SELECTION FOR RESISTANCE, REVERSION TOWARD SUSCEPTIBILITY, AND SYNERGISM OF CHLORANTRANILIPROLE AND SPINETORAM IN OBLIQUEBANDED LEAFROLLER, *CHORISTONEURA ROSACEANA* (LEPIDOPTERA: TORTRICIDAE)

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Abstract

Larvae of the obliquebanded leafroller, *Choristoneura rosaceana*, derived from a laboratory colony, were selected for resistance to two reduced-risk insecticides chlorantraniliprole and spinetoram. Significant levels of resistance to each insecticide were observed after twelve generations of selection. In the absence of selection pressure, susceptibility of a subset of larvae from both chlorantraniliprole- and spinetoram-selected populations reverted to pre-selection levels after five and six generations, respectively, indicating that resistance to both chlorantraniliprole and spinetoram was unstable in *C. rosaceana*. In synergist bioassays performed after twelve generations of selection, S,S,S-tributylphosphoro trithioate (DEF) and piperonyl butoxide (PBO) synergized the toxicity of chlorantraniliprole and spinetoram, respectively; suggesting the involvement of esterases in chlorantraniliprole resistance and that of mixed-function oxidases in spinetoram resistance. These findings suggest that

chlorantraniliprole and spinetoram could be incorporated into *C. rosaceana* resistance management programs by using rotational strategies.

Key words: Obliquebanded leafroller, chlorantraniliprole, spinetoram, resistance, selection, reversion, synergism

Introduction

Obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) is a polyphagous insect with a broad host range including members of Rosaceae and Cornaceae [1]. It is native to North America [2, 3], and is an economic pest of deciduous tree fruits throughout major tree fruit growing regions in the United States and Canada [2, 4-6]. Broad-spectrum insecticides such as organophosphates (OPs) have been widely used to control major pests of tree fruits including *C. rosaceana*. The reliance on repeated applications of conventional neurotoxic insecticides has resulted in the development of resistance to those insecticides and cross-resistance to other insecticides in *C. rosaceana* [7-15], and in some cases to the new classes of insecticides prior to them being applied in the field [13-17].

The occurrence of resistance, cross- and multiple-resistance in *C. rosaceana* necessitates the introduction of resistance management strategies in order to ensure adequate control and conserve chemical control options [18, 19] which are scarce resources, indeed. Additionally, the implementation of Food Quality Protection Act of 1996 has put restrictions on the use of conventional broad-spectrum insecticides, and expedited the registration of reduced-risk insecticides [20]. Chlorantraniliprole and spinetoram are two of the reduced-risk insecticides

recently registered as OP alternatives for use in tree fruit production. Chlorantraniliprole is a member of a new class of insecticides, the anthranilic diamides, which selectively binds to the ryanodine receptors in insect muscles resulting in an uncontrolled release of internal calcium stores from the sarcoplasmic reticulum, causing impaired regulation of muscle contraction leading to feeding cessation, lethargy, paralysis, and death of the target insect [21, 22]. Spinetoram is a recently developed spinosyn which primarily activates the nicotinic acetylcholine receptors by acting on a unique and yet unknown site [23-26]. Both chlorantraniliprole and spinetoram have shown high efficacy against *C. rosaceana* [27, Brunner unpublished data].

Resistance management strategies aimed at prolonging the efficacy of newly introduced insecticides are critical components of sustainable pest management. Once resistance has developed, tactics such as rotation of insecticides across generations are practiced to maintain the efficacy of the new insecticides [19, 28, 29]. However, the success of rotation as a strategy to delay the onset of resistance may strongly depend on the magnitude of the fitness costs associated with resistance, and the pattern of cross-resistance among the alternated insecticides [28-33]. Although chlorantraniliprole and spinetoram are new chemistries, the evidence of pre-existing resistance to chlorantraniliprole and positively correlated cross-resistance between spinetoram and spinosad in *C. rosaceana* [17] may reduce the effectiveness of resistance management programs for *C. rosaceana*. Further understanding of mechanisms conferring resistance to these new insecticides and whether the resistance of *C. rosaceana* to these chemicals would be stable in the absence of selection pressure seems necessary to develop effective resistance management strategies and to determine if rotation of these chemicals would be an appropriate tactic.

