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INSECTICIDE-TOLERANT BACTERIAL POPULATION IN *Eisenia fetida*'s GUT AND VERMICAST EXPOSED TO CHLORANTRANILIPROLE AND FIPRONIL

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ABSTRACT

An experiment was conducted to evaluate the impact of chlorantraniliprole and fipronil on the bacterial population of gut of *Eisenia fetida* and its vermicast. Thirty adult earthworms were exposed to chlorantraniliprole- and fipronil-treated cow dung in 30 L capacity tub. Enumeration was done by spread plate technique and the bacteria were identified on the basis of morpho-biochemical characteristics. Studies revealed that *E. fetida* survived even after the exposure to high concentration of chlorantraniliprole and fipronil, perhaps due to the presence of tolerant bacteria in the earthworm's gut. The chlorantraniliprole and fipronil largely affected the bacterial population in earthworm's gut and its vermicast. Chlorantraniliprole proved more harmful than fipronil. Maximum reduction in total viable tolerant bacterial colonies in earthworm gut and vermicast was 2.42 and 2.95%, respectively, when exposed to the mixed concentration of chlorantra-niliprole and fipronil (5.00 and 8.34 mg kg^{-1}, respectively). Overall, the vermicast had more tolerant bacterial colonies than the gut of *E. fetida*.

Keywords: Chlorantraniliprole, *Eisenia fetida,* fipronil, tolerant bacterial colonies, vermicast

INTRODUCTION

The indiscriminate use of insecticides has not only reduced the soil fertility but also negatively impacted the soil organisms. Residual traces of insecticides have been reported in food materials making them unfit for human and animal consumption (Chatterjee and Thirumdasu 2014; Garrido *et al.*, 2000). So, there is a need for sustainable agriculture development, and the earthworms play a crucial role in this process (Kumar *et al.*, 2020; Kumar *et al.*, 2022). Earthworms are "farmer's friend" or "nature's ploughman" because they affect the physical and chemical and biological properties of soil. They breakdown the organic matter and increase the surface area for microbial degradation. The cast released by them also possess beneficial microbes that enhance the organic matter degradation. Earthworm and its gut microflora symbiotically digest the ingested food material (Maboeta and Van Rensburg, 2003). Vermicasts act as microsites for nitrogen fixing bacteria and earthworms ingest plant growth promoting rhizobacteria such as *Azospirillium, Rhizobium*, *Pseudomonas*, *Bacillus*, *Azotobacter*, etc. along with rhizospheric soil, and generally the bacteria get activated due to the perfect micro-environment of worm's gut (Sinha *et al*., 2010; Kiyasudeen *et al.*, 2015). Earthworm activities enhance the population of plant growth promoting rhizobacteria in soil (Sinha *et al*., 2010). Earth-worms occupy almost 80% of soil invertebrate's biomass and serve as the preferred food material of other organisms. Thus, the chances of insecticide biomagnification are greater in higher animals (Gupta *et al.,* 2010; Kumar *et al.,* 2020; Krishnaswamy *et al.* 2021). Earthworm also acts as a model organism to study the eco-toxic effects of insecticides (Chen *et al.*, 2011; Kavitha *et al*., 2020).

The excessive use of insecticides inflicts severe damage to earthworm growth, gut microbiome (Krishnaswamy *et al.* 2021) and its reproductive potential. The epigeic earthworm species (*E. fetida*) are largely affected by insecticide use (Gupta *et al.,* 2010), mainly by soil applicable insecticides like chlorantraniliprole and fipronil (Lai and Su, 2011; Qin *et al.*, 2014). These insecticides are used against lepidopteran and non-lepidopteran insects. These insecticides may accumulate in earthworm and affect their survivability and reproductive capability (Kasai *et al*. 2016; He *et al*., 2019; Kumar *et al.,* 2020). As a consequence, earthworms and beneficial microflora for plants are greatly reduced, thereby impacting soil fertility. The present study was aimed to evaluate the effects of chlorantraniliprole and fipronil insecticides on the epigeic earthworm *E. fetida. E. fetida* is considered ecologically important to evaluate the ecologically toxic effects of these insecticides and for its potential of being used in the production of vermicompost (Fracchia et al., 2006; Sinha et *al.* 2010). The purpose was to enumerate the insecticides tolerant bacterial populations in *E. fetida*'s gut and its vermicast following the exposure to known concentration of the insecticides for a stipulated time.

