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Genotyping the Susceptibility of Philippine Geographic Populations of the Diamondback Moth, *Plutella xylostella* Linn. (Lepidoptera: Plutellidae), to Flubendiamide

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The diamondback moth (DBM), *Plutella xylostella*, is considered as the most notorious insect pest species of crucifers due to its rapid ability to develop resistance against various kinds of insecticides, including diamide – a novel class of insecticide that targets the insect ryanodine receptor (RyR). Recently, a diamide-resistant DBM population from Sudlon, Cebu was reported to exhibit an irreversible G4946E mutation in the RyR. Thus, in order to monitor and design an efficient insecticide resistance management strategy for diamide, the relative potency of flubendiamide to DBM populations from various cabbage-growing areas in the Philippines was estimated using the leaf-dip bioassay method. The DBM populations were then genotyped for the presence of target-site mutation G4946E in the RyR using pyrosequencing technology. Our bioassay results revealed the continuing presence of high levels of flubendiamide resistance in the DBM Sudlon strain (LC_{50} = 270.6 ppm). The DBM populations from Majayjay, Laguna were also found to be flubendiamide-resistant (LC_{50} = 218.195 ppm), as well as strains from Calauan, Laguna (LC_{50} = 200.3 ppm), and Buguias, Benguet (LC_{50} = 135.02 ppm). Genotyping results confirmed the presence of the RyR target-site mutation G4946E in these resistant populations. Both Calauan and Cebu strains were 100% homozygous for the resistance allele while the Majayjay strain is composed of 90% and 10% homozygous and heterozygous for the resistance allele, respectively. On the other hand, the Buguias strain exhibited 33% homozygosity and 44% heterozygosity for the resistance allele and 22% homozygosity for the susceptible allele. Our data suggest that diamide resistance allele for RyR mutation G4946E is still spreading, and therefore, requires immediate attention to design efficient insecticide resistance management programs on diamide use against DBM.

Key Words: diamondback moth, flubendiamide, G4946E RyR mutation, insecticide resistance, leaf-dip bioassay method, *Plutella xylostella,* pyrosequencing

Abbreviations: DBM – diamondback moth, gDNA – genomic DNA, HS – susceptible *P. xylostella* strain, IRAC – Insecticide Resistance Action Committee, LC_{50} – the concentration that causes 50% mortality of test subjects, MoA – mode of action, RyR – ryanodine receptor, SNP – single nucleotide polymorphism

INTRODUCTION

Plutella xylostella L., commonly known as diamondback moth (DBM), is the most serious pest of cruciferous vegetables in Southeast Asia (Talekar et al. 1986). In the Philippines, DBM may cause at least 60% yield loss (University of Illinois Extension 2011) to 100% crop loss if not effectively controlled (Morallo-Rejesus and Sayaboc 1990). *P. xylostella* is known as a notorious species to almost all classes of insecticide used for its control, since it can easily adapt to and develop resistance against a new chemistry (Talekar and Shelton 1993; Teixeira and Andaloro 2013). Among the insecticides to which DBM developed resistance are malathion, methyl parathion, DDT, diazinon, mevinphos, dichlorvos, and carbaryl (Barroga and Rejesus 1976); cypermethrin, triazophos, *Bacillus thuringiensis*, cartap, fenvalerate, deltamethrin (Magallona 1986; IRAC 2009); insect growth regulators and fipronil (IRAC 2009); and

recently, flubendiamide (IRAC 2011).

Flubendiamide is the first representative of a novel class of insecticides – the benzene dicarboxamides or phthalic acid diamides (Nauen 2006). It is classified under IRAC Group 28, which acts as ryanodine receptor modulators (IRAC 2016). It is a selective conformation sensitive activator of the insect ryanodine receptor (RyR) in the endo/sarcoplasmic reticulum of nerve and muscle tissue (Cordova et al. 2006; Ebbinghaus-Kintscher et al. 2006; Lümmen et al. 2007; Sattelle et al. 2008). There is an increasing demand for this novel insecticide as diamides had a total global end-user sale share of 8% that amounted to USD 1,411 million in 2013 (Agranova 2014).

