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Characterization of the Spectrum of Activity of IPD079Ea: A Protein Derived From *Ophioglossum pendulum* (Ophioglossales: Ophioglossaceae) With Activity Against Western Corn Rootworm [*Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)]

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Abstract

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is a major pest of corn in both North America and Europe and as such presents significant challenges for farmers. IPD079Ea protein is encoded by the *ipd079Ea* gene from *Ophioglossum pendulum* (a species of fern) and was found to have activity against western corn rootworm in multiple corn events transformed to express the IPD079Ea protein. In chronic laboratory hazard studies, IPD079Ea protein was fed to eleven species in the order Coleoptera and four species in the order Lepidoptera to assess the spectrum of activity. Activity was observed on certain species of the Chrysomelidae and Coccinellidae families, with western corn rootworm as the most sensitive insect tested. No adverse effects on mortality or other sublethal endpoints were observed on any species within Lepidoptera. Overall, IPD079Ea protein appears not to have broad insecticidal properties and has potential value as an effective trait to control western corn rootworm in agricultural systems.

Keywords: western corn rootworm, spectrum of activity, insecticidal protein, Coleoptera, *Ophioglossum pendulum*

The western corn rootworm, (WCR) (*Diabrotica virgifera virgifera* LeConte), is one of the most significant and influential pests of corn in both North America and Europe. Economic losses associated with WCR in North America alone have been estimated between $1 and $2 billion annually (Gray *et al.* 2009, Wechsler and Smith 2018). As such, management of WCR can significantly influence decision making by farmers such as choice of which crop to plant, when to plant it, and what pest control technologies to use to maintain yield and return on investment. Historically, crop rotations along with deploying soil, seed, and/or foliar applied chemicals, along with the use of genetically modified (GM) corn hybrids, have all been utilized to manage WCR pressure (Prasifka *et al.* 2013, Johnson *et al.* 2017, Pereira *et al.* 2019). Despite the variety of management options, WCR have evolved resistance to crop rotations (Levine *et al.* 2002, Crowder *et al.* 2005) and numerous chemistries and traits (Gassmann et al. 2014, 2019; Pereira *et al.* 2015) particularly in areas where a diversified management strategy was not employed (Fishilevich *et al.* 2016).

More comprehensive and integrated management strategies have been promoted to delay the evolution of resistance in WCR and extend the durability of individual management options when they are deployed collectively (Onstad *et al.* 2003, Gassmann *et al.* 2019). One component of more fully integrated management plans is new modes of action to diversify the toolbox available to growers facing high WCR pressure. Historically, GM corn events have utilized several classes of proteins from *Bacillus thuringiensis* (Bt), to protect plants from WCR damage (Moellenbeck *et al.* 2001, Carriere *et al.* 2020). More recently, other actives targeting WCR

have been developed including several dsRNA events (Baum *et al.* 2007, Bachman *et al.* 2013, Hu et al. 2016, 2019) and one protein from *Pseudomonas chlororaphis*, IPD072Aa (Schellenberger *et al.* 2016, Boeckman *et al.* 2019), and these traits are currently working through global regulatory approval before they can be cultivated commercially. Further complementing these new actives, IPD079Ea protein, encoded by the *ipd079Ea* gene from *Ophioglossum pendulum* (a species of fern), was found to have activity against WCR in multiple corn events transformed to express the IPD079Ea protein (Allen *et al.* 2018).

IPD079Ea protein will diversify the toolbox available to growers in regions impacted by WCR; however, prior to commercial release, an extensive characterization of the potential environmental and safety risks will need to be conducted. The process used to evaluate the potential environmental risks is well established with numerous classes of chemistries and traits having been evaluated under this framework (USEPA 1998, Romeis *et al.* 2013, Layton *et al.* 2015). Further, given the relatively recent advancement of different modes of action noted above, this process has again been evaluated for its robustness to evaluate traits from non-Bt sources and found to be sufficiently flexible and informative for the risk assessment (USEPA 2016). As such, one of the steps in the risk assessment process is to understand the spectrum of activity (Tabashnik 2016, Boeckman and Layton 2017). This information is helpful to better inform the necessity and direction of safety testing with nontarget organisms that provide a beneficial ecosystem service in agroecosystems. Traits with narrow activity profiles practically require fewer hazard studies, whereas other traits with either uncertain modes of action or broader activity profiles may require more hazard studies to reduce uncertainty and better inform the risk assessment (Romeis *et al.* 2013).

