Sublethal Effects of Spinosad on Some Biochemical Parameters of *Xanthogaleruca luteola* (Müller) (Coleoptera: Chrysomelidae)

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Abstract

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The effect of spinosad on some biochemical characteristics of *Xanthogaleruca luteola* was investigated. LC_{30} and LC_{50} of spinosad for the third instar larvae were determined as 1.6 and 2.9 ppm, respectively. Biochemical tests were carried out on the 3rd instar larvae treated with LC_{30} and LC_{50} of spinosad. The LC_{30} concentration decreased carbohydrate and glycogen content and increased lipid content significantly. There was a significant decrease in carbohydrate, glycogen, and protein contents following the exposure to LC_{50} concentration. Available energy of the larvae exposed to LC_{30} of spinosad increased significantly, whereas it decreased significantly at LC_{50}. However, the energy consumed was increased at both concentrations significantly. Cellular energy allocation of larvae treated with LC_{50} concentrations of spinosad decreased significantly. Glutathione-*S*-transferase and esterase activities were increased significantly at LC_{50}. However, lipase activity was decreased at both concentrations.

Keywords: elm leaf beetle; spinosad; cellular energy allocation; glutathione-*S*-transferase and esterase activities; LC_{30} concentrations; LC_{50} concentrations

The elm leaf beetle, *Xanthogaleruca luteola* (Müller) (Coleoptera: Chrysomelidae), is one the major defoliator pests of elm trees in Iran. This beetle attacks elm trees in both larval and adult stages by feeding on the leaves. In addition to defoliation and morphological changes, this pest causes physiological stress which increases the elm susceptibility to secondary pest and Dutch elm diseases (Arbab *et al.* 2001). The chemical control has been considered as a major solution for controlling the elm leaf beetle. Due to the widespread plantation of elm trees in urban areas, the application of pesticides against *X. luteola* poses some special risks. Therefore, the application of pesticides with high selectivity to the pest and low toxicity to humans and environment is highly appreciated (Defago *et al.* 2006).

Spinosad is a novel mode-of-action selective biological insecticide (Saunders & Bret 1997) with proved efficacy for controlling a wide range of pests including Lepidoptera, Diptera, Thysanoptera, Coleoptera, Orthoptera, and Hymenoptera (Sparks *et al.* 1995). This pesticide is obtained from the soil-borne bacteria, *Saccharopolyspora spinosa* (Mertz & Yao 1990), by fermentation and contains two active spinosoids: spinosyn A and spinosyn D (at an approximate ratio of 17 : 3) (Mertz & Yao 1990). Spinosad exerts its toxic effects on insects by affecting their nicotinic acetylcholine (nAChRs) and gamma-aminobutyric acid (GABA) receptors (Salgado 1997). Spinosad also inhibits acetylcholinesterase (Rabea *et al.* 2010). This insecticide has low mammalian toxicity with weak environmental impact (Cleveland *et al.* 2002),

which makes it suitable for application in urban areas and integrated pest management (IPM) programs (Cleveland 2007).

Traditionally, the acute toxicity of chemicals to insects has been expressed as LC_{50} or LD_{50}. However, in addition to mortality induced by pesticides, their sublethal effects on detoxification enzyme and energy allocation of the pests must be considered. The biochemical responses of some insects on exposure to sublethal concentrations of spinosad have been well documented. Hussain *et al.* (2009) revealed a decrease in the protein content of *Tribolium castaneum* (Herbst) compared to the control, when treated with LC_{30} and LC_{50} concentrations of spinosad. Nehad *et al.* (2008) also showed that total carbohydrate and protein contents of *Spodoptera littoralis* (Boisduval) larvae treated with spinetoram (a new generation of spinosyn group) at LC_{50} concentration declined significantly while the acetylcholinesterase activity was increased. Glutathione-*S*-transferase (GST) activity was relatively decreased after treatment of the 5th instar larvae of *S. littoralis* with spinetoram according to the results of Nedal and Hassan (2009). However, the exposure of *Helicoverpa armigera* (Hübner) larvae to LC_{50} concentration of spinosad caused an insignificant increase in the GST activity (Wang *et al*. 2009). Spinosad at LC_{50} concentration is reported to cause a minor decrease in the α-esterase activity of a laboratory strain of *S. littoralis* larvae. On the contrary, the β-esterase activity of the larvae was increased compared to the control (Abd El-Mageed & Elgohary 2006).

