

## Article

# First Report of *Helicoverpa armigera* (Lepidoptera: Noctuidae) Resistance to Flubendiamide in Brazil: Genetic Basis and Mechanisms of the Resistance

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**Abstract:** The cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) is a significant pest in the world and it was identified in Brazil in 2013, causing severe economic losses. Recent studies showed a significant decrease in the susceptibility of *H. armigera* to diamide insecticides in Brazil. Understanding the genetic basis and mechanisms of the resistance are essential to develop proactive resistance management strategies. A laboratory strain of *H. armigera* resistant to the phthalic acid diamide flubendiamide (Flub-R) was selected from a field-collected population to characterize the resistance. The resistance ratio of the Flub-R strain was >50,000-fold. The inheritance pattern of the resistance was characterized as an autosomal dominant trait. Flub-R showed no cross-resistance to the anthranilic diamides chlorantraniliprole, cyantraniliprole or cyclaniliprole. Susceptible strain larvae that fed on flubendiamide-treated soybean leaves at field-recommended rates were killed while heterozygotes and Flub-R larvae showed a high survival and no reduction in the leaf consumption, confirming the functional dominance of the resistance. No indication of metabolic resistance was detected. The partial sequencing of ryanodine receptor (RyR) genes covering the transmembrane II to VI did not show any amino acid mutations, indicating the presence of a non-common resistance mechanism to diamide insecticides in the Flub-R strain.

**Keywords:** cotton bollworm; diamide; inheritance; IRM; ryanodine receptor



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## 1. Introduction

*Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a severe agricultural pest in the world and this pest was officially identified in Brazil in 2013 [1,2]. This species is widely distributed in the world [3,4], and besides Brazil, it was reported in many other countries of the Americas such as Argentina [5], Paraguay [6], Uruguay [6,7], and the United States of America [8]. *H. armigera* has a high capacity of dispersion, reproduction, and a wide range of plant hosts [3,9,10]. These characteristics favor the evolution of resistance due to intense selection pressure with the use of insecticides, mainly under tropical agricultural production systems with successive crop cultivation throughout the year. In the Michigan Resistance Database, 891 cases of *H. armigera* resistant to 48 different active ingredients were reported in the world [11], however, so far there is no case of resistance to diamide insecticides.

The diamide insecticides act as ryanodine receptor modulators (IRAC MoA group 28). This chemical class is divided into two subclasses: the phthalic acid, including flubendiamide, and the anthranilic acid, including chlorantraniliprole, cyantraniliprole, and cyclaniliprole. The first class discovered was phthalic acid, developed in the 90s by Nihon Nohyaku Co. Ltd. (Tokyo, Japan) Later, in collaboration with Bayer Crop Science, it resulted in the development of flubendiamide, a molecule with high insecticidal activity on

lepidopteran pests, launched in the market in 2007 [12]. This insecticide class affects the homeostasis of calcium ( $\text{Ca}^{2+}$ ) into the muscle cells by binding to ryanodine receptors (RyR). The calcium channels are kept open, and uncontrolled calcium efflux from the sarcoplasmic reticulum stores of muscle cells results in paralysis due to muscle contraction, cessation of feeding, and consequently insect death [13,14].

The resistance to diamide insecticides is frequently associated with amino acid alteration in the region C-terminal, which contains the transmembrane I to VI of the ryanodine receptors (RyR) [14,15]. These alterations promote different resistance magnitudes, and they may be associated with one or more mutations in the ryanodine receptor gene [15]. Some of these mutations can confer cross-resistance between diamide subclasses, such as G4946E [16] and I4790M [17,18]. Moreover, other mechanisms can lead to insect resistance to diamides, such as the enzymatic detoxification by cytochromes P450 (P450), carboxylesterases (CE), and glutathione S-transferases (GST) [15]. Resistance to flubendiamide has already been detected in major lepidopteran pests, with cross-resistance to other diamide insecticides in most cases [15,19–22].

Because of intense use of insecticides to control *H. armigera* in Brazil, a significant decrease in the susceptibility to diamide insecticides was recently reported [23]. Thus, to evaluate the resistance risk evolution to diamide insecticides and implement Insect Resistance Management (IRM) strategies, we selected a laboratory strain of *H. armigera* resistant to the phthalic acid diamide flubendiamide from a field-collected population to (i) characterize the genetic basis of the resistance of *H. armigera* to flubendiamide; (ii) evaluate cross-resistance between flubendiamide and the anthranilic diamides chlorantraniliprole, cyantraniliprole, and cyclaniliprole; (iii) assess functional dominance based on the survival and leaf consumption of larvae of different genotypes (flubendiamide-resistant, heterozygous, and susceptible) on flubendiamide-treated soybean leaves; (iv) evaluate possible resistance mechanisms by conducting synergist tests and sequencing the main region of the ryanodine receptor (RyR) gene responsible for diamide resistance in other insect pests.

## 2. Materials and Methods

### 2.1. Insects

The susceptible reference strain (SUS) was obtained in September 2013, a few months after the first report of *H. armigera* in Brazil. Approximately 1000 insects were collected in the field from a dry bean crop at the municipality of Luiz Eduardo Magalhães, Bahia state, Brazil ( $12^{\circ}05'58''$  S and  $45^{\circ}47'54''$  W). The SUS strain was reared in the absence of selection pressure with insecticides since 2013. The flubendiamide-resistant strain (Flub-R) was selected from a field-collected population in a soybean crop in January 2016 from the same location of our SUS strain. The Flub-R strain was obtained by selecting surviving larvae at the diagnostic dose ( $2.64 \mu\text{g a.i. cm}^{-2}$ ) of flubendiamide (Belt<sup>®</sup>, Bayer S.A., São Paulo, SP, Brazil  $480 \text{ g a.i. L}^{-1}$ ) [23] for seven generations, and then at  $157.89 \mu\text{g a.i. cm}^{-2}$  for an additional seven generations.

