Indian Journal of Entomology Online published Ref. No. e23199 DoI. No.: 10.55446/JE.2023.1199

MONITORING THE TOXICITY OF CYANTRANILIPROLE TO THE FIELD POPULATIONS OF *SPODOPTERA LITURA* (F.) FEEDING COLE CROPS

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ABSTRACT

***Spodoptera litura*, a significant insect pest of tropical agriculture, devastates more than 112 cultivated plants. It has become challenging to manage with insecticides alone as it develops resistance quickly. Cyantraniliprole, a newer insecticide under diamide, was introduced a decade ago and is widely used to manage several lepidopteran and hemipteran insects. This study was taken to look after the field efficiency of cyantraniliprole against *S. litura* and the possible mechanism of resistance development in cole crop regions of Tamil Nadu. The maximum LC_{50} obtained was 0.20 mg/ ℓ in Theni population and the least (0.11 mg/ ℓ) in Dindigul, with no significant difference between the field populations. The resistance ratio observed in the field populations were between 1.89 – 1.09, indicating that the field populations were susceptible. Further, all three detoxifying enzymes, mixed function oxidase, glutathione S-transferase, and carboxylesterase, were found to differ irrespective of the time of insecticide exposure and the populations examined, indicating that they have no role against cyantraniliprole.**

Key words: Cole crops, cyantraniliprole, diamides, MFO, GST, CarE, *Spodoptera litura*, LC_{50}, resistance ratio, toxicity.

*Spodoptera litura (*F.) the tobacco caterpillar*,* is an extensive polyphagous feeder widely distributed across South and East Asia and Oceania. It infests at least 40 different plant families (EFSA, 2022). The early instars of *S. litura* cause damage to leaves by scrapping the mesophyll. In contrast, the later instars feed the whole leaves (Dhaliwal et al., 2010), destroying plants in case of severe incidence (Channakeshava et al., 2020). Over a few decades, chemical insecticides have been the primary method for managing this pest (Huang and Han, 2007). Nevertheless, in recent days, insecticide sprayings have become ineffective in managing *S. litura*. All along its exposure, this pest has gained resistance to 42 insecticides of various classes, including newer insecticides like indoxacarb, metaflumizone, chlorantraniliprole, spinosad, etc. (APRD, 2022). Their resistance to various insecticides leads to pest outbreaks and immense crop damage (Ahmad et al., 2007). High reproductive capacity, several overlapping generations in a year, high migrating ability, and nocturnal behaviour highlight that it can evolve resistance against insecticides (Kranthi et al., 2001). India is the second largest producer of cole crops next to China (FAO, 2022), with the high productivity of cabbage (23.2 MT/ ha) and cauliflower (19.64 MT/ ha) (Indiastat, 2022). However, these crops are prone to many leaf-feeding insect pests. Diamides are the newer class of insecticides currently marketed in India and include three commercially registered compounds: flubendiamide, chlorantraniliprole, and cyantraniliprole. Of these, cyantraniliprole was commercialized in 2008. Diamides are widely used to manage lepidopteran insect pests as they differ in their mode of action from other insecticides. A decade after the commercialization and use of diamides, many insects have developed resistance against them (Richardson et al., 2020). Like any other pest, several field populations of *S. litura* have developed resistance to flubendiamide and chlorantraniliprole in India (Grace et al., 2019) and worldwide (Su et al., 2012). However, the *S. litura* field populations' resistance to cyantraniliprole in India needs an indepth study. Thus this study was carried out to monitor the cyantraniliprole toxicity to the *S. litura* field populations thriving in brassica crop growing regions in Tamil Nadu and to assess the functional role of detoxification enzymes, mixed function oxidases (MFO), glutathione S-transferases (GST), and carboxylesterases (CarE) in resistance development against cyantraniliprole.

