https://doi.org/10.17221/46/2022-PPS

Biological impact, oxidative stress and adipokinetic hormone activities of *Agrotis ipsilon* in response to bioinsecticides

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Citation: Moustafa M.A.M., Elmenofy W.H., Osman E.A., El-Said N.A., Awad M. (2022): Biological impact, oxidative stress and adipokinetic hormone activities of *Agrotis ipsilon* in response to bioinsecticides. Plant Protect. Sci., 58: 326–337.

Abstract: The use of biological control products enhances the reduction of harmful pressures on the environment caused by the use of conventional pesticides. Therefore, this study aims to evaluate the toxicity of eight bioinsecticides on the black cutworm *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) to understand the relationships between insecticide exposure and insect response at the individual and population levels. The bioassay results showed that emamectin benzoate had high toxicity against *A. ipsilon* second instar larvae with an LC_{50} of 0.007 (mg/L). Sublethal concentration (LC_{30}) of emamectin benzoate, spinosyn group and *Bacillus thuringiensis* formulation (Dipel 2X) had significantly prolonged pupal period. In addition, emamectin benzoate significantly decreased the number of eggs laid per female (135.3 ± 6.919) compared with the control treatment. Thus, the formulation of chlorfenapyr showed a 1.26-fold reduction in hatching rate. Meanwhile, the activities of oxidative stress enzymes (catalase, superoxide dismutase, lipid peroxidase, and glutathione reductase) were significantly affected due to exposure to sublethal concentration. Changes in adipokinetic hormone (AKH) transcriptional regulation were detected via SQ-PCR via using cDNA synthesized from mRNA isolated from treated *A. ipsilon* larvae. The results showed a higher transcription rate of AKH in spinotram-treated larvae with a 1.42-fold increase over untreated larvae. Our results provide useful information for integrated pest management programs for *A. ipsilon* by using bioinsecticides.

Keywords: toxicity; black cutworm; bioinsecticde; enzyme activity; AKH

Black cutworm, *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae), poses a serious threat to many crops (Williamson & Potter 1997). *A. ipsilon* larvae cause crop deficiency in unprotected crop fields and cause huge economic losses (Yu et al. 2012; Du et al. 2013). Therefore, chemical insecticides have been used as the most common control strategy. However, the regular use of these insecticides could cause a resistance problem; *A. ipsilon* has developed resistance to organophosphates, carbamates, and pyrethroids because of the highly frequent use of these conventional chemical insecticides at high concentrations and without active substance rotation (Yu et al. 2012; Xu et al. 2016). Therefore, dur-

Supported by the Science, Technology & Innovation Funding Authority (STDF) (Grant No. 28938).

https://doi.org/10.17221/46/2022-PPS

ing the past three decades (from the 1990s), a new range of insecticides has been developed to reduce the risk of resistance development (Horowitz & Ishaaya 2004; Sparks et al. 2019). A new group of insecticides, bioinsecticides, including microbial and fermentation products, could represent promising alternative compounds synergy with other biological control methods (Urbaneja et al. 2012). Bioinsecticides based on *Bacillus thuringiensis* (Bt) are used worldwide for pest control, with sales accounting for over 90% of all bioinsecticide (Sayed & Behle 2017). Bt harbors several kinds of insecticidal proteins, such as crystalline (Cry) proteins (Moustafa et al. 2013) used against several insect pests. This Cry toxin binds to specific receptors in the midgut and derange the uptake of nutrients or cause perforation of the gut wall leading to general sepsis and death (Bravo et al. 2004).

Spinosyn group, including spinosad and spinetoram are fermented metabolite products of the soil actinomycete *Saccharopolyspora spinosa* (Thompson et al. 1997). Spinosad is a mixture of spinosyn A and D, whereas spinetoram, the second generation of spinosyn group, is composed of spinosyn J and L (Bacci et al. 2016). Spinosyn group has strong insecticidal activity against a wide spectrum of insect pests (Bret et al. 1997; Sparks et al. 1998). It affects γ-aminobutyric acid (GABA) or glutamate receptors (Duce et al. 1995) as a primary site of action and persistent activation of nicotinic acetylcholine receptors as a secondary site on other subunits different from all other nicotinic agonists (Salgado et al. 1997). In addition, emamectin benzoate is composed of two macrocyclic lactones metabolites, avermectin B_{1a} and B_{1b} (Mushtaq et al. 1997), produced through the fermentation process of the soil microorganism, *Streptomyces avermilitis* (Crouch et al. 1997). Furthermore, emamectin benzoate has an insecticidal activity against lepidopteran insect pests, including *Helicoverpa zea* (Boddie), *Spodoptera littoralis* (Boisd.) and *Mamestra brassicae* (Linnaeus) (López et al. 2010; El-Sheikh et al. 2015; Moustafa et al. 2016). It affects the glutamate- or GABA-gated chloride channels, causing the hyperpolarization action of a neuronal cell (Jansson et al. 1997; Grafton-Cardwell et al. 2005). However, chlorfenapyr is specifically a pro-insecticide, activated after oxidative removal of the *N*-ethoxymethyl group by mixed-function oxidases. It was derived from a natural product dioxapyrrolomycin, isolated from *Streptomyces fumanus* actinomycete (Ahmed & Mehmood 2015). Chlorfenapyr works by disrupting the mitochondrial uncoupling (Black et al. 1994), effective against lepidopteran pest, thrips and mites.

