Philipp Ent 30 (2) : 159-170 ISSN 0048-3753 October 2016

BASELINE SUSCEPTIBILITY OF EGGPLANT FRUIT AND SHOOT BORER, *Leucinodes orbonalis* Guenée IN THE PHILIPPINES TO A *Bacillus thuringiensis* CRY1Ac INSECTICIDAL PROTEIN[1]

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[1] Part of the manuscript was presented as poster during the 4th South Asia Biosafety Conference held at Taj Krishna, Hyderabad, India on September 19-21, 2016

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ABSTRACT

The baseline susceptibility of eggplant fruit and shoot borer (*Leucinodes orbonalis)* populations in the Philippines to a *Bacillus thuringiensis* Cry1Ac protein was evaluated using a diet-overlay bioassay. Infested fruits with larvae were collected from nine eggplant growing provinces in the Philippines during the 2012-2013 crop growing seasons. *L. orbonalis* larvae were reared to adults on semi-synthetic diet, allowed to mate in the laboratory and produce eggs. The neonate larvae, from eggs of the field collected parents, were exposed to eight concentrations of Cry1Ac along with an untreated control and then assessed for mortality. The results showed that all *L. orbonalis* populations tested were susceptible to the Cry1Ac protein obtained from a bio insecticide that was used in the assay. Probit analyses of 35 bioassays of EFSB larvae collected from nine provinces showed that the median lethal concentrations (LC_{50}) ranged from 0.45 to 2.07 ng/cm^2 while LC_{95} values ranged from 65.10 to 1501.00 ng/cm^2. Interpopulation variation in susceptibility was 4.6-fold and 23-fold at the LC_{50} and LC_{95} values, respectively. Linear Mixed Model and pairwise mean comparison analysis allowed pooling of the 35 bioassays data into four (4) groups. Effects of season and province for lethal concentrations (LCs) and the slope for each group were insignificant. The median lethal concentrations (LC_{50}) ranged from 0.88 to 1.54 ng/cm^2 and the LC_{95} values ranged from 119.23 – 470.73 ng/cm^{2}which represented a <2-fold and 4-fold variation in susceptibility, respectively. The results obtained in this study will be continued to generate more reliable estimates of the LC_{99} values which can be considered for development of diagnostic concentrations in resistance monitoring.

Key words: baseline susceptibility, Bt Cry1Ac, eggplant shoot and fruit borer, *Leucinodes orbonalis*,

INTRODUCTION

Eggplant is one of the most important vegetables in the Philippines, contributing PHP 2.5 billion annually in agriculture (PSA-BAS, 2015). The primary insect pest of eggplant in the Philippines is the eggplant fruit and shoot borer (EFSB) (*Leucinodes orbonalis* Guenée) (Lepidoptera: Crambidae). EFSB damages the shoots, flowers and fruits resulting in yield loss of up to 80% of the crop (Francisco, 2009).

There is no conventionally-bred commercial variety available with an acceptable level of host plant resistance against this pest. As a result, eggplant is heavily treated with insecticides to control EFSB (Francisco, 2009 & 2014; Quicoy, 2010 & 2014; Chupungco, 2011). Because EFSB is only vulnerable to applications of contact insecticides for few hours after hatching and before the larvae bore into the soft tissues of shoots, flowers and fruits, farmers are forced to spray insecticides as often as every 2-3 days to ensure needed control (Mainali, 2014). A good alternative to chemical insecticides is the use of Bt transgenic plants as demonstrated by the introduction of Bt corn against the Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée) .

