Selection of *Aphis craccivora* (Hemiptera: Aphididae) for Resistance to Thiamethoxam Insecticide under Laboratory Conditions[1]

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Abstract The cowpea aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae), is a polyphagous pest that causes yield losses in a variety of crops worldwide. Its management relies mainly on the use of chemical insecticides. This study followed the development of resistance to thiamethoxam in *A. craccivora* under laboratory conditions for 24 generations. The initial median lethal concentration (LC_{50}) of thiamethoxam in a susceptible laboratory population of *A. craccivora* was 2.62 ppm. Exposure of surviving aphids in each generation to thiamethoxam caused an increase in the LC_{50} with each successive generation. After 24 generations, the LC_{50} had increased to 225.83 ppm, an 86.2-fold increase over the initial LC_{50}. Resistance, as indicated by the increasing LC_{50}, appeared as early as the second generation with a 1.4-fold increase over the initial generation. By the 14th generation, the LC_{50} had increased by 29-fold, and by the 16th generation, it had quickly increased by 69.4-fold. Our results indicate that *A. craccivora* populations repeatedly exposed to thiamethoxam in a cropping system can lead to rapid development of resistance to the insecticide. Any overuse or other misuse of the pesticide may lead to an even more rapid development of high levels of resistance to thiamethoxam within a short period of time. Elevated levels of the detoxifying enzymes, carboxylesterase, glutathione-*S*-transferase, and mixed function oxidases, in aphids exhibiting resistance as compared with the initial susceptible population indicated that the detoxifying enzymes, especially the carboxylesterases, are likely involved in facilitating the development of resistance to thiamethoxam in *A. craccivora*.

Key Words resistance, thiamethoxam, aphids, detoxifying enzymes

The cowpea aphid, *Aphis craccivora* Koch (Aphididae: Homiptera), is a polyphagous pest of about 50 crops worldwide, representing 19 plant families (Obopile and Ositile 2010, Radha 2013). *Aphis craccivora* has reportedly caused 20–40% yield losses in Asia and approximately 35% losses in Africa (Singh and Allen 1980). Up to 100% yield loss has been reported in various bean and pea crops (Ganguli and Roychaudhury 1984).

Currently, management practices for aphids rely on the use of synthetic chemical insecticides of the neonicotinoid group (Tang et al. 2013). Neonicotinoids are

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registered for use in more than 120 countries and found to be effective against pests with sucking feeding habits (e.g., hemipterans, homopterans, thrips) on various crops. Ubiquitous use, however, has imposed a mounting selection pressure that facilitates resistance development, and in several species, resistance has now reached levels that compromise the efficacy of these insecticides (Bas et al. 2015).

Thiamethoxam is one of the neonicotinoids of thionicotinyl group showing promise against approximately 100 economically important aphid species. Among them, approximately 20 species have developed at least one known insecticide resistance mechanism (Foster et al. 2017). In India, resistance to thiamethoxam has yet to be reported in *A. craccivora* under field conditions. Therefore, we undertook this study with the objective of estimating the potential magnitude of the development of resistance in *A. craccivora* to thiamethoxam under laboratory conditions with artificially imposed selection pressure. We also aimed at defining the biochemical mechanisms underlying this development of resistance.

Materials and Methods

The laboratory experiment was conducted at the Insecticide Toxicology Laboratory of the Department of Entomology, College of Agriculture, Odisha University of Agriculture and Technology (OUAT), Bhubaneswar, India, during 2018–2019. This facility is situated at 20.2647°N latitude and of 85.8141°E longitude at an elevation of 25.9 m above sea level and 64 km west of the Bay of Bengal.

Aphid collection and rearing. *Aphis craccivora* specimens were collected from a field of cowpea, *Vigna unguiculata* (L.) Walpers, grown at the Central Research Station Farm at OUAT. The crop had not been treated with or exposed to any insecticide. The aphids were transported to the department and reared in well-ventilated cages in an insectary on cowpea seedlings in 15-cm-diameter pots filled with soil and maintained at ambient conditions. This initial population of aphids was reared continuously without being exposed to any insecticides throughout the study period. From this colony, apterous adults were used for the initial bioassay, thus, establishing a toxicity baseline for susceptibility to thiamethoxam. The initial generation of aphids was treated with thiamethoxam at a concentration that was 60% over the initial median lethal concentration (LC_{50}), as determined by bioassay. Leaf dip bioassays were used to estimate the LC_{50} of each successive generation, and those aphids within a generation that survived the treatment were then subjected to a selection pressure of 60% over the previous generation's LC_{50} in every two generations.