Due to the lack of knowledge of spinosad toxicity to the elm leaf beetle and responses of its esterase and GST activities to spinosad, this paper presents the toxicity of spinosad to *X. luteola* larvae and its sublethal effects on esterase and GST activities of the elm leaf beetle. Also, sublethal effects of spinosad on the energy budget of the elm leaf beetle were evaluated.

MATERIAL AND METHODS

Chemicals. Methanol, anthrone, sulphuric acid, phosphoric acid, maltose, triton X-100, bovine serum albumin, α-naphthyl acetate (α-NA), β-naphthyl acetate (β-NA), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), Tris and acetic acid were purchased from Merck (Darmstadt, Germany). Glycogen and vanillin were purchased from Wako (Tokyo, Japan). Fast blue RR salt was obtained from Fluka (Buchs, Switzerland). *p*-Nitrophenyl acetate was purchased from Sigma Aldrich (Stenheim, Germany).

Laboratory mass culture of** X. luteola.** The elm leaf beetle adults were collected from Rasht (Guilan province) in the north of Iran. Insects were reared under laboratory conditions at 25 ± 2°C, 16 : 8 (L : D) and 75 ± 10 RH. Transparent plastic jars (10 × 20 cm) in which the lids contained holes were used for the rearing of larvae and adults. Moisture was provided by placing pieces of wet cotton at the corner of each jar. Fresh leaves were provided daily for feeding. Eggs laid by adults were used to maintain the culture. In order to obtain larvae of the same age for bioassay tests, each pair of male and female adults was kept in similar plastic jars and the laid eggs were transported to a new container daily.

Pesticide. Spinosad (Entrust naturalyte®, 80% spinosad mixture of spinosyn A and D and 20% of other ingredients) was obtained from Dow Agrosciences (Indianapolis, USA).

Bioassay tests. Bioassay tests were carried out on one-day-old third instar larvae. Five concentrations (1, 1.8, 3.2, 5.6, 10 ppm) of spinosad were used for determination of LC_{30} and LC_{50}. Triton X-100 was added as an emulsifier at 0.08% to all concentrations. Elm leaves were dipped in the desired concentration for 30 s and dried in the air for 30 min before being offered to *X. luteola* larvae. Control leaves were dipped in water containing triton X-100 (0.08%) and dried as mentioned above. Four replications (each replication with 10 larvae) were used for each treatment and control. Mortality of the larvae was recorded 48 h after treatment and the LC_{30} and LC_{50} values were calculated using the Polo-PC software (Leora Software 1987).

Biochemical tests. Biochemical tests were carried out using one-day-old 3rd instar larvae (whole body) fed for 48 h on elm leaves treated with LC_{30} and LC_{50} concentrations of spinosad.

Carbohydrate, lipid, and glycogen assays. Two larvae were homogenised in a sodium sulphate solution (Na_2SO_4 2%) and the homogenates were centrifuged in 469 µl of chloroform: methanol (1 : 2 v/v) solution at 8000 rpm for 10 min at 4°C. Carbohydrate and lipid assays were carried out using a supernatant based on Singh and Sinha (1977) and Yuval *et al*. (1998) methods, respectively. Glycogen content was measured according to Yuval *et al.* (1998) method using the resultant pellet.

Protein assays. Protein content was measured based on Bradford (1976) method with bovine serum albumin as standard.

Determination of energy consumed. The energy consumed (Ec) was estimated by measuring the elec-

tron transport system activity (ETS) according to De Coen *et al.* (1995). Cellular energy allocation (CEA) was calculated by determination of the total energy reserve in an insect body as energy available (Ea) and the activity of electron transport system (ETS) as energy consumed (Ec) according to the following formula:

Ea (available energy) = (glucose + lipid + glycogen + + protein) (J/insect)

Ec (energy consumption) = ETS activity (J/insect/min)

CEA (cellular energy allocation) = Ea/Ec (J/insect)

Esterase activity measurement. Esterase activity was measured according to van Asperen (1962) using α-NA and β-NA as substrates. Larvae were homogenised on ice in 100 µl of phosphate buffer (pH 7.0, 20mM) containing 0.01% Triton X-100. The homogenates were centrifuged at 10 000 *g* for 15 min at 4°C and 13 µl of the supernatant was transferred to a microplate containing 112 µl of phosphate buffer (pH 7.0, 20mM) per well. In order to start the reaction, 25 µl of the 10mM substrate solution was added per well. Fifty µl of the fast blue RR salt (1mM) was added to the microplate per well and absorbance was measured at 450 and 500 nm for α-NA and β-NA, respectively, with a microplate reader (Stat Fax® 3200; Awareness Technology Inc., Palm City, USA).

