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Research

Ecotoxicology

Characterization of the Spectrum of Insecticidal Activity for IPD072Aa: A Protein Derived from *Psuedomonas chlororaphis* with Activity Against *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)

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Abstract

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) presents significant pest management challenges for farmers in both North America and Europe. IPD072Aa, a protein derived from *Pseudomonas chlororaphis*, has previously been shown to have activity against western corn rootworm. In the current study, the spectrum of activity of IPD072Aa was evaluated in controlled laboratory diet bioassays. IPD072Aa was fed at high concentrations in subchronic or chronic bioassays to 11 different insect species, representing 4 families within Coleoptera, and an additional 4 species representing four families of Lepidoptera. No adverse effects were noted in the Lepidoptera species. Within the order Coleoptera, western corn rootworm was the most sensitive species tested. A range of responses was observed within each of the four families of Coleoptera evaluated that included either no-observed effects or reduced growth, developmental delays, and/or reduced survival. These data will help inform the environmental risk assessment of genetically modified plants that express the IPD072Aa protein for western corn rootworm control.

Key words: western corn rootworm, spectrum of activity, insecticidal protein, Coleoptera, Pseudomonas

The western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte) is one of the most significant pests of corn (*Zea mays* L.) in several countries (Blandino et al. 2017, Campbell et al. 2017) with economic losses estimated to be in excess of $1 billion annually (Gray et al. 2009). WCR larvae feed upon corn root tissue which can lead to significant yield loss driven through impaired physiological processes within the plant and lodging as a result of the loss of root tissue (Campbell et al. 2017, Johnson et al. 2017 and references therein). WCR pressure can significantly influence decisions made by growers, such as their choice of crop, when to plant that crop, and use of various pest control options. Typical WCR control strategies include crop rotation, use of soil, seed, and foliar applied chemicals and/or genetically modified (GM) corn hybrids containing proteins designed to control WCR feeding (Prasifka et al. 2013, Blandino et al. 2017, Johnson et al. 2017) and GM solutions involving dsRNA should be available in the near future (Bachman et al. 2013). Given the historical difficulty controlling this pest and the corn rootworm complex in general (Pereira et al. 2017a,b; Wang et al. 2017), increasingly holistic approaches considering a fully integrated pest management (IPM) strategy have been developed (Kuhlmann and Van der Burgt 1998, Onstad et al. 2003). However, as a component of the broader IPM and insect resistance management strategy, new insecticidal modes of action are needed to diversify the toolbox from which growers choose control options. This is especially important considering the evolution of resistance to crop rotation and chemistries and traits currently on the market (Gassmann et al. 2011, 2014; Pereira et al. 2015; Schellenberger et al. 2016).

The environmental risks of new insecticidal modes of action are assessed prior to commercial use as part of the regulatory review process. The science-based framework used to guide the environmental risk assessment (ERA) process has been used for decades, and numerous classes of control agents have been evaluated under this process (US-EPA 1998, Garcia-Alonso et al. 2006, Romeis et al. 2013, Layton et al. 2015). One important component of the ERA process for new transgenic traits is to characterize the spectrum of insecticidal activity of the trait (Tabashnik 2016, Boeckman and Layton 2017). Information on the spectrum of activity can help drive the necessity and direction of safety testing with nontarget organisms (Bachman et al. 2013). Romeis et al. (2013) suggest for traits with

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well-understood modes of action and known narrow spectrums of activity, relatively fewer hazard studies need be conducted, whereas novel traits may require more hazard studies to sufficiently inform assessment of risk.

In accordance with the above guidance, the purpose of the current study was to characterize the spectrum of activity for IPD072Aa, a protein derived from *Pseudomonas chlororaphis* (Guignard and Sauvageau, Pseudomonadales: Pseudomonadaceae) with activity against WCR (Schellenberger et al. 2016). As *Psuedomonas* has a ubiquitous distribution in soil and a long history of use in agricultural settings as frequent root colonizers and use as biopesticides (Anderson et al. 2018), further characterization in this study focused on the insecticidal spectrum of activity within the order Coleoptera, as well as four families within Lepidoptera. These data additionally help inform further hazard testing with beneficial insects that may be exposed to IPD072Aa in agroecosystems where the trait is expected to be deployed (Bachman et al. 2013).

