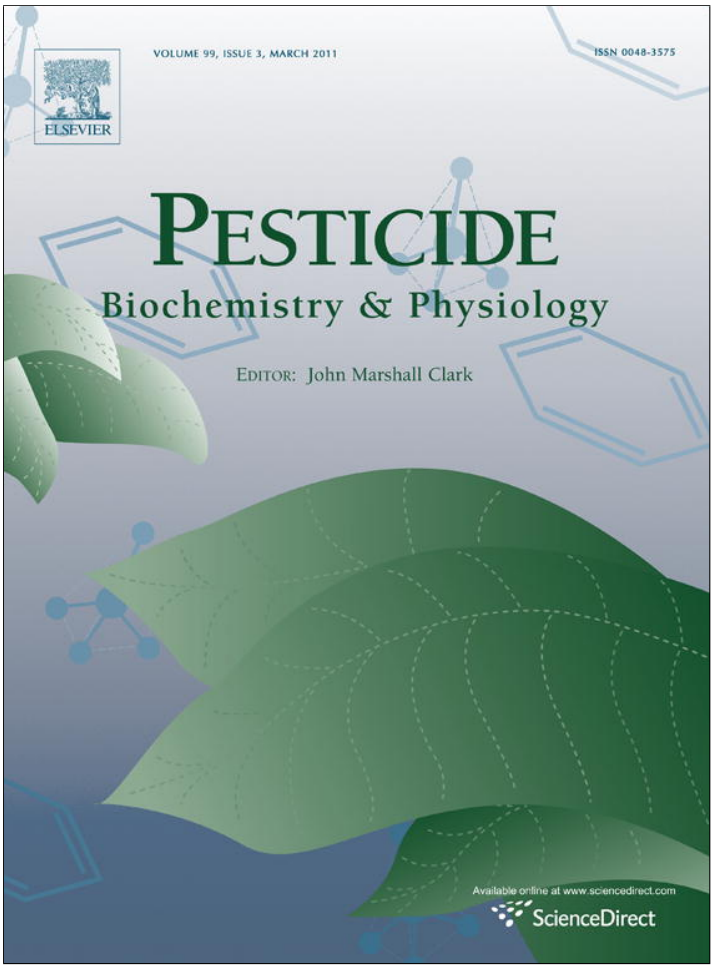

Pesticide Biochemistry and Physiology 99 (2011) 274–279

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Biochemical characterization of chlorantraniliprole and spinetoram resistance in laboratory-selected obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae)

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ARTICLE INFO

Article history:
Received 9 September 2010
Accepted 9 January 2011
Available online 28 January 2011

Keywords:
Obliquebanded leafroller
Chlorantraniliprole
Rynaxypyr
Spinetoram
Resistance mechanisms
Esterases
Mixed-function oxidases
Glutathione-*S*-transferase

ABSTRACT

Neonate larvae of obliquebanded leafroller, *Choristoneura rosaceana*, from a laboratory colony were exposed to two reduced-risk insecticides, chlorantraniliprole and spinetoram. After nine generations of selection, significant levels of resistance to each insecticide were observed. Biochemical assays were performed on third instars to determine potential resistance mechanisms. Enzyme assays indicated that esterase activity was significantly increased in the chlorantraniliprole-selected colony, whereas mixed-function oxidase levels were elevated in the spinetoram-selected colony as compared to the unselected colony. No difference in glutathione-*S*-transferase activity was seen in either of the insecticide-selected colonies. These results indicate the potential involvement of esterases and mixed-function oxidases as detoxification mechanisms responsible for resistance to chlorantraniliprole and spinetoram, respectively. Furthermore, the results of this study suggest that chlorantraniliprole and spinetoram are not detoxified by similar mechanisms and could therefore be incorporated into resistance management programs in tree fruit leading to sustainable management of *C. rosaceana*.

1. Introduction

The obliquebanded leafroller (OBLR), *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), is one of the major pests of pome fruits in the state of Washington [1]. Broad-spectrum insecticides, such as organophosphates (OPs), have been used to control OBLR for over four decades, leading to the development of resistance to OPs, and cross-resistance to other classes of insecticides [2–7]. OBLR has been reported to develop resistance to new insecticides, such as spinosad, only 6 years after its introduction into the field [7]; and in some cases high levels of resistance were documented for OBLR to chemicals that have never been used for insect control in tree fruits (i.e. indoxacarb) [5,7,8]. The development of insecticide resistance as well as regulatory actions such as Food Quality Protection Act of 1996 have prioritized the development of reduced-risk insecticides [9].

