OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE) RESISTANCE TO INSECTICIDES IN MICHIGAN APPLE AND CHERRY ORCHARDS

By

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ABSTRACT

OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE) RESISTANCE TO INSECTICIDES IN MICHIGAN APPLE AND CHERRY ORCHARDS

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Field populations of *Choristoneura rosaceana* (Harris) were collected from commercial apple and cherry orchards in western Michigan. **A baseline toxicity study**, using a diet overlay bioassay, of eight insecticides was conducted on 12-24h old larvae of the *C. rosaceana* populations. The commercial cherry population showed resistance to bifenthrin, spinetoram, emamectin benzoate, and indoxacarb with 4.9, 4.1, 5.8, and 21-fold resistance respectively. The commercial cherry population was not resistant to phosmet, methomyl, chlorantraniliprole, and novaluron. The commercial apple population showed resistance to phosmet, bifenthrin, spinetoram, emamectin benzoate, chlorantraniliprole, and indoxacard with 5, 5, 4.3, 6.3, 4.7, and 620.4-fold resistance respectively. The commercial apple population was not resistant to methomyl and novaluron. This difference in resistance levels between the apple and cherry populations is worthwhile and requires further study to identify the source of this difference. **Synergism and metabolic studies** were conducted to identify the detoxification mechanism against indoxacarb in the tested *C. rosaceana* field populations. In the diet incorporation bioassay, in both susceptible and cherry populations, only DEF significantly synergized indoxacarb with a synergism ratio (SR) of 6.5 and 22.6-folds respectively. In the apple population all synergists PBO, DEM, and DEF significantly synergized indoxacarb with SR of 9.6, 7.7, and 285.6-folds respectively indicating a complex resistance case with the possible involvement of all three metabolic resistance mechanisms. In the in vitro metabolic study,

indoxacarb (DPX-JW062) was very rapidly bio-transformed within 5 min into small molecules in the lower portion of the metabolic pathway when it reacted with the 12,000g midgut supernatant of each population. In the second part of the in vitro study, the bio-transformation of DPX-JW062 remarkably was decreased when it reacted with the pre-inhibited (by DEF) 12,000g midgut supernatant of each population. Additionally, the degradation of metabolites in the upper portion of the metabolic pathway remarkably decreased, which resulted in accumulation of DCJW and MP819 metabolites. The accumulation of DCJW metabolite treatment provided a persuasive explanation of the synergistic impact of esterase inhibitor DEF on indoxacarb toxicity in *C. rosaceana*. **Field-based residual bioassay and residue analysis** were conducted to assess the field performance and toxicity longevity of the insecticides that had previously been associated with significant resistance levels in the baseline study. 12-24h old larvae of *C. rosaceana* were exposed to the leaf samples that were collected at different post-application (DPA) intervals. In apple and cherry trials, the order of residual longevity of insecticides that effectively controlled the tested populations was: bifenthrin and spinetoram (apple: 14, cherry 21 DPA), phosmet (apple: 7, cherry 14 DPA), chlorantraniliprole (apple: 7 DPA), and indoxacarb and emamectin benzoate (apple: 1, cherry 7 DPA). The previously documented significant resistance levels in the tested populations resulted in a measurable loss of field performance only in the cases of emamectin benzoate, chlorantraniliprole, and indoxacarb at 7, 21, and all DPA intervals respectively in the apple trials while in cherry trial just indoxacarb at 7 DPA. In term of long lasting residues, only chlorantraniliprole and indoxacarb maintained measurable leaf residues over all DPA intervals. These findings can help fruit growers make adjustments to spray/re-spray intervals and optimally utilize important chemical tools in their control programs.

I dedicate my dissertation work to my grandparents **Dafer bin Ali Al Shobrami Al gahtani** and **Norah bent Ali Al Mugram Al gahtani** who passed away while I was thousands of miles away from them. This dedication is an appreciation of their lifelong, unwavering support and kindness from the time I was a young boy, up until the last day of their lives.

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KEY TO ABBREVIATIONS

K.A. apple population

F.C. cherry population

S.S. susceptible population

a.i. active ingredient

ppm parts per million

LC Lethal concentration

RR Resistance ratio

SR Synergism ratio

95% FL 95% fiducial limits

s.e. standard error

df degrees of freedom

RCBD randomized complete block design

DPA day post-application

DPX-JW062 indoxacarb

DCJW (IN-JT333) decarbomethoxyllated metabolite

KT413 carboxylic acid of DPX-JW062

MP819 decarboxylation and rearrangement product from KT413 metabolite

LOD Limit of detecting a peak

LOQ Limit of quantifying a peak

CHAPTER 1: BACKGROUND & STATE-OF-THE-ART

Introduction

The apple-production of United States of America was 5,113,537 metric tons in 2016 with an estimated value of ≈ $2.2 billion annually (United States Department of Agriculture-National Agricultural Statistics Service USDA-NASS 2017). Michigan is the second largest state in apple-production in the USA (www.michiganapples.com). The tart-cherry-production of USA was 149,368 metric tons in 2016 with an estimated value of ≈ $85.6 million annually (USDA-NASS 2017). Michigan is the largest state in tart-cherry-production in the USA (www.choosecherries.com). These valuable crops require effective control programs to protect them from various pests and diseases.

Obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), is an important pest in apple and cherry orchards. *C. rosaceana* is a North American pest of tree fruit that has a broad host range including many members of the families Rosaceae and Cornaceae, with a preference the Rosaceae family members. Over the course of a year, *C. rosaceana* has two generations, one from overwintering larvae and one late summer generation. The larva is the injurious stage of the *C. rosaceana* and develops through five to six larval instars. Damage includes feeding on flower buds, leaves, and developing fruit (Sanderson and Jackson 1909, Reissig 1978). The *C. rosaceana* was long considered to be a minor pest in apple and cherry orchards until outbreaks occurred in the 1970s (Sial and Brunner 2010a).

In apple, *C. rosaceana* is a foliage and fruit injury pest causing remarkable damage especially by the summer generation. While in cherry, the foliage and fruit injury that caused by *C. rosaceana*

is less serious compared to apple. However, *C. rosaceana* is more critical pest in cherry late season when it can be a contaminant pest in harvested cherries, thus causing high risk for load rejection due to the U.S. Department of Agriculture zero-tolerance mandate (USDA 1941, Mason and Huber 2002, Wise and Whalon 2009).

After becoming a primarily pest in fruit orchards, over the following four decades *C. rosaceana* was targeted directly by control programs. The control programs mainly relied on conventional pesticides especially organophosphates. Eventually, resistance to the conventional insecticides was documented in *C. rosaceana* populations throughout the North American fruit-producing regions (Waldstein and Reissig 2000, Ahmad et al. 2002, Sial and Brunner 2012b).

In Michigan apple orchards *C. rosaceana* populations were resistant to organophosphates compounds with moderate resistance against Azinphos-methyl and Chlorpyrifos, and low resistance against Phosmet, while it showed negligible level of resistance against the pyrethroids, including cypermethrin, zeta-cypermethrin, bifenthrin, deltamethrin and esfenvalerate. Both Organochlorine and carbamates compounds, including endosulfan, thiodicarb, methomyl and carbaryl, had almost no toxicity against susceptible and resistance populations and that suggests these compounds are ineffective against the *C. rosaceana* populations in the control programs (Ahmad et al. 2002). Similarly, the *C. rosaceana* populations, in apple orchards of Washington State and Canadian Provinces: British Columbia, Ontario, and Quebec, were resistant against the organophosphates and carbamates compounds (Sial and Brunner 2012a, Smirle et al. 2002, Smirle et al. 2003).

As a result of insecticide resistance development and in order to fulfill the safety requirements of the Food Quality Protection Act of 1996 (FQPA), apple and cherry growers have shifted to use

reduced-risk insecticides including insect growth regulators (IGRs). These new chemical classes have novel mode of action, and can be used in rotation with other insecticides as part of resistance management while helping to ensure the safety of non-target organisms and the environment (Smirle et al. 2003, Sial and Brunner 2010b, Sial and Brunner 2010c, Sial et al. 2010, Sial and Brunner 2012a, Sial and Brunner 2012b).

Reduced-risk insecticides and insect growth regulators (IGRs) including emamectin benzoate, indoxacarb, novaluron, spinetoram, and chlorantraniliprole have been registered for use in many fruit crops in the U.S. in 1999, 2000, 2001, 2007, and 2008 respectively (USEPA 2015). At first, these compounds were promising and highly effective but resistance of *C. rosaceana* populations to some of these new compounds have been reported, and the level of resistance ranged between slight to high (Ahmad et al. 2002, Smirle et al. 2002, Sial et al. 2010).

In Michigan apple orchards, the *C. rosaceana* populations were low resistant to the chlorinated pyrrole chlorfenapyr compound. Spinosad and emamectin benzoate were highly toxic against *C. rosaceana* populations with almost no resistance against these compounds (Ahmad et al. 2002). Similar results were found in Washington State apple orchards. Where, the reduced-risk insecticides chlorantraniliprole and spinetoram were highly toxic to the *C. rosaceana* populations, which had almost no resistance against these two compounds (Sial and Brunner 2012a, Sial et al. 2010). Also, the *C. rosaceana* field populations, from British Columbia apple orchards, in south-west Canada, found to be susceptible to spinosad (Smirle et al. 2003).

However, in the last decade some cases of resistance against these reduced-risk insecticides were documented in some parts of the United State and Canada. For instance, the *C. rosaceana* field populations from apple orchards in Okanagan and Similkameen Valleys in British Columbia had

significant resistance levels against the two benzoylhydrazine insect growth regulators tebufenozide and methoxyfenozide (Smirle et al. 2002). In Michigan apple orchards, although the indoxacarb compound was not used in the control programs, the field population was very high resistant against it. That supported the notion of cross-resistance from other chemical classes specially the conventional compounds (Ahmad et al. 2002). Likewise, in Washington State apple orchards one *C. rosaceana* field population out of nine populations was slightly resistant to chlorantraniliprole compound, even though this compound was not applied in the control programs by that time. Regardless of this low level of resistance against chlorantraniliprole, the concern of developing higher levels of resistance is expected; especially when this compound is applied consistently (Sial et al. 2010).

This concern of developing resistance had studied in a laboratory selective pressure study using the reduced-risk insecticides chlorantraniliprole and spinetoram on a laboratory *C. rosaceana* population. The *C. rosaceana* neonate larvae developed resistance after six generations by 6.58 and 3.64 fold to chlorantraniliprole and spinetoram respectively. Despite the fact of the level of this resistance is still considered as a low resistance level, the development of resistance after repeated the exposure to these compounds should be an alert for the growers to manage the control programs carefully to maintain the efficiency of these new compounds (Sial and Brunner 2012a).

Indoxacarb, an oxadiazine insecticide, is one of the new insecticides which discovered by the E.I. DuPont Co. in 1992 and introduced to the market in 2000 (McCann et al. 2001, IRAC 2017, USEPA 2017). Indoxacarb is a pro-insecticide where it undergoes hydrolase based bio-activation, due to the action of carboxylesterases, to yield the potent sodium channel blocker decarbomethoxyllated (DCJW) metabolite (Wing et al. 1998). Ingestion is the major route of

entry where indoxacarb is highly active, with limited documented cases of contact activity, when applied topically (Alves et al. 2008, Nehare et al. 2010).

Outstanding activity of indoxacarb was documented against several lepidopteran species, including some members of the Tortricidae family, even though some of these field populations were resistant to other insecticide classes such as pyrethroids, organophosphates, carbamates, and insect growth regulators (Wing et al. 2000). However, *C. rosaceana* appears to be an anomaly case in this context where different *C. rosaceana* field populations showed high resistance levels against indoxacarb even before some field populations had been exposed through commercial use (Ahmad et al. 2002, Smirle et al. 2002, Dunley et al. 2006).

This variation in susceptibility to indoxacarb in different species is thought to be correlated to the variation in the metabolic rate in those different species (Wing et al. 2000). Metabolism of xenobiotic compounds, including insecticides, in arthropods is mainly modulated by cytochrome P450s, hydrolases, and glutathione-S-transferases enzymes resulting in either detoxification or activation of those xenobiotic compounds. In the case of insecticide metabolism both will determine the abundance of active form of each insecticide in the insect body and the resistance level against the insecticide (Fu-Gen et al. 2014). For many targeted insects indoxacarb is activated at a high rate but this is not the case in some other targeted or non-targeted species. In these less frequent cases indoxacarb breaks-down to metabolites other than DCJW or they have high rates of DCJW breaking down to non-toxic metabolites in a way that overcomes the timeframe of the toxic effect of DCJW (Wing et al. 1998, Alves et al. 2008).

The rate of indoxacarb bio-activation or detoxification could be a critical factor towards understanding the variation in its effectiveness for different lepidopteran species as well as the

resistance phenomenon in general (Wing et al. 2000). Previous studies describe the role of carboxylesterases in the bio-activation of indoxacarb to yield the potent sodium channel blocker DCJW as well as the rate of this biotransformation. However, Gondhalekar et al. (2016) were the first to investigate the indoxacarb metabolic pathway beyond this point in insects where both indoxacarb and DCJW were metabolized to more polar derivatives in the German cockroach.

While laboratory studies including, baseline and resistance mechanism studies, are important to document and measure insecticide resistance plus identify the resistance mechanism in *C. rosaceana* from commercial fruit orchards, results from such research are often difficult for growers to interpret. Field-based residual bioassays can provide temporal performance data that help demonstrate to growers how various degrees of resistance is expressed under semi-field conditions (Mota-Sanchez et al. 2008). Concurrent residue analysis, in turn, can determine the insecticide longevity under normal field conditions. This temporal dimension of the insecticide field performance can help growers to identify adjustments to spray/re-spray intervals in their control programs (Brunner et al. 2005, Wise et al. 2006, Wise et al. 2007, Sial and Brunner. 2010b).

In general, the laboratory performance of an insecticide is expected to be a good indicator of its field performance (Bruck et al. 2011). This concept has been proven in different studies. For example methoxyfenozide, lambda-cyhalothrin, and acetamiprid against *Cydia pomonella* (Linnaeus) (Mota-Sanchez et al. 2008), methoxyfenozide and tebufenozide against *Paralobesia viteana* (Clemens) (Isaacs et al. 2005), spinosad, imidacloprid, and thiacloprid against *Rhagoletis mendax* Curran (Liburd et al. 2003, Barry and Polavarapu 2005, Barry et al. 2005), bifenthrin, malathion, and spinetoram against *Drosophila suzukii* (Bruck et al. 2011) showed equally effectiveness under laboratory and field conditions. However, other studies have

documented differences between the insecticides efficacy under laboratory and field conditions. For instance, clothianidin, thiacloprid, and azinphos-methyl against *Cydia pomonella* (Linnaeus) (Brunner et al. 2005, Mota-Sanchez et al. 2008), imidacloprid and spinosad against *Rhagoletis pomonella* (Walsh) (Yee and Alston 2006) showed lower effectiveness under field conditions compared to the laboratory conditions. By contrast, thiacloprid against *Rhagoletis pomonella* (Walsh) showed higher efficacy under field conditions compared to the laboratory conditions (Reissig 2003).

Based on the above, current study aimed to extend the knowledge of the resistance in *C. rosaceana* field populations, from apple and cherry orchards, by studying different aspects of this phenomenon as the following:

(Chapter 2) Baseline study

This study aimed to determine the resistance levels in Michigan *C. rosaceana* field populations from apple and cherry orchards against commonly used insecticides.

(Chapter 3) Resistance mechanism study

This study aimed to (i) identify the mechanism resistance in *C. rosaceana* field populations against indoxacarb (ii) determine the rate and direction of indoxacarb metabolism and the abundance of parent compound and four metabolites in the *C. rosaceana* tested populations at different incubation times and in the presence and absence of esterase inhibitor S,S,S-tributyl phosphorotrithioate (DEF).

(Chapter 4) Insecticide field-performance study

This study aimed to:

1) Identify the performance of different insecticides against *C. rosaceana* field populations in apple and cherry orchards using a field-based residual bioassay and compare it with their performance in laboratory-based bioassays.

2) Assess the toxicity longevity of the different insecticides in apple and cherry orchards using the field-based residual bioassays and residues analysis.

3) Determine the correlation between the toxicity and the residues of each insecticide at different post-application intervals.

CHAPTER 2: OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE) RESISTANCE TO INSECTICIDES IN MICHIGAN APPLE AND CHERRY ORCHARDS

Abstract

Field populations of *C. rosaceana*, were collected from one commercial apple and one commercial cherry orchard in Kent and Newaygo Counties in western Michigan, respectively. A *C. rosaceana* laboratory susceptible population was used as a reference for these field populations. A baseline toxicity study, using a diet overlay bioassay, of eight insecticides including phosmet, bifenthrin, methomyl, indoxacarb, chlorantraniliprole, spinetoram, emamectin benzoate, and novaluron, was conducted on 12-24h old larvae of the three *C. rosaceana* populations. The resistance levels were low (<10-fold in all cases except in indoxacarb for both populations). The commercial cherry population showed various levels of resistance to bifenthrin, spinetoram, emamectin benzoate, and indoxacarb with 4.9, 4.1, 5.8, and 21-fold resistance respectively. The commercial cherry population was not resistant to phosmet, methomyl, chlorantraniliprole, and novaluron. Generally, the apple population showed resistance to more compounds than the cherry population. The commercial apple population showed various levels of resistance to phosmet, bifenthrin, spinetoram, emamectin benzoate, chlorantraniliprole, and indoxacard with 5, 5, 4.3, 6.3, 4.7, and 620.4-fold resistance respectively. The commercial apple population was not resistant to methomyl and novaluron., This difference in resistance levels between the apple and cherry populations is worthwhile and requires further study to identify the source of this difference. The levels of resistance against these insecticides should be monitored periodically for further increases in resistance levels. A

state-wide survey of more commercial orchards would help determine the extent of insecticide resistance across Michigan's five tree fruit production regions.

Key words: *C.rosaceana*, insecticide, resistance, pest management, cherry, apple.

Introduction

The native North American pest, Obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae), is widely distributed and it has a broad range of hosts, over 50 hosts, but the Rosaceae family members are more preferable (Sanderson and Jackson 1909, Larocque et al. 1999). The polyphagous larva is the injurious stage of *C. rosaceana*, feeding on flower buds, leaves, and developing fruit (Sanderson and Jackson 1909, Reissig 1978). In cherry, the foliage and fruit injury caused by *C. rosaceana* is less serious compared to apple. However, the principal impact of *C. rosaceana* in cherries is as a contaminant at harvest, thus causing high risk for load rejection due to the U.S. Department of Agriculture zero-tolerance mandate (USDA 1941, Mason and Huber 2002, Wise and Whalon 2009).

Historically, *C. rosaceana* was considered a minor pest in fruit orchards but it was recognized as a serious pest causing significant damage in fruit orchards when outbreaks were noted in the late 1970's (Sial and Brunner 2010a, Sial and Brunner 2012b). The occurance of many cases of insecticide resistance in *C. rosaceana* populations against conventional insecticides has made this pest even more problematic (Ahmad et al. 2002, Smirle et al. 2002, Smirle et al. 2003, Sial and Brunner 2012a). In fact, the conventional insecticides, especially the organophosphates, were the backbone of control programs at the time when the resistance phenomenon among *C. rosaceana* populations against these compounds was leading to field

failures in commercial apple control programs (Bostanian et al. 1985, Waldstein and Reissig 2000, Ahmad et al. 2002, Sial and Brunner 2012b).