In the Philippines, Bt corn has been grown extensively for more than a decade (Aldemita et al., 2015). Recently, Bt eggplants containing event 'EE-1', which expresses a Cry1Ac protein, were developed to control EFSB. Results from field trials of Bt eggplants demonstrated high levels of control of shoot (98.6-100%) and fruit damage (98.1-99.7%) and reduced EFSB larval infestations (95.8-99.3%) (Hautea et al., 2016). Furthermore, studies have shown that Bt eggplant has no significant adverse impacts on species abundance, diversity and community dynamics of beneficial non-target organisms (Navasero et al., 2016.) These results suggest that this technology will benefit farmers and the environment because a significant reduction in insecticide use is expected. However, one of the potential consequences in the cultivation of any Bt crop is the possibility of development of field resistance to the Bt protein by the target insect pest (Bates et al., 2005). Use of Bt sprays, including those containing Cry1Ac protein, are rare on eggplant in the Philippines because of their ineffectiveness, due to their short residual period and lack of good coverage. Thus, there has been little or no prior exposure of EFSB to Cry1Ac, making it a good candidate for a Bt crop.

To promote the durability of Bt eggplant, an Insect Resistance Management (IRM) plan should be in place before commercial cultivation (Bates et al., 2005; Tabashnik et al., 2008; Macintosh, 2009). The IRM strategy includes the development of effective resistance surveillance and/or monitoring programs capable of early detection of resistance development, allowing the implementation of appropriate and timely management decisions (Marcon et al., 1999). The initial steps in implementing such programs include development of appropriate bioassay techniques and establishment of baseline susceptibility data among populations across the geographic range of the target pest species (Siegfried et al., 2000). In the Philippines, similar data on susceptibility of Asian corn borers were generated for monitoring resistance evolution in the pest (Alcantara et al. 2011; Tan et al., 2011). In programs to monitor for resistance after commercialization, bioassay results can be compared with the baseline data to establish whether a significant change in susceptibility has occurred. This will be accomplished through a surveillance program utilizing a continuous, systematized insect collection and performing bioassays and properly interpreting their results.

A few studies have been reported on the baseline susceptibility of *L. orbonalis* populations to Cry1Ac but all of them were conducted only in India (M/S Mahyco et al., 2009; Ghante, 2012; Ranjithkumar et al., 2013; Salunke et al., 2014). Therefore, this study was conducted to determine the baseline susceptibility of nine populations of *L. orbonalis* in the Philippines to the Cry1Ac protein.

MATERIALS AND METHODS

Field collection

Fruits infested with *L. orbonalis* larvae were collected from nine distinct eggplant production areas in the Philippines between 2012-2013 (Table 1, Figure 1). The nine populations originated from eggplant growing provinces in the main island of Luzon: Pangasinan, Isabela, Nueva Ecija, Laguna, Batangas, Quezon and Camarines Sur, and one population each from the Visayas (Iloilo) and Mindanao (Cotabato) regions (Figure 1). A minimum of 250 larvae from infested fruits were collected for each population.

Rearing of test insects

Infested fruits with *L. orbonalis* larvae were transported to the CSC-IPB Entomology Laboratory in the University of the Philippines Los Baños, Laguna. The larvae were reared on a modified soybean-based multiple lepidopteran species diet (Southland Products, Inc., Lake Village, AZ, USA) supplemented with bee pollen and 10% powdered non-Bt eggplant calyx (De Guzman et al., 2012). The field-collected larvae were allowed to pupate then placed in a container until moth emergence. For each colony, five pairs of newly-emerged, healthy adults were placed inside an oviposition cage with a fine nylon net cover and provided with 10% honey solution. The moths were allowed to mate randomly. The fine nylon net cover containing the egg masses was placed inside a plastic container with the lid lined with a paper towel and kept at 25°C and 70% RH until the eggs hatched.

Table 1. *Leucinodes orbonalis* larvae collected from eggplant production areas in the Philippines during 2012-2013 cropping seasons.

Region	Province	Municipality	Geographical Coordinates	Number of EFSB Larvae Recovered
I	Pangasinan	Sta. Maria	15° 58' 51" N, 120° 42' 1" E	1,300
II	Isabela	Echague	16° 42' 9" N, 121° 38' 54" E	250
III	Nueva Ecija	San Leonardo	15° 22' 0" N, 120° 58' 0" E	250
IV-A	Laguna	Los Banos	14° 10' 44" N, 121° 13' 32" E	750
IV-A	Batangas	Balete	14° 1' 47" N, 121° 5' 52" E	250
IV-A	Quezon	Tiaong	13° 57' 13" N, 121° 18' 58" E	300
V	Camarines Sur	Pili	13° 35' 0" N, 123° 18' 0" E	300
VI	Iloilo	Sta. Barbara	10° 49' 23" N, 122° 32' 4" E	250
XII	North Cotabato	Kabacan	7° 7' 0" N, 124° 49' 0" E	300
Total				3,950

