2015

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Isaac O. Oyediran
Syngenta Crop Protection, LLC, Research Triangle Park, NC, isaac.oyediran@syngenta.com

Phillip Matthews
Syngenta Crop Protection, LLC, Research Triangle Park, NC

Narendra Palekar
Syngenta Crop Protection, LLC, Research Triangle Park, NC

Wade French
USDA ARS, North Central Agricultural Research Laboratory, Brookings, SD

Jared Conville
Syngenta Crop Protection, LLC, Research Triangle Park, NC

See next page for additional authors

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Oyediran, Isaac O.; Matthews, Phillip; Palekar, Narendra; French, Wade; Conville, Jared; and Burd, Tony, "Susceptibility of northern corn rootworm Diabrotica barberi (Coleoptera: Chrysomelidae) to mCry3A and eCry3.1Ab Bacillus thuringiensis proteins" (2015). Publications from USDA-ARS / UNL Faculty. Paper 1537.
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SHORT COMMUNICATION

Susceptibility of northern corn rootworm *Diabrotica barberi* (Coleoptera: Chrysomelidae) to mCry3A and eCry3.1Ab *Bacillus thuringiensis* proteins

Isaac O. Oyediran¹, Phillip Matthews¹, Narendra Palekar¹, Wade French², Jared Conville¹ and Tony Burd¹

¹Syngenta Crop Protection, LLC, Research Triangle Park, NC 27709, USA and ²USDA ARS, North Central Agricultural Research Laboratory, Brookings, SD 57006, USA

Abstract The susceptibility of the northern corn rootworm *Diabrotica barberi* (Smith & Lawrence) to mCry3A and eCry3.1Ab proteins derived from *Bacillus thuringiensis* (Bt) was determined using a diet bioassay. Northern corn rootworm neonates were exposed to different concentrations of mCry3A and eCry3.1Ab, incorporated into artificial diet. Larval mortality was evaluated after 7 d. The mCry3A and eCry3.1Ab proteins were found to be toxic to the northern corn rootworm larvae. The LC₅₀ and LC₉₀ values for mCry3A were 5.13 and 2482.31 μg/mL, respectively. For eCry3.1Ab, the LC₅₀ and LC₉₀ values were 0.49 and 213.01 μg/mL. Based on the estimated lethal concentrations, eCry3.1Ab protein was more efficacious to northern corn rootworm larvae than mCry3A. These lethal concentration values will be used as diagnostic doses for routine annual monitoring for change in susceptibility of field collected northern corn rootworm to mCry3A, and eCry3.1Ab toxins.

Key words *Bacillus thuringiensis*; *Diabrotica barberi*; eCry3.1Ab; mCry3A; susceptibility

Introduction

The western *Diabrotica virgifera virgifera* LeConte and northern corn rootworms, *Diabrotica barberi* Smith & Lawrence (Coleoptera: Chrysomelidae), are major pests of maize, *Zea mays* L., in certain regions of the U.S. Corn Belt, causing damage to maize by feeding directly on the roots (Chiang, 1973). The soil-inhabiting larval stage is essentially monophagous on corn roots, but also can survive on other grass species (Oyediran et al., 2004, 2008). Because the damage by the corn rootworm complex has such a negative impact on corn production, several management tactics, such as soil-applied insecticides, crop rotation, and foliar sprays (to reduce beetle populations) are used to control these pests (Krysan & Miller, 1986). However, extensive use of these tactics has resulted in the selection of insect resistance to several insecticides in the western corn rootworm (Ball & Weekman, 1962; Meinke et al., 1998; Wright et al., 2000). Crop rotation also has selected for northern corn rootworm individuals that have an extended egg diapause and can overwinter two or more years (Krysan et al., 1984; Krysan & Miller, 1986). The occurrence of prolonged diapause in *D. barberi* eggs helps account for larval root damage observed in first-year cornfields (Bigger, 1932; Chiang, 1965, 1973; Krysan et al., 1984, 1986; Levine et al., 1992, 2002; Steffey et al., 1992; Gray et al., 1998; French et al., 2014). The failure of these primary control strategies for the northern and western corn rootworm has created a need that might be addressed by new technologies. Seed companies have developed maize hybrids containing genes from the soil bacterium *Bacillus thuringiensis* C⃝2015 Institute of Zoology, Chinese Academy of Sciences