However, due to misuse or overuse by farmers, resistance to flubendiamide was reported in a Philippine DBM population 5 years after its registration in 2006. A resistance ratio of >1,000-fold was observed in the DBM population in Sudlon, Cebu (Troczka et al. 2012). Molecular analysis revealed that the resistance was due to a target site mutation at G4946E of the *Plutella* RyR. This mutation resulted in glycine to glutamic acid substitution (Troczka et al. 2012; Steinbach et al. 2015). The mutation was homozygous in nature, similar to that of the DBM strain in Guangdong, Guangxi, and Fujian, China (Wang et al. 2012; Steinbach et al. 2015).

A baseline susceptibility data of an insect species to any novel insecticide is a primary requirement in the formulation of an efficient monitoring system for insecticide resistance prevention, as well as effective insecticide resistance management programs. In addition, baseline susceptibility of an insect species from a broad geographical range provides information regarding the insect natural variation in response to insecticidal action. This information may also aid in predicting whether or not an insect population is more likely to develop resistance to the insecticide. Likewise, any knowledge on the mechanisms of resistance development can help foresee when and where mutation is likely to occur (Constant and Bass 2017).

As of 2009, other than the DBM Sudlon (Cebu) strain, no other local population has been reported to be resistant to flubendiamide. Other areas with vast cabbage production in the country include Ilocos and Benguet in Luzon in addition to Bukidnon and Davao in Mindanao. Diamide overdosing with no insecticide rotation was a common practice in these areas (IRAC International MOA Working Group 2011), thus, prompting the need to assess the possible existence of the *Plutella* RyR resistance allele in these cabbage-growing areas. In addition, although a range of different mechanisms underlying resistance to different classes of insecticide had been described in *P. xylostella* (Noppun et al. 1989; Baxter et al. 2010; Sonoda 2010), very little is known about the mechanism of diamide resistance in lepidopteran pests (Lai et al. 2011; Sial et al. 2011).

This study therefore provides a baseline data on the susceptibility or the resistance of geographically distinct DBM populations to flubendiamide as supported by bioassay and genotype analysis studies. Specifically, this study aimed to: 1) assess the response of DBM to the recommended rate of flubendiamide; b) establish the LC_{50} values of flubendiamide; c) determine the residual toxicity of flubendiamide; d) determine the type of mutation in the identified resistant DBM strains; and e) confirm diamide-cross resistance reported by Troczka et al. (2012) and Steinbach et al. (2015).

MATERIALS AND METHODS

Insects

Representative samples of *P. xylostella* were collected from major cabbage-growing areas in the Philippines. These included populations from the provinces of Laguna (Majayjay and Calauan), Benguet (Buguias) and Cebu (Sudlon) (Fig. 1). The collection sites were composed of three randomly selected sites per locality. Approximately 1,000 individuals composed of last instar larvae and pupae were collected from the field and then reared in the laboratory until they reached the F_1 generation. Insect rearing followed the protocol described by Ebuenga (1992) using cabbage (var. Kale) as host plant. Insect collections were done in the following months and years: Sudlon, Cebu (March 2013); Buguias, Benguet (May 2013); Calauan and Majayjay, Laguna (February 2014), when large areas were planted to cabbage, thus assuring the collection of highly heterogeneous sample populations.

Susceptibility Test

Susceptibility tests were done following the leaf-dip bioassay protocol described in the Insecticide Resistance Action Committee (IRAC) Method No. 007 (IRAC 2011). Commercial formulation of flubendiamide (Fenos™

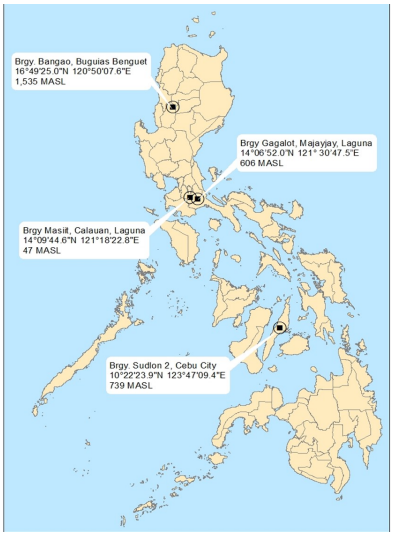

Fig. 1. Selected collection sites for flubendiamide efficacy on *Plutella xylostella*, diamondback moth (DBM), populations from Buguias, Benguet; Majayjay, Laguna; Calauan, Laguna; and Sudlon, Cebu.

