Molecular basis of acaricide resistance in *Tetranychus truncatus* Ehara (Prostigmata: Tetranychidae) infesting vegetable crops

By

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KERALA, INDIA

2018

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2

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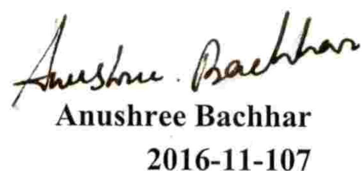

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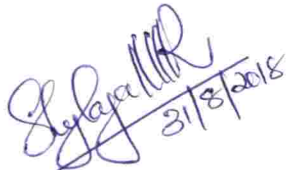

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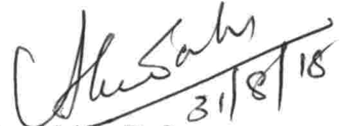

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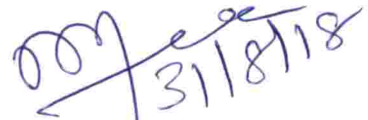

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List of Abbreviations

%	Percentage
@	At the rate
<	Less than
=	Equal to
>	Greater than
µg	Microgram
µl	Microlitre
BLAST	Basic Local Alignment Search Tool
bp	base pair
CPBMB	Centre for Plant Biotechnology and MolecularBiology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
DTT	Di thio thretol
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
l	Litre
M	Molar
mg	Milligram

ml	Millilitre
mM	Milli mole
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
ng	Nanogram
ºC	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PMSF	Phenyl methane sulfonyl fluoride
PTU	Phenyl thio urea
RNA	Ribonucleic acid
rpm	Revolutions per minute
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts

Introduction

1. Introduction

Spider mites of the family Tetranychidae are considered as one of the most serious sucking pests of vegetable crops worldwide. Acaricides play a major role in management of mites in vegetable ecosystems. The intensive use of acaricides has led to the development of resistance in many mite species around the globe making mite management difficult. In view of this, since 1990's, several novel acaricides with unique chemical structure and mode of action were introduced and commercialized for mite management. However, mite populations developed resistance to newly introduced compounds after few years of use (Vassiliou and Kitsis, 2013).

The mite is difficult to manage because of its ability to quickly develop resistance to acaricides (Dittrich, 1975; Cranham and Helle, 1985). High reproductive potential, the extremely short life cycle and arrhenotokous parthenogenesis, facilitate rapid resistance development in mites to many pesticides compared with insects, often after only a few applications (Stumpf and Nauen, 2001; Ay and Gurkan, 2005). The spider mite, *Tetranychus urticae* Koch is currently considered as the 'most resistant' in terms of the total number of pesticides to which populations have become resistant (Van Leeuwen *et al.*, 2010).

Among the spider mites, *Tetranychus truncatus* Ehara is the major species infesting different vegetable crops of Thrissur district (Bennur *et al.*, 2015). The mite colonises the lower surface of leaf in large numbers and desap leading to white speckling initially followed by yellowing and drying of leaves. Different novel acaricide molecules are currently in use in the vegetable tracts of Thrissur against the mite. Of late, several farmers raised concern over the poor efficacy of the novel acaricides against mite pests on vegetable crops. As spider mites have the ability to rapidly develop resistance to a compound after continuous exposure, it has become necessary to

determine the susceptibility status of *T. truncatus* to the commonly used acaricides in this region.

The resistance mechanism in spider mites mainly involves enhanced detoxification through the enzymatic activity of esterases, glutathione-S-transferases and P450 monooxygenases (Van Leeuwen *et al.*, 2009; Tirello *et al.*, 2012). Identification of mechanisms conferring resistance to particular acaricides is essential to control the development and spread of resistant populations as well as to evolve suitable management strategy against the mite. This involves characterisation of detoxification enzymes involved in resistance, as well as identification of specific changes at the genomic level.

Biochemical and molecular mechanism underlying the resistance to pesticides have been studied by several workers in the polyphagous two spotted spider mite, *T. urticae.* However no work has been carried out in this line on *T. truncatus*, a predominant mite species in Kerala. It is in this context that the present study was undertaken with the following objectives.