Larvae of both strains were fed with an artificial diet based on beans, brewer's yeast, soy protein, wheat germ, and casein [24]. The adults were maintained in PVC cages closed at the top with fabric for egg-laying. The fabric-containing eggs was replaced every two days. Newly hatched larvae were transferred to 100 mL plastic cups containing an artificial diet. The insects were maintained under controlled conditions ( $25 \pm 1^{\circ}\text{C}$ ,  $70 \pm 10\%$  relative humidity, and photophase of 14 h) in all development stages.

### 2.2. Characterization of Flubendiamide Resistance

Third-instar larvae from SUS and Flub-R strains were subjected to dose–response bioassays with the insecticide flubendiamide (Belt<sup>®</sup>, Bayer S.A.;  $480 \text{ g a.i. L}^{-1}$ ). Seven to nine logarithmically distributed doses were tested, between  $0.003$  to  $0.088 \mu\text{g a.i. cm}^{-2}$  for susceptible strains, and at  $15.79$  to  $5052.63 \mu\text{g a.i. cm}^{-2}$  for resistant strains, with larval mortality from 5 to 95%. To obtain these doses the insecticide was diluted in distilled water

and added with the surfactant Triton<sup>®</sup> at 0.1% to obtain a uniform solution spread over the diet surface. The control treatment consisted of distilled water and Triton<sup>®</sup> only. The ingestion bioassays were performed in 24-well acrylic plates (Costar<sup>®</sup>, Washington, DC, USA) with 1.25 mL of artificial diet per well (area of 1.9 cm<sup>2</sup>). An amount of 30 µL of the insecticide solution was added per well by using an electronic repeating pipette. After drying out, one larva was added to each well, and at least four replicates with 24 larvae each were tested per dose. The trays were sealed with their covers and kept under controlled conditions (25 ± 1 °C, 70 ± 10% relative humidity, and photophase of 14 h). Mortality was evaluated at 96 h after transferring the larvae in the bioassay plates by considering the ones that did not show coordinated movements when probing with fine paint brush to be dead.

### 2.3. Inheritance of Flubendiamide Resistance

Reciprocal crosses between susceptible and resistant strains were performed to generate the heterozygotes, H1 (♀SUS × ♂Flub-R) and H2 (♀Flub-R × ♂SUS), using 20 individuals of each strain. Dose–mortality bioassays were conducted with the third instar larvae from the F1 progeny. The larvae were exposed to eight doses of flubendiamide spaced on a logarithmic scale, between 15.79 to 5052.63 µg a.i. cm<sup>-2</sup> of flubendiamide. Dose–mortality bioassays were subjected as outlined in the characterization of flubendiamide resistance.

### 2.4. Dominance of Resistance

Dose–mortality data outlined in Section 2.2 were used. According to Stone et al. method [25], the average degree of dominance is given by the equation:

$$D = (2X_F - X_R - X_S) / (X_R - X_S) \quad (1)$$

where the coefficients  $X_F$ ,  $X_R$ , and  $X_S$  are logarithms of the lethal dose that kills 50% of larvae (LD<sub>50</sub>), which were estimated for the heterozygotes, Flub-R, and SUS strains, respectively. For the values of dominance (D): D = 1 indicates completely dominant, 0 < D < 1 indicates incompletely dominant, -1 < D < 0 indicates incompletely recessive, and D = -1 indicates completely recessive. The standard error (SE) of D was calculated based on Lehmann and Roman [26], as described by Robertson et al. [27].

The dominance of resistance was also analyzed using the method proposed by Bourguet et al. [28] given by Equation (2), where dominance was calculated to each tested dose.

$$D_{ML} = (ML_{RS} - ML_{SS}) / (ML_{RR} - ML_{SS}) \quad (2)$$

where  $ML_{RR}$ ,  $ML_{SS}$ , and  $ML_{RS}$  represent the mortality of the resistant, susceptible, and heterozygote strains to each dose tested, respectively. The values of effective dominance ( $D_{ML}$ ) are:  $D_{ML} = 1$  indicates completely dominant,  $D_{ML}$  close to 1 indicates incompletely dominant, close to 0 indicates incompletely recessive, and  $D_{ML} = 0$  indicates completely recessive.

### 2.5. Functional Dominance of Resistance and Leaf Consumption

Susceptible (SUS), flubendiamide-resistant (Flub-R) strains, and heterozygotes H1 (♀SUS × ♂Flub-R) and H2 (♀Flub-R × ♂SUS) were used to assess the functional dominance. The strains were evaluated on untreated or flubendiamide-treated soybean leaves at a concentration of 0.336 µg a.i. mL<sup>-1</sup> (recommended field concentration for soybean). The bioassay method was a leaf dip for five seconds. The treated leaf was transferred to a Petri dish, and three third-instar larvae were carefully placed over the treated leaf and then covered with the lid. A Petri dish with three third-instar larvae was a replicate. Each treatment was replicated 10 times, totaling 30 larvae per treatment. After 96 h, the larval survival was evaluated by touching the larvae with a fine paint brush. Larvae with coordinated movements were considered to be alive. Leaf consumption (cm<sup>2</sup>) was assessed with the average consumption of the three larvae per replicate. The leaves of

treatments were digitalized by a scanner, and the consumed area was estimated using ImageJ software [29].