In this paper we describe results of experiments investigating the effect of selection of *C. rosaceana* for resistance in the laboratory to chlorantraniliprole and spinetoram, the stability of resistance after removal of selection pressure, and the effect of metabolic synergists on toxicity of chlorantraniliprole and spinetoram. This information would enable growers and pest management consultants to develop rational resistance management strategies by incorporating these novel reduced-risk insecticides into tree fruit pest management programs leading to sustainable management of *C. rosaceana*. It would also be useful in suggesting resistance mechanisms and in improving effectiveness of chlorantraniliprole and spinetoram spray programs in commercial orchards.

Materials and Methods

Insects. The *C. rosaceana* larvae were obtained from a laboratory colony which was established in 1990 from larvae collected from apple orchards in Mattawa, WA. This colony has been reared continuously since their collection on a pinto bean diet following the method of Shorey and Hale [34] under constant conditions of temperature (23 ± 2°C), relative humidity (RH, 70%), and photoperiod (16:8, L:D) and without exposure to insecticides.

Chemicals. The insecticides used were chlorantraniliprole (Rynaxypyr™/Altacor® 35WG, E.I. du Pont de Nemours & Co., Wilmington, DE) and spinetoram (Delegate® 25WG, Dow AgroSciences, Indianapolis, IN). The synergists tested with chlorantraniliprole and spinetoram were DEF (S,S,S-Tributylphosphoro trithioate, Chem Service, West Chester, PA), DEM (Diethyl maleate, 97% technical, Aldrich, Milwaukee, WI), and PBO (Piperonyl butoxide, 90% technical, Aldrich, Milwaukee, WI).

Selection for Resistance. Selection for resistance to chlorantraniliprole and spinetoram was performed in the laboratory as described in Sial and Brunner [35]. Briefly, cohorts of larvae from a susceptible laboratory colony were selected with chlorantraniliprole (RYN) or spinetoram (SPIN) while another cohort treated in the same way, but without exposure to insecticides, served as unselected control (LAB). In the first selection, neonate larvae were exposed to LC_{70} of the baseline established for the LAB colony. After 4 d of exposure, surviving larvae were transferred to untreated pinto bean diet, and reared in the laboratory under conditions described above. The concentration of chlorantraniliprole and spinetoram used to select each subsequent generation was the $\approx LC_{70}$ based on the results of bioassays from the previous generation. The number of neonate larvae used for each generation varied (1,000-2,000) depending on availability. Selection of some generations was deferred because sufficient numbers of progeny were not available.

Reversion toward Susceptibility: After six generations of selection and after resistance had been documented, a subset of *C. rosaceana* larvae from each of the selected RYN and SPIN populations was removed from selection to establish two new populations, RYN-Rev and SPIN-Rev, respectively. The main objective of establishing these colonies was to determine whether or not the resistance in the selected populations was stable. The *C. rosaceana* larvae in RYN-Rev and SPIN-Rev populations were reared in the laboratory without any further exposure to insecticides. Susceptibility of neonate larvae from the RYN-Rev and SPIN-Rev populations was assessed and compared with that of the neonate larvae from the unselected LAB populations at each generation using a diet incorporation bioassay.

Bioassays. Depending on availability of larvae, diet incorporation bioassays were performed as described by Sial et al. [17] on the RYN and SPIN populations after each

selected generation, and on the RYN-Rev and SPIN-Rev populations at each generation without selection to determine their susceptibility to chlorantraniliprole or spinetoram. Every time a bioassay was performed on RYN, SPIN, RYN-Rev, or SPIN-Rev, *C. rosaceana* larvae from the unselected LAB colony were tested using the same experimental materials in order to minimize the experimental error. Bioassays were not performed on G2 and G4 of the RYN population, and G3 and G5 of the SPIN population because sufficient larvae were not available. The *C. rosaceana* larvae of the RYN population at G10 were not tested because selection had not been performed on their parental generation due to unavailability of enough neonate larvae.