- To detect the status of acaricide resistance in *Tetranychus truncatus* infesting vegetable crops
- To investigate the biochemical basis of acaricide resistance in *T. truncatus*
- To elucidate the molecular basis of acaricide resistance in *T. truncatus*

Review of Literature

2.Review of literature

Spider mites cause severe damage to vegetable crops both under open and protected cultivation. The ill effects associated with the indiscriminate use of conventional acaricides had led to the development of novel acaricide molecules for mite management. It has been reported recently that several novel acaricides have become ineffective against mites due the development of resistance. However, reports on resistance in spider mites to novel acaricide molecules from India are very scanty. In the present study, the status of acaricide resistance and the molecular mechanism of resistance in *Tetranychus truncatus* to commonly used novel acaricides were assessed. The literature pertaining to the studies on acaricide resistance in spider mites are reviewed below

2.1 Resistance to acaricides in mites

Modern agricultural practices, over use of fertilizers, synthetic and organic pesticides during and post-World War II created favourable condition for spider mites to increase to extremely high densities and experience outbreaks (Van de Vrie *et al.,* 1972). Management of mite pests has become difficult as mites develop resistance quickly on exposure to acaricides (Dittrich, 1975; Cranham and Helle, 1985).

In England, *T. urticae* infesting hops was reported to have developed resistance to four METI- acaricides after a short history of tebufenpyrad use in mite management. In the laboratory bioassay, the mite recorded resistance factor of 46, 346, 168 and 77, respectively for the acaricides, tebufenpyrad, pyridaben, fenazaquin and fenpyroximate compared to an METI acaricide-resistant reference strain (Devine *et al*., 2001).

Resistance in mites against acaricides of different genera were reported from all corners of the world. Resistance in *Tetranychus* species to

METI-acaricides has been reported from Belgium, Japan and Australia (Bylemans and Meurrens 1997, Ozawa 1994, Herron and Rophail, 1998). Rapid development of resistance in *T. urticae* to four novel acaricides viz., fenpyroximate, pyridaben, tebufenpyrad, and pyrimidifen was reported soon after their release in market (Uesugi *et al.*, 2002).

Resistance and control failures against *T. urticae* have been reported for organophosphates, organotins (Edge and James, 1986; Flexner *et al.*, 1988), bifenthrin (Farnham *et al.*, 1992), hexythiazox (Herron & Rophail 1993), fenpyroximate (Stumpf and Nauen 2001). Significant level of resistance in *T. truncatus* to three acaricides, pyridaben, fenpyroximate and tebufenpyrad was reported by Stumpf and Nauen (2001).

Low to moderate level of resistance to fenazaquin was reported in *T. urticae* on tomato crop from Bangalore (5 -32 fold) and Kolar districts (8-25 fold) of Karnataka. However, the strain from Kolar district recorded 79 fold resistance to diafenthiuron (Aji *et al.*, 2007).

Tetranychus urticae in particular has been documented to have evolved resistance to over 95 acaricidal/insecticidal active ingredients (Van Leeuwan *et al.,* 2010) from 60 countries and made it world's one of the most resistant pests (Van Leeuwan *et al.,* 2010).

The factors favouring development of resistance to a number of acaricides in spider mites are their short life cycle, the reproduction system and high biotic potential (Stumpf and Nauen, 2001; Van Pottelberge *et al.*, 2009; Nicastro et al., 2013). There are reports of cross and multiple resistant strains in *T. urticae* from several countries (Sato *et al.*, 2005; Kim *et al.*, 2006; Kwon *et al.*, 2010; Nicastro *et al.*, 2010).

Khajihali *et al.* (2011) reported that a strain of *T urticae* collected from rose greenhouses of Netherlands showed varied level of resistance to

the acaricides bifenazate, acequinocyl, milbemectin, abamectin, cyflumetofen, bifenthrin spiromesifen and etoxazole. Adult females of *T. urticae* collected from green houses of Italy on rose were reported to have developed reistance to tebufenpyrad and fenpyroximate (Tirello *et al.*, 2012).