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Berliner (Bt) that code for production of insecticidal proteins that have high levels of antibiosis to neonates of the rootworm complex. Currently, there are 4 Bt toxins registered in the United States that are active against Diabrotica species and are produced either singly (Cry3Bb1, Cry34/35Ab1, mCry3Aa, and eCry3.1Ab) or in pyramids (Cry3Bb1 + Cry34/35Ab1, mCry3Aa + Cry34/35Ab1, and mCry3Aa + eCry3.1Ab) (USEPA, 2005a, 2010, 2011 and 2014).

The widespread planting of crops genetically engineered to produce these insecticidal toxins places intense selective pressure on pest populations to evolve resistance. For example, in continuous maize fields the western corn rootworm is often managed through planting of Bt maize. And during 2009 and 2010, fields were identified in Iowa in which western corn rootworm imposed severe injury to maize producing Bt toxin Cry3Bb1. Subsequent bioassays revealed Cry3Bb1 resistance in these populations (Gassmann et al., 2014).

Insect resistance management programs have been an integral part of delaying resistance in pest populations to maize plant incorporated protectants (PIPs) since their first U.S. commercial sales in 1996. Prior to the commercial release of a PIP containing maize hybrid, different resistance management tactics were evaluated and proposed. When transgenic corn for rootworm control was first registered in February 2003, an insect resistance management (IRM) plan, based on mandatory refuges, was required by the U.S. Environmental Protection Agency (USEPA, 2005b). The U.S. Environmental Protection Agency (USEPA, 2002) has required a resistance monitoring plan for all of the Bt insect protected products. The ability to effectively detect the evolution of insecticide resistance before a control failure is an integral component of resistance management strategies for transgenic plants that express Bt toxins and a regulatory requirement for registering Bt-expressing corn hybrids in the United States (USEPA, 1998, 2002; ILSI, 1998). The first step in implementing such programs include developing appropriate bioassay techniques and estimating baseline susceptibility to the Bt protein among insect populations across the geographic range of the target species.

Syngenta has received regulatory approvals to sell Agrisure RW (MIR604) and Agrisure Duracade (5307) containing mCry3A and eCry3.1Ab proteins derived from B. thuringiensis (Bt). As a condition of registration by U.S. Environmental Protection Agency, Syngenta is required to conduct annual monitoring of the northern corn rootworm to determine change in susceptibility of the insect to the 2 Bt toxins. The objective of this study was to determine the susceptibility of northern corn rootworm to mCry3A and eCry3.1Ab proteins.

Materials and methods

Insects

In this study, because of the difficulty in collecting field populations of northern corn rootworm, we used laboratory colony of northern corn rootworm to study the susceptibility of these insects to 2 proteins mCry3A and eCry3.1Ab. Northern corn rootworm eggs were obtained from a 1-year diapausing laboratory population at the USDA, ARS, North Central Agricultural Research Laboratory in Brookings, SD. The original beetles were collected near Brookings in 1996 and complete 1 generation each year. These beetles are reared similarly to D. v. virgifera LeConte (Jackson, 1986; Branson & Jackson, 1988; Branson et al., 1988; Kim et al., 2007; French & Hammack, 2010) except the egg dishes contain several small, 0.25–1.0 cm soil clods in addition to the 80 mesh soil. Upon arrival, the eggs were incubated at 28 ± 2 °C and 60% RH for 20 d to initiate hatching. The neonate larvae that hatched from the eggs were used for the diet bioassay. Because egg hatching is not as synchronized, perhaps relating to quantitative genetic variation of the diapause trait (French et al., 2014), in northern corn rootworm as it is with the western corn rootworm, the eggs did not hatch at the same time making it difficult to get enough larvae to run more replicates of the diet assay at the same time.