Determining the sublethal effects of bioinsecticides is important (Vojoudi et al. 2017) to prolong the long-term efficiency of these insecticides in pest management. Therefore, the sublethal effect of bioinsecticides has been recorded in several insect pests, including *A. ipsilon* (He et al. 2019), *Plutella xylostella* (Linnaeus) (Han et al. 2012; Su & Xia 2020), *M. brassicae* (Moustafa et al. 2016), *S. littoralis* (Moustafa et al. 2021a), *Helicoverpa armigera* (Hűbner) (Vojoudi et al. 2017) and *Tuta absoluta* (Meyrick) (Kandil et al. 2020a). Thus, insecticides with different chemical classes could induce the reactive oxygen species (ROS) (Abd El-Aziz & Fahmy 2015), which are generated from the oxidative metabolism of cells (Bagchi et al. 1995). Functionally, oxidative stress (OS) occurs because of the imbalance between higher levels of ROS and the cellular antioxidant defense (Ilhan et al. 2005). In insects, the antioxidant enzyme system, which comprises enzymes such as catalase (CAT) and superoxide dismutase (SOD) or non-enzymatic antioxidants such as glutathione reductase (GR), can stop the deleterious effect of ROS (Felton 1995; Rindler et al. 2013). Activation of the antioxidant mechanism is a complex process under hormonal control (Lu et al. 1991) of adipokinetic hormones (AKHs) (Kodrík et al. 2007; Večeřa et al. 2007). AKH is a synthesized neuropeptide, released from the corpora cardiaca, and recognized as the main antioxidant stress hormone in insects (Kodrík et al. 2015). A main function of AKHs is to control the metabolism of insects (Gäde et al. 1997). They act as typical stress hormones through catabolic stimulation (mobilize lipids, carbohydrates, and certain amino acids), providing more energy and inhibiting structural reactions. This increases the concentration of energy substrates in the hemolymph (lipids, carbohydrates, proline), inhibits synthetic reactions (lipid, protein, RNA), and control of many supportive actions, including motility stimulation (Kodrík & Socha 2005).

In this context, the current work aims to assess the sublethal effects of certain bioinsecticides on the development and reproductive activity parameters (fecundity and fertility) of *A. ipsilon*. In addition, we investigate the effect of these bioinsecticides on changes in the activity of oxidative stress

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enzymes and AKH to show their role in resolving critical situations disturbing the homeostasis on *A. ipsilon*.

MATERIAL AND METHODS

Agrotis ipsilon culture. We reared *A. ipsilon* in the laboratory according to Moustafa et al. (2021b). The culture was maintained in a rearing room at 27 ± 2 °C, 55 ± 5% relative humidity under a reversed 16L : 8D (light : dark) regime. The larvae from the 3rd instar (He et al. 2019) were reared in small plastic cups (7.0 cm in diameter, 3.5 cm in height) individually with a castor bean leaf to prevent cannibalism. The emerged moths were transferred to glass jars (5 L), with 10% sugar solution as source of feeding (Kandil et al. 2020a) and a piece of black fabric mesh netting to lay eggs.

Tested insecticides. Common name, trade name, and mode of action of tested insecticides are detailed in Table 1.

Bioassay. Toxicity of the above bioinsecticides was assessed on the 2nd larval instar of *A. ipsilon* using the leaf dipping technique (Moustafa et al. 2021b). Five different concentrations of each bioinsecticide formulation were used (Bt formulations: from 1 600 to 200 mg/L, spinosyns formulations: from 5 to 0.012 5 mg/L, proclaim formulation: from 1.25 to 0.000 125 mg/L and Challenger formulation: from 50 to 0.125 mg/L). First, castor bean leaves were dipped in each concentration for 20 s, while untreated leaves were dipped in water for the control, then both treatments were air-dried for 30 minutes. Next, both treated and untreated leaves were transferred into a glass container (0.25 L) and ten larvae with five replications were added and left to feed for 24 h. After four days (96 h) post-treatment, the mortality percentage was taken to calculate the lethal and sub-lethal concentrations of each formulation on *A. ipsilon* larvae. The bioassay experiments were repeated twice.

Sublethal effects of bioinsecticides on development of A. ipsilon. We exposed the 2nd larval instar of *A. ipsilon* to the sublethal concentration of LC_{30} for the tested bioinsecticides as described above. Three replicates, with 50 larvae each, were performed. Seven days after the exposure (Awad et al. 2022), surviving larvae were transferred individually to clean plastic cups to record larval duration, mortality % and pupation %. Each pupa was sexed, weighed and maintained individually with moist cotton to record the total pupal duration and emergence %. After emergence, five females and seven males in three replicates were transferred to clean glass jars (5 L) as described by Moustafa et al. (2016). Egg clusters were collected daily and transferred to a clean jar with a piece of wet cotton until hatching to calculate the fecundity (total number of eggs laid/female) and fertility (total number of larvae/egg cluster).