### 2.6. Cross Resistance

Third-instar larvae from *H. armigera*-susceptible and flubendiamide-resistant strains were used in dose–mortality bioassays with the commercial insecticides chlorantraniliprole (Premio<sup>®</sup>, FMC, Campinas, SP, Brazil; 200 g a.i. L<sup>-1</sup>), cyantraniliprole (Benevia<sup>®</sup>, FMC; 100 g a.i. L<sup>-1</sup>) and cyclaniliprole (Goemon<sup>®</sup>, ISK, Indaiatuba, SP, Brazil; 50 g a.i. L<sup>-1</sup>) to establish a cross-resistance profile. Susceptible and resistant flubendiamide strains were exposed to several doses of chlorantraniliprole (0.002 to 0.884 µg a.i. cm<sup>-2</sup>), cyantraniliprole (0.005 to 1.579 µg a.i. cm<sup>-2</sup>) and cyclaniliprole (0.0003 to 0.03 µg a.i. cm<sup>-2</sup>). At least four replicates with 24 larvae were tested per dose. Dose–mortality bioassays were obtained as previously described in Section 2.2.

### 2.7. Synergist Bioassays

Dose–response bioassays were performed on third-instar larvae of Flub-R strains. Artificial diet–overlay dose assays were conducted in 12-well plates as described in Section 2.2, with a dose near to LD<sub>50</sub> of Flub-R strain of 505.28 µg a.i. cm<sup>-2</sup>. The larvae were topically applied with 1 µL acetonic solutions of piperonyl butoxide (PBO, Sigma-Aldrich, St. Louis, MO, USA, 90%), diethyl maleate (DEM, Sigma-Aldrich, 97%) and S,S,S-tributyl phosphorotrithioate (DEF, Chem Service, West Chester, PA, USA, 97.2%) two hours prior to insecticide exposure. Synergist solutions were prepared in acetone (Labsynth, Diadema, SP, Brazil, 100%) and applied onto the larval pronotum using a micro applicator (Burkard Manufacturing CO, Rickmansworth, UK). Synergists PBO, DEM and DEF were applied at the dose of 1 µg a.i. larva<sup>-1</sup>. Acetone alone served as solvent control. The highest non-lethal concentration of each synergist was established in preliminary bioassays. Four replicates of approximately 12 larvae were used for each treatment, totaling 48 larvae. After infestation, the larvae were kept under controlled conditions (25 ± 1 °C, 70 ± 10% relative humidity, and photophase of 14 h). Larvae were scored for mortality after 96 h.

### 2.8. Sequencing Partial RyR

Total RNA was extracted from the thorax of *H. armigera* adults of the SUS and Flub-R strain. RiboZol<sup>™</sup> RNA Extraction Reagent (VWR Life Science, Radnor, PA, USA), according to its protocol, was used for RNA separation, followed by DNase I purification (Thermo Scientific, Waltham, MA, USA). The purified RNA was quantified by spectrophotometer (Epoch, Ellicott City, MD, USA) and its integrity was assessed by 1.5% agarose gel electrophoresis. The RNA was normalized to 500 ng µL<sup>-1</sup>, and 1 µg total RNA was used in a 20 µL reaction for cDNA synthesis using GoScript<sup>™</sup> Reverse Transcriptase and oligo (dT) (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Primer pairs were designed manually based on the cDNA sequences of the RyR of *H. armigera* (GenBank KF641862.1 and KJ573634.1) covering the transmembrane II to VI (Table 1). Primer pairs were used to amplify fragments, overlapping each other, and covering the 1553 bp *H. armigera* RyR fragment. The PCR reaction contained 100 ng of cDNA, 500 nM of each primer, 10 mM Tris-Cl<sup>+</sup> 50 mM KCl (PCR buffer 10x, Sinapse), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP (Sinapse), 1 U of Taq DNA Polymerase (Invitrogen (5 U µL<sup>-1</sup>)) and MilliQ water to complete 25 µL. The reaction was subjected to cycling conditions of 10 s at 98 °C followed by 35 cycles at 98 °C for 3 s, 57 °C for 35 s, and 72 °C for 50 s, and a final extension step at 72 °C for 3 min in the thermal cycle (SimpliAmp, ThermoFisher, Waltham, MA, USA). The PCR product was verified by 1% agarose gel electrophoresis, purified by an EasyPure PCR purification kit (Trans, Haidian District, BJ, China), and then sent to Sanger-sequencing at the Laboratory of Molecular Biology of Plants (Department of Biological Sciences–ESALQ/USP), using the forward and reverse primers described in Table 1. The 1553 bp of *H. armigera* partial RyR obtained from the Sanger-sequencing was translated and aligned with the RyR of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae)

(GenBank AET09964) and RyR of *H. armigera* (GenBank AHB33498) amino acid sequence to find any alteration.

**Table 1.** List of primers used in sanger sequencing.

Primer	Sequence 5'-3'	Amplification Length (bp)
HA1-F HA1-R	CTCAAGAGAGGATGGAAGC AGAAGGAAGTCTTCTCCATGC	728
HA2-F HA2-R	TCGCTCGTAAGCTGGAAT AGACCCTGGAGAATAGCG	756
HA3-F HA3-R	TTCCGCAAGTTCTACGTGC TTCGGAAACAATCTCCCACG	510

### 2.9. Data Analysis

The dose–mortality data were corrected with controls (Natural Mortality) by Abbott’s formula [30]. The data corrected were subjected to Probit analysis with MASS package [31] by function glm with a binomial distribution and probit or logit links in R software [32] to estimate the LD<sub>50</sub> values. The resistance ratio (RR) was determined by dividing the LD<sub>50</sub> value of the Flub-R strain by LD<sub>50</sub> value of the SUS strain. The likelihood ratio test (LRT) [31] was performed to verify the variation between strain responses. In addition, parallelism, and equality tests ( $p < 0.05$ ) were performed to test the equality hypotheses between regression lines of each strain [31].