480SC; Bayer Philippines) was diluted in aqueous 0.1% Triton X-100 (v/v). Six application rates (100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm and 350 ppm) were used to estimate the LC_{50} values. Leaves were individually dipped in the insecticide solutions, placed on a filter paper in petri plates, and air-dried in a fume hood. The leaves were then infested with 20 third-instar (5- to 6-d-old) larvae. Each concentration was replicated thrice, and three trials were done. Insects were allowed to feed on the leaves at 25 °C, at 60% relative humidity and 16:8 light/dark photoperiods. Larval mortality was recorded 24 h after exposure to the treatments. The leaves that were treated with 0.1% Triton X-100 in sterile distilled water were used as the control group. The susceptibility data for the susceptible *P. xylostella* strain (HS), that was being maintained for 20 years without the use of selection pressure, was provided by Dr. D. Steinbach using the six application rates namely, 0.00256 ppm, 0.0128 ppm, 0.064 ppm, 0.32 ppm, 1.6 ppm, and 8 ppm.

Statistical Analysis

The LC_{50} values and the corresponding 95% confidence limits of the Philippine DBM populations and the HS strain as well as the likelihood ratios were estimated using Polo Plus Probit and Logit Analysis version 2.0 (Le Ora Software Copyright© 2002–2015). Heterogeneity was estimated by dividing the chi-square value by the degrees of freedom (Robertson et al. 2007). The resistance ratio (RR) was calculated by dividing the LC_{50} value of the field populations by the LC_{50} of the susceptible strain (HS) (Troczka et al. 2012).

Pyrosequencing for Single Nucleotide Polymorphism (SNP) Analysis

The genomic DNA (gDNA) of the five populations (15 to 20 individuals per population) was isolated following the protocol of the Animal and Fungi DNA Preparation Kit/ DNA Extraction kit (Jena Biosciences, Germany) and then sent to the Resistant Management Facility at Bayer AG, Division CropScience, Monheim, Germany. The polymerase chain reaction (PCR) condition for pyrosequencing and pyrosequencing were carried out as described below. A 50 ng aliquot of gDNA was used as PCR template for the primer pair 494-for (5′GCCGCTCATCTGTTGGACGT-3′) and 536-rev_btn (5′-[btn] TCCCRTTATGYRTGACRGAC-3′) as recently described in Steinbach et al. (2015). A short gene fragment of 79 bp was amplified using a protocol of 40 PCR cycles with 0.5 μM forward and biotinylated reverse primer in 50 μL reaction mixtures containing 1 × Taq enzyme reaction mix (RedTaq Jumpstart Master Mix, Sigma Aldrich) and cycling conditions of 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C for 45 s, and a final elongation step at 72 °C for 5 min (Steinbach et al. 2015). The pyrosequencing reaction (including the sequence-primer for genotyping) was carried out as described in Troczka et al. (2012). A total of 70 samples composed of 18, 15, 20 and 17 samples for Baguio, Cebu, Majayjay and Calauan, respectively, was analyzed.

RESULTS AND DISCUSSION

Susceptibility Test

The toxicity and relative potency of flubendiamide on the third instar DBM larvae are presented in Table 1. The highest LC_{50} was observed in the Sudlon DBM population, while the lowest was in the Calauan DBM population. The ranking of the populations in decreasing order of resistance was as follows: Sudlon (270.6 ppm) > Majayjay (218.2 ppm) > Calauan (200.37 ppm) > Buguias (135.02 ppm). Consequently, the highest relative potency or resistance ratio observed was in the Sudlon DBM strain, which is 12,885.71-fold higher than that of the susceptible strain, HS (LC_{50} = 0.021 ppm), followed by the Majayjay (>10,390.24 ppm), Calauan, (>9,541.24 ppm), and lastly, Benguet Buguias (>6,429.52 ppm) strains.