Synergist Bioassays. A diet overlay bioassay as described by Ahmad and Hollingworth [36] was used to test the effect of synergists DEF, DEM and PBO on toxicity of chlorantraniliprole and spinetoram to RYN and SPIN (resistant), and the LAB (susceptible) populations of *C. rosaceana*. Diet was prepared from dry diet premix (Stonefly Heliothis Diet, Ward's Natural Science, Rochester, NY) as described by Sial et al. [17]. A small portion of the diet (≈5-8 cm^3) was added to one-ounce plastic cups (Solo Cup Company, Highland Park, IL). Synergists were dissolved in 100% acetone and added to distilled water. The final solutions consisted of six concentrations of insecticides each with 20 µl per liter synergist in 5% solution of acetone in distilled water for a total volume of 200 ml. A concentration of 20 µl per liter of synergists was used because this was the maximum concentration that could be used without significant (>5%) mortality to *C. rosaceana* neonates [16, 37]. For each synergist and colony, 5 larvae were tested for each of the 6-10 replications of six concentrations for a total of 180-300 larvae tested. Mortality of neonates was assessed after 7 d.

Data Analysis. Median lethal concentration (LC_{50}) values and their corresponding 95% fiducial limits (FL) were estimated using POLO [38]. Lethal concentration ratios (LCR) at LC_{50} and their corresponding 95% confidence limits (CL) were calculated using lethal concentration ratio significance test [39]. A laboratory colony (LAB) that was not selected with any of the insecticides but otherwise treated the same way served as the reference susceptible population for comparison purposes and was assigned a ratio of 1.0. LCRs were considered significant ($\alpha = 0.05$) if their 95% CL did not include the value of 1.0. Similarly, synergism ratios (SR) at LC_{50} (LC_{50} of insecticide/LC_{50} of insecticide + synergist) and their corresponding 95% CL were calculated using lethal concentration ratio significance test [39]. SRs were considered significant ($\alpha = 0.05$) if their 95% CL did not include the value of 1.0.

Results

Our results indicate that the toxicity of both chlorantraniliprole (Fig. 1) and spinetoram (Fig. 2) to *C. rosaceana* neonate larvae was significantly decreased as a result of selection for resistance in the laboratory. However, the level of resistance against chlorantraniliprole was higher than spinetoram after the same number of selected generations. After 12 generations of selection, the LC_{50} values of chlorantraniliprole and spinetoram to *C. rosaceana* were increased to 8.5- and 5.3-fold, respectively, compared to the unselected LAB colony (Fig. 1 and Fig. 2).

After six generations of selection to chlorantraniliprole and spinetoram resistance levels of 7-fold and 4-fold had been documented, and a subset of larvae from the RYN and SPIN populations were used to establish two new colonies RYN-Rev and SPIN-Rev, respectively. The RYN-Rev and SPIN-Rev populations were reared in laboratory without exposure to

insecticides. In the absence of insecticide selection, both the RYN-Rev and SPIN-Rev populations reverted to susceptibility after five and six generations, respectively (Fig. 3 and Fig. 4). Although level of chlorantraniliprole resistance in the RYN-Rev population at the beginning of the reversion experiment was higher than that of spinetoram resistance in the SPIN-Rev population, it took five generations for the RYN-Rev population to return to susceptibility statistically similar to the unselected LAB population (Fig. 3) whereas a similar return to susceptibility took six generations for the SPIN-Rev population (Fig. 4).

To determine the potential effects of metabolic synergists on toxicity of chlorantraniliprole and spinetoram on *C. rosaceana* neonate larvae from the RYN, SPIN, and susceptible LAB populations, diet overlay bioassays, with and without synergists (DEF, DEM, and PBO), were performed. The toxicity of chlorantraniliprole to both the LAB (susceptible) and the RYN (resistant) population was increased by the addition of DEF (Table 1) suggesting the involvement of esterases as a potential mechanism for chlorantraniliprole resistance. The LC_{50} of chlorantraniliprole to the RYN population was significantly decreased (2.54-fold) whereas there was only a slight decrease (1.44-fold) in LC_{50} of the chlorantraniliprole to the LAB population. The addition of DEM or PBO did not result in any significant change in LC_{50} of chlorantraniliprole to the LAB or RYN populations (Table 1).

In contrast to the results above, the toxicity of spinetoram to both the LAB (1.8-fold) and the SPIN (3.6-fold) populations was significantly increased by the addition of PBO (Table 2) indicating the involvement of oxidases as a potential mechanism for spinetoram resistance. There was also a decrease, though not significant, in LC_{50} of spinetoram to the SPIN population (1.66-fold) by the addition of DEM, but not in the LAB population, pointing toward a possible role of glutathione-*S*-transferases in spinetoram detoxification as a

secondary mechanism. The susceptibility of the LAB or the SPIN populations to spinetoram was not affected by DEF (Table 1).