Materials and Methods

Test Substance

The IPD072Aa protein was expressed in an *E. coli* strain BL21 (DE3) through fermentation as a fusion protein with an N-terminal His tag and a Factor Xa cleavage site. The protein expression was controlled under an inducible promoter and the induction was done with 1mM IPTG at 16°C. The His-tagged protein was purified from the *E. coli* cell lysates using Ni-NTA affinity chromatography. The fusion tag was then cleaved by immobilized trypsin and removed by further Ni-NTA affinity chromatography followed by additional purification with Q Sepharose-based anion exchange column chromatography. Tangential flow filtration was used to exchange the buffer to 50 mM ammonium bicarbonate. The protein was then lyophilized and stored at −80°C.

The concentration of the lyophilized protein was determined by amino acid composition analysis and showed 0.80 mg protein/mg lyophilized powder. The purity was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis, which revealed >95% purity on a total protein basis (Supp. Fig. 1 [online only]). Additional characterization of the protein was conducted using western blotting, N-terminal sequencing, mass spectrometry for intact mass determination and peptide mapping, 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 redetermined by amino acid composition analysis.

Bioassay Design

As is typical for laboratory bioassays with protein actives (Romeis et al. 2011), attention was focused on incorporating the IPD072Aa protein into a suitable diet for each test species. All studies were conducted under good laboratory practices 40 CFR part 160. The IPD072Aa protein concentration, stability, and homogeneity in the test diet(s) were confirmed using western blots (see supplemental details). Absence of IPD072Aa protein in control diet was also confirmed using western blot. In all cases, fresh diets with intended concentrations of IPD072Aa were provided as frequently as the organism would allow without exceeding acceptable levels of control mortality (no more frequently than once per day), or prior to a decline in protein stability under bioassay conditions as measured by loss or weakening protein band intensity on western blots. In most cases, fresh diets were provided at least every 3–4 d and in one case daily (Table 1). Based on the historical performance of each organism in laboratory experiments, bioassay acceptability criteria were established as ≤20% mortality in the negative controls with ≥80% mortality observed with a positive control conducted with each bioassay. These criteria are generally well accepted within invertebrate toxicity testing (Rose 2007, Romeis et al. 2011); however for WCR, the criterion of ≤30% negative control mortality was employed given the reported performance of this species in laboratory bioassays on artificial diet (Ludwick et al. 2018). All test species were exposed to a concentration of 1,000 ng/mg IPD072Aa and in some cases additional treatments of 100 and 500 ng/mg IPD072Aa were used. All concentrations are reported as ng IPD072Aa per mg of diet. Concentrations were selected to exceed a dose causing mortality to the target insect, WCR (Schellenberger et al. 2016 and see below).

Table 1. Species and life stage tested, bioassay durations, frequency of diet replacements, and positive control substances and concentration for bioassays with IPD072Aa

Order	Family	Species	Life stage tested[a]	Bioassay duration (days)	Diet refresh frequency	Positive control and concentration (ng/mg)
Coleoptera	Chrysomelidae	*Diabrotica virgifera virgifera*	Neonate	7	3–4 d	NA[b]
	Chrysomelidae	*Diabrotica undecimpunctata*	Neonate	7	3–4 d	Boric acid (10,000)
	Chrysomelidae	*Leptinotarsa decemlineata*	Neonate	7	3–4 d	Cryolite (5,000)
	Tenebrionidae	*Tenebrio molitor*	Neonate	14	3–4 d	Boric acid (12,500)
	Tenebrionidae	*Zophobas morio*	Neonate	14	3–4 d	Boric acid (12,500)
	Tenebrionidae	*Tribolium castaneum*	Neonate	7	3–4 d	Boric acid (10,000)
	Coccinellidae	*Epilachna varivestis*	Neonate	7	3–4 d	Boric acid (1,250)
	Coccinellidae	*Hippodamia convergens*	Neonate	28	3–4 d	Boric acid (15,000)
	Coccinellidae	*Coleomegilla maculata*	Neonate	28	3–4 d	Cryolite (10,000)
	Coccinellidae	*Cryptolaemus montrouzieri*	Adult	7	3–4 d	Boric acid (37,500)
	Staphylinidae	*Dalotia coriaria*	Neonate	7	daily	Boric acid (37,500)
Lepidoptera	Crambidae	*Ostrinia nubilalis*	Neonate	7	3–4 d	Boric acid (3,750)
	Noctuidae	*Helicoverpa zea*	Neonate	7	3–4 d	Boric acid (3,125)
	Nymphalidae	*Vanessa cardui*	Neonate	7	3–4 d	Boric acid (2,500)
	Tortricidae	*Cydia pomonella*	Neonate	7	3–4 d	Boric acid (5,000)