GST activity measurement. GST activity was measured based on Habig *et al.* (1974) method and CDNB was used as substrate. Larvae were homogenized in 100 µl of phosphate buffer (pH 7.0, 20mM) without Triton X-100. The homogenates were centrifuged at 12 000 *g* for 12 min at 4°C and 10 µl of the supernatant was transferred to a microplate followed by the addition of 100 µl GSH (10mM) and 80 µl CDNB (100mM). Absorption was measured at 340 nm using a microplate reader (Stat Fax® 3200; Awareness Technology Inc.).

Lipase activity measurement. Lipase assay was performed using *p*-nitrophenyl acetate as substrate. Enzyme preparations were carried out similar to that of GST with Triton X-100. Ten µl of the supernatant was added to a microplate already supplied with 172 µl of Tris-sulphate buffer (20mM, pH 7.75) per well followed by the addition of 18 µl (50mM) substrate solution. Absorption was measured at 405 nm using the plate reader.

Data analysis. Data obtained from the experiments were analysed using analysis of variance (Proc ANOVA, SAS 8.2.). Means were compared by Tukey's student range test, accepting significant differences at $P < 0.05$.

Table 1. Determination of sublethal concentrations of spinosad for the third instar larvae of *Xanthogaleruca luteola*

	Concentration (ppm)	CL
LC_{30}	1.6	1.1–2.1
LC_{50}	2.9	2.3–3.6

CL (confidence limits) which have been calculated with 95% confidence

RESULTS

LC_{30} and LC_{50} values for spinosad in the treated 3rd instar larvae are presented in Table 1. The LC_{30} and LC_{50} values were determined as 1.6 and 2.9 ppm, respectively.

Biochemical effects

Energy reserves. Results showed significant difference between carbohydrate, glycogen, and lipid contents of the larvae treated with LC_{30} of spinosad in comparison with the control group (Table 2). At LC_{30} concentration, carbohydrate and glycogen contents were decreased by 29.1 and 31.9%, respectively, while lipid content was increased by 61.7% compared to the control. However, protein content of larvae treated with LC_{30} concentration showed a non-significant elevation (12.5%) compared to the control.

The results obtained from the treatment of larvae with LC_{50} concentration were different (Table 2).

Table 2. Effects of spinosad on the energy reserve of the 3rd instar larvae of *Xanthogaleruca luteola*

Treatment	Concentrations (µg/larva) ± SD			
	carbohydrate	protein	glycogen	lipid
Control	251.7 ± 4.2^a	35.8 ± 1.3^a	69.8 ± 2.8^a	242.6 ± 21.0^b
LC_{30}	178.9 ± 10.2^b	40.3 ± 1.9^a	47.2 ± 3.2^b	631.7 ± 11.9^a
LC_{50}	124.2 ± 3.7^c	27.2 ± 2.5^b	31.3 ± 0.8^c	195 ± 19.0^b

Within columns, means followed by the same letter do not differ significantly (Tukey's test, $P \leq 0.05$)

Table 3. Effects of spinosad on the cellular energy allocation in the 3rd instar larvae of *Xanthogaleruca luteola*

Treatment	Ea (J/insect) ± SD	Ec (J/insect/min) ± SD	CEA (J/insect) ± SD
Control	14.7 ± 0.6[b]	0.3 ± 0.01[c]	54.4 ± 2.1[a]
LC_{30}	28.1 ± 0.4[a]	0.5 ± 0.01[a]	57.1 ± 1.5[a]
LC_{50}	10.3 ± 0.8[c]	0.4 ± 0.02[b]	24.3 ± 1.0[b]

Within columns, means followed by the same letter do not differ significantly (Tukey's test, $P \leq 0.05$); Ea – available energy; c – energy consumed; CEA – cellular energy allocation

Carbohydrate, glycogen, and protein contents were reduced significantly in comparison with those of the untreated larvae (50.6, 55.1, and 22.9%, respectively), while a non-significant decrease in lipid content (19.4%) was observed.

The Ea value of larvae treated with LC_{30} concentration of spinosad increased significantly by 47.6% compared to the control, whereas it showed a significant decrease by 30.3% at LC_{50}. The Ec value of larvae following their treatment with both LC_{30} and LC_{50} concentrations of spinosad was increased compared with the control. Ec was increased by 45.1% and 36% at LC_{30} and LC_{50} concentrations, respectively. Consequently, CEA of larvae exposed to LC_{50} concentrations of spinosad decreased significantly by 55.3% compared to the control (Table 3).

