

TIER I TESTING OF Cry1Ab PROTEIN ON *Micraspis discolor* (Fab.) (Coleoptera: Coccinellidae) AND EVALUATION OF ITS FUNCTIONAL RESPONSE TO DIFFERENT PREY TYPES

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ABSTRACT

Micraspis discolor (Fab.) is a ubiquitous and abundant predaceous insect in corn fields. Hazard from its exposure to Cry1Ab protein was evaluated by laboratory toxicity test using both maximum hazard dose and functional response assays. Mortality resulting from exposure to Cry1Ab protein was evaluated using artificial diet assays. Larvae and adults of *M. discolor* did not exhibit significant mortality after treatment for 7 days with Cry1Ab protein with concentration 20x higher than the concentration of Cry1Ab found in Bt corn pollen. The functional response to different prey types was evaluated in the laboratory by offering to *M. discolor*, that has been fed with Bt corn pollen, corn leaf aphid *Rhopalosiphum maidis* Fitch and Asian corn borer *Ostrinia furnacalis* Guenee in varying numbers or densities. The type of functional response reflected in each set of predation data was analyzed by logistic regression followed by curve fitting to model equation describing a particular type of functional response. *M. discolor* exhibited a Type III functional response to all prey types tested which remained unaltered after ingestion of Bt corn pollen. Attack rates for all prey types were density-dependent which is typical of a Type III functional response.

Key words: *Micraspis discolor*, functional response, Bt corn pollen, non target organism

INTRODUCTION

The sustainability of transgenic crops is dependent on many factors, one of which is the effects on nontarget organisms. Nontarget effects can be evaluated through experiments designed to identify hazard, determine level and route of exposure, and assess if hazard constitutes a risk. Risk can be defined as the combined probabilities of exposure and insecticidal effect (Sears et al., 2001). The potential value of a nontarget species as biological control agent can be used as a selection criterion for a risk assessment study (Hilbeck et al., 1998; Down et al., 2000). After the nontarget species has been identified, studies to determine sensitivity to transgenic protein and

assess the risk from its ingestion are required. Schuler et al. (2000) recommends a tiered approach with increasing level of complexity.

The coccinellid beetle *Micraspis discolor* occupies the third trophic level of the food chain in the corn ecosystem. The insect has an important role in regulating the population of herbivorous insect pests. Current commercially propagated Bt corn in the Philippines contains Cry1Ab protein from *Bacillus thuringiensis*. This protein is highly toxic to the Asian corn borer *Ostrinia furnacalis*. *M. discolor* exposure to Bt corn is high because the insect is an omnivore, feeding on both corn pollen and herbivorous insect prey that might have ingested the transgenic Cry protein. *M. discolor* is also the most frequently observed insect predator in corn fields. Tier testing is generally used to assess both nontarget effects of conventional insecticides and the potential of transgenic crops to adversely affect nontarget arthropods. After a risk hypothesis is formulated, toxicity tests to selected nontarget species are conducted within experimental tiers that are started with high dose exposures and proceeding to more realistic scenarios of exposure to transgenic plants if toxicity data exceed specified threshold values (Romeis et al., 2008).

Tier I testing was conducted to determine acute toxicity of Cry1Ab protein to *M. discolor* larvae. In addition, feeding assays were conducted to determine if ingestion of Bt corn pollen will significantly diminish *M. discolor* attack rate and alter the type of functional response to the corn leaf aphid *Rhopalosiphum maidis* Fitch and Asian corn borer *Ostrinia furnacalis* Guenee. Functional response curves refer to the change in the number of preys consumed per predator per unit of time, as a function of prey density (Gitonga et al., 2002). Such curves reveal prey-predator interactions, permitting the prediction of suitability of a predator as a biological control agent (Wiedenmann and Smith, 1997). Holling (1959) considered three major types of functional response: Type I where prey mortality due to predation is constant, Type II where prey mortality declines with prey density and Type III where prey mortality increases with prey density.

METHODOLOGY

Tier 1 testing. Feeding assays were carried out with both adults and immatures of *M. discolor* to estimate the maximum hazard dose. Feeding rate was indicated as the maximum hazard dose of 20x the maximum Cry1Ab protein concentration in YieldGard® Bt corn pollen. The estimated concentration of Cry1Ab protein in YieldGard® Bt corn pollen is 45 ppm. *M. discolor* adults were forced-fed (droplet feeding) with the solution of activated Cry1Ab protein (900 ppm) in 50% honey solution. *M. discolor* 4th instar larvae were fed with solution of activated Cry1Ab

protein mixed with artificial diet purchased from BioServ, Inc., USA. Two positive controls were provided to ensure treatment sensitivity of the bioassay setup. The first positive control was provided by feeding neonate *O. furnacalis* with artificial diet mixed with solution of Cry1Ab protein. This assay would confirm the integrity of the insecticidal Cry1Ab protein. The second positive control was provided by feeding *M. discolor* larvae and adults with potassium arsenate mixed with artificial diet. Neonate larvae of *O. furnacalis* were also treated with potassium arsenate mixed with corn borer artificial diet. This assay would confirm that the test substance was ingested by the test insect. Mortality data from all bioassays were recorded after 5 days. Purified activated Cry1Ab protein was provided by Monsanto, USA. Significant difference between treatments was determined by analysis of variance (ANOVA).