Leaf dip bioassay. The baseline and successive generation toxicity levels were determined using the leaf dip bioassay of Moores et al. (1996). Six to seven concentrations of thiamethoxam were prepared by serial dilution from a stock solution of 1% thiamethoxam based on the preliminary and repeated response of adult aphids. Each concentration was replicated three times. Freshly excised cowpea leaves collected from untreated plants were immersed in the respective thiamethoxam solutions for 10 s; controls were immersed in water only. Upon removal from the solutions, the leaves were dried for 30 s under circulating air and placed with the abaxial surface upward in 60-mm-diameter plastic containers lined with moistened filter paper and with a mesh cap. Twenty adult aphids from the susceptible colony were placed in each container using a camel hair brush. The containers with

the aphids were maintained in an incubator operated at 28 ± 2°C and 75–80% relative humidity. Aphid mortality was assessed after 24 h. Aphids that were unable to right themselves within 10 s once turned on their backs were considered moribund and counted as dead. Mortality data were subjected to corrections following Abbott (1925) if control mortality was observed and were subjected to probit analysis (Finney 1971) using Ldp line software (version 0, release date 9 May 2013, Cairo, Egypt).

Resistance selection and data analysis. The LC_{60} obtained from the baseline toxicity for the susceptible laboratory colony served as the first-generation selection pressure of thiamethoxam against the aphid population. Adult apterous aphids were released on untreated cowpea plants in separate cages in the insectary 24 h prior to application of the insecticide at the LC_{60} level. These plants colonized with aphids were sprayed with LC_{60} of the parental susceptible generation. Neonates from the surviving adults continued to be reared on the same treated plants until the next generation. The LC_{50} was determined every two generations using the leaf dip bioassay. The LC_{60}, based on the previous generation LC_{50}, was used for each successive generation. The magnitude of resistance development (resistance ratio) was calculated by dividing the LC_{50} of the selected generation by the LC_{50} of the susceptible laboratory colony. The selection continued through 24 generations.

Detoxification enzymes activity. Several biochemical parameters were determined among the generations and compared with the susceptible laboratory colony. Protein content was determined by using bovine serum albumin as the standard (Bradford 1976). Esterase activity in the resistant and susceptible colonies were assayed with α-naphthyl acetate as substrate following the procedure of Van Asperen (1962). Activity of the mixed function oxidases (MFO; monooxygenases) were analyzed according to the protocol of Hansen and Hodgson (1971). The glutathione-*S*-transferase (GST) was measured as described by Habing et al. (1974). Acetylcholinesterase activity was measured following Ellman et al. (1961) in both resistant and susceptible *A. craccivora* populations.

Results and Discussion

Initially, various concentrations of thiamethoxam were used repeatedly based on the response of adult apterous aphids from the leaf dip bioassay. The final concentrations of thiamethoxam used in determining the baseline toxicity were 0.5, 1.0, 3.0, 5.0, 7.0, 9.0, and 10.0 ppm. The probit analysis determined the LC_{50} value of 2.62 ppm, with fiducial limits of 1.91 to 3.43 ppm. The slope of the probit mortality line was 1.60 ± 0.22, with an intercept of 4.327. The X^2 value was 10.13, with a tabulated value of 11.1.

The LC_{50} values for the remaining generations increased to 3.61 ppm in the 2nd generation, to 7.11 ppm in the 4th generation, 23.49 ppm in the 6th generation, 31.32 ppm in the 8th generation, 47.08 ppm in the 10th generation, 48.76 ppm in the 12th generation, 75.92 ppm in the 14th generation, 181.73 ppm in the 16th generation, 196.58 ppm in the 18th generation, 199.57 ppm in the 20th generation, 207.21 ppm in the 22nd generation, and 225.83 ppm in the 24th generation (Table 1). In 24 generations of selection pressure with applications of thiamethoxam, we obtained a population of *A. craccivora* showing 86.19-fold resistance to thiamethoxam as compared with the initial parental line. The progression of resistance over the 24 generations gradually increased from a 1.4-fold to 29-fold from the 2nd to the 14th generation.

Table 1. Development of resistance to thiamethoxam in *Aphis craccivora* under laboratory conditions selected for 24 generations.

