A RAPID AND EASY BIOASSAY METHOD FOR STINGLESS BEES
TETRAGONULA TRAVANCORICA SHANAS AND FASEEH

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ABSTRACT

An attempt was made to develop an effective bioassay method for toxicological studies for the stingless bee, *Tetragonula travancorica* Shanas and Faseeh. The developed bioassay employed stingless bees sampled from the forest area with no history of past insecticide exposure and vegetable ecosystem having frequent insecticide exposure with insecticides viz., chlorantraniliprole and thiamethoxam. A residual film bioassay using pre-treated conical flasks and transferring the bees to the treated flasks in the field itself was easy and less time-consuming. Stingless bees survived for 12 hours in the control flasks with the bioassay method III. Whereas mortality was significantly low in bioassay method I and II within 3h and 6h, respectively. Chlorantraniliprole was less toxic (LC_{50} – 10.98 ppm to 17.30 ppm) to stingless bees than thiamethoxam (LC_{50} – 0.30 ppm to 0.78 ppm). Thiamethoxam was highly toxic (LC_{50} – 0.30 ppm to 0.37 ppm) to bees from the forest ecosystem, whereas bees in the vegetable ecosystem were more tolerant (LC_{50} – 0.67 ppm to 0.78 ppm). The findings may help carry out ecotoxicology and risk assessment studies in stingless bees, a key pollinator of many crops, more rapidly and easily.

Key words: *Tetragonula travancorica*, chlorantraniliprole, thiamethoxam, bioassay, residue film, vegetables, forest, toxicity, stingless bee, ecotoxicology, risk assessment

Plants need pollination to reproduce, and many cross-pollinated crops rely on bees as pollinators (Potts et al., 2010). Bees account for one out of every three bites of food we consume (MSU Extension, 2021). Bees visit >90% of the world's most significant crop types, and pollinate around 70% of all cultivated plant species (Ricketts et al., 2008; Slaa et al., 2006). Stingless bees (Meliponini) are increasingly being recognised as viable commercial pollinators with their remarked biological traits that make them suitable for supervised pollination. The genus *Trigona* with more than 130 species, is the largest and most widespread among stingless bees (Michener, 2013). Chlorantraniliprole and thiamethoxam are the widely used new-generation insecticides in vegetables against lepidopteran and sucking pests, respectively. Chlorantraniliprole is a novel anthranilic diamide insecticide that acts as a selective ryanodine receptor agonist and thiamethoxam is a neonicotinoid that acts on postsynaptic nicotinic acetylcholine receptors (nAChRs) (Ihara and Matsuda, 2018). The recent decline in pollinator diversity and abundance due to unsustainable agricultural landscapes and excessive pesticide usage has prompted concerns about pollination services' long-term viability (Dively and Kamel, 2012). So diversifying crop pollinators with stingless bees can improve pollination services. Despite

this, research in ecotoxicology and risk assessment of insecticides, stingless bees receive little consideration (Tomé et al., 2015). There is a scarcity of information on toxicological guidelines for stingless bees, thus research to establish the effects of insecticides on these bees is clearly needed (Boyle et al., 2019; Padilha et al., 2020). In this context, the impact of two insecticides, chlorantraniliprole, and thiamethoxam, was tested on the stingless bee, *Tetragonula travancorica* Shanas and Faseeh, collected from the forest and vegetable ecosystem using a simple and quick residual film bioassay method.

MATERIALS AND METHODS

The study for comparing insecticide tolerance of stingless bees on exposure was carried out in the College of Agriculture, Kerala Agricultural University, Vellanikkara during 2018 to 2021. Three bioassay methods were carried out with stingless bees collected from a feral colony in Kerala Agricultural University campus (N 10°32'.49.7544''; E 76°16'57.20412'') with no history of past insecticide exposure and a domesticated bee hive maintained at the vegetable ecosystem of Krishi Vigyan Kendra, Thrissur, (N 10°32'.48.79428''; E 76°16'4.70064'') with frequent insecticide exposure.

Bees collected were identified morphologically using taxonomic keys as *Teragonula trvancorica* followed by barcoding and sequences deposition in GenBank with submission ID - SUB10957255 and accession number OM293512. Technical grade analytical standards of chlorantraniliprole and thiamethoxam from Sigma-Aldrich were used to prepare the stock solution with acetone as solvent. For the initial broad range bioassay, concentrations viz., 10, 5, 1 ppm chlorantraniliprole and 50, 10, 5, 1 ppm for thiamethoxam were taken from the stock. From the wide range bioassay, the insecticide concentrations for a narrow range were selected. Three different bioassay methods were compared to find out the most effective one in a completely randomized design of six replications in 250 ml conical flasks. The residue film method was adopted for determining contact toxicity in stingless bees. From each insecticide concentration, 2 ml was taken using a micropipette and transferred to conical flasks followed by rotating the flasks so as to evenly coat the insecticide as a thin film without a runoff on the walls of flasks. The flasks were then allowed to air dry for two hours and the mouth of conical flasks was covered using a white muslin cloth. Acetone (2 ml) was used as a control in all bioassay methods.