Chlorantraniliprole and spinetoram are reduced-risk insecticides which were recently registered for use in tree fruits as alternatives to OPs. Chlorantraniliprole is an anthranilic diamide which belongs to insecticide resistance action committee (IRAC) mode of action class 28 [10]. Anthranilic diamides selectively bind to ryanodine receptors (RyR) in insect muscles resulting in an uncontrolled release of calcium from internal stores in the sarcoplasmic reticulum [11,12], causing impaired regulation of muscle contraction leading to feeding cessation, lethargy, paralysis, and death of target organisms. Anthranilic diamides have very low vertebrate toxicity due to a >500-fold differential selectivity toward insect over mammalian RyR [12]. Spinetoram is a recently developed spinosyn belonging to IRAC mode of action class 5 [10]. Spinosyns primarily activate the nicotinic acetylcholine receptors by acting on a unique and yet unknown binding site [13–15]. Both chlorantraniliprole and spinetoram are highly effective against OBLR in both laboratory and field trials [16, Brunner unpublished data].

For successful production of tree fruits on a sustainable basis, it is critical for growers to incorporate reduced-risk insecticides into OBLR management programs. However, the development of resistance, and the possibility of cross-resistance, to previously used insecticides are major concerns with all new insecticides. The rational development of resistance management strategies for newly developed insecticides may not be possible without identification of mechanisms conferring resistance to those particular insecticides. Mechanisms responsible for resistance to broad-spectrum insecticides including azinphosmethyl, chlorpyrifos, cypermethrin, chlorfenapyr, indoxacarb, and the insect growth regulators tebufenozide and methoxyfenozide have been investigated in OBLR [17–20]. Biochemical mechanisms responsible for resistance to

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doi:10.1016/j.pestbp.2011.01.006

spinosad have been reported in other species of insects [21–26], but not for OBLR. Although resistance to chlorantraniliprole and spinetoram in some field-collected populations of OBLR has been reported [27], mechanisms conferring resistance to these chemicals have not been investigated.

In this paper, we report the results of our studies designed to characterize potential biochemical mechanisms of resistance to chlorantraniliprole and spinetoram in OBLR. This information will enable growers to incorporate these reduced-risk insecticides into resistance management programs based on scientific knowledge, leading to successful control of OBLR on a sustainable basis.

2. Materials and methods

2.1. Insects

C. rosaceana were obtained from a laboratory colony that was established in 1990 from larvae collected from apple orchards in Mattawa, WA, and maintained at WSU Tree Fruit Research and Extension Center (Wenatchee, WA). This colony has been reared continuously since their collection on a pinto bean diet following the method of Shorey and Hale [28] under constant conditions of temperature (23 ± 2 °C), relative humidity (RH, 70%), photoperiod (16:8, L:D), and without exposure to insecticides.

2.2. Chemicals

The insecticides used were chlorantraniliprole (Rynaxypyr™/Altacor® 35WG) obtained from E.I. du Pont de Nemours & Co., Wilmington, DE 19898, USA; and spinetoram (Delegate® 25WG) obtained from Dow AgroSciences (Indianapolis, IN). Bovine serum albumin (BSA), Cytochrome c from bovine heart (⩾95%), 3,3′,5,5′-tetramethyl benzidine dihydrochloride [TMBZ] (⩾98%), alpha-naphthyl acetate [αNA] (⩾98%), Fast Garnet GBC sulfate salt, L-glutathione reduced [GSH] (⩾98%), 1-chloro-2,4-dinitrobenzene [CDNB] (97%), and 30% hydrogen peroxide were obtained from Sigma–Aldrich® (St. Louis, MO).

2.3. Bioassays

Toxicity of chlorantraniliprole and spinetoram to neonate *C. rosaceana* larvae was estimated by using a diet incorporation bioassay [27]. A stock solution of each insecticide was prepared by diluting it at 10× the highest concentration to be used in the bioassay. Serial dilutions were then prepared from the stock solution at 10× of each of the target concentrations to be used in the bioassay. Insecticide incorporated diet was prepared by mixing insecticide dilution (insecticide + water), water, vinegar, and dry diet premix (Stonefly *Heliothis* Diet Ward's Natural Science, Rochester, NY) at a ratio of 10:61:4:25. An untreated control was prepared by mixing water, vinegar, and dry diet premix at a ratio of 71:4:25. Enough insecticide incorporated diet was prepared prior to the start of the bioassays, so that all tests were run on the same diet mixtures, and new diet was prepared for each replication.