Serial Number	Selected Generations (F_n)	Median Lethal Concentration (ppm)	Fiducial limits (ppm)		Intercept	Regression Coefficient (Slope) $b \pm$ SE	Regression Equation	Resistance Ratio
			Lower Limit	Upper Limit				
1	F_0	2.62	1.91	3.44	4.32	1.60 ± 0.22	$Y = 1.60X + 4.32$	—
2	F_2	3.61	2.54	4.69	4.07	1.65 ± 0.28	$Y = 1.65X + 4.07$	1.37
3	F_4	7.11	5.49	8.83	3.35	2.04 ± 0.27	$Y = 2.04X + 3.35$	2.71
4	F_6	23.49	19.03	28.66	2.09	2.19 ± 0.30	$Y = 2.19X + 2.09$	8.96
5	F_8	31.32	24.10	38.72	1.85	2.14 ± 0.28	$Y = 2.14X + 1.85$	11.95
6	F_{10}	47.08	37.43	56.97	1.25	2.34 ± 0.32	$Y = 2.34X + 1.25$	17.96
7	F_{12}	48.76	38.24	59.87	1.46	2.12 ± 0.30	$Y = 2.12X + 1.46$	18.61
8	F_{14}	75.92	63.05	89.28	0.19	2.59 ± 0.41	$Y = 2.59X + 0.19$	28.97
9	F_{16}	181.73	157.13	209.41	−1.83	3.08 ± 0.63	$Y = 3.08X - 1.83$	69.36
10	F_{18}	196.58	174.04	216.87	−4.63	4.18 ± 0.78	$Y = 4.18X - 4.63$	75.02
11	F_{20}	199.57	177.08	220.45	−4.41	4.14 ± 0.78	$Y = 4.14X - 4.41$	76.17
12	F_{22}	207.21	189.24	225.75	−6.24	4.99 ± 0.83	$Y = 4.99X - 6.24$	79.08
13	F_{24}	225.83	204.45	244.41	−7.36	5.20 ± 0.98	$Y = 5.20X - 7.36$	86.19

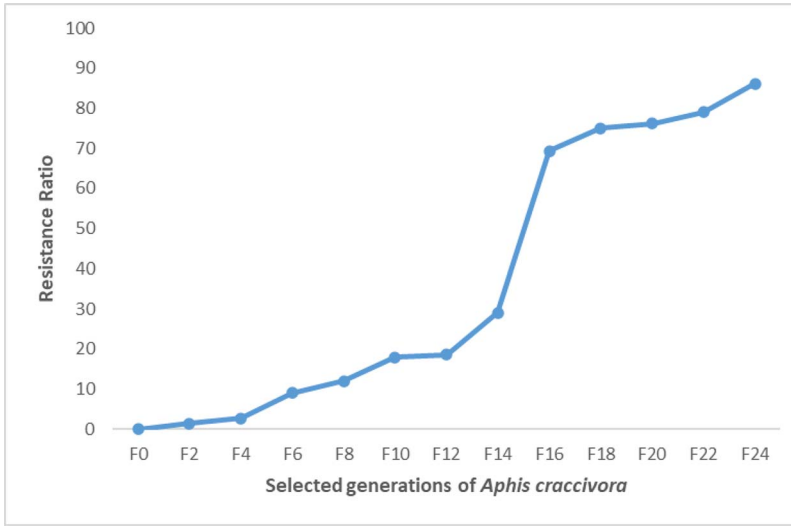

Fig. 1. Resistance ratio to thiamethoxam in different generations of selection in *Aphis craccivora*.

There was a marked increase from the 14th to the 16th generation when the level of resistance increased to 69.4-fold larger than the susceptible laboratory colony (Fig. 1). Thereafter, the increase was more gradual until it reached a level that was 86.2-fold greater than the baseline level (Fig. 1).

