

SELECTION OF *Plutella xylostella* (L.) (LEPIDOPTERA: PLUTELLIDAE) TO CHLORFENAPYR RESISTANCE: HERITABILITY AND THE NUMBER OF GENES INVOLVED¹

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ABSTRACT - The *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is a worldwide pest of Brassicaceae. Resistance has evolved against various insecticides including chlorfenapyr one of the most recently registered molecules to control this pest. The failure of chlorfenapyr to control this pest could be related to resistance in *P. xylostella* in the state of Pernambuco (Brazil), but there is currently no information on its heritability. Here, we estimated the heritability of resistance of *P. xylostella* to chlorfenapyr and the number of genes involved in the resistance in a field-derived population (PxClf-SEL). A field population was selected in the laboratory with increasing doses of chlorfenapyr (for five generations), and the LC_{50s} were estimated for every generation using the leaf dip bioassay. The selection increased resistance to chlorfenapyr in the PxClf-SEL as the LC₅₀ shifted from 27.6 (F₁) to 256.5 (F₅) mg chlorfenapyr/L. As a result, the resistance ratio (RR) increased from 33-fold (F₁) to 310-fold (F₅). The heritability of resistance of *P. xylostella* to chlorfenapyr was 0.90 (h^2), and the number of generations needed for a 10-fold increase in the resistance to chlorfenapyr was 5.20 (G). Other methods have shown different numbers of genes (0.64 and 1.88) involved in resistance of *P. xylostella* to chlorfenapyr. There was sufficient variation regarding resistance in the field population to account for a high realized heritability influenced mainly by additive genetic factors. Therefore, there is a high risk of chlorfenapyr resistance in the field.

Keywords: Genetics. Resistance management. Evolution of resistance. Toxicity.

SELEÇÃO DE *Plutella xylostella* (L.) (LEPIDOPTERA: PLUTELLIDAE) PARA RESISTÊNCIA A CLORFENAPIR: HERDABILIDADE E O NÚMERO DE GENES ENVOLVIDOS

RESUMO – A *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) é uma praga mundial de Brassicaceae. A resistência tem evoluído para vários inseticidas incluindo clorfenapir, uma das moléculas registradas recentemente para o controle desta praga. As falhas de clorfenapir para controlar esta praga podem ser relacionadas com a resistência em *P. xylostella* no Estado de Pernambuco (Brasil), mas atualmente não há informações sobre sua herdabilidade. Aqui, foi estimada a herdabilidade da resistência de *P. xylostella* para clorfenapir e o número de genes envolvidos na resistência em uma população derivada do campo (PxClf-SEL). A população de campo foi selecionada no laboratório com doses crescentes de clorfenapir (por cinco gerações) e as CL_{50s} foram estimadas para todas as gerações usando bioensaio de imersão de folha. A seleção aumentou a resistência para clorfenapir na PxClf-SEL, como também deslocou a CL₅₀ de 27,60 (F₁) para 256,50 (F₅) mg de clorfenapir/L. Como resultado, a razão de resistência (RR) aumentou de 33 (F₁) para 310 vezes (F₅). A herdabilidade da resistência de *P. xylostella* para clorfenapir foi 0,90 (h^2) e o número de gerações necessário para aumentar em 10 vezes a resistência foi 5,20 (G). Outros métodos tem mostrado diferente número de genes (0,64 e 1,88) envolvidos na resistência de *P. xylostella* para clorfenapir. Houve variação suficiente na população de campo em relação à resistência para justificar a alta herdabilidade, influenciada principalmente por fatores genéticos aditivos. Portanto, existe um alto risco de resistência no campo.

Palavras-chave: Genética. Manejo da resistência. Evolução da resistência. Toxicidade.