[a]A target of 30 insects per treatment were used in each bioassay.

[b]No positive control was necessary for the dose–response bioassays conducted with WCR.

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Species Selection

Species were selected for testing with IPD072Aa based on several criteria: relatedness to WCR, established laboratory bioassay methodologies, availability of laboratory reared insects, a known suitable diet, and laboratory performance, and reproducibility of the response variables based on method development experiments prior to study conduct (Table 1). In some cases, no previously established laboratory methodologies using artificial diet existed; therefore, method development prior to experimentation focused on establishing a suitable diet and environmental conditions that enabled robust bioassay performance and establishment of acceptability criteria as noted above. When possible, in addition to recording insect survival, endpoints of weight at the end of the bioassay and/or development time to adult were also recorded for surviving insects. Bioassays were conducted in environmental chambers (Percival Scientific, Perry, IA) with environmental loggers (Onset Computer Corporation, Bourne, MA) providing records of temperature, relative humidity, and light intensity. Temperature, photoperiod, and relative humidity were held constant during the full duration of each bioassay based on previously established performance criteria for each insect. Bioassays were conducted at temperatures ranging from 21 to 30°C, light regime ranged from continuous dark to a photoperiod of 16:8 (L:D) h and relative humidity was generally >65% again based on the performance of each species. Occasional deviations from these metrics occurred while transferring organisms to fresh diets; however, the magnitude and duration of these deviations were very limited. Table 1 provides species specific information on bioassay durations, life stage tested, positive control substances used, and the frequency insects were moved to fresh diets of the appropriate treatment.

Lepidoptera

European corn borer (*Ostrinia nubilalis* Hubner Lepidoptera: Crambidae, ECB), codling moth (*Cydia pomonella* Linnaeus, Lepidoptera: Tortricidae, CM), painted lady (*Vanessa cardui* Linnaeus, Lepidoptera Nymphalidae, PL), and corn earworm (*Helicoverpa zea* Boddie, Lepidoptera: Noctuidae, CEW) were selected to represent four different families within the order Lepidoptera and test for cross-order activity. ECB were sourced from an in-house colony (Johnston, IA). CM and CEW were supplied by Benzon Research (Carlisle, PA) and PL were sourced from Carolina Biological Supply Company (Burlington, NC). The diet for each of the four Lepidopterans consisted of Stonefly Heliothis Diet (SHD, Ward's Science, Rochester, NY). IPD072Aa was diluted in ultra-pure water to the targeted nominal concentration and incorporated into SHD as a liquid dosing solution. The diet was mixed by hand to achieve uniform consistency and provided to organisms housed individually in 12-well Falcon culture plates (Fisher Scientific, Hampton, NH) and then covered with a vented film lidding material. Boric Acid (Sigma–Aldrich, St. Louis, MO) served as a positive control substance for each lepidopteran and was solubilized and mixed into the diet as described for the IPD072Aa protein.

Coleoptera

Eleven different species, representing four different families within Coleoptera were used in laboratory studies to evaluate the spectrum of activity of IPD072Aa. Bioassays with Coleoptera were also initiated with unfed neonates less than 24-h old except for *Cryptolaemus montrouzieri* Mulsant, Coleoptera: Coccinellidae (MBD), which was initiated with adults (Table 1). Bioassays with yellow mealworm (*Tenebrio molitor* Linnaeus, Coleoptera: Tenebrionidae, MWM) and *Zophobas morio* Fabricius, Coleoptera: Tenebrionidae (SWM), both sourced from Carolina Biological, were similar to the conditions described for the lepidopteran insects except the duration was extended to 14 d. Despite WCR showing effects after 3–4 d of exposure to IPD072Aa, bioassays with MWM and SWM were extended as previous studies with these insects had shown a 14-d duration was required for the positive control substance to elicit an organismal response.