A comparison of log concentration–probit mortality regression lines (Fig. 2) indicated that the line for the thiamethoxam-resistant population gradually shifted to the right as selection progressed and was distinctly separated from that of the parental population,

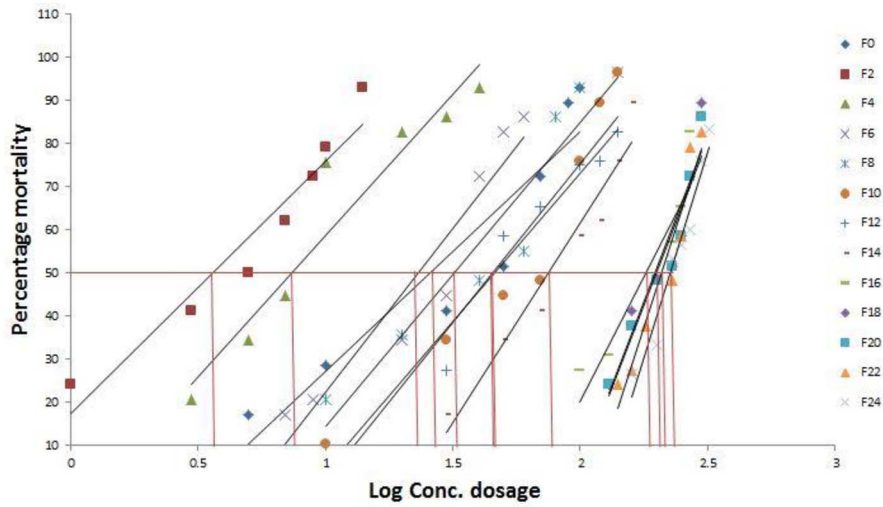

Fig. 2. Log concentration versus probit mortality regression lines.

indicating a definite increase in resistance. The almost parallelism of the regression lines shown in Fig. 2 suggests that the method of selection over those generations was efficient in the removal of more susceptible insects from the parental population and had produced a homogenous thiamethoxam-resistant population of *A. craccivora*. Resistance development was affected by the following: (a) the parental population was heterogeneous, thus ensuring the presence of resistant genotypes; (b) the selection for resistance to thiamethoxam was facilitated by applying the systemic insecticide (translaminar) on the plants infested with insects so that all the developmental stages of the insects fed on the insecticide-contaminated plant sap and that the survivors of the treatment alone had a chance to propagate; and (3) the selection was intensified by maintaining a selection pressure of 60% in each successive generation.

The slope of the log concentration–probit mortality regression line showed a gradual increase from 1.6 ± 0.22 in the susceptible parental generation to 5.20 ± 0.98 in the 24th generation of selection, with significant variation among the generations (Table 1). Very little increase in the slope occurred from 4th (2.04 ± 0.27) to 14th generation (2.59 ± 0.41).

Our results indicate that thiamethoxam, like other neonicotinoids, has great potential to impart resistance in *A. craccivora*. Wang et al. (2002) reported that *A. gossypii* showed 8.1-fold in resistance to imidacloprid (a related neonicotinoid insecticide of the chloronicotinyl group having a similar mode of action as the thionicotinyl insecticide thiamethoxam) after being selected for 13 generations. According to Mokbel (2007), resistance to the neonicotinoid insecticide dinotefuran in *A. craccivora* was slow and reached 4.13-fold in comparison to the parent population after 10 generations of intense selection. Koo et al. (2014) found that field populations of *A. gossypii* exhibited extremely high resistance ratios to the neonicotinoids acetamiprid, clothianidin, thiacloprid, and imidacloprid. Resistance to thiamethoxam was about 48-fold greater when a population of *A. craccivora* was selected for 12 generations (Abdallah et al. 2016). A strain of *A. gossypii* with 13.8-fold resistance to thiamethoxam also was reported by Wei et al. (2017). Pan et al. (2018) determined that thiamethoxam resistance in *A. gossypii* was associated with the presence of multiple Uridine 5'-Diphosphoglucuronosyltransferases.

Resistance to neonicotinoid insecticides has been reported in many insects other than *A. craccivora*. Prabhaker et al. (1997) selected a field-collected population of silverleaf whitefly, *Bemisia argentifolii* Bellows and Perring, with the nicotinyl compound imidacloprid over 32 generations and concluded that resistance increased by only 82-fold. In the brown planthopper, *Nilaparvata lugens* Stål, laboratory selection increased imidacloprid resistance by 11.35 times in 25 generations, as the resistance ratio reached 72.8 compared with a laboratory susceptible strain (Liu et al. 2003). It may be inferred from this information that development of resistance to neonicotinoids, irrespective of the insect species, is slower and more gradual than with the synthetic pyrethroids, which is very high within a few generations of selective pressure. Misra and Bhatia (1997), for example, determined a 201.7-fold increase in seven generations of selection to fenvalerate for *Tribolium castaneum* Herbst.

