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Susceptibility to acaricides and detoxifying enzyme activities in the red spider mite, *Oligonychus coffeae* Nietner (Acari: Tetranychidae)

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**ABSTRACT**

Susceptibility of red spider mite, *Oligonychus coffeae* Nietner (Acari: Tetranychidae), collected from conventionally-managed (synthetic acaricide usage) versus an organically-managed (no acaricide usage) tea plantations in Assam, India, to five synthetic acaricides was determined in laboratory bioassays. Activity of three principal detoxifying enzymes of these mite populations was also assayed. The median lethal concentrations (LC$_{50}$) of ethion, dicofol, propargite, fenpropathrin, and fenazaquin were 1049.75, 599.21, 232.03, 11.44, and 6.75 ppm, respectively. Field rates of these acaricides were compared with 95% lethal concentration (LC$_{95}$ in ppm) values, and a decrease in the susceptibility of the test population to ethion, propargite, dicofol and fenpropathrin was observed. There was no change for fenazaquin which was effective at lower doses than the recommended dose. Of all the acaricides tested, fenazaquin was the most toxic and ethion was the least toxic. General esterases (GES), glutathione-S-transferase (GST), and cytochrome P450 monoxygenases exhibited a higher activity in mite population from the conventionally-managed tea plantation as compared with the activity in mites from the organically-managed tea plantation. These findings may be helpful in the selection of acaricides and in developing resistance management strategies for an effective management program for this major tea pest.

**Keywords** acaricides, detoxifying enzymes, *Oligonychus coffeae*, tolerance, tea

**Introduction**

Red spider mite, *Oligonychus coffeae* Nietner (Acari: Tetranychidae), is a highly pervasive polyphagous spider mite pest that infests nearly 133 crops cultivated worldwide (Roy et al. 2014a, Migeon et al. 2011). In India, the most preferred and principal host of *O. coffeae* is tea, *Camellia sinensis* L. (O. Kuntze), in which damage by this mite is attributed a 17 to 46% loss (Roy et al. 2014a). *O. coffeae* causes the maintenance foliage to turn red which reduces the photosynthetic capacity of leaves, ultimately resulting in defoliation of these infested leaves which, in turn, leads to reduced growth of young shoots (Gotoh et al. 2001). For many decades, synthetic acaricides, namely organochlorines, organophosphates, and synthetic pyrethroids, have been routinely used to manage the pest in tea production. However, repeated applications of the acaricides combined with the mite’s high reproductive capacity and short life cycle have facilitated the development of resistance of these commonly-used chemistries (Roy et al. 2014a).

Globally, there are approximately 800 examples of acaricide resistance in phytophagous mites (Arthropod Pesticide Resistance Database [APRD] 2017), and 93 % of those involve tetranychids (Ullah and Gotoh 2013). Acaricide resistance in mite pest can result from
the selection of one or more mechanisms including modification of behavior, alteration in integument, sequestrations, metabolic resistance and molecular mutations (Namin 2017).

Furthermore, mite populations exhibit differing susceptibilities to acaricides from different regions. For example, *O. coffeae* collected from Japan was less susceptible to pyrrole, bifenazate, and fenbutatin oxide groups of acaricides than populations from other locations (Gotoh *et al.* 2001), while the susceptibility of populations from the northern part of Bengal (India) to organochlorine and organophosphate groups of insecticides was considerably less than other regions (Roy *et al.* 2012). Tea growers from northeast India report that management of *O. coffeae* is increasingly difficult, presumably due to development of acaricide resistance. Use of synthetic acaricides is the most widely practiced mite control strategy there (Gurusubramanian *et al.* 2008).

Metabolic resistance, or enhanced detoxification of acaricides, is a mechanism of acaricide resistance development, involves metabolism of the pesticide before it reaches the target site resulting from quantitative or qualitative changes in major detoxification enzymes for example esterases (GE), glutathione-S-transferases (GST) and Cytochrome P450 monoxygenases (CYP450), caused by the up-regulation of detoxification enzyme genes or gene duplication (Leeuwen *et al.* 2010). Modification of acaricides through enzymatic activity is one of the main mechanisms of resistance which may lead to cross-resistance among different classes of insecticides (Ranson *et al.*, 2011)

Detoxification enzymes such as GE, GST and CYP450 monoxygenase are involved in this process of metabolic detoxification of pesticides and, thus, confer resistance to almost all groups of pesticides. Although resistance mechanisms in *Tetranychus urticae* Kochare was well documented (Kumral *et al.* 2009), such information is limited for *O. coffeae*, especially from tea plantations of northeast India. Knowledge on the activity of detoxification enzymes in *O. coffeae* may provide a basis for abating or minimizing the development of resistance to effective acaricides.