Discussion

Chlorantraniliprole and spinetoram are two recently registered insecticides that are highly effective against *C. rosaceana* [27, Brunner unpublished data]. However, reports of pre-existing resistance to these insecticides in the field populations of *C. rosaceana* and cross-resistance between spinosad and spinetoram [17], present a major risk to the effective life of these insecticides in the field. The effective life of new insecticides can be prolonged by the implementation of a resistance management program [40]. The success of such programs depends on better understanding of different characteristics of resistance such as the relative risk of resistance evolution, stability of resistance, and resistance mechanisms. In an attempt to further our understanding of chlorantraniliprole and spinetoram resistance, we selected *C. rosaceana* neonate larvae for resistance in the laboratory. Significant levels of resistance to both chlorantraniliprole and spinetoram were observed soon after selection was initiated but selection continued through 12 generations. These results indicate that high levels of resistance against these new chemistries could occur in relatively shorter period of time in the field where selection pressures are likely to be much higher than those imposed in our laboratory selection and where populations are likely to be more heterogeneous [35].

For the first time we demonstrate two new characteristics of chlorantraniliprole and spinetoram resistance in *C. rosaceana*: the instability of resistance in the absence of selection pressure, and the synergism of toxicity of chlorantraniliprole and spinetoram by DEF and PBO, respectively. It is evident from the results of reversion experiments where, in the

absence of selection pressure, both of the selected populations reverted to being susceptible that the chlorantraniliprole and spinetoram resistance in *C. rosaceana* was unstable. These findings are encouraging for resistance management programs aimed at slowing the process of resistance evolution against chlorantraniliprole and spinetoram, and prolonging the useful life of these new insecticides against *C. rosaceana* in the field. Our results suggest that chlorantraniliprole and spinetoram resistance could revert in *C. rosaceana* in the field when selection pressure was relaxed, however, it could take several generations for this to occur. One of the operational strategies that can be used to reduce selection pressure is rotation of chlorantraniliprole and spinetoram treatments with other chemicals that do not have cross-resistance to these insecticides. Reversion of resistance to pre-selection levels has been demonstrated in *C. rosaceana* [41] and other species [42, 43], and is sometimes cited as a pre-requisite for the success of rotational strategies for resistance management in the field [44].

The reasons for reversion toward susceptibility are unclear, but it likely reflects fitness costs associated resistance development. Fitness costs associated with resistance have been demonstrated in *C. rosaceana* [33] and other species [45-49]. Azinphosmethyl resistance mediated by elevated activity of esterases resulted in lower larval weights, reduced pupal weights, and longer development times in *C. rosaceana* [33]. Fitness disadvantages associated with resistance are particularly relevant to insecticide resistance management. Once resistance has evolved, the use of substitute chemicals that do not have cross-resistance will halt or even revert the evolution of resistance [28, 30] as witnessed in the current study.

The fitness cost associated with resistance increases with the degree of resistance [33, 50] which was evident in the current study where the RYN population had higher level of resistance than SPIN population in the beginning of reversion experiment, but reverted to

being susceptible faster than the SPIN population. Based on the faster occurrence of reversion, we hypothesize that the fitness costs associated with chlorantraniliprole resistance would be higher than those of spinetoram resistance in *C. rosaceana*. This hypothesis was further strengthened by the results of synergism studies where chlorantraniliprole resistance was mediated by esterases, which have been shown to be costlier than oxidases [19], which were responsible for spinetoram resistance.

The synergism of the toxicity of chlorantraniliprole and spinetoram primarily by DEF and PBO, respectively, suggests that chlorantraniliprole resistance was mediated by esterases whereas oxidases were the primary mechanism responsible for spinetoram resistance in *C. rosaceana*. In other species, PBO has been previously reported to synergize the toxicity of spinosad [51-53] indicating possible involvement of oxidases in resistance to spinosad, which is a spinosyn just like spinetoram. Additionally, a small degree of synergism of spinetoram toxicity in the SPIN population by DEM is indicative of a possible role of glutathione-S-tranferases as a secondary mechanism in spinetortam resistance. In contrast, the involvement of esterases has been reported as a secondary mechanism involved in spinosad detoxification in other species [51-53]. These differences might be due to species difference because earlier studies have characterized spinosad resistance in other species and not in *C. rosaceana*.