Enzyme assays

Total esterase activity. As demonstrated in Table 4, treating the larvae with LC_{50} concentrations of spinosad enhanced α-esterase activity by 49.7%, whereas α-esterase activity at LC_{30} concentration did not show a significant difference from that of the control. Furthermore, β-esterase activity was increased significantly at LC_{50} concentration by 27.6% compared to the control (Table 4).

GST activity. Following the treatment of larvae with LC_{30} and LC_{50} concentrations, GST activity was increased by 25.9 and 49.5 %, respectively, compared to that of the untreated ones which was significant at LC_{50} (Table 4).

Lipase activity. Lipase activity declined sharply and significantly at both LC_{30} and LC_{50} concentrations by 79.9% and 77.8%, respectively, compared to the control. However, no significant difference in lipase activity at LC_{30} and LC_{50} was observed (Table 4).

DISCUSSION

Results of this research revealed a decline in the level of energy sources (carbohydrate, glycogen, and protein) of the larvae following their treatment with LC_{30} and LC_{50} concentrations of spinosad. Nutritional deficiencies along with the increase in metabolic activities for detoxification process during the exposure to pesticides are among the main reasons for the reduced energy level (De Coen & Janssen 1997; Verslycke *et al*. 2003).

According to our results, the protein contents of *X. luteola* larvae decreased following their treatment with LC_{50} concentration of spinosad. Hussain *et al.* (2009) also showed a reduction in the protein content of *T. castaneum* adults when treated with spinosad.

The exposure to pesticides can affect carbohydrate metabolism in different species of insects by either decreasing or increasing its content (Mansingh 1972). In this study, carbohydrate content of the elm leaf

Table 4. Effects of spinosad on enzyme activities of the 3rd instar larvae of *Xanthogaleruca luteola*

Treatment	Enzyme activity (μmol/min/mg protein) ± SD			
	α-esterase	β-esterase	GST	lipase
Control	379.9 ± 6.2[b]	62.9 ± 2.3[b]	3.5 ± 0.4[b]	3406 ± 49.6[a]
LC_{30}	381.3 ± 6.3[b]	57.4 ± 1.1[b]	4.7 ± 0.3[b]	683.7 ± 32.0[b]
LC_{50}	755.2 ± 7.1[a]	86.8 ± 1.6[a]	6.9 ± 0.7[a]	756 ± 51.1[b]

Within columns, means followed by the same letter do not differ significantly (Tukey's test, $P \leq 0.05$); GST – glutathione-*S*-transferase

beetle larvae dropped when they were treated with spinosad. These results coincide with others which reported a decrease in carbohydrate contents of different insect species as a response to some insecticides (Mandal & Chaudhuri 1992; Gamil *et al*. 2011).

Glycogen is one of the essential nutrient reserves in insect which can also be affected by pesticide treatment (Nedal & Hassan 2009). Our results revealed a significant drop in the glycogen level of the *X. luteola* when treated with LC_{30} and LC_{50} concentrations of spinosad. The decline in the level of glycogen is probably due to its breakdown in the fat bodies and its release in the haemolymph as trehalose under stress conditions (Nath 2003). Our results are in agreement with the results of Piri *et al.* (2014), who showed that spinosad significantly decreased the glycogen content of the fifth larval instar of *Glyphodes pyloalis* (Walker).

Results of this study demonstrated a significant elevation in the lipid content of the larvae of the elm leaf beetle following their exposure to LC_{30} concentration of spinosad. However, at LC_{50} treatment, the lipid content dropped, which was not significant. According to some studies, the exposure to pesticides affects synthesis and storage of lipids more than their breakdown. Ali *et al*. (2011) showed that the lipid content of *Rhyzopertha dominica* (Fabricius) adults increased dramatically in response to the exposure to Talstar, a pyrethroid insecticide. A similar result was reported by Abdul Mujeeb *et al.* (2011) for *Trogoderma granarium* (Everts) larvae treated with chlorpyrifos, whereas Bagheri *et al.* (2010) reported a decrease in the lipid content of *Brachynema germari* larvae treated with pyriproxyfen. They reported that a decrease in lipid content is due to the enhanced metabolic activity of the larvae resulting from pesticide stress.

Ea of *X. luteola* was affected by spinosad significantly. Other studies have also shown that pesticides may interfere with available energy of the insects. Bagheri *et al.* (2010) reported that pyriproxyfen affected the Ea amount in *B. germari*. They showed a decreasing trend of Ea when the concentration of pyriproxyfen was elevated. Our results also showed a significant decrease of Ea at the higher concentration (LC_{50}) of spinosad.