Following this framework, the purpose of the current study was to characterize the spectrum of activity of IPD079Ea protein. Given the observed activity against WCR, the target pest of IPD079Ea protein, significant focus was applied to other organisms in the order Coleoptera, as these species are more closely taxonomically related to WCR. Additional testing with surrogate species from four families in the order Lepidoptera were also included as an outgroup and to understand if cross-order activity within Lepidoptera was observed. These data will further inform additional hazard studies with organisms that provide a beneficial ecosystem service in agroecosystems and have a rational exposure pathway identified.

Methods

Test Substance

In order to generate IPD079Ea protein for the studies, the protein was expressed in an *Escherichia coli* protein expression system as a fusion protein with an N-terminal His tag. The tagged protein was purified using Ni-NTA affinity chromatography. The fusion tag was cleaved with thrombin and the thrombin was removed using heparin Sepharose column chromatography. Prior to lyophilization, tangential flow filtration was used to exchange the buffer to 50 mM ammonium bicarbonate. The protein was then lyophilized and stored in a –80°C freezer until use.

The concentration of IPD079Ea protein in the lyophilized powder was determined by amino acid composition analysis. Briefly, three lyophilized protein samples were solubilized in 70% formic acid. Sub-samples were hydrolyzed in 6N hydrochloric acid for 24 h under argon in a 110°C oven. Hydrolyzed samples were diluted and then isotopically labeled amino acid internal standards were added to a final concentration of 0.5 µg/ml. Isotopically labeled amino acid

internal standards were added to amino acid calibration solutions to a final concentration of 0.5 µg/ml. Amino acids in diluted hydrolysate samples and calibration solutions were separated with a Waters Acuity UPLC column (2.1 × 150 mm). The eluent from the column was directed into an electrospray source and analyzed using a Waters Xevo TQ mass spectrometer through multiple reaction monitoring (MRM) for both the non-labeled amino acids and isotopically labeled internal standards. The quantification of the individual amino acids was performed using Waters QuanLynx software. The individual amino acid concentration was used to determine the protein concentration based on the frequency of the specific amino acid in the protein sequence. The concentration of the protein was determined to be 0.63 milligrams of protein per mg of lyophilized powder. The purity of the protein was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which showed > 95% purity on a total protein basis (Supp Fig. 1 [online only]). Additional characterization of IPD079Ea was conducted using western blotting, N-terminal sequencing, intact mass determination and peptide mapping by mass spectrometry, glycosylation staining, and insecticidal activity using WCR. Prior to use in insect bioassays, the lyophilized protein was solubilized in ultrapure water. The protein concentration of the resulting stock solution was determined by amino acid composition analysis.

Bioassay Design and Species Selection

Species were selected for testing based on phylogenetic relatedness to WCR, amenability to laboratory bioassays, availability of specific life stages from commercial vendors, and reproducibility and robustness of each observation endpoint (Table 1). In some cases, established standardized bioassay methods were not available; thus, bioassays were developed in-house and validated prior to study. Due to the known activity of IPD079Ea protein against WCR within the order Coleoptera, primary focus was applied to this order to further characterize the spectrum of activity across different families within Coleoptera. Additionally, four families from Lepidoptera were also tested as an outgroup and to assess for cross order activity.

As the IPD079Ea protein is only active via oral ingestion, the protein was incorporated into artificial diets specific for each insect. IPD079Ea protein stock solution was diluted in ultrapure water and mixed with dry diet ingredients, as described below, to support the growth and development of organisms for the duration of the bioassays. Each insect was provided diet incorporated with multiple concentrations of IPD079Ea as shown in Table 2. All studies were conducted under good laboratory practices 40 CFR part 160 with concentration, stability, and homogeneity verification of the test substance characterized in each bioassay using ELISA. Some diets were prepared in bulk and stored frozen for up to 30 d. If applicable, protein stability in the stored diets was also assessed under frozen storage using both a specific antibody-based detection method such as Western blot or enzyme-linked immunoassay (ELISA), and a bioactivity study with a known sensitive insect to demonstrate that the IPD079Ea protein was present and active at the expected potency after the full duration of storage.