Functional dominance and leaf consumption data were analyzed using software R [32]. Mortality data were modeled by the GAMLSS package with the gamlss function [33], with a binomial distribution, probit link, and sigma adjustment. Results were submitted to a post-hoc test to verify significant differences in the mean by function emmeans [34] with the Tukey’s test,  $\alpha = 5\%$  and  $p < 0.05$ . The consumption data were submitted to the LM model with package MASS [31]; after this, the residual was tested for normality [35], homoscedasticity [36], and independency [37] by the package lmtest [38]. The ANOVA and Tukey’s test were subjected to verify significant differences in means with  $\alpha = 5\%$  and  $p < 0.05$ . The data describing the mortality of the synergist bioassay was subjected to the analysis of variance (ANOVA) and means were compared using the Tukey’s test,  $\alpha = 5\%$  and  $p < 0.05$ .

## 3. Results

### 3.1. Characterization of Flubendiamide Resistance

The LD<sub>50</sub> of SUS strain was 0.018  $\mu\text{g a.i. cm}^{-2}$  of flubendiamide, and the LD<sub>50</sub> of Flub-R strain was 871.83  $\mu\text{g a.i. cm}^{-2}$  of flubendiamide (Table 2). The resistance ratio (RR) of the Flub-R strain was >50,000-fold. The null hypothesis of equality and parallelism was rejected with  $\chi^2(1) = 17.19$ ,  $p < 0.05$  between the slopes and intercepts of the strains.

**Table 2.** Dose–mortality of flubendiamide to susceptible (SUS) and resistant (Flub-R) strains of *H. armigera* and heterozygotes from reciprocal crosses.

Strain	$n^a$	Slope ( $\pm$ SE)	LD <sub>50</sub> (95% CI) <sup>b</sup>	$\chi^2(p)^c$	d.f. <sup>d</sup>	RR <sup>e</sup>
SUS	864	1.98 ( $\pm$ 0.12)	0.017 (0.015–0.019)	8.70 (0.88)	6	-
Flub-R	662	1.58 ( $\pm$ 0.11)	871.84 (744.74–1020.62)	1.96 (0.14)	5	>50,000
H1 ( $\text{♀SUS} \times \text{♂Flub-R}$ )	707	1.14 ( $\pm$ 0.08)	509.43 (412.26–629.48)	10.68 (0.90)	6	29,966.47
H2 ( $\text{♀Flub-R} \times \text{♂SUS}$ )	709	1.09 ( $\pm$ 0.08)	504.36 (403.78–630.00)	10.67 (0.90)	6	29,647.06

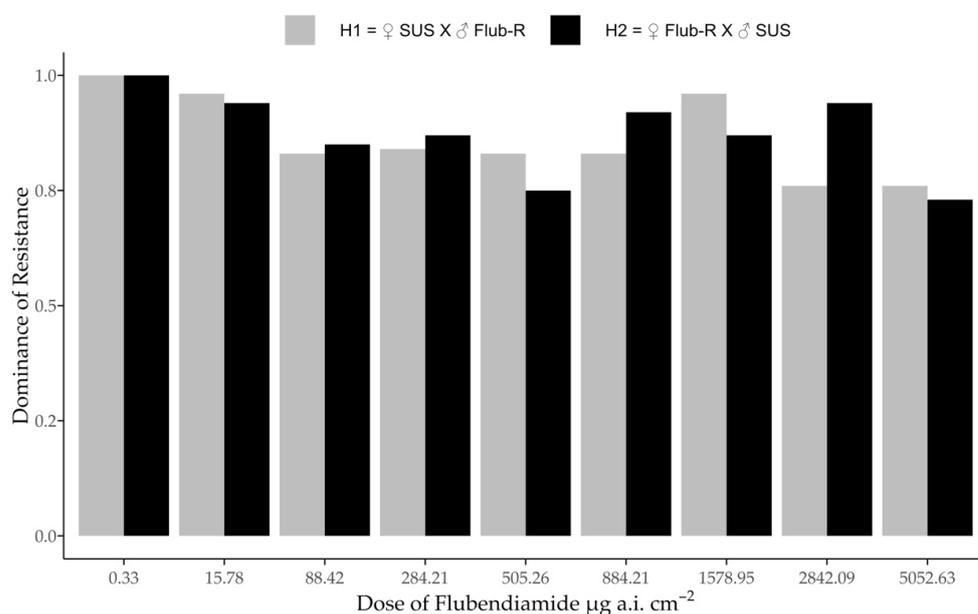
<sup>a</sup> Number of tested insects; <sup>b</sup> Dose of flubendiamide required to kill 50% of larvae ( $\mu\text{g a.i. cm}^{-2}$ ) and 95% confidence interval; <sup>c</sup> Chi-squared and  $p$ -value of goodness of fit; <sup>d</sup> Degrees of freedom; <sup>e</sup> Resistance ratio.