SHD served as the primary diet component for WCR, southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber, Coleoptera: chrysomelidae, SCR), Colorado potato beetle (*Leptinotarsa decemlineata* Say, Coleoptera: chrysomelidae, CPB), and Mexican bean beetle (*Epilachna varivestis* Mulsant, Coleoptera: Coccinellidae, MBB) with a proprietary mixture of additional ingredients added for each species. The additional ingredients were added to stimulate feeding and/or allow for biological maturation of these insects. WCR and SCR bioassays were conducted in 24-well Falcon culture plates with one insect per well. WCR were sourced from an internal colony (Johnston, IA) and SCR from Crop Characteristics (Farmington, MN). Four independent dose–response bioassays were conducted with WCR to characterize the median lethal concentration (LC_{50}) and associated 95% CIs. Each bioassay was initiated on different days with different batches of WCR to capture variability in WCR response to IPD072Aa. This initial LC_{50} also provided context for concentrations to use in subsequent testing with other organisms. WCR were exposed to diets containing 2, 4, 8, 16, 32, 64, and 128-ng/mg IPD072Aa.

CPB and MBB bioassays were conducted in 12-well plates as described for the Lepidoptera bioassays, with CPB and MBB obtained from French Agricultural Research, Inc. (Lamberton, MN) and the State of New Jersey Department of Agriculture (Trenton, NJ), respectively.

Red flour beetle (*Tribolium castaneum* Herbst, Coleoptera: Tenebrionidae, RFB) sourced from Carolina Biological Supply were housed individually in 30-ml plastic cups (Cater Supply Direct, Denton, MD) with lids and provided with a lyophilized flour and yeast mixture as the diet. IPD072Aa was first solubilized in ultrapure water, which was then applied to the diet and vortexed to achieve uniform consistency. The prepared diet was then divided into several containers and lyophilized until dry. After lyophilization, the diet was ground and pooled, then aliquoted into single use tubes and stored at −80°C until use.

Lyophilized *Ephestia kuehniella* Zeller, Lepidoptera: pyralidae eggs (Beneficial Insectary, Redding, CA), served as the diet for *Dalotia coriaria* Kraatz, Coleoptera: Staphylinidae (RVB), *Cryptolaemus montrouzieri* (MBD) (Mulsant, Coleoptera: Coccinellidae), and convergent lady beetle (*Hippodamia convergens* Guerin-Meneville, Coleoptera: Coccinellidae, CNV). RVB (Rincon-Vitova Insectaries, Ventura, CA) were exposed to diet containing IPD072Aa in 30-ml plastic cups as described for the RFB bioassay with fresh diet provided daily. MBD (Beneficial Insectary) and CNV (Carolina Biological Supply, Burlington, NC) were both housed individually in 60 × 15-mm Petri dishes (Fisher Scientific) containing the treated diet and a source of moisture. *Coleomegilla maculata* De Geer, Coleoptera: Coccinellidae (CMAC; internal colony) were provided with dosed and lyophilized brine shrimp eggs every 3–4 d as the diet, generally following Li et al. (2011) and housed in Petri dishes as noted above.

Either concurrently with or at the end of each spectrum of activity bioassay, the biological activity of the diets containing IPD072Aa was confirmed by incorporation of those diets into the standard diet for WCR. WCR experiments were conducted for 7-d with survival

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and growth inhibition endpoints confirming the biological activity (Supp Table 1 [online only]).

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% CIs 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 exact test (SAS PROC MULTTEST) to determine whether 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 was dependent upon the number of treatments evaluated. For experiments with only one test entry, if the normality assumption was satisfied by the data distributions of the test and control entries, a two-sample *t*-test (SAS PROC TTEST) was conducted to assess if the test diet caused growth inhibition. If the equality of variance assumption was satisfied by an *F*-test; the *t*-test based on pooled variance across the treatment groups was used. Otherwise, the two-sample unpooled *t*-test for unequal variances (also known as Welch's *t*-test) was used. In cases where more than one treatment was used in the study, a linear mixed model analysis was conducted to test if exposure to the test diet caused growth inhibition. 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% CIs, and the statistical comparisons between means.