Enzyme activity determination

Sample preparation. The second larval instar of *A. ipsilon* was fed on castor bean leaves dipped in LC_{30} equivalent concentration for each formulation as described above. The treatment was repeated three times. Approximately 100 mg of the surviving

Table 1. Tested bioinsecticides and their mode of action

Bioinsecticides		Trade names (producer)	Formulation (a.i. %)	Mode of action*
Bacillus thuringiensis		Diple 2x (Valent Biosciences, USA)	6.4% WP	Microbial disruptors of insect midgut membranes
		Diple DF (Valent Biosciences, USA)	6.4% WP	
		Protecto (Institute of Plant Protection, Egypt)	9.4% WP	
		Biotect (Organic Biotechnology, Egypt)	9.4% WP	
Spinosyns	spinosad	Tracer (Wadi El-Nile, Egypt)	24% SC	Nicotinic acetylcholine receptor allosteric modulators – site I
	spinetoram	Radiant (Shoura, Egypt)	12% SC	
Emamectin benzoate		Proclaim (Syngenta, Switzerland)	5% SG	Glutamate-gated chloride channel allosteric modulators
Chlorfenapyr		Challenger (BASF, USA)	24% SC	Uncouplers of oxidative phosphorylation via disruption of the proton gradient

SC – suspension concentration; SG – water soluble granules; WP – wettable powder

*Insecticide Resistance Action Committee (IRAC 2020)

https://doi.org/10.17221/46/2022-PPS

A. ipsilon larvae were transferred to a clean Eppendorf tube and kept at –20 °C prior to the biochemical assays. Untreated larvae were subjected to the same procedure. The frozen *A. ipsilon* larvae were homogenized in ice-cold potassium phosphate buffer (50 mM, pH 7.0) in a ratio of 30 µL buffer per 1 mg body weight, and centrifuged at 7 000 *g* for 15 min at 4 °C, and the supernatant was placed into a new tube and kept on ice for the next step.

Enzyme assay. SOD activity was evaluated according to the method described by Nishikimi et al. (1972) using a Biodiagnostic Kit (Biodiagnostics Company, Egypt). CAT activity was measured using a Biodiagnostic Kit according to Aebi (1984) at an absorption rate of 510 nm. Lipid Peroxidase Assay Kit (Biodiagnostics Company, Egypt) was used to monitor malondialdehyde (MDA) formation at 534 nm (Ohkawa et al. 1979). Glutathione reductase activity was measured by a Biodiagnostic kit according to Goldberg & Spooner (1983). The estimation of Glutathione reductase was based on the reduction of glutathione (GSSG) in the presence of NADPH, which is oxidized to $NADPH^+$ at 340 nm.

Determination of gene expression of AKH

Dissection and tissue homogenization. The gut tissue of treated *A. ipsilon* larvae was extracted after treatment using eight different bio-agents as mentioned above. The head and the last abdominal segment of the *A. ipsilon* larva was cut off; the midgut and the content with a peritrophic membrane was separated from the midgut tissue devoid of Malpighian tubules. The midgut tissue was rinsed in the Ringer saline, dissected in liquid nitrogen and stored at –80 °C for the downstream work.

RNA purification and cDNA synthesis. Total RNA was isolated from the midgut tissue of *A. ipsilon* larvae using Gene JET RNA purification kit following the manufacturer's instruction (ThermoFisher, USA). The purity was estimated and approximate concentration of total RNA extracted using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA) and the quality of isolated RNA was checked by electrophoresis. Prior to mRNA quantification, all RNA samples were treated using DNaseI to eliminate genomic DNA contamination. We unified about 1 µg of total RNA and used it to generate first-strand cDNA as the initial step of a two-step RT-PCR protocol using RevertAid first-strand cDNA synthesis kit (Thermo Scientific, USA) according to the manufacturer's instruction.

Semi quantitative RT-PCR. Primers were designed for target gene sequences using Primer Quest and obtained from Integrated DNA Technologies (https://www.idtdna.com/Scitools/Applications/Primerquest/). We designed six degenerate primer sets and synthesized based on AKH gene sequences published in the GenBank (http://www.ncbi.nlm.nih.gov). All primer sets used for AKH are listed in Table 2. We performed the RT-PCR reaction using 1 µL of the first-strand cDNA synthesis in a total volume of 25 µL containing the following components: 1 µL of the forward primer (10 pmol/µL), 1 µL of the reverse primer (10 pmol/µL), and 12.5 µL of Emerald-Amp Max PCR Master Mix (Takara Cat# RR320A). We performed negative control by omitting reverse transcriptase. We set up atypical touch-down PCR program as follows: 94 °C for 3 min, followed by nine cycles of 94 °C for 45 s, 71–62 °C for 45 s in a touch-down manner (1 °C/cycle) and 72 °C for 45 seconds. We then used

Table 2. Primers used for PCR amplification of AKH gene fragment using *A. ipsilon* treated larvae

Nr	Name	Primer seq. 5'–3'	From–to (nucleotides)	Optimal annealing	PCR (bp)
1	AGIP-AKH1_F1	ACTTCATCATGGGGTGGTGGTA	(1/2)	51.2	132
2	AGIP-AKH1_R1	TGATTTTTGTTGGCAGATGATG			
3	AGIP-AKH1_F2	ACTTCATCATGGGGTGGTGGTAA	(3/2)	51.2	132
4	AGIP-AKH1.1_R1	CAGCTTCGTTTTGGATCAGTTTGT	(3/4)	52.5	106
5	AGIP-AKH1.1_R2	GCAGTCTTTCAGCTTCGTTTTG	(1/5)	52.0	115
6	AGIP-AKH2_F1	GAATCTTCATCGTGCTGCTGGTG	(6/7)	53.0	123
7	AGIP-AKH2_R1	AGTCGTCGTTGATTTGTTCTGATG			
8	AGIP-AKH2_R2	AGTCGTCGTTGATTTGTTCTGA	(6/8)	51.9	123

Table contains primers' nucleotide sequencing; each pair of primers used for amplification, optimal annealing temperature, and the expected PCR product

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the following annealing temperature for 21 cycles: 55 °C to each 30 cycles with a final extension at 72 °C for 5 minutes. Subsequently, we loaded 10 µL of the RT-PCR product on 1.7% agarose gel in 1x TAE buffer stained with ethidium bromide and visualized under UV light and photographed using a Gel Doc-XR$^+$ (Bio-Rad, USA) system with image lab software Bio-Rad.