MATERIALS AND METHODS

Collection of test animal and substrate

The earthworm species *E. fetida* was collected from the vermicomposting unit of the Department of Zoology & Aquaculture, CCSHAU, Hisar, Haryana, India. The earthworms were maintained properly in cow dung and their 3rd generation was used to avoid the pre-exposure or residual effects of pesticides. Mature and fully clitellated *E. fetida* were used for assessing the impact of insecticides on earthworm's gut bacterial colonies and its vermicast. The cow dung was collected from the farm yard CCSHAU, Hisar and utilized as substrate. The dung was pre-decomposed for 15 days prior, to avoid toxic gases and an increase in temperature during vermicomposting.

Procurement of insecticides and media

The insecticides used were chlorantraniliprole (0.4%, w/w GR) (5-bromo-*N*-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-2-(3-chloropyridin-2-yl)pyrazole-3-carboxamide) and fipronil (0.3%, w/w GR) (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)pyrazole-3-carbonitrile), available under trade names of "Ferterra®' and "Regent®', respectively. These were purchased from Kisan Seva shop Hisar, Haryana, India. Jensen's medium, King's B medium, malate medium and nutrient agar medium were procured from Hi-Media while YEMA (yeast extract mannitol agar) was procured from Central Drug House (CDH), India.

Preparation of media

YEMA, Jensen's, *Azospirillium* and King's B media were prepared as per the manufacturer's instructions and used for growing *Rhizobium*, *Azotobacter*, *Azospirillium* and *Pseudomonas,* respectively, whereas nutrient agar culture medium was used for the enumeration of total viable count in vermicast and gut of earthworm. The media were sterilized by autoclaving at 6.804 kg /121^{o}C for 20 min and poured into sterilized petri-plates.

Experimental set up

Thirty adult *E. fetida* were exposed to chlorantraniliprole- and fipronil-treated cow dung in 30 L capacity tubs. The experiment was conducted in a completely randomized design; and each treatment was replicated three times. Selection and standardization of different concentrations were done strictly as per the descriptions given by the Directorate of Plant Protection Quarantine & Storage, India (*http://ppqs.gov.in*). The treatments, besides an untreated control, comprised of chlorantraniliprole (0.4% GR) tested @ 5.0, 7.5 and 10.0 mg kg^{-1}; fipronil (0.3% GR) tested @ 8.34, 12.51 and 16.6 mg kg^{-1}; chlorantraniliprole + fipronil tested @ 2.5 + 4.17, 3.75 + 6.26, and 5.0 + 8.34 mg kg^{-1}. After 90 days, the enumeration of different bacteria in vermicast and gut of earthworm was carried out by spread plate technique. The earthworms were surface sterilized by 70% ethanol to avoid any contamination. vermicast and gut contents of earthworm (0.1 g each) were transferred to 0.9 mL distilled water separately and shaken properly. The dilutions up to 10^{-4} were prepared. After proper shaking, 0.1 mL aliquots of 10^{-4} dilution were spread on different media plates. These plates were incubated at 28±2ºC for 24-72 h in inverted position and thereafter, the colony forming units (CFU) were counted carefully. Isolation and partial characterizations of bacteria were based on morphological and biochemical tests (Garrity *et al.* 2005a,b). The number of bacterial colonies so obtained was used for calculating the total population of bacteria.

Data analysis

The experimental design for screen house and laboratory studies were completely randomized block (CRD) with three replicates. Critical difference (CD) was calculated between the various treatments by using Software 'OPSTAT', developed at the Computer Centre, College of Basic Sciences and Humanities, CCSHAU, Hisar (Sheoran, *et al.*, 1998).