Regardless of numbers of treatments, when the normality assumption was not satisfied, nonparametric Wilcoxon two-sample tests were conducted to test if exposure to the test diet caused growth inhibition. The Siegel–Tukey test was conducted to further assess for differences in scale between the two treatments (Gibbons and Chakraborti 1992). The Siegel–Tukey and Wilcoxon two-sample tests were conducted with SAS PROC NPAR1WAY.

Data for days to adult emergence were not normally distributed; therefore, nonparametric Wilcoxon two-sample tests were conducted to examine if exposure to the test diet caused developmental delay. The Siegel–Tukey test was again conducted to further assess for differences in scale as noted above.

RESULTS

Spectrum testing was primarily focused within the order Coleoptera given the demonstrated activity of IPD072Aa on WCR (Schellenberger et al. 2016), though several lepidopterans were also screened to test for cross-order activity. IPD072Aa protein stability, homogeneity in the diet, and biological activity was confirmed in each study (Supp. Table 1 [online only]). Bioassay control mortality met the acceptability criterion of ≤20% (≤30% for WCR) in each study. Positive controls conducted within each individual bioassay also met the acceptability criterion showing at least 80% mortality for each insect except for CEW. Mortality of CEW exposed to the positive control reached 46.7% with a mean weight of 0.3 mg compared with 109 mg for the control organisms. Despite not achieving the previously set criteria of 80% mortality, given the difference in mortality and weight between the control and positive control treatments, the bioassay indicated exposure to the test substance incorporated into the diet. Further, WCR was used as a sensitive insect to demonstrate the bioactivity of the IPD072Aa protein fed to each of the insects above. Mortality of WCR in theses confirmatory bioassays was >75% with each of the diets fed to the other species with <30% WCR mortality in control diets with no IPD072Aa (Supp. Table 1 [online only]).

Table 2 provides detailed observations on each endpoint collected for each organism; however, a high-level summary is provided below. For each lepidopteran tested, there was no statistically significant difference in survival ($P > 0.873$) or weight ($P > 0.070$) between insects fed control diet or 1,000 ng/mg IPD072Aa (Table 2). There was a range of responses observed for the various coleopterans tested.

Within the Chrysomelidae family, WCR was the most sensitive species tested. WCR median lethal concentrations from the four independent bioassays ranged from 12 to 39 ng/mg IPD072Aa with overall value of 26 ng/mg (95% CI: 16–37; Table 3).

The closely related SCR also exhibited sensitivity to IPD072Aa. About 75% survival of SCR was observed at 1,000 ng/mg IPD072Aa; however, reductions in weight were observed and statistically significant at 100, 500, and 1,000 ng/mg ($P < 0.001$). No effects were observed on the survival or growth of CPB at concentrations up to 1,000 ng/mg.

There was a range of responses to IPD072Aa within the Tenebrionidae family. There was no significant effect on survival ($P = 0.755$) or growth ($P = 0.088$) of RFB fed 1,000-ng/mg IPD072Aa. Survival was unaffected in SWM when fed 100, 500, or 1,000-ng/mg IPD072Aa ($P = 0.754$, 0.090, and 0.051, respectively); however, mean weight was statistically significantly lower for SWM exposed to each concentration when compared with the control treatment ($P = 0.003$, 0.011, and $P < 0.001$, respectively). Survival and growth of MWM was unaffected at 100 ng/mg IPD072Aa ($P = 0.365$ and $P = 0.779$, respectively). At a concentration of 500 ng/mg IPD072Aa, a statistically significant difference was observed in weight ($P = 0.006$) but not survival ($P = 0.063$). For MWM fed 1,000-ng/mg IPD072Aa, statistically significant differences were observed for both survival ($P = 0.002$) and growth ($P < 0.001$).