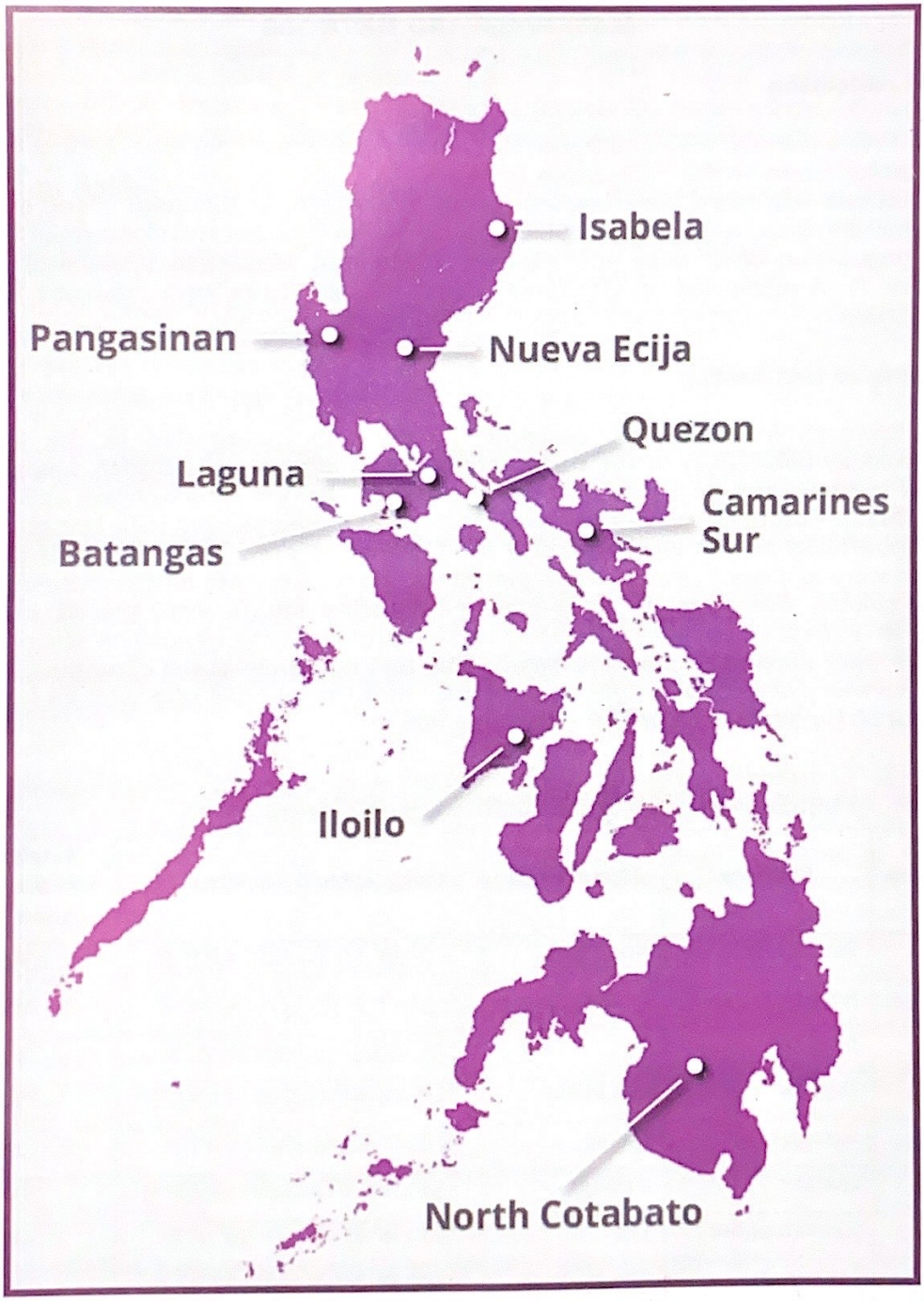

Figure 1. Sampling sites for eggplant fruit and shoot borer (EFSB) populations in the Philippines for baseline susceptibility evaluation.