Our findings that the resistance to chlorantraniliprole and spinetoram in *C. rosaceana* is unstable and that these two new insecticides appear to be detoxified by different enzyme systems suggest that chlorantraniliprole and spinetoram could be incorporated into *C. rosaceana* management programs, and management of resistance may be possible with rotational strategies. The information that chlorantraniliprole and spinetoram was esterase- and oxidase-based will also be helpful in making sound choices regarding the best alternation

of materials to be used in such management programs. Furthermore, synergism of chlorantraniliprole and spinetoram by DEF and PBO, respectively, also indicates that DEF and PBO could be useful in improving the efficacy of these compounds. However, prior to such use, further studies should be conducted to determine the effects of metabolic synergists on toxicity of these insecticides to field populations of *C. rosaceana*.

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Tables and Figures

Table 1. Toxicity of chlorantraniliprole to *C. rosaceana* neonate larvae from a colony (RYN) subjected to selection for resistance to chlorantraniliprole for 12 generations and the unselected laboratory (LAB) colony after synergism.

Colony	Compound	N	Slope (± SE)	LC_{50} (ppm) (95% FL)[1]	SR^2 (95% CL)[3]
LAB	Chlorantraniliprole	180	1.51 (0.18)	0.12 (0.08-0.18)	
	Chlorantraniliprole + DEF	180	1.59 (0.20)	0.09 (0.06-0.12)	1.44 (0.86-2.39)
	Chlorantraniliprole + DEM	180	1.44 (0.18)	0.13 (0.09-0.19)	0.95 (0.56-1.61)
	Chlorantraniliprole + PBO	180	1.71 (0.26)	0.13 (0.08-0.20)	0.94 (0.54-1.64)
RYN	Chlorantraniliprole	180	2.60 (0.24)	1.05 (0.31-1.91)	
	Chlorantraniliprole + DEF	180	1.65 (0.25)	0.41 (0.26-0.62)	2.54 (1.41-4.6)*
	Chlorantraniliprole + DEM	180	1.63 (0.29)	1.08 (0.51-1.90)	0.97 (0.53-1.78)
	Chlorantraniliprole + PBO	180	1.63 (0.25)	0.99 (0.41-2.02)	1.07 (0.59-1.92)

n = number of larvae assayed.

[1] 95% fiducial limits estimated using POLO (LeOra Software 1987).

[2] SR, synergistic ratio = LC_{50} (without synergist)/LC_{50} (with synergist).

[3] 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* Indicates that synergistic ration was significant ($\alpha = 0.05$) (Robertson et al. 2007).

Table 2. Toxicity of spinetoram to *C. rosaceana* neonate larvae from a colony (SPIN) subjected to selection for resistance to spinetoram for 12 generations and the unselected laboratory (LAB) colony after synergism.

Colony	Compound	*n*	Slope (± SE)	LC_{50} (ppm) (95% FL)[1]	SR^2 (95% CL)[3]
LAB	Spinetoram	300	1.86 (0.18)	0.06 (0.03-0.14)	
	Spinetoram + DEF	300	2.56 (0.44)	0.07 (0.05-0.09)	0.95 (0.65-1.40)
	Spinetoram + DEM	300	2.11 (0.29)	0.06 (0.04-0.08)	1.11 (0.75-1.64)
	Spinetoram + PBO	300	1.71 (0.16)	0.04 (0.02-0.06)	1.83 (1.26-2.64)*
SPIN	Spinetoram	300	2.71 (0.34)	0.34 (0.27-0.42)	
	Spinetoram + DEF	300	2.58 (0.32)	0.33 (0.26-0.41)	1.05 (0.76-1.44)
	Spinetoram + DEM	300	1.99 (0.19)	0.21 (0.12-0.38)	1.66 (0.68-2.31)
	Spinetoram + PBO	300	1.76 (0.27)	0.10 (0.04-0.16)	3.58 (2.28-5.61)*

n = number of larvae assayed.

[1] 95% fiducial limits estimated using POLO (LeOra Software 1987).

[2] SR, synergistic ratio = LC_{50} (without synergist)/LC_{50} (with synergist).

[3] 95% confidence limits estimated using lethal concentration ratio significance test (Robertson et al. 2007).