Functional response assays. Two sets of treatments were used for the feeding assays. One set consisted of individual 4th instar larvae of *M. discolor* separately fed with different densities of adult corn leaf aphid *Rhopalosiphum maidis* and neonate larvae of Asian corn borer *Ostrinia furnacalis*. Each density of prey type was contained in a glass test tube sealed with cotton plugs. The other set consisted only of 4th instar *M. discolor* larvae previously fed with Bt corn Mon810 pollen. After 24 h, prey consumption data were fitted to a logistic equation (Juliano 2001) to determine the type of functional response. The data were fitted to a polynomial function that describes the relationship between N_a/N_o and N_o where N_a is the number of prey consumed and N_o is the number of prey offered to the predator:

$$N_a/N_o = \exp(P_o + P_1N_o + P_2N_o^2 + P_3N_o^3) / (1 + \exp(P_o + P_1N_o + P_2N_o^2 + P_3N_o^3))$$

where N_a/N_o is the proportion of prey eaten and N_o is the number of prey offered. P_o , P_1 , P_2 and P_3 represent the intercept, linear, quadratic and cubic coefficients, respectively. If $P_1 > 0$ and $P_2 < 0$ (i.e., a negative value), the proportion of prey consumed is positively density-dependent thus describing a type III functional response. If $P_1 < 0$, the data describe a Type II functional response (Juliano 2001). In the second step, the data were fitted to a non-linear equation describing Type III functional response (Hassell 1978):

$$N_e = N_o \{1 - \exp[-(d + bN_o)(T_h * N_e - T)/(1 + cN_o)]\}$$

where N_e is the number of prey attacked, N_o is the initial prey density, T_h is handling time per prey and T is the length of time the predator and prey are exposed to one another (Işikber 2005). The attack rate was calculated using the equations:

$$a = (d + bN_o)/(1 + cN_o)$$

where N_0 is the initial prey density and b , c , d are constants (Schenk and Bacher 2002). Curve fitting was carried out using SigmaPlot 10.

RESULTS AND DISCUSSION

The tiered approach is being advocated as the standard method to evaluate the potential risks of insect-resistant transgenic crops to non-target arthropods (Romeis et al., 2008). A key principle of the approach is that a particular study is conducted only when it will serve to reduce uncertainty in the risk assessment (Romeis et al. 2008). The result of Tier 1 testing is shown in Figure 1. There was no significant difference in mortality between treated and untreated *M. discolor* adults or larvae. Mortalities in the control and treated insects were less than 10%. The lack of significant mortality of *M. discolor* caused by Cry1Ab protein was attributed to the absence of midgut receptors necessary to facilitate the mode of action of the insecticidal protein leading to intoxication (Piggot and Ellar, 2007). Also, the negative Tier I test suggests that it is highly unlikely that field exposure to Cry1Ab Bt corn will cause adverse effects on *M. discolor*. Positive controls were provided in the experiment to ensure that the test protein (i.e., Cry1Ab protein) and bioassay design were properly working. The Cry1Ab protein used in the experiment was highly toxic to *O. furnacalis*. This result suggests that *M. discolor* ingested a functional Cry1Ab protein and a negative response of the insect to the Cry protein cannot be attributed to loss of activity of the Cry1Ab protein.