Acceptability criteria for all bioassays were determined prior to initiating each study and required less than or equal to 20% mortality in the bioassay control with the exception of WCR and southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber, Coleoptera: chrysomelidae, SCR), which required less than or equal to 30% mortality due to the greater variation in laboratory studies with these organisms (Ludwick *et al.* 2018). To demonstrate exposure to the test substance through the diet and the sensitivity of each organism tested,

Table 1. Species tested with vendor, bioassay duration, and endpoints observed

Order	Family	Species	Common name (acronym)	Vendor	Bioassay duration (days)	Endpoints
Coleoptera	Chrysomelidae	*Diabrotica virgifera virgifera*	Western corn rootworm (WCR)	Internal colony (Johnston, IA)	7	Survival, weight
	Chrysomelidae	*Diabrotica undecimpunctata*	Southern corn rootworm (SCR)	Crop Characteristics, (Farmington, MN)	7	Survival, weight
	Chrysomelidae	*Leptinotarsa decemlineata*	Colorado potato beetle (CPB)	French Agricultural Research, Inc., (Lamberton, MN)	7	Survival, weight
	Tenebrionidae	*Tenebrio molitor*	Yellow mealworm (MWM)	Carolina Biological Supply (Burlington, NC)	14	Survival, weight
	Tenebrionidae	*Zophobas morio*	Superworm (SWM)	Carolina Biological Supply (Burlington, NC)	14	Survival, weight
	Tenebrionidae	*Tribolium castaneum*	Red flour beetle (RFB)	Carolina Biological Supply (Burlington, NC)	7	Survival, weight
	Coccinellidae	*Epilachna varivestis*	Mexican bean beetle (MBB)	New Jersey Dept. of Ag. (Trenton, NJ)	7	Survival, weight
	Coccinellidae	*Hippodamia convergens*	Convergent lady beetle (CNV)	Carolina Biological Supply (Burlington, NC)	28	Survival, weight, development
	Coccinellidae	*Coleomegilla maculata*	Pink spotted lady beetle (CMAC)	Internal colony (Johnston, IA)	28	Survival, weight, development
	Staphylinidae	*Dalotia coriaria*	Rove beetle (RVB)	Rincon- Vitova Insectaries, (Ventrua, CA)	7	Survival
Lepidoptera	Crambidae	*Ostrinia nubilalis*	European corn borer (ECB)	Internal colony (Johnston, IA)	7	Survival, weight
	Noctuidae	*Helicoverpa zea*	Corn earworm (CEW)	Benzon Research (Carlisle, PA)	7	Survival, weight
	Nymphalidae	*Vanessa cardui*	Painted lady (PL)	Carolina Biological Supply (Burlington, NC)	7	Survival, weight
	Tortricidae	*Cydia pomonella*	Codling moth (CM)	Benzon Research (Carlisle, PA)	7	Survival, weight

a positive control was added to each bioassay. Either boric acid or cryolite served as a positive control as described in Supp Table 1 (online only). Generally, at least 80% mortality associated with the positive control was considered acceptable. Further, to demonstrate the biological activity of the IPD079Ea protein after incorporation into artificial diets, a sensitive insect bioassay was conducted alongside each bioassay. A portion of each diet fed to the test organisms either concurrently with or at the conclusion of each bioassay was combined with WCR diet and fed to WCR. Bioactivity of the IPD079Ea protein in each of the insect diets was confirmed by greater than 80% mortality of WCR in these sensitive insect bioassays.

All bioassays were conducted in small environmental chambers (Percival Scientific, Perry, IA). The specific environmental conditions varied for each organism as noted below. Studies with yellow mealworm (*Tenebrio molitor* Linnaeus, Coleoptera: Tenebrionidae, MWM), *Zophobas morio* Fabricius, Coleoptera: Tenebrionidae (SWM), Mexican bean beetle (*Epilachna varivestis* Mulsant, Coleoptera: Coccinellidae, MBB), and each of the lepidopterans were conducted at 25°C, 65% relative humidity, and no light for 7 d except MWM and SWM which used a 14-d duration to allow time for the positive control to show effectiveness. *Coleomegilla maculata* De Geer, Coleoptera: Coccinellidae (CMAC), and convergent lady beetle (*Hippodamia convergens* Guerin-Meneville, Coleoptera: Coccinellidae, CNV) bioassays were both conducted at 27°C, 65% relative humidity, and 16:8 light:dark h photoperiod. The *Dalotia coriaria* Kraatz, Coleoptera: Staphylinidae (RVB) bioassay was conducted at 25°C, 65% relative humidity, and 16:8 light:dark h photoperiod. WCR and SCR were conditions were 21°C, 65% relative humidity, and no light and the red flour beetle (*Tribolium castaneum* Herbst, Coleoptera: Tenebrionidae, RFB) bioassay was conducted at 30°C, 70% relative humidity, and no light.