RESULTS AND DISCUSSION

Various tolerant bacterial colonies were observed in earthworm's gut and vermicast even after the exposure to chlorantraniliprole and fipronil. The isolated bacterial colonies on the basis of morpho-biochemical characterization were identified as *Rhizobium*, *Azotobacter*, *Azospirillum* and *Pseudomonas* (Fig. 1).

Fig. 1: Bacterial colonies on different media; a) *Rhizobium* b) *Azotobacter* c) *Azospirillium*, and d) *Pseudomonas*

Effect of insecticides on gut tolerant bacterial population

A maximum of log 6.84 ± 0.01 cfu mL^{-1} of tolerant bacterial colonies were observed in *Azospirillum* while the minimum of log 6.72 ± 0.01 cfu mL^{-1} was found in *Pseudomonas* sp. The total viable count was log 7.02 ± 0.01 cfu mL^{-1} in control (Table 1). *Rhizobium* colonies decreased in worm's gut from log 6.81 to log 6.54 (3.96%) after treatment with chlorantraniliprole along with fipronil @ 5.00 + 8.34 mg kg^{-1} in comparison to control. The minimum reduction *Rhizobium* population was 0.73% (from log 6.81 to log 6.76) in fipronil treated worms @ 8.34 mg kg^{-1}. The maximum reduction in *Azotobacter* population in comparison to control was from log 6.77 to log 6.37 (5.91%) after treating the earthworms with a combined concentration of chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1}. While minimum reduction in population of *Azotobacter* was 0.89% (from log 6.77 to log 6.71) with fipronil treatment @ 8.34 mg kg^{-1}. The *Azospirillum* colonies decreased from log 6.84 to log 6.63 (3.07%) after treatment with chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1}. Minimum reduction was 0.58% (from log 6.84 to log 6.80) in worms treated with fipronil @ 8.34 mg kg^{-1}. The maximum decrease in *Pseudomonas* population was from log 6.72 to log 6.35 (5.5%) in chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1} treatment, whereas minimum reduction was 0.45% (from log 6.72 to log 6.69) in fipronil @ 8.34 mg kg^{-1} treatment.

Table 1: Effect of insecticides, chlorantraniliprole and fipronil, on gut tolerant bacterial population (log CFU mL^{-1}) of *E. fetida*'s gut

Treatments (mg kg^{-1})	Total viable count	*Rhizobium* sp.	*Azotobacter* sp.	*Azospirillum* sp.	*Pseudomonas* sp.
Control	7.02 ± 0.01	6.81 ± 0.01	6.77 ± 0.01	6.84 ± 0.01	6.72 ± 0.01
Chlorantraniliprole [CA] (5.0)	6.90 ± 0.00	6.74 ± 0.00	6.58 ± 0.02	6.74 ± 0.02	6.62 ± 0.02
CA (7.5)	6.89 ± 0.02	6.68 ± 0.02	6.54 ± 0.01	6.70 ± 0.01	6.56 ± 0.02
CA (10.0)	6.86 ± 0.03	6.67 ± 0.01	6.52 ± 0.02	6.68 ± 0.02	6.54 ± 0.06
Fipronil (8.34)	7.01 ± 0.00	6.76 ± 0.01	6.71 ± 0.01	6.80 ± 0.02	6.69 ± 0.04
Fipronil (12.51)	6.99 ± 0.01	6.75 ± 0.02	6.66 ± 0.02	6.76 ± 0.01	6.64 ± 0.01
Fipronil (16.68)	6.92 ± 0.01	6.71 ± 0.01	6.57 ± 0.02	6.73 ± 0.01	6.63 ± 0.03
CA + Fipronil (2.50 + 4.17)	6.89 ± 0.00	6.67 ± 0.01	6.44 ± 0.02	6.67 ± 0.02	6.58 ± 0.01
CA + Fipronil (3.75 +6.26)	6.86 ± 0.01	6.55 ± 0.03	6.37 ± 0.03	6.64 ± 0.01	6.39 ± 0.01
CA + Fipronil (5.00 + 8.34)	6.85 ± 0.02	6.54 ± 0.01	6.37 ± 0.02	6.63 ± 0.01	6.35 ± 0.01
S.E(m)	0.01	0.02	0.02	0.02	0.03
$CD_{0.05}$	0.04	0.05	0.05	0.05	0.08