Figure 2 shows the graphical representations of the toxicity response of different DBM populations to flubendiamide. The heterogeneity factor of the different DBM populations, in ascending order was: HS (0.029) > Sudlon (0.175) > Buguias (0.385) > Calauan (0.682) > Majayjay (2.9496). Results showed that the Majayjay DBM population had a value greater than 1, indicating that the population did not fit the statistical model (Robertson et al. 2007). Macatula (2010) noted that the conformity of the data to the model is important, since it indicates whether the samples are more or less homogeneous (low value of heterogeneity). The Majayjay population used in the study, based on the heterogeneity value, had a large gene pool variation, suggesting that more assays should be done to generate an acceptable data set.

The slope of the dose-mortality response indicates rejection of the hypothesis of equality. It means that the dose-mortality response lines of the strains used in this study are significantly different at the 0.5 level of significance. On the other hand, the likelihood ratio (LR) test of parallelism, which compares whether or not the slopes of the line are similar (Robertson et al. 2007), indicates that the different DBM populations had relatively the same response to flubendiamide.

A comparison of the baseline susceptibility data from other studies showed that the DBM strains analyzed in this study are highly resistant as reported in the monitoring data of Troczka et al. (2012) and Steinbach et al. (2015). Furthermore, the levels of resistance had increased as indicated by the lower LC_{50} values observed in the DBM strains compared with the population

Table 1. Median lethal concentration estimates (LC_{50}) of flubendiamide against diamondback moth, *Plutella xylostella*, populations (Sudlon, Majayjay, Calauan and Buguias) from selected cabbage-growing areas in the Philippines compared with the susceptible strain (HS). The resistance ratios of *P. xylostella* Philippine populations to flubendiamide were also estimated using HS as the benchmark

Strain/ Population	LC_{50} (95% Confidence Limits)	Slope (± SD)	χ^2	df	Heterogeneity	Resistance Ratio
Susceptible strain (HS)	0.021 (0.017 to 0.028)	2.487 ± 0.367	1.156	4	0.029	-
Sudlon, Cebu	270.6 (242.081 to 312.958)	2.5 ± 0.35	0.698	4	0.175	12, 885.71
Majayjay, Laguna	218.195 (154.874 to 321.472)	2.408 ± 0.338	5.6938	4	2.9496	10, 390.23
Calauan, Laguna	200.366 (175.235 to 227.445)	2.143 ± 0.327	1.54	4	0.682	9, 541.23
Buguias, Benguet	135.02 (111.671 to 153.951)	2.455 ± 0.335	2.728	4	0.385	6, 429.52

Hypothesis of Equality (equal slopes, equal intercepts): REJECTED (P < 0.05) (chi-square: 485, degrees of freedom: 8, tail probability: 0.000)
Hypothesis of Parallelism (equal slopes): NOT REJECTED (P > 0.05) (chi-square: 0.82, degrees of freedom: 4, tail probability: 0.936)

previously assayed by Troczka et al. (2012). This result indicates that a stable resistance is present in the DBM populations collected from the different areas.

Our findings further emphasize the importance of RyR target-site mutation G4946E as it is present in the highly diamide-resistant strains. It is comparable to some of the known recorded incidence that showed rapid insecticide resistance development in diamondback moth, for instance, (1) spinosad resistance development 2 years after it was released in the market in Hawaii (Zhao et al. 2002; Sparks et al. 2012); and (2) chlorantraniliprole resistance development 3 years after commercialization in China (Wang et al. 2012). The observed rapid insecticide resistance in diamondback moth was due to lack of an alternative insecticide that can be used in rotation or the exclusive and continuous use of the selected active ingredient.