Concurrently with the aggravation of resistance problems, the US Congress in 1996 passed the Food Quality Protection Act (FQPA) which restricted or prevented the use of many conventional insecticides. As a consequence of resistance problems and FQPA, fruit growers were forced to replace many of the conventional insecticides with new reduced-risk insecticides that have novel modes of action (Smirle et al. 2003, Sial and Brunner 2010b, Sial and Brunner 2010c, Sial et al. 2010, Sial and Brunner 2012a).

Most of these new insecticides showed high efficiency against *C. rosaceana* populations (Smirle et al. 2003, Sial and Brunner 2010b, Sial and Brunner 2010c, Sial et al. 2010, Sial and Brunner 2012a, Sial and Brunner 2012b). However, some cases of resistance in *C. rosaceana* populations were recorded against some of the new insecticides even when *C. rosaceana* field populations had not been previously exposed to the new compounds (Ahmad et al. 2002, Smirle et al. 2002, Sial et al. 2010).

The escalating concern about resistance development in *C. rosaceana* field populations has reinforced the need for continued resistance monitoring and identification of effective tools for integrated pest management (IPM) programs (Waldstein and Reissig 2001, Wise et al. 2006, Wise et al. 2007, Mota-Sanchez et al. 2008, Hoffmann et al. 2009, Wise and Whalon. 2009, Sial and Brunner. 2010b, VanWoerkom et al. 2014). Therefore, the current study aimed to determine the resistance levels in Michigan *C. rosaceana* field populations against commonly used insecticides for control of this pest.

Materials and Methods

Insects

In the summer of 2013, two *C. rosaceana* field populations (K.A. and F.C.) were collected from one commercial apple and one commercial cherry orchard in Kent and Newaygo Counties in western Michigan, respectively. A *C. rosaceana* laboratory susceptible strain (S.S.) originally obtained from an isolated abandoned apple orchard in Kalamazoo County, with no history of commercial insecticide use, was used as a reference to these field populations. The colony from the susceptible strain was established in 2000 by Ahmad et al. 2002 and has been continuously reared at the Michigan State University's Trevor Nichols Research Center (TNRC) in Fennville, MI since that time. The three *C. rosaceana* populations were maintained under constant conditions (25(±1) °C and 16:8h light:dark photoperiod). For each population, freely mating adults were held in cylindrical plastic oviposition chambers (9 × 33 cm) with waxed paper on the inside surface for oviposition and a 5% aqueous sucrose solution provided as a food and water source for emerging adults. Egg masses on wax paper were placed in 120 ml (4 fl oz.) cups containing 10 ml of an artificial diet described by Ahmad et al. 2002. Hatching larvae were moved from the egg masses into the diet to feed until the pupal stage, and then pupae were collected and transferred into the plastic cylinder oviposition chamber where the adults, emerged, mated, and continued the rearing cycle.

Insecticides

The insecticides evaluated in the baseline toxicity study were eight common compounds in apple and cherry control programs, each belonging to a different chemical class: phosmet 700g kg^{-1} WP (70% Imidan®; Gowan), bifenthrin 10DF (10% Bifenture™; UPI Inc., USA),

methomyl 900g kg^{-1} SP (29% Lannate®; DuPont), indoxacarb 300g kg^{-1} WG (30% Avaunt®; Dupont), chlorantraniliprole (Rynaxypyr™/ 35% Altacor® 35WG; E.I. du Pont de Nemours & Co., Wilmington, DE), spinetoram (25% Delegate® 25WG; Dow AgroSciences, Indianapolis, IN), emamectin benzoate (5% Proclaim® 5SC; Syngenta Crop Protec- tion Inc., Greensboro, NC), novaluron (9.3% Rimon® 0.83EC; Chemtura Corporation, Middlebury, CT).

Bioassay

Baseline toxicity studies for each compound and each of the *C. rosaceana* strains were conducted when a sufficient number (≥ 200) of 12-24h old *C. rosaceana* larvae were available to complete an assay. For each insecticide, we prepared a range of six to 13 concentrations of each insecticide in deionized water. Deionized water was used as a control. One hundred microliters of one concentration of an insecticide was applied onto the surface of 3 ml of diet in 30 ml (1 fl oz) clear plastic soufflés cups (Dart Container Corporation. 500 Hogsback Road Mason, MI 48854 USA). Cups were rotated to insure the solution covered the entire diet surface. Treated cups were allowed to dry for 35 to 45 min, and then five 12-24h old *C. rosaceana* larvae were placed in each cup. Five to ten cups were used for each concentration of each compound. For the compounds phosmet, bifenthrin, methomyl, indoxacarb, and spinetoram, mortality was recorded 120h after the larvae were placed on the treated diet while the mortality for chlorantraniliprole and novaluron were recorded after 168h. Larvae that failed to move when prodded with soft camel's hair brush were recorded as dead.

Statistical analysis

In order to determine the LC_{50} and LC_{90} values, fiducial limits 95%, and slope values +SE, the mortality data for each insecticide and each strain was analyzed by Probit Analysis using SAS 9.4 (SAS Institute 2013). Prior to the Probit analysis mortality was adjusted for the mortality in the control using formula (Abbott 1925).

The LC_{50} and LC_{90} values of the two *C. rosaceana* field strains were compared to the LC_{50} and LC_{90} values of the laboratory susceptible strain to assign the resistance ratio (RR) for each strain. The field strain was considered significantly different from the susceptible strain if the 95% fiducial limits do not overlap.

Results

The cherry (F.C.) and apple (K.A.) *C. rosaceana* field strains showed varied levels of resistance, ranging from none to 620-fold, against the eight compounds. Out of the eight tested insecticides, the field strains were statistically resistant to four (cherry strain) and six (apple strain) compounds. In general, there were more cases of insecticide resistance for the apple than the cherry population. In addition, the new insecticides were more toxic than the conventional insecticides against the tested strains.

Phosmet

Based on the fiducial limits 95% values with the susceptible strain, the cherry strain was not significant different. While, the apple strain was significant different based on the fiducial limits 95% values with the susceptible strain with level of resistance of 5-fold to phosmet (Table 1).

Bifenthrin

Based on the fiducial limits 95% values with the susceptible strain, both cherry and apple strains were significant different with levels of resistance of 4.9 and 5-fold respectively to bifenthrin (Table 1).

Methomyl

Based on the fiducial limits 95% values with the susceptible strain, the cherry and apple strains were not significant different (Table 1).

Indoxacarb

The cherry strain at LC_{50} showed moderate resistance to indoxacarb with 21-fold when treated with indoxacarb. Whereas, the apple strain was highly resistant to indoxacarb at LC_{50} with 620.4-fold (Table 1).

Spinetoram

Based on the fiducial limits 95% values with the susceptible strain, both cherry and apple strains were significant different with levels of resistance of 4.1 and 4.3-fold respectively to spinetoram (Table 1).

Emamectin benzoate

Based on the fiducial limits 95% values with the susceptible strain, both cherry and apple strains were significant different with levels of resistance of 5.8 and 6.3-fold respectively to emamectin benzoate (Table 1).

Chlorantraniliprole

Based on the fiducial limits 95% values with the susceptible strain, the cherry strain was not significant different. While, the apple strain was significant different based on the fiducial limits 95% values with the susceptible strain with level of resistance of 4.7-fold to chlorantraniliprole (Table 1).

Novaluron

At the LC_{50} level none of the two cherry and apple field strains found to be significantly resistant to novaluron when compared to the susceptible strain (Table 1).

Table 1 Baseline toxicity of eight insecticides on *C. rosaceana* 12/24h old larvae of two field strains Apple (K.A.) and Cherry (F.C.) compared to susceptible strain (S.S.)

Treatment[a]	Population	n[b]	$LC_{50\ ppm}$[ce]	95% FL[de]	RR_{50}[f]	$LC_{90\ ppm}$[ce]	95% FL[de]	RR_{90}[f]	Slope ±SE	X^2
Phosmet	S.S.	256	59.8	(22.1, 124.2)	1.0	317.2	(151.2, 1060)	1.0	1.8 (± 0.4)	95
	F.C.	260	152.2	(86.5, 467.2)	2.5	569.8	(254.9, 14817)	1.8	2.2 (± 0.5)	9
	K.A.	405	301.4	(231.8, 382.3)	5.0	1,809	(1325, 2724)	5.7	1.7 (± 0.2)	11
Bifenthrin	S.S.	275	0.2	(0.2, 0.4)	1.0	0.7	(0.4, 3.0)	1.0	2.8 (± 0.6)	16
	F.C.	267	1.2	(0.9, 1.8)	4.9	5.4	(3.2, 14.3)	7.6	2.0 (± 0.3)	8
	K.A.	341	1.2	(0.9, 1.7)	5.0	9.2	(6.1,17.6)	12.9	1.5 (± 0.2)	9
Methomyl	S.S.	493	233.9	(122.8, 470.1)	1.0	5,054	(1591, 85063)	1.0	1.0 (± 0.2)	33
	F.C.	303	28.0	(1.7, 156.9)	0.1	222.9	(58.5, 500885)	0.0	1.4 (± 0.4)	9
	K.A.	320	92.3	(46.3, 170.4)	0.4	721.4	(351.4, 2600)	0.1	1.4 (± 0.2)	21
Indoxacarb	S.S.	288	0.9	(0.6, 1.1)	1.0	1.9	(1.7, 2.2)	1.0	1.3 (± 0.2)	4
	F.C.	263	18.6	(8.6, 36.9)	21.0	1,197	(443.8, 5749)	629.9	0.7 (± 0.1)	2
	K.A.	339	548.1	(280.9, 1053)	620.4	96,914	(21414, 2518821)	50998	0.6 (± 0.1)	2
Spinetoram	S.S.	256	0.2	(0.07, 0.4)	1.0	1.7	(0.7, 33.4)	1.0	1.3 (± 0.3)	13
	F.C.	294	0.7	(0.5, 1.0)	4.1	6.4	(3.9, 13.1)	3.7	1.4 (± 0.2)	5
	K.A.	361	0.8	(0.6, 1.0)	4.3	5.3	(3.5, 9.5)	3.1	1.5 (± 0.2)	7
Emamectin	S.S.	327	0.2	(0.2, 0.3)	1.0	0.9	(0.7, 1.4)	1.0	2.3 (± 0.3)	5
benzoate	F.C.	364	1.4	(1.2, 1.6)	5.8	3.8	(3.0, 5.4)	4.1	3.1 (± 0.4)	5
	K.A.	276	1.6	(1.4, 1.8)	6.3	3.9	(3.1, 5.6)	4.3	3.2 (± 0.4)	5
Chlorantraniliprole	S.S.	417	0.9	(0.3, 1.9)	1.0	11.0	(4.3, 90)	1.0	1.2 (± 0.2)	27
	F.C.	262	0.9	(0.5, 1.6)	1.1	16.7	(8.8, 40.9)	1.5	1.0 (± 0.1)	8
	K.A.	419	4.0	(2.6, 6.1)	4.7	65.8	(35.2, 164.2)	6.0	1.1 (± 0.1)	3
Novaluron	S.S.	337	31.8	(5.2, 88.5)	1.0	2,139	(624, 33620)	1.0	0.7 (± 0.1)	10
	F.C.	287	168.7	(85, 324.5)	5.3	12,330	(4462, 60743)	5.8	0.7 (± 0.1)	3
	K.A.	394	75.5	(38.5, 143.9)	2.4	18,093	(6113, 88113)	8.5	0.5 (± 0.1)	7

[a] Mortality was recorded after 120h of exposure to insecticides (except for chlorantraniliprole and novaluron after 168h)

[b] n = number of tested larvae.

[c] LC= Lethal concentration.

[d] 95% FL: 95% fiducial limits estimated using SAS9.4 software (SAS Institute 2013).

[e] Field strains were considered significantly more resistance than the susceptible strain if their 95% FL did not overlap.

[f] Resistance ratio (RR)= LC value of Field strain/ LC value of susceptible strain.

Discussion

Variable levels of resistance were observed in the two *C. rosaceana* field populations against the eight tested insecticides. Generally when compared the LC_{50} and LC_{90} values of both field populations, the commercial apple population was found to express low levels of resistance to more compounds than the commercial cherry population, except to indoxacarb.

Conventional insecticides

Organophosphates

Taking into account the historical long-term use of organophosphates in apple orchards, high levels of resistance to organophosphates in *C. rosaceana* field populations were highly expected (Mota-Sanchez et al. 2008). Indeed high levels of resistance to organophosphates compounds have been recorded in *C. rosaceana* populations throughout United States and Canada (Sial and Brunner 2012b, Smirle et al. 2002, Smirle et al. 2003). However, this study recorded a low resistance to phosmet in the apple field population and no resistance in the cherry population compared to the susceptible population. These results of Michigan field populations were consistent with the results of a previous study (Ahmad et al. 2002) where Michigan *C. rosaceana* populations had moderate resistance against azinphos-methyl and chlorpyrifos, and low resistance against phosmet. Maintaining a low level of resistance in *C. rosaceana* field populations against an old chemical compound like phosmet over the last decade may be evidense of the effectiveness of the IPM programs in Michigan apple and cherry orchards. Even so, phosmet should be avoided for *C. rosaceana* control programs in Michigan apple and cherry orchards because of a likelihood of rapid build-up of resistance if selection pressure is resumed.

Carbamates

Similarly to the organophosphates, high levels of resistance against carbamates were expected in *C. rosaceana* field populations since both classes have the same mode of action as acetylcholinesterase (AChE) inhibitors (IRAC 2017). In other words, applying each one of those two classes will not only develop resistance in the field populations against the same class but also will develop resistance against the other class and this phenomenon is known as cross-resistance. The cross-resistance between these two classes was documented in apple orchards in Ontario, Canada (Pree et al. 2001) and Michigan (Ahmad et al. 2002) where *C. rosaceana* organophosphate-resistant field populations were found to be highly resistant to the carbamates methomyl and carbaryl. However, our study has recorded no resistance to the carbamate insecticide methomyl in the *C. rosaceana* field populations compared to the susceptible laboratory population. This surprising result may be a consequence of growers excluding carbamate insecticides from the IPM programs in Michigan fruit orchards and the reduction of organophosphates applications per season in the same control programs over the past decade (Wise et al. 2015). None-the-less carbamate insecticides are not recommended for control of *C. rosaceana* field populations.

Pyrethroids

The *C. rosaceana* field populations in this study had low resistance to bifenthrin which is consistent with previous work in Michigan (Ahmad et al. 2002) where a low resistance level was recorded in the *C. rosaceana* field populations against the pyrethroid insecticides cypermethrin, zeta-cypermethrin, bifenthrin, deltamethrin and esfenvalerate. A lack of reduction in the

effectiveness of pyrethroids likely reflects the effectiveness of IPM programs in Michigan apple and cherry orchards to limit resistance development over the last decade. However, periodical monitoring is required to detect further increase in the resistance level against pyrethroid insecticides, and these are not recommended as first choice for *C. rosaceana* control.

Reduced-risk insecticides

Cherry and apple field populations showed similar levels of resistance to the reduced-risk insecticides, except chlorantraniliprole. Both populations had very low resistance to spinetoram and emamectin benzoate, and no resistance to novaluron. In the chlorantraniliprole treatment, the apple field population showed very low resistance while the cherry field population was not resistant. The very low levels or the absence of resistance against the reduced-risk insecticides were expected since they are relatively new compounds, with emamectin benzoate, novaluron, spinetoram, and chlorantraniliprole were first registered in the US in 1999, 2001, 2007, and 2008 respectively (USEPA 2017). However, the results of the current study showed a slight shift in the resistance levels against some of reduced-risk insecticides compared to previous studies. *C. rosaceana* field populations from apple orchards in Washington and Michigan were not found to be resistant to spinetoram, emamectin benzoate, novaluron, and chlorantraniliprole (Ahmad et al. 2002, Sial et al. 2010, Sial and Brunner. 2012b), except one field population from Washington State which was very low resistant to chlorantraniliprole. Although the resistance levels in the current study are still considered negligible, this shift towards resistance for these new insecticides should be a warning to monitor control programs carefully; especially when these compounds are applied repeatedly, to prevent loosing effective chemical tools due to resistance development.

Indoxacarb

Indoxacarb was first registered in the US in 2000 (USEPA 2017). In this study, the *C. rosaceana* apple and cherry field populations were highly and moderately resistant to indoxacarb, respectively. Similar results were reported 15 years ago in a *C. rosaceana* field population from a Michigan apple orchard with no history of indoxacarb exposure (Ahmad et al. 2002). Similarly, the *C. rosaceana* field populations, from apple orchards in the Okanagan and Similkameen Valleys in British Columbia, Canada, had high levels of resistance to indoxacarb (Smirle et al. 2002). These observations were from populations that had no history of indoxacarb exposure, which supports the notion of cross-resistance from other chemical classes. Indoxacarb is not currently labeled for *C. rosaceana* control, but is applied for control of other pests at timings when *C. rosaceana* larvae are present. Thus, further study to identify the mechanisms that play a role in indoxacarb resistance is warranted.

Resistance management future insights

Studying the resistance status in two different populations of same species from two different crops will provide valuable information in terms of the sources of variation in resistance levels and as an evaluation of the Michigan IPM programs in both crops. However, the findings are limited by the spatial frame of collection sites of these populations, and thus may not reflect the patterns of resistance across the entire state. Therefore, a state-wide survey of more commercial orchards would help determine the extent of insecticide resistance across Michigan's five tree fruit production regions, and result in having a database of determined resistance cases by time and location. In addition to this, a database of the control programs of each location

should be established including the types of applied chemical materials, types of applications, time of application, number of applications, and the related circumstances e.g. weather conditions. Studying the correlation between these two databases would help specialists in resistance management to create prediction models of what type of resistance, when, and where it is predicted to occur.

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CHAPTER 3: IDENTIFYING THE MECHANISM OF INDOXACARB DETOXIFICATION IN POPULATIONS OF *CHORISTONEURA ROSACEANA* (HARRIS) (LEPIDOPTERA: TORTRICIDAE)

Abstract

Synergism and metabolic studies were conducted to identify the detoxification mechanism against indoxacarb in two *Choristoneura rosaceana* (Harris) field populations compared to a laboratory susceptible population. The synergism study was carried out using diet incorporation bioassay for indoxacarb and the three synergists PBO, DEM, and DEF. While for each *C. rosaceana* population and at different incubation times, the metabolic study consists of indoxacarb in vitro reaction with fifth instar larvae 12,000g midgut supernatant or with pre-inhibited (in vivo by the esterases inhibitor DEF) fifth instar larvae 12,000g midgut supernatant. In both susceptible laboratory and cherry field populations, only DEF significantly synergized indoxacarb with a synergism ratio (SR) of 6.5 and 22.6-folds respectively indicating an involvement of esterase enzymes in the tolerance and resistance of both populations. In the apple population all synergists PBO, DEM, and DEF significantly synergized indoxacarb with SR of 9.6, 7.7, and 285.6-folds respectively indicating a complex resistance case with the possible involvement of all three metabolic resistance mechanisms with consideration of the central role of esterase enzymes based on the highest synergism of DEF. In the in vitro metabolic study, indoxacarb (DPX-JW062) was very rapidly bio-transformed within 5 min into small molecules in the lower portion of the metabolic pathway when it reacted with the 12,000g midgut supernatant of each population. None of the metabolites in the upper portion of the metabolic pathway were detected at any incubation time including the potent sodium channel blocker DCJW metabolite. The two field populations showed significantly higher rates of DPX-JW062 bio-transformation compared to the laboratory population at five min of incubation and that may

explain the presence of indoxacarb resistance in the field populations. The DPX-JW062 bio-transformation of each field population was not significantly different between the incubation times; indicating enzyme function completion within 5 min. Shorter incubation times would possibly reveal greater differentiation between the metabolic capabilities of the three strains. In the second part of the in vitro study, the bio-transformation of DPX-JW062 remarkably was decreased when it reacted with the pre-inhibited (by DEF) 12,000g midgut supernatant of each population. Additionally, the degradation of metabolites in the upper portion of the metabolic pathway remarkably decreased, which resulted in accumulation of DCJW and MP819 metabolites. The accumulation of DCJW metabolite under the pre-inhibited midgut supernatants treatment provided a persuasive explanation of the synergistic impact of esterase inhibitor DEF on indoxacarb toxicity in *C. rosaceana*.