Preparation of Cry1Ac test concentrations

The commercial bio-insectide (MVP II®, Mycogen, San Diego, CA, USA) (Soares and Quick, 1990) was used as the source of Cry1Ac protein. MVP II® contains 20% (volume/weight) of Cry1Ac and does not contain spores or any other potentially toxic ingredients (Gould et al., 1995). The original stock solution, was prepared from MVP II® following the protocol of Marcon et al. (2000). A volume of 3.125 ml of the washed pellet solution was diluted to a final volume of 100 ml deionized water to make a 6.250 mg/ml solution. This solution was serially diluted to produce eight Cry1Ac test concentrations: 11.504, 2.876, 0.719, 0.179, 0.044, 0.0112, 0.0028 and 0.0007 µg mL-1 (ppm).

Laboratory bioassay and data collection

Assays were conducted by applying the Cry1Ac solution onto the diet surface. EFSB diet was prepared according to De Guzman et al. (2012). Approximately 5.0 ml of the diet was dispensed in a 30-ml plastic cup and was allowed to solidify. A volume of 0.8 ml of the Cry1Ac test solution with 0.1% Triton-X-100 was dispensed uniformly onto the diet surface (7.07 cm^2) and allowed to air-dry for 2-3 hrs inside a laminar-flow cabinet. Control treatment consisted of diet treated with deionized water with 100µl of 0.1% Triton®-X-100 only. Five starved EFSB neonates (<24 hr old) were transferred onto the solidified diet in each of the bioassay cups using a fine-hair brush and then capped. Eight concentrations (1369.472 and 0.083 ng/cm^2 as the highest and lowest concentrations, respectively) of Cry1Ac were used in every assay and each assay was replicated 3-6 times (with 25-50 larvae per replicate assay). All bioassays were conducted in the laboratory and bioassay cups were kept at 25°C and 70% RH. The mortality of the larvae was recorded on seventh day. A larva was considered dead when it did not respond when prodded or when it weighed ≤0.1 mg.

Statistical and Probit analyses

The total number of dead larvae at each concentration (ng/cm^2) was used in the Probit analysis (Finney, 1971) following the PROC Probit procedure by SAS® University Edition (SAS, 2016). The count data were subjected to logarithmic transformation prior to analysis.

A series of linear mixed model (LMM) analyses was performed using SAS® PROC MIXED with assays within provinces as the random model to evaluate whether there was interaction between the factors, season and province for each parameter. The variation among the baseline estimates of slope, LC_{50}, and LC_{95} with season and province as fixed effects was tested for significance at α=0.05 to provide a basis for decisions on pooling data. Pairwise mean comparison using the LSD (least significant difference) test was done among seasons and provinces for each lethal concentration (LC) and slope to group the provinces based on their similarity (=similar letter grouping).

Baseline bioassay data were reanalyzed by combining data sets of all similar EFSB populations tested during 2012-2013 to increase sample size and minimize

fiducial limits after the LMM analysis showed no significant variation across seasons, year and province at $\alpha = 0.05$ for each group of provinces. For each group, the pooled mortality data were analyzed using PROC Probit in SAS, similar to that initially done.

RESULTS AND DISCUSSION

EFSB sample collection from 2012-2013

EFSB larvae were collected from nine eggplant production provinces in the Philippines (Table 1, Figure 1). The largest number of collections was made in Pangasinan because this province accounts for the biggest share (19%) of the eggplant production area in the country (PSA-BAS, 2015). Other provinces in Regions 3, 4, 5 in Luzon, Region 6 in the Visayas and Region 12 in Mindanao were also sampled.