Four different organisms from Coccinellidae were selected for testing with IPD072Aa. Statistically significant effects on survival at 500- and 1,000-ng/mg IPD072Aa ($P < 0.001$) were observed with MBB. Reduced growth was observed in MBB fed 100-ng/mg IPD072Aa ($P < 0.001$). No effects on survival were observed in experiments with CNV exposed to 100- and 500-ng/mg IPD072Aa ($P = 1.000$); however, survival was reduced at 1,000 ng/mg ($P < 0.001$). CNV exposed to 500 and 1,000 ng/mg IPD072Aa showed reduced growth ($P < 0.001$) and required longer to emerge as adults ($P < 0.001$). CNV fed 100-ng/mg IPD072Aa weighed on average 1.1 mg less than control organisms ($P = 0.024$); however, there was no significant increase in days to adult emergence ($P = 0.055$). For CMAC, there was no significant effect of IPD072Aa at 100 ng/mg on survival, growth, or days to adult emergence. Statistically reduced survival was observed in CMAC at the 500 and 1,000 ng/mg concentrations ($P = 0.045$ and 0.006, respectively). CMAC growth was

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Table 2. Concentration and endpoint observations with associated statistical analysis for bioassays with IPD072Aa

Species	Concentration (ng IPD072Aa per mg diet)[b]	Mortality (%)	*P*-value[c]	Mean weight (mg)	*P*-value	Mean days to adult emergence	*P*-value
Diabrotica undecimpunctata	0	3.3	—	0.862	—		
Southern corn rootworm	100	3.3	0.754	0.300	<0.001		
(SCR)	500	17.2	0.090	0.192	<0.001		
	1,000	25.0	0.020	0.170	<0.001		
Leptinotarsa decemlineata	0	0	—	24.6	—		
Colorado potato beetle (CPB)	100	0	1.00	20.8	0.111		
	500	6.7	0.246	25.9	0.665		
	1,000	3.3	0.500	30.3	0.968		
Tenebrio molitor	0	13.3	—	0.858	—		
Yellow mealworm	100	20.0	0.365	0.913	0.779		
(MWM)	500	33.3	0.063	0.665	0.006		
	1,000	50.0	0.002	0.580	<0.001		
Zophobas morio (SWM)	0	3.33	—	1.11	—		
	100	3.33	0.754	0.886	0.003		
	500	17.2	0.090	0.921	0.011		
	1,000	20.0	0.051	0.729	<0.001		
Tribolium castaneum	0	3.57	—	0.370	—		
Red flour beetle (RFB)	1,000	3.57	0.755	0.304	0.088		
Epilachna varivestis	0	3.33	—	1.23	—		
Mexican bean beetle (MBB)	100	16.7	0.097	0.436	<0.001		
	500	96.6	<0.001	0.500	—		
	1,000	100	<0.001	—	—		
Hippodamia convergens	0	0	—	19.7	—	13.8	—
Convergent lady beetle (CNV)	100	0	1.00	18.6	0.024	14.1	0.055
	500	0	1.00	11.3	< 0.001	17.4	< 0.001
	1,000	56.7	< 0.001	8.63	< 0.001	22.3	< 0.001
Coleomegilla maculata	0	6.90	—	12.0	—	14.6	—
(CMAC)	100	6.67	0.707	12.1	0.543	14.7	0.153
	500	26.7	0.045	10.7	0.007	16.6	0.002
	1,000	36.7	0.006	10.4	0.001	15.9	0.009
Cryptolaemus montrouzieri[a]	0	20.0	—	3.02	—		
(MBD)	100	26.7	0.381	2.54	0.025		
	500	13.3	0.851	2.82	0.167		
	1,000	36.7	0.126	3.02	0.468		
Dalotia coriaria (RVB)	0	0	—				
	100	3.33	0.500				
	500	10.0	0.119				
	1,000	6.67	0.246				
Ostrinia nubilalis	0	6.67	—	6.70	—		
European corn borer (ECB)	1,000	0	1.00	6.72	0.510		
Helicoverpa zea	0	0	—	109	—		
Corn earworm (CEW)	1,000	0	1.00	97.5	0.070		
Vanessa cardui	0	16.7	—	18.2	—		
Painted lady butterfly (PL)	1,000	10.0	0.873	21.9	0.914		
Cydia pomonella	0	6.67	—	5.65	—		
Codling moth (CM)	1,000	3.33	0.881	5.58	0.426		

