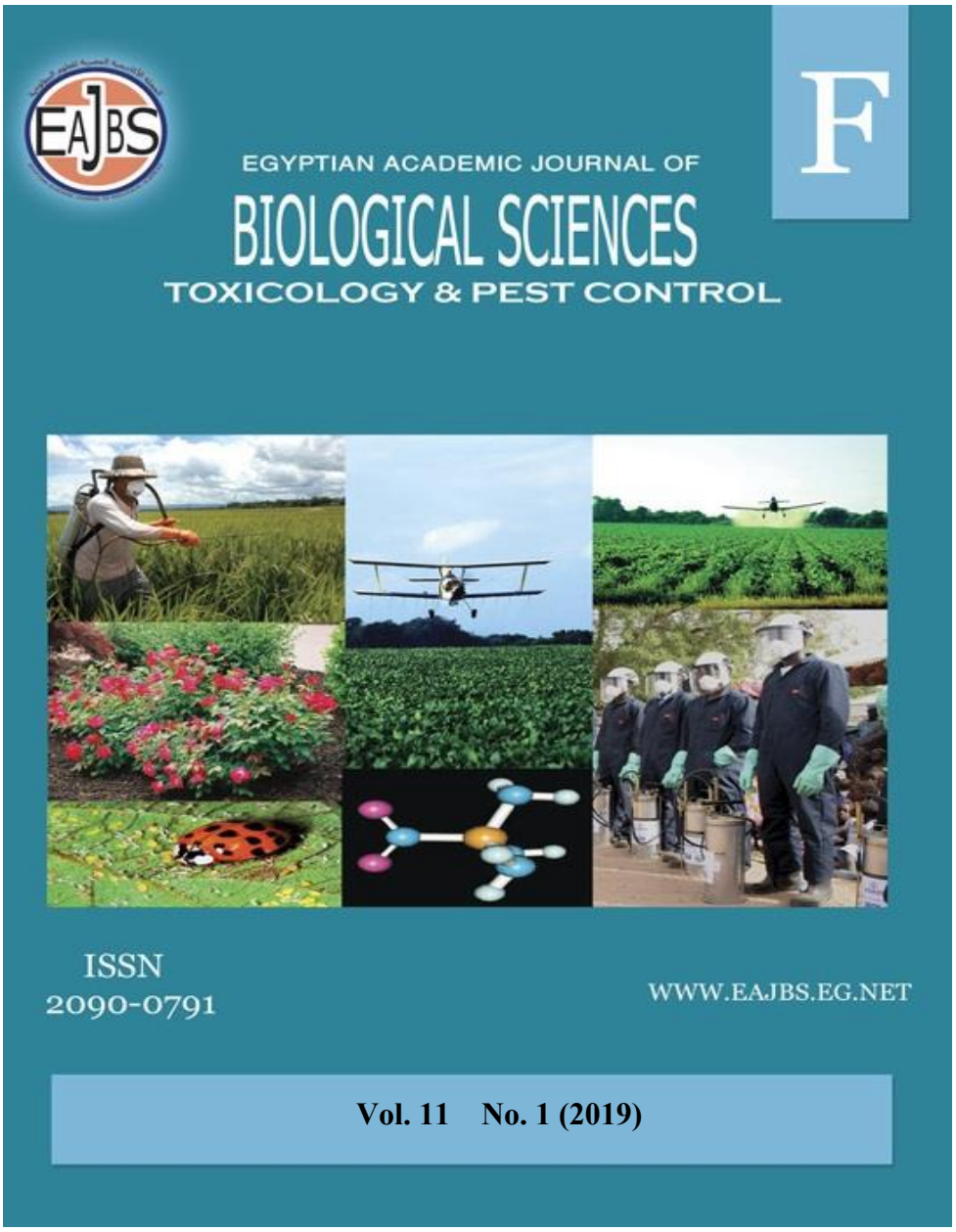

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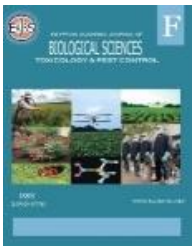

Disturbing Effects of the Chitin Synthesis Inhibitors, Novaluron and Diofenolan, on the Phosphatase Activity in the Pink Bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae).