A small portion of insecticide incorporated diet (approximately 8.0 cm^3) was added to a plastic 50 × 9 mm Petri dish (Becton Dickinson and Company, Franklin Lakes, NJ). The diet was pushed firmly along the edges of the dish, and scored with a pin so that *C. rosaceana* neonates could readily colonize the diet. Petri dishes were chosen randomly, and five 1-day-old *C. rosaceana* larvae were transferred into each dish using a camel's hair brush. Six to ten dishes were prepared for each treatment (30–50 larvae/treatment) depending on the availability of neonate larvae. These dishes were placed in growth chambers at constant conditions of temperature (23 ± 2 °C), relative humidity (RH, 70%), and photoperiod (16:8, L:D). Larval mortality in each bioassay was evaluated after 7 d. Larvae were recorded as dead if they did not move when probed with camel's hair brush. To ensure that offspring of different females were assayed, larvae emerging from any given egg mass were systematically distributed among various concentrations so that a maximum of 5–10 larvae per egg mass were exposed to any one concentration.

2.4. Selection for resistance

Based on results from initial bioassays, cohorts of larvae from the laboratory colony were selected with chlorantraniliprole (RYN) or spinetoram (SPIN) using diet incorporation bioassays, while the unselected control (LAB) was treated identically but without exposure to insecticides. In the first round of selection, neonate larvae were exposed to insecticides at concentrations corresponding to the LC_{70} of the baseline established for the laboratory colony. After 4 d 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 LC_{70} based on the results of bioassays from the previous generation. The number of neonate larvae used for each generation varied (1000–2000) depending on availability.

2.5. Enzyme assays

Esterase, oxidase, and glutathione-*S*-tranferase activities were determined according to the methods of Martin et al. [29] with modification as described below. For enzyme assays, 30 third-instar *C. rosaceana* (10–15 mg each) from the susceptible colony (LAB) and each of the selected resistant colonies (RYN and SPIN) were used. Individual insects were homogenized in 200 µl ice cold potassium phosphate buffer (0.1 M, pH 7.2), and then spun in a microfuge at ~21,000*g* for 2 min. All reactions were carried out in disposable 96-well microplates (Greiner Bio-One, VWR International, West Chester, PA), as detailed below.

2.6. Esterase assay

Esterase activity was measured using αNA as a substrate. Hydrolysis of alpha-naphthyl acetate [αNA] was performed by incubating 10 µl homogenate with 90 µl of phosphate-buffered saline plus 1% Triton X-100 [PBS] (pH 6.5) for 10 min at room temperature. One hundred microlitres solution containing αNA (60 mM) in PBS was added and the mixture was incubated for 30 min at room temperature. The reaction was stopped by adding 100 µl Fast Garnet salt (2.4 mM) and the mixture was incubated for 10 min at room temperature. Absorbance was read at λ = 550 nm in a PowerWave 340 microplate spectrophotometer (BioTek, Winooski, VT) against blanks and converted to esterase activity (nmol/min/µg protein) based on the standard curve. The standard curve of α-Naphthol product was linear with an r^2 value of 0.9997.

2.7. Oxidase assay

Total oxidase activity was measured using 3,3′,5,5′-tetramethyl benzidine dihydrochloride (TMBZ) as a substrate. The reaction mixture consisted of 50 µl of 100 mM potassium phosphate buffer (pH 7.2), 50 µl of larval homogenate, 200 µl of 1.3 mM TMBZ 25% methanol, 250 mM sodium acetate buffer (pH 5.0). Then 25 µl of hydrogen peroxide (3.0%) was added, giving a final volume of 325 µl. Absorbance was read at λ = 630 nm against blanks (wells containing all reaction components except larval homogenate) in a PowerWave 340 microplate reader after 30 min incubation at room temperature. Total oxidase activity was expressed as pmol

equivalent cyt-P450/mg protein. The standard curve of cytochrome c was linear with an r^2 value of 0.9947.