[a] Mean weight measured as gain in weight over the 7-d bioassay duration

[b] Underlined concentration represents No Observed Effect Concentration (NOEC) for the survival endpoint

[c] All p-values are in reference to the control.

significantly reduced (by <2 mg) in the 500 and 1,000 ng/mg concentrations (P = 0.007 and 0.001, respectively) and days to emerge as adults was increased roughly 1–2 d (P = 0.002 and 0.009, respectively). For MBD, there was no statistically significant decrease in survival at any concentration of IPD072Aa tested (P > 0.126) with 27, 13, and 37% mortality in the 100, 500, and 1,000 ng/mg concentrations, respectively. Weight gain was statistically lower for MBD in the 100 ng/mg concentration (P = 0.025); however, no effects were observed at 500 (P = 0.167) and 1,000 ng/mg (P = 0.468) IPD072Aa.

One representative from the Staphylinidae family was tested for potential sensitivity to IPD072Aa. There was no effect on survival for RVB fed 100, 500, or 1,000 ng/mg IPD072Aa as compared with control organisms (P > 0.119).

Discussion

GM crops providing protection from insect damage currently rely exclusively on various Cry proteins derived from the common soil bacterium *Bacillus thuringiensis*. (Berliner, Bacillales: Bacillaceae)

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Table 3. WCR median lethal concentrations (LC_{50}) from four independent bioassays

Bioassay	LC_{50}	SE	95% CI	Slope
1	12	3.9	4.1–19	3.49
2	39	4.7	29–48	8.99
3	30	4.5	21–39	2.76
4	26	3.9	19–34	4.13
Mean	26	5.6	16–37	NA

These Cry proteins have a well-established spectrum of activity (Fabrick et al. 2009, van Frankenhuyzen 2009, Romeis et al. 2011, Li et al. 2014,) due in part to their long history of use in both GM crops and in biopesticide spray formulations. Cry proteins, however, represent only one class of insecticidal proteins that have the potential to control pest damage when deployed in GM crops (Gatehouse and Gatehouse 1998, Anderson et al. 2018). IPD072Aa represents an alternative protein for WCR control and importantly diversifies the landscape of available control options to help growers manage WCR damage in their fields. Spectrum of activity data are important as they provide a context for further safety evaluation. By understanding the specificity of an active, hazard studies conducted with valued nontarget species may be prioritized based on the established context of hazard and an understanding of which species are likely to be exposed to the trait (Romeis et al. 2013). In the current study, spectrum testing was focused primarily within the order Coleoptera to better define activity of IPD072Aa against species related to WCR. In total, four families of Coleopterans were evaluated for sensitivity to IPD072Aa. Additionally, four families from Lepidoptera were tested to broaden the assessment.

Overall, the results from the bioassays show that IPD072Aa activity within the order Coleoptera is restricted to certain species and no activity was observed within the order Lepidoptera. The nature of the effects observed within Coleoptera was generally sublethal in nature at concentrations less than 1,000-ng/mg IPD072Aa except for WCR and MBB, two pests of agricultural crops.

Given the previously demonstrated potency of IPD072Aa against western corn rootworm (Schellenberger et al. 2016), activity was expected within the Chrysomelidae. However, both SCR and CPB exhibited less sensitivity to IPD072Aa than WCR, with reduced growth observed for SCR and no observed effects of IPD072Aa on CPB at the concentrations used in this study.

Within the Tenebrionidae, MWM, SWM, and RFB were all shown to be sensitive to IPD072Aa, though less so than WCR. IPD072Aa elicited no effects in RFB at concentrations tested in this study. Effects observed in SWM were restricted to reductions in growth. For MWM, effects on growth and/or survival were observed at concentrations greater than the WCR LC_{50}.