In the first method, stingless bees collected in plastic bottles from the feral colony and vegetable ecosystem colony were brought to the lab and transferred to each conical flask of various concentrations with six replications two hours after impregnating insecticides. The second bioassay method was according to Botina et al. (2020), with slight modifications in which stingless bee workers collected at the hive entrance using glass bottles were brought to the laboratory immediately and anaesthetized in a -20 °C deep freezer for 10 minutes. The duration of ten minutes in the deep freezer was standardized after exposing the bees for 1, 3, 5, 7, and 10 minutes. Ten minutes was the appropriate time where the bees got anaesthetized completely. Five anaesthetized bees were transferred to each conical flask using a camel hairbrush. In the third bioassay method, pre-treated 250 ml conical flasks were carried to the field, and bees were collected directly from the hive entrance to the treated flasks at a rate of 5 bees per flask two hours after treatment and bees were then returned to the laboratory. In all three bioassay methods, a cotton ball dipped in 10 % honey solution was hung in the conical flask as food for the bees. Mortality of bees was recorded at 3, 6, 9 and 12h after insecticide exposure and mortality data were analysed using POLO PLUS software. The survival % of bees in the control was observed for all three methods after 3, 6, 9 and 12h to assess their efficiency. The data on the survival of bees was analysed as a Completely Randomized Block design using GRAPES software (Gopinath et al., 2020).

RESULTS AND DISCUSSION

The % survival of stingless bees *T. travancorica* in control was only 13.33% after 3 hr in bioassay method I, and complete mortality was observed subsequently in both feral and vegetable ecosystem-based colonies. In the bioassay method II, the % survival of bees for the feral colony was 90 % after 3 hr which gradually decreased to 46.67, 26.67, 6.67 % by 6, 9, 12 hr, respectively; for those bees from the vegetable ecosystem, it was 86.67% which decreased to 10.00% by 12 hr. The bees from both the colonies survived in bioassay method III for 12 hr, and remained active even for 48 hr in the flasks (Table 1). On comparing the three bioassay methods, method I was significantly inferior. The bees carried to the laboratory in plastic bottles from the feral and vegetable ecosystem could not live longer. Moreover, transferring active bees from collected plastic bottles to each treated flask @ 5 bees/ flask was difficult, time-consuming, and damaged the delicate bees. Oliveira et al. (2012) also recorded low activity followed by the death of stingless bees in plastic containers because of excessive moisture inside.

Table 1. Comparison of % survival of stingless bees in control under three bioassay methods

Method	3 h		6 h		9 h		12 h	
	Feral	Vegetable ecosystem	Feral	Vegetable ecosystem	Feral	Vegetable ecosystem	Feral	Vegetable ecosystem
Bioassay I	13.33[b] (21.85)	13.33[b] (21.85)	0.00[c] (12.92)	0.00[c] (12.92)	0.00[c] (12.92)	0.00[c] (12.92)	0.00[c] (12.92)	0.00[c] (12.92)
Bioassay II	90.00[a] (70.26)	86.67[a] (67.98)	46.67[b] (42.73)	33.33[b] (34.82)	26.67[b] (30.62)	13.33[b] (21.85)	6.67[b] (17.31)	10.00[b] (19.58)
Bioassay III	100.00[a] (77.08)	100.00[a] (77.08)	100.00[a] (77.08)	100.00[a] (77.08)	100.00[a] (77.08)	100.00[a] (77.08)	100.00[a] (77.08)	100.00[a] (77.08)

*Figures in parentheses denote arc sign transformed values

Bioassay method II and III did not differ significantly for the first three hours in both feral and vegetable ecosystem-based colonies. However, after 6, 9 and 12 hr, bioassay method III was significantly superior over method II, which caused freezing injury to the bees while exposing the bees at -20° C for 10 min before transferring the collected bees to pretreated conical flasks. Botina et al. (2020) used plastic cups for bioassay, whereas this study used glass bottles. The higher exposure of 10 min used now was due to the use of glass bottles for the bioassay, as the glass bottles took more time to cool than plastic ones. Once anaesthetized, transferring the bees to the insecticide coated flasks damaged the bees as they were sticking to the camel hairbrush. As % of bees survived in the control flask after 3 hr decreased drastically in bioassay II, it could be utilized only for the bioassay of those insecticides with very high contact toxicity. Therefore, bioassay method III was the most appropriate, as errors were negligible. The survival of bees for 48 hr would enable bioassay for insecticides with weak contact toxicity. Moreover, the chances of injury during the transfer of bees to insecticide coated flasks could be avoided in this method.