Hence, our objectives in this study were to (1) compare the susceptibility of *O. coffeae* populations collected from conventionally-managed versus organically-managed tea plantations of Assam (India), (2) determine the relative susceptibility of these mite populations to five commonly-used acaricides, and (3) assess the comparative activity of detoxifying enzymes in these *O. coffeae* populations. Our overall goal was to provide information for effective management of *O. coffeae* on tea while developing strategies for minimizing development of resistance to the chemistries.

**Materials and methods**

Two *O. coffeae* colonies were established for this study. One was collected in 2016 from infested tea bushes growing on a conventionally-managed tea plantation located at Hoolungooree TE of Jorhat (26.7465°N; 94.2026°E), Assam, in northeast India. This plantation had a history of applying synthetic acaricides, i.e., ethion, dicofol, propargite, for controlling *O. coffeae*. The second colony was established from *O. coffeae* collected from organically-managed tea plantation in Hatikhuli TE of Bokakhat region (26.6215°N; 93.6116°E), Assam. This plantation had never used any synthetic pesticides in its history; thus, the mite populations were considered to be susceptible to acaricides.

A detached leaf culture method (Roy *et al.* 2014b) was adopted to maintain the colonies in the laboratory on the susceptible tea clone, TV1. Adult mites were transferred to fresh tea leaf discs (6 cm²) which were placed on moistened cotton pads (approximately 1.5 cm thick) in plastic trays measuring 42 × 30 × 6.5 cm. The rearing trays were maintained at 27 ± 2°C, 75 ± 5% relative humidity, and on a 14L:10D photo regime. Leaves were replaced with fresh ones at 3-d intervals.

**Acaricides:** Five acaricides were obtained from authorized vendors and used for this study. These were ethion 50 EC (aliphatic organothiolephosphate: Tafethion®, Rallis India
Ltd., Mumbai, India), dicofol 18.5 EC (diphenylaliphatics organochlorine: Klin XL®, Krishi Rasayan India Ltd., Delhi, India), fenpropathrin 30 EC (fourth generation pyrethroid ester: Meothrin®, Sumitomo Chemicals India Pvt. Ltd., Mumbai, India), fenazaquin 10 EC (quinazoline: Magister®, Gowan Company LLC, USA) and propargite 57 EC (organosulphurs: Mastamite®, Chemtura Chemical India Pvt. Ltd).

Toxicity bioassays: Bioassays were conducted using the leaf dip method recommended by the Insecticide Resistance Action Committee (ARAC) and described previously by Roy et al. (2010). Each acaricide was serially diluted using deionized water to establish five to nine concentrations (ppm). Preliminary bioassays were performed to establish a series of concentrations of each acaricide that caused 10 to 90% mite mortality.

Mature tea leaves of TV1 clones were cut into 4-cm diameter discs. A single disc was immersed for 5 s in the appropriate acaricide concentration to ensure complete coverage of the disc. Dipped leaf discs were then air dried for 15 min and then they were placed, ventral surface down, individually on a water-saturated cotton pad (1 x 1 cm) in a Petri dish (90 mm diameter). Twenty adult mites from one of the two colonies were released on each leaf disc using a camel-hair brush. The acaricide concentrations along with an untreated control were replicated three times. Bioassays were repeated until mortality data in the range of 10 to 90% were observed after 24 or 72 h depending on speed of action of the test compound. Mortality was assessed by touching mites with a fine brush. When there was no response or movement, the mite was considered dead. Percentage mortality data were corrected for control mortality using Abbott’s formula (Abbott 1925). The resistance coefficient (RC) of a given population of *O. coffeae* was calculated following methods described by Wegorek et al. (2009).