Mortality Response of EFSB to Cry1Ac

The mortality responses for nine EFSB populations exposed to Cry1Ac are presented in Table 2. For all populations, 100% mortality was observed at the highest concentration used in the bioassays. The median lethal concentrations (LC_{50}) ranged from 0.45 to 2.07 ng/cm^2, which is less than a 5-fold variation. The lowest and highest LC_{50} values were observed among Isabela and Batangas populations, respectively. The LC_{95} values obtained ranged from 65.10 to 1501.00 ng/cm^2, which represent a 23-fold variation. The very high numbers and wider variation obtained for the LC_{95} upper fiducial limit could be attributed to the unbalanced number of assays for each season and year for each province.

Determination of Diagnostic Concentrations

The LMM was used to determine whether pooling of mortality data is possible and allows the analysis of unbalanced data. Test for interaction between seasons and provinces ranged from 0.0862 to 0.1276 for $LC_{50\ and}$ LC_{95} and the slope was 0.0964 (α=0.05). Hence, individual fixed effects of year, season and province were tested on all lethal concentrations and the slope. All effects were significant for all parameters except for the year and season at LC_{50}.

Pairwise mean comparisons grouped the provinces into four, namely: Group 1: Batangas, Laguna, Camarines Sur, Iloilo and Cotabato; Group 2: Isabela and Pangasinan; Group 3: Quezon and Group 4: Nueva Ecija. Only assays with chi-square values of $P \geq 0.05$ were included in the pooling. A re-analysis using LMM showed insignificant effects of season and province for lethal concentrations (LCs) and the slope for each group.

After pooling mortality data per group, probit results generated one value for slope, LC_{50}, and LC_{95} per group of provinces (Table 3). The median lethal concentrations (LC_{50}) ranged from 0.88 to 1.54 ng/cm^2 and the LC_{95} values ranged from 119.23 – 470.73 ng/cm^2 which represented <2-fold and 4-fold variation in susceptibility, respectively. All groups had overlapping fiducial limits for all lethal concentrations.

Table 2. Mortality response to Cry1Ac (LC^a, ng/cm^2) of *L. orbonalis* populations collected during 2012-2013.