Representatives of the Coccinellidae showed varying levels of sensitivity to IPD072Aa in this study. MBB was the most sensitive insect tested except WCR. Both WCR and MBB (Nottingham et al. 2016) are pests of various agricultural crops. Ladybird beetles (CMAC and CNV) were both less sensitive to IPD072Aa than WCR and MBB. Reduced survival, growth, and/or increased time to adult emergence was observed at concentrations of ≥500 ng/mg. No biologically relevant effects of IPD072Aa were observed on MBD at concentrations evaluated in this study.

One representative of the Staphilinidae family, RVB, was evaluated in this study and no effects were observed of IPD072Aa up to a concentration of 1,000-ng/mg IPD072Aa. Further, no effects of IPD072Aa were observed at 1,000 ng/mg on several representatives of the order Lepidoptera covering four families.

Overall, from the observations of this study, it appears activity of IPD072Aa is limited to the order Coleoptera. Within the Coleoptera families evaluated, WCR was the most sensitive organism tested followed by MBB, at concentrations approximately 10 times greater than those required to elicit mortality in WCR. Where effects on other species were observed, they occurred only when exposed to greater concentrations of IPD072Aa protein and were generally sublethal in nature (i.e., weight reduction or developmental delays).

These data build upon the results provided in Schellenberger et al. (2016), confirming that IPD072Aa is primarily active against WCR and closely related Coleoptera. The organisms selected for testing at this stage focused primarily on those species related to WCR and species for which laboratory methods existed or could be developed and validated to meet established acceptability criteria. It is important to note that species presented here may not have ecological relevance with regard to the agroecosystem; however, they do provide information on the relative potency characteristics of IPD072Aa, and they provide a basis for guiding further safety testing. For instance, MWM, SWM, and RFB are grain pests not known to inhabit agricultural fields (Ramos-Elorduy et al. 2002, Aribi et al. 2006, Tribolium Genome Sequencing Consortium 2008) and are not typically used as a representative nontarget organism in the safety assessment process. They did however provide several representatives within Tenebrionidae and broadened the understanding of IPD072Aa potency within the order Coleoptera. Conversely, the two ladybird beetles tested, CMAC and CNV, are predators and are commonly found in agroecosystems feeding upon aphids and other prey species (Rodriguez-Saona and Miller 1999, Li et al. 2011); thus, they provide a beneficial ecosystem service. As characterization of IPD072Aa advances, relevant exposure information will be used to generate conservative estimates of ladybird beetle exposure to IPD072Aa and compare that information with the hazard data presented here. Additional testing with other orders will occur as part of the conventional ERA process using representatives of organisms that provide beneficial ecosystem services within agroecosystems.

In conclusion, spectrum of activity testing was conducted in this study and focused on species related to the target pest of interest, WCR. For all organisms evaluated, the observed potency of IPD072Aa, where effects were observed, was less than that for WCR. No effects were observed in representatives from the order Lepidoptera. Within Coleoptera, where sensitivity to IPD072Aa was observed the effects were generally at high concentrations of IPD072Aa and predominantly involved reduced growth and or developmental delays. These data confirm and build upon the early characterization information previously reported for IPD072Aa (Schellenberger et al. 2016) and will help guide testing on species that provide a valued ecosystem service and may be exposed to the trait under relevant agricultural practices.

Supplementary Data

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

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Corrigendum

Corrigendum to "Characterization of the Spectrum of Insecticidal Activity for IPD072Aa: A Protein Derived from *Pseudomonas chlororaphis* with Activity Against *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)"

Correction of "Boeckman C. J., E. Huang, K. Sturtz, C. Walker, R. Woods, and J. Zhang. 2019. Characterization of the Spectrum of Insecticidal Activity for IPD072Aa: A Protein Derived from *Psuedomonas chlororaphis* with Activity Against Western Corn Rootworm [*Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae)]. J. Econ. Entomol."

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This article has been updated with the following corrections:
In the title, "*Psuedomonas*" was corrected to "*Pseudomonas*."
In Table 1, "neonoate" was corrected to "adult" for the lifestage tested for *Dalotia coriaria*.
On page 1192, under the "Coleoptera" heading, *Dalotia coriaria* was added to the list of insects tested as adults.
On page 1193, under the "Statistical Analysis" heading, the first sentence was corrected to refer to "Table 2" instead of "Table 1."
The authors regret these errors.

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