MATERIALS AND METHODS

Surveys were conducted in different Tamil Nadu districts, which are cole crops growing regions (viz., Coimbatore, Dharmapuri, Dindigul, Krishnagiri, Theni, and Tiruppur) to collect the field populations (coded as CBE, DHA, DIN, KRI, THE and TIR, respectively) to collect *S. litura* (either egg masses or neonates) in 2021 and 2022. The susceptible culture of *S. litura* obtained from the National Bureau of Agricultural Insect Resources (NBAIR) was maintained at a different laboratory away from the field-collected populations. Both field strains and susceptible populations were maintained under regulated temperature (27± 2° C), relative humidity (50-70%), and photoperiod (16:8 – L:D). Adults of field and susceptible populations were reared in separate cages (45× 45× 45 cm) supplemented with Vit. E enriched with the honey solution and allowed to lay eggs on fresh, cleaned *Nerium* leaves. The hatched larvae were raised in a semi-synthetic chickpea flour-based diet fortified with sorbic acid, ascorbic acid, multivitamins, yeast, agar agar, streptomycin sulfate, etc. (Gupta et al., 2005). Using commercially available cyantraniliprole, IRAC (Insecticide Resistance Action Committee) susceptibility test method no: 018 was used to conduct leaf dip bioassay. Washed pests and pesticide-free cauliflower (*Brassica oleracea var. botrytis*) leaf discs (4.5 cm diameter) were dipped (10 sec) in different concentrations of cyantraniliprole solutions prepared in distilled water containing Triton X-100 (1g/ ℓ). The control was prepared by treating leaf discs with distilled water containing Triton X-100 (1g/ ℓ), and the treated leaves were allowed to air dry for 10 mins and placed in the breeding dish. About 10 pre-starved second-instar larvae were released in each treatment, and each treatment was replicated thrice.Then the larval mortality was assessed after 72 hr, and those that did not move when disturbed with a brush were considered dead.

The MFO, GST, and CarE were analyzed at three different times (viz., 3, 24, and 48 h) in the two field populations (THE and KRI) showing significant resistance, and the susceptible population after exposing the second instar larvae to the sub-lethal insecticide concentration (LC_{25}) of cyantraniliprole. About 15 mg of live larvae after insecticide treatment were homogenized with respective buffers (0.1M Tris buffer, 10 mM glutathione reduced, pH 8.0) for GST; 50 mM ice-cold tris buffer, 1.15% KCl, and 1.0 mM EDTA for MFO; (0.1M phosphate buffer, pH 7.5, containing 0.1% Triton X-100 for CarE) under ice-cold conditions using pestle and mortar and centrifuged at 10000 rpm, at 4°C for 12 min. The supernatant was stored at -20°C. Based on Bradford (1976), the protein was estimated using bovine serum albumin as a standard, and the absorbance was measured spectrophotometrically at 595 nm using BioSpectrometer®.MFO was evaluated using *p*-nitroanisole as the substrate, with a few modifications from Yasoob et al. (2018). 50 mM *p*-nitroanisole (in ethanol) and 200 ml of 10.0 mM NADPH were added to 100 µl of enzyme source, and it was incubated in darkness for 3 min at room temperature.The end product *p*-nitrophenol was measured at 405 nm at 30-sec intervals for 3 min and calculated using the extinction coefficient of 3.32 $mM^{-1}cm^{-1}$ (Sharma, 2017), expressed as nmol *p*-nitrophenol released x min^{-1} x mg^{-1} protein. Using CDNB (1-chloro-2, 4-dinitrobenzene) as a substrate, based on Kao et al. (1989), GST was estimated. To 100 µl of enzyme source, 0.1M tris buffer and 0.1 M CDNB were added, and at 340 nm, the absorbance was recorded for 3 min at 30-sec intervals. Based on the extinction coefficient 9.6 mM^{-1} cm^{-1} of CDNB, the activity of GST was calculated and expressed as µmol 2, 4-dinitrophenyl glutathione formed x min^{-1} x mg^{-1} protein. To quantify CarE, based on Gong et al. (2013),100 µl of enzyme source was mixed with 450 µl of chilled phosphate buffer (0.04 M) and 1.80 ml of 1-naphthyl acetate (0.3 mM). The mixture was then left to incubate at room temperature for 30 min. To the incubated mixture, a coupling reagent was added (two parts of fast blue RR salt (1%) and five parts of sodium dodecyl sulfate (5%)). Further, the optical density was recorded after 15 min at the wavelength of 600 nm, and the activity of CarE was calculated using the extinction coefficient of 1-naphthyl acetate (2.22 $mM^{-1}cm^{-1}$) as used by He (2003).