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ABSTRACT

The pink bollworm *Pectinophora gossypiella* is one of the most destructive insects attacking cotton fields worldwide. It acquired resistance against most of the conventional pesticides. Therefore, the present study was carried out aiming at the investigation of disturbing effects of Novaluron (LC_{50}: 0.765 ppm) and Diofenolan (LC_{50}: 0.036 ppm) on the phosphatase activity in homogenates of larvae (6 hr post-treatment) as well as in early- aged pupae (1-day old), mid-aged pupae (3-day old) and late-aged pupae (7-day old), after treatment of full-grown larvae. After treatment with Novaluron and Diofenolan, both compounds enhanced the larvae to attain remarkably elevated acid phosphatase activity (ACP). In the pupae, ACP activity was predominantly promoted by both compounds, regardless the age. The Alkaline phosphatase (ALP) activity was tremendously declined in the treated larvae. Novaluron enhanced pupae to gain slightly or considerably increasing activity, regardless the age. On the other hand, Diofenolan exhibited a diverse effect since the enzyme activity was pronouncedly declined in the early-aged pupae but remarkably enhanced in the mid- and late-aged pupae.

INTRODUCTION

In insects, acid phosphatase (ACP, E.C.3.1.3.2) and alkaline phosphatase (ALP, E.C.3.1.3.1) are responsible for cytolysis of tissues during the insect development (Dadd, 1970) and may act as hydrolases during the final stages of digestion (Cheug and Low, 1975), gonad maturation and the final stages of metamorphic moults (Tsumuki and kanehisa, 1984). 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). Also, ALP is an important synthesizing enzyme of tyrosine which is known to take part in the control of levels of insect developmental hormones (Rauschenbach *et al*., 2007). In general, ACP and ALP are responsible for removing phosphate groups from many types of molecules under acidic and alkaline conditions, respectively (Janda and

Benesova, 1991; Zibaee *et al*., 2011).

Insecticide resistance involves three major mechanisms, i.e., enhance detoxification, decreased penetration and target site insensitivity (Ahmad and Mccaffery, 1999). Enzyme detoxification is an important mechanism against the conventional pesticides. Detoxification enzyme in insects is generally demonstrated as the enzymatic defending agent against the foreign compounds and plays significant role in the maintenance of their normal physiological functions (Li and Liu, 2007). Insects use detoxification enzymes to reduce the toxicity of poison (Visetson and Milne, 2001). The ineffectiveness of an insecticide for controlling the insect pests, and subsequently the development of insecticide resistance, may be due to the action of enzymes which are either insensitive to the insecticide or able to degrade it into less toxic metabolites (Biddinger *et al*., 1996). In general, the detoxifying enzymes, which react against insecticides, or compounds exhibiting insecticidal activities, include general esterases, glutathione S-transferase, cytochrome, P450, monooxygenases and phosphatases (Ahmad *et al*., 2007; Zibaee *et al*., 2011). Phosphatases have been included in the list of detoxifying enzymes of insecticides; mostly of organophosphorus (Oppenoorth, 1985), however, fenvalerate and cypermethrin resistant larvae of *Helicoverpa armigera* showed higher activities of esterases, phosphatases and methylparaoxon hydrolase compared with susceptible larvae (Srinivas *et al*., 2003). In addition, Abdel-Hafez *et al*. (1985) recorded changes in the phosphatase activities in *Pectinophora gossypiella* during the course of insecticide poisoning. Changed phosphatase activities were also reported in *Tribolium castaneum* by exposure to some insecticides (Ahmed *et al*., 2004).

The pink bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) is one of the most destructive insect pests that cause terrible damage to the cotton in the world because it is difficult to be controlled by conventional insecticides (Patil, 2003; Lykouressis *et al*., 2005). Its larvae damage the floral outgrowths, flowers, bolls, developing seeds within bolls and deteriorate the staple length and strength of lint. The termination of boll growth results in boll rotting and premature or partial boll opening (Abbas *et al*., 2016). In Egypt, this pest causes serious loss of cotton arising to one million qantar annually (El-Aswad and Aly, 2007; Kandil *et al*., 2012). Moreover, *P. gossypiella* has been reported to develop resistance against the transgenic cotton varieties in Arizona (USA)(Fabrick and Tabashnik, 2012) and different districts of India (Monsanto, 2010). In Egypt, this pest has recently developed resistance to several categories of insecticides currently used in cotton fields because of its ability to detoxify these chemicals (Abd-Elhady and Abd El-Aal, 2011).