Keywords: metabolism, resistance mechanisms, cytochrome P450, esterase, glutathione S-transferases, indoxacarb.

Introduction

The native North American pest obliquebanded leafroller, *Choristoneura rosaceana* (Harris), is widely distributed and has over 50 hosts including many members of the Rosaceae and Cornaceae families (Sanderson and Jackson 1909, Reissig 1978). Despite the wide distribution and host range, *C. rosaceana* was long considered to be a minor pest in fruit orchards until the late 1970s where it became major pest after the occurrence of outbreaks in the field after nearly two decades of intensified insecticide use targeting the other major pests in fruit orchards (Sial and Brunner 2010a).

In *C. rosaceana* control programs throughout the North American fruit-producing regions, most newly labeled insecticides showed high efficiency against *C. rosaceana* field populations (Smirle et al. 2003, Sial and Brunner 2010b, Sial and Brunner 2010c, Sial et al. 2010, Sial and Brunner 2012a, Sial and Brunner 2012b). However, cases of resistance have been documented in *C. rosaceana* populations against some of these new insecticides (Ahmad et al. 2002, Smirle et al. 2002, Sial et al. 2010).

Indoxacarb, an oxadiazine insecticide, is a new insecticide discovered by the E.I. DuPont Co. in 1992 and introduced to the market in 2000 (McCann et al. 2001, IRAC 2017, USEPA 2017). Indoxacarb is a pro-insecticide, undergoes hydrolase based bio-activation, due to the action of carboxylesterases, to yield the potent sodium channel blocker decarbomethoxyllated (DCJW) metabolite (Wing et al. 1998). Ingestion is the major route of entry where indoxacarb is highly active, with limited documented cases of contact activity, when applied topically (Alves et al. 2008, Nehare et al. 2010).

Outstanding activity of indoxacarb has been documented against several lepidopteran species, including members of the Tortricidae family, even though some of these field populations were resistant to other insecticide classes such as pyrethroids, organophosphates, carbamates, and insect growth regulators (Wing et al. 2000). However, *C. rosaceana* appears to be an anomaly case in this context where different *C. rosaceana* field populations showed high resistance levels against indoxacarb even before some field populations had been exposed through commercial use (Ahmad et al. 2002, Smirle et al. 2002, Dunley et al. 2006).

This variation in susceptibility to indoxacarb in different species is thought to be correlated to the variation in their metabolic rate (Wing et al. 2000). Metabolism of xenobiotic compounds, including insecticides, in arthropods is mainly modulated by cytochrome P450s,

hydrolases, and glutathione-S-transferases enzymes resulting in either detoxification or activation of those xenobiotic compounds. In the case of insecticide metabolism both will determine the abundance of the active form of each insecticide in the insect body and the resistance level against the insecticide (Fu-Gen et al. 2014). For many targeted insects indoxacarb is activated at a high rate but this is not the case in some other targeted or non-targeted species. In these less frequent cases indoxacarb breaks-down to metabolites other than DCJW or they have high rates of DCJW breaking down to non-toxic metabolites in a way that overcomes the timeframe of the toxic effect of DCJW (Wing et al. 1998, Alves et al. 2008).

The rate of indoxacarb bio-activation or detoxification could be a critical factor accounting for the variation in its effectiveness for different lepidopteran species as well as the resistance phenomenon in general (Wing et al. 2000). Previous studies describe the role of carboxylesterases in the bio-activation of indoxacarb to yield the potent sodium channel blocker DCJW as well as the rate of this biotransformation. However, Gondhalekar et al. (2016) were the first to investigate the indoxacarb metabolic pathway beyond this point in insects where both indoxacarb and DCJW were metabolized to more polar derivatives in the German cockroach. Thus, this study aimed to (i) identify the resistance mechanism in *C. rosaceana* field populations against indoxacarb (ii) determine the rate and direction of indoxacarb metabolism and the abundance of parent compound and four metabolites in the *C. rosaceana* tested populations at different incubation times and in the presence and absence of the esterase inhibitor S,S,S-tributyl phosphorotrithioate (DEF).

Materials and Methods

Insects

Two *C. rosaceana* field populations, which were collected from Michigan commercial orchards, one originating from an apple orchard (K.A.) and one from a cherry orchard (F.C.), and a laboratory susceptible strain (S.S.), were tested in this study. All three *C. rosaceana* populations were maintained and reared under constant conditions (25(±1) °C and 16:8h light:dark photoperiod). All three *C. rosaceana* populations were not exposed to any kind of chemicals since they were collected.

Chemicals

Insecticides

Indoxacarb 300g kg-1 WG (30% Avaunt®; Dupont) and indoxacarb technical active ingredient (DPX-JW062; R/S isomer-ratio 50/50) were obtained from Dupont Company, Delaware, United States.

Parent compound and its metabolites

Analytical reference standards of indoxacarb, (DPX-JW062), IN-JT333 (N-decarbomethoxyllated (DCJW)), IN-U8E24 (carboxylic acid of JT333), IN-KT413 (carboxylic acid of JW062), and IN-MP819 (the rearrangement product of IN-KT413) were obtained from Dupont Division of Agricultural Technology, Delaware, United States.

Synergists

Three common synergists were tested in this study: esterases inhibitor 98.1% S.S S-tributyl phosphorotrithioate "DEF" (Sigma-Aldrich 3050 Spruce St. Saint Louis, MO 63103 USA), glutathione S-transferase inhibitor 97% diethyl maleate "DEM" (Sigma-Aldrich 3050 Spruce St. Saint Louis, MO 63103 USA), and mixed-function oxidases inhibitor 91.3% piperonyl butoxide "PBO" (SynerPro™ Control Solutions Inc. 5903 Genoa- Red Bluff Pasadena, TX 77507).

Synergism study

Diet incorporation bioassay

Following the methods of Sial et al. (2010), with some modifications, I conducted diet incorporation bioassay with four treatments: indoxacarb, indoxacarb plus piperonyl butoxide (PBO) synergist, indoxacarb plus S, S, S-tributyl phosphorotrithioate (DEF) synergist, and indoxacarb plus diethyl maleate (DEM) synergist for each population. The indoxacarb plus synergists treatments were a combination of the tested concentration of indoxacarb plus the maximum non-lethal concentration of each synergist as determined in bioassays (described below). Where, the maximum non-lethal concentration of each synergist was used the same for each insecticide concentration. Stock solutions were prepared for each treatment by diluting the tested chemicals in deionized water except the DEF synergist which was diluted first in acetone then in deionized water with a final combination of 5% acetone and 95% deionized water. Serial concentrations (8 to 11) of each treatment were prepared by mixing the tested chemicals with pinto bean artificial diet, described by Ahmad et al. (2002), (weight/weight), (tested

compound/diet), parts per million (ppm). Two controls were used for the indoxacarb plus synergists treatments. In control-1 the diet was mixed with the solvent (all treatments: deionized water except the treatment of DEF: combination of 5% acetone and 95% deionized water), and in control-2 the diet was mixed with the maximum non-lethal concentration of each synergist. Only one control was used for the treatment of indoxacarb alone by mixing the diet with deionized water. After mixing the diet for each treatment and each concentration, 3 ml of the diet was placed in 30 ml (1 fl oz) clear plastic soufflés cups (Dart Container Corporation. 500 Hogsback Road Mason, MI 48854 USA). The cups were allowed to cool down for 35 to 45 min, and five 12-24h old *C. rosaceana* larvae were placed in each cup. Six to eight replications (cups) were used for each concentration per treatment per population. The larval mortality was recorded 120 h after the larvae were placed on the diet. Larvae that failed to move when prodded with soft camel's hair brush were record as dead.

Maximum non-lethal concentration

The maximum non-lethal concentration for each synergist on 12-24h old *C. rosaceana* larvae was determined using the diet incorporation bioassay to identify the highest concentration of each synergist that did not cause any mortality in the tested 12-24 h old *C. rosaceana* larvae. The maximum non-lethal concentrations for DEF, DEM, and PBO synergists were 30ppm, 30ppm, and 100ppm respectively.

Metabolic study

Enzyme extraction

For each population and following the method of Fu-Gen et al. (2014), with some modifications, midguts were obtained from 250 dissected *C. rosaceana* fifth instar larvae in iced-cold sodium phosphate buffer (pH=7.6) in waxed petri dishes. The contents of obtained midguts was removed gently then the midguts were transferred into 2 ml microcentrifuge tube containing iced-cold sodium phosphate buffer (pH=7.6) with a ratio of 1gut/ 10µl buffer. The contents of each microcentrifuge tube were homogenized manually (PELLET PSTLE W TB 2 ML plastic homogenizer, Fisher Scientific Company) then vortexed briefly under iced-cold conditions (IKA® Vortex 3). Midgut homogenates were centrifuged at 12,000g for 10 min at 4 ºC (Eppendorf™ 5424 R Microcentrifuge). Supernatant from each centrifuged midgut homogenate was collected into new 2 ml microcentrifuge tubes and stored at -20 ºC as an enzyme source.

Pre-inhibited enzyme extraction

For each population and 6 h prior to dissection, *C. rosaceana* fifth instar larvae were transferred into treated diet cups, where the diet was incorporated with the maximum non-lethal concentration of DEF (weight/weight) as described in the synergism study. After 6 h of feeding on treated diet, larvae were observed individually and any larva that did not consume sufficient amount of treated diet was excluded from the analysis. The successfully feeding larvae were dissected and midguts were obtained and processed to end up with supernatant as described above.

In vitro indoxacarb/midgut supernatant reaction

The indoxacarb/midgut supernatant for all treatments were subjected to three different incubation times: 5, 15, and 30 min. In each 2 ml microcentrifuge tube the reaction mixture consisted of 40 µl of the supernatant, 450 µl sodium phosphate buffer (pH=7.6), and 10 µl indoxacarb (stock solution= 0.005 M) with a total volume of 500 µl where the final concentration of indoxacarb was 100 µM. Two controls were used for each treatment, an enzyme solution control (without substrate) and a substrate control (without enzyme). Each treatment was replicated three times. At the end of each incubation time, the reaction was stopped by vortexing the reaction mixture with 500 µl of ice-cold acetonitrile high-performance liquid chromatography (HPLC) grade. The mixture was evaporated to dryness under a gentle current of N_2. Dried samples were re-dissolved by vortexing the dried contents with 500 µl of acetonitrile HPLC grade (two times) then collected into 2 ml HPLC glass vials (Agilent Technologies Inc., Santa Clara, CA). The 2 ml HPLC glass vials were stored at 4 C° until the samples were analyzed by HPLC.

HPLC conditions

Samples were analyzed for indoxacarb and metabolites residues with a Waters HPLC-MSD (Waters Co. Milford, Massachusetts) using mobile phase solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) were used for this study. These were initially held at 75% solvent A and 25% solvent B and followed by a gradient shown in Table 2.

Table 2 The gradient mobile phase flow used for HPLC-MS residue analysis

A.I.	Time (min)	Flow rate (μ min^{-1})	Solvent A[y] (%)	Solvent B[z] (%)
Indoxacarb (DPX-JW062)		0.30	75.0	25.0
	5.00	0.30	10.0	90.0
	7.00	0.30	10.0	90.0
	7.50	0.30	75.0	25.0
	12.00	0.30	75.0	25.0

[y] Water with 0.1% formic acid.
[z] Acetonitrile with 0.1% formic acid.

To calculate the percentage of bio-transformation from the initial mass of parent compound (DPX-JW062) for each treatment, the detected mass of each metabolite was first converted to its equivalent parent compound mass using the following equation:

$$\text{Equivalent mass of DPX} - \text{JW062} = \frac{\text{Molecular weight of DPX} - \text{JW062}}{\text{Molecular weight of metabolite}} \times \text{detected mass of metabolite}$$

Followed by:

$$\text{Percentage of bio} - \text{transformation} = \left(\frac{\text{equivalent mass of DPX} - \text{JW062}}{\text{initial mass of DPX} - \text{JW062}}\right) \text{x } 100$$

Protein assay

Protein concentration for each 12,000g midgut supernatant was determined (Table 3) using the method of Lowry et al. (1951).

Table 3 Protein concentration for each 12,000g midgut supernatant using the method of Lowry et al. (1951)

12,000g midgut supernatant	Protein concentration (mg protein/ml)
SS	4.68 ±0.52
FC	4.36 ±0.44
KA	4.54 ±0.28
DSS	4.47 ±0.39
DFC	4.78 ±0.47
DKA	4.98 ±0.67

SS: 12,000g supernatant of susceptible *C. Rosaceana* population (mean ± SE)

FC: 12,000g supernatant of cherry *C. Rosaceana* field population (mean ± SE)

KA: 12,000g supernatant of apple *C. Rosaceana* field population (mean ± SE)

DSS: in vivo pre-inhibited by DEF 12,000g supernatant of susceptible *C. Rosaceana* population (mean ± SE)

DFC: in vivo pre-inhibited by DEF 12,000g supernatant of cherry *C. Rosaceana* field population (mean ± SE)

DKA: in vivo pre-inhibited by DEF 12,000g supernatant of apple *C. Rosaceana* field population (mean ± SE)

Statistical analysis

To determine the LC_{50} and LC_{90} values for each treatment/ each population in the Synergism study, the mortality data was analyzed by Probit Analysis using SAS 9.4 (SAS Institute 2013). In each population, the LC_{50} and LC_{90} values of indoxacarb treatment were compared to the LC_{50} and LC_{90} values of the treatment of indoxacarb plus each synergist. Each synergist was considered as having significantly synergized/ antagonizes the toxicity of indoxacarb if the 95% fiducial limits of the LC_{50} and LC_{90} values of the indoxacarb treatment and the indoxacarb plus that synergist treatment did not overlap. The mortality was corrected by the mortality in the control using the formula of Abbott, 1925. Control mortality was recorded only in the treatment of inoxacarb alone against apple population with less than 5% mortality.

In the Metabolic study, the percentage mass detected (means ± SE) of indoxacarb (DPX-JW062) and its metabolites were compared at each incubation time for *C. rosaceana* population's 12,000 g midgut supernatants in the presence and absence of DEF. The percentage mass detected (means ± SE) of indoxacarb (DPX-JW062) and its metabolites were compared between incubation times for each *C. rosaceana* population's 12,000 g midgut supernatant in the presence and absence of DEF ($P < 0.05$, PROC GLM, LSMEANS) using SAS 9.4 (SAS Institute 2013).

Results

Synergism study

Based on the LC_{50} values, the mixed-function oxidase inhibitor PBO and glutathione S-transferase inhibitor DEM did not synergize nor antagonize the toxicity of indoxacarb against both susceptible (S.S.) and cherry (F.C.) populations, while the esterase inhibitor DEF significantly synergized the toxicity of indoxacarb against both populations with a low (6.5 fold) and moderate (22.6 fold) synergism ratios (SR) respectively (Table 4). This finding indicates that both mixed-function oxidase and glutathione S-transferase enzymes probably were not involved in the tolerance (SR= 6.5-fold) and the moderate resistance (SR= 22.6-fold) of indoxacarb alone treatment in the S.S. and F.C. populations, respectively (Table 4).

Conversely, at the LC_{50} values the synergists PBO, DEM, and DEF all significantly synergized the toxicity of indoxacarb against the apple (K.A.) population with a low (9.6 fold), low (7.7 fold), and high (285.6 fold) synergism ratios (SR), respectively (Table 4). The association between the three tested synergists and the synergism of indoxacarb toxicity against the K.A. population indicates an involvement of mixed-function oxidases, glutathione reactions,

and esterases enzymes in the resistance phenomenon in this population and that means a complex case of resistance against indoxacarb with the presence of more than one resistance mechanism. The esterase inhibitor DEF caused the highest level of indoxacarb toxicity synergism, indicating a major role of esterases enzymes not only in the bio-activation of indoxacarb but also in the detoxification of this compound and its toxic metabolite DCJW in the resistance populations of OBLR. In addition, the presence of the association between DEF synergist and synergism of indoxacarb toxicity in all tested OBLR populations, including the susceptible population, indicates that the esterases are an integral part of the nature of the tolerance or resistance of OBLR individuals against indoxacarb.

Table 4 Toxicity of indoxacarb in combination with synergists on *C. rosaceana* 12/24h old larvae of Apple (K.A.), Cherry (F.C.), and susceptible (S.S.) populations.

Population	Treatment[a]	n[b]	LC_{50}[c]	95% FL[de]	SR_{50}[f]	LC_{90}[c]	95% FL[de]	SR_{90}[f]	Slope ±SE	X^2
S.S.	Indoxacarb	292	9.6	(6.7, 13.1)	--	62.9	(43.6, 102.9)	--	1.6 (± 0.2)	10
	Indoxacarb + PBO	488	5.5	(3.0, 8.6)	1.7	77.6	(53.1, 126.7)	0.8	1.1 (± 0.1)	3
	Indoxacarb + DEM	498	6.4	(4.2, 9.0)	1.5	96.3	(63.3, 170.9)	0.7	1.1 (± 0.1)	6
	Indoxacarb + DEF	286	1.5	(0.6, 3.3)	6.5	24.2	(9.6, 116.7)	2.6	1.1 (± 0.2)	11
F.C.	Indoxacarb	294	22.6	(16.8, 29.1)	--	106.3	(77.6, 163.2)	--	1.9 (± 0.2)	4
	Indoxacarb + PBO	493	15	(10.6, 20.7)	1.5	246.9	(152.7, 473.2)	0.4	1.1 (± 0.1)	6
	Indoxacarb + DEM	441	9.4	(0.02, 30.9)	2.4	200.5	(51.0, 628154935)	0.5	1.0 (± 0.3)	19
	Indoxacarb + DEF	457	1.0	(0.6, 1.6)	22.6	47	(25.6, 105.3)	2.3	0.8 (± 0.1)	5
K.A.	Indoxacarb	279	88.3	(58.5, 143.2)	--	1,948	(847.6, 7647)	--	1.0 (± 0.1)	7
	Indoxacarb + PBO	533	9.2	(5.6, 13.6)	9.6	240.5	(148.7, 465.3)	8.1	0.9 (± 0.1)	3
	Indoxacarb + DEM	429	11.5	(3.5, 26.9)	7.7	297.6	(94.0, 5759)	6.5	0.9 (± 0.2)	13
	Indoxacarb + DEF	447	0.3	(0.1, 0.6)	285.6	4.5	(2.1, 17.7)	432.8	1.1 (± 0.2)	11

[a] Mortality was recorded after 120h of exposure; [b] n = number of larvae. [c] LC= Lethal concentration.

