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Tolerance development in termites: A case study of *Microtermes obesi* Holmgren (Blattodea: Termitidae) from the tea plantations of Darjeeling foothills, West Bengal (India)

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Abstract

Microtermes obesi belong to one such new clad of termite species which damages tea plantations of Darjeeling foothills. To control tea pests, planters use synthetic insecticides that have lead to the much bigger problem of insecticide tolerance in many tea pests. Present study contemplates to investigate the tolerance status of the pest species, collected from organically and conventionally managed tea plantations of Darjeeling foothills to three different commonly used insecticides – Imidacloprid 17.8% SL, Cypermethrin 35% EC and Chlorpyrifos 20% EC, along with the activity of the three major detoxifying enzymes, General Esterases(GE), Glutathion S Transferase (GST) and Cytochrome oxydases (CYP450). Results obtained showed higher level of mean lethal concentration (LC_{50}) for Imidacloprid 17.8% SL = 63.66 ppm, Cypermethrin 35% EC = 31.99 ppm, and Chlorpyrifos 20% EC = 18.57 ppm in *M. obesi* population collected from conventionally managed tea plantations, as compared to that from organically managed plantations. Even observations on detoxifying enzymes reflected a corresponding enhancement in their activities which were noted as: GE = 2.25 mM mg $^{-1}$ protein; GST = 120.86 µM min^{-1} mg^{-1} protein; CYP450 = 0.75 n mol min^{-1} mg protein^{-1} in population of pesticide managed conventional plantation, and at a lower level (GE = 0.23 mM mg $^{-1}$ protein; GST = 16.73 µM min^{-1} mg^{-1} protein; CYP450 = 0.26 n mol min^{-1} mg protein^{-1}) in population of organically managed tea plantation. Hence, developing an enzyme-based marker for *M. obesi* populations may be of practical use in titrating an effective pesticide dosage under IPM/IRM strategies.

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Introduction

Tea *Camellia sinensis* (L.) O. Kuntze. It is an intensively managed perennial monoculture crop cultivated on large and small-scale plantations situated between latitudes 41ºN and 16ºS.

It is grown on over 5 million hectares (ha) in more than 46 countries across Asia, Africa, Latin America, and Oceania (Harden, 2021) to produce 28 million tonnes of tea (FAO, 2023). The global tea market size was valued at $49 billion in 2021, and is projected to reach $93.2 billion by 2031, growing at a CAGR of 6.7% from 2022 to 2031 (Jain and Deshmukh, 2023). The crop hence attracts a large array of arthropod pests leading to a substantial crop loss. According to Hazarika *et al.* (2009), globally there are 1031 arthropod species associated with tea, out of which 300 species of insect pests are recorded from India and specifically 167 species from North-East India (Das, 1965). Pests which attack the shoot system of tea bush deserve more attention because of their perceptible and permanent damage symptoms. Therefore acting against them with appropriate control measures is imperative.

There are pests like termites or cockchafer grubs who often surreptitiously attack tea plants underground without facing any trouble from planters. Termites are dominant soil dwelling animals and play a significant role in tropical terrestrial ecosystems, especially in the decomposition process (Lee and Wood, 1971; La Fage and Nutting, 1978; Wood and Sands, 1978; Griffits *et al.*, 2029). Termites are polymorphic, eusocial insect which depend on varied sources of cellulose as food. Their dependence on cellulose is the major reason why some of them are considered as serious pests. Out of 2933 living species of termites only about 12.4% (371 species) has been recognised as structural pests and only 3.5 per cent (104 species) are considered as serious field pests (Wood, 1996; Krishna *et al.*, 2013). Despite low number of species, termites can cause significant damage to many crops and wooden structures resulting into loss estimated in billions of dollars annually (Su and Scheffrahn, 1998).