In general, the intensive and discriminate uses of many conventional pesticides have lead to several drastic problems, such as the environmental pollution, hazards to human and animals like birds, fishes and mammals, destruction of the pollinators and other non-target insects as well as the natural enemies, like parasites and predators (Miles and Lysandrou, 2002; Abo-El Ghar *et al*., 2005; Aydin and Gurkan, 2006; Davies *et al*., 2007; Costa *et al*., 2008; Relyea, 2009; Mosallanejad and Smagghe, 2009). Therefore, alternative materials have been initiated recently to minimize the environment hazards and the serious toxicological problems to humans and animals (Derbalah *et al.*, 2014) as well as to delay the resistance development in *P. gossypiella* (Dahi *et al*., 2009; Hussain, 2012; Salama *et al*., 2013; Sabry and Abdel-Aziz, 2013). At present, using insect growth regulators (IGRs) is considered as the possible alternative way of synthetic insecticides for controlling this pest.

IGRs can be classified according to their modes of action as chitin synthesis inhibitors (CSIs) and substances that interfere with the action of insect hormones (i.e. juvenile hormone analogues, ecdysteroids) (Tunaz and Uygun, 2004). **Novaluron** is a relatively

new benzoylphenyl urea CSI with a good activity against several insect pests, such as the Colorado potato beetle (Cutler *et al*., 2005a,b, 2007; Alyokhin *et al*., 2009), *Spodoptera littoralis* (Ghoneim *et al*., 2015; Hamadah *et al*., 2015, 2016; Tanani *et al*., 2016; Basiouny *et al*., 2016) and *P. gossypiella* (Ghoneim *et al*., 2017), as well as it has only low mammalian toxicity (Barazani, 2001; Ishaaya *et al*., 2002, 2003). Its residues tend to dissipate with a half-life of 2.08 days and the safe use of it was established on various crops in Egypt (Malhat *et al*., 2014). However, Cutler and Scott-Dupree (2007) reviewed some prospects and limitations Novaluron in insect pest management. Diofenolan is a CSI used for the control of several pests, such as lepidopterous species and scale insects (Paloukis and Navrozidis, 1995; Dhadialla *et al*., 1998), *Papilio demoleus* (Singh and Kumar, 2011), *Musca domestica* (Ghoneim *et al*., 2001, 2003), *Rhynchophorus ferrugineus* (Ghoneim *et al*., 2004) and *Schistocerca gregaria* (Ghoneim *et al.*, 2012; Hamadah *et al*., 2012; Tanani *et al*., 2012). This compound did not affect the survival of beneficial parasitoids and predators of some pests, such as *Chrysoperla carnea* (Sechser *et al*., 1994). The main objective of the present study was to evaluate the disturbing effects of the CSIs, Novaluron and Diofenolan, on the activities of acid and alkaline phosphatases in both larvae and pupae of *P. gossypiella*.

MATERIALS AND METHODS

Experimental Insect:

A culture of *P. gossypiella* was originated by a sample of newly hatched larvae from the susceptible culture maintained for several generations in Plant Protection Research Institute, Giza, Egypt. It was reared under constant conditions (27±2°C and 75±5% R.H.) at the Department of Zoology and Entomology, Faculty of Science, Al-Azhar University, Cairo. Larvae were provided with an artificial diet as described by Abd El-Hafez *et al*. (1982). For rearing details and manipulation of all developmental stages under laboratory controlled conditions, see Ghoneim *et al*. (2017).

The CSIs and Larval Treatment:

The tested compounds in the present study were the chitin synthesis inhibitors, Novaluron and Diofenolan. Novaluron (Rimon) [1-[chloro-4-(1,1,2-trifluoromethoxyethoxy) phenyl] -3- (2,6-difluorobenzoyl) urea] has the molecular formula $C_{17}H_9ClF_8N_2O_4$. Diofenolan (Aware®) (2S,4R)-2-Ethyl-4-[(4-phenoxyphenoxy) methyl]-1,3-dioxolane has the molecular formula $C_{18}H_{20}O_4$. Both compounds were purchased from Sigma-Aldrich Chemicals. In a preliminary experiment on full-grown larvae of *P. gossypiella*, LC_{50} values were estimated in 0.765 and 0.036 ppm of Novaluron and Diofenolan, respectively. Four replicates of full-grown larvae (10/replicate) were transferred into Petri dishes (one replicate/dish). Each replicate was sprayed with one of the prepared concentrations using an atomizer. Control replicates were treated with distilled water only using the same technique.