Due to the aforementioned difficulty of timing of egg hatch of this species, the laboratory colony was utilized instead of the field collected populations.

Source of protein

The Cry proteins, mCry3A and eCry3.1Ab were produced in recombinant Escherichia coli culture, and were provided by Syngenta Crop Product Safety (Research Triangle Park, NC, USA). The initial stock solution of mCry3A protein was prepared by solubilizing in purified water, 10% ethanol, 50 mmol/L Tris, 2 mmol/L EDTA, pH 10.5 buffer. The stock solution for eCry3.1Ab was prepared by solubilizing the protein in 10 mmol/L ammonium bicarbonate, purified water, 10% ethanol, 10 mmol/L Tris, 0.4 mmol/L EDTA, 0.1% Tween 20, pH 10 buffer. Dilutions were made in 50 mmol/L Tris, 2 mmol/L EDTA, pH 10.5 buffer and 10 mmol/L ammonium bicarbonate, pH 10 buffer for mCry3A and eCry3.1Ab. Lyophilized protein powder for mCry3A and eCry3.1Ab was added to the buffer, to make a stock solution of 2000 μg/mL for mCry3A and 1000 μg/mL. The resulting stock solutions were used in making dilutions for the diet assays.
Bioassay

The standard diet incorporation method was used to measure the susceptibility of northern corn rootworm to mCry3A and eCry3.1Ab toxins. A range of ten concentrations of lyophilized protein powder of mCry3A (0, 0.4, 1, 5, 10, 50, 100, 300, 600, and 1000 μg/mL), and 9 concentrations of eCry3.1Ab (0, 0.015, 0.03, 1, 5, 10, 25, 50, and 300 μg/mL) were incorporated into diet. The artificial diet used in all bioassays was prepared as described by Marrone et al. (1985), and antibiotics were added as described by Chen and Stacy (2006). For diet incorporation assays, the Cry proteins were individually suspended in buffer solutions. To obtain final concentrations for the 2 toxins, 500 μL of protein sample was mixed with an equal volume of 2× molten diet and mixed to obtain a final concentration of 1000 μg/mL as the highest dose for mCry3A and 300 μg/mL for eCry3.1Ab, 1 mL amount of the different concentrations of mCry3A and eCry3.1Ab diets were dispensed into each 47-mm sterilized Petri dishes (Millipore, Billerica, MA, USA) representing all the concentrations tested and allowed to dry. Ten to 12 neonate northern corn rootworm larvae (<24-h old) were placed in each Petri dish. The bioassay dishes were held in an environmental chamber maintained at 28 ± 2 °C, 60% RH, and a photoperiod of 16 : 8 (L : D) h. Larval mortality for each dish recorded on the 7th day after inoculation.

Data analysis

Bioassays were conducted in duplicate on 3 different dates; mortality and the 50% lethal concentration (LC50) and 99% LC99 were determined using the Environmental Protection Agency, Probit Analysis Software (version 1.5; Washington, DC, USA).

Results and discussion

The susceptibility of northern corn rootworm neonates exposed to proteins of mCry3A and eCry3.1Ab are presented in Table 1. The LC50 value for mCry3A was 5.13 μg/mL, and the LC99 value was 2482.31 μg/mL (Table 1). The LC50 value for eCry3.1Ab was 0.49 μg/mL, and the LC99 value was 213.01 μg/mL (Table 1).