2.8. Glutathione-S-transferase assay

Glutathione-S-Transferase (GSH) activity was measured using 1-chloro-2,4-dinitrobenzene [CDNB] as a model substrate. The reaction mixture contained 100 µl of 10 mM GSH in 100 mM sodium phosphate buffer (pH 6.5) and 100 µl of larval homogenate. The reaction was initiated by the adding 100 µl CDNB (6 mM in methanol), resulting in a final volume of 300 µl. The plates were immediately transferred to a microplate reader. The reactions were allowed to continue for 5 min and absorbance readings were taken at $\lambda = 340$ nm automatically once per min against blanks (wells containing all reaction components except larval homogenate). The increase in absorbance was linear throughout the 5-min reading interval. An extinction coefficient of 9.6 mM^{-1} cm^{-1} was used to calculate the amount of CDNB conjugated.

2.9. Protein assay

Protein contents were determined by the method of Bradford [30] using Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA) with BSA as a standard. Three replicates of 10 µl were assayed for each larval homogenate. Absorbance was read at $\lambda = 595$ nm against blanks and converted to protein concentration based on the standard curve.

2.10. Data analysis

Median lethal concentration (LC_{50}) values and their corresponding 95% fiducial limits (FL) were estimated [31]. Lethal concentration ratios (LCR) at LC_{50} and their corresponding 95% confidence limits (CL) were calculated using lethal concentration ratio significance test [32]. A laboratory colony (LAB) that was not selected with any of the insecticides but otherwise treated the same served as the reference susceptible population for comparison purposes and was assigned a ratio of 1.0. Lethal concentrations of the selected colonies RYN and SPIN, and the unselected LAB colony were considered significantly different if the 95% CL of their corresponding LCR-LC_{50} did not include the value of 1.0 (32). Mean enzyme activities recorded in larvae from the RYN and SPIN selected colonies were compared with those from larvae in the unselected LAB colony using *t*-test. Significance was accepted at $\alpha = 0.05$ in all statistical tests used in this study.

3. Results

Bioassays show that both chlorantraniliprole and spinetoram are toxic to the OBLR LAB colony, with LC_{50} values of 0.12 ppm and 0.06 ppm, respectively (Table 1 and Table 2). After nine generations of selection for resistance, susceptibility of RYN and SPIN selected colonies significantly decreased in diet incorporation bioassays with both chlorantraniliprole and spinetoram, with LC_{50} values of 0.96 ppm and 0.28 ppm, respectively (Table 1 and Table 2). Diet incorporation bioassays showed an 8-fold increase in the LC_{50} value of the RYN colony after nine consecutive generations of selection as compared to the unselected LAB colony (Table 1). Similarly, a 5-fold increase was observed in the LC_{50} value of the SPIN colony after selection for the same number of generations as compared to the unselected LAB colony (Table 2).

Table 2
Results of probit analyses for diet incorporation bioassays of spinetoram with *C. rosaceana* neonate larvae from spinetoram-selected (SPIN) colony after nine generations of selection for resistance and the unselected laboratory (LAB) colony.

Colony	*N*	Slope (± SE)	χ^2	LC_{50} (ppm) (95% FL)[a]	LCR[b] (95% CL)[c]
LAB	300	2.07 (0.20)	9.34	0.06 (0.03–0.10)	
SPIN	300	2.60 (0.29)	4.76	0.28 (0.17–0.29)	4.99 (3.61–6.90) *

n = number of larvae assayed.
[a] 95% fiducial limits estimated using POLO (LeOra Software 1987).
[b] LCR, lethal concentration ratio = LC_{50} (SPIN colony)/LC_{50} (LAB colony).
[c] 95% confidence limits estimated using lethal concentration ratio significance test, Robertson et al. [32].
* Indicates that Lethal Concentration Ratio was significant ($\alpha = 0.05$), Robertson et al. [32].

To determine the potential role of detoxification enzymes in resistance to chlorantraniliprole and spinetoram in the selected OBLR colonies, enzyme assays to measure the levels of esterases, oxidases, and glutathione-S-transferases were performed. The activity of non-specific esterases was significantly higher in third-instar OBLR from the RYN colony compared to the LAB colony ($t = -3.00$; df = 55; $p = 0.004$) when determined using αNA as substrate (Fig. 1), indicating the possible involvement of esterases in conferring resistance to chlorantraniliprole. For spinetoram selected OBLR however, there was no significant difference in the esterase activity in OBLR larvae from the SPIN colony compared to the LAB colony ($t = -0.81$; df = 54; $p = 0.420$) (Fig. 1).