Homogenate Preparation:

After treatment of full-grown larvae, homogenate samples of larvae (6 hr post-treatment) and pupae of three ages: 1-day old (early-aged pupae), 3-day old (mid-aged pupae) and 7-day old (late-aged pupae) were prepared. The treated and control larvae were homogenized in saline solution (50 larvae/5 ml saline solution) using a fine electric homogenizer. Homogenates were centrifuged at 4000 r.p.m. for 15 min. under 2ºC in a refrigerated centrifuge. The supernatant was used directly or stored at -20ºC until the use. For the determination of the enzyme activities in pupae, treated and control pupae of each age were homogenized in saline solution (50 pupae/5 ml saline solution) using a fine electric homogenizer. Homogenates were centrifuged and the supernatant was manipulated as done with larvae.

Determination of Phosphatase Activities:

Acid phosphatase activity was determined in the larval and pupal homogenates according to the method of Tietz (1986) and using a kit of Biodiagnostics. Alkaline phosphatase activity was determined according to the method of Klein *et al*. (1960) using a kit of Biodiagnostics. The enzyme was measured at wavelength 510 nm by a 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 the difference between means.

RESULTS

The activities of phosphatases (Acid phosphatase, ACP and Alkaline phosphatase, ALP) were determined in full-grown larvae of *P. gossypiella* (6 hr post-treatment) as well as in early- aged pupae (1-day old), mid-aged pupae (3-day old) and late-aged pupae (7-day old). Depending on data assorted in Table (**1**), Novaluron and Diofenolan enhanced the larvae to gain remarkably elevated activity of ACP (152.67±5.51 and 229.67±13.65 U/L, after treatment with Novaluron and Diofenolan, respectively, *vs*. 24.33±5.77 U/L enzyme activity in control larvae). As clearly observed, Diofenolan exerted stronger enhancing action on the enzyme activity in larvae than Novaluron.

With regard to the altered activity of the enzyme in the developed pupae, data of the same table obviously revealed predominant enhancing actions of the tested compounds, regardless the age. For some detail, the promoting action of Novaluron gradually declined with the pupal age (1118.92, 819.10 and 269.04% activity increments, in early-, mid- & late-aged pupae, respectively). A similar trend of the promoting action was easily detected after treatment with Diofenolan (274.78, 253.21 and 126.01% activity increments, in early-, mid- & late-aged pupae, respectively). As obviously shown, Novaluron exerted stronger enhancing action on the enzyme activity in pupae than Diofenolan.

Table (1): Acid phosphatase activity in the body homogenate of *P. gossypiella* as influenced by treatment of full-grown larvae with LC_{50} values of CSIs.

CSI		Full-grown larvae *	Pupal age		
			1-day old pupae	3-day old pupae	7-day old pupae
Novaluron	Mean (U/L)±SD	152.67±5.51 d	451.0±18.08 d	242.0±14.18 d	123.0±13.12 d
	Change (%)	+527.50	+1118.92	+819.10	+269.04
Diofenolan	Mean (U/L)±SD	229.67±13.65 d	138.67±7.64 d	93.0±8.19 d	75.33±8.08 c
	Change (%)	+843.98	+274.78	+253.21	+126.01
Control	Mean (U/L)±SD	24.33±5.77	37.0±5.0	26.33±5.51	33.33±8.08

Mean±SD followed by letter (a): not significantly different (P>0.05), (b): significantly different (P<0.05), (c): highly significantly different (P<0.01), (d): very highly significantly different (P<0.001).
*: 6 hr post-treatment.

Table (2): Alkaline phosphatase activity in the body homogenate of *P. gossypiella* as influenced by treatment of full-grown larvae with LC_{50} values of CSIs.

CSI		Full-grown larvae *	Pupal age		
			1-day old pupae	3-day old pupae	7-day old pupae
Novaluron	Mean (IU/L)±SD	2434.0±12.00 d	1889.0±43.14 a	503.67±21.36 d	138.67±18.77 b
	Change (%)	-45.61	+1.32	+323.25	+54.08
Diofenolan	Mean (IU/L)±SD	2347.67±32.32 d	1175.67±55.63 d	671.67±55.63 d	175.67±14.43 d
	Change (%)	-47.54	-36.94	+464.43	+95.19
Control	Mean (IU/L)±SD	4475.0±49.00	1864.33±18.77	119.0±6.93	90.0±6.93

a, b, d, *: see footnote of Table (**1**).