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INTRODUCTION

The *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is a highly destructive pest to Brassicaceae and many factors contribute to its success as an agricultural pest. The short generation time, high fecundity and broad host usage within Brassicaceae as well as its remarkable ability to rapidly evolve insecticide resistance are among these factors (TALEKAR; SHELTON, 1993; SAYYED et al., 2008). Several field populations of *P. xylostella* have evolved resistance to novel insecticides including chlorantraniliprole (RIBEIRO et al., 2014), spinosad (SAYYED; WRIGHT, 2006) and indoxacarb (NEHARE et al., 2010). Nearly two decades ago, the annual cost of controlling *P. xylostella* on a worldwide basis was estimated to be US\$ 1 billion. This increased more recently to US\$ 4 -5 billion (ZALUCKI et al., 2012; FURLONG; WRIGHT; DOSDALL, 2013) with resistance likely accounting for most of this cost.

Chlorfenapyr is one of the most recently deployed insecticide to control *P. xylostella* in the state of Pernambuco (Brazil). This insecticide is a pro-insecticide, and oxidative removal of the N-ethoxymethyl group of chlorfenapyr by mixed function oxidases leads to a toxic form that uncouples oxidative phosphorylation in the mitochondria (RAGHAVENDRA et al., 2011). Consequently, ATP production is disrupted followed by a loss of energy leading to cell disjunction and eventual death of the organism (RAGHAVENDRA et al., 2011).

Farmers from the Pernambuco state (Jupi and Bezerros municipalities) (Brazil) have recently reported control failure by chlorfenapyr against *P. xylostella*, and this suggests the development of resistance to chlorfenapyr in those areas. Our hypothesis is that the resistance (a pre-adaptive phenomenon) to chlorfenapyr in *P. xylostella* is invariably controlled by at least a gene that is present in the environment, which upon artificial selection may increase its frequency in the population leading to reduced susceptibility in agroecosystems.

The successful management of insecticide resistance depends ultimately on a detailed knowledge of its genetic basis and the mechanisms involved in the resistance (BALASUBRAMANI; SAYYED; CRICKMORE, 2008). In this sense, studies of quantitative genetics can improve the monitoring, detection, risk assessment and management of resistance particularly before field resistance is detected (SAYYED; AHMAD ; SALEEM, 2008). Thus, the realized heritability (h^2) (or narrow sense heritability, which consider only the additive genetic effect) is an important parameter for assessing the risk of resistance evolution. It is the ratio between additive genetic variance (V_A) and the phenotypic variance (V_P) (BROOKFIELD, 2012). Thus, the narrow sense heritability determines how

much of this phenotypic variation is due to additive genetic effects (JALLOW; HOY, 2006) or the proportion of the phenotypic variance that is a result of genetic factors. The heritability is based on the statistics of 'partitioning the variance', where we can see the major variables, which influence the phenotypic variance of the population. This variance may arise from different causes (BROOKFIELD, 2012).

Artificial selection of resistant populations is important because it is possible to characterize the genetic basis of resistance and consequently the risk assessment of development of resistance (LIU et al., 2015). In this sense, the realized heritability and number of genes can explain the extent of genetic makeup to chlorfenapyr resistance in *P. xylostella*. Therefore, the objective of this study was to estimate the resistance heritability (in the narrow sense) of *P. xylostella* to chlorfenapyr in a pool of field populations and assess the number of genes that influence this resistance.

MATERIAL AND METHODS

Insects. The field populations of *P. xylostella* were collected from *Brassica oleracea* L. var. *capitata*-producing areas in the state of Pernambuco (Jupi, Bezerros and Boas Novas municipalities) (Brazil). Larvae, adults and pupae were maintained at the Laboratory of Insect-Toxicant Interactions (LIIT, Recife, Brazil), where larvae were fed on insecticide-free *B. oleracea* var. *acephala* leaves, and adults were fed with 10% honey solution according to Barros and Vendramim (1999). The conditions were 27 ± 0.2 °C, $65 \pm 5\%$ relative humidity (RH), and a photoperiod of 12:12 (L:D) h.