The energy consumption which is measured based on ETS activity showed a significant increase following the treatment with LC_{30} and LC_{50} concentrations of spinosad. These results coincide with the results of Verslycke *et al*. (2004) for chlorpyrifos-treated *Neomysis integer* (Leach). Their results revealed that the increase of Ec can be a sign of the higher rate of oxygen consumption. Watanabe and Tanaka (2000) also demonstrated an increase in the *Aulacophora nigripennis* (Motschulsky) respiration rate when treated with pyriproxyfen.

Cellular energy allocation is the ratio of available energy, which is the sum of protein, sugar, and lipid reserves, to the energy consumption. According to our results, CEA of the larvae treated with LC_{50} decreased significantly and it showed a non-significant difference at LC_{30} concentration compared to the control. Among the energy reserves of the larvae, only the lipid showed a significant increase at LC_{30} exposure. It can be concluded that the CEA is in direct relation with the lipid content and its increase was due to the accumulation of lipids. However, CEA decreased significantly at LC_{50} exposure, which indicates the utilization of energy reserves by the larvae when exposed to a higher concentration of spinosad and thus a decrease in Ea. Verslycke *et al*. (2004) reported a decline of CEA in *N. integer* at higher concentrations of chlorpyrifos.

Esterases are a large group of hydrolases and enzymes metabolizing exogenous and endogenous substrates with the ester bond (Devorshak & Roe 1998). Results of our study showed an increase in α- and β-esterase activities of the treated larvae which was significant at LC_{50} and not significant at LC_{30} compared to the control. Hussain *et al.* (2009) reported an insignificant effect of spinosad at LC_{10} and LC_{20} concentrations on the esterase activity of susceptible strains of *T. castaneum*. Piri *et al.* (2014) showed no significant differences in the activities of α- and β-esterases in *Glyphodes pyloalis* larvae treated with LC_{20}, LC_{30}, and LC_{40} concentrations of spinosad and the control. However, *S. littoralis* larvae treated with indoxacarb showed a significant increase in α and β-esterase activities (Gamil *et al.* 2011). They suggested that this result was due to the blocking of the action potential of the nervous system caused by the toxic effect of indoxacarb on the larvae.

GSTs are another group of detoxifying enzymes which catalyse the conjugation of the reduced form of glutathione (GSH) to electrophile xenobiotic substrates (Habig *et al.* 1974). Surprisingly, results of this study showed that GST activity was increased after the treatment of the larvae with LC_{50} concentration. This result demonstrates that GSTs of the elm leaf beetle are involved in the detoxification of spinosad. Also, the spinosad and its oxidative metabolite can react with glutathione-*S*-transferase to form a cysteine conjugate in rat (Domoradzki *et al.* 1995).

H. armigera larvae treated with spinosad showed an insignificant increase in GST activity compared to the control (Wang *et al*. 2009). The enzyme activity was increased significantly in *S. littoralis* larvae treated with LC_{50} concentration of indoxacarb (Gamil *et al.* 2011). They concluded that maybe an overproduction of this enzyme occurred as a result of treatment. Sarita *et al*. (2010) showed similar results. However, Rumpf *et al.* (1997) revealed that sublethal concentrations of fenoxycarb decreased the GST activity in *Micromus tasmaniae* (Walker) larvae.

Lipase is a hydrolytic enzyme which catalyses the hydrolysis of ester bonds in lipids (Aha *et al*. 2000). Lipase plays an essential role in the storage and movement of lipids in insects. Our result showed a significant decrease in the lipase activity of the larvae treated with both concentrations of spinosad. Similarly, treating the elm leaf beetle larvae with *Thymus vulgaris* (L.) reduced the lipase activity (Khosravi & Jalali Sendi 2013).

Oxidases have an important role in the metabolism of spinosad which can be investigated in a further study. Awan *et al.* (2012) showed that piperonyl butoxide (a microsomal oxidase inhibitor) resulted in a significant synergism with spinosad, which indicates the important role of oxidases in *T. castaneum* resistance to the pesticide. Similar results were reported by Wang *et al.* (2009) on *H. armigera*. They revealed that spinosad can increase the activity of *p*-nitroanisole-*O*-demethylase (ODM), which indicates the role of cytochrome P450 monooxygenase in the metabolism of spinosad.

In conclusion, our results indicated that spinosad showed high toxicity to the third instar larvae of *X. luteola* with a low LD_{50}. Sublethal concentrations of spinosad affected some biochemical parameters of *X. luteola*. Spinosad presents a significant effect on insect regular metabolism and detoxification enzymes in the larvae of *X. luteola* and this pesticide has been proved to be effective against this serious defoliator pest.

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