In the light of data arranged in Table (**2**), ALP activity was tremendously declined in the treated full-grown larvae (6 hrs. post-treatment) of *P. gossypiella*, as a response to the inhibitory effects of Novaluron and Diofenolan. Diofenolan estimated this dramatic reduction in the enzyme activity as 45.61% by Novaluron and 47.54%, i.e., Diofenolan was stronger than Novaluron for prohibiting the enzyme activity in larvae.

In contrast, Novaluron slightly or considerably enhanced the developed pupae to gain elevated ALP activity, regardless the age (1.32, 323.25 and 54.08%-elevated activity, in early-, mid- and late-aged pupae, respectively). On the other hand, a diverse effect on the enzyme activity in pupae was exhibited by Diofenolan since the activity was pronouncedly reduced in early-aged pupae (36.94% reduction) but remarkably induced in mid- and late-aged pupae (464.43 and 95.19% increments, respectively). Based on the intensity of the enhancing action on the mid-aged pupae, Diofenolan was stronger than Novaluron (464.43 and 323.25% increments, respectively).

DISCUSSION

Disturbed Acid Phosphatase in *P. gossypiella* by Novaluron and Diofenolan:

According to the currently available literature, increasing activity of acid phosphatase (ACP) was reported in larvae and/or pupae of many insect species after larval treatment with several insect growth regulators (IGRs)(including chitin synthesis inhibitors, CSIs), such as *P. gossypiella* by pyriproxyfen (Anan *et al*., 1993); *Spodoptera littoralis* by Hexaflumuron (Sokar, 1995), Chlorfluazuron, Flufenoxuron and Pyriproxyfen (Abdel-Aal, 2003), Diflubenzuron, Hexaflumuron, Flufenoxuron, Chlorfluazuron, Lufenuron and Tebufenozide (Anwar and Abd el-Mageed, 2005), Chlorfluazuron (Zohry, 2006), Novaluron and Cyromazine (Hamadah *et al*., 2016); *Earias insulana* by pyriproxyfen (Anan *et al*., 1993); *Agrotis ipsilon* by pyriproxyfen (El-Sheikh, 2002); *Culex pipiens* by Cyromazine (Assar *et al*., 2012); *Spodoptera litura* by Methoxyfenozide (Jian-jun and Tian, 2009); *Musca domestica* by Buprofezin, Hexaflumuron, Lufenuron, Tebufenozide and Pyriproxyfen (Assar *et al*., 2010); *Ephestia kuehniella* by pyriproxyfen (Sharifi *et al*., **2013**); *etc*. To a great extent, increasing activity of ACP in larvae and pupae of *P. gossypiella*, as recorded in the present study, are in agreement with those previously reported results, since treatment of full-grown larvae with LC_{50} values of Novaluron (0.765 ppm) and Diofenolan (0.036 ppm) resulted in elevated activity of ACP in homogenates of larvae (6 hr post-treatment) and pupae (of all ages: 1-, 3- and 7-day old). Diofenolan was stronger than Novaluron for promoting larvae and pupae to gain a high

level of the enzyme activity. In contrast, the present results disagree with those reported results of declined ACP activity in larvae of some insects as response to various IGRs, such as *S. littoralis* by Pyriproxyfen (Mostafa, 1993), Triflumuron (El-Bermawy, 1994), Chlorfluazuron (Zohry, 2006), Flufenoxuron (Bakr *et al*., 2010), Tebufenozide and Lufenuron (Bakr *et al*., 2013), Diofenolan (Hamadah *et al*., 2016); *M. domestica* by Triflumuron or Pyriproxyfen (El-Bermawy, 1994; Hassanein *et al*., 1996); *Bombyx mori* by Pyriproxyfen (Etebari *et al*., 2007); *etc*.

The elevated activity of ACP in larvae and pupae of *P. gossypiella*, in the current work, may be attributed to increasing lysosome number as a response to the tested CSIs (Novaluron and Diofenolan), as suggested for ecdysone which is responsible for the increase of lysosomal ACP enzyme (Bassal and Ismail, 1985). This result can be, also, interpreted since ACP, directly or indirectly, interferes with the food digestion and absorption (Smirle *et al*., 1996; Senthil Nathan *et al*., 2004).