The total viable count (TVC) showed maximum decrease from log 7.02 to log 6.85 (2.42%) in gut-tolerant bacterial colonies in *E. fetida* exposed to chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1}; while minimum reduction was 0.14% (from log 7.02 to log 7.01) in fipronil treatment at 8.34 mg kg^{-1}. In comparison to the control, the maximum and minimum reduction was observed in TVC of *Azotobacter* (from log 6.77 to log 6.37; 5.91%) and *Azospirillum* (from log 6.84 to log 6.63; 3.07%), respectively, when the worms were treated with chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1}.

Effect of insecticides on vermicast tolerant bacterial population

A maximum of log 6.86 ± 0.01 cfu mL^{-1} of tolerant bacteria were observed in case of *Azospirillum* and a minimum of log 6.75 ± 0.01 cfu mL^{-1} was in case of *Pseudomonas*. The total viable count was log 7.11 ± 0.01 cfu mL^{-1} in control (Table 2). Maximum decrease in *Rhizobium* population (from log 6.79 to log 6.50; 4.27%) was found in the earthworms treated with chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1}, while minimum reduction was 0.59% (from log 6.79 to log 6.75) in fipronil treatment @ 8.34 mg kg^{-1}. *Azotobacter* exhibited maximum decrease in population from log 6.78 to log 6.40 (5.6%) in chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1} treatment, while minimum reduction was 0.88% (from log 6.78 to log 6.72) in fipronil treatment @ 8.34 mg kg^{-1}. In case of *Azospirillum* the treatment of chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1} exhibited maximum decrease in population from log 6.86 to log 6.65 (3.06%) while minimum reduction was 0.29% (from log 6.86 to log 6.84) in fipronil treatment @ 8.34 mg kg^{-1}. In *Pseudomonas* the deleterious effects were maximum in chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1} treatment (from log 6.75 to log 6.39) (5.33%), whereas minimum reduction was 0.74% (from log 6.75 to log 6.70) in fipronil treatment at 8.34 mg kg^{-1} in comparison to control. The maximum decrease from log 7.11 to log 6.90 (2.95%) in total viable count of tolerant bacteria was noted in vermicast treated with chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1}, whereas minimum reduction (log 7.11 to log 7.05) was noted in vermicast fipronil @ 8.34 mg kg^{-1} treatment. Maximum and minimum reduction in tolerant bacterial population from log 6.78 to log 6.40 (5.6%) and log 6.86 to log 6.65 (3.06%) was observed in *Azotobacter* sp. and *Azospirillum* sp., respectively, in vermicast of worms treated with chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1}.

Table 2: Effect of different concentrations of insecticides on vermicast tolerant bacterial population (log CFU mL^{-1}) of *E. fetida*

Treatments (mg kg^{-1})	Total viable count	*Rhizobium* sp.	*Azotobacter* sp.	*Azospirillum* sp.	*Pseudomonas* sp.
Control	7.11 ± 0.01	6.79 ± 0.02	6.78 ± 0.01	6.86 ± 0.01	6.75 ± 0.01
Chlorantraniliprole [CA] (5.0)	6.95 ± 0.01	6.72 ± 0.03	6.64 ± 0.01	6.76 ± 0.01	6.64 ± 0.03
CA (7.5)	6.94 ± 0.00	6.67 ± 0.02	6.60 ± 0.01	6.72 ± 0.04	6.57 ± 0.03
CA (10.0)	6.92 ± 0.03	6.66 ± 0.03	6.56 ± 0.02	6.67 ± 0.02	6.51 ± 0.07
Fipronil (8.34)	7.05 ± 0.01	6.75 ± 0.01	6.72 ± 0.01	6.84 ± 0.01	6.70 ± 0.02
Fipronil (12.51)	7.02 ± 0.02	6.73 ± 0.03	6.70 ± 0.01	6.79 ± 0.03	6.64 ± 0.01
Fipronil (16.68)	6.99 ± 0.01	6.69 ± 0.03	6.64 ± 0.03	6.73 ± 0.02	6.63 ± 0.01
CA + Fipronil (2.50 + 4.17)	6.94 ± 0.01	6.60 ± 0.03	6.49 ± 0.02	6.71 ± 0.03	6.47 ± 0.07
CA + Fipronil (3.75 + 6.26)	6.92 ± 0.00	6.52 ± 0.02	6.45 ± 0.02	6.68 ± 0.02	6.41 ± 0.04
CA + Fipronil (5.00 + 8.34)	6.90 ± 0.01	6.50 ± 0.02	6.40 ± 0.01	6.65 ± 0.00	6.39 ± 0.01
S.E(m)	0.01	0.03	0.02	0.02	0.04
$CD_{0.05}$	0.04	0.07	0.05	0.06	0.11