[d] 95% fiducial limits (FL) estimated using SAS9.4 software (SAS Institute 2013).

[e] Field strains were considered significantly more resistance than the susceptible strain if their 95% FL did not overlap.

[f] Synergism ratio (SR)= LC value of indoxacarb alone treatment / LC value of indoxacarb in combination with synergist treatment.

Metabolic study

In the in vitro indoxacarb bio-transformation experiment the *C. rosaceana* fifth instar larvae 12,000g midgut supernatant showed a high rate of indoxacarb bio-transformation, where after five min of incubation 68.7, 77.2, and 77.6% of the initial mass of parent compound were bio-transformed when reacted with the 12,000g midgut supernatant of the susceptible (S.S.), cherry (F.C.), and apple (K.A.) populations, respectively (Figure 1A). The two field populations, F.C. and K.A., showed a significantly higher rate of indoxacarb bio-transformation compared to the laboratory population S.S. at the five min of incubation (Figure 1A) and that may explain the higher indoxacarb LC_{50} values for these field populations compared to the laboratory population (Table 4). It is likely that there would be higher differences in the rate of indoxacarb bio-transformation between the field populations and laboratory population at incubation time less than five min. The indoxacarb bio-transformation of field populations at 15 and 30 min of incubation were not significantly different from the indoxacarb bio-transformation of laboratory population, probably because by then the reaction was almost complete for all three populations. In addition, the indoxacarb bio-transformation of each field population were not significantly different through the different incubation times of 5, 15, and 30 min and that means the enzyme has the ability to bio-transform the initial mass of parent compound within 5 min. (Figure 1A).

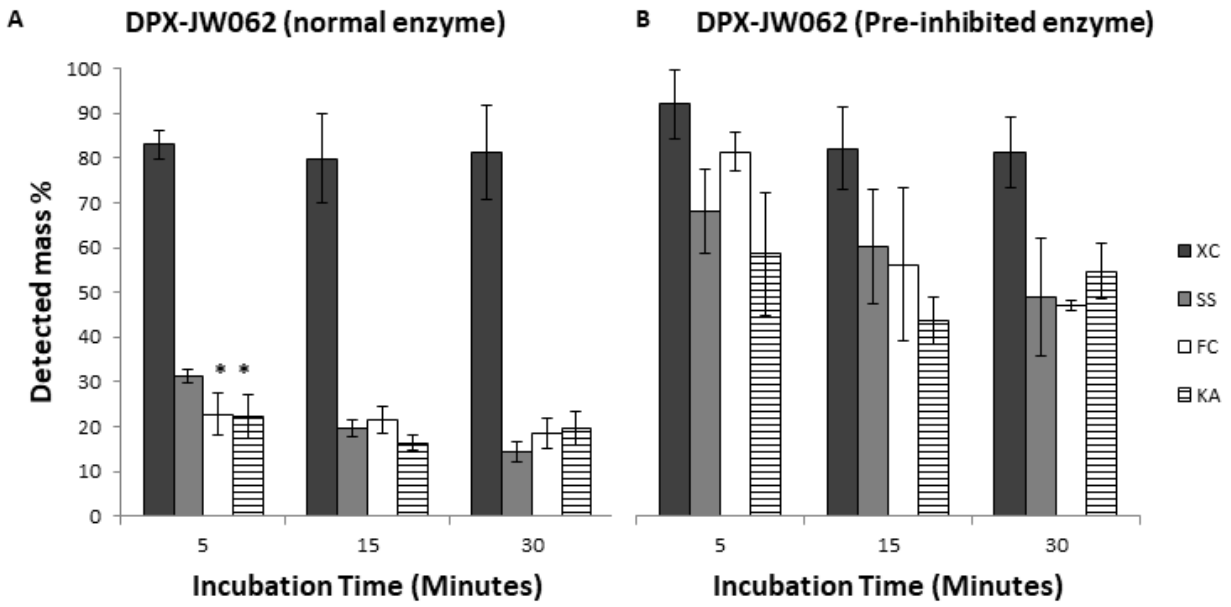

Figure 1 Detected mass percentage of indoxacarb (DPX-JW062) after different incubation times of in vitro reaction with: **A.** 12,000g midgut homogenates supernatants from three different *C. Rosaceana* (mean ± SE). **B.** in vivo pre-inhibited (by DEF) 12,000g midgut homogenates supernatants from three different *C. Rosaceana* (mean ± SE). XC: Substrate control (without enzyme). SS: 12,000g supernatant of susceptible population. FC: 12,000g supernatant of field cherry population. KA: 12,000g supernatant of field apple population. *Indicate significance difference between the detected mass percentages of field population and susceptible population treatments at each incubation time. ($P < 0.05$, PROC GLM, LSMEANS)

Even though most of the initial mass of parent compound was bio-transformed, the large metabolites of the upper portion of the indoxacarb metabolic pathway in the insect (Figure 2) were not detected at any of the different incubation times. Thus, 100% of the bio-transformed parent compound rapidly degraded into small molecules in the lower portion of the metabolic pathway (Figure 3A).

In the second part of the in vitro indoxacarb bio-transformation experiment, the *C. rosaceana* fifth instar larvae 12,000g midgut supernatants were inhibited in vivo by the esterase inhibitor DEF. The indoxacarb biotransformation rate of the pre-inhibited *C. rosaceana* fifth

instar larvae 12,000g midgut supernatant was remarkably decreased in all tested populations at all incubation times (Figure 1B) compared to the indoxacarb biotransformation rate of the normal 12,000g midgut supernatant (Figure 1A). At five min of incubation 31.8, 18.6, and 41.5%, at 15 min of incubation 39.9, 43.8, and 56.2%, and at 30 min of incubation 51, 52.9, and 45.3% of the initial mass of parent compound were bio-transformed when reacted with the pre-inhibited 12,000g midgut supernatant of susceptible (S.S.), cherry (F.C.), and apple (K.A.) populations, respectively (Figure 1B). The indoxacarb bio-transformation of the two field populations, F.C. and K.A., were not significantly different from the indoxacarb bio-transformation of the laboratory population S.S. at each one of the incubation times (Figure 1B).

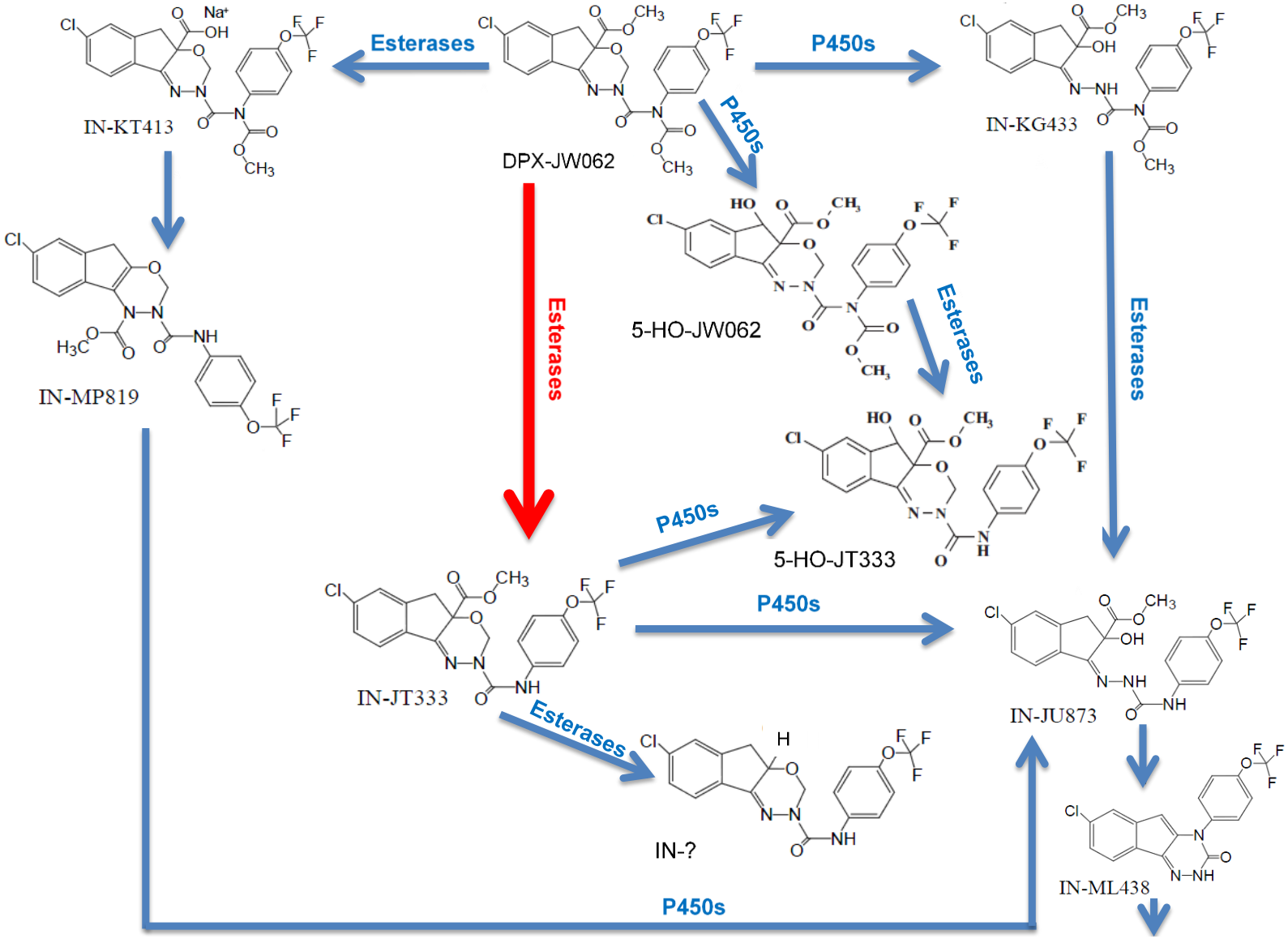

Figure 2 Possible metabolic pathway of indoxacarb in insect.

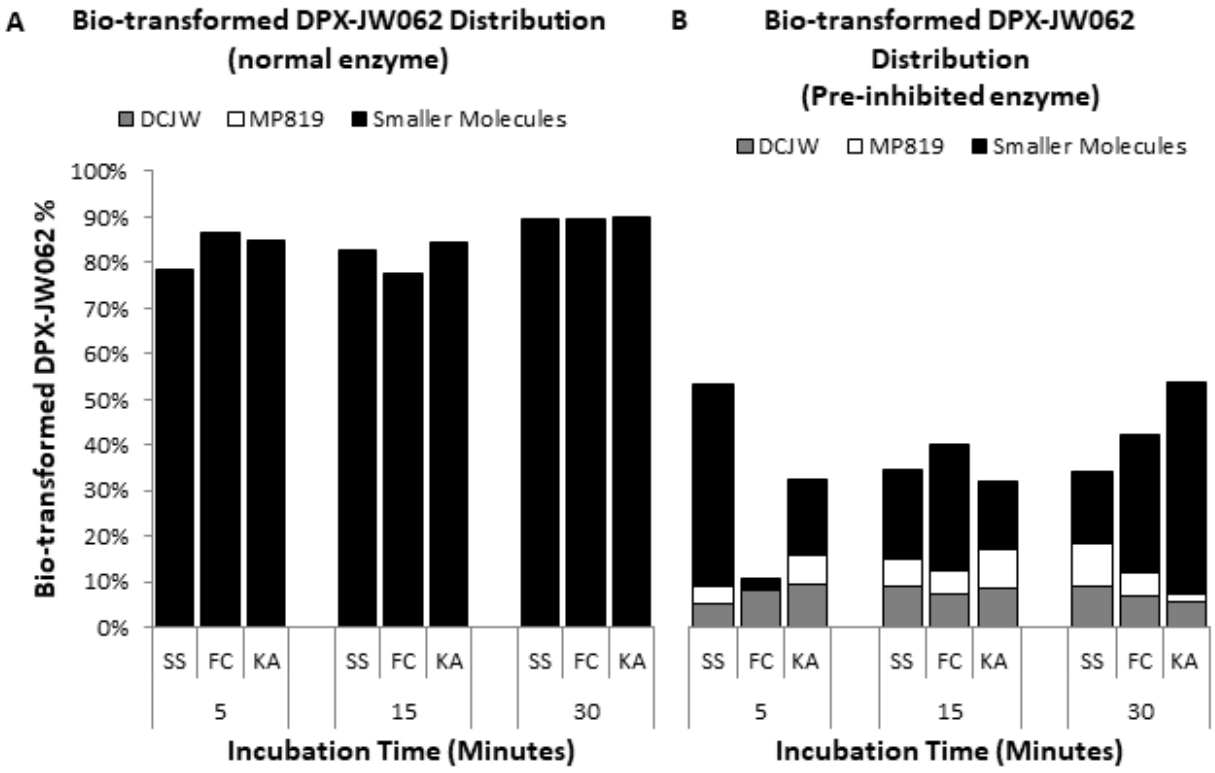

Figure 3 Bio-transformed DPX-JW062 Distribution for normal and pre-inhibited enzyme

$$\text{Bio-transformed DPX-JW062 \%} = \left(\frac{\text{DPX-JW062 initial mass-DPX-JW062 detected mass}}{\text{DPX-JW062 initial mass}}\right) \text{x } 100$$

$$\text{Each metabolite \%} = \left(\frac{\text{detected equivalent mass of DPX} - \text{JW062}}{\text{DPX} - \text{JW062 initial mass}}\right) \text{x } 100$$

Where:

$$\text{Equivalent mass of DPX-JW062} = \frac{\text{Molecular weight of DPX-JW062}}{\text{Molecular weight of metabolite}} \times \text{detected mass of metabolite}$$

SS: DPX-JW062 reaction with the 12,000g supernatant of susceptible population.

FC: DPX-JW062 reaction with the 12,000g supernatant of field cherry population.

KA: DPX-JW062 reaction with the 12,000g supernatant of field apple population.

Although the bio-transformed amount of parent compound was significantly decreased in the pre-inhibited 12,000 g midgut supernatant experiment, the two indoxacarb metabolites DCJW and MP819 were detected in significant amounts. At five min of incubation, 9.7, 11.5, and 8.7%, at 15 min of incubation 8.5, 8.0, and 6.2%, and at 30 min of incubation 7.0, 6.7, and 7.8% of parent compound were bio-transformed into the DCJW active metabolite when reacted with the pre-inhibited 12,000g midgut supernatant of susceptible (S.S.), cherry (F.C.), and apple (K.A.) populations, respectively (Figure 4A). At five min of incubation, 4.0, 0.0, and 7.1%, at 15 min of incubation 6.7, 5.4, and 9.5%, and at 30 min of incubation 10.2, 5.3, and 2.0% of parent compound were bio-transformed into the MP819 metabolite when reacted with the pre-inhibited 12,000g midgut supernatant of susceptible (S.S.), cherry (F.C.), and apple (K.A.) populations, respectively (Figure 4B).

By calculating the percentage of parent compound that was bio-transformed into DCJW and MP819 from the total bio-transformed parent compound, 17.1, 77.3, and 49.4% at five min of incubation, 43.2, 30.5, and 53.7% at 15 min of incubation, and 54.5, 28.2, and 13.6% at 30 min of incubation of the total amount of bio-transformed parent compound were located in the upper portion of the indoxacarb metabolic pathway in the insect (Figure 2) when reacted with the pre-inhibited 12,000g midgut supernatant of susceptible (S.S.), cherry (F.C.), and apple (K.A.) populations, respectively (Figure 3B).

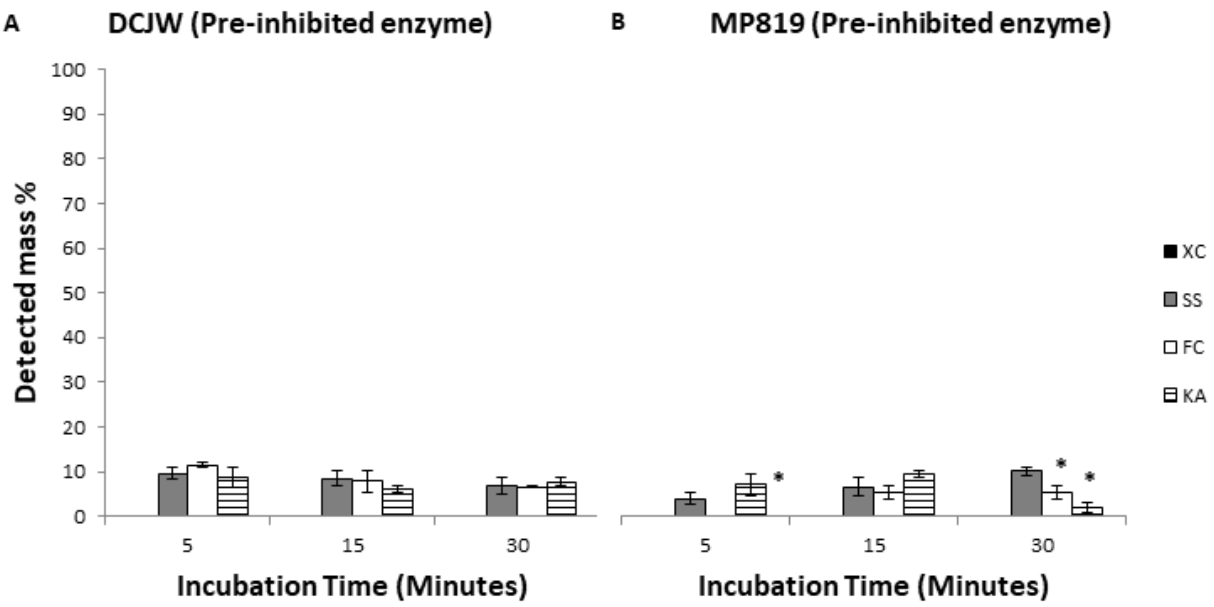

Figure 4 Detected equivalent mass percentage of indoxacarb (DPX-JW062) that was bio-transformed to **A.** DCJW and **B.** MP819 metabolites after different incubation times of in vitro reaction with in vivo pre-inhibited (by DEF) 12,000g midgut homogenates supernatants from three different C. Rosaceana (mean ± SE).

$$\text{Equivalent mass of DPX-JW062} = \frac{\text{Molecular weight of DPX-JW062}}{\text{Molecular weight of metabolite}} \times \text{detected mass of metabolite}$$

XC: Substrate control (without pre-inhibited enzyme). SS: pre-inhibited 12,000g supernatant of susceptible population. FC: pre-inhibited 12,000g supernatant of field cherry population. KA: pre-inhibited 12,000g supernatant of field apple population. *Indicate significance difference between the detected mass percentages of field population and susceptible population treatments at each incubation time. ($P < 0.05$, PROC GLM, LSMEANS)

Discussion

The critical role of the metabolic balance between indoxacarb bio-activation and detoxification was demonstrated in the current study. In addition, the previously considered unexpectedly large synergistic impact of DEF on the toxicity of indoxacarb was explained with solid evidence in the current study as described and discussed below.