Year/ Season	Province	Slope ± S.E.	LC_{50} ng/cm^2	95% Fiducial limits		LC_{95} ng/cm^2	95% Fiducial limits		χ^2
				Lower	Upper		Lower	Upper	
2012	Batangas	0.79±0.08	1.23	0.69	2.05	152.35	66.61	496.43	0.45
Dry		0.77±0.07	1.04	0.61	1.65	138.22	64.41	397.20	0.89
	Camarines Sur	0.61±0.08	1.50	0.64	3.04	780.59	228.47	5,483.00	0.31
	Cotabato	0.73±0.08	1.28	0.69	2.19	230.98	95.37	824.62	0.41
		0.78±0.10	1.17	0.58	2.10	145.20	57.09	596.95	0.63
		0.73±0.08	0.99	0.52	1.72	183.60	75.67	664.31	0.98
		0.72±0.08	0.75	0.38	1.32	146.69	59.94	544.20	0.33
		0.82±0.09	1.33	0.78	2.18	131.84	57.02	448.09	0.28
	Laguna	0.82±0.08	1.35	0.84	2.10	138.53	66.52	377.54	0.21
		0.69±0.09	1.50	0.71	2.83	362.85	124.67	1,876.00	0.26
		0.67±0.09	0.72	0.30	1.44	210.50	72.19	1,135.00	0.38
	Pangasinan	0.52±0.11	0.98	0.08	5.02	1,501.00	128.57	1,142,672.00	0.98
		0.54±0.08	0.45	0.08	1.50	538.50	89.31	18,914.00	0.12
		0.60±0.07	0.71	0.29	1.49	399.01	121.80	2,406.00	0.38
	Quezon	0.60±0.09	1.20	0.45	2.62	645.18	171.41	5,916.00	0.67
		0.57±0.10	1.32	0.26	4.16	1,016.00	155.37	64,047.00	0.60
2012	Batangas	0.73±0.09	2.07	1.05	3.77	364.51	132.69	1,674.00	0.04*
Wet	Camarines	0.72±0.09	1.58	0.77	2.92	305.36	110.19	1,440.00	0.19
	Sur	0.78±0.08	1.08	0.60	1.81	140.51	60.96	465.80	0.77
		0.79±0.07	1.13	0.68	1.77	132.57	62.87	369.78	0.63
	Iloilo	0.77±0.08	1.66	0.94	2.76	232.03	99.05	778.15	0.08
		0.80±0.10	1.44	0.75	2.55	160.40	63.93	637.65	0.25
		0.76±0.09	1.33	0.66	2.42	194.95	74.38	838.75	0.39
	Pangasinan	0.62±0.07	0.94	0.41	1.92	417.75	132.19	2,337.00	0.87
	Quezon	0.69±0.09	1.50	0.71	2.84	365.53	125.67	1,888.00	0.26
2013	Pangasinan	0.68±0.10	1.01	0.29	2.83	259.21	57.76	4,077.00	0.98
Dry		0.67±0.11	0.90	0.20	2.90	253.07	48.05	7,108.00	0.83
2013	Isabela	0.66±0.09	0.85	0.23	2.39	263.91	57.79	4,291.00	0.71
Wet		0.67±0.08	1.29	0.61	2.51	361.59	124.35	1,728.00	0.48
		0.71±0.08	0.70	0.33	1.35	146.33	53.53	637.87	0.30
	Nueva	0.80±0.07	1.65	1.01	2.57	189.34	89.90	522.34	0.04 *
	Ecija	0.87±0.08	1.59	1.01	2.42	120.97	60.76	307.80	0.04 *
		0.95±0.11	1.21	0.68	2.02	65.10	29.02	217.65	0.49
		0.89±0.08	1.80	1.16	2.72	128.81	65.34	321.42	0.01
		0.90±0.09	1.32	0.80	2.09	87.33	41.61	248.43	0.26
Magnitude difference of lowest and highest values (x-fold)			4.54			23.06			

[a] LC:lethal concentration of Cry1Ac needed to kill 50 and 95% of test neonate larvae per population during the 7-day observation period
* Significant at $P \leq 0.05$

Table 3. Mortality response to Cry1Ac (LC[a], ng/cm^2) of *L. orbonalis* based on pooled bioassay grouping.

Group	Province	Slope ± SE	LC_{50} ng/cm^2	95% Fiducial Limit		LC_{95} ng/cm^2	95% Fiducial Limit		x^2
				Lower	Upper		Lower	Upper	
1	Batangas Laguna Iloilo Camarines Sur North Cotabato	0.75±0.02	1.23	1.08	1.41	193.48	152.59	250.68	0.99
2	Pangasinan Isabela	0.65±0.04	0.88	0.60	1.26	309.89	170.69	639.34	0.22
3	Quezon	0.65±0.06	1.36	0.77	2.25	470.73	195.39	1590.00	0.41
4	Nueva Ecija	0.87±0.04	1.54	1.25	1.88	119.23	83.80	179.13	1.00
Magnitude difference of lowest and highest values (x-fold)			1.74			3.95			

[a] LC: lethal concentration of Cry1Ac needed to kill 50 and 95% of test neonate larvae per population during the 7-day observation period
[b] Chi square value is significant at $P \leq 0.05$

Similar studies had been conducted, mostly in India, to determine the baseline susceptibility of EFSB (Ghante, 2012; Ranjithkumar et al., 2013; Salunke et al., 2014) to Bt Cry1Ac protein using Bt protein incorporated into the semi-synthetic diet. Those studies demonstrated that *L. orbonalis* is highly susceptible to Cry1Ac. Previously reported LC_{50} values ranged from 0.026 to 0.104 ug/ml for *L. orbonalis* populations from Southern India (Ghante, 2012; Ranjithkumar et al., 2013; Salunke et al., 2014) and LC_{95} values ranged from 0.0440 to 1.543 ug/ml (Ranjithkumar et al., 2013; Salunke et al., 2014). Interpopulation variation in susceptibility for LC_{50} and LC_{95} values (1- to 4-fold variability) were reported for populations in Southern India (Ghante, 2012; Ranjithkumar et al., 2013; Salunke et al., 2014) and a 12-fold variability in LC_{50} values for 29 populations all over India (M/S et al., 2009)

In this study, diagnostic LC_{50} and LC_{95} concentrations were determined for each group. However, because of the very high numbers and high variation observed in the LC_{95} values, it created challenges in developing a diagnostic concentration at this time. More data will be generated in the future so that reliable estimates of the LC values can be developed, and will be considered for development of diagnostic concentrations in resistance monitoring.