The increasing occurrence of insecticide resistance causes the depletion of the available commercial insecticide tools. Discovery of new insecticides for rotational use to delay resistance development entails tedious screening protocols that require high monetary cost (Sparks and Nauen 2015) and time. Thus, an efficient and strictly implemented insecticide resistance program is necessary to prevent the loss of newly discovered insecticides.

Identification and Analysis of the SNPs

Comparison of RyR genotypes. The pyrosequencing diagnostic assay allowed the identification of the three RyR genotypes in *P. xylostella*, either homozygous wildtype, homozygous susceptible, heterozygous wildtype or heterozygous susceptible, based on its observed genotypic frequency and specific amino acid substitution (Table 2). Figure 3 shows the successful detection of

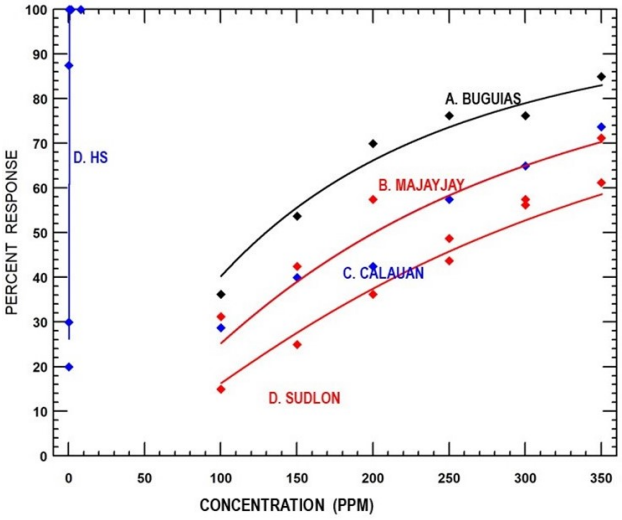

Fig. 2. Probit lines for flubendiamide efficacy on *Plutella xylostella*, diamondback moth (DBM), populations from (A) Buguias, Benguet; (B) Majayjay, Laguna; (C) Calauan, Laguna; (D) Sudlon, Cebu; and (E) HS (sensitive strain).

polymorphism in the codon positioned at G4946E. Genotype variation among the populations was observed considering that the samples were collected within the Philippines with almost the same climatic conditions. The reference strain, HS, exhibited homozygosity for the wildtype allele, GGG – a codon encoding for glycine (Troczka et al. 2012). Surprisingly, among the four DBM strains used in this study, only the Calauan DBM strain exhibited the same genotype and genotypic frequency as the previously studied flubendiamide-resistant Sudlon strain (Troczka et al. 2012). Both DBM strains were 100% homozygous for the GAA-resistant allele, which resulted in glycine to glutamic acid substitution.

Meanwhile, the other DBM populations namely,

Buguias and Majayjay, exhibited a different G4946E genotypic percentage and genotype. The Buguias strain was only 33% homozygous for the resistant allele GAA, 22% homozygous for the wild-type allele GGG, and 44% heterozygous for the wild-type allele GGA. On the other hand, the Majayjay strain had a higher degree of 90% homozygosity for the GAA-resistant allele and a 10% heterozygosity for GAG, a resistant allele that also resulted in substitution of glycine to glutamic acid. Interestingly, our results indicate that the two closely neighboring DBM strains may exhibit genotypic variation as implied by their genotype composition as well as genotypic frequencies.

In addition, Sudlon and Calauan strains, both with G4946E substitution, further confirm the findings of Steinbach et al. (2015), in their analysis of Philippine and Thailand DBM strains, that diamide cross-resistance could have evolved independently in two different geographic locations. The present data also demonstrated the existence of flubendiamide resistance in these four locations where high diamide use was recorded (IRAC 2011).