In contrast to the results above, the activity of oxidases was significantly higher in third-instar OBLR from the SPIN colony compared to the LAB colony ($t = -2.11$; df = 54; $p = 0.039$) when determined using TMBZ as substrate (Fig. 2), suggesting a possible role of oxidases in conferring resistance to spinetoram. For chlorantraniliprole selected OBLR, there was no significant differences in oxidase activity in OBLR larvae from the RYN colony when compared to the LAB colony ($t = 0.06$; df = 47; $p = 0.950$) (Fig. 2).

The laboratory colonies of OBLR selected for resistance to chlorantraniliprole and spinetoram were also assessed for glutathione-S-transferase activity. In each case, there was no significant differences in the activity of glutathione-S-transferases in third-instar OBLR from either the RYN colony ($t = 0.78$; df = 55; $p = 0.441$) or the SPIN colony ($t = -1.97$; df = 54; $p = 0.054$), when compared with the LAB colony (Fig. 3). These results indicate that glutathione-S-transferases probably have no role in detoxification of chlorantraniliprole or spinetoram, at least in these laboratory-selected colonies of *C. rosaceana*.

Table 1
Results of probit analyses for diet incorporation bioassays of chlorantraniliprole with *C. rosaceana* neonate larvae from chlorantraniliprole-selected (RYN) colony after nine generations of selection for resistance and the unselected laboratory (LAB) colony.

Colony	*n*	Slope (± SE)	χ^2	LC_{50} (ppm) (95% FL)[a]	LCR[b] (95% CL)[c]
LAB	180	1.59 (0.19)	11.83	0.12 (0.06–0.20)	
RYN	180	1.65 (0.29)	5.20	0.96 (0.32–1.95)	7.95 (3.81–16.59)*

n = number of larvae assayed.
[a] 5% fiducial limits estimated using POLO (LeOra Software 1987).
[b] LCR, lethal concentration ratio = LC_{50} (RYN colony)/LC_{50} (LAB colony).
[c] 95% confidence limits estimated using lethal concentration ratio significance test, Robertson et al. [32].
* Indicates that Lethal Concentration Ratio was significant ($\alpha = 0.05$), Robertson et al. [32].

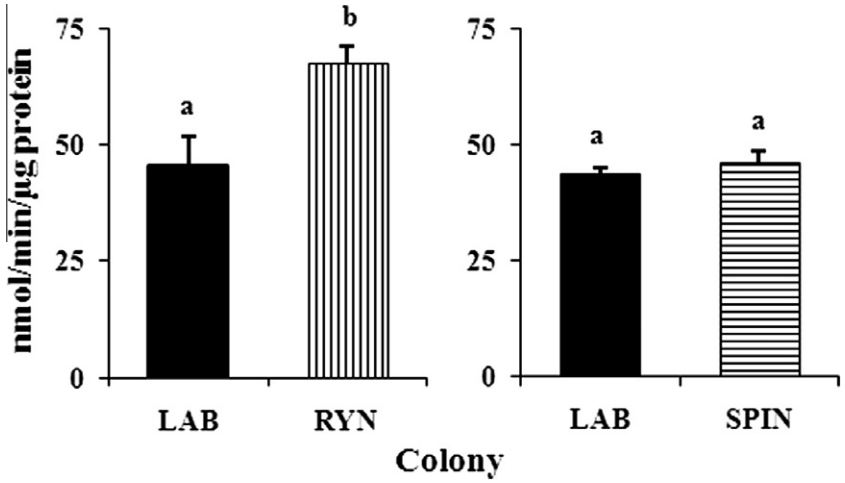

Fig. 1. Activity of esterases (Mean + SEM) in chlorantraniliprole-selected (RYN) and spinetoram-selected (SPIN) colonies after nine generations of selection for resistance in laboratory, and the unselected laboratory (LAB) colony of *C. rosaceana*. Graph bars containing similar letters on the top are not significantly different ($\alpha = 0.05$, *t*-test).