Lepidopteran larvae of many economically important target species, including *Heliothis virescens, Helicoverpa zea, Spodoptera eridania, Spodoptera exigua, Spodoptera frugiperda, Trichoplusia ni, Manduca sexta, and Heliothis virescens,* rapidly convert parent compound "JW062" to the potent sodium channel blocker DCJW bio-activated metabolite, the major metabolite of JW062 and a persistent compound that is rarely metabolized to further molecules (Wing et al. 1998, Wing et al. 2000, Alves et al. 2008). The *C. rosaceana* 12,000g midgut supernatant in the current study rapidly converted the parent compound similar to lepidopteran species in previous studies; however it also rapidly metabolized the big molecule metabolites in the upper portion of the indoxacarb metabolic pathway. This includes DCJW, which was found to be easily metabolized to further smaller metabolites by *C. rosaceana*. Thus, the fact that enzyme function was already almost complete at 5, 15, and 30 min suggests that *C. rosaceana* has the ability to bio-transform the initial mass of parent compound at a very rapid rate.

Similar to the current study, Gondhalekar et al. (2016) found that parent compound and DCJW metabolite were further metabolized to more polar non-toxic metabolites in the German cockroach (Blattella germanica L.). However, Gondhalekar et al. (2016) found that the greater the indoxacarb disappearance the greater the formation of DCJW. In the current study the loss of indoxacarb did not lead to increased formation of DCJW as a result of a high rate of DCJW degradation, indicating that it had overcome its rate of formation. This means the rate of

indoxacarb detoxification in *C. rosaceana* is higher than the rate of indoxacarb activation, leading to low susceptibility to indoxacarb. This may explain the high efficacy of indoxacarb against these tested lepidopteran species in the previous studies versus poor efficacy of indoxacarb against *C. rosaceana* in the current study and other studies of *C. rosaceana* (Pree et al. 2003, Ahmad and Hollingworth 2004). Also the finding of the current study emphasizes the critical influence of the metabolic rate of each species on its susceptibility to indoxacarb (Wing et al. 2000).

In insects, the metabolic rate is usually mediated by the major metabolic enzymes including cytochrome P450 monooxygenases (P450s), carboxylesterases (esterases) and glutathione transferases (GSTs) (Tiwari et al. 2011, Pang et al. 2012, Fu-Gen et al. 2014, Gondhalekar et al. 2016). Different synergism and kinetic enzymatic studies were conducted in order to identify the resistance mechanism, in different species, that is responsible for the indoxacarb resistance. However, the findings of these studies were highly variable and a specific metabolic resistance mechanism cannot be concluded as the closely linked mechanism to indoxacarb metabolic resistance. Where, in *Plutella xylostella* L. (Lepidoptera: Plutellidae) (Nehare et al. 2010), *Sitophilus zeamais* (Coleoptera: Curculionidae) (Haddi et al. 2015), and *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) (current study; Ahmad and Hollingworth 2004) it was concluded that all three major metabolic enzymes P450 monooxygenases, esterases, and glutathione S-transferases were involved in the indoxacarb resistance. While, in *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) (Sayyed et al. 2008), *Phenacoccus solenopsis* Tinsley (Homoptera: Pseudococcidae) (Afzal and Shad 2016), and *Plutella xylostella L.,* (Sayyed and Wright 2006) P450 monooxygenases and esterases, but not glutathione S-transferases, were involved in the indoxacarb resistance. In *Spodoptera exigua*

(Hübner) (Lepidoptera: Noctuidae) (Gao et al. 2014) and *Helicoverpa assulta* Guenee (Lepidoptera: Noctuidae) (Pang et al. 2012) esterases and glutathione S-transferases, but not P450 monooxygenases, were involved in the indoxacarb resistance. In *Musca domestica* (Diptera: Muscidae) (Shono et al. 2004) and *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Bird 2017) only P450 monooxygenases was involved in the indoxacarb resistance. In *Spodoptera frugiperda (Lepidoptera: Noctuidae)* Yu and McCord (2007) found that none of the three major metabolic enzymes were involved in the indoxacarb resistance.

This high variability in the metabolic resistance mechanisms of indoxacarb is more likely due to one or more of the following factors: species differences, the level (e.g. intensive) and type (e.g. directly targeted) of long-term exposure of the field populations to different insecticide control programs, and the nutritional role in metabolic activity including activation and detoxification. Species differences and the selection pressure as a result of exposure to different insecticides seem to be obvious and easy to be linked to the variability of metabolic resistance mechanisms. For example, the tested *C. rosaceana* field populations, which were collected from apple orchards in Michigan, in the current study and in the study of Ahmad and Hollingworth (2004), showed the same indoxacarb metabolic resistance pattern where all three major metabolic enzymes were involved in the indoxacarb resistance in the tested field populations in both studies. However, in the Ahmad and Hollingworth (2004) study P450 monooxygenases found to be the major metabolic mechanism resistance which was indicated by the highest synergism ratio (51-folds) in the treatment of indoxacarb + PBO while in the current study the carboxylesterases were the major metabolic mechanism resistance with the highest synergism ratio of 285.6-fold in the treatment of indoxacarb + DEF.

The dissimilarity in the levels of involvement of the three metabolic enzymes in indoxacarb resistance in the tested *C. rosaceana* field populations may be due to the exposure to different insecticide regimes in the two different periods of time. The *C. rosaceana* field population in the study of Ahmad and Hollingworth (2004) was collected from a commercial apple orchard in 2000 and thus a history of primary exposure to conventional insecticides including organophosphates, carbamates, and pyrethroids with minimal or no exposure to the newly introduced reduced-risk insecticides. The *C. rosaceana* field population in the current study was collected from a commercial apple orchard in 2013 with nearly a decade since the new reduced-risk insecticides were introduced and replacement of many of the conventional insecticides in the *C. rosaceana* control programs. This would result in different selection pressures on each population and eventually development of different metabolic resistance mechanisms based on the causative chemical agents in the selection pressure of each case.

In addition, the role of nutrition in resistance development, has not received enough attention in many studies of this area. Where, in different studies the feeding strategies for the species have evidently impacted the activities and abundance of the metabolic enzymes (Yu 2014). Usually, polyphagous insects are more capable of evolving an efficient detoxifying system than the monophagous insects (Krieger et al. 1971). The finding of the current study is in line with conclusion of Krieger et al. 1971 that the polyphagous insect *C. rosaceana*, which has a wide range of hosts over 50 species (Sanderson and Jackson 1909), has evolved all three major metabolic enzymes and this result in a high level of resistance against indoxacarb.

The synergistic impact of the esterase inhibitors DEF, PBO, and triphenyl phosphate (TPP) on the toxicity of indoxacarb in the synergism part of the current study and similar studies (Ahmad and Hollingworth 2004, Sayyed and Wright 2006, Sayyed et al. 2008, Nehare et al.

2010, Pang et al. 2012, Gao et al. 2014, Haddi et al. 2015, Afzal and Shad 2016) was an unexpected result since indoxacarb is a pro-insecticide that requires hydrolase based bio-activation by the carboxylesterases to yield the potent toxic metabolite DCJW. Therefore, the inhibition of esterases was expected to reduce the toxicity of indoxacarb rather than increase it. Thus, the analytical part of this study was conducted to track indoxacarb and its metabolites in the in vitro indoxacarb reaction with midgut supernatant and pre-inhibited midgut supernatant by DEF at different incubation times aiming to determine the reason for the synergistic impact of DEF on the toxicity of indoxacarb, especially when the expected antagonistic impact of DEF on the toxicity of indoxacarb has been reported in the study of Alves et al. (2008). Alves et al. (2008) found that the DEF treatment in the in vitro and in vivo experiments had reduced indoxacarb biotransformation and the formation of DCJW metabolite which resulted in decreasing indoxacarb toxicity, i.e. an antagonistic impact, on *Ostrinia nubilalis* (Hübner). The reducing indoxacarb biotransformation following the DEF treatment was consistent with the finding of Alves et al. (2008). However, we observed that DEF also decreased the degradation of the DCJW metabolite. This resulted in the accumulation of DCJW which might be an evidence of the synergistic effect of DEF on the toxicity of indoxacarb in the current study and similar other studies. Similarly to the case of DCJW accumulation under DEF treatment, the MP819 metabolite also builds up following the DEF treatment. However, the reason for the accumulation of these two metabolites in the DEF treatment is still unknown. It could be due to the inhibitory effect of DEF on esterases and P450 monooxygenases enzymes with a lesser extent, which is in agreement with Ahmad and Hollingworth (2004), since the degradation of the parent compound and its metabolites, in the upper portion of the indoxacarb metabolic pathway, is mediated by either esterase or P450 monooxygenases enzymes (Gondhalekar et al. 2016). This

assumption should be limited to the species level and is in agreement with Alves et al. (2008) that any kind of generalization should be avoided, especially when three similar metabolic studies have produced three different results relative to the impact of DEF on the indoxacarb toxicity. While the current study found a synergistic effect of DEF, Alves et al. (2008) reported an antagonistic effect of DEF and Gondhalekar et al. (2016) had reported no effect of DEF on the indoxacarb toxicity in *C. rosaceana*, *Ostrinia nubilalis* (Hübner), and *Blattella germanica* L. respectively.

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CHAPTER 4: EVALUATING THE FIELD PERFORMANCEAND TOXICITY LONGEVITY OF DIFFERENT INSECTICIDES ON OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE) IN MICHIGAN APPLE AND CHERRY ORCHARDS.

Abstract

Field-based residual bioassay and residue analysis were conducted to assess the field performance and toxicity longevity of different insecticides that had previously been associated with significant resistance levels in a baseline study of obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) populations collected from apple and cherry orchards. All insecticides were applied at the recommended field rate using an FMC 1029 Airblast sprayer and water only was applied as control treatment. Leaf samples were collected from the apple and cherry orchards at different post-application (DPA) intervals. 12-24h old larvae of field-collected apple and susceptible laboratory populations were exposed to the apple leaf samples and 12-24h old larvae of field-collected cherry and susceptible laboratory populations were exposed to the cherry leaf samples at all DPA intervals. In apple and cherry trials, the order of residual longevity of insecticides that effectively controlled the tested populations was: bifenthrin and spinetoram (apple: 14, cherry 21 DPA), phosmet (apple: 7, cherry 14 DPA), chlorantraniliprole (apple: 7 DPA), and indoxacarb and emamectin benzoate (apple: 1, cherry 7 DPA). The field performance was higher for all tested compound in the cherry trial compared to the apple trial possibly due to the differences between the previously documented resistance levels in the tested populations, the rate of precipitation, and plant substrate of both trials. The previously documented significant resistance levels in the tested populations resulted in a measurable loss of field performance only in the cases of emamectin benzoate, chlorantraniliprole, and indoxacarb at 7, 21, and all DPA intervals respectively in the

apple trials while in cherry trial just indoxacarb at 7 DPA. In term of long lasting residues, only chlorantraniliprole and indoxacarb maintained measurable leaf residues over all DPA intervals while the leaf residues of the other compounds had degraded within the first 7 days. These findings can help fruit growers make adjustments to spray/re-spray intervals and optimally utilize important chemical tools in their control programs.

Key words: insecticide, resistance, integrated pest management, cherry, apple.

Introduction

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris), is an important economic pest of apple and cherry (Sial et al. 2010). Even though *C. rosaceana* has a wide host range, with over 50 species (Sanderson and Jackson 1909), for a long time it was considered as minor pest with limited damage in fruit orchards (Ahmad et al. 2002). However, this perception changed in the late 1970's when outbreaks of *C. rosaceana* populations occurred and it became a serious pest causing economical damage in tree fruit orchards (Sial and Brunner 2010a, Sial and Brunner 2012b).

Since becoming a primarily pest in fruit orchards, *C. rosaceana* has been routinely targeted directly by control programs. The control programs mainly have relied on conventional insecticides especially organophosphates. Eventually, resistance to the conventional insecticides was documented in *C. rosaceana* populations throughout the North American fruit-producing regions (Waldstein and Reissig 2000, Ahmad et al. 2002, Sial and Brunner 2012b).

In the mid 1990s fruit growers replaced many of the older conventional insecticides with new reduced-risk insecticides to combat resistance problems and due to changes imposed by the 1996 Food Quality Protection Act (FQPA) which restricted or prevented the use of many

conventional insecticides (Smirle et al. 2003, Sial and Brunner 2010b, Sial and Brunner 2010c, Sial et al. 2010, Sial and Brunner 2012a).

Many of the new insecticides that replaced the older chemistries were highly effective against the *C. rosaceana* populations (Smirle et al. 2003, Sial and Brunner 2010b, Sial and Brunner 2010c, Sial et al. 2010, Sial and Brunner 2012a, Sial and Brunner 2012b). However, recent studies have recorded resistance levels, ranged between low to high, in the *C. rosaceana* populations against some of the new insecticides even though some of the *C. rosaceana* field populations were not previously exposed to the compounds (Ahmad et al. 2002, Smirle et al. 2002, Sial et al. 2010).

While baseline laboratory studies are important to document and measure insecticide resistance in *C. rosaceana* from commercial fruit orchards, results from such research are often difficult for growers to interpret. Field-based residual bioassays can provide temporal performance data that help demonstrate to growers how various degrees of resistance are expressed under semi-field conditions (Mota-Sanchez et al. 2008). Concurrent residue analysis, in turn, can determine the insecticide longevity under normal field conditions. This temporal dimension of the insecticide field performance can help growers to identify adjustments to spray/re-spray intervals in their control programs (Brunner et al. 2005, Wise et al. 2006, Wise et al. 2007, Sial and Brunner 2010b).

In general, the laboratory performance of an insecticide is expected to be a good indicator of its field performance (Bruck et al. 2011). Examples of compounds with documented equal effectiveness under laboratory and field conditions include: methoxyfenozide, lambda-cyhalothrin, and acetamiprid against *Cydia pomonella* (Linnaeus) (Mota-Sanchez et al. 2008), methoxyfenozide and tebufenozide against *Paralobesia viteana* (Clemens) (Isaacs et al. 2005),

spinosad, imidacloprid, and thiacloprid against *Rhagoletis mendax* Curran (Liburd et al. 2003, Barry and Polavarapu 2005, Barry et al. 2005), and bifenthrin, malathion, and spinetoram against *Drosophila suzukii* (Bruck et al. 2011). However, studies have documented differences between the insecticides efficacy under laboratory and field conditions. For instance, clothianidin, thiacloprid, and azinphos-methyl against *Cydia pomonella* (Linnaeus) (Brunner et al. 2005, Mota-Sanchez et al. 2008), imidacloprid and spinosad against *Rhagoletis pomonella* (Walsh) (Yee and Alston, 2006) showed lower effectiveness under field conditions compared to the laboratory conditions. By contrast, thiacloprid against R*hagoletis pomonella* (Walsh) showed higher efficacy under field conditions compared to the laboratory conditions (Reissig 2003).

Therefore this study aimed to:

1) Identify the performance of different insecticides against *C. rosaceana* field populations originating from apple and cherry using a field-based residual bioassay and compare it with their performance in laboratory-based bioassays.

2) Assess the toxicity longevity of the different insecticides against *C. rosaceana* field populations in apple and cherry using the field-based residual bioassays and residues analysis.

3) Determine the relationship between the toxicity and the residues of each insecticide at different post-application intervals.

Materials and Methods

Insects

Three *C. rosaceana* populations were tested in the bioassays. Two *C. rosaceana* field populations were collected in summer 2013, one from a commercial apple and one from a

commercial cherry orchard in Kent and Newaygo Counties in western Michigan, respectively. The third population was a laboratory susceptible population which was established in 2000 (Ahmad et al. 2002) and has been continuously reared at the Michigan State University Trevor Nichols Research Center (TNRC) in Fennville, MI. The susceptible population was originally obtained from an isolated abandoned apple orchard in Kalamazoo County, with no history of insecticide application, and it has been used as a reference for field populations (Ahmad et al. 2002). The three *C. rosaceana* populations were maintained, and assessed under constant conditions (25(±1) °C, 16:L8D). Freely mated adults were held for each population in cylindrical plastic oviposition chambers (9 × 33 cm) that were lined with waxed paper as a surface for oviposition. A 5% aqueous sucrose solution was provided as a food and water source for emerging adults. Egg masses on wax paper were collected and then placed in 120 ml plastic cups containing 10 ml of an artificial diet (Ahmad et al. 2002), as food for the hatching larvae. Once the larvae completed their feeding and development, the pupae were collected and transferred into the plastic cylinder oviposition chamber.

Field residual activity trials

Apple and cherry field trials were conducted at June 9 and July 16, 2015 respectively at the TNRC in Fennville, Michigan (42°35'42.4"N, 86°09'22.0"W). In the apple trial, six treatments of insecticides (phosmet, bifenthrin, spinetoram, chlorantraniliprole, indoxacarb, and emamectin benzoate, for full details see Table 5) plus a control treatment (water only) were applied to 28 year-old semi-dwarf Red Delicious apple trees (Malus Miller; Rosaceae) with six-meter row spacing and three-meter tree spacing (6m × 3m). In the cherry trial, five treatments of insecticides (phosmet, bifenthrin, spinetoram, indoxacarb, and emamectin benzoate, for full

details see Table 5) plus a control treatment (water only) were applied to 21-year old Montmorency cherry trees (Sare Montmorency) with six-meters row spacing and 4.5-meters tree spacing (6m × 4.5m). The treatments were selected based on the results of a toxicity baseline study (Chapter 2), choosing compounds with none to a high level of resistance. Insecticides were applied using an FMC 1029 airblast sprayer (Jonesboro, AK) sprayer calibrated to deliver material and water diluent at 935 liters/ ha (100 gallons per acre).

Five single tree replications were used for each treatment and were arranged in a randomized complete block design (RCBD). In order to prevent insecticide drift between the adjacent treatments, one buffer (untreated) row was used to separate the treatment blocks and two buffer trees were used to separate the treated trees in each block. To obtain a representative sample, each replication was divided into sides representing the four cardinal directions and each side was divided into two levels (upper and lower). At 1, 7, 14, and 21 d post-application, a total of 48 young and fully expanded leaves were collected from each replication (12 from each side/ six from each level). Collected samples were placed in tight sealing plastic bags (the six leaves from each level of each side were collected in a separate bag for a total of eight bags per replicate), placed in coolers and transferred to the lab to conduct laboratory assays.

Table 5 The details of compounds that were tested in apple and cherry field trials.