Meanwhile, although the Buguias DBM was only 33% homozygous for the resistant allele, a very high resistance ratio was observed (> 6,000-fold higher than HS), thus, we suggest a revalidation study. This finding is in contrast with the results of Troczka et al. (2012) on the Thai S strain with homozygous wild-type allele (G4946E) and two heterozygotes for mutant allele (E4946). They noted that mutations at very low frequency would have little or no impact on diamide resistance as revealed in their bioassay studies. Nonetheless, they also suggested that the mutation that occurred in populations from at least two sites in Thailand could eventually spread to adjacent areas. On the other hand, Sukonthabhirom et al. (2011) found different levels of resistance development as measured by the resistance factor (RF) in the DBM Thailand populations such as Sai Noi (RF = 407.2), Tha Muang, Kanchanaburi (RF = 4,817.4) and Lat Lum Kaew, Pathum Thani (RF = 26, 602). Whether or not this phenomenon would also occur in the country still needs to be verified.

Table 2. Genotypic frequency for the ryanodine receptor (*RyR*) G4946E mutation (GGG [Glycin] → GAA or GAG [Glutamic]) in amplified gDNA fragments of *P. xylostella* from selected cabbage-growing areas in the Philippines.

Strain	Genotype Frequency (%)			
	Homozygous		Heterozygous	
	GGG/ GGG	GAA/ GAA	GGA/ GAA	GAG/ GAA
Sensitive strain (HS)	100			
Buguias, Benguet	22	33	44	
Sudlon, Cebu		100		
Gagalot, Majayjay		90		10
Mainit, Calauan		100		

Sensitive wildtype GGG/GGA
Resistant mutation GAG/GAA

Our results further confirm the notoriety of *P. xyllostella* in developing resistance against new insecticidal chemistry. Sukonthabhirom et al. (2011) identified the key factors that led to diamide resistance in Thailand DBM populations, that include the overdependency on a single mode of action, minimal crop rotation due to continuous planting of crucifers, under-dosing of insecticide to save on cost, irrigation practices that led to excessive product wash-off that provided opportunities for insect exposure to sub-lethal doses, and lack of any coherent insecticide resistance management strategies. In addition, Troczka et al. (2017) noticed that Thai farmers used flubendiamide, mixed with other insecticides, more than four to five times per cropping season to simultaneously control DBM and other pests as well as to reduce labor costs on spraying. This heavy use of flubendiamide against DBM created high selection pressure in the field leading to resistance. Aside from farmers' malpractices, the rapid insecticide resistance development observed in DBM may also be due to several inherent biological attributes such as genetic plasticity, rapid generation time, and high fecundity (Troczka et al. 2017).

Any mutation that occurs through a series of steps involving duplication, insertion or deletion will result in new alleles that will increase the resistance or the fitness of the insect (Constant and Bass 2017). In addition, the genotype of the insect will also determine the strength of the resistant gene it carries, giving rise to survival variation of the insect such as those with heterozygous RS male, which had higher mating success compared with those with homozygous (RR) resistant male (Constant and Bass 2017).

Insecticide resistance due to mutations resulting in a change in insect genotype is widely assumed to affect fitness as observed in *Tribolium castaneum* (Arnaued et al. 2002) and *Anopheles gambiae* (Assogba et al. 2016). Those individuals carrying a heterozygous resistant gene had improved mating success compared with those carrying a homozygous resistant gene, while those carrying a homozygous gene for target site resistance carries a cost in reduced mating success. Also, those individuals with heterozygous resistance gene had intermediate phenotypes showing lower resistance and fitness costs, while those with homozygous resistance gene had increased resistance and fitness costs (Assogba et al. 2016). Whether or not Calauan and Sudlon suffered higher fitness cost such as reduced mating success compared with Majayjay and Buguias needs to be