Detoxifying enzymes assays

Sample preparation

Samples for analysis for detoxifying enzymes were prepared by homogenizing *O. coffeae* (n = 50) adults in 500µl of 0.1M phosphate buffer (pH 7.0) in an ice bath and then centrifuged at 10,000g for 15 min at 4°C. The supernatant was then stored at -20°C for future analysis.

Determination of esterase activity

Activity level of general esterases was estimated using 30 mM of α-napthyl acetate as substrate, following the method of van Asperen (1962). The enzyme supernatant (20µl) was incubated with substrate (200 µl) (1:100) for 15 min at 25°C. Reaction was stopped by 50 µl of a staining solution [0.1% fast blue BB salt: 5% sodium dodecyl sulphate (SDS): 2:5] and kept for 5 min. The absorbance was recorded at 570 nm on microplate reader (Thermo Scientific: Multiskan Go). General esterase activity was expressed as micromoles of the α-napthol per minute per milligram of protein.

Determination of glutathione-S-transferase activity

Glutathione-S-transferase activity was measured using 1-chloro-2, 4- dinitrobenzene (CDNB) as the substrate (Habig et al., 1974). A reaction mixture of 50 µl of CDNB (50 mM in ethanol) and 150 µl of reduced glutathione (50 mM in 0.1M PBS, pH 6.5) were mixed with 2.78 ml of sodium phosphate buffer (0.1M, pH 6.5 containing 1 mM EDTA). 20 µl of enzyme supernatant was then added and shaken well. Absorbance of the 300 µl of each reaction mixture at 340 nm was recorded 10 min employing kinetics (time scan) menu on microplate reader. The GST activity was calculated using the formula CDNB-GSH conjugate (µM/min/mg protein) = (Absorbance increase in 5 min × 0.3 × 1000) / (9.6* × 5 × mg of protein) (*9.6 mM/cm is the extinction coefficient for CDNB-GSH conjugate at 340 nm).

Determination of Cytochrome P450 activity

As heme protein is a major constituent of the majority of Cytochrome P450 (CYP450), its activity was calculated by estimating heme peroxidase activity (Penilla et al., 2007, Tiwari et
al., 2011). 20µl of enzyme homogenate was incubated with 200µl of TMBZ solution (0.01g of TMBZ in 5ml of methanol+15ml of 0.25M sodium acetate, pH 5.0) and 80µl of 0.0625M PBS (pH 7.2) and 25µl of 3% H2O2 for 30 minutes at 25°C. Absorbance was recorded at 630nm 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 milligram protein per minute.

**Protein estimation**

Protein present in enzyme suspension was measured following the method of Lowry et al. (1951).

**Statistical Analysis**

Data were statistically analyzed using InStat GraphPad software (www.graphpad.com) (GraphPad Software, Inc., La Jolla, CA, USA). Unpaired t-tests compared treatment means with the level of significance set at P ≤ 0.05. Welch’s correction (Welch 1949) was applied if standard deviations were found to be unequal. Median lethal concentrations (LC50) were calculated using the SPSS version 15.0 (SPSS Inc., Chicago, IL, USA), based on Finney’s probit analysis method (Finney 1973) and expressed in ppm.

**Results**

**Toxicity bioassay**

Relative toxicities of ethion, dicofol, fenpropathrin, propargite and fenazaquin regarding median lethal dose (LC50) to both conventional and susceptible *O. coffeae* populations are summarized in Table 1. The LC50 (ppm) values of ethion, dicofol, fenpropathrin, propargite and fenazaquin against *O. coffeae* population from conventional plantation were found to be considerably higher than the LC50 values of susceptible *O. coffeae* population. Their resistance factor (RF) values showed 10.74 times for ethion, 7.71 times for dicofol and 11.94 times for propargite. The RF value was lowest for fenpropathrin and fenazaquin. In terms of relative toxicity, fenazaquin was the most toxic acaricide for both strains.

**Enzyme assays**

A statistically significant enhanced detoxifying enzyme activity was observed in conventionally-managed population than that of susceptible population of *O. coffeae* (Table 2). The GE, GST, and CYP450 activity levels observed in the population from the conventionally-managed plantation were 2.83, 1.68, and 1.31 higher than the levels observed in the susceptible population obtained from the organically-managed plantation.