Microtermes obesi Holmgren is one of such live wood eating termite species which severely affects many crops and in particular tea bushes. As a subterranean pest, it hollows the stem of the plant by devouring pith and associated wood from inside leaving minimum vitality left for the plant to sustain for some time. Like many other tea pests, termite fauna has also adapted to pesticide managed conventional tea plantations. Earlier records suggest that *M. obesi* is one of the most prevalent pest species in tea plantations of North East region including the Darjeeling foothills and terai plains (Choudhury *et al.*, 2001; Choudhury *et al.*, 2005; Singha *et al.*, 2012, 2014; Biswa, 2018). This may be due to the selection process by synthetic chemicals, especially pesticides that have been primarily used in tea pest control.

There have also been many attempts of controlling these pests with biological control measures such as by using entomopathogenic fungi or bacteria (Verma *et al.*, 2009; Singha *et al.*, 2012, 2014; Debnath *et al.*, 2012) however, these seem to have limited success. As Chouvenc *et al.* (2011) claimed that in last 50 odd years despite boom in biological control research against termites, they have failed miserably. To defend the tea crop against pest attack, in recent past large quantities of chemical agents are being used. However, this has turned out to be burden to planters as well as to environment. With more exposure of pests to insecticides, their resistance to these chemicals are becoming the major obstacle to their control. This worldwide problem has been documented for over 500 arthropod species, particularly in flies, caterpillars, beetles and mites (Georghiou, 1990). Although, among termites, reports on developing resistance is scanty, despite the fact that they are chiefly controlled by insecticides. Due to their social structure, typical resistance development theory doesn't fit well with termites, unlike other insects (Mahapatro, 2017). However, recent works on termites do show a development of reasonable amount of tolerance towards xenobiotics (Osbrink *et al.*, 2001; Osbrink and Lax, 2002, 2003; Valles *et al.*, 1998; Valles and Woodson, 2002). Though eusocial structure of termite colony plays a major role in the tolerance against toxic and harmful chemicals (De Souza *et al.*, 2001), yet their physiological adaptability to toxic environment can't be denied (Roisin and Korb, 2011).

Tea plantations are primarily maintained either organically or conventionally. Organic plantation uses eco-friendly and non-chemical pest control strategies, whereas, conventionally managed plantations use chemical insecticides for pest control. This study was undertaken on one of the severe termite pests of tea, *Microtermes obesi* to see any differential physiological adaptation that may be an attribution of absence or presence of chemical inputs (insecticides) in above mentioned types of tea plantations.

Materials and methods

Study area: Northern part of West Bengal has three important ecological zones (Hill slopes, Terai foothills and Plains) which cover a total area of 21,763.0 sq km. Tea is grown mainly in the foothill regions of North Bengal dooars comprising districts of Alipurduar, Jalpaiguri, Kalimpong and terai in Darjeeling. The study was carried out in the tea plantations of the dooars, terai and Darjeeling hill slopes which spread over an area of 10,629 sq km.

Termite collections: Termites were collected from several tea plantations of Darjeeling foothills. Infested and dying tea logs were located, collected and brought back to laboratory. At times modified perforated pit fall traps provided with moist carton pieces were also used for collection. Logs were later opened to collect fresh worker and soldier castes. The termites were identified as the live wood eating termite, *M. obesi*, based on key taxonomic characters and character states of the soldier caste (Chhotani, 1997). Collected samples were maintained at constant temperature of 25 ± 2°C and Relative Humidity of 75 - 80%.

Insecticide Bioassay: Termites were kept in BOD and let them get acclimatized before proceeding for the bioassay (Gurusubramanian *et al.*, 1999). Worker castes were treated with varied concentrations of three commercial insecticides (Cypermethrin 10% EC, Cholrpyrifos 20% EC and Imidacloprid 17.8% SL) by "Surface coating method" after Kranthi (2005). At least five concentrations of these insecticides were prepared by serial dilution in distilled water and each dilution was replicated thrice. A set of 30 termite workers were placed in each petridish (n=30) having a filter paper treated with a particular concentration of insecticide.