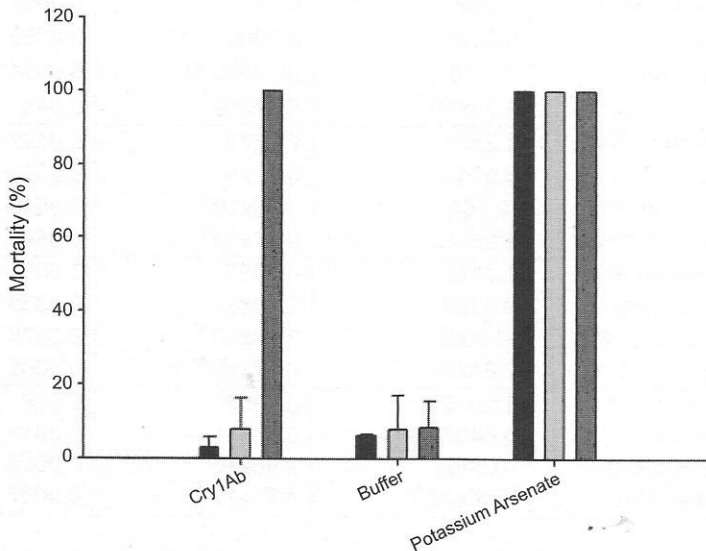


Figure 1. Mortality response of *Micraspis discolor* to Tier I test using maximum hazard concentration of Cry1Ab protein (900 ppm). Difference in treatment means from 3 replicates were not significant as determined by ANOVA ($P > 0.05$). Thirty larvae or adults were tested per replicate. ■, *Micraspis discolor* adults; ■, *Micraspis discolor* 4th instar larvae; ■, *Ostrinia furnacalis* neonate larvae.

Potassium arsenate was also highly toxic to *M. discolor* and *O. furnacalis*. This result also suggests that the route of exposure (oral) chosen to deliver the Cry1Ab protein was working properly.

It has been reported that arthropod predators collected from Bt corn contained significant amounts of transgenic Cry1Ab protein which indicates that long term exposure occurs in the field (Harwood et al., 2005). To the best of the author's knowledge, the effect of Cry1Ab protein exposure on the functional response of arthropod predators has not been reported. Functional response is a key factor that controls the population dynamics of predator-prey systems (Pervez and Omkar, 2005). Predators that exhibit Type III response are considered as efficient biological control agents because they can manage prey population in the field (Fernandez-Arhex and Corley, 2003). Table 1 shows that *M. discolor* exhibits a Type III functional response because the estimated linear and quadratic parameters P_1 and P_2 which are significant ($P < 0.05$) are always > 0 and < 0 , respectively, for all treatments and prey types tested. This observation is in accordance with previous reports on another predatory Coccinellid species (Haji-Zadeh & Biat-Asadi, 1993; Sohrabi & Shishehbor, 2007).

Table 1. Parameter estimates^a for logistic regressions of the proportion of prey killed against the number of prey offered (N_0) for the 4th instar larvae of *Micraspis discolor* for 24 h.

Prey	Parameter	Estimate	SE ^b	P ^c
<i>R. maidis</i>	Constant (P)	1.2991	0.2517	0.0067
	Linear (P1)	0.0278	0.0060	0.0099
	Quadratic (P2)	-0.0001	0.0000376	0.0314
	Cubic (P3)	1.73×10^{-7}	6.17×10^{-8}	0.048
<i>R. maidis</i> + Bt pollen	Constant (P)	1.2374	0.2879	0.0127
	Linear (P1)	0.0243	0.0079	0.0368
	Quadratic (P2)	-0.0001	5.36×10^{-5}	0.0649
	Cubic (P3)	2.26×10^{-7}	9.17×10^{-8}	0.0690
<i>O. furnacalis</i>	Constant (P)	1.3529	0.2859	0.0091
	Linear (P1)	0.0380	0.0063	0.0038
	Quadratic (P2)	-0.0002	3.77×10^{-5}	0.0078
	Cubic (P3)	2.84×10^{-7}	6.07×10^{-8}	0.0095
<i>O. furnacalis</i> + Bt pollen	Constant (P)	1.2578	0.2362	0.006
	Linear (P1)	0.0407	0.005	0.0013
	Quadratic (P2)	-0.0002	2.96×10^{-5}	0.0028
	Cubic (P3)	2.86×10^{-7}	4.71×10^{-8}	0.0037

^a/Parameter estimates were obtained by curve fitting the predation data to the logistic regression equation described by Juliano, 2001.

^bStandard error

^cP is the statistical probability of rejecting the null hypothesis at $\alpha=0.05$. Parameter estimates are significant when $P < 0.05$.