Treatment				
Trial	Trade name	Active ingredient	AI/Acre (lb)	Company
Apple and Cherry	Imidan 70W	phosmet	3	Gowan Company, Yuma, AZ
Apple and Cherry	Bifenture 10DF	bifenthrin	1	United Phosphorus, Inc. King of Prussia, PA
Apple and Cherry	Delegate 25WG	spinetoram	0.375	Dow AgroSciences, Indianapolis, IN
Apple	Altacor 35WG	chlorantraniliprole	0.281	I.E. du Pont De Nemours and Co., Wilmington, DE
Apple and Cherry	Avaunt 30WG	indoxacarb	0.375	I.E. du Pont De Nemours and Co., Wilmington, DE
Apple and Cherry	Proclaim 5SG	emamectin benzoate	0.3	Syngenta Crop Protec- tion Inc., Greensboro, NC

100 Gallons per acre (GPA)

Field-based residual bioassay

Following the method of VanWoerkom et al. (2014), residual toxicity bioassays were conducted as soon as the samples arrived in the lab. One leave was selected randomly from each bag; each one representing a level and side of each tree, for a total of eight leaves per replicate. Using a cork borer, a 20-mm-diameter disc was cut from each leaf. The cork borer was dipped in acetone between each sample to minimize cross contamination. The eight discs were placed in a 50-mm-diameter Falcon® disposable petri dish (Corning Inc.- Life Science. Durham, NC) padded with moistened (by deionized water) 55-mm-diameter Whatman™ filter paper (Whatman Limited, Buckinghamshire, UK). Five 12-24h old *C. rosaceana* larvae were placed in each petri dish. Five petri dishes were assigned to each treatment (one for each replication). The petri dishes were maintained under constant conditions (25(±1) °C and 16L:8D). The *C. rosaceana* larvae from the apple population were exposed to treated apple leaves, the *C. rosaceana* larvae from the cherry population were exposed to treated cherry leaves and the *C. rosaceana* larvae from susceptible population were exposed to the apple treated leaves in the apple trial and to the cherry treated leaves in the cherry trial. The larval mortality was recorded 120h after the larvae were placed in the petri dishes for the treatments: phosmet, bifenthrin, spinetoram, indoxacarb, and emamectin benzoate while the larval mortality of the non-neurotoxic insecticide chlorantraniliprole treatment was recorded after 168h, because it has slower mode of action compared to neurotoxic insecticides. Any larva that failed to move when prodded with soft camel's hair brush was recorded as dead.

Residues Analysis

When the samples arrived at the lab, 20 g ±1 (approximately 40 leaves) were removed from each replication/ each treatment (total of three replications for each treatment) for residual analysis. Leaves were stored it in 120 ml glass jars (Qorpak, Bridgeville, PA) containing 8.0 g Mg_2SO_4 to absorb water in the sample and 2.0 g NaCl to push ionized compounds into the water. Then HPLC grade dichloromethane was added until the entire sample was submerged (approximately 50-100 ml) and the jars were stored at 4 C° until the samples were processed.

After 2-4 weeks, the contents of each jar were filtered into a 250 ml round bottom flask through 185-mm-diameter filter paper containing 20g of anhydrous sodium sulfate to remove the water from the sample. After completing filtration, the 250 ml round bottom flasks were connected to an R-114 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) to remove the dichloromethane. Two ml of HPLC grade acetonitrile was added to dissolve the remaining dry extract residue and the flask was rotated for 3 min. The dissolved extract residue was collected with a 3 ml syringe (BD LuerLok Tip) then filtered through a 45-mm Acrodisc 33 mm syringe filter (Pall, East Hills, NY) into 2 ml HPLC glass vial (Agilent Technologies Inc., Santa Clara, CA). The purpose of this filtration was to remove the remaining particulates. The 2 ml HPLC glass vials then were stored at 4 C° until the samples were analyzed. High-performance liquid chromatography (HPLC) was used to analyze the samples for spinetoram and emamectin benzoate treatments and gas chromatography (GC) to analyze the samples for phosmet, bifenthrin, chlorantraniliprole, and indoxacarb treatments. Limit of detecting a peak (LOD) and Limit of quantifying a peak (LOQ) values for each treatment compound are presented in Table 6.

Statistical analysis

All mortality due to insecticide exposure was adjusted by the mortality of control treatment using Abbott's (1925) formula. For each *C. rosaceana* population per each treatment, the correlation between the mortality and the residue in the samples of post-application days was determined by linear regression analysis using SAS 9.4 (SAS Institute 2013).

Table 6 LOD and LOQ values for each treatment compound

Chemical	LOD (µg/g)	LOQ (µg/g)
Phosmet	0.001	0.005
Bifenthrin	0.005	0.016
Spinetoram	0.121	0.400
Chlorantraniliprole	0.015	0.050
Indoxacarb	0.001	0.002
Emamectin benzoate	0.010	0.050

LOD = Limit of detecting a peak
LOQ = Limit of quantifying a peak

Results

The main objective of the current study was to determine how resistance affects field performance of insecticides used for control of *C. rosaceana* in relation to the associated residual activity of each compound. The results for each compound were analyzed and compared separately for apple and cherry collected populations.

Apple trial

Phosmet

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and apple *C. rosaceana* populations when exposed to the phosmet-

treated apple leaves at all post-application intervals 1, 7, 14, and 21 day post-application (Figure 5). This indicates that the documented 5-fold resistance in the field *C. rosaceana* apple population in the baseline study (Chapter 2) may not result in measurable loss of field performance at the labeled field rate.

Phosmet toxicity to both *C. rosaceana* populations were high for the first 7 d post-application, with a loss of performance following closely the gradual decline of surface residues over 21 d (Figures 5 and 6). The efficacy of phosmet declined significantly after 7d post-application with only 48.3 ±25.9 and 48.7% ±14.7 larval mortality in susceptible and apple *C. rosaceana* populations, respectively. By 21d post-application its efficacy was diminished completely with 5.5 ±5.5 and 4.1% ±4.1 larval mortality in susceptible and apple *C. rosaceana* populations, respectively (Figure 5).

Gradual degradation was recorded in the phosmet leaf residues over the post-application intervals 1, 7, 14, and 21 days post-application (Figure 6). The major residual degradation occurred within the first 7 days, with only 22.2% of the 1d post-application leaf residue detected at 7d post-application (Figure 6).

Bifenthrin

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and apple *C. rosaceana* populations when exposed to the bifenthrin-treated apple leaves at all post-application intervals 1, 7, 14, and 21 d (Figure 5). This indicates that the documented 5-fold resistance in the field *C. rosaceana* apple population in the baseline study (Chapter 2) may not result in measurable loss of field performance with the labeled field rate of bifenthrin.

Bifenthrin toxicity to *C. rosaceana* populations was high for the first 14 d post-application, despite the gradual decline of surface residues (Figures 5 and 6). Its efficacy against both populations required nearly three weeks to show the first significant decline at 21d post-application with 40.5 ±30.4 and 15.6% ±10.0 larval mortality in susceptible and apple *C. rosaceana* populations, respectively (Figure 5).

Gradual degradation was recorded in the bifenthrin leaves surface residues over the post-application intervals 1, 7, 14, and 21 day post-application (Figure 6). The major residual degradation occurred within the 7 days, with only 22.8% of the 1d post-application surface residue detected 7d post-application (Figure 6).

Spinetoram

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and apple *C. rosaceana* populations when exposed to the spinetoram-treated apple leaves at all post-application intervals 1, 7, 14, and 21 day post-application (Figure 5). This indicates that the documented 4.3-fold resistance in the field *C. rosaceana* apple population in the baseline study (Chapter 2) may not result in measurable loss of field performance with the labeled field rate of spinetoram treatment.

Spinetoram toxicity to *C. rosaceana* tested populations was high for 21 d post-application, despite the significant sharp degradation of leaf residues (Figures 5 and 6), with high efficacy against both *C. rosaceana* tested populations for nearly three weeks post-application. The first significant loss of performance was observed at 21d post-application with 47.7 ±10.5 and 49.3% ±8.2 larval mortality in susceptible and apple *C. rosaceana* populations, respectively (Figure 5).

Sharp degradation was recorded in the spinetoram leaf residues over the post-application intervals 1, 7, 14, and 21 d (Figure 6). Negligible spinetoram residues were detected at 7, 14, and 21 d post-application (Figure 6). The most dramatic residue degradation occurred within the first 7 days, with only 5.5% of the 1d post-application surface residue detected 7d post-application (Figure 6).

Chlorantraniliprole

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and apple *C. rosaceana* populations when exposed to the chlorantraniliprole-treated apple leaves at all post-application intervals 1, 7, and 14 day post-application (Figure 5). However, at 21d post-application a significant difference in chlorantraniliprole performance was detected between the susceptible and apple *C. rosaceana* populations where the larval mortality was significantly lower for the apple population compared to the susceptible population when both were exposed to the 21d post-application treated apple leaves (Figure 5).

Chlorantraniliprole toxicity to *C. rosaceana* tested populations was high for the first 7 d post-application, with a loss of performance following closely the gradual decline of surface residues over 21 d (Figures 5 and 6). Its efficacy against both *C. rosaceana* populations declined significantly after 7d post-application with only 60 ±18.7 and 62.6% ±9.6 larval mortality in susceptible and apple *C. rosaceana* populations, respectively (Figure 5).

Gradual degradation was recorded in the chlorantraniliprole leaf residues over the post-application intervals 1, 7, 14, and 21 day post-application (Figure 6). The major residual

degradation occurred within the first 7 days, with only 57.2% of the 1d post-application surface residue detected 7d post-application (Figure 6).

Indoxacarb

In the field-aged residue bioassays significant differences in indoxacarb performance were detected between the susceptible and apple *C. rosaceana* populations at all post-application intervals 1, 7, and 14 d (Figure 5). The larval mortality of the apple *C. rosaceana* population was significantly lower than that of the susceptible *C. rosaceana* population at all post-application intervals (Figure 5).

With the exception of the 85% ±9.6 larval mortality recorded for susceptible *C. rosaceana* population at 1 d post-application, low toxicity of indoxacarb to *C. rosaceana* tested populations was recorded at all post-application intervals (Figure 5).

Relatively flat degradation was recorded in the indoxacarb leaf residues over the post-application intervals 1, 7, and 14 day post-application with no major residual declines at any post-application interval (Figure 6).

Emamectin benzoate

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and apple *C. rosaceana* populations when exposed to the emamectin benzoate-treated apple leaves at 1d post-application (Figure 5). However, at 7d post-application a significant difference in emamectin benzoate performance was detected between the susceptible and apple *C. rosaceana* populations where the larval mortality was significantly lower for the apple population compare to the susceptible population (Figure 5). No larval

mortality was observed in the susceptible and apple *C. rosaceana* populations when both were exposed to the 14 d post-application treated apple leaves (Figure 5).

Emamectin benzoate toxicity was high 1 d post-application and for the first 7 days to susceptible and apple *C. rosaceana* population (Figure 5), with a loss of performance following closely the rapid decline of surface residues (Figures 5 and 6). Its efficacy against the susceptible laboratory *C. rosaceana* population declined significantly after 7 d post-application with 0.0% ±0.0 larval mortality (Figure 5), while efficacy against the apple population declined significantly after 1 d post-application with 40.0% ±0.0 larval mortality (Figure 5).

Emamectin benzoate showed rapid decline of surface residues under field conditions, with leaf residues detected only at 1 d post-application and no leaves surface residues detected at 7 and 14 d post-application (Figure 6).

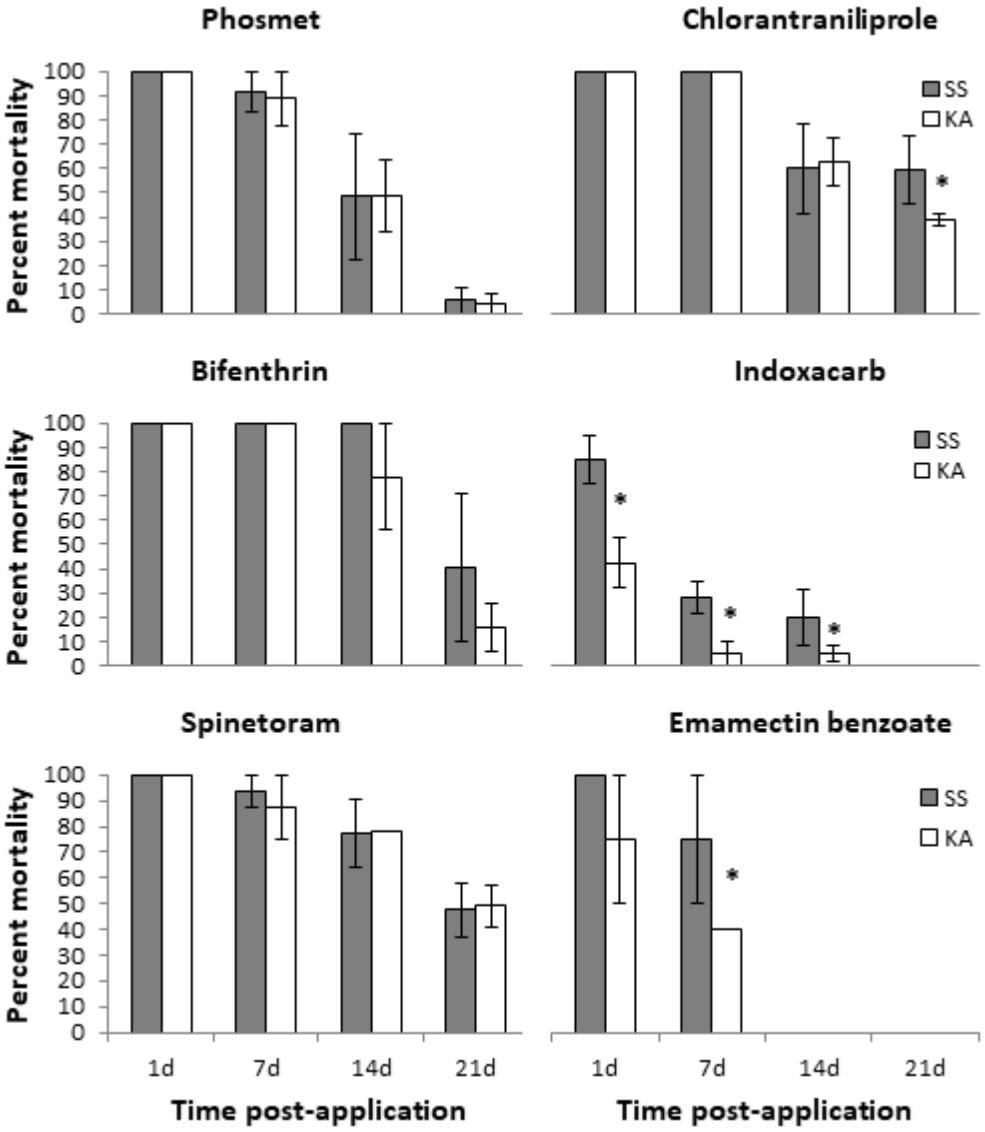

Figure 5 Mortality means (± SE) of *C. rosaceana* 12-24h larvae of apple population (KA) and susceptible population (SS) when exposed to apple foliage collected at various times post application.

* means the mortality of apple population is significantly different from the mortality of susceptible population at a given post-application time interval (α=0.05).

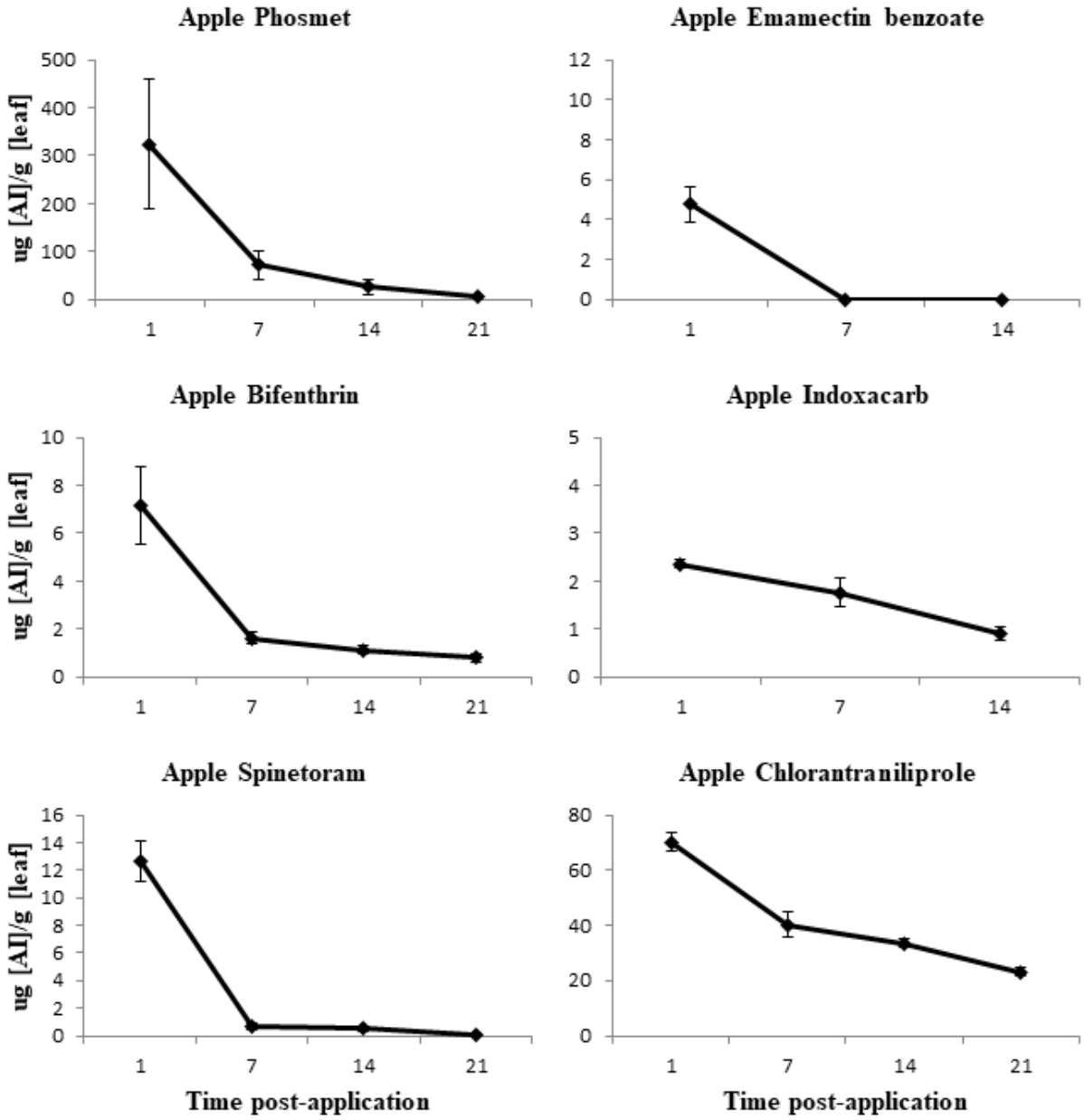

Figure 6 Residues means (SE±) measured in micrograms per gram of active ingredient per leaf taken at 1, 7, 14, and 21d post-application.

Cherry trial

Phosmet

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and cherry *C. rosaceana* populations when exposed to the phosmet-treated cherry leaves at all post-application intervals 1, 7, 14, and 21 d (Figure 7). Phosmet toxicity to *C. rosaceana* populations were high for the first 7 d post-application, with a loss of performance following closely the gradual decline of leaf residues over 21 d (Figures 7 and 8). Its efficacy against both *C. rosaceana* populations declined significantly after 7 d post-application, with only 82.2 ±8.0 and 62.2% ±13.6 larval mortality in susceptible and cherry *C. rosaceana* populations, respectively (Figure 7). No further significant loss of performance was recorded beyond 7 days. The larval mortality of both *C. rosaceana* populations at 21 d post-application had not declined significantly compared to their larval mortality at 14 d post-application (Figure 7).

Gradual degradation was recorded in the phosmet leaf residues over the post-application intervals 1, 7, 14, and 21 d (Figure 8). The major residual degradation occurred within the first 7 days, with only 14.4% of the 1 d post-application residue was detected at 7 d post-application (Figure 8).

Bifenthrin

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and cherry *C. rosaceana* populations when exposed to the bifenthrin-treated cherry leaves at all post-application intervals 1, 7, 14, and 21 d (Figure 7). This indicates that the documented 4.9-fold resistance in the field *C. rosaceana* cherry population in the baseline study (Chapter 2) may not result in a measurable loss of field performance at the labeled field rate of bifenthrin.