Data analyses. Larval mortality percentage was calculated by using Abbott's formula (Abbott 1925) and corrected according to Finney (1971) by using Probit analysis (LDP-line Probit analysis program) to determine the lethal and sublethal concentration values (LC_{30}, LC_{50} and LC_{90}) for each bioinsecticide (with 95% confidence limits).

The biological and enzymatic parameters were proceeded to assess the significant differences of all possible treatments according to control and treated larvae involved on eight levels of tested bioinsecticides by using One Way ANOVA through the SPSS software (version 27). The estimating differences among means of the treatments was performed depending on significance level ($P \leq 0.05$) by using Duncan's multiple range tests (Duncan 1955). Subsequently, the assumption of regular completely randomized design (CRD) as a one Factor included nine of treatments with three replicates was done according to Snedecor & Cochran (1989). The normality of obtained data was checked by the Kolmogorov-Smirnov test. The normality test of the percentage traits namely pupation percentage, sex ratio, emergence and hatchability percentages, which tested by $P < 0.05$ was significant. Therefore, Arc sine transformation (Gomez & Gomez 1984) was performed before analysis of data by Chi square (χ^2) at $P < 0.05$. The figures of reproductive activity and enzymatic activity were designed using Graph Pad Prism statistical analysis software (version 9).

RESULTS

Acute toxicity of bioinsecticides on A. ipsilon. Toxicity of eight bioinsecticide formulations following treatment of 2nd larval instar of *A. ipsilon* are presented in Table 3. After 96 h post-treatment, the LC_{50} values ranged from 0.007–890.5 mg/L. The LC_{50} values were 185.5, 477.3, 718.2, and 890.5 mg/L for Bt formulations (Dipel 2X, Dipel DF, Protecto and Biotect, respectively). The LC_{50} values for spinosad formulation (Tracer), spinotram

Table 3. The calculated lethal and sublethal values of bioinsecticides in *A. ipsilon* larvae

Common name	Trade names	LC_{30} (mg/L) (95% confidence limits)	LC_{50} (mg/L) (95% confidence limits)	LC_{90} (mg/L) (95% confidence limits)	χ^2	Slope ± SE	Toxicity index
Bacillus thuringiensis	Dipel 2X	140.1 (108.9–166.6)	185.5 (154.7–221.0)	368.5 (294.7–546.5)	0.12	4.30 ± 0.74	0.003 7
	Dipel DF	235.2 (94.11–341.9)	477.3 (322.9–675.3)	2 692.4 (1 447.9–16 185.2)	0.48	1.70 ± 0.44	0.001 4
	Protecto	546.1 (431.9–627.9)	718.2 (624.2–835.5)	1 402.664 (1 113.9–2 315.0)	1.13	4.41 ± 0.91	0.000 9
	Biotect	671.2 (562.8–770.0)	890.5 (775.6–1132.4)	1 777.126 (1 317.9–3 746.9)	2.40	4.27 ± 0.97	0.000 8
Spinosad	Tracer	0.089 (0.041–0.146)	0.244 (0.152–0.385)	2.806 7 (1.354–11.50)	6.65	1.21 ± 0.22	2.86
Spinotram	Radiant	0.043 (0.028–0.062)	0.157 (0.108–0.236)	3.770 3 (1.965–9.132)	2.71	0.93 ± 0.08	4.45
Emamectin benzoate	Proclaim	0.002 (0.001–0.004)	0.007 (0.003–0.014)	0.150 9 (0.057–0.837)	2.49	0.94 ± 0.16	100
Chlorfenpyr	Challenger	0.338 (0.185–0.537)	1.389 (1.175–4.339)	43.90 (24.74–96.09)	2.79	0.85 ± 0.08	0.503

Castor bean leaves were dipped in five different concentrations of each bioinsecticide. Second instar larvae of *A. ipsilon* were fed on the treated leaves for 24 h and after 96 h; the lethal and sublethal values were calculated. Treatments were performed in five replicates; each one has ten larvae

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formulation (Radiant), emamectin benzoate formulation (Proclaim), and chlorfenapyr formulation (Challenger) were 0.244, 0.157, 0.007 and 1.389 mg/L, respectively. Based on the values denoted, the decreasing order of toxicity to *A. ipsilon* was emamectin benzoate > spinotram > spinosad > chlorfenapyr > Bt formulations (Dipel 2X > Dipel DF > Protecto > Biotect).

Effects of bioinsecticides on biological parameters of *A. ipsilon*.