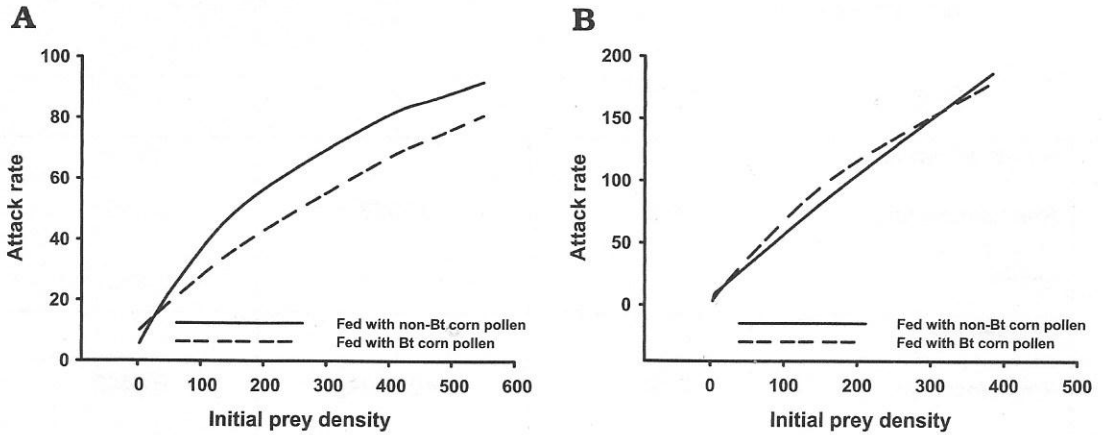


Figure 2. Attack rates of Bt corn pollen-fed 4th instar larvae of *Micraspis discolor* (Fab.) on A) corn leaf aphid *Rhopalosiphum maidis* (Fitch) and B) Asian corn borer *Ostrinia furnacalis* Guenee. Note: Each curve is a mean of three replicates. The curves are not significantly different from each other due to overlapping 95% confidence intervals (data not shown).

Attack rate of *M. discolor* on different prey types was not adversely affected by prior ingestion of Bt pollen (Fig. 2). There was no significant difference in attack rate to both *R. maidis* and *O. furnacalis* by Bt pollen-fed *M. discolor*. Comparing attack rates of *M. discolor* between the two preys, results showed that *O. furnacalis* as prey type elicited higher attack rate response from *M. discolor*. Lower attack rate of *M. discolor* to *R. maidis* might be due to the role of alarm pheromone from this prey resulting in effective predator avoidance (Montgomery and Nault, 1977). Handling time, (T_h) is the time a predator spends in identifying, chasing, killing, eating and other related time-consuming activities (Holling, 1959; Holling, 1966). As shown in Table 2, the handling times (T_h) observed in Bt pollen-fed *M. discolor* preying on *R. maidis* (2.68 h) and *O. furnacalis* (10.56 h) were comparatively shorter than those of the non-Bt pollen-fed predator preying on *R. maidis* (9.05 h) and *O. furnacalis* (20.96 h). Regardless of type of pollen exposure of the predator, T_h was longer in *O. furnacalis* probably due to the more mobile nature of the neonate larvae of this prey. Prey size may also affect handling time (Aljetlawi et al., 2004) but the role of this parameter (i.e. prey size) could not be determined because size difference between the two preys was not measured in this study. A noticeably shorter prey T_h was observed in Bt pollen-fed *M. discolor* larvae.

Table 2. Effect of ingesting Bt corn pollen on prey handling time^{a/} of *Micraspis. discolor* (Fab.) on different prey types.

Prey	T _h (h) ^b	P ^c	r ²
Non-Bt pollen-fed <i>Rhopalosiphum maidis</i>	9.05	0.0032	0.9957
Bt pollen-fed <i>Rhopalosiphum maidis</i>	2.68	<0.0001	0.8442
Non-Bt pollen-fed <i>Ostrinia furnacalis</i>	20.96	0.0043	0.9926
Bt pollen-fed <i>Ostrinia furnacalis</i>	10.56	0.0015	0.9968

^{a/} Parameter estimates were obtained from curve fitting of predation data to the Hassell equation (1978) describing Type III functional response.

^{b/} T_h, handling time.

^{c/} P, statistical probability of rejecting the null hypothesis at $\alpha=0.05$. Parameter estimates are significant when $P < 0.05$.

SUMMARY, CONCLUSION AND RECOMMENDATION

Tier I test was conducted to determine toxicity of Cry1Ab protein to *Micraspis discolor* larvae. Maximum hazard dose of the protein did not significantly result in mortality. Functional response assays of *M. discolor* to corn leaf aphid *Rhopalosiphum maidis* and Asian corn borer *Ostrinia furnacalis* both showed a Type III functional response. Ingestion of Bt corn pollen by *M. discolor* larvae prior to prey feeding did not alter the Type III functional response.

Direct exposure to Cry1Ab protein does not pose a significant hazard to the predatory coccinellid beetle *M. discolor*. Tier testing is a straightforward approach to evaluate potential risk of insect-resistant transgenic crops such as Cry1Ab Bt corn to the predator.

ACKNOWLEDGMENT

The author wishes to thank Ms. Gliceria Hirao and Mr. Ariel Hirao for technical assistance. Funding for this study was provided by Program for Biosafety Systems-USAID.

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