### 3.2. Inheritance of Flubendiamide Resistance

The heterozygote strain H1 ( $\varphi$ SUS  $\times$   $\sigma$ Flub-R) showed an LD<sub>50</sub> equal to 509.43, 95% CI [412.26–629.48]  $\mu\text{g a.i. cm}^{-2}$  of flubendiamide, and the heterozygote strain H2 ( $\varphi$ Flub-R  $\times$   $\sigma$ SUS) showed an LD<sub>50</sub> equal to 504.36, 95% CI [403.78–630.00]  $\mu\text{g a.i. cm}^{-2}$  of flubendiamide. The null hypothesis of equality and parallelism was not rejected between the slopes and intercepts of strains H1 and H2 with  $\chi^2(1) = 0.001$ ,  $p = 0.969$ . Moreover, the null hypothesis of the likelihood ratio test of LD<sub>50</sub> between strains H1 and H2 was not rejected with  $\chi^2(1) = 0.173$ ,  $p = 0.863$ . Therefore, this finding confirms the hypothesis of autosomal inheritance of the resistance of *H. armigera* to flubendiamide and ruled out the hypothesis of sex-linked or mitochondrial inheritance.

### 3.3. Dominance of Resistance

The analysis of dominance was assessed by two methods. According to Stone's method [25], the degree of dominance calculated by LD<sub>50</sub> and approximate 95% confidence interval for reciprocal crosses were  $D = 0.903$ ,  $SE = 0.053$  CI ( $D \pm 2 SE = (0.795-1.007)$ ) for H1 ( $\varphi$ SUS  $\times$   $\sigma$ Flub-R) and  $D = 0.899$ ,  $SE = 0.055$  CI ( $D \pm 2 SE = (0.790-1.009)$ ) for H2 ( $\varphi$ Flub-R  $\times$   $\sigma$ SUS). Considering the standard errors calculated using Lehmann's formula [26] for each value of D and the approximate 95% confidential intervals for D ( $D \pm 2 SE$ ), the resistance to flubendiamide is completely dominant. According to Bourguet et al. [28] method, by which dominance for each tested dose was calculated, the dominance values were higher than 0.73 for the higher doses, indicating an incomplete dominance (Figure 1), except at the lowest tested dose, that is, the dose recommended in the field when the dominance is completely dominant (Figure 1). Thus, the dominance values decreased as the flubendiamide dose increased.

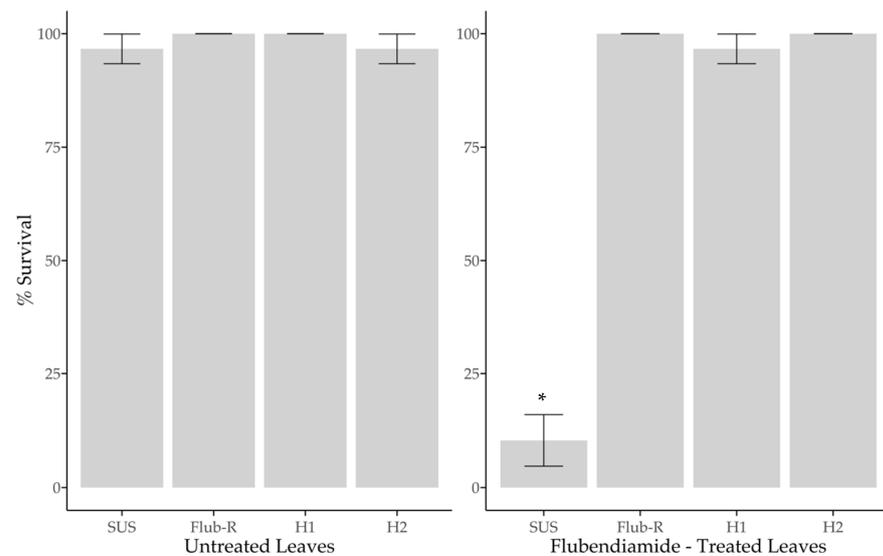


**Figure 1.** Dominance of resistance to flubendiamide in *H. armigera* as a function of dose. Reciprocal crosses represented by H1 ( $\varphi$ SUS  $\times$   $\sigma$ Flub-R) and H2 ( $\varphi$ Flub-R  $\times$   $\sigma$ SUS).

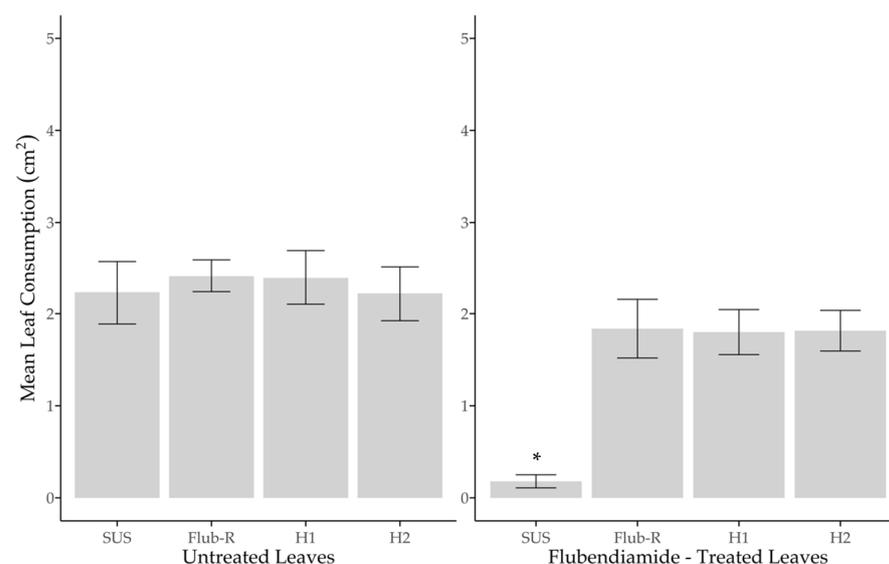
### 3.4. Functional Dominance of Resistance and Leaf Consumption