* Indicates that synergistic ration was significant ($\alpha = 0.05$) (Robertson et al. 2007

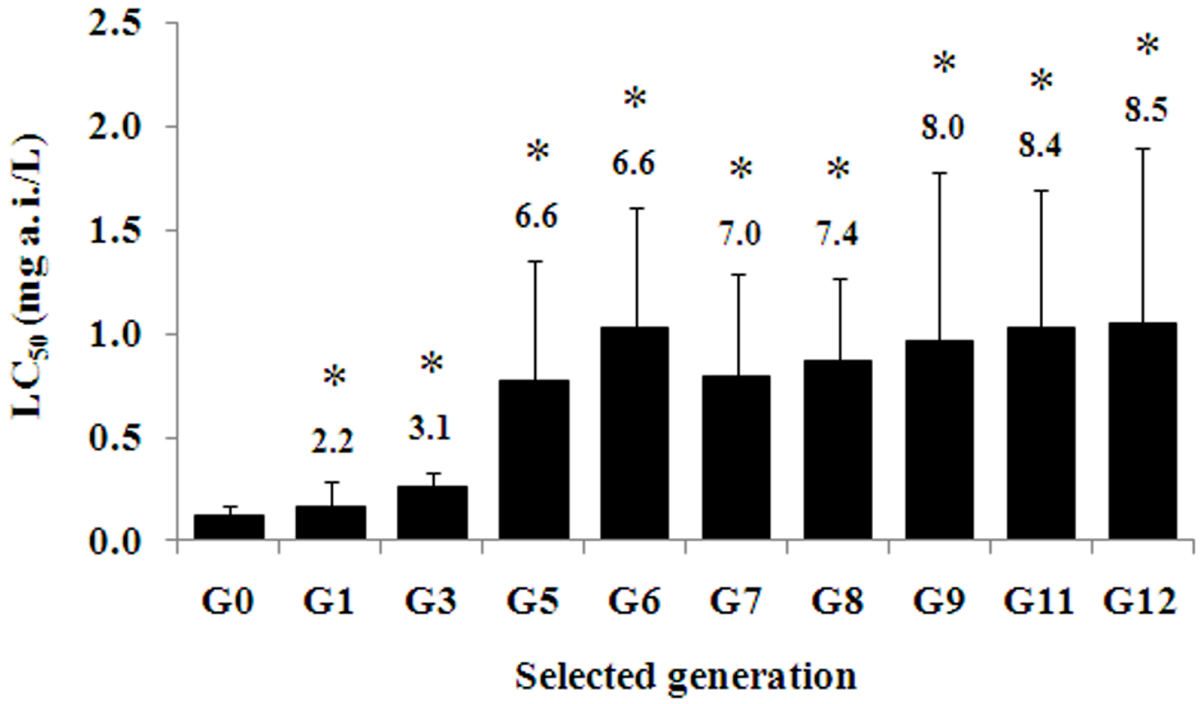

Fig. 1. Toxicity of chlorantraniliprole (LC_{50} + 95% CL) to *C. rosaceana* neonate larvae from a colony (RYN) subjected to artificial selection for resistance to chlorantraniliprole in laboratory for 12 generations. Numbers on top of the graph bars represent resistance ratios (RR); and *Indicates that the RR is significant ($\alpha = 0.05$).