Control was set with 90 worker termites divided equally into three replicates (n=30 each) having a filter paper treated with distilled water. Termites under assay were maintained at 25°C ± 2°C temperature and 80-85% of RH in BOD incubator. The mortality count was recorded after 24 hours. Bioassay data was pooled and mean lethal concentrations (LC_{50}) were computed by Probit analysis (Finney, 1971) aided by computing software. Moribund insects were counted as dead (Gurusubramanian and Bora, 2007).

General Esterase Activity: General esterase activity was measured by using α-naphthyl acetate (α NA) as substrates after the method of van Asperen (1962) with few modifications. 20 µl of supernatant equivalent to one adult insect was taken in each well of the 96-well microplate reader in duplicate. 200 µl α NA (30 mM) was added to each well for reaction.

The reaction was stopped after 10 minutes by adding 50 µl of staining solution containing 0.1% Fast BB salt and 5% SDS (2:5). The plate was left for five minutes for equilibration and absorbance was recorded at 590 nm. The change in absorbance was converted to end product formation from a standard curve of α naphthol (5-500 nM). Blanks were set at the same time using reaction mixture without protein extracts.

Glutathione S-transferase (GST): The activity of GST was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) and reduced glutathione (GSH) in conjugation reaction as described by Habig *et al.* (1974). The reaction mixture contained 150 µl of GSH (1.0 mM) and 50 µl CDNB (1.0 mM). The microplate was left for 3 minutes for equilibration and reaction.

The absorbance was recorded at 340 nm continuously for 5 minutes. An extinction coefficient 9.6 $mM^{-1}cm^{-1}$ was used to convert the change in absorbance per minute to rate of conjugate formation.

Cytochrome P450 monoxygenase: As heme protein is a major constitute of the majority of Cytochrome P450, its activity was calculated by estimating heme peroxidase activity (Penilla *et al.*, 2007; Tiwari *et al.*, 2011). 20 µl of enzyme homogenate was incubated with 200 µl of TMBZ solution (0.01 g of TMBZ in 5 ml of methanol + 15 ml of 0.25 M sodium acetate, pH 5.0) and 80 µl of 0.0625 M PBS (pH 7.2) and 25 µl of 3% H_2O_2 for 30 minutes at 25°C. Absorbance was recorded at 630 nm on microplate reader. The standard curve of heme peroxidase activity was prepared using Cytochrome c from horse heart type IV. Total Cytochrome P450 was expressed as nmoles of Cytochrome P450 equivalent units (EUs) per mg protein per minute.

Results

Comparison of LC_{50} values of three different pesticides tested on population of termites collected from organically and conventionally (pesticide) managed plantations strongly suggests that there is an enhanced tolerance status developed in the population of *M. obesi* sampled from conventionally managed tea plantations. Chlorpyrifos seemed to be more effective in controlling susceptible *M. obesi* population collected from organically managed tea plantations (LC_{50} = 3.29 ppm), whereas Imidacloprid seemed to have less effect on populations collected from conventionally managed tea plantations with a high LC_{50} value of 63.66 ppm. It is also clearly evident that termite population of organic maintained plantations are still susceptible with a significantly low level of tolerance against these pesticides in comparison to that of the populations of conventionally managed plantations. The difference in the LC_{50} values between the populations of organically managed and conventionally managed plantations significantly differed at p = 0.05 level of probability based on Tukey's HSD analysis (Table 1).

Similar observations were obtained in defence enzyme activities of *M. obesi*. Three assayed enzymes namely General Esterases, Glutathione S-transferases and Cytochrome P450 exhibited elevated levels of expression when assayed for the population of conventionally managed tea plantations (Table 2). Whereas, populations collected from organically managed tea plantations responded with low activity of these enzymes (Table 2). This may be due to the absence of the use and exposure of the pest species to chemical insecticides that are largely related to build up of hyperactivity of these enzymes.