When we compared the survival of strains on untreated leaves, SUS 96.7%, Flub-R 100%, H1 100% and H2 96.7%, there was no significant statistical difference ( $F(3,36) = [0.002]$ ,  $p = 0.999$ ) (Figure 2), when in treated leaves there were significant statistical differences between strains ( $F(3,36) = (7.243)$ ,  $p < 0.05$ ). Tukey's test for pairwise comparisons showed that SUS 10.34% differed from Flub-R 100%  $t(36) = [15.85]$ ,  $p < 0.05$ , two-tailed, H1 96.66%  $t(36) = [13.20]$ ,  $p < 0.05$ , two-tailed, and H2 100%  $t(36) = [15.85]$ ,  $p < 0.05$ , two-tailed) (Figure 2). In terms of the mean leaves' consumption, the strains in untreated leaves SUS

( $M = 2.23 \text{ cm}^2$ ), Flub-R ( $M = 2.41 \text{ cm}^2$ ), H1 ( $M = 2.40 \text{ cm}^2$ ) and H2 ( $M = 2.22 \text{ cm}^2$ ) did not show significant statistical differences between strains ( $F(3,36) = (0.136)$ ,  $p = 0.938$ ) (Figure 3); otherwise in treated leaves there was a significant statistical difference between strains ( $F(3, 36) = (12.47)$ ,  $p < 0.05$ ). The Tukey's test for multiple comparisons found that in treated leaves the strain SUS ( $M = 0.18 \text{ cm}^2$ ) differed from Flub-R ( $M = 1.83$ ) ( $p < 0.05$ , 95% CI = (0.77–2.54)), H1 ( $M = 1.80 \text{ cm}^2$ ) ( $p < 0.05$ , 95% CI = (0.74–2.50)) and H2 ( $M = 1.81 \text{ cm}^2$ ) ( $p < 0.05$ , 95% CI = (0.75–2.52)) (Figure 3). In summary, the survival of heterozygotes and the leaf consumption of treated leaves are not different from that of the resistant strain. Thus, we demonstrated that the resistance of *H. armigera* to flubendiamide at a field rate is functionally dominant.



**Figure 2.** Proportion of larval survival by susceptible (SUS), resistant (Flub-R) and heterozygous H1 ( $\varphi$ SUS  $\times$   $\sigma$ Flub-R) and H2 ( $\varphi$ Flub-R  $\times$   $\sigma$ SUS) *H. armigera* in soybean leaves untreated and treated with flubendiamide ( $0.33 \mu\text{g a.i. cm}^{-2}$ ). \* Significant difference between strains according to the Tukey's test ( $p < 0.05$ ).



**Figure 3.** Mean leaf consumption by susceptible (SUS), resistant (Flub-R) and heterozygous H1 ( $\varphi$ SUS  $\times$   $\sigma$ Flub-R) and H2 ( $\varphi$ Flub-R  $\times$   $\sigma$ SUS) *H. armigera* larva in soybean leaves untreated and flubendiamide-treated ( $0.33 \mu\text{g a.i. cm}^{-2}$ ). \* Significant difference between strains according to the Tukey test ( $p < 0.05$ ).

### 3.5. Cross Resistance

The flubendiamide-resistant strain (Flub-R) showed no cross-resistance to the anthranilic diamides. The Flub-R showed an LD<sub>50</sub> of 0.090, 95% CI [0.077–0.106] µg a.i. cm<sup>-2</sup>, 0.193, 95% CI [0.158–0.237] µg a.i. cm<sup>-2</sup> and 0.004, 95% CI [0.003–0.005] µg a.i. cm<sup>-2</sup> for chlorantraniliprole, cyantraniliprole and cyclaniliprole, respectively (Table 3). When compared with the SUS LD<sub>50</sub> of each insecticide tested, Flub-R showed low resistance ratio to chlorantraniliprole (4.50-fold), cyantraniliprole (4.94-fold) and cyclaniliprole (1.33-fold) (Table 3).

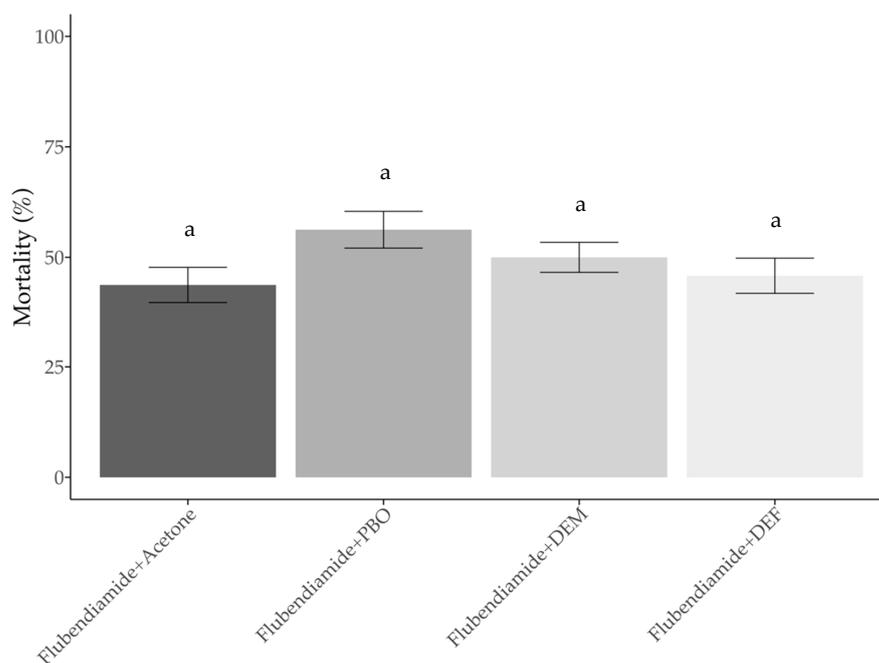
**Table 3.** Dose–mortality data for evaluating cross resistance between flubendiamide and anthranilic diamides in *H. armigera*.