The obtained mortality data were subjected to Probit analysis to estimate LC_{50}, LC_{95}, fiducial limits, chi-square, degrees of freedom, and slope. The resistance ratio (RR) was calculated according to Tamilselvan et al. (2021) to classify the insect population as susceptible (RR <3), less susceptible (RR= 3.1-5.0), low resistance (RR= 5.1-10.0), moderate resistance (RR= 10.1- 40.0), high resistance (RR= 40.0-160.0) and very high resistance (RR>160). Data were analyzed using one-way analysis of variance (ANOVA) ($p < 0.05$), and the mean values were compared using Tukey's test (Tukey, 1977) with IBM SPSS® Statistics 22.0.

RESULTS AND DISCUSSION

The susceptible population obtained from NBAIR exhibited the lowest median lethal concentration mortality (0.09 mg/ ℓ) to cyantraniliprole. Among the field populations, THE population had a 50 percent mortality at a higher lethal concentration of 0.20 mg/

ℓ. It was followed by KRI (0.17 mg/ ℓ) and CBE (0.14 mg/ ℓ). Least LC_{50} was observed in DIN (0.11 mg/ ℓ). Based on the LC_{50} value, all the field-collected *S. litura* populations recorded a lower resistance ratio of 1.89 – 1.09, indicating that all the field populations were susceptible to cyantraniliprole (Table 1). There is no significant difference between the field populations in their lethal concentrations, as their fiducial limits were on par. However, the probit model had a very excellent match to the concentration-mortality responses of *S. litura* to cyantraniliprole. Cyantraniliprole is a newer diamide insecticide widely used to manage several lepidopteran and hemipteran insect pests (Lahm et al., 2009). A report from China indicates that five out of seventeen field populations of *S. litura* collected from crops like *Colocasia* and *Nelumbo* showed moderate resistance to cyantraniliprole, with the RR ranging from 10.9 to 16.1 (Sang et al., 2016). There were reports of *S. litura* getting moderate resistance to another diamide molecule, chlorantraniliprole, with a resistance ratio of 22.3 (Wang et al., 2019), 24.4 (Su et al., 2012), and 12.4 (Muthusamy et al., 2014). There were reports of cross-resistance between chlorantraniliprole and cyantraniliprole in several insects like *P. xylostella* (Liu et al., 2015), *S. litura* (Sang et al., 2016) and *S. frugiperda* (Bolzan et al., 2019). Since these insecticides act on the same RyR, cross-resistance development for these compounds is possible. We found that field populations of *S. litura* feeding cole crops are still susceptible to the insecticide cyantraniliprole.