Organisms were provided with fresh diets on a frequency based on the stability of the IPD079Ea protein under bioassay conditions and the needs of the insect. Generally, fresh diet was provided every 3 to 4 d with the exception of Colorado potato beetle (*Leptinotarsa decemlineata* Say, Coleoptera: Chrysomelidae, CPB) and MBB which were provided fresh diet every other day. For WCR, SCR, MWM, SWM, MBB, and each of the lepidopterans, diets were prepared fresh using freshly prepared dosing solutions. Diet for CPB was prepared using a dosing solution that was stored for the duration of the bioassay at 4°C with appropriate IPD079Ea protein stability characterization.

Stonefly heliothis diet (SHD, Ward's Science, Rochester, NY) served as a base diet for all insects with the exception of CMAC, CNV, RVB, and RFB. A proprietary mixture of additional ingredients was added to SHD for some insects to promote growth and development of each species. The SHD based diets were mixed with water or a dosing solution containing the IPD079Ea protein prior to feeding. CMAC were fed lyophilized brine shrimp eggs following Li *et al.* (2011), and both RVB and CNV were fed lyophilized *Esphestia kuehniella* Zeller, Lepidoptera: Pyralidae eggs (Beneficial Insectary, Redding, CA). RFB were fed a lyophilized flour-based diet. For those diets that were lyophilized, the IPD079Ea protein was applied to the diet and mixed by hand to a uniform consistency. Then the diet was aliquoted and lyophilized, and after lyophilization, the aliquots were pooled and again mixed by hand and subsequently placed in a −80°C freezer until used in the bioassay.

MWM, SWM, CPB, MBB, and all lepidopteran bioassays were conducted in 12-well Falcon tissue culture plates (Fisher Scientific, Hampton, NH) arranged in a generalized randomized design. A single neonate was placed in each well and the plates were sealed with a heat sealing film with a small hole over each well providing ventilation. The WCR and SCR bioassays were conducted as

Table 2. Concentration and endpoint observations for bioassays with IPD079Ea

Species	Concentration (ppm)	Mortality (%)	P-value	Mean weight (mg)	P-value	Mean days to adult emergence	P-value
Diabrotica undecimpunctata	0	16.7	–	0.616	–		
	100	56.7	0.0014	0.123	<0.001		
	200	86.7	<0.0001	0.08	<0.001		
	400	89.3	<0.0001	0.07	0.003		
	800	100	<0.0001	–	–		
Leptinotarsa decemlineata	0	10.0	–	18.2	–		
	100	13.3	0.5000	18.7	0.5683		
	200	3.4	0.9398	17.0	0.3396		
	400	13.3	0.5000	16.4	0.2699		
	800	13.3	0.5000	18.7	0.5653		
Tenebrio molitor	0	0.0	–	0.897	–		
	100	10.0	0.1186	0.911	0.5882		
	200	10.0	0.1186	0.956	0.8179		
	400	16.7	0.0261	0.948	0.7809		
	800	3.3	0.5000	0.931	0.7054		
Zophobas morio	0	4.17	–	1.27	–		
	100	12.5	0.3043	1.27	0.5181		
	200	8.33	0.5000	1.29	0.5622		
	400	8.33	0.5000	1.38	0.8056		
	800	8.33	0.5000	1.30	0.6016		
Tribolium castaneum	0	0.0	–	0.425	–		
	100	11.5	0.1048	0.443	0.6070		
	200	0.0	1.0000	0.476	0.7860		
	400	6.90	0.2544	0.407	0.3937		
	800	6.90	0.2544	0.333	0.0808		
Epilachna varivestis	0	0.00	–	1.34	–		
	1	3.33	0.5000	1.09	0.038		
	5	0.00	1.0000	0.717	<0.001		
	10	6.67	0.2458	0.564	<0.001		
	50	66.7	<0.0001	0.340	<0.001		
	100	93.3	<0.0001	0.200	0.010		
Hippodamia convergens	0	6.67	—	16.5	–	15.0	–
	100	16.7	0.2119	18.2	0.9559	14.2	0.9381
	200	6.90	0.6811	17.6	0.8711	14.1	0.9512
	400	10.0	0.5000	17.7	0.8797	14.4	0.7697
	800	10.0	0.5000	16.0	0.2874	14.7	0.5072
Coleomegilla maculata	0	3.85	—	14.8	–	13.6	–
	100	17.9	0.1135	14.4	0.2628	13.9	0.0942
	200	6.90	0.5416	14.4	0.2729	14.0	0.0308
	400	3.70	0.7641	15.0	0.5851	13.7	0.1665
	800	10.3	0.3482	14.4	0.2620	14.2	0.0111
Dalotia coriaria	0	16.7	–				
	100	13.3	0.7642				
	200	16.7	0.6347				
	400	13.8	0.7469				
	800	6.67	0.9486				
Ostrinia nubilalis	0	10.0	–	7.0	–		
	100	10.0	0.6646	8.3	0.9791		
	200	0.0	1.000	7.7	0.8964		
	400	10.3	0.6482	8.9	0.9989		
	800	13.3	0.5000	7.2	0.6294		
Helicoverpa zea	0	0.0	–	77.7	–		
	100	3.3	0.5000	93.7	0.9527		
	200	3.6	0.4828	85.3	0.7815		
	400	0.0	1.0000	98.1	0.9836		
	800	6.7	0.2458	82.2	0.6794		
Vanessa cardui	0	13.3	–	24.2	–		
	100	0.0	1.0000	23.5	0.3922		
	200	3.3	0.9739	28.3	0.9280		
	400	10.0	0.7881	23.8	0.4368		
	800	10.0	0.7881	27.9	0.9001		
Cydia pomonella	0	6.7	–	4.89	–		
	100	6.7	0.6940	4.48	0.219		
	200	10.0	0.5000	5.04	0.613		