**Table 1** Relative toxicity of different acaricides against adults of *Oligonychus coffeae*.

<table>
<thead>
<tr>
<th>Acaricide</th>
<th>df</th>
<th>Population collected from conventionally-managed tea plantation</th>
<th>Population collected Organically-managed tea</th>
<th>df</th>
<th>Population collected from conventionally-managed tea plantation</th>
<th>Population collected Organically-managed tea</th>
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<tr>
<td></td>
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<td>95% FL± of LC50 (ppm)</td>
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<td>Slope ± SE</td>
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<td>RT (χ2)</td>
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<td>LC50 (ppm)</td>
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<td>95% FL± of LC50 (ppm)</td>
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<td>Slope ± SE</td>
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<td>Upper</td>
<td>Lower</td>
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<td>Upper</td>
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<tr>
<td>Ethion</td>
<td>6</td>
<td>2.56 ± 0.001</td>
<td>1049.75</td>
<td></td>
<td>1252.89</td>
<td>879.55</td>
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<tr>
<td>Dicofol</td>
<td>6</td>
<td>2.32 ± 0.001</td>
<td>599.21</td>
<td></td>
<td>723.56</td>
<td>496.23</td>
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<tr>
<td>Fenpropathrin</td>
<td>6</td>
<td>0.82 ± 0.005</td>
<td>6.75</td>
<td></td>
<td>11.44</td>
<td>3.98</td>
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<tr>
<td>Propargite</td>
<td>6</td>
<td>1.29 ± 0.002</td>
<td>232.03</td>
<td></td>
<td>332.72</td>
<td>161.80</td>
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<td></td>
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<tr>
<td>Fenazaquin</td>
<td>6</td>
<td>2.08 ± 0.002</td>
<td>2.80</td>
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<td>3.55</td>
<td>2.21</td>
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</tbody>
</table>

LC50 = lethal concentration for 50%; LC95 = lethal concentration for 95%; df = Degrees of freedom; FL = Fiducial limit; RT = Relative toxicity (RT) = LC50 of the ethion/LC50 of each acaricide; RC = Resistance coefficient (RC) = LC50/recommended field dose; RF = Resistance Factor (RF) = LC50 of the conventional field-collected population/LC50 of the susceptible population.
Table 2 Detoxifying enzyme activities of *Oligonychus coffeae* in tea plantations of Assam, north east India.

<table>
<thead>
<tr>
<th>Population collected from</th>
<th>Detoxifying Enzyme</th>
<th>General esterases</th>
<th>Glutathione-S-transferases</th>
<th>Cytochrome P450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean values ± SE</td>
<td>Mean values ± SE</td>
<td>Mean values ± SE</td>
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<td></td>
<td>(μmoles α-</td>
<td>(CDNB-GSH</td>
<td>(nmol P450</td>
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<td></td>
<td></td>
<td>napthol/min/mg</td>
<td>conjugate/min/mg</td>
<td>equivalent/min/mg</td>
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<td></td>
<td></td>
<td>protein)</td>
<td>protein)</td>
<td>protein)</td>
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<tr>
<td>Conventionally managed tea plantation</td>
<td></td>
<td>1.081± 0.116a</td>
<td>10.589±1.292a</td>
<td>0.00017±0.00003a</td>
</tr>
<tr>
<td>Organically managed tea plantation</td>
<td></td>
<td>0.382±0.065b</td>
<td>6.295± 0.554b</td>
<td>0.00013±0.00002b</td>
</tr>
</tbody>
</table>

*ab* Mean values within a column are significantly different at *P* = 0.05 by unpaired *t*-test. *b* Activity ratio = Enzyme activity of conventional field-collected population/enzyme activity of susceptible population.

### Discussion

The development of resistance/tolerance is related to the history of exposure of the target pest to commonly-applied insecticides or pesticides similar chemical groups (Zhu *et al.* 2011, Das *et al.* 2017). Our results were in agreement with those observations. Among the acaricides tested in our study, fenazaquin showed the highest toxicity to *O. coffeae* adults. Its LC$_{50}$ of 17.42 ppm (slope = 2.08 ± 0.002) for conventionally-managed population was significantly lower than that of the other acaricides (Table 1) and was lower than the concentrations recommended for management of the mite pest. The LC$_{50}$s of fenazaquin and fenpropathrin were not significantly different for either the conventionally or organically managed populations, but both were significantly lower than the other three acaricides for both populations.