The susceptibility of the northern corn rootworm to the mCry3A and eCry3.1Ab derived from B. thuringiensis (Bt) varied significantly. The eCry3.1Ab toxin was more toxic to northern corn rootworm larvae than mCry3A based on the estimated lethal concentrations from our diet incorporated assays. These lethal concentration values will be used as diagnostic doses for routine monitoring of change in susceptibility of northern corn rootworm to mCry3A and eCry3.1Ab. The average percent mortality among northern corn rootworms treated with mCry3A ranged from 15% to 85%, and the mortality of larvae exposed to eCry3.1Ab ranged from 16% to 98%. The average percent mortality for the larvae in the control treatments was 16%.

For resistance management programs to be effective; monitoring, surveillance, and early detection of resistance are important prerequisites. Regular monitoring for resistance development helps to detect the emergence of resistant phenotypes in order to initiate timely remedial measures. Resistance monitoring also enables the evaluation of the effectiveness of resistance management strategies. Traditionally, log dose probit assays, and recently diagnostic dose assays have been routinely used to monitor development of insect resistance to insecticides (Forrester et al., 1993; Kranthi et al., 1997). The log dose probit assays are used to calculate resistance ratios (LC50 of the field strain ÷ LC50 of the susceptible reference strain), whereas the diagnostic dose assays help to discriminate between resistant and susceptible phenotypes. The LC50 values, and slope estimates can be used to distinguish resistant phenotypes only at a high frequency, and are not adequately sensitive enough to detect lower levels of resistance (Roush & Miller, 1986). Georghiou and Taylor (1976) emphasized the importance of diagnostic dose assays in detection of resistance to insecticides. After examining the genetic and logistical implications of resistance monitoring methods, Roush and Miller (1986) concluded that discriminating dose assays were more efficient than the log dose probit regression assays in monitoring for resistance. Sims et al. (1996) estimated diagnostic doses for Heliothis virescens (Fabricius) and Helicoverpa zea (Bodie). The determination of baseline susceptibility data is the first step in the development of a monitoring program designed to detect changes in susceptibility that may result from repeated and prolonged exposure to Bt toxins. These data may provide information that will allow development of diagnostic bioassays that would be more efficient in detection of resistant populations.

In conclusion, the lethal concentrations determined for mCry3A and eCry3.1Ab to detect change in Bt susceptibility will be used as diagnostic concentrations in monitoring northern corn rootworm resistance in corn growing regions. However, it should be noted that because the toxins are not high dose against the northern corn rootworm, using the LC99 as the diagnostic dose may be difficult to achieve because of the large amounts of purified proteins that would be required for testing large field populations.
Table 1  Susceptibility of northern corn rootworm neonates derived from laboratory population exposed to mCry3A and eCry3.1Ab toxins.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>n</th>
<th>Slope ± SE</th>
<th>LC₅₀ (95% CI)</th>
<th>LC₉₉ (95% CI)</th>
<th>χ²</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCry3A</td>
<td>460</td>
<td>0.86 ± 0.12</td>
<td>5.13 (2.6–8.7)</td>
<td>2482.31 (702.6–22144.1)</td>
<td>9.49</td>
<td>9</td>
</tr>
<tr>
<td>eCry3.1Ab</td>
<td>444</td>
<td>0.88 ± 0.11</td>
<td>0.49 (0.2–0.9)</td>
<td>213.01 (75.9–1155.1)</td>
<td>14.07</td>
<td>8</td>
</tr>
</tbody>
</table>

†Concentrations of mCry3A and eCry3.1Ab that produced 50% mortality by probit analysis.
‡mCry3A and eCry3.1Ab (in μg/mL) incorporated into the diet.

Acknowledgments

We would like to thank Chad Nielson at USDA, ARS, NCARL in Brookings, SD for rearing the corn rootworm eggs. We thank Lauren Shaw of Syngenta Crop Protection, Product Safety for providing the protein used for the study. We also thank our Syngenta colleagues for reviewing this manuscript. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Disclosure

The authors declare that they have no conflicts of interest.

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Accepted May 27, 2015