Table 2. Continued

Species	Concentration (ppm)	Mortality (%)	*P*-value	Mean weight (mg)	*P*-value	Mean days to adult emergence	*P*-value
	400	3.3	0.8814	5.49	0.877		
	800	3.3	0.8814	4.44	0.194		

No observed effect concentration (NOEC) for survival endpoint is noted in bold text.

above except in 24-well tissue culture plates. The CMAC and CNV bioassays were conducted in small Petri dishes (60 × 15 mm, Fisher Scientific) containing a single neonate on day 0 and water source. The dishes were stacked in a randomized complete block design. The RVB and RFB bioassays were conducted in 30-ml plastic cups with lids (Cater Supply Direct, Denton, MD) and provided with an agar moisture source in a randomized complete block design.

Four separate concentration-dependent bioassays were conducted with WCR to characterize the median lethal concentration (LC_{50}) and associated 95% confidence intervals for the target insect. Each bioassay was conducted on different days with separate WCR neonates to better characterize the variability in response of the target insect to IPD079Ea protein. In each bioassay, a target of 30 individual WCR were used per concentration with five IPD079Ea protein concentrations and a negative control (six total treatments). The LC_{50} also provided context for concentrations to use in subsequent testing with the other organisms noted in this study and provided context for concentrations necessary to achieve in the sensitive insect bioassays to confirm activity of IPD079Ea protein.

Statistical Analysis

Statistical analyses were conducted using SAS software, Version 9.4 (SAS Institute Inc., Cary, NC) separately for each bioassay for the response variables shown in Table 1. For the WCR median lethal concentration, a log-logistic regression model was utilized to analyze the dose-response curve data. SAS PROC NLMIXED was used to fit the log-logistic regression model for each independent bioassay and the model parameter estimates and associated standard errors were obtained using maximum likelihood methods. A random effects meta-analysis approach (Normand 1999) was used in SAS PROC MIXED to derive the estimate of the overall mean LC_{50} and associated 95% confidence interval across the independent bioassays. For all other studies, statistical comparisons were made between test diets and the bioassay control diet for each response variable with significance declared if the *P*-value was < 0.05.

For all insects other than WCR, statistical analysis of survival data was conducted using Fisher's exact test (SAS PROC MULTTEST) to determine if the survival observed for each test diet was less than the survival observed with the bioassay control diet.

The statistical analysis methods used for weights of surviving insects were dependent upon validity of statistical assumptions for each data set. For most experiments, a linear model analysis was conducted to test if exposure to the test diet caused growth inhibition as compared to the control diet. Assumptions of independent errors that were normally and identically distributed were confirmed by inspection of residuals from the fitted model. SAS PROC GLIMMIX was used for linear mixed model analysis and to generate estimated treatment means, 95% confidence intervals, and the statistical comparisons between means.