Effects on the life-table characteristics of A. ipsilon. Effect of the LC_{30} value as a sublethal concentration for the eight bioinsecticide formulations on life-table parameters of *A. ipsilon* are presented in Table 4. The results showed no statistically significant differences were found in the larval duration, pupation %, sex ratio and emergency %. In addition, the duration of the pupal stage was significantly longer under LC_{30} sublethal concentration of chlorfenapyr, emamectin benzoate and Bt formulations (Dipel 2X and Dipel DF) by 20.35, 19.73, 19.71 and 19.60 day, respectively than in control (18.30 days). In contrast, female pupal weight showed a statistically significant increase after the 2nd instar larvae were treated with Bt formulations (Dipel DF, Biotect, Protecto and Diple 2X) and spinosad.

Fecundity and fertility. A sublethal concentration of emamectin benzoate significantly reduced fecundity per female (135.33 ± 6.919) compared with control (280.50 ± 31.85) [df = 8, $F_{(8,18)} = 3.35$, $P = 0.015\ 7$], where the percentage of reduction in the number of eggs laid by one female was about 2.07 fold (Figure 1A). In comparison with control, fecundity per female have been significantly decreased when treated with spinotram, chlorfenpyer and Bt formulations (Protecto and Biotect) by 146.07, 149.07, 191.33, 202.33 egg/female, respectively. No significant differences were detected in the hatchability compared with the control group with all the tested bioinsecticides.

Sublethal effects on enzyme activity of A. ipsilon. Data in Figure 2 shows that exposures to Bt formulations, spinosad formulation (Tracer), spinotram formulation (Radiant), emamectin benzoate formulation (Proclaim) and chlorfenapyr formulation (Challenger) caused changes in the level of the enzyme activity of OS enzyme. The results showed that exposures to spinotram, chlorfenapyr, Bt formulation (Dipel 2X), emamectin benzoate, *B. thuringiensis* formulation (Dipel DF), spinosad and Bt

Table 4. Life history traits (mean ± SE) on second larval instar of *A. ipsilon* exposed to sublethal concentration (LC_{30}) of different bioinsecticides

Common name	Trade names	Larval duration (days)	Pupation (%)	Pupal duration (days)	Pupal weight (g)		Sex ratio		Emergence (%)
					female	male	female	male	
Control		20.40 ± 0.17	100.0 ± 0.00	18.30^{e} ± 0.29	0.39^{e} ± 0.020	0.367^{d} ± 0.015	60.93 ± 7.13	39.07 ± 7.04	98.61 ± 1.39
Bacillus thuringiensis	Dipel 2X	21.99 ± 0.33	94.06 ± 3.81	19.71^{b} ± 0.27	0.44^{b} ± 0.023	0.413^{b} ± 0.018	34.04 ± 7.04	65.96 ± 7.04	98.33 ± 1.66
	Dipel DF	21.92 ± 0.34	93.63 ± 2.58	19.60^{b} ± 0.18	0.53^{a} ± 0.022	0.45^{a} ± 0.021	46.41 ± 4.72	53.59 ± 4.72	100.0 ± 0.00
	Protecto	21.95 ± 0.33	90.45 ± 6.95	19.26^{cd} ± 0.32	0.45^{b} ± 0.021	0.37^{d} ± 0.018	47.92 ± 6.13	52.08 ± 6.13	98.04 ± 1.96
	Biotect	21.79 ± 0.27	94.19 ± 2.91	19.01^{d} ± 0.23	0.48^{b} ± 0.021	0.406^{bc} ± 0.021	63.48 ± 9.13	36.52 ± 9.08	96.29 ± 3.70
Spinosad	Tracer	21.84 ± 0.26	93.19 ± 4.80	19.01^{d} ± 0.29	0.47^{b} ± 0.023	0.393^{bcd} ± 0.021	43.91 ± 5.71	56.09 ± 5.71	100.0 ± 0.00
Spinotram	Radiant	21.81 ± 0.33	94.66 ± 1.33	19.45^{bc} ± 0.11	0.37^{c} ± 0.016	0.373^{d} ± 0.012	54.17 ± 4.29	45.83 ± 5.55	97.22 ± 1.39
Emamectin benzoate	Proclaim	22.03 ± 0.30	98.55 ± 1.45	19.73^{b} ± 0.18	0.38^{c} ± 0.016	0.376^{cd} ± 0.016	44.24 ± 1.23	55.76 ± 7.50	98.67 ± 1.33
Chlorfenpyr	Challenger	21.83 ± 0.25	98.81 ± 1.19	20.35^{a} ± 0.15	0.36^{c} ± 0.018	0.390^{bcd} ± 0.012	60.89 ± 7.04	39.07 ± 7.04	98.85 ± 1.15

$^{a–e}$Means of column (different treatments effect of each study trait) followed by the same letters are not significantly different at 0.05 level of significance

Each bioinsecticide was applied in sublethal concentration of LC_{30} value. The second larval instar were exposed to the bioinsecticides-treated leaves for 24 h and then transferred to untreated leaves to assess developmental parameters. Treatments were performed in three replicates; each one contained 50 larvae at the beginning

Actual data were tabulated, but transformed ones were used in statistical analysis and Duncan's multiple range tests calculation for both male and female sex ratio