Studies conducted on stability of resistance, cross-resistance and monitoring of chlorfenapyr resistance in *T. urticae* showed that chlorfenapyr resistance was stable in the absence of selection pressure under laboratory conditions. The results indicated possible positive cross resistance between chlorfenapyr and the acaricides abamectin, propargite and etoxazole, while no cross resistance was detected for the acaricides: milbemectin, fenpyroximate and diafenthiuron. Susceptiblity studies of *T. urticae* collected from different hosts showed that susceptibility of the different strains to chlorfenapyr was variable, with percentages of resistant mites ranging from 0.0 to 86.5 per cent (Nicastro *et al.*, 2013).

The level of resistance in *T. urticae* collected from field and green house crops in Cyprus was evaluated in the laboratory following leaf disk bioassay, in comparison to a German susceptible reference strain. Resistance of *T. urticae* was detected to abamectin, acrinathrin, fenazaquin, and pirimiphos methyl. The highest resistance ratio was recorded by abamectin followed by acinathrin both in the field as well as in greenhouse, suggesting that the use of the two acaricides should be avoided or minimized for the control of *T. urticae* populations in indoor and outdoor environments (Vassiliou and Kitsis, 2013).

Spiromesifen resistance in *T urticae* was reported from Jordan on cucumber which showed 17.96-fold resistance (Mohamed *et al.*, 2012). Ullah and Gotoh (2013) reported development of resistance in *T. truncatus* to two acaricides, spirodiclofen and fenpyroximate from Bangladesh. Resistance to spiromesifen (32.13 fold) in laboratory reared *T. urticae*

population was reported from Kangra, Himachal Pradesh (Kumari *et al.*, 2015).

Sato *et al.* (2016), reported spiromesifen resistance in *T. urticae* on several crops in Brazil. In order to generate information on spiromesifen resistance management program in *T. urticae*, studies were conducted on artificial selections, stability of resistance and monitoring of resistance in the mite population collected from commercial chrysanthemum fields in Brazil. In the laboratory, after 20 cycles of selection for spiromesifen resistance, *T. urticae* recorded a resistant ratio of 121. It was also found that the spiromesifen resistance was unstable in the absence of selection pressure. The susceptibility of different strains of *T. urticae* collected from different crops showed varying level of susceptibility to spiromesifen with percentages of resistant mites ranging from 0.0 to 81.5 per cent.

Resistance to acaricides was reported very recently from Punjab, India, for a number of acaricides viz., fenpyroximate, spiromesifen, fenazaquin and propargite in *T. urticae* (Sharma and Bhullar, 2018).

2.2 Biochemical mechanism of resistance to pesticides

The mechanism of resistance in mites also involves detoxification by the activity of enzymes viz., esterases, glutathione -S-transferases and P450 monooxygenases as in the case of insects (Van Leeuwen *et al.*, 2010). The enhanced production of detoxifying enzymes that sequester the insecticide and mutations in target proteins, making them less sensitive to the insecticide are considered as the two major mechanisms involved in development of resistance in mites (Panini *et al.*, 2016).

To withstand the plant toxins (allelochemicals) such as alkaloids, terpenes and phenols, insects evolve by producing detoxifying enzymes

(Gatehouse, 2002; Despres *et al.*, 2007; War *et al.*, 2012; Heidel-Fischer and Vogel, 2015). In many cases, development of metabolic resistance against insecticides in insects has direct or indirect botanical origin (Isman, 2006). Resistant insects possess detoxifying enzymes with a higher catalytic rate and also in large amount, as a result of increased transcription or gene amplification.

The elevated activity of esterase is reported to be a major resistance mechanism in pyrethroid (Young *et al.*, 2006) and organophosphate resistant in insects (Mohan *et al.*, 2007). Carboxylesterase is an important enzyme associated with organophosphate and pyrethroid resistance (Zhang *et al.*, 2010) Glutathione S-transferase is specifically involved in organophosphate metabolism via conjugation (Huang *et al.,* 1998). The cytochrome P450 monooxygenases family enzymes are vital oxidative enzymes involved in pyrethroid detoxification (Huang and Han, 2007).