Selection with chlorfenapyr. For selection experiments, second-instar larvae of *P. xylostella* were exposed to collard leaf discs (6 cm diameter) treated with chlorfenapyr (Pirate[®], Concentrated Solution, 240 g a. i./L, BASF Brazil) formulation through leaf dip bioassays. The population of *P. xylostella* used for selecting the chlorfenapyr-resistant subsequent offspring was a synthetic population (pool of three subpopulations: Jupi, Bezerros, and Boas Novas) named PxClf-SEL. These populations were the most tolerant population seen in a previous susceptibility study (LIMA NETO et al., 2016). In each selected generation, 2,000 to 2,500 larvae were exposed to increasing concentrations of chlorfenapyr. Treated leaves were provided to larvae only once, and an untreated leaf was provided as needed until pupation. A side-by-side experiment was done with 100 larvae to assess the percentage of survival in each generation. The concentrations were 40, 56, 75, 100 and 150 mg of chlorfenapyr/L for generations F₁, F₂, F₃, F₄ and F₅, respectively. These concentrations were based on confidence intervals of LC₅₀ of curves that were

previously estimated. The concentration recommended by manufacturer to control of *P. xylostella* is 240 mg of chlorfenapyr/L.

Bioassays. Leaf dip bioassays (made for each selection with chlorfenapyr) were developed to estimate the chlorfenapyr concentration-mortality curves to *P. xylostella* populations. Previously, preliminary tests established the chlorfenapyr concentration ranges (six to eight concentrations) between the “all or none” response. The chlorfenapyr formulation was diluted in water + Triton X-100 (0.01%) as surfactant that was used in all the bioassays. Leaf discs (6 cm in diameter) of *B. oleracea* var. *acephala* were dipped in the insecticide or control [water + Triton X-100 (0.01%)] treatments for 10 s and then dried at room temperature for 1-2 h (SILVA et al., 2012). The leaf discs were then transferred to individual Petri dishes (6 cm diameter) lined with filter paper misted with water. Thereafter, the second-instar larvae (at least 10/ Petri dish) were placed on each treated leaf disc. The bioassays were kept at 27 ± 0.2 °C, $65 \pm 5\%$ relative humidity (RH), and a photoperiod of 12:12 (L:D) h. Mortality was assessed after 48 h of exposure. Larvae were considered dead if they failed to make coordinated movements. All bioassays were repeated twice.

Statistical Analysis. The concentration-mortality data were subjected to Probit analysis (FINNEY, 1971) using Polo-Plus software (LeOra Software Co., Petaluma, CA, USA) with correction for the natural mortality in the control treatment (ABBOTT, 1925). The narrow sense heritability (h^2) of resistance to chlorfenapyr was estimated using the formula $h^2 = R/S$, where R is the response to selection, and S is the differential selection (FALCONER; MACKAY, 1996). The 10-fold increase in the resistance to chlorfenapyr was estimated using the formula $G = R^{-1}$. The response to selection (R) was calculated by the formula $R = (L_f - L_i)/n$, where L_f and L_i are the log LC_{50} of the 1st and 5th generations and n is the number of generations under selection, respectively. The differential selection (S) was estimated using the formula $S = i \cdot \sigma_F$, where i is the selection intensity and σ_F is the phenotypic standard deviation. The selection intensity (i) was estimated for p, which is the percentage of individuals surviving the selection based on the properties of normal distribution using the tables presented in Falconer and MacKay (1996). The phenotypic standard deviation (σ_F) was estimated using the formula $\sigma_F = \frac{1}{2}(b_i + b_f)^{-1}$, where b_i and b_f are the initial and final slopes of the concentration-mortality curves, respectively (FALCONER; MACKAY, 1996).

Number of independent genes. Two methods were used to estimate the number of independent genes with additive effects in the expression of insecticide resistance (quantitative trait). According to Raymond, Pasteur and Geoghio (1987), the

number of genes (n_E) estimated for each generation was $n_E = \log_{10}(\% \text{ survivors})/\log_{10}(1/2)$. The second method was performed according to Lande (1981).

$$n_E = [\sum_{i=1}^N \sigma^2] / \sum_{i=1}^N (\sigma^2)^2$$

Here, s^2 = genetic variance of insecticide-selected estimated as $(\text{slope}^{-1})^2$; N = number of generations.