The insecticides negatively affected the earthworms. The colonies of *Azotobacter*, *Azospirillum*, *Pseudomonas* and *Rhizobium* were found tolerant to the test insecticides. Tolerant bacteria help in food digestion in earthworm's gut. The earthworms were able to survive even in such harsh conditions (Kavitha *et al*., 2020). Bacterial colonies showed concentration-dependent decrease both in *E. fetida*'s gut and vermicast exposed to chlorantraniliprole and fipronil. As the dose of insecticides increased, the tolerant colonies decreased with maximum decrease of 2.42 and 2.95%, in total viable tolerant bacteria in gut and vermicast, respectively, when earthworms were treated with chlorantraniliprole + fipronil @ 5.00 + 8.34 mg kg^{-1}.

Symbiotic relationship between earthworm and gut microbes indicates that they help one another even in polluted soil (Fracchia *et al*., 2006). Pesticides interfere in the metabolic activities of soil bacteria (Singh and Walker, 2006). Shaikh *et al.* (2016) observed that chlorantraniliprole affected the digestive enzyme of *Eudrilus eugeniae* which result in metabolic disturbance in animal. Salokhe *et al.* (2014) observed that there was a successive decrement in bacterial colonies, amylase, cellulase, invertase and pectinase from the gut of *E. eugeniae* as the concentration of fipronil was increased. The changes in the metabolic and physiological activities may have caused the deleterious effect on *E. fetida*'s gut and its vermicast bacterial population. Kavitha *et al*. (2020) also reported that in *Lampito mauritii* gut microbial population decreased even at 0.093 and 0.311 ppm kg^{-1} monocrotophos. Wang *et al*. (2007) reported that the combined use of cadmium and butachlor significantly affected the diversity of microbial community. The combined use of pesticides with fertilizer also significantly affects soil microbial diversity and activity. The lower viable bacterial counts in gut as compared to vermicast after insecticide exposure could be due to the digestion activities on ingested material in earthworm's gut (Kadam and Pathade, 2017; Kavitha *et al*., 2020). Ramteke and Hans (1992) and Verma *et al.* (2006) reported that the pesticide-tolerant bacteria in earthworm's gut help in pesticide degradation as a result the earthworms were able to survive the harsh condition imposed by pesticides. Yadav *et al.* (2017) reported similar observations in *E. fetida* after exposure to chlorpyrifos. Bacterial colonies decreased in earthworm's gut as the insecticide concentration increased and comparatively more colonies were observed in vermicast than gut of *E. fetida*.

Conclusions: Vermicast of *E. fetida* had more tolerant bacterial colonies than its gut. Insecticides greatly affected the earthworm bacterial colonies. Chlorantraniliprole caused more damage to earthworms as compared to fipronil. Insecticides (chlorantraniliprole and fipronil) when used in combination had a severe and synergistic impact on earthworms and its vermicast that's why more bacterial colonies were reduced. This study showed that both chlorantraniliprole and fipronil have a negative impact on bacterial colonies of *E. fetida* gut and its vermicast.

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