Some scientists have used differences in regression slopes to make inferences about the progression of resistance. Initially, Hoskins and Gordon (1956) stated that the slope of the concentration–mortality curve from pesticide bioassays was greatest both at low and high levels of resistance because genetic variation was

Table 2. Activity of detoxifying enzymes in resistant and susceptible populations of *Aphis craccivora*.

Serial Number	Detoxification Enzymes	Mean ± SE Specific Activity† (nmol/min/mg protein)		Activity Ratio††
		Susceptible	Resistant	
1	Mixed function oxidase (nmol/min/mg protein)	4.20 ± 1.76	29.61 ± 6.49*	7.05
2	Carboxylesterase (nmol/min/mg protein)	0.03 ± 0.02	1.20 ± 0.60**	42.02
3	Glutathione-*S*-transferase (nmol/min/mg protein)	6.24 ± 3.01	18.80 ± 5.87	3.01

* Significantly different at $P = 0.05$.
** Significantly different at $P = 0.01$.
† Mean (±SE) specific activity determined over 15 replicates.
†† Activity ratio = activity in resistant population/activity in susceptible population.

lowest at these two extremes. Hoskins (1960) also proposed that the slope of dose–mortality curve represents the phenotypic variations in susceptibility in a population including both environmental and genetic components. Chilcut and Tabashnik (1995) subsequently tested the hypothesis on an exhaustive scale and opined that slope was not a good indicator of the genetic variation of susceptibility, and for most pests and pesticide combinations, slopes were not highest at the two extremes. Hence, slope values obtained in our study have no inferences on the progression of resistance in relation to genetic variability.

We also saw that the level of activity of some detoxifying enzymes increased in those populations of *A. craccivora* that were resistant to thiamethoxam (Tables 2 and 3). The mean activity of MFO was 4.20 ± 1.76 nmol/min/mg protein in susceptible population, whereas the activity of 29.61 ± 6.49 nmol/min/mg protein was significantly ($P = 0.05$) higher in the resistant population. Carboxylesterase (CbE) activity was also significantly ($P = 0.05$) higher in resistant (1.20 ± 0.60 nmol/min/mg protein) than in susceptible (0.03 ± 0.021 nmol/min/mg protein) populations. The GST activity was three times higher in resistant (18.80 ± 5.87 nmol/min/mg protein) than in the susceptible population (6.24 ± 3.0 nmol/min/mg protein). Of the enzymes monitored, Ach activity was the only one that did not differ between susceptible and resistant populations.

Table 3. Mean (±SE) activity of acetyl cholinesterase in the thiamethoxam susceptible and resistant populations of *Aphis craccivora*.

Population	Specific Activity (μmol/min/mg)*	Activity Ratio**
Susceptible	8.27 ± 1.98	Not applicable
Resistant	8.52 ± 1.08	1.03

* Activity determined with 15 replicates.
** Activity ratio = activity in resistant population/activity in susceptible population.

Abdallah et al. (2016) reported similar results with *A. craccivora*, although recorded CbE activity as 30 times greater in the thiamethoxam-resistant strain than in the susceptible strain. We saw a 42 times difference. In addition, they also found that GST and MFO activity levels increased by only 3.7 and 2.7 times, respectively, in resistant versus susceptible strains, while the differences we saw were three times greater with GST and seven times greater with MFO. These differences in the magnitude of the enzyme activity could be attributed to a number of factors, including the degree of selection pressure and the length of time of the selection.

Fouad et al. (2016), after monitoring the resistance in three field populations of *A. craccivora* to seven insecticides (organophosphates, carbamates, and neonicotinoids) and measuring enzyme activity levels for each, opined that resistance in *A. craccivora* to those insecticides may be due to the higher activity of the CbEs. Cao et al. (2008) proposed that increased CbE detoxification is due to gene overexpression accounting for omethoate resistance in laboratory-selected *A. gossypii*.

In conclusion, the results of our study indicate that there is a high probability of the development of resistance in *A. craccivora* to thiamethoxam when the toxicant is used repetitively. Continuous selection through use will likely further increase that likelihood. We also determined that CbE and related detoxifying enzymes, such as GST and MFO, are higher in resistance than in susceptible populations of *A. craccivora* and are likely, at least partially, responsible for the observed increased level of resistance to thiamethoxam.

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