The activities of detoxifying enzymes, MFO, GST, and CarE were analyzed in susceptible and the two field populations, which had comparatively high LC_{50}than other field populations. The concentrations of all three enzymes were found to fluctuate irrespective of the assay time studied in the post-insecticide exposure period. The concentration of MFO in the susceptible population at 3 hours after treatment (HAT) of cyantraniliprole was 9.71 nmol mg of protein^{-1}min^{-1}, which raised to 12.22 nmol mg of protein^{-1}min^{-1} at 48 HAT, indicating a significant increase in its concentration (Fig. 1A). The concentration of MFO was found to decrease substantially in resistant THE population from 13.36 (at 3 HAT) to 11.71 (at 48 HAT) nmol mg of protein^{-1}min^{-1} while, in KRI, the concentration decreased significantly at 24 HAT (7.77 nmol mg of protein^{-1}min^{-1}) and had a significant increase at 48th HAT (18.22 nmol mg of protein^{-1}min^{-1}). However, the concentration of MFO was higher in the susceptible population than in the field population at 24 HAT (both THE and KRI) or 48 HAT (THE alone). There were no significant differences in the GST concentration between different HAT (at 3, 24, 48 HAT) in the susceptible population (Fig. 1B). In THE population, though, there was no significant difference in the GST levels between 3 and 24 HAT, it increased significantly (3.50 µmol mg of protein^{-1} min^{-1}) at 48 HAT. However, in the KRI population, the concentration of GST increased considerably to 3.30 µmol mg of protein^{-1}min^{-1} at 24 HAT itself. However, the GST concentration was higher in susceptible at 3 and 24 HAT than in both the field populations. The concentration of CarE increased significantly in the susceptible populationat 24 and 48 HAT compared to 3 HAT and reached the maximum (1.00 µmol mg of protein^{-1}min^{-1}) at 48 HAT (Fig. 1C). However, no significant variation was encountered in the CarE concentration in THE population. Nevertheless, in KRI, a notable increase in the concentration of CarE was observed at 24 HAT (0.73 µmol mg of protein^{-1}min^{-1}). Regardless of these variations, the concentration of CarE was higher in the susceptible than in the field populations tested at all three different HAT examined. Overall, the concentrations of detoxifying enzymes were found to vary indistinctly, irrespective of the time of insecticide exposure. Further, the enzyme concentrations were higher in the lab maintained susceptible than in the field

Table 1. Log dose probit mortality for cyantraniliprole tested against second instar larvae of field-collected *Spodoptera litura*

Location	Population code	*S. litura* Source crop	n	Slope± SE	χ^2 (df)	LC_{50} (mg/ ℓ) (95% FL)	LC_{95} (mg/ ℓ) (95% FL)	RR
Susceptible	-		180	1.446± 0.265	3.43 (3)	0.09 (0.019 -0.17)	1.21 (0.49-34.60)	-
Coimbatore	CBE	Cabbage	180	1.020± 0.310	0.52 (3)	0.14 (0.06-0.22)	5.69 (1.53-863.96)	1.27
Dharmapuri	DHA	Cauliflower	180	0.694± 0.196	2.20 (3)	0.13 (0.04-0.27)	23.07 (4.64-3287.5)	1.44
Dindigul	DIN	Cauliflower	180	1.514± 0.292	2.23 (3)	0.11 (0.071-0.16)	1.37 (0.72-5.30)	1.22
Krishnagiri	KRI	Cabbage & Cauliflower	180	0.913± 0.271	1.77 (3)	0.17(0.08-0.30)	10.45 (2.24-3008.64)	1.89
Theni	THE	Cauliflower	180	1.161± 0.267	2.85 (3)	0.20 (0.13-0.35)	5.10 (1.58-102.08)	1.82
Tiruppur	TIR	Cauliflower	180	1.389± 0.321	1.34 (3)	0.12 (0.06- 0.16)	1.74 (0.82-11.71)	1.09

n: No. of larvae tested; df: degrees of freedom; FL: Fiducial limits; RR, Resistance Ratio - the LC_{50} of laboratory maintained susceptible population utilized as factor divisor.