Assessment of the residual plots indicated that the assumptions of the linear model were not satisfied for MBB and SCR weight data; therefore, non-parametric Wilcoxon two-sample tests were conducted to test if exposure to each test diet caused growth inhibition as compared to the control diet. The Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY.

Data for days to adult emergence were not normally distributed; therefore, non-parametric Wilcoxon two-sample tests were conducted to examine if exposure to the test diet caused developmental delay.

Results

IPD079Ea protein concentrations, homogeneity in diet, and stability under bioassay conditions were all confirmed for each diet and bioassay. Confirmation of the bioactivity of IPD079Ea protein in diet was verified by greater than 80% mortality of WCR observed with each study (Supp Table 2 [online only]). Mortality associated with the negative control met the acceptability criterion of ≤ 20% (≤ 30% for WCR and SCR) in each study, (Table 2), and mortality associated with the positive controls also exceeded the 80% acceptability criterion, (Supp Table 1 [online only]), confirming exposure to the test substance via the diet.

Table 2 provides detailed observations on each endpoint collected for each organism; however, a high-level summary is provided below. WCR, the target pest, showed median lethal concentrations ranging from 3.4 ng IPD079Ea/mg diet to 7.9 ng IPD079Ea/mg diet (Table 3) over the course of four separate and independent bioassays, culminating in a summary LC_{50} of 5.6 ng IPD079Ea/mg diet (95% CIs of 2.9–8.3 ng IPD079Ea/mg diet). WCR was the most sensitive insect tested followed by MBB with a survival No Observed Effect Concentration (NOEC) of 10 ng IPD079Ea/mg diet, though weight was significantly lower in the 10 ng IPD079Ea/mg diet treatment compared with the control (*P* < 0.001, Table 2). SCR also showed sensitivity to IPD079Ea protein with greater than 50% mortality observed at a concentration of 100 ng/mg (Table 2). These three species, showing sensitivity to IPD079Ea protein, are pests of agricultural crops, primarily corn for WCR and SCR, and MBB is a pest of various legumes (Dobrin and Hammond 1983).

For all other species tested, there were no effects on mortality or weight associated with IPD079Ea protein at concentrations up to 800 ng/mg except for MWM (Table 2). Observed mortality for MWM exposed to 400 ng IPD079Ea/mg diet reached 16.7% compared with 0% for the negative control which was statistically significantly different (*P* = 0.026). Given there was no dose-dependent response for mortality of MWM exposed to the other IPD079Ea protein concentrations, the observed mortality was below the 20% acceptability criteria for the negative control, and there was no apparent effect on weight of MWM, the result was deemed non-biologically relevant, and no further studies were performed.

Days to adult emergence were observed for CNV and CMAC, both of which are potential predators in agricultural fields. There was no effect of IPD079Ea protein on CNV days to adult emergence; however, CMAC exposed to both 200 and 800 ng IPD079Ea/mg diet were statistically significantly different from the control (*P* = 0.031 and *P* = 0.011, respectively). Given mean adult emergence

Table 3. Western corn rootworm median lethal concentration and slope with associated standard error and 95% confidence intervals

Bioassay	LC$_{50}$ estimate (ng/mg)	Standard error	95% confidence interval	Slope	Standard error	95% Confidence interval
1	3.4	1.7	0.0086–6.7	1.4	0.37	0.68–2.2
2	5.7	2.2	1.3–10	3.7	1.5	0.72–6.6
3	4.7	8.8	0–22	26	920	0–1900
4	7.9	1.8	4.4–11	2.2	0.35	1.6–2.9
Overall	5.6	1.4	2.9–8.3	2.0	0.41	1.2–2.8

in the control was 13.6 d compared with 14 and 14.2 d in the 200 and 800 ng IPD079Ea/mg diet treatments, respectively, (Table 2), this represents roughly a 14-h difference between the treatments and control. As there was no dose-dependent response, no concomitant increase in mortality or decrease in weight, and given the relatively small 14-h difference in adult emergence between the negative control and these treatments, this result was considered non-biologically relevant, and no further studies were performed.

Overall, of the coleopteran families tested, only certain organisms in the family Chrysomelidae and Coccinellidae showed sensitivity to IPD079Ea protein at concentrations up to 800 ng/mg, but not every species tested in those families were affected. Further, there was no indication that IPD079Ea protein had any effect on mortality or weight of the four lepidopteran families tested.