Bifenthrin toxicity to *C. rosaceana* cherry and susceptible populations was high for 21 d post-application, despite the gradual decline of leaf residues (Figures 7 and 8). Its efficacy against both *C. rosaceana* tested populations did not significantly decline even after 21 d post-application with 80.0 ±20 and 79.2% ±11.4 larval mortality in susceptible and cherry *C. rosaceana* populations, respectively (Figure 7).

Significant gradual degradation was recorded in the bifenthrin leaf residues from days 1 to 21 post-application (Figure 8). The major residual degradation occurred within the 7 d post-application, with only 25% of the 1 d post-application residue detected at 7 d post-application (Figure 8).

Spinetoram

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and cherry *C. rosaceana* populations when exposed to the spinetoram-treated cherry leaves at all post-application intervals 1, 7, 14, and 21 d (Figure 7). This indicates that the documented 4.1-fold resistance in the field *C. rosaceana* cherry

population in the baseline study may not result in measurable loss of field performance at the labeled field rate of spinetoram.

Spinetoram toxicity to *C. rosaceana* cherry and susceptible populations was high for 21 d post-application, despite the sharp degradation of residues (Figures 7 and 8), with high efficacy against both *C. rosaceana* tested populations for nearly three weeks post-application. The first significant loss of performance was observed at 21d post-application with 75.7 ±19.4 and 69.6% ±18.3 larval mortality in susceptible and cherry *C. rosaceana* populations, respectively (Figure 7).

Significant sharp degradation was recorded in the spinetoram leaf residues over the post-application intervals 1, 7, 14, and 21 d (Figure 8). Negligible spinetoram leaf surface residues were detected at 7, 14, and 21 d post-application (Figure 8). The major residual degradation occurred within the 7d post-application in which only 8.6% of the 1d post-application residue was detected at 7d post-application (Figure 8).

Indoxacarb

In the field-aged residue bioassays significant differences in indoxacarb performance were detected between the susceptible and cherry *C. rosaceana* populations at 7 day post-application (Figure 7). The larval mortality of the cherry *C. rosaceana* population was significantly lower than the larval mortality of the susceptible *C. rosaceana* population when both were exposed to the indoxacarb treated cherry leaves at 7 d post-application (Figure 7). No significant differences were found between the larval mortality of susceptible and cherry *C. rosaceana* populations when they were exposed to the indoxacarb treated cherry leaves at 1 and 14 d post-application (Figure 7). This indicates that the documented 21-fold resistance in the

field *C. rosaceana* cherry population in the baseline study (Chapter 2) resulted in measurable loss of field performance with the labeled field rate of indoxacarb treatment at 7d post-application.

Indoxacarb toxicity was high to susceptible and cherry *C. rosaceana* population at 1d and 7d post-application, despite the relatively flat degradation of residues (Figure 7 and 8). Its efficacy against the susceptible *C. rosaceana* population declined significantly after 7 d post-application with 40.9% ±16.3 larval mortality (Figure 7), while efficacy against cherry *C. rosaceana* tested population declined significantly after 1 d post-application with 55.3% ±11.4 larval mortality (Figure 7).

Slow degradation was recorded in the indoxacarb leaf residues over the post-application intervals 1, 7, and 14 d post-application with no major residual degradation at any post-application interval (Figure 8).

Emamectin benzoate

In the field-aged residue bioassays no significant differences were found between the larval mortality of susceptible and cherry *C. rosaceana* populations when exposed to the emamectin benzoate-treated cherry leaves at all post-application intervals 1, 7, and 14 d (Figure 7). This indicates that the documented 5.8-fold resistance in the field *C. rosaceana* cherry population in the baseline study (Chapter 2) may not result in measurable loss of field performance at the labeled field rate of emamectin benzoate.

Emamectin benzoate toxicity to cherry and susceptible *C. rosaceana* populations was high for the first 7 d post-application, with a loss of performance following closely the rapid decline of residues over 21 d (Figures 7 and 8). Its efficacy against both *C. rosaceana*

populations declined significantly after 7 d post-application, with only 65.4 ±10.4 and 41.9% ±17.5 larval mortality in susceptible and cherry *C. rosaceana* populations, respectively (Figure 7).

Emamectin benzoate showed rapid decline of leaf residues under the field conditions, with residues detected .at 1 d post-application, but no residues detected at 7 and 14 d post-application (Figure 8).

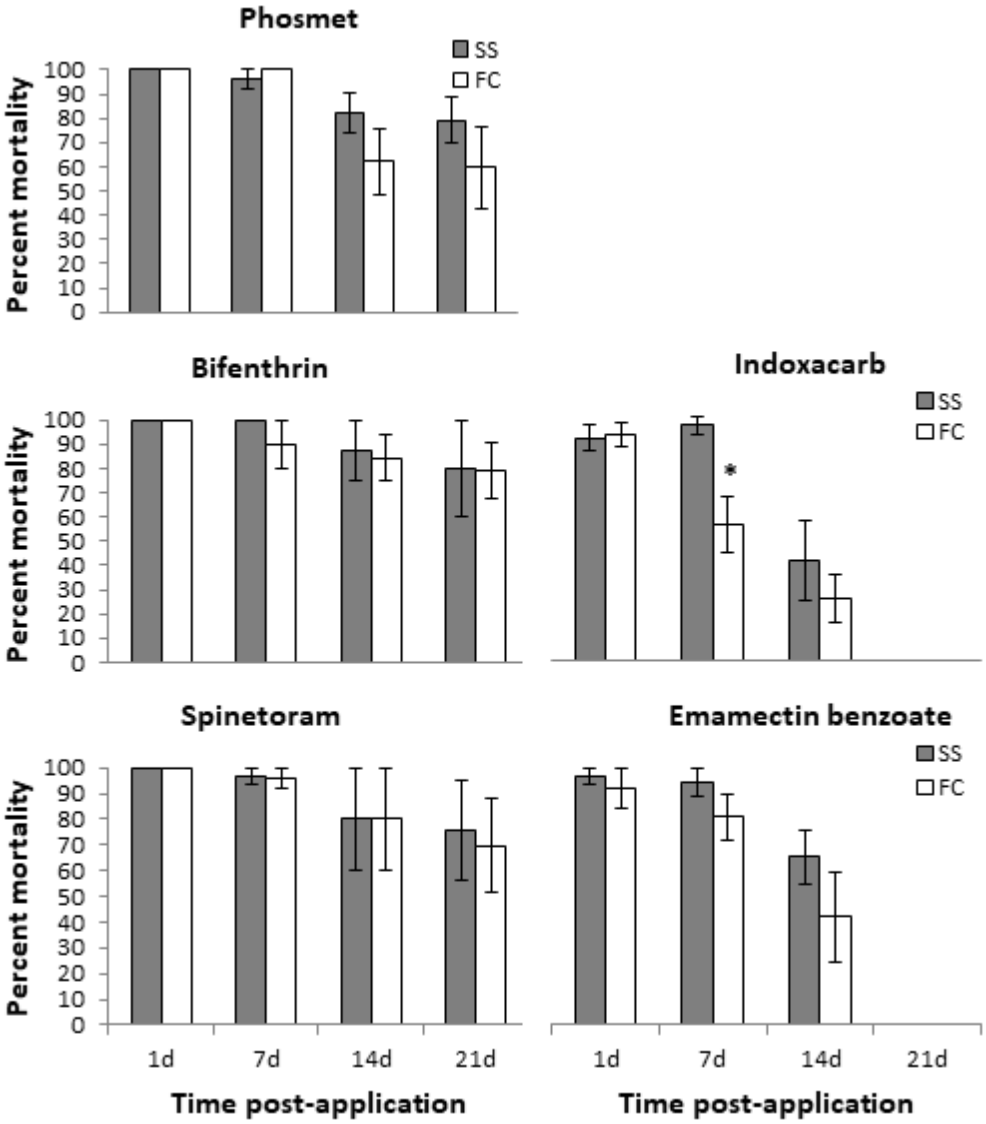

Figure 7 Mortality means (± SE) of *C. rosaceana* 12-24h larvae of cherry population (FC) and susceptible population (SS) when exposed to cherry foliage collected at various times post application.

* means the mortality of cherry population is significantly different from the mortality of susceptible population at a given post-application time interval (α=0.05).

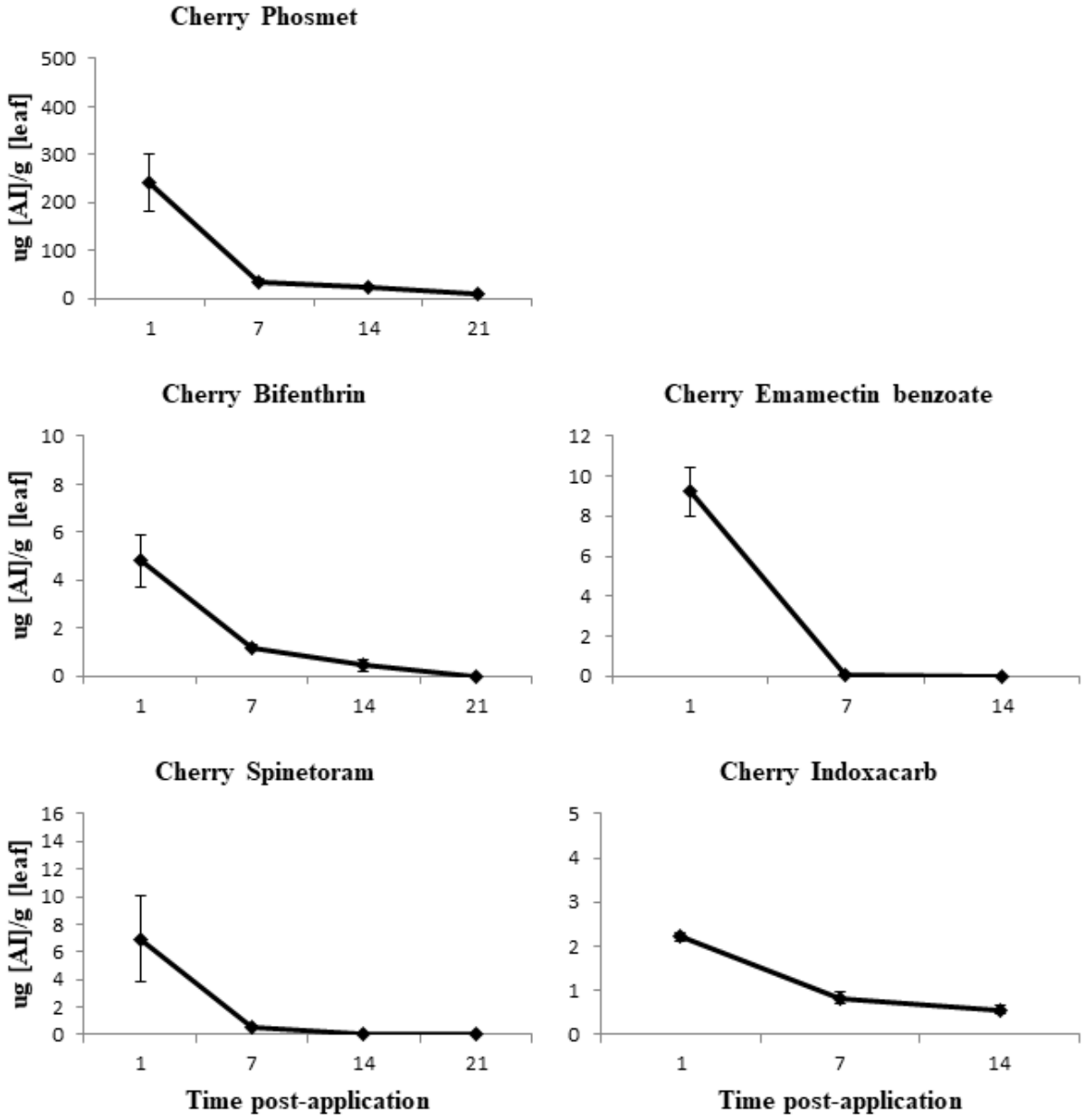

Figure 8 Residues means (SE±) measured in micrograms per gram of active ingredient per leaf taken at 1, 7, 14, and 21d post-application.

Discussion

The current study demonstrated how various degrees of laboratory-documented resistance are expressed under semi-field conditions. The results for compounds in this study fall into two categories in terms of how resistance is expressed under field conditions, 1) reduced longevity of control, 2) no evidence for loss of lethality of the compound. For example, phosmet, spinetoram, and bifenthrin were associated with significant levels of resistance in the *C. rosaceana* field populations in the baseline laboratory bioassay but the field-based residual bioassay revealed that these laboratory-documented significant levels of resistance did not result in measurable loss of field performance with the labeled field rate of those compounds. While the principles of resistance management would justify efforts to rotate to insecticides with different modes of action, our study suggests that low-level resistance of *C. rosaceana* may not be readily recognized under grower field conditions.

The differences in the field performance of the same compound under apple and cherry field trial are expected to be due to one or more of the following three factors. First, the tested apple and cherry *C. rosaceana* field populations showed different levels of resistance against the tested insecticides under the baseline study (Chapter 2). Second, both field trials were conducted at different times during the same season with slightly different weather conditions that could impact residues. For example, the apple field trial received 0.0, 2.4, 4.6, and 4.9 milliliters of precipitation over 1, 7, 14, and 21-day post-application respectively while the cherry field trial received 0.1, 0.7, 0.7, and 1.6 milliliters of precipitation over 1, 7, 14, and 21-day post-application respectively. Third, differences in physiology or morphology of apple and cherry leaves may affect for example larvae consumption; insecticide solution adhesion, insecticide penetration etc. (Chowdhury et al. 2001).

In addition, the residue sample preparation methods did not grind leaf substrate within the solvent, thus depending on the compound some portions of sub-cuticular residues from internal leaf tissues may have remained unmeasured. This can likely explain the cases where temporal lethality continued beyond the dates of detectible residues such as phosmet, bifenthrin, and spinetoram treatments.

Phosmet

This non-systemic organophosphate insecticide is a broad-spectrum and effective compound in fruit orchards, including apple and tart cherry, to wide range of pests. Phosmet is slightly soluble in water, has relatively low vapor pressure, and is stable to photolysis (Pesticide Information Profiles 1996, EPA 2000). Despite its slight solubility in water, phosmet is highly susceptible for wash-off from precipitation (Wise et al. 2017). However, its plant systemic capability of cuticle penetration helps to reserve a portion of phosmet active ingredient in the plant leaves and fruit cuticle (Bostanian et al. 2012).

The ability of this wash-off susceptible compound to maintain longevity of control over 14 d post-application possibly due to its nature as a fast acting contact neurotoxin where the lethal effect is accumulated through the mobility of larvae over the treated surface (Wise et al. 2017) and possibly due to its plant systemic capability of cuticle penetration (Bostanian et al. 2012).

Similar field performance of phosmet and some other organophosphate insecticides has been documented in previous studies. For example, azinphos-methyl and phosmet effectively controlled first-instar oriental fruit moth larvae for 14d post-application when they were exposed to treated peach foliage (Pree 1979), phosmet effectively controlled apple maggot larvae when it

were exposed to treated apple fruit (Wise et al. 2009), and azinphos-methyl effectively controlled plum curculio adult when it were exposed to treated tart cherry fruit (Hoffmann et al. 2010).

In contrast, different field performance of phosmet and some other organophosphate insecticides has also been documented in previous studies. For example, high field performance of azinphos-methyl and phosmet was recorded in tart cherry field study where both controlled plum curculio larvae for more than 30 d post-application when the larvae were exposed to treated cherry fruit (Hoffmann et al. 2009). Chlorpyrifos and azinphos-methyl in two separate apple field studies showed low field performance where both failed to control *C. rosaceana* and codling moth neonate larvae for 10 and 14d post-application respectively (Waldstein and Reissig 2001, Mota-Sanchez et al. 2008). Similarly, malathion showed low field performance by controlling *drosophila suzukii* for only the first 7d post-application when the flies were exposed to treated blueberry fruit (Bruck et al. 2011). But the same compound showed poor field performance when it failed to control *drosophila suzukii* even for the first 7d post-application when the flies were exposed to treated strawberry fruit (Bruck et al. 2011).

The current study, showed phosmet as an effective chemical tool to control *C. rosaceana* field populations in Michigan apple and cherry orchards. However, this compound with long history of use in Michigan apple and cherry orchards should be avoided for *C. rosaceana* control programs due to the high potential of rapid build-up of resistance. If circumstances necessitate applying phosmet, a 14 d post-application should be considered in the re-entry, re-spray, and pre-harvest intervals for this compound.

Bifenthrin

Bifenthrin is a non-systemic, broad-spectrum, and widely used insecticide. The high field-performance of bifenthrin, under the current study, is compatible with its physical properties of being relatively insoluble in water, stable to hydrolysis, and minimal volatility (Johnson et al. 2010). Bifenthrin is also moderately susceptible to wash-off from precipitation (Wise et al. 2017) but its plant systemic capability of cuticle penetration helps to reserve a portion of its active ingredient in the plant leaves and fruit cuticle (Bostanian et al. 2012) which may provide an addition support to its physical properties to show this high field-performance.

Variable field performance of bifenthrin and some other pyrethroid insecticides have been documented in previous studies. For example, in berry crops field studies bifenthrin and fenpropathrin showed poor field performance when both failed completely to control *drosophila suzukii* at 7d post-application when the flies were exposed to blueberry and strawberry treated fruit (Bruck et al. 2011). Similarly, esfenvalerate showed poor field performance when it failed to control *C. rosaceana* at 10d post-application when the neonate larvae were exposed to treated apple foliage (Waldstein and Reissig 2001). Permethrin in turn showed moderate field performance by effectively controlling the oriental fruit moth over 14d post-application when first-instar larvae were exposed to treated peach foliage (Pree 1979). Lambda-cyhalothrin also showed moderate field performance by effectively controlled codling moth until its efficacy significantly declined when the neonate larvae were exposed to 21d post-application treated apple fruit (Mota-Sanchez et al. 2008).

The variation in the field performance of bifenthrin and some other pyrethroid insecticides documented previously and in the current study, is assumed to be due to the

differences in the field and application conditions, tested species, tested life-stage, levels of resistance, and plant substrate.

The high field performance of bifenthrin that was documented in the current study indicates that this insecticide is an effective chemical tool to control *C. rosaceana* field populations in Michigan apple and cherry orchards. However, this compound belongs to a chemical class that has long history of use in Michigan apple and cherry orchards. Therefore, bifenthrin should be avoided for *C. rosaceana* control programs due to the high potential of rapid build-up of resistance. If circumstances necessitate applying bifenthrin, a 21 d post-application should be considered in the re-entry, re-spray, and pre-harvest intervals for this compound.

Spinetoram

Spinetoram is a broad-spectrum and widely used insecticide. It has low solubility in water and low vapor pressure, but this semi-synthetic compound, isolated from fermentation of Saccharopolyspora spinose, degrades rapidly by photolysis (United States Environmental Protection Agency (EPA) 2009a). Despite the photolysis sensitivity, spinetoram has excellent translaminar movement where this insecticide has the ability to penetrate the leaf cuticle, move into and across leaf tissues (DuPont Technical Information 2008). This means the compound's active ingredient is protected from photolysis inside the leaf tissue and this is probably the main reason for the long-lasting control of this compound under the field conditions.