After 3 hr of exposure, the LC_{50} values of chlorantraniliprole and thiamethoxam in bioassay methods II and III showed that the LC_{50} of chlorantraniliprole was 10.98 and 12.53 ppm, respectively, with bioassay methods II and III for the feral colony (Table 2). The LC_{50} values of vegetable ecosystem-based colony with chlorantraniliprole was 13.30 and 17.30 ppm, respectively. The fiducial limits for the bioassay methods II and III overlap for the feral colony and vegetable ecosystem-based colony, indicating the comparative efficacy of bioassay

method III with the already reported bioassay method II. With the neonicotinoid thiamethoxam, the LC_{50} values were 0.30 and 0.37 ppm with the feral colony and 0.67 and 0.78 with the vegetable ecosystem-based colony in bioassay methods II and III, respectively. As with chlorantraniliprole, the bioassay methods had comparable fiducial limits with thiamethoxam too, with bees from both the colonies. In the vegetable ecosystem, two to three sprays of chlorantraniliprole and thiamethoxam were given. The susceptibility of stingless bees from feral and vegetable ecosystem colonies to chlorantraniliprole were comparable. In contrast, bees from the feral colony were more susceptible to thiamethoxam than the bees from the vegetable ecosystem- based colony; and frequent exposure of these to thiamethoxam could be the reason for their lower susceptibility. This indicates that stingless bees exhibited tolerance to thiamethoxam even with two to three sprays.

With the bioassay method III and at a concentration of 3 ppm, the mortality of bees from vegetable ecosystem colony was 96.67% with thiamethoxam, and it was only 10% with chlorantraniliprole. The exposure of bees from feral colony to 1 ppm thiamethoxam resulted in complete mortality. But, with chlorantraniliprole, the mortality was only 33.33% at 10 ppm, and at 30 ppm, the mortality was 96.67%. Thus, chlorantraniliprole is less toxic to *T. tranvancorica* compared to thiamethoxam. Tomé et al. (2015) also reported a low risk of chlorantraniliprole with insignificant mortality to stingless bees compared to neonicotinoids. According to Williams et al. (2020), 72 hr of exposure of *Apis mellifera* to chlorantraniliprole was not acutely hazardous to honey bees. Thiamethoxam is reported to be highly toxic to various other stingless bees such as

Table 2. Susceptibility of *T. travancorica* to chlorantraniliprole and thiamethoxam
(bioassay method II and III)

Colony type	Treatment	Slope		Chi- square		LC_{50} (ppm)		95 % Fiducial limit	
		Bioassay method II	Bioassay method III	Bioassay method II	Bioassay method III	Bioassay method II	Bioassay method III	Bioassay method II	Bioassay method III
Feral	Chlorantraniliprole	4.53	4.83	2.81	0.13	10.98	12.53	9.16-13.48	10.95-15.14
	Thiamethoxam	3.88	3.06	3.72	3.90	0.30	0.37	0.09-0.42	0.23-0.53
Vegetable ecosystem	Chlorantraniliprole	2.29	1.61	0.58	0.35	13.30	17.30	9.6-50.53	11.07-63.31
	Thiamethoxam	3.12	3.06	1.92	0.49	0.67	0.78	0.48-0.84	0.64-0.92

*Based on 3 hr exposure

Scaptotrigona bipunctata (Moreira et al., 2018) and *T. angustula* (Jacob et al., 2019). The sensitivity ratio, R (LC_{50}a/LC_{50}s) between *A. mellifera* (a) and stingless bee species (s) calculated by Arena and Sgolastra (2014) compared the sensitivity of the two bee species to pesticides. Stingless bees had a sensitivity ratio >1, indicating the higher sensitivity of stingless bees to thiamethoxam than *A. mellifera*. The acute oral LC_{50} for thiamethoxam was 0.227 ng a.i./ µl (ppm) in laboratory tests with female workers of *A. mellifera* and 0.0543 ng a.i./ µl (ppm) with stingless bee, *Melipona scutellaris* at 24 hr (Miotelo et al., 2021). These findings support the assertion that stingless bees are more sensitive to thiamethoxam than honey bees. Bee depletion is causing increasing public and political worry around the world. The loss of habitat and food supplies, pesticide exposure and climate change contribute to bee decline. The newly standardised bioassay method can help carry out ecotoxicology assays of *T. travancorica* more easily and effectively.

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