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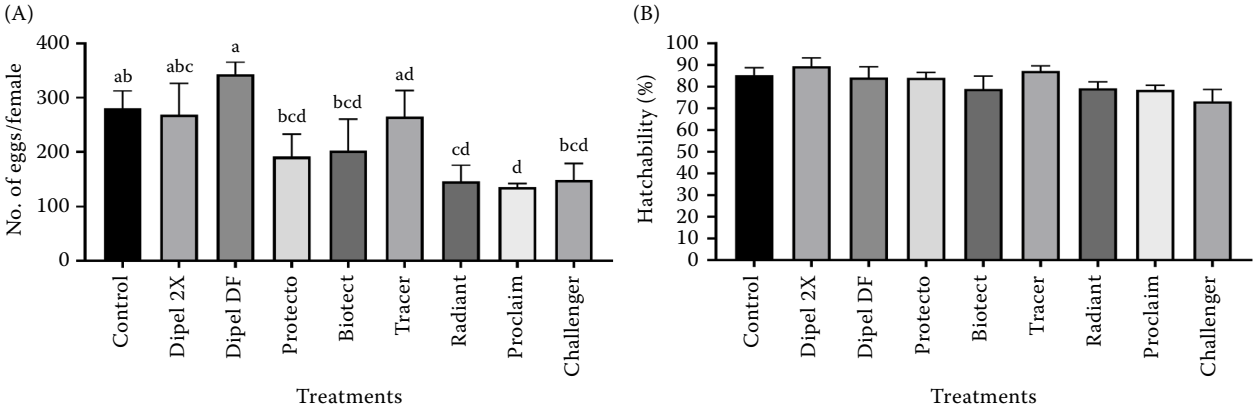

Figure 1. Effect of larval exposure to tested bioinsecticides on the fecundity (eggs/female) (A) and hatchability % (B) of *A. ipsilon* after treatment with LC_{30} value

[a–d]Means of column (different treatments effect of each study trait) followed by the same letters are not significantly different at 0.05 level of significance

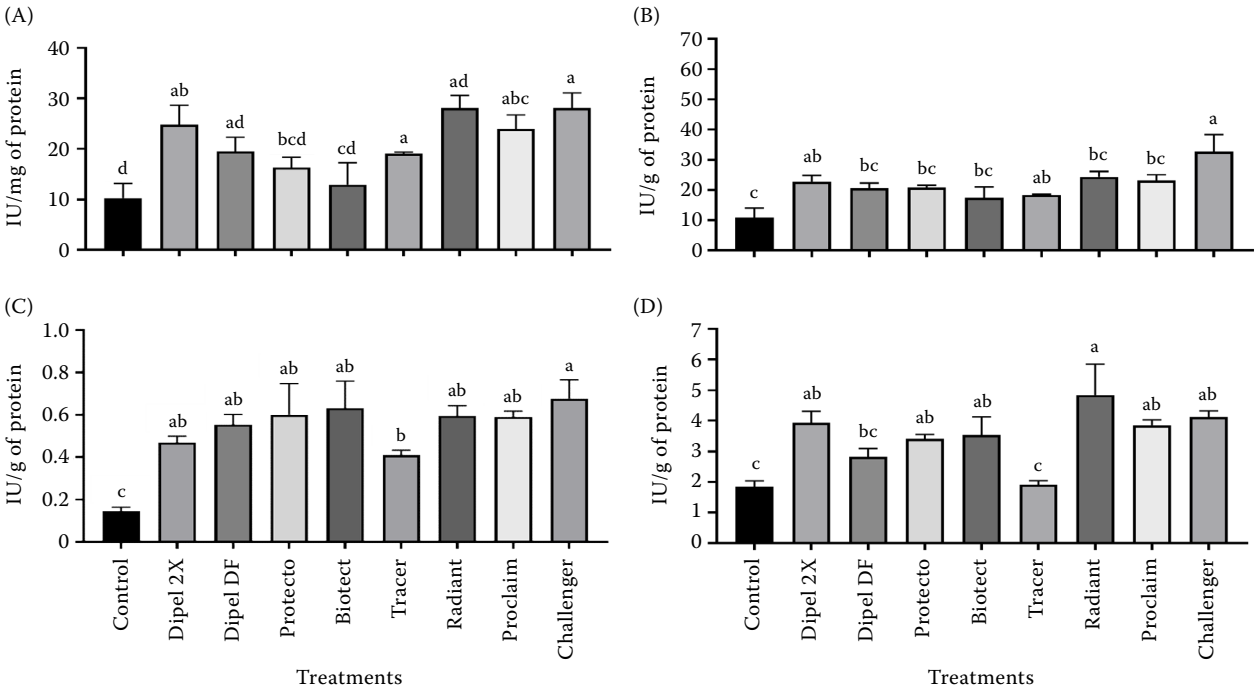

Figure 2. Mean ± SE of catalase (A), superoxide dismutase (B), lipid peroxidase (C) and glutathione reductase (D) enzymes activities of *A. ipsilon* after exposure of second larval instar to LC_{30} value of tested bioinsecticides