Comparison of the concentration-mortality responses between conventionally-managed population and organically-managed population showed that conventionally-managed population is resistant to ethion (RF = 10.74) and propargite (RF = 11.94) and difference in susceptibility between resistant and susceptible populations is at least ten fold (Mota-Sanchez and Bills 2002). A population is said to be tolerant to a chemical if relative toxicity level against same chemical between their tolerant and susceptible population differ by less than 10 folds (Mota-Sanchez and Bills 2002),wich was observed with fenpropathrin (RF = 1.23) and dicofol (RF = 7.71) (Table 1).

All the five acaricides have different mode of action. Ethion is an acetylcholinesterase (AChE) inhibitor, propargite impedes mitochondrial ATP synthase activity, fenpropathrin acts on the sodium channel modulators, fenazaquin is a mitochondrial complex I electron transport inhibitor, and mode of action of dicofol is unknown or uncertain. The poor performance of ethion and dicofol against *O. coffeae* observed in our laboratory bioassays was reported earlier by Roy *et al.* (2010, 2012) from North Bengal tea plantations. Fenpropathrin resistance in *O. coffeae* was also observed by Amsalingam *et al.* (2012, 2017) in south Indian tea plantations. Ethion, propargite, dicofol, and fenpropathrin have been extensively used for *O. coffeae* management for more than 3 decades (Roy *et al.* 2014a); whereas, fenazaquin was recently registered for use only in tea. The latter is also a quinazoline acaricide with a novel mode of action (e.g., mitochondrial electron transport inhibitor) (Kumar *et al.* 2004, Ware and Whitacre 2004). The high reproductive potential and short duration of the lifecycle of *O. coffeae*, combined with frequent application of ethion, propargite, dicofol, and fenpropathrin and the possibility of induction of cross-resistance may have driven the observed development of resistance and tolerance to these compounds. Therefore, based on the results of these toxicity bioassays, fenpropathrin and dicofol should be carefully deployed and used to avoid or, at least, delay further development of resistance to these compounds and their chemical relatives. And, the apparent resistance of *O. coffeae* in conventionally-managed tea plantations to recommended concentrations of ethion and propargite indicates that these compounds are ineffective in managing the mite pest in tea plantations in Assam.
Our results further show that metabolic detoxification plays an important role in the observed development of resistance/tolerance of *O. coffeae* to the acaricides. And, these results are in agreement with those of Amsalingam *et al.* (2012, 2017). Alterations in the activity of metabolic detoxification enzymes are attributed to the development of resistance in mite or insect populations (Van Leeuwen *et al.* 2005, Van Leeuwen and Tirry 2007, Amsalingam *et al.* 2012, 2017, Das *et al.* 2017). The link between esterase activity and resistance to organophosphate insecticides has been observed in many arthropod pests (Ay and Gurkan 2005). GE and CYP450 play major roles in the hydrolysis of various chemicals that contain ester linkages, thereby degrading various organophosphate and synthetic pyrethroid pesticides with carboxyl (or) amide groups (Devonshire and Field 1991; Bass and Field 2011, Wu *et al.*, 2011; Bharati *et al.* 2016, Mukhopadhyay *et al.* 2016, Das *et al.* 2017), while GST conjugates polar products with various endogenous compounds, such as sugars, sulphate, phosphate, amino acids, or glutathione (Yu 2008) to increase pesticide resistance (Nehare *et al.* 2010), especially to organochlorine, organophosphate, and pyrethroid groups (Hemingway *et al.* 2004, Das *et al.* 2017).

Resistance or tolerance in insect and mite populations increases when pesticides are used and, thus, exert high selective pressure on resistant genotypes, but the resistance development is not observed in populations left unexposed to pesticides (Forrester 1990, Roy *et al.* 2010). We observed the same phenomenon herein. This suggests that the frequent use of excessively high concentrations and the reliance only on pesticides can exacerbate problem of resistance development and underscores the need for comprehensive insecticide-resistance management strategies. If not, it is predicted that target pest populations will be resistant to new materials within a few generations or growing seasons after their registration and release. Our results support the immediate need for planning to mitigate the problem of resistance of *O. coffeae* to acaricides in Assam tea plantations.

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