RESULTS AND DISCUSSION

This study showed that laboratory selection of the PxClf-SEL strain with chlorfenapyr increased the resistance quite steadily to a very high level. The background of this strain derived from three *P. xylostella* populations had already evolved low to moderate resistance to chlorfenapyr in the field. The steep increase in the resistance suggests a great additive effect of a gene or genes contributing to observed result. All of the concentration-mortality curves estimated in each generation of selection were fitted to the Probit model (χ^2 not significant $P > 0.05$) (Table 1). The LC_{50} (mg a. i./L) value varied from 27.6 (10.2 - 44.7) in the F₁ generation to 256.5 (137.6 - 384.7) in the F₅ generation for PxClf-SEL, which resulted in an increase of the resistance ratio (RR) from 33.4-fold to 309.9-fold compared with the susceptible strain [Rec-SUS, $LC_{50} = 0.83$ (0.4 - 1.4)] (Table 1). The slopes were 4.13 ± 0.85 and 1.60 ± 0.34 for the initial (F₁) and final generations of selection (F₅), respectively (Table 1). Such discrepancy on initial and final slopes may be because of the reduced dose relative to the doses in the field. These were used in the beginning of the selection to preserve insects to maintain the next generations.

The percentage of survivors in the selected F₁ and F₅ generations were 54 and 62% at 40 and 150 mg of chlorfenapyr/L, respectively (Table 1). The selection intensity (I), selection response (R) and the differential selection (S) were 0.61, 0.19 and 0.21, respectively (Table 2). The phenotype standard deviation (σ) was 0.35 (Table 2). We verified a high realized heritability of resistance to chlorfenapyr ($h^2 = 0.90$), which represents an increase of 10-fold in the level of resistance over ~5 generations (Table 2). In a recent study, the heritability of resistance to chlorfenapyr was $h^2 = 0.12$ in *Oxycarenus hyalinipennis* Costa (Hemiptera: Lygaeidae) (ULLAH; SHAH; SHAD, 2016). These authors reported a lower genetic interference in resistance to chlorfenapyr when compared with the present study. This agrees with the high heritability found for *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) resistant to spinosad ($h^2 = 0.71$) (CAMPOS et al., 2014) and for *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) resistant to malathion ($h^2 = 0.60$) (ASSIÉ et al., 2007).

Table 1. Selection of *P. xylostella* and dose-response curve of chlorfenapyr at each generation.

Strain	G ^a	N ^b	$\chi^2(df)^c$	LC ₅₀ (CI95%) ^d	Slope ± SE ^e	RR ₅₀ ^f (CI95%)
Rec-SUS	F ₃₄₀	221	7.5 (6)	0.83 (0.4 - 1.4)	1.41 ± 0.19	1 (0.65 - 1.55)
PxClf-SEL	F ₁	184	5.9 (5)	27.6 (10.2 - 44.7)	4.13 ± 0.85	33.4 (20.1 - 55.4)*
	F ₂	164	1.9 (4)	34.6 (18.1 - 53.8)	2.11 ± 0.51	41.9 (23.6 - 74.3)*
	F ₃	244	0.7 (4)	117.1 (87.2 - 162.4)	1.34 ± 0.18	141.3 (91.4 - 218.3)*
	F ₄	200	7.5 (5)	118.2 (51.5 - 67.1)	1.34 ± 0.17	142.9 (90.0 - 226.9)*
	F ₅	211	2.5 (5)	256.5 (137.6 - 384.7)	1.60 ± 0.34	309.9 (176.8 - 542.9)*

^aNumber of generations. ^bNumber of individuals used in bioassays. ^cChi-square and degree of freedom. ^dConfidence Interval at 95%. ^eStandard error. ^fResistance ratio calculated as in Robertson et al. (2007) and significant (*) when the confidence interval does not bracket the value 1.0.

