *et al.*, 2014). The activities of some detoxification enzymes, involved in the metabolism of xenobiotics, increased (such as ACP) as response to the PGR, kinetin, in the mustard aphid *Lipaphis erysimi* (Rup *et al.*, 2002, 2006), the fruit fly *Zaprionus paravittiger* (Sharma *et al.*, 1997) and the melon fly *Bactrocera cucurbitae* (Kaur and Rup, 2003).

To explicate the predominantly induced ACP activity in the 5th and 7th instar larvae of *G. mellonella* after force-feeding of 3rd instar larvae on diets supplemented with IAA, IBA, 2,4-D and 6-BA, in the present study, these PGRs might promote the ecdysone production in *G. mellonella*, that is responsible for the increase of lysosome number as a lysosomal ACP enzyme (Bassal and Ismail, 1985).

The increasing ACP activity could be, also, understood because ACP activity, directly or indirectly, interferes with the digestion, absorption and positive transport of nutrient in the midgut (Smirle *et al.*, 1996; Senthil Nathan *et al.*, 2004). Also, increasing ACP activity in *G. mellonella* larvae, in the current study, might indicate a physiological adaptability to compensate for the PGRs-induced oxidative stress (Altuntaş, 2015) or might be related to an inhibition of lipid peroxidation process and physiological response mechanism against the PGRs' toxicity for cellular detoxification (Altuntaş *et al.*, 2016).

In contrast, all PGRs exhibited reducing effects on the ACP activity in pupae of different ages (early-, mid- and late-aged pupae), after continuously force-feeding of 3rd instar larvae of *G. mellonella* on diets mixed with these compounds, in the current study.

These results were consistent with some reported results of reduced activity of ACP in larvae of some insects by certain plant-derived compounds, such as the grasshopper *Euprepocnemis plorans* after treatment with some neem limonoids (Al-Dali, 2007); the house fly *Musca domestica* after treatment with Margosan-O (Azadirachtin preparation) or Jojoba oil (Ghoneim *et al*., 2008); *S. littoralis* after treatment with Azadirachtin (Ayyangar and Rao, 1990) or essential oils of some aromatic plants (Ibrahim and Abd El-Kareem, 2018); the desert locust *Schistocerca gregaria* after treatment with Neemazal (a neem preparation)(Hamadah, 2009); the tobacco cutworm *Spodoptera litura* after treatment with Andrographolide (Edwin *et al*., 2016); *Rhizopertha dominica* after treatment with hexane extract of *Capparis deciduas* (Upadhyay, 2013); *Tribolium castaneum* after treatment with various doses of different extracts of *Melia azedarach*, *Nicotiana tabacum, Azadirachta indica* and *Colosynthus citrullus* (Ali *et al*., 2015). After treatment of 4th instar larvae of *S. littoralis* with LC_{50} of garlic, peppermint, eucalyptus, and lavender oils, ACP activity was significantly inhibited (Ibrahim and Abd El-Kareem, 2018). Datta *et al.* (2021) fed larvae of *Spodoptera litura* on diet treated with the ethyl acetate extract of *Alpinia galangal* and determined the activity of ACP, after 48 and 96 hrs of feeding. They recorded an inhibitory effect of this extract on the activity of this enzyme.

Table 4. Alkaline phosphatase activity (IU/L) in the homogenized whole body of *G. mellonella* pupae after force-feeding of 3rd instar larvae on diet mixed with LC_{50} concentrations of plant growth regulator (PGR).

PGR		Pupal age		
		Early-aged pupae	Mid-aged pupae	Late-aged pupae
Indole-3-Acetic Acid	Mean±SD	71.8±3.0 d	78.1±1.4 d	82.7±2.7 d
	Change (%)	-63.3	-65.2	-69.6
Indole-3-butyric acid	Mean±SD	2.5±0.3 d	4.7±0.1 d	8.9±0.6 d
	Change (%)	-98.7	-97.9	-96.7
2,4-Dichlorophenoxy acetic acid	Mean±SD	13.5±2.3 d	20.7±1.7 d	26.6±0.8 d
	Change (%)	-93.1	-90.9	-90.2
6-Benzyladenine	Mean±SD	18.2±1.0 d	21.0±1.1 d	23.0±0.6 d
	Change (%)	-90.7	-90.6	-91.5
Control	Mean±SD	196.0±6.5	225.0±5.0	272.3±22.5

(d): see footnote of table (1).

For the interpretation of decreasing activity of ACP in G. mellonella pupae of different ages, after force-feeding of 3rd instar larvae on diets mixed with IAA, IBA, 2,4-D and 6-BA, in the present study, it is important to point out that the ineffectiveness of a toxic material for controlling the insect pests, and subsequently the development of resistance against that material, are due to the action of detoxifying enzymes which are either insensitive to the toxic material or able to degrade it to less toxic metabolites (Biddinger *et al*., 1996). Therefore, decreasing activity of ACP, in pupae, in the present study, indicated that this enzyme play no role in detoxification of the tested PGRs and failure of these pupae to decrease their toxicities or detoxify them. Also, the declination of ACP activity in *G. mellonella* pupae, as a response to treatment with PGRs, might be due to strong inhibition of ecdysone which is followed by subsequent decrease in number of lysosomes and in turn declined level of ACP (Hassan, 2002). In addition, decreasing ACP activity might be due to the reduced phosphorus liberation for energy metabolism

and decreased rate of metabolism, as well as decreased rate of transport of metabolites (Senthil Nathan *et al*., 2005).