[a–d]Means of column (different treatments effect of each study trait) followed by the same letters are not significantly different at 0.05 level of significance

formulations (Protecto and Biotect) caused a significant increase in CAT activity after 96 h post-treatment at the LC_{30} (2.7, 2.7, 2.4, 2.3, 1.8, 1.5 and 1.2 fold, respectively). The activity of SOD showed a similar pattern of change: the SOD activity significantly increased when the larvae were exposed to chlorfenapyr, spinotram, emamectin benzoate and Bt formulation (Dipel 2X) by (32.77 ± 5.56, 24.37 ± 1.77, 23.15 ± 1.89 and 22.71 ± 2.12 IU/g protein, respectively) compared with the control treatment 10.87 ± 3.19 IU/g protein. The lipid peroxidase activity of LC_{30} was more significant than the control treatment with all insecticides tested when the larvae were exposed to chlorfenapyr, Bt formulations (Biotect and Protecto), spinotram, emamectin benzoate, Bt formulation (Dipel DF and Dipel 2X) and spinosad by 4.5, 4.2, 4.0, 4.0, 3.9, 3.7, 3.1 and 2.7 fold, respectively). Glutathion reductase activi-

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ties were significantly affected by Radiant, Challenger, Diple 2X and Proclaim after 96 h post-treatment at the LC_{30} by 2.6, 2.2, 2.1, and 2.08-fold, respectively compared with the control treatment [df = 8, $F_{(8,18)}$ = 5.120, P = 0.001 9]. On the other hand, there were no significant differences in Biotech, Protein, Dipel DF and Tracer compared with the control group.

Determination of gene expression of AKH. In order to compare the transcriptional regulations of AKH hormone of treated *A. ipsilon* larvae treated with different bio-agent types as mentioned above, the treated larvae were subjected to gut-tissue dissection, total RNA isolation, cDNA synthesis, and semi-quantitative reverse transcription PCR (SQ-PCR) analysis. Six different primers were examined to amplify AKH gene fragments using the synthesized cDNA from treated larvae. Only one set of primers (AKH2-F1/AKH2R2) out of six has shown the AKH PCR product expected fragment size of 123 bp; however, the remaining sets of primers did not show any amplified PCR products (data not shown). The produced PCR fragments were subjected to semi-quantitative analysis using ImageJ software package (32-bit, https://imagej.nih.gov).

As shown in Figure 3, the results present the produced AKH gene fragments generated by RT-PCR after treating *A. ipsilon* larvae with different bio-agents. The intensity of each band was measured using ImageJ analyzer software. The volume density (pixel) was measured and proportionally correlated to the AKH gene transcript using the AKH cDNA for untreated larvae as an internal control. The results showed that the recorded volume density (pixel) ranged from 28 338.392 to 52 454.990 pixels, which were for Tracer and Radiant, bio-agents treated *A. ipsilon*, larvae, respectively. The Tracer proportion made up 0.76-fold (28 338.392/36 870.463), and the Radiant proportion made up 1.42-fold (52 454.990/36 870.463) of the AKH gene transcripts compared with the untreated larvae. In the same context, the Dipel 2X volume density was 52 220.040 and made up a proportion of 1.41-fold (52 220.040/36 870.463). In addition, the volume density of Biotect formulation was 46 214.999 and made up a proportion of 1.25-fold (46 214.999/36 870.463), while the volume density of the Protecto was 40 197.099 and made up a proportion of 1.09-fold (40 197.099/36 870.463). The volume density of Dipel DF was 38 388.756 and made up a proportion of 1.04-fold (38 388.756/36 870.463), the volume density of Challenger was 34 081.049 and made up a proportion of 0.92-fold (34 081.049/36 870.463), and finally, the volume density of Proclaim was 30 717.220 and made up a proportion of 0.83-fold (30 717.220/36 870.463).

DISCUSSION

In this paper, we characterized and investigated the insecticidal effect of eight bioinsecticides following treatment of second larval instar of *A. ipsilon*, following oxidative stress enzymes activity and finally gene expression of AKH. The high toxicity of emamectin benzoate against *A. ipsilon* may be because of its mode of action correlated with the target site that activates the GABA that induces presynaptic neurons to release excessive GABA (Kandil et al. 2020b). Generally, bioinsecticides influence insect populations by affecting insect longevity, fecundity, and physiological parameters (Desneux et al. 2007; Su & Xia 2020). Therefore, to develop sustainable pest management programs, an understanding of biological and biochemical parameters following sublethal exposure to insecticides is required. The sublethal concentration LC_{30} for the tested bioinsecticides was not statistically significant in the elongation of the larval developmental time of *A. ipsilon*. However, the larval duration was reduced on *S. littoralis* exposed to sublethal concentration of emamectin benzoate (El-Helaly et al. 2020). In contrast, studies reported

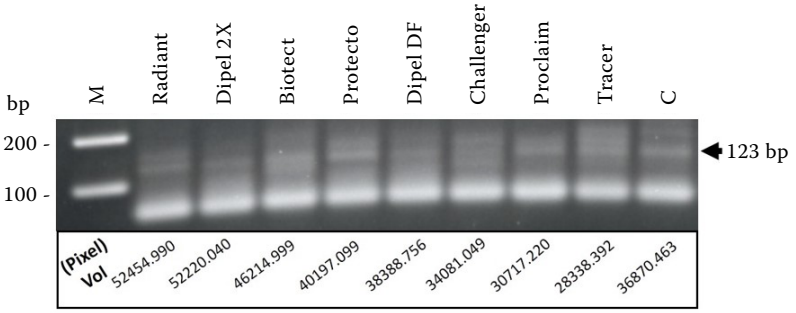

Figure 3. Semi-quantitative RT-PCR of transcripts encoding AKH hormone of *A. ipsilon* larvae under abiotic stresses using different bio-agents
C – internal control in which *A. ipsilon* larvae were untreated; M – 1kb DNA ladder
Each PCR reaction was optimized for 30 cycles to achieve non-saturated gel image