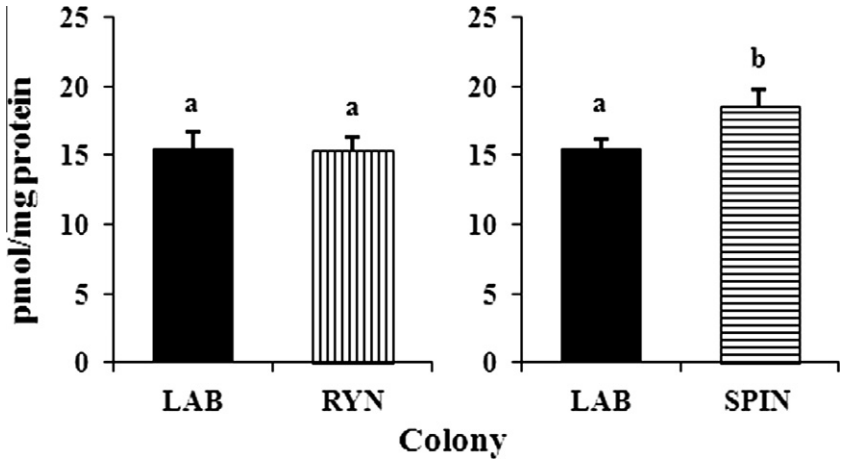

Fig. 2. Level of oxidases (pmol equivalent cytochrome-P450 U) (Mean + SEM) in chlorantraniliprole-selected (RYN) and spinetoram-selected (SPIN) colonies after nine generations of selection for resistance in laboratory, and the unselected laboratory (LAB) colony of *C. rosaceana*. Graph bars containing similar letters on the top are not significantly different ($\alpha = 0.05$, *t*-test).

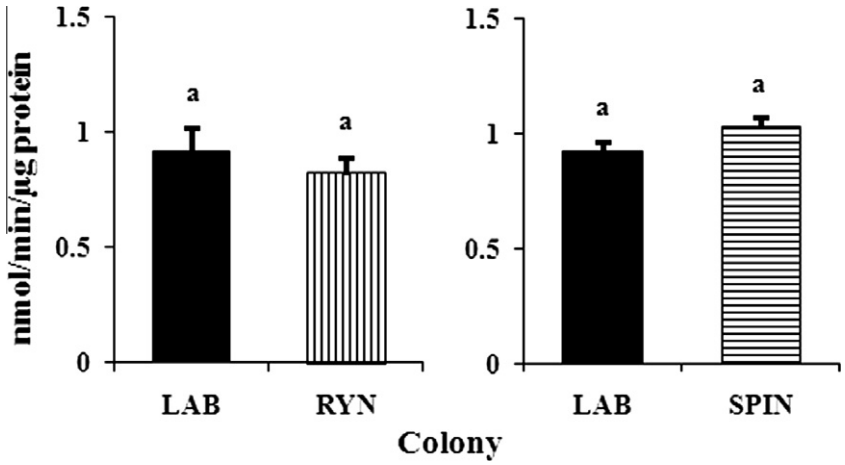

Fig. 3. Activity of glutathione-*S*-transferases (Mean + SEM) in chlorantraniliprole-selected (RYN) and spinetoram-selected (SPIN) colonies after nine generations of selection for resistance in laboratory, and the unselected laboratory (LAB) colony of *C. rosaceana*. Graph bars containing similar letters on the top are not significantly different ($\alpha = 0.05$, *t*-test).

4. Discussion

Insecticide resistance presents a major risk to the sustainability of integrated pest management (IPM) programs for *C. rosaceana*. Resistance management strategies could slow the development of resistance only if implemented in a timely manner. However, the effectiveness of resistance management strategies may be reduced without the knowledge of biochemical mechanisms conferring resistance to insecticides used in IPM programs. In this study, we selected *C. rosaceana* neonates from a laboratory colony for resistance to two recently registered insecticides, chlorantraniliprole and spinetoram.

Our results show that artificial selection for resistance in the laboratory resulted in development of significant levels of resistance to both chlorantraniliprole and spinetoram in OBLR. Moreover, the level of resistance observed after nine generations of selection was higher for chlorantraniliprole (8-fold) than spinetoram (5-fold). These results suggest that the levels of resistance to chlorantraniliprole could be higher than spinetoram under similar circumstances in field populations of *C. rosaceana* [33].