Insecticide	Strain	<i>n</i> <sup>a</sup>	Slope (±SE)	LD <sub>50</sub> (CI 95%) <sup>b</sup>	χ <sup>2</sup> ( <i>p</i> ) <sup>c</sup>	d.f. <sup>d</sup>	RR <sup>e</sup>
Chlorantraniliprole	SUS	499	2.28 (±0.22)	0.020 (0.016–0.025)	7.19 (0.79)	5	-
	Flub-R	767	1.59 (±0.11)	0.090 (0.077–0.106)	1.78 (0.12)	5	4.50
Cyantraniliprole	SUS	497	1.51 (±0.12)	0.039 (0.032–0.047)	3.65 (0.40)	5	-
	Flub-R	637	1.29 (±0.09)	0.193 (0.158–0.237)	4.25 (0.48)	5	4.94
Cyclaniliprole	SUS	475	1.54 (±0.12)	0.003 (0.002–0.004)	7.11 (0.78)	5	-
	Flub-R	461	2.00 (±0.14)	0.004 (0.003–0.005)	2.60 (0.23)	5	1.33

<sup>a</sup> Number of tested insects; <sup>b</sup> Dose required to kill 50% of larvae (µg a.i. cm<sup>-2</sup>) and 95% confidence interval; <sup>c</sup> *Chi-squared* and *p*-value of goodness of fit; <sup>d</sup> Degrees of freedom; <sup>e</sup> Resistance ratio.

### 3.6. Synergist Bioassays

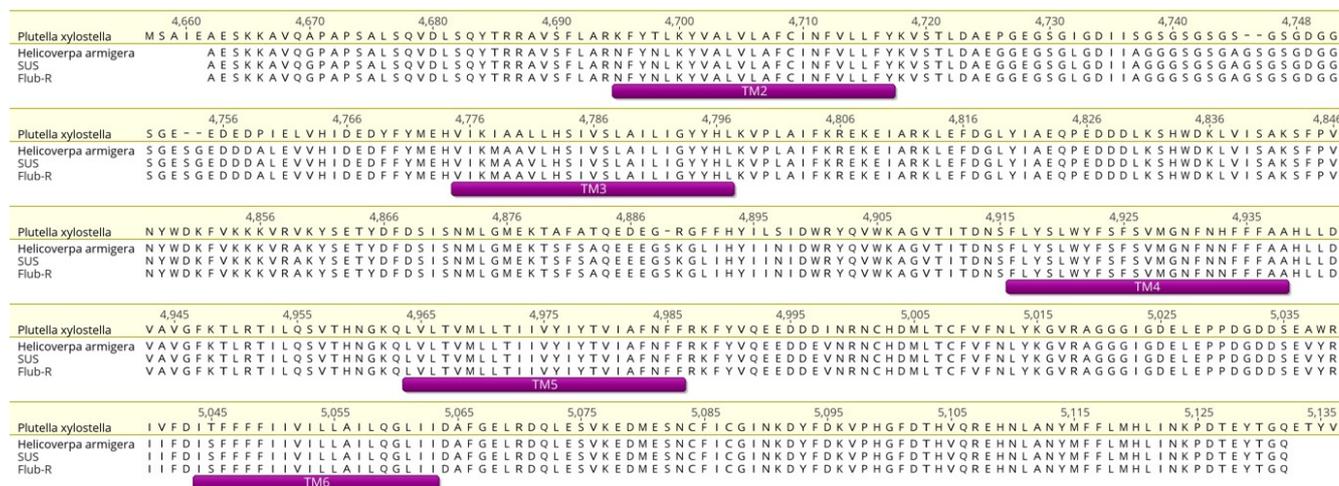
Third instar larvae of Flub-R strain subjected to controls (acetone plus flubendiamide) showed a mortality of 43.8 ± 3.98%. The treatment that larvae were pretreated with, PBO, and then treated with flubendiamide presented a mortality value of 56.2 ± 4.16%. The synergist DEM applied before flubendiamide resulted in a similar mortality compared to the control, which showed 50 ± 3.40%. The treatment with DEF showed mortality values of 45.8 ± 3.98%. The analysis of variance (ANOVA) showed no significant variation among treatments, (F(3,12) = (2), *p* = 0.168) (Figure 4).



**Figure 4.** Effect of synergists (PBO, DEM and DEF) on *H. armigera* (third-instar larvae) mortality to flubendiamide. Bars followed by the same letter do not differ from the treatment control (flubendiamide + acetone) according to the Tukey's test (*p* > 0.05).

### 3.7. Sequencing Partial RyR

The amplified PCR products that overlap each other were sequenced to identify non-synonymous mutations related to diamides resistance. The amplified PCR product translates approximately 470 amino acids from RyR, covering transmembrane domains II to VI. The amplified partial RyR of *H. armigera* SUS and Flub-R showed a high similarity (91% pairwise identified at amino acid level) to the respective stretch of the *P. xylostella* (GenBank AET09964). Analysis obtained from the Flub-R strain did not show any change in terms of amino acids (Figure 5) when compared to SUS strain and *H. armigera* reference sequence from GenBank in National Center for Biotechnology Information (NCBI).