Discussion

The gene encoding for the IPD079Ea protein originated from the fern *Ophioglossum pendulum*. The protein was found to have activity against WCR in multiple corn events transformed to express the IPD079Ea protein (Allen *et al.* 2018). As current commercial GM corn events that provide protection from WCR damage exclusively rely on Cry proteins from the *Bacillus thuringiensis* genome, and as WCR continue to evolve resistance to these proteins, IPD079Ea protein represents an important new tool to maintain corn yields for global grain trade and extend the durability of traits currently on the market. Prior to commercial release; however, an extensive characterization is required of the IPD079Ea protein focused in part on the environmental safety.

Part of that environmental safety characterization includes understanding the activity spectrum of the trait of interest. Over time and with broader use, traits develop a particular familiarity and empirical studies add little new information to the risk assessment for these traits. Such is the case today with traditional Cry proteins mentioned above. However, as technology developers delve into other sources for new actives, laboratory studies focused on characterizing activity spectra are an important part of the risk assessment process (Boeckman and Layton 2017, Roberts *et al.* 2020).

Given that IPD079Ea protein was shown to be active against WCR, it was logical to focus a large part of the spectrum of activity effort on other species in the order Coleoptera, under the hypothesis that related species are more likely to share sensitivity to the IPD079Ea protein than more distantly related species (Romeis *et al.* 2011, Carstens *et al.* 2014). Additional considerations for selection of species used in laboratory studies included: organism (and specific life stage) availability from commercial vendors, established bioassay protocols and methods, an artificial diet into which IPD079Ea protein could be incorporated for oral exposure, and reproducibility and robustness of the endpoints observed with each species meeting generally standard performance criteria for survival over the duration of the study. At this stage in the risk assessment process, protection of valued ecosystem services is not necessarily considered. As such, many of the species used to understand spectrum of activity are typically considered pest species. For instance,

of the species reported here, only CMAC, CNV, and RVB would be considered beneficial species in agroecosystems, with each providing a predatory ecosystem service that would be important to evaluate and preserve in later stages of the full environmental risk assessment. All other species used to evaluate IPD079Ea protein and reported here are either stored grain or agricultural pests and thus, not necessarily a desired species to conserve from an environmental risk assessment context.

Overall, results of the studies detailed here demonstrate that the IPD079Ea protein does not appear to have broad activity against surrogate species from four families within the orders Coleoptera and Lepidoptera. Activity was observed within certain species of the Chrysomelidae family, with WCR showing the greatest sensitivity to the IPD079Ea protein, though not every Chrysomelid was sensitive. CPB showed no adverse effects when exposed to IPD079Ea protein concentrations up to 800 ng/mg. IPD079Ea protein was also active within certain species from the Coccinellidae family. MBB was the second most sensitive species tested with a survival NOEC of 10 ng/mg, yet again not every Coccinellid was sensitive. CNV showed no effect on survival, time to adult emergence, or adult weight at concentration up to 800 ng/mg. CMAC did show modest increases in time to adult emergence at greater concentrations of IPD079Ea protein; however, as the delay was marginal at 14-h difference between the control and IPD079Ea treatment, this observation is not likely to be biologically meaningful particularly under more realistic exposure scenarios resulting from the potential cultivation of corn events expressing IPD079Ea to control WCR. Further information characterizing the concentration of IPD079Ea protein in various tissues from corn events would be helpful to better characterize the potential risk to predatory Coccinellids.

There were no adverse effects observed for any of the surrogate species from four families of Lepidoptera. These lepidopteran surrogates were specifically selected to diversify at the family level the spectra of species tested. Additional testing from a broader array of insect orders will be conducted as part of the full environmental risk assessment; however, from the studies described herein, IPD079Ea protein appears not to have broad spectrum activity across the orders Coleoptera and Lepidoptera. Based on the testing conducted to date, IPD079Ea protein appears to be most potent against its intended target pest, WCR and future characterization with a greater diversity of valued non-target organisms will complement these data to fully evaluate any potential risk associated with cultivation of corn events expressing IPD079Ea protein. As the evolution of resistance to current technologies and practices used to control WCR continues, additional traits such as IPD079Ea protein become ever more important, not only as a new mode of action but also to help extend the durability of those currently commercial technologies.

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Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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