Table 1. Comparative median lethal concentration (LC_{50}) values of three insecticides against *Microtermes obesi* populations collected from organically and conventionally (pesticide) managed tea plantations

Insecticide used	Management type	*LC_{50} (Mean[†] ± SE)	95% FL of LC_{50}		RF	Regression value (Y)		X^2	*LC_{95}
			Lower limit	Upper limit		bx	a		
Imidacloprid 17.8% SL	Organic	5.50^a ± 0.346	3.86	7.84	1.40	1.28	0.20	1.58	107.00
	Conventional	63.66^c ± 26.129	47.21	85.95	16.24	1.55	-2.37	3.18	693.65
Cypermethrin 35% EC	Organic	5.67^a ± 0.231	4.24	7.59	1.45	1.69	-1.35	3.68	54.05
	Conventional	31.99^c ± 7.183	23.41	43.81	8.16	1.65	-2.36	1.55	450.58
Chlorpyrifos 20% EC	Organic	3.92^a ± 0.058	1.62	5.36	1	1.60	-0.74	0.14	42.59
	Conventional	18.57^b ± 2.098	13.96	24.75	4.74	1.61	-1.86	3.96	209.41

*values are expressed in parts per million (ppm), [†] Means followed by different superscripts denote significant difference in LC_{50} values at p = 0.05 based on Tukey's Honest Significant Difference (HSD) Post Hoc test

It is well explained by different workers how a continuous selection can occur and more tolerant variety of insects emerges under insecticidal pressure (Brogdon and McAllister, 1998; Karaağaç, 2012; Kunz and Kemp, 1994; Basnet *et al.*, 2017). Regression scatterplot was prepared to verify if dependence of resistance status on detoxifying enzyme level stands true also in *M. obesi*. The regression of resistance factor (RF) on activity ratio of detoxifying enzymes (AR) was checked for all these three enzymes by performing simple linear regression analysis which furnished a significant R^2-value of 0.879 and F-value of 36.19 at $p < 0.05$ for GE. The result showed 87.9% dependence of RF values on AR indicating a significant linear relationship between them (Fig. 1 A). Linear regression analysis of GST showed R^2 = 0.742 and F-value of 14.4 at $p < 0.05$ indicating at least 74.2% dependency of RF on AR values (Fig. 1 B). A similar R^2 value of 0.841 and F = 26.51 at $p < 0.05$ were obtained for CYP450 (Fig. 1 C).

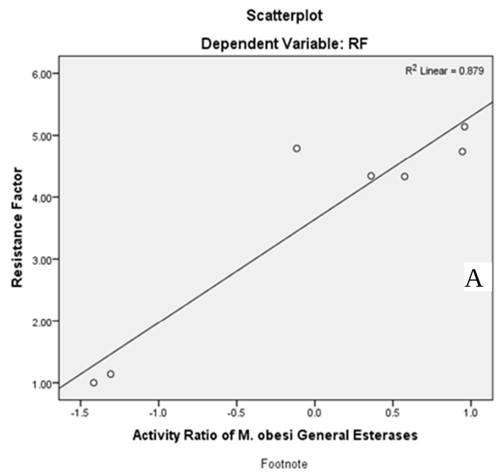
Table 2. Defense enzyme (GE, GST and CYP450) activities (pooled) in *Microtermes obesi* populations collected from organically and conventionally managed tea plantations of Terai-Dooars regions