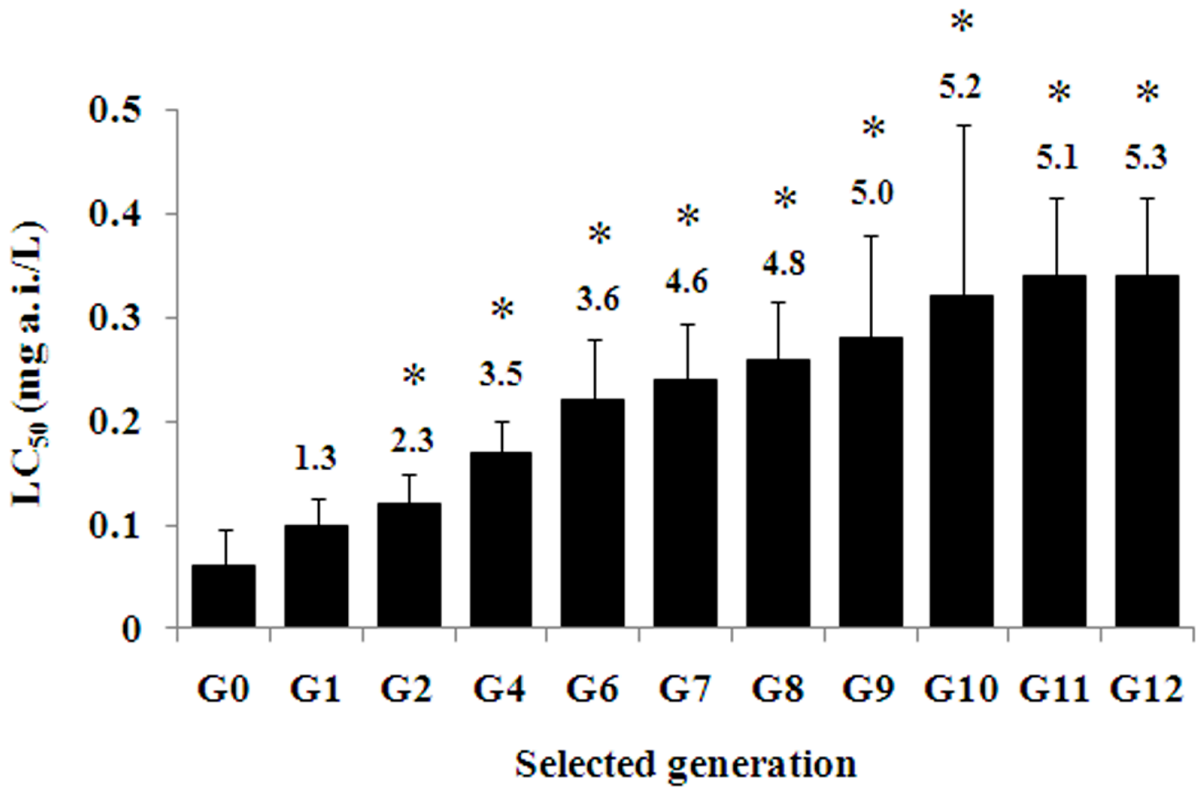

Fig. 2. Toxicity of spinetoram (LC_{50} + 95% CL) to *C. rosaceana* neonate larvae from a colony (SPIN) subjected to artificial selection for resistance to spinetoram in laboratory for 12 generations. Numbers on top of the graph bars represent resistance ratios (RR); and *Indicates that the RR is significant ($\alpha = 0.05$).