Other crambid species like the ACB and the European corn borer (ECB), *Ostrinia nubilalis* (Hübner), have been extensively studied on Bt corn to determine their susceptibility to Bt proteins (Marcon et al., 1999; Sigfried et al., 2000; Alcantara et al., 2011; Tan et al., 2011). Their findings showed the high level of sensitivity of these species and low levels of variability in susceptibility to Cry1Ab. Several workers in various countries have used LC_{99} estimates to establish a diagnostic concentration for monitoring possible development of insect resistance to Bt proteins (Roush and Miller, 1986; Sims et al., 1996; Marcon et al., 2000; Wu et al., 2002; Alcantara et al., 2011). Although the current bioassay-based technique is convenient to use for monitoring changes in susceptibility, for early detection of resistance, it has its limitations and surveillance is preferred (Vennette et al., 2002). However, it continues to be a useful technique and has revealed that ECB populations appear to remain susceptible to Cry1Ab, which has been available to growers in transgenic Bt corn hybrids since 1996 (Siegfried et al., 2006).

CONCLUSION AND RECOMMENDATIONS

The use of Bt technology promises to be an innovative option for effective management of EFSB. The new Joint Department Circular No.1 series of 2016 of the Philippine government that governs the rules and regulations for genetically-modified plant and products derived from modern biotechnology requires technology developers to submit an appropriate IRM plan prior to commercial release of GM crops. It is very important to develop specific IRM strategies for Bt eggplant to prolong product durability and delay the development of rare resistant individuals in the pest population. The collection of baseline data on the susceptibility of EFSB to Bt Cry1Ac represents an important step toward the development of an IRM plan, to support a monitoring program designed to detect changes in EFSB susceptibility to Cry1Ac that may result from repeated and prolonged exposure to Bt eggplant. The results of this study show that prior to commercial cultivation of Bt eggplant, the variability in Cry1Ac susceptibility among widespread geographic populations of EFSB in the Philippines is limited. The LC_{50} values generated for each group of provinces could be used in monitoring (or detecting possible) changes in susceptibility to Cry1Ac of EFSB in Bt eggplants. However, Philippine regulators recommend LC_{99} estimates to establish a diagnostic concentration for monitoring possible development of insect resistance to Bt proteins. Future work should focus on additional collections of EFSB populations from other eggplant productions area and further refinements in the assay procedure including narrower range of test concentrations for the bioassay, so that reliable estimates of the LC_{99} dose can be developed.

ACKNOWLEDGEMENTS

This research was funded thrugh the United States Agency for International Development (USAID) Cooperative Agreement GDG-A-00-02-00017-00 Agricultural Biotechnology Support Project II (ABSPII) and AID-OAA-A-15-00052 Feed the Future South Asia Eggplant Improvement Project) to Cornell University and the matching funds from the Crop Science Cluster-Institute of the Plant Breeding, College of Agriculture, University of the Philippines Los Baños (UPLB). We also acknowledge the assistance of the UPLB Foundation Inc., the executing agency for the ABSPII project in

the Philippines. The authors also gratefully appreciate the reviews by N. Storer, A.M. Shelton and J.E. Huesing. The authors acknowledge the assistance of R.P. Urriza and E.R. Maligalig in insect rearing, diet preparation and bioassay; A.P.L. Masanga, M.G.S. Sagarbarria, J.C. Marasigan and R.N. Candaño in the initial data analyses; and V. Bartolome for the statistical analyses and advice.

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