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that emamectin benzoate and *B. thuringiensis* formulation (Dipel 2X) extends the larval duration of different species of Noctuidae, such as *M. brassica* and *T. absoluta* (Moustafa et al. 2016; Kandil et al. 2020a). The LC_{30} of chlorfenapyr, emamectin benzoate and *B. thuringiensis* formulations (Dipel 2X and Dipel DF) significantly elongated the pupal phase. Emamectin benzoate has an ovicidal activity in some lepidopteran pests, including *M. brassica* and *T. absoluta* (Moustafa et al. 2016; Kandil et al. 2020a). This could be attributed to the lethality of emamectin benzoate against Noctuidae species, including *H. zea* (López et al. 2010), *T. absoluta* (Gacemi & Guenaoui 2012) and *H. armigera* (Dagar et al. 2020). Alternatively, it could be related to physiological effects (Yin et al. 2008) caused by insecticides, including fecundity and fertility (Liu & Trumble 2005).

Sublethal exposure may lead to changes in the physiological characteristics of insects (Desneux et al. 2007). Several studies reported that sublethal concentrations or doses of pesticides altered enzyme activities (Li et al. 2018). The increase in CAT enzymatic and SOD activities could be attributed to SOD and CAT directly eliminating surplus ROS in a coordinated way. SOD removes O_2^- through the process of dismutation to O_2 and H_2O_2, and then H_2O_2 is sequentially reduced to H_2O and O_2 by CAT (Kashiwagi et al. 1997; Zhang 2014). Malondialdehyde (MDA) is the final lipid oxidation product and is an important biomarker of oxidative stress. A significantly elevated level of lipid peroxidase enzyme in *A. ipsilon* was observed in response to the tested bioinsecticide. MDA concentration was decreasing in order of Challenger > Biotect > Protecto > Radiant > Proclaim > Dipel DF > Dipel 2X after 96 h post-treatment at the LC_{30}. In insects, lipid peroxidase is harmful because lipids are components of cell membranes and play an important role in insect growth and reproductive physiology (Karthi et al. 2018). Glutathione reductase (GR) is one of the main enzymes responsible for the detoxification of insecticides or pathogens by insects (Claudianoc et al. 2006). GR was expressed in *A. ipsilon* larvae after being treated with Radiant, Challenger, Dipel 2X and Proclaim showed a significant increase in enzyme activity compared with control.

The changes that have been detected in oxidative stress enzymes activity may be attributed to the change that happened to the AKH regulation after sublethal exposure to bioinsecticide. The changes in AKH transcription regulation were detected via SQ-PCR via cDNA that was synthesized using mRNA isolated from treated *A. ipsilon* larvae. The results showed that transcripts of AKH increased after sublethal exposure to Radiant, compared with the untreated larvae. This is in accordance with the activity of the CAT enzyme, which increased significantly after 96 h post-treatment with both Dipel 2X and Radiant, respectively. In addition, this was under the expression of the SOD activity, which significantly increased when the larvae were exposed to Dipel 2X and Radiant by 22.71 and 24.36 protein, respectively. Therefore, the transcriptional regulation of AKH transcripts detected using SQ-PCR may strongly support the increase of SOD, CAT and GR enzymes after sublethal exposure of *A. ipsilon* larvae to Radiant and Dipel 2X bioinsecticides. On the other hand, the exposure of *A. ipsilon* larvae to the Challenger and Proclaim have shown relatively lower AKH transcripts of 0.92 and 0.83-fold change, for Challenger and Proclaim respectively, compared with the untreated larvae. This may be attributed to the low AKH transcripts that could not be differentiated from the control larvae. In the same context, the Biotect, Protecto and Dipel DF bioinsecticides showed moderate up regulation of AKH transcripts of 1.25, 1.09, and 1.04-fold, respectively. Here, the effect of various bioinsecticides on AKH gene regulation and subsequently on the oxidative stress enzymes was confirmed. AKHs are neurohormones that have been shown to stimulate defensive mechanisms in insects. They are also responsible for mediating anti-stress reactions, such as those that counteract oxidative stress (Gäde et al. 1997).

CONCLUSION

In summary, emamectin benzoate and spinosyn group bioinsecticides showed acute toxicity to *A. ipsilon* when second-instar larvae were treated. In addition, both bioinsecticides showed sublethal effect on pupal stage characteristics, fecundity and altered the enzymatic balance that may further affect the immunity of *A. ipsilon*. Our results showed the possibility of use these bioinsecticides against *A. ipsilon* but their adverse effects on non-target organisms should be studied in more detail before they can be used as a component of inte-

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grated pest management in fields/agricultural practice. In contrast, there was no effect of other tested bioinsecticides on the other biological parameters. These results showed that *A. ipsilon* was more tolerant than other Noctuidae species.

Acknowledgement: We would like to thank the Egyptian Knowledge Bank (EKB) for improving the manuscript considerably, including English language and grammar. Thus, our special thanks to Stephen Giles (English Language Support Manager, Harper Adams University, UK) for improving the manuscript.

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Received: April 12, 2022
Accepted: July 26, 2022
Published online: August 29, 2022