As a result of changes in the quantity or quality of major detoxification enzymes (esterases, P450 monooxygenases and glutathione-S-transferases), insecticides either get metabolised or sequestered before reaching the target site. Also, sensitivity of the target site varies due to point mutations (Oakeshott *et al.*, 2005; Feyereisen, 2005; Enayati *et al.*, 2005 and Li *et al.*, 2007).

Stumpf and Nauen (2001) investigated the biochemical mechanism of Mitochondrial Electron Transport Inhibitor-acaricide (METI) resistance in *T. urticae* by studying two resistant populations namely, Japanese and English populations. The METI resistant strains showed enhanced activity of cytochrome P450 compared to susceptible strain. They also found that the monooxygenase-inhibitor, piperonyl butoxide could suppress the detoxification of the METI-acaricides in the resistant mite population.

Carboxylesterase constitute a class of enzyme esterases, contain functional groups as carboxylic acid ester, amide and thioester, which are widely distributed in microbes, plants and animals. These enzymes hydrolyse chemicals containing carboxylic esters to the corresponding component alcohols and acids (Satoh *et al.*, 2006). Increased esterase activity through up-regulation esterase transcription and point mutations within esterase genes are two known mechanisms of esterase-mediated insecticide resistance (Guo and Gao, 2009).

In blow flies, a single amino acid substitution resulted in increase in activity in carboxyl esterase which detoxifies the pyrethroid more effectively (Newcomb *et al.*, 1997). A large number of esterases were linked to pyrethroid resistance in *Rhipicephalus microplus* (Chevillion *et al.*, 2007).

A field-collected strain of *T. urticae* which exhibited high level of resistance to bifenthrin, dicofol and fenbutatin oxide when evaluated in the laboratory in comparison with a susceptible laboratory strain, showed cross-resistance to a number of acaricides. In the laboratory, the strain recorded escalated activity of mono-oxygenases (MO) and esterases linked with resistance and cross-resistance in the strains (Va Leeuwen and Tirry, 2005).

Ghadamyari and Sendi (2008) studied the resistance mechanisms in Iranian strains of *T. urticae*. The strains which recorded significant level of resistance to oxydemeton-methyl showed enhanced esterase activity compared to susceptible strain.

Increase in MFO, GST and esterase activity was reported in *T. urticae* population on rose in green house of Netherlands (Khajihali *et al.*, 2011). They studied the efficacy of eight major acaricides to *T. urticae* strains collected at different places in the Dutch rose cultivation. Susceptiblility of the strains to different acaricides was evaluated in the laboratory. To

correlate the resistance status and detoxifying activity of enzymes, the activity of esterases, glutathione-S-transferases (GSTs) and cytochrome P450 monooxygenases (MFOs) was evaluated in selected strains. They found that, the levels of susceptibility varied between strains and acaricides, but resistance was detected in most strains. The activity of cytochrome P 450 mono oxygenase was found to have increased in field populations compared to the susceptible line. In addition, notable differences in the activities of esterase activity and GST were also identfied in resistant lines.

Elevated activities of cytochrome P450 monooxygenases (MFOs) and glutathione-S-transferases (GSTs) were reported to be associated with resistance in *T. urticae* on green house rose in Italy (Tirello *et al.*, 2012). Similarly, increased activities of both mixed-function oxidases and esterases were found to contribute to the fenpyroximate resistance in *T urticae* (Kim *et al.*, 2005).

2.3 Genetic bases of acaricide resistance

The studies on molecular elucidation of target-site resistance in mites have not progressed much, as compared to insects. Recent studies have documented resistance mutations in genes of major acaricide targets such as acetylcholinesterease (Khajehali *et al.*, 2010; Kwon *et al.*, 2010a), the para sodium channel (Kwon *et al.*, 2010b; Tsagkarakou *et al.*, 2009), the glutamate-gated chloride channel (Kwon *et al.*, 2010c) and cytochrome b (Van Leeuwen *et al.*, 2008, 2011). These genes and their proteins are the target sites of different class of insecticides (Van Leeuwen *et al.*, 2010).