This study represents the first report on the mechanisms involved in resistance to chlorantraniliprole and spinetoram in *C. rosaceana*. Chlorantraniliprole is the first anthranilic diamide registered for use in *C. rosaceana* control programs. Chlorantraniliprole or other diamides have never been used for insect control in tree fruit in the past, but we have reported significant levels of resistance to chlorantraniliprole in some of the field populations of *C. rosaceana* even before its first field application [27]. However, specific mechanisms responsible for chlorantraniliprole resistance have not been reported in any other insects. The higher activities of esterases in *C. rosaceana* larvae from the RYN colony are indicative of the possible involvement of esterases in conferring resistance to chlorantraniliprole. Detoxification by esterase enzymes has been reported in *C. rosaceana* resistance to azinphosmethyl [18,20] and other tortricid moths, such as light brown apple moth, *Epiphyas postvittana* [34]. The azinphosmethyl-resistance in *C. rosaceana* mediated by general esterases usually extends to several types of organophosphates, carbamates, and other classes of insecticides [20], and has been associated with cross-resistance to pyrethroids [35–37].

Spinetoram is a second generation spinosyn which was recently registered for *C. rosaceana* control in tree fruit. Spinosad, the first spinosyn registered, has been used in tree fruit since 1998 and *C. rosaceana* populations developed resistance to this compound only 6 years after its introduction [7]. In a recent study, we reported low levels of resistance to spinetoram in some field populations of *C. rosaceana*, which were highly correlated with resistance to spinosad, even before its registration [27]. Resistance to spinosad has been characterized in several species of insects; however, mechanisms responsible for spinetoram-resistance have not yet been reported. Significant elevation in the level of oxidases in *C. rosaceana* larvae from the SPIN colony suggests that resistance to spinetoram in this laboratory-selected colony was mediated by oxidases. Our findings are in agreement with the previous studies reporting the involvement of oxidases as a mechanism for resistance to spinosad in *Musca domestica* [21], *Spodoptera exigua* [22], and *Helicoverpa armigera* [25], an anticipated result since spinosad and spinetoram are both spinosyns.

Insecticide resistance management in *C. rosaceana* in tree fruit orchards is a challenge for growers especially at a time when broad-spectrum insecticides such as OPs are being phased out and a wide range of newer insecticides with different modes of action are available to control this pest. Both chlorantraniliprole and spinetoram are highly effective against *C. rosaceana* in preliminary studies in laboratory and field situations [16,Brunner unpublished data], and this study indicates that they do not share detoxification mechanisms. These findings support our contention that chlorantraniliprole and spinetoram could be incorporated into a resistance management program leading to sustainable management of *C. rosaceana* in tree fruit. Unfortunately the evidence of pre-existing resistance to chlorantraniliprole and cross-resistance between spinosad and spinetoram in some field populations of *C. rosaceana* [27] coupled with a high potential for resistance evolution witnessed in selection experiments may make resistance management more difficult. Therefore, chlorantraniliprole and spinetoram must be used wisely in the framework of a well thought-out resistance management program taking into account all other insecticide options and avoiding the use of those detoxified by the similar enzyme systems against consecutive generations of *C. rosaceana*.

The mechanisms responsible for resistance to the same chemical may vary from one population to another [18]. Therefore, detoxification mechanisms responsible for chlorantraniliprole and spinetoram resistance observed in the laboratory-selected populations in the current study may not be the same as those present in the field populations. Further studies are required to determine the mechanisms of resistance in field-collected populations. Additionally, glutathione-*S*-transferase activity in this study was measured by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) alone which revealed no significant differences between any of the selected colonies and the unselected colony. However, further studies should be conducted using both CDNB as well as DCNB (1,2-dichloro-4-nitrobenzene) to determine whether or not glutathione-*S*-transferases are involved in detoxification, especially if one substrate CDNB or DCNB fails to detect the significant differences in the activity of glutathione-*S*-transferases.

Acknowledgments

This study was sponsored by the Washington Tree Fruit Research Commission, the Washington State Commission on Pesticide Registration, and Western Sustainable Agriculture Research and Education (Project No. GW10-003). We thank Drs. John Brown (Washington State University), and Thomas Unruh and Lisa Neven (USDA-ARS) for their comments on the manuscript; Dr. John Dunley (Washington State University, Tree Fruit Research and Extension Center Wenatchee) for his guidance and advice on the selection experiments, Mike Doerr (WSU) for his assistance in the standardizing diet incorporation bioassays, Kathy Pierre (WSU) for her help in maintaining colonies of *C. rosaceana* in the laboratory, and Robert McCurdy and Laura Willett (USDA-ARS) for their assistance in enzyme assays.

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