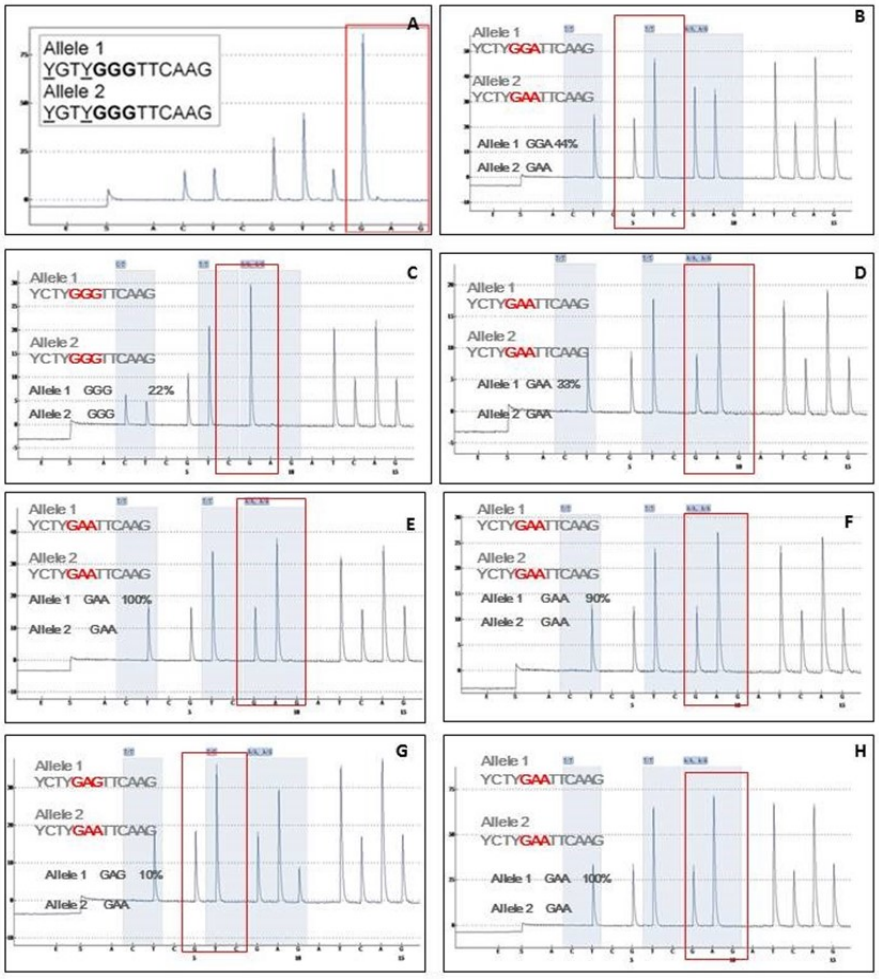

Fig. 3. Single nucleotide polymorphism (SNP) assay results for the ryanodine receptor (RyR) G4946E mutation (GGG [glycine] → GAA or GAG [glutamic acid]) in amplified gDNA fragments of *P. xylostella*. (A) Sensitive strain HS, gDNA, homozygous GGG, genotype SS (Steinbach et al. 2015). (B) Buguias, Benguet strain with heterozygous GGA (sensitive strain) with 44% genotypic frequency. (C) Buguias, Benguet strain with homozygous GGG (sensitive strain) with 22% genotypic frequency. (D) Buguias, Benguet strain with homozygous GAA (resistant strain) with 33% genotypic frequency showing G4946E mutation. (E) Sudlon, Cebu strain with homozygous GAA (resistant strain) with 100% genotypic frequency showing G4946E mutation. (F) Majayjay, Laguna strain with homozygous GAA (resistant strain) with 90% genotypic frequency showing G4946E mutation. (G) Majayjay, Laguna strain with with heterozygous GAG (resistant strain) with 10% genotypic frequency showing G4946E mutation. (H) Calauan, Laguna strain with homozygous GAA (resistant strain) with 100% genotypic frequency showing G4946E mutation.

verified. Further studies to understand the variation on fitness cost that will help to predict the nature of resistance among the four Philippine DBM populations and between the sexes can be done through gene editing, gene knock-out, and exploration of resistance-associated mutations to delay the flubendiamide resistance development.

Due to the continuing expansion of insecticide resistance, limited insecticide tools have been available since in most cases, the available insecticides were mostly pest-crop-region dependent plus the increasingly expensive and complex process of discovering new insecticide control product. Thus, any new insecticide options, especially those bringing forward new Modes of Action (MoAs), should be treated as limited, finite resources that need to be used with care. If these new MoAs will be overused or misused, rapid development of resistance and consequent loss of efficacy and utility is not far from happening. Our results also suggest the utility of diamondback moth as an appropriate insect monitoring tool for insecticide resistance, especially for new insecticide chemistry, due to its history of rapid resistance development.