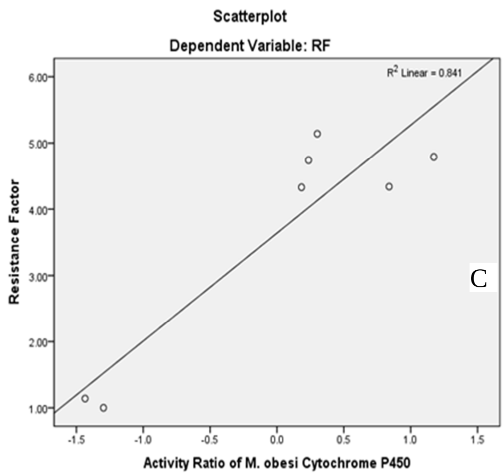
Management type	‡RF	GE (mM mg $^{-1}$ protein) (Mean*± SE)	†AR	GST (µM min^{-1} mg^{-1} protein) (Mean*± SE)	AR	CYP450 (n mol min^{-1} mg protein^{-1}) (Mean*± SE)	AR
Organic	1.00	$0.23^a \pm 0.03$	1.00	$16.73^a \pm 1.20$	1.00	$0.26^a \pm 0.01$	1.00
Conventional	16.24	$2.25^b \pm 0.51$	9.68	$120.86^b \pm 21.93$	7.23	$0.75^b \pm 0.07$	2.89

‡RF = Resistance factor, is the ratio of Median Lethal Concentration (LC_{50}) of more tolerant population to the least tolerant organic population

*mean values with different superscript alphabets in columns denote significant difference at 0.05% level of probability

†AR = Activity Ratio, is the ratio of defence enzyme activity of a population to the activity of the most susceptible organic population





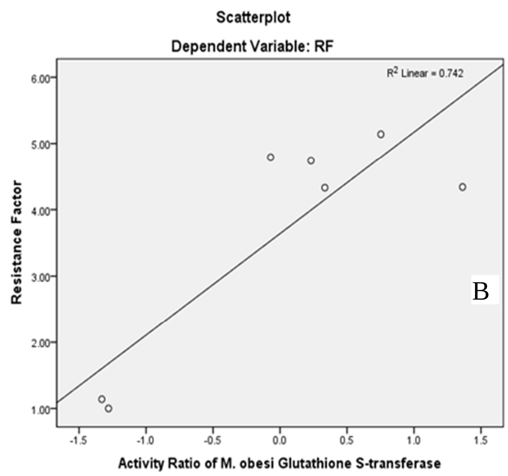


Fig. 1. Scatterplot of Linear Regression with R^2 values between Resistance Factor (RF) and Activity Ratios of (A) General Esterases; (B) Glutathione *S*-transferases and (C) Cytochrome P450 in *Microtermes obesi*

An insect has defensive mechanisms that involve physiological machinery for insecticides detoxification. This machinery can either metabolically neutralize the toxicity of an insecticide before it reaches its target or the target site itself gets altered to become insensitive to these insecticides. In an organism the metabolic detoxification is principally achieved with the help of enzyme system such as, general esterases (GE), glutathione *S*-transferases (GSTs) or cytochrome P450 monoxygenases which are expressed excessively in tolerant group of insects in comparison to susceptible ones (Brown and Brogdon, 1987; Hemingway, 1989; Hemingway *et al.*, 1995).

Conclusion

In the present study all the results based on LC_{50} values and detoxifying enzymes suggested that there is an insecticide mediated artificial selection process operative on the termite population commensurating with the enhancement of their physiological capability for tolerating pesticide load. So, more use of pesticides would force the selection of potential pest populations to higher tolerance level. Development of such a selection process is strongly suggested by the significant linear relationship between these two said factors. However, the variability in the populations might have been due to the amount, interval and intensity of pesticide used in different plantations to control pests. Similar observations have been made for many other insect pests where use of xenobiotics and consequently highly stressed environment have been related to difference in the expression of the detoxifying enzymes (Biswa and Mukhopadhyay, 2014; Nardini *et al.*, 2012; Ishaaya, 1993). Therefore, hyperactivity of detoxifying enzymes in termite populations can be used as an indicator of higher tolerance levels in *M. obesi*. Such findings may help planning better pest management or resistance management strategies.

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