Similar to the current study, high field performance of spinetoram and spinosad has been documented in previous studies. For example, spinosad showed high field performance with high levels of mortality through 21 d post application in *C. rosaceana, P. pyrusana, L. subjuncta,* and *C. pomonella* when the larvae were exposed to treated apple fruit and leaves (Brunner et al.

2005). Spinosad bait as well showed high field performance when the 14 d post-application treated cherry leaves killed 100% of *Rhagoletis indifferens* adults (Yee and Alston. 2006). Higher spinetoram field performance, compared to its field performance in the current study, was documented by Sial and Brunner (2010), who reported that spinetoram-treated apple foliage caused 100% mortality in the *C. rosaceana* neonate larvae (<48 h old) over 59 d post-application. Since both studies had tested the same compound (spinetoram), species (*C. rosaceana*), life-stage (<48 h old neonate larvae) and plant substrate (Red Delicious apple leaves), the sources of the difference in spinetoram field performance between both studies were due to differences in the field conditions, application conditions, levels of resistance, and time of exposure (120h in the current study and 168h in Sial and Brunner 2010 study).

In contrast, spinetoram showed low field performance by controlling *drosophila suzukii* and apple maggot for only the first 7d post-application when the flies were exposed to treated blueberry and apple fruit respectively (Bruck et al. 2011, Yee et al. 2007). The same compound showed poor field performance when it failed to control *drosophila suzukii* even for the first 7 d post-application when the flies were exposed to treated strawberry fruit (Bruck et al. 2011).

Based on the high field performance of spinetoram that was documented in the current study, this insecticide appears to be excellent chemical tool to control *C. rosaceana* field populations in Michigan apple and cherry orchards. However, resistance development against this insecticide should be monitored periodically for further increases in the resistance level. This can be accomplished once annually, if possible, by collecting *C. rosaceana* field populations from the targeted orchards then determine the resistance levels in them against spinetoram. In the spinetoram application, a 21 d post-application should be considered in the re-entry, re-spray, and pre-harvest intervals for this compound.

Chlorantraniliprole

Chlorantraniliprole is a broad-spectrum insecticide with activity a wide range of pests in many crops. It has very low water solubility, has low vapor pressure, and is stable to photolysis (except in water) (EPA 2008). Chlorantraniliprole is highly rainfast plus it has translaminar activity in which both surface and inner residues are relatively protected, allowing it to provide long lasting crop protection (Bostanian et al. 2012, Wise et al. 2017).

The long-lasting leaf residues documented in the current study over all post-application intervals is consistent with the findings reported by Wise et al. (2017). The absence of significant differences in larval mortality between susceptible and apple *C. rosaceana* populations at all post-application intervals (except 21 d) was expected to be due to the high efficacy and long lasting residues of chlorantraniliprole overcoming the documented 4.7-folds resistance of apple field *C. rosaceana* population in the baseline study (Chapter two) over nearly three weeks until 67.1% of the 1d post-application leaf residues had been degraded This means the presence of nearly ≥ 40% of chlorantraniliprole field rate residues is enough to control the *C. rosaceana* field populations effectively.

Poor field performance of chlorantraniliprole was previously documented when this compound failed to reduce the apple maggot larval emergence significantly, compared to the untreated control, in the chlorantraniliprole-treated apple fruit even at 1 d post-application (Wise et al. 2009).

In contrast, higher chlorantraniliprole field performance that recorded in the current study was documented by Sial and Brunner (2010), who reported that chlorantraniliprole-treated apple foliage caused 100% mortality in *C. rosaceana* neonate larvae (<48 h old) over 38 d post-

application. Since Sial and Brunner (2010) and the current studies had tested the same compound (chlorantraniliprole), species (*C. rosaceana*), life-stage (<48 h old neonate larvae), time of exposure (168h), and plant substrate (Red Delicious apple leaves), the source of the difference in chlorantraniliprole field performance between both studies is likely due to the differences in the levels of resistance in the tested populations, field conditions, and application conditions Where for example, Sial and Brunner (2010) had tested a laboratory susceptible population of *C. rosaceana* with no level of resistance while the current study had tested in addition to the susceptible population a field population with a resistance ratio of 4.7-fold compared to the susceptible population.

The moderate lethality in the current study should not be considered as a final conclusion of the ability of chlorantraniliprole to provide fruit protection. The current study evaluated chlorantraniliprole only based on the lethal activity; however the compound has lethal and sublethal effects e.g. delaying oviposition (Teixeira et al. 2009). Based on the results of the current study, 14 days post-application should be considered as the recommended period of re-spray, and pre-harvest in the chlorantraniliprole application.

Indoxacarb

Indoxacarb is a broad-spectrum and effective insecticide against a wide range of pests, especially lepidopteran larvae, in many crops (Wing et al. 2000). It is a non-volatile compound with low vapor pressure, low water solubility, and a complex degradation profile (except in soil where it has rapid degradation rate) (Moncada 2003). Indoxacarb is also moderately susceptible to wash-off from precipitation but its plant systemic capability of cuticle penetration helps to

reserve a portion of its active ingredient in the plant leaves and fruit cuticle (Bostanian et al. 2012).

The failure of indoxacarb to control *C. rosaceana* in the field, especially in the apple trial, was likely due to the high documented resistance levels in the tested *C. rosaceana* populations uncovered in the baseline study, despite the flat degradation in leaf residues over time. Wise et al. (2006) also found that ndoxacarb residues remained stable over time (up to two weeks) in a sufficient quantity to cause lethal effects.

Unlike the poor field performance in the current study, indoxacarb showed good field efficacy with 14 d post-application control of *Forficula auricularia* when the adults were exposed to treated apple foliage (Vogt et al. 2009). Similarly, indoxacarb showed good field performance with control of plum curculio over 14 d post-application when the adults were exposed to treated apple fruit (Wise et al. 2006). A previous trial in tart cherry was similar to the current cherry trial where indoxacarb showed low field performance and provided fruit protection for only 7 d post-application against plum curculio when the adults were exposed to treated cherry fruit (Hoffmann et al. 2010).

The differences in the field performance of indoxacarb that were documented in different studies, including the current study, is assumed to be due to the differences in the field conditions, application conditions, tested species, tested life-stage, levels of resistance, and plant substrate.

Even though indoxacarb is not labeled to use in the *C. rosaceana* control programs in fruit orchards, growers should take this indoxacarb field failure into account when using this compound in the control programs of other pests e.g. plum curculio and not expect incidental control of the leafroller.

Emamectin benzoate

The emamectin benzoate insecticide is a derivative of abamectin which is effective against numerous pests. It is a semi-synthetic compound which is isolated from the soil actinomycete: *Streptomyces avermitilis* fermentation (Jansson et al. 1997, EPA 2009b). Emamectin benzoate has very low solubility in water and low vapor pressure, and is sensitive to photo degradation (Jansson et al. 1997, EPA 2009b). Despite the photolysis sensitivity, emamectin benzoate has translaminar activity in which it is able to penetrate the leaf cuticle and move into and across leaf tissues which means the compound's active ingredient is protected from photolysis inside the leaf tissues (EPA 2009b).

The low field performance in the cherry and apple trials were not due only to the documented resistance levels in the *C. rosaceana* field populations against this compound in the baseline study, but also due to the short residual of this compound as a foliar application. The rapid decline of surface residues under the field conditions was expected since emamectin benzoate is highly sensitive to photolysis. However, the lack of detectable residues at 7 d sample and after may in part been a result of the residue sample preparation methods used in the current study, where some portions of sub-cuticular residues from internal leaf tissues may have remained unmeasured.

Higher emamectin benzoate field performance, compared to its field performance in the current study, was documented by Sial and Brunner (2010) for *C. rosaceana* and for codling moth and oriental fruit moth (Ioriatti et al. 2009) when neonate larvae were exposed to treated apple foliage or fruit. Sial and Brunner 2010 found that emamectin benzoate-treated apple foliage caused 100% mortality in the *C. rosaceana* neonate larvae (<48 h old) up to 10 d post-

application. Since both studies had tested the same compound (emamectin benzoate), same species (*C. rosaceana*), same life-stage (<48 h old neonate larvae), and same plant substrate (Red Delicious apple leaves, in the case of current apple trial), the sources of the difference in emamectin benzoate field performance between both studies are expected to be due to the tested populations.

Emamectin benzoate is registered as a foliar spray in the U.S. against numerous pests in apples (Wise et al. 2012). However, based on the documented low field performance of emamectin benzoate as a foliar spray in the current study and other previous studies, we recommend the application of this compound by tree-injections, where, it controlled *C. rosaceana* effectively over two growing seasons (VanWoerkom et al. 2014).

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CHAPTER 5: CONCLUSION

According to the Insecticide Resistance Action Committee (IRAC) the resistance is: "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (www.irac-online.org). This definition of resistance is somehow narrow and the consideration of the resistance occurrence was linked to the failure of a product to control the targeted pest species.

In fact, the ability of any product to control the targeted pest species is not a reliable parameter in determining the resistance cases. For example, in the current study significant levels of resistance were detected in the *C. rosaceana* field populations to phosmet, bifenthrin, and spinetoram under precise laboratory bioassays. This means the susceptibility of field populations was decreased to these insecticides. However, the documented significant levels of resistance did not reflect in a failure of these insecticides to achieve the expected level of control when they were applied according to the label recommendation for the targeted species *C. rosaceana*. Where, the three insecticides controlled the *C. rosaceana* field populations effectively without any significant differences in the mortality compared to the *C. rosaceana* susceptible population. Therefore, the decrease in susceptibility of any species to any product is an essential parameter in the detection of the occurrence of resistance in that species to that product. And any definition of resistance should mainly consider this parameter as a core of that definition such as defining the resistance as "genetically based decrease in susceptibility to a pesticide" according to Tabashnik et al. (2014).

Resistance management in turn, is an essential part of the integrated pest management (IPM) programs (Onstad 2014). Where, it is integrated strategies include diluting the resistance gene (providing refuge for susceptible individuals), use rotation of different chemical classes

with different mode of actions, use new pesticides with novel mode of actions, use natural enemies with high level of resistance against the applied pesticides, use synergists to enhance the toxicity of the applied insecticides etc. (Simon 2014). Through this integrated strategies, resistance management aims to reduce resistance level in the pest population or at least delay or prevent the resistance development (Onstad 2014). As a shown in the current study, a combination of integrated field and laboratory studies can provide a solid base for the resistance management decision-making.

Resistance current and future status

Studying the resistance status in two different populations of same species from two different crops will provide valuable information in terms of the sources of variation in resistance levels and as an evaluation of the Michigan IPM programs in both crops. However, the findings are limited by the spatial frame of collection sites of these populations, and thus may not reflect the patterns of resistance across the entire state. Therefore, a state-wide survey of more commercial orchards would help determine the extent of insecticide resistance across Michigan's five tree fruit production regions, and result in having a database of determined resistance cases by time and location. In addition to this, a database of the control programs of each location should be established including the types of applied chemical materials, types of applications, time of application, number of applications, and the related circumstances e.g. weather conditions. Studying the correlation between these two databases would help specialists in resistance management to create prediction models of what type of resistance, when, and where it is predicted to occur.

Conventional insecticides

Three conventional insecticides phosmet, bifenthrin, and methomyl were tested in the baseline study. They belong to the most common and oldest conventional chemical classes in the fruit control programs: organophosphate, pyrethroids, and carbamates respectively. This study provided a recent and direct assessment of the resistance levels in the *C. rosaceana* field populations against those conventional insecticides, which reflected at the same time their efficacy. It provided as well an evaluation of the integrated pest management (IPM) programs in Michigan fruit orchards in the last decade since the last similar study (Ahmad et al. 2002) was conducted more than one decade ago. It also assessed the impact of the Food Quality Protection Act (FQPA, 1996) on the efficacy of the chemical tools of control programs in the fruit production. Where, it restricted or prevented the use of many conventional insecticides and this has coincided with the introduction of the new reduced-risk insecticides, which in turn replaced many of the conventional insecticides.

The result of this study showed good efficacy of the tested conventional insecticides against the *C. rosaceana* field populations. This means it maintained its good efficacy over the last 15 years where the detected low resistance levels in the *C. rosaceana* field populations were similar to that documented in Ahmad et al. (2002) study. This also is a sign of the effectiveness of IPM programs in the fruit orchards where it successfully manage the use of insecticides (e.g. insecticides rotation), as a part of the insecticide resistance management (IRM), in which preventing further resistance development. FQPA (1996) also positively contributed in maintaining the high efficacy of conventional insecticides by reducing the selective pressure in the targeted pests, without omission the cross resistance between the different chemical classes. This reduction of selective pressure was achieved by preventing the use of many of conventional insecticides, restrict the use of some others of these insecticides (e.g. reduce the number of its

applications per season), and expanded the cycle of insecticides rotation by adding more chemical classes that have new and different mode of actions.

Worth mentioning, in spite of its good efficacy, conventional insecticides with long history of use in fruit orchards have a high likelihood of rapid build-up of resistance if the selection pressure slightly increased and therefore they are not recommended as a first choice for the *C. rosaceana* control programs in fruit orchards.

Reduced-risk insecticides

Five reduced-risk insecticides were tested in the baseline study including spinetoram, emamectin benzoate, novaluron, chlorantraniliprole, and indoxacarb. They belong to different chemical classes with different mode of actions including spinosyns, avermectins, benzoylureas, diamides, and oxadiazines respectively. Except indoxacarb, the tested reduced-risk insecticides found to be effective against the *C. rosaceana* field populations with some cases of low resistance levels which are practically considered as a tolerance rather than resistance. However, this low resistance levels should be an alert for the fruit growers. Where, it occurred after a short period of time, nearly one decade, from the introduction of these reduced-risk insecticides in the market. Without omission the cross resistance between the different chemical classes, it also occurred in the presence of the other conventional chemical classes in the *C. rosaceana* control programs. Where, the remaining permitted conventional insecticides are subjected to be phased-out in the near future. This will makes control programs rely completely on this reduced-risk insecticides and therefore the direct selective pressure is expected to increase dramatically in the pest populations which may develop higher levels of resistance against those insecticides.

Maintaining the efficacy of these reduced-risk insecticides requires monitoring the resistance levels periodically in the targeted pest populations and enhancing the other IPM program control tactics e.g. biological control. The documented failure of indoxacarb in this study to control the *C. rosaceana* field populations will be discussed in the mechanism study section below.

New aspects of indoxacarb resistance mechanism

In the baseline study, high resistance levels were detected against indoxacarb in the tested *C. rosaceana* field populations. Similar resistance level was detected in a *C. rosaceana* field population 15 years ago (Ahmad et al. 2002). At the time of Ahmad et al. (2002) study the indoxacarb had never been used in Michigan fruit orchards and the tested *C. rosaceana* field population was not previously exposed to indoxacarb which was a conclusive evidence of cross-resistance case. This increased the importance of the indoxacarb resistance phenomenon in the *C. rosaceana* field populations. Where, the indoxacarb resistance, due to the involvement of cross-resistance, is not important only for indoxacarb application in the control programs of fruit orchards but it also important for the applications of other chemical classes in which it may cause failure of indoxacarb and one or more other insecticides in the same control program and eventually failure of the entire control program.

Indoxacarb is a pro-insecticide where it undergoes hydrolase based bio-activation, due to the action of carboxylesterases. This bio-activation yield the potent sodium channel blocker decarbomethoxyllated (DCJW) metabolite which is responsible for the lethal effect of indoxacarb. Synergism and metabolic studies were conducted to identify the detoxification mechanism against indoxacarb in the tested *C. rosaceana* field populations.

Based on the result of synergism study, an involvement of mixed-function oxidases, glutathione S-transferase, and esterases enzymes in the resistance phenomenon in the apple *C. rosaceana* field population and that mean a complex case of resistance against indoxacarb with the presence of more than one resistance mechanism. While only esterases enzymes were involved in the tolerance and moderate resistance in the *C. rosaceana* susceptible and cherry field population respectively.

The involvement of esterases enzymes in the *C. rosaceana* resistance against indoxacarb was determined by synergistic impact of the esterases inhibitor DEF on the toxicity of indoxacarb. This synergistic impact of DEF was previously considered as unexpected result. However, it explained with solid evidence in the metabolic study. Where, based on the result of metabolic study, the 12,000g midgut supernatant of all tested *C. rosaceana* populations showed rapid indoxacarb bio-transformation within five min. However, the indoxacarb biotransformation rate of the pre-inhibited (in vivo by DEF) 12,000g midgut supernatant was remarkably decreased in all tested *C. rosaceana* populations at all incubation times compared to the indoxacarb biotransformation rate of the normal 12,000g midgut supernatant. But at the same time a significant reduction in the degradation rate of the DCJW metabolite was observed under the pre-inhibited 12,000g midgut supernatant treatment. This resulted in an accumulation of DCJW and therefore increases the toxicity of indoxacarb.

The current study has revealed that the metabolic balance between the indoxacarb bio-activation (formation of DCJW) rate and detoxification (degradation of DCJW) rate is the core of detoxification mechanism against indoxacarb in *C. rosaceana*. Also, the explanation of the synergistic impact of DEF on the indoxacarb toxicity should be limited to a species level and we agree with Alves et al. (2008) that any kind of generalization in this matter should be avoided.

Further studies are needed to extend our understanding of indoxacarb metabolic resistance and to fully identify the metabolic pathway in insects.

Field implementation of the scientific findings

Apple and cherry field studies, consist of field-based residual bioassay and residue analysis, were conducted to assess the field performance and toxicity longevity of different insecticides, which were associated with significant resistance levels in the tested *C. rosaceana* populations in the baseline study. The tested compounds under the field studies include phosmet, bifenthrin, spinetoram, chlorantraniliprole, indoxacarb and emamectin benzoate.

The documented significant resistance levels in the baseline study resulted in a measurable loss of field performance only in the cases of emamectin benzoate, chlorantraniliprole, and indoxacarb at 7, 21, and all day post-application (DPA) intervals respectively in the apple trials while in cherry trial just indoxacarb case at 7 DPA. In term of residues long lasting, only chlorantraniliprole and indoxacarb maintained measurable leaf residues over all DPA intervals while the leaf residues of the other compounds had degraded within the first 7 days.

While the principles of resistance management would justify efforts to rotate to insecticides with different modes of action, our study suggests that low-level resistance of *C. rosaceana* may not be readily recognized under grower field conditions. Laboratory bioassay can provide important information by detecting the resistance in early stage but it can’t capture the temporal dimension of the field-performance of the tested insecticides without an associated field study.

In addition, tree-injection is a promising technology, environmentally safer, and in general provides longer protection compared to the foliar spray in fruit orchards. Therefore, further studies are needed to expand the tree-injection insecticides list especially those compounds that show low field-performance as a foliar spray and effectively long control as a tree-injection application e.g. emamectin benzoate (the current study, VanWoerkom et al. 2014).

APPENDIX

APPENDIX 1: RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: _2017-07_____

Author:

Abdulwahab Mohammed Hafez

Title of thesis:

OBLIQUEBANDED LEAFROLLER (LEPIDOPTERA: TORTRICIDAE) RESISTANCE TO INSECTICIDES IN MICHIGAN APPLE AND CHERRY ORCHARDS

Museum(s) where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Family	Genus-Species	Life Stage	Quantity	Preservation
Tortricidae	*Choristoneura rosaceana*	adult	10♀ 10♂	pinned

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