**Figure 5.** Multiple alignments of partial ryanodine receptors, spanning transmembrane II to VI. This region harbors the main mutations related to diamide resistance. Alignment was performed based on the *H. armigera* sequence (GenBank AHB33498). No mutations were found in the flubendiamide-resistant strain (Flub-R). The sites are numbered according to the sequence of *P. xylostella* (GenBank AET09964).

### 4. Discussion

In this study, a very high resistance ratio of *H. armigera* to flubendiamide (>50,000-fold) was obtained after laboratory selection of a field-collected population in the Western Bahia State, Brazil in 2016. So far, there have been no records of resistance of *H. armigera* populations to diamide insecticides in Brazil. However, previous studies conducted by Pereira et al. [23] have already indicated significant reductions in susceptibility to diamide insecticides in *H. armigera* populations throughout the crop seasons in Brazil, with survival rates increasing from 0% in the 2014 crop season up to 51.99% in the 2018 crop season based on diagnostic dose bioassays. A high resistance ratio to flubendiamide was also reported for *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) [19] and *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) [39] collected from same region. This region is characterized by an intensive agriculture and climatic conditions that favor the development of many pest generations during the entire year and the high use of insecticides to pest control may have led to the selection of resistant alleles.

The inheritance pattern of *H. armigera* resistant to flubendiamide was defined as an autosomal and completely dominant trait. This finding eliminates the hypothesis of sex-linked and maternal influence on the resistance of *H. armigera* to flubendiamide. The resistance of *S. frugiperda* [19], *T. absoluta* [39] and *P. xylostella* (L.) [40] to diamide insecticides was characterized as autosomal and incompletely recessive. The resistance of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) was autosomal and incompletely recessive to anthranilic diamides and completely recessive to flubendiamide [16]. Different from these works, our Flub-R strain showed a dominant trait. The dominance level has a great impact on pest control, given that the resistance allele tends to be fixed in the

population when inheritance is dominant. The completely dominant trait contributes to the rapid evolution of resistance due to the survival of heterozygotes. These heterozygotes are the main carriers of resistance alleles in the field [41,42].

The heterozygote larvae that fed on soybean leaves treated with flubendiamide at the field-recommended rates did not differ in leaf consumption and survival when compared to the resistant strain. These results indicate that the resistance at field rates of flubendiamide is completely dominant trait. In fact, dominance is characterized as the relationship between phenotypes and genotypes (homozygous resistant, homozygous susceptible and heterozygous) that can vary according to environmental conditions [28]. Thus, the use of doses below those that are field-recommended promotes the maintenance of heterozygotes in the area, increasing the frequency of resistance alleles in the population. Therefore, it is necessary to implement proactive Insect Resistance Management (IRM) strategies to delay the evolution of resistance and, consequently, the fixation of resistance alleles in the populations [41].

Our results showed the potential risk of the development of diamide resistance under applied field conditions. However, it is important to note that the selected strain with flubendiamide did not show cross-resistance to chlorantraniliprole, cyantraniliprole or cyclaniliprole (anthranilic diamides). This result is different from other published studies showing cross-resistance between diamide insecticides due to a mutation in the ryanodine receptor genes [16,17,20,43]. Synergists inhibiting detoxification enzyme families such as cytochromes P450 (P450), carboxylesterases (CE), and glutathione S-transferases (GST) did not increase flubendiamide efficacy in the Flub-R strain, suggesting the absence of metabolic resistance. Some studies show that detoxifying enzymes could be associated with resistance to diamide insecticides [44–46], but they promote low, or no resistance levels compared to mutations at the target site [15].

The partial sequencing of the ryanodine receptor genes of *H. armigera*-resistant strains to flubendiamide showed no mutations in the region that covers the transmembrane regions II to VI. This C-terminal region was formerly described to contain target site mutations [15,47]. The primary lepidopteran resistance mechanism to diamides is the mutation in RyR, which can promote very high resistance ratios [15]. Some mutations such as G4946E and I4790M are responsible for cross-resistance between flubendiamide, chlorantraniliprole, and cyantraniliprole [16,17,20,43], as observed in *S. frugiperda* [48] and *P. xylostella* [49]. Otherwise, *S. exigua* resistance to diamides was related to InDels, which showed a 29-bp insertion close to the intron associated with the resistance mutation [50]. The fact that our sequencing did not find mutations in the main region could explain the lack of cross-resistance between the diamides tested. Our results indicate that resistance of *H. armigera* to flubendiamide in Brazil may be related to a non-common mutation in another region of the ryanodine receptor gene or a different mechanism of resistance. We are conducting studies such as genotype–phenotype association to investigate the possible mechanisms responsible for *H. armigera* resistance to flubendiamide and we hope to clarify this question.

This is the first report of resistance of *H. armigera* to flubendiamide. Our results reveal the high risk of resistance evolution of *H. armigera* to flubendiamide and the importance of implementing resistance management strategies to preserve the lifetime of this insecticide to control *H. armigera* in Brazil.

## 5. Conclusions

Our results show the potential risk for the rapid evolution of flubendiamide resistance in *H. armigera* based on a completely dominant trait. In addition, no cross-resistance was found between flubendiamide (phthalic acid diamide), chlorantraniliprole, cyantraniliprole or cyclaniliprole (anthranilic diamides) in *H. armigera*. We did not find any reported mutations in transmembrane regions II to VI of the ryanodine receptor genes, nor indication of metabolic resistance in the flubendiamide-resistant strain of *H. armigera*.

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