SUMMARY AND CONCLUSION

In 2006, flubendiamide, a novel class of insecticide that selectively targets the ryanodine receptor (RyR) of insects, has dominated the local market for the management of *P. xylostella*. Recently, however, resistance to flubendiamide in the *P. xylostella* from Sudlon, Cebu was reported. Flubendiamide resistance was due to the G4946E mutation that resulted in the substitution of glycine with glutamic acid residue. In this study, the relative potency of flubendiamide against four DBM populations from cabbage-growing areas was assessed using the leaf-dip method. The genotypes present in each DBM were also estimated using pyrosequencing analysis to identify the possible mechanism of flubendiamide resistance.

Results of the dose-response analysis of flubendiamide on the local DBM populations indicated that 2 years after the first reported flubendiamide resistance in DBM Sudlon strain, a high level of resistance was still evident not just in Sudlon (LC_{50} = 270.6; 2.5± 0.35 SD), but also in other DBM populations from Majayjay, Laguna (LC_{50} = 218.195; 2.4 ± 0.3 SD), Calauan, Laguna ((LC_{50} = 200.3; 2.1 ± 0.3 SD), and Buguias, Benguet (LC_{50} = 135.02; 2.46 ± −0.3 SD). The resistance of the four selected

populations ranged from 6,000 to 10,000-fold higher compared with the susceptible DBM strain, HS (Troczca et al. 2012). In addition, the resistance ratio observed in the Sudlon strain collected in 2014 (12,886-fold) was 12 times higher than in the Sudlon strain collected in 2012 (1,000-fold) compared with the susceptible strain. The heterogeneity factor estimate of the Majayjay population (2.9496) indicated larger gene pool variation compared with the gene pool of the Calauan, Buguias and Sudlon populations. The observed variation can be explained by the comparative genotypic frequencies among these populations.

The presence of the flubendiamide resistance allele in the four DBM populations was confirmed by the genotyping by pyrosequencing results. Further analysis revealed that only the Calauan strain shared the same genotype and genotypic frequency with the previously studied Sudlon strain, with 100% homozygous GAA resistance allele. The Buguias strain showed only 33% homozygous resistance allele, indicating that mutation was at a very low frequency. Interestingly, the Buguias and Majayjay strains showed different G4946E genotypic percentages and genotypes suggesting the existence of diamide cross-resistance. However, our data indicates that mutation occurs in populations and may eventually spread to adjacent areas. This flubendiamide resistance phenomenon, which was first reported in the DBM population in Sudlon, Cebu in 2012, was observed just after a couple of years in other cabbage-growing areas in Laguna and Benguet.

The data showed that the presence of stable resistance among the DBM populations in a wide geographical range in the Philippines such as in Sudlon, Calauan, Majayjay and Buguias resulted in genetic mutation exhibiting different insecticide potent response and genotypic frequencies. With this, an efficient insecticide resistance management (IRM) program must be delivered to the farmers to prevent vast occurrence of resistance. In addition, constant monitoring (baseline susceptibility and SNP monitoring) must be done among different geographic locations especially in those areas with production and heavy pesticide use. Factors that increase the selection pressure of pesticide in the environment that is usually farmers' practice must be corrected. These farmers' practices include (1) the use of a cocktail of different pesticides to target several pests to lower the cost and labor that leads to cross/multiple resistance among different classes of pesticides; (2) overdependence on a single mode of action of pesticide for several cropping seasons; (3) minimal crop rotation; and (4) minimal knowledge on the IRM and even the mode of action of insecticides or how they can apply or where they can find in the label the mode of action. Also, our findings regarding the dependence of fitness cost on the genotype of the insect and the dependence of the level of potency on the genotypic frequency contradict the findings of Troczka et al. (2012), thus the conclusion stating that mutations at very low frequency would have little or no impact on diamide resistance must be verified.

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