Disturbed Alkaline Phosphatase in *P. gossypiella* by Novaluron and Diofenolan:
After treatment of full-grown larvae of *P. gossypiella* with LC_{50} values of Novaluron or Diofenolan, in the present study, the alkaline phosphatase (ALP) activity was tremendously declined in larvae. Also, pronouncedly suppressed enzyme activity was recorded only in the early-aged (1-day old) pupae after treatment with Diofenolan. These results corroborated with those reported results of inhibited ALP activity in certain tissues of some developmental stages of some insects by various IGRs, such as *C. pipiens* by Diflubenzuron (Yan and Wu, 1990) or Cyromazine (Assar *et al*., 2012); *B. mori* by pyriproxyfen (Etebari *et al*., 2007); in haemolymph of *S. littoralis* larvae Chlorfluazuron or Flufenoxuron (Anwar and Abd el-Mageed, 2005) and in fat bodies of *S. littoralis* larvae by Novaluron, Cyromazine or Diofenolan (Hamadah *et al*., 2016). However, inhibition of ALP activity in larvae and pupae of *P. gossypiella*, in the present investigation, may be attributed to the effects of tested CSIs, directly or indirectly, on the juvenile hormone and ecdysone regulation (Sridhara and Bhat, 1963). It may be, also, explicated by some developmental disturbance as appreciably suggested by Wu (1990) for *C. pipiens* after treatment with Diflubenzuron.

On the other hand, the increasing ALP activity had been recorded in pupae of *P. gossypiella*, in the present study. After treatment of full-grown larvae with the tested CSIs, ALP activity was slightly or considerably elevated in the successfully developed pupae, of all ages, by Novaluron while Diofenolan enhanced the mid- and late-aged pupae to attain remarkably high ALP activity. The present results are, to some extent, in agreement with those reported results of increasing ALP activity in certain tissues of some developmental stages of the same insect after treatment with pyriproxyfen (Mostafa, 1993) as well as other insects by different IGRs, such as *S. littoralis* by Pyriproxyfen (Abdel-Aal, 2003), Diflubenzuron or Lufenuron (Anwar and Abd el-Mageed, 2005), Novaluron, Cyromazine or Diofenolan (Hamadah *et al*., 2016); *E. insulana* (Anan *et al*., 1993) and *E. kuehniella* (Sharifi *et al*., 2013) by pyriproxyfen; *M. domestica* by Buprofezin, Hexaflumuron, Lufenuron or Tebufenozide (Assar *et al*., 2010); *Schistocerca gregaria* by pyridalyl (Teleb *et al*., 2012); *etc*. However, increasing ALP activity in pupae of *P. gossypiella* after treatment of the full-grown larvae with Novaluron and Diofenolan, in the present study, may indicate the involvement of this enzyme in detoxification process against these CSIs (Shekari *et al*., 2008).

It is important to point out that the detoxification enzymes, including acid and alkaline phosphatases, in insects are generally demonstrated as the enzymatic defence against foreign compounds and play significant roles in maintaining their normal physiological functions (Li, and Liu, 2007). There is a relationship between the increase of insecticide

resistance and the activity of detoxification enzymes (Xin-Ju and Hui-Min, 2011). Inhibition of the detoxifying enzymes indicates that these enzymes play no role in the detoxification of tested compounds and may increase the susceptibility of the insect pest against these compounds (Abd-Elaziz and El-Sayed, 2009). On the other hand, increasing activities of these phosphatases denote the insecticide insensitivity in many insect species (Zhou *et al*., 2002) or even indicate an increasing capability of the target insect to detoxify the tested compounds (Sharifi *et al*., 2013). Therefore, the ineffectiveness of IGRs in controlling insect pests is attributed to the increased levels of enzymatic detoxification (Biddinger *et al*., 1996).

Conclusion:

Because the induction of detoxification metabolic system plays an important role in insect's detoxification mechanism, the prevalent increasing activities of ACP and ALP in larvae and pupae of *P. gossypiella* by Novaluron and Diofenolan, in the present study, denotes an increasing capability of it to detoxify these compounds. Therefore, the tested compounds unfortunately, may not be effective agents in the integrated management of this pest.

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