The number of genes involved in the chlorfenapyr resistance following Raymond, Pasteur and Geoghiou (1987) was nearly 1.0 for F₁ and less than 1.0 for other generations (Table 3), indicating that chlorfenapyr-resistance is controlled by only one gene. Using the Lande's (1981) formula, the number of genes segregating across five generations was estimated as 1.88 genes for chlorfenapyr resistance (Table 3). These different results may be due to the Lande's (1981) method to have more variables indicating that the process of selection to chlorfenapyr used a secondary gene. This may be due to increasing concentrations used in the laboratory selection (< 150 mg chlorfenapyr/L), which favored more than one allele. However, in the field, the concentrations are quite high (240 mg chlorfenapyr/L), which potentially favor a monofactorial resistance development. The number of genes with chlorfenapyr resistance in *P. xylostella* can use the direct test across the mortality. This was estimated to progeny obtained via backcrossing. The

results indicated a high risk of resistance development in *P. xylostella* to chlorfenapyr. However, it is important to determine the degree of dominance of resistance for the best estimated risk assessment.

The heritability of chlorfenapyr resistance estimated here was higher than those observed with flubendiamide ($h^2 = 0.15$) (YAN et al., 2014), chlorantraniliprole ($h^2 = 0.26$) (GONG et al., 2014), spinosad ($h^2 = 0.02$) (SAYYED; WRIGHT, 2006), indoxacarb ($h^2 = 0.40$) (NEHARE et al., 2010) and *Bacillus thuringiensis* subsp. *kurstaki* ($h^2 = 0.19$) and *Bacillus thuringiensis* subsp. *aizawai* ($h^2 = 0.21$) (SAYYED et al., 2000). However, we highlight that all of these features were based on the selection of laboratory-established colonies or colonies presenting very low initial frequencies. Here, our results were based on a field colony with a pre-existing moderate level of resistance or sufficient variation to quickly develop resistance under selection.

Table 2. Parameters of the estimate of heritability held (h^2) to chlorfenapyr resistance at *P. xylostella* and the generation number (G) to increase the resistance to 10-fold based on the initial LC₅₀.

Parameters	Generation (F ₁ - F ₅)	
Estimate of response	Log ₁₀ (Initial LC ₅₀)	1.45
	Log ₁₀ (Final LC ₅₀)	2.41
	Response to selection (R)	0.19
Differential selection estimate	Survival after selection (S)	62 %
	Selection Intensity (I) ³	0.61
	Initial slope	4.13
	Final slope	1.60
	Phenotype standard deviation (σ)	0.35
	Differential Selection (S)	0.21
	h^2	0.90
G	5.20	

³According to Falconer and Mackay (1996).

The estimated realized heritability of resistance to chlorfenapyr may be explained by the selection pressure during the field control of *P. xylostella*. Furthermore, the various *Brassica* crops near each other contribute to low environmental variance in the areas of Jupi, Bezerros and Boas

Novas. These areas produce various Brassicaceae along the year, and contribute to environmental stability. This might explain the ratio of the majority phenotypic variation to be influenced by additive genetic variation (90.0%).

Table 3. Estimation of the number of genes contributing to chlorfenapyr-resistance in *P. xylostella* for different generations.

Generation	Concentration (mg a.i./L)	Survival (%)	Nº of genes (n)
F ₁	40	54	0.94
F ₂	56	65	0.62
F ₃	75	66	0.60
F ₄	100	75	0.42
F ₅	150	62	0.62
Mean	-	62.66	0.64

The environmental variance is dependent on the conditions of culture or management. Thus, more variable conditions reduce the heritability (FALCONER; MACKAY, 1996). Therefore, continuous pressure with insecticides especially chlorfenapyr after failure of other molecules has generated uniform conditions to quickly develop resistance to chlorfenapyr. In this sense, monitoring resistance and multiple attack approaches with different modes of action should mitigate the problems of chemical control in the *P. xylostella* field populations.

CONCLUSION

The heritability of chlorfenapyr resistance was likely influenced by additive genetic effects. Therefore, resistance to chlorfenapyr can develop quickly in *P. xylostella*. Different methods of estimating heritability showed different number of genes involved in resistance of *P. xylostella* to chlorfenapyr.

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