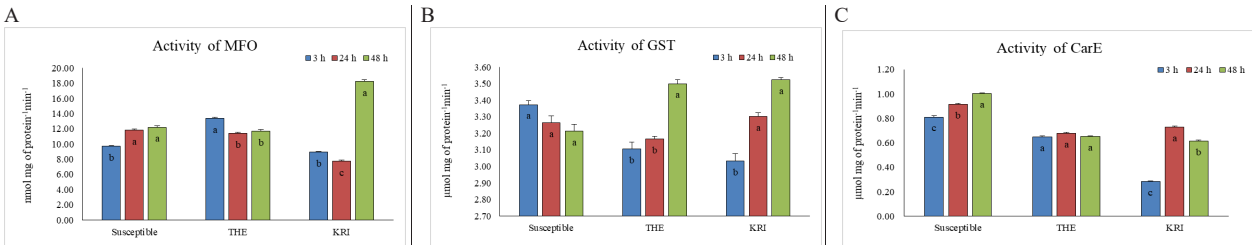

Fig. 1. Activities of detoxifying enzymes in the laboratory maintained susceptible and two field populations of *Spodoptera litura* at 3,24, and 48hours after treatment (HAT) to cyantraniliprole. A:MFO, Mixed function oxidase (expressed as nmol/ min/ mg of protein); B: GST, Glutathione S transferase (expressed as µmol/ min/ mg of protein); C: CarE, Carboxyl esterase (expressed as µmol/min/mg of protein)

populations then and there. Hence, the role of detoxifying enzymes in *S. litura* for resistance development against cyantraniliprole is nullified.

Generally, insects have sufficient detoxification mechanisms to overcome xenobiotic encounters (Gao et al., 2022). After sublethal exposure to cyantraniliprole, the activities of cytochrome P450 monooxygenases (CYP450) and CarE were found to get increased significantly in small brown planthopper *Laodelphax striatellus* (Wang et al., 2022). Similarly, the concentration of detoxification enzymes like GST, MFO, and CarE or esterase was found to increase either after sublethal exposure to diamides in *P. xylostella* (Hu et al., 2014) or diamide-resistant field populations (flubendiamide and chlorantraniliprole) like *S. litura* (Muthusamy et al., 2014), *P. xylostella* (Zhang et al., 2016) and *Ostrinia furnacalis* (Cui et al., 2017). In contrast, there is a report that the resistance of insects to diamide is not metabolism-mediated (Ribeiro et al., 2017). In another study, the point mutation at the RyR, where the diamide insecticide compounds act,was reported in mediating resistance (Troczka et al., 2017). The mutation site (G4946E), which weakened the toxicity of diamides, was confirmed by establishing a cloned Sf9 (*S. frugiperda*) cell lines expressing the muted (G4946E) RYR constantly, showed decreased efficacy to flubendiamide and chlorantraniliprole (Troczka et al., 2015).The genome-edited strain of *S. exigua,* containing a mutation at the RyR G4946E site, exhibited a higher resistance ratio to flubendiamide (RR>1000), cyantraniliprole (RR= 336) and chlorantraniliprole (RR= 223) (Zuo et al., 2017). In the case of field-collected *S. litura*, the absence of these mutations may be responsible for their susceptibility to diamides, especially cyantraniliprole, as the activities of detoxifying enzymes varied irrelevant to the time of insecticide exposure and between the populations tested. Since *S. litura* collected from Tamil Nadu has developed resistance to other diamide compounds like flubendiamide and chlorantraniliprole (Muthusamy et al., 2014), there is a possibility of resistance development to cyantraniliprole sooner or later. So far, from the results of the present study, the *S. litura* field populations are still susceptible to cyantraniliprole. However, for the safer side, the rotation of insecticides with different modes of action is appreciable in delaying the resistance development process.

ACKNOWLEDGEMENTS

The authors thank the Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, for providing facilities and technical support.

FINANCIAL SUPPORT

No funding sources were involved in the research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

K. E: For collection, analysis, and interpretation of data; drafting the manuscript. M. M: Advisor for the research work and for drafting the manuscript. S. V. K: Advisor for the research work and providing research facilities. N. S: Advisor for the research work. D. V: Advisor for the research.

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(Manuscript Received: March, 2023; Revised: May, 2023;
Accepted: May, 2023; Online Published: May, 2023)
Online First in www.entosocindia.org and indianentomology.org Ref. No. e23199