Disturbed ALP activity in G. mellonella by PGRs

Many controversial effects of several botanicals on ALP activity are reported in the available literature (Senthil-Nathan *et al*., 2005; Basiouny *et al*., 2010; Ghoneim *et al*., 2016). In the current investigation, the force-feeding of 3rd instar larvae of *G. mellonella* on diets supplemented with the PGRs, IAA, IBA and 2,4-D resulted in a considerable increase of ALP activity in haemolymph of 5th and 7th instar larvae. This result was, to a great extent, in accordance with the reported results of increasing ALP activity in some insects after treatment with some plant-derived products. For example, the lectins significantly increased the level of ALP activity in the bright-line brown-eye *Lacanobia oleracea* larvae (Fitches and Gatehouse, 1998). Induced activity of ALP was determined in the yellow fever mosquito *Aedes aegypti* larvae after treatment with Neemazal (an Azadirachtin preparation) (Koodalingam *et al*., 2014). Water dilutions of Biostop Moustiques® were applied on 4th instar larvae of susceptible and resistant strains of the major malaria vector mosquito *Anopheles gambiae*. The ALP activity was significantly induced in both strains (Ahadji-Dabla *et al*., 2015). Also, ALP activity was reported to be induced in some insects after treatment with extracts of certain plants, such as in larvae of the cabbage white butterfly *Pieris rapae* by methanolic extract of *Silybium marianum* (Hasheminia *et al*., 2013); in haemolymph of newly emerged adults of the desert locust *Schistocerca gregaria* by different extracts of the Khella *Ammi visnaga* fruits (Ghoneim *et al*., 2014) and in haemolymph of *S. gregaria* nymphs and adults by certain extracts of *Nigella sativa* (Ghoneim *et al*., 2016). In the 4th instar larvae of *S. littoralis*, ALP activity significantly induced by LC_{50} of peppermint and eucalyptus oils (Ibrahim and Abd El-Kareem, 2018). After feeding of the whitefly *Bemisia tabaci* adults on tomato seedlings sprayed with different concentrations of IAA, activity of ALP increased in the treated adults (Di *et al*., 2014).

The induced ALP activity in 5th and 7th instar larvae of *G. mellonella* after force-feeding of 3rd instar larvae on diets supplemented with the PGRs, IAA, IBA and 2,4-D, in the present study, might indicate the involvement of this enzyme in detoxification process against these PGRs and an increasing capability of larvae to detoxify them (Sharifi *et al*., 2013). Also, the increased ALP activity could be due to a juvenoid effect of the tested PGRs, since juvenile hormone leads to increasing ALP level in insects (Omar, 2010). In general, the induced ALP activity in *G. mellonella* larvae could be a protective physiological response against the action of the tested PGRs (Ahadji-Dabla *et al*., 2015) or indicated a physiological adaptability to compensate for PGRs-induced oxidative stress (Altuntaş, 2015).

On the contrary, the force-feeding of 3rd instar larvae of *G. mellonella* on diets supplemented with 6-BA led to remarkably reduced ALP activity in the 5th and 7th instar larvae, in the present study. Also, all PGRs, IAA, IBA, 2,4-D and 6-BA, exhibited predominant reducing effects leading to decreasing ALP activity throughout the pupal stage. These results were, to some extent, in corroboration with those results of declined ALP in some insects after treatment with some PGRs or other botanicals. For example, decreased ALP activity was determined in *L. erysimi* (Rup *et al*., 2002, 2006), *Z. paravittiger* (Sharma *et al*., 1997) and *B. cucurbitae* (Kaur and Rup, 2003) after treatment with the PGR, kinetin. The inhibitory effects on ALP activity were reported for extracts of some plants on some insects, such as hexane extract of *C. deciduas* on *R. dominica* (Upadhyay, 2013); different extracts of *Curcuma longa* on *T. castaneum* (Umadevi and Sujatha, 2013); *A. visnaga* seed extracts on last instar nymphs of *S. gregaria* (Ghoneim *et al*., 2014); different extracts of *M. azedarach, N. tabacum, A. indica* and *C. citrullus* on *T. castaneum* adults (Ali *et al*., 2015); LC_{50} of *Acorus calamus* (essential oil) extracts or Biosal (a neem preparation) on *Callosobruchus analis* (Arif *et al*., 2015). After treatment of *S. litura* with Andrographolide, activity of ALP had been remarkably inhibited in larvae (Edwin *et al*., 2016).

Also, ALP activity was decreased when *S. littoralis* 4th instar larvae were treated with garlic and lavender oils (Ibrahim and Abd El-Kareem, 2018). Datta *et al.* (2021) fed larvae of *Spodoptera litura* on diet treated with different concentrations of the ethyl acetate extract of *Alpinia galangal* and determined the activity of ALP, after 48 and 96 hrs of feeding. They recorded an inhibitory effect of this extract on the activity of this enzyme.

In the present study, the remarkable reduction of ALP activity in *G. mellonella* larvae, after treatment with 6-BA, and in pupae, after treatment with IAA, IBA, 2,4-D and 6-BA, could be explicated as previously mentioned for ACP reduction in the same insect by the same PGRs. However, the modulation of ALP could be interpreted rather in terms of a specific response towards a given stressor, like PGRs, in the current investigation, than in terms of a non-specific protective response.

Conclusion

Depending on the present study, the tested PGRs, *viz.*, IAA, IBA, 2,4-D and 6-BA, exhibited predominant inducing effects on the *G. mellonella* larvae to attain increasing phosphatase activities but predominant reducing effects on the enzymatic activities in pupae. Because the induction of detoxification metabolic system plays an important role in insect's detoxification mechanism, increasing activities of ACP and ALP in larvae denoted an increasing capability of *G. mellonella* to detoxify the tested PGRs. Therefore, these PGRs may be ineffective compounds for the IPM program of G. mellonella, dangerous pest of Apiculture.

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