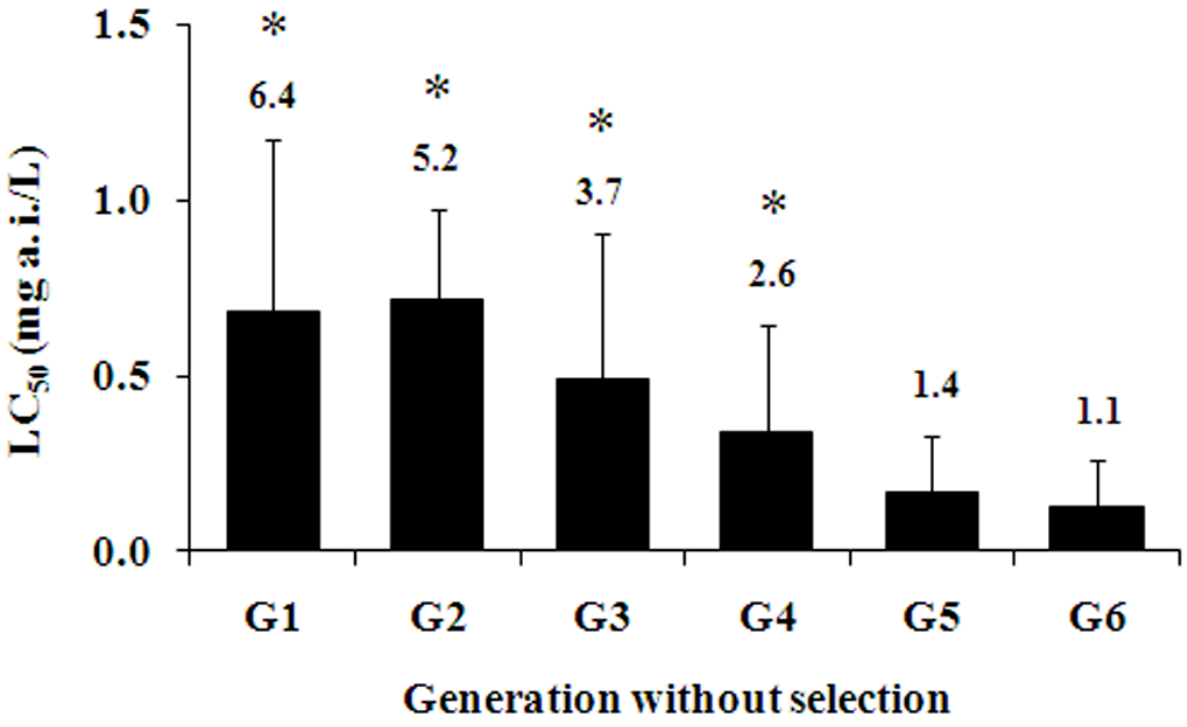

Fig. 3. Toxicity of chlorantraniliprole (LC_{50} + 95% CL) to *C. rosaceana* neonate larvae from chlorantraniliprole-selected colony when reared in the absence of selection pressure (RYN-Rev). Numbers on top of the graph bars represent resistance ratios (RR); and *Indicates that the RR is significant ($\alpha = 0.05$).

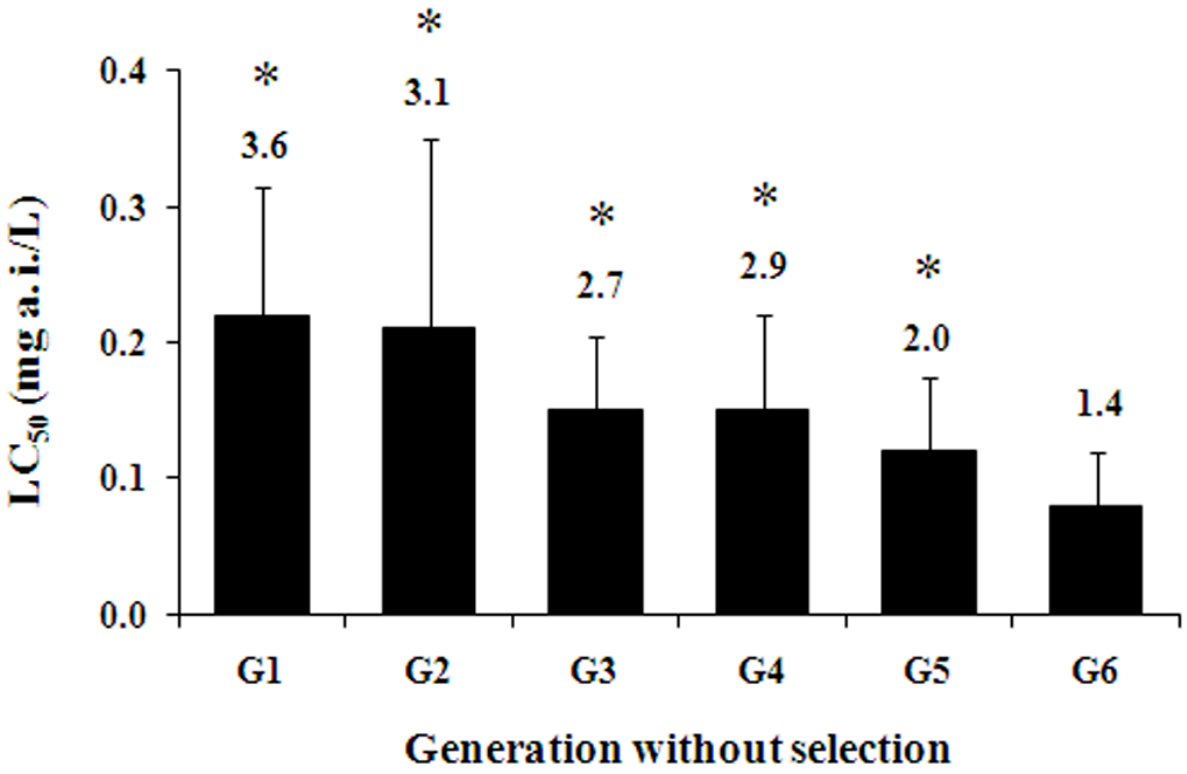

Fig. 4. Toxicity of spinetoram (LC_{50} + 95% CL) to *C. rosaceana* neonate larvae from spinetoram-selected colony when reared in the absence of selection pressure (SPIN-Rev). Numbers on top of the graph bars represent resistance ratios (RR); and *Indicates that the RR is significant ($\alpha = 0.05$).