Grbic *et al.* (2011) sequenced the *T. urticae* genome which is the first completely sequenced and annotated chelicerate genome. They found strong signatures of polyphagy and detoxification in gene families associated with

feeding on different hosts and in new gene families acquired by lateral gene transfer. Eighty-six cytochrome P450 (CYP) genes were identified in the *T. urticae* genome similar to insects but with an expansion of *T. urticae* - specific intron less genes of the CYP2 clan. The carboxyl/cholinesterases (CCEs) gene family contained 71 genes, with a single acetylcholinesterase gene (Ace1) but two new clades at the root of the neurodevelopmental class of CCEs, representing 34 and 22 CCEs, respectively.

Identification of genetic markers confering resistance to new acaricides in spider mites gained momentum with the recent completion of *T. urticae* genome analysis (Grbic *et al.,* 2011; Van Leeuwen *et al.,* 2013). Several qualitative markers have been identified in the acetylcholinesterase, voltage sensitive sodium channel, glutamate-gated chloride channel, chitin synthase 1 and cytochrome b genes (Feyereisen *et al.*, 2015; Van Leeuwen *et al.,* 2015). Five point mutations were identified in the catalytic triad and peripheral anionic sites of actyl choline esterase gene (Khajehali *et al.,* 2010; Anazawa *et al.,* 2003; Kwon *et al.*, 2012; kwon *et al.*, 2010a). Three point mutations associated with pyrethroid resistance were found on voltage sensitive sodium channel gene (Kwon *et al.,* 2010b; Tsagkarakou *et al.,* 2009). Two point mutations in two different types of glutamate-gated chloride channels were identified to confer abamectin resistance (Dermauw *et al.*, 2012; Kwon *et al.*, 2010c). A point mutation associated with etoxazole resistance was identified in chitin synthase 1 gene (Van Leeuwen *et al.,* 2012). Five point mutations associated with bifenazate resistance were identified in cytochrome b gene (Van Leeuwen *et al.*, 2008; Van Nieuwenhuyse *et al.*, 2009).

Study done by Khajehali *et al.* (2011) showed that there was significant level of resistance associated mutations in para sodium channel (pyrethroid) and cytb (bifenazate and acequinocyl resistance) linked with

acaricide resistance in *T urticae* strains collected from rose greenhouses in the Netherlands.

Shi *et al*. (2016) identified a carboxylesterase gene *TCE2* gene which was over-expressed in resistant mites of *Tetranychus cinnabarinus* (Boisduval). In the laboratory bioassay, it was found that the resistant levels to three acaricides viz., abamectin, fenpropathrin, and cyflumetofen significantly decreased after the down-regulation of *TCE2*, indicating a correlation between the expression of *TCE2* and the acaricide-resistance in *T. cinnabarinus*. *TCE*2 gene when reengineered in *Escherichia coli*, the activity of caboxylesterase could be subpressed by abamectin, fenpropathrin, and cyflumetofen. The study identified *TCE*2 as a functional gene involved in acaricide resistance in *T. cinnabarinus*.

Bajda *et al*. (2017) identfied a mutation in METI-I resistant Strain of *T. urticae*. The position of the mutation was located in a stretch of amino acids, previously photo-affinity labeled by fenpyroximate. They also confirmed the involvement of the mutation in METI-I resistance through marker-assisted back-crossing. In addition, QTL analysis identified the genomic region of pyridaben resistance, which included the *PSST* gene.

Material and Methods

3. Material and Methods

The present study was carried out at the Centre of Plant Biotechnology and Molecular Biology (CPBMB) and All India Network Project on Agricultural Acarology (AINPAA), Department of Agricultural Entomology, College of Horticulture, Vellanikkra, Kerala Agricultural University during 2017-18. The objectives of the study were to investigate the status, biochemical and molecular bases of acaricide resistance in *Tetranychus truncatus* Ehara infesting vegetable crops.

The methods and techniques adopted for conducting various experiments based on the objectives set forth in the study are presented hereunder.

3.1 Collection and rearing of *Tetranychus truncatus*

3.1.1 Field survey

Purposive surveys were conducted in vegetable fields of College of Horticulture, KAU campus (viz., the seed production plots and experimental fields of the Departments of Vegetable Science and Seed Technology) and KVK, Vellanikkara, Thrissur district during the months of April- December, 2017 and January, 2018, (Plate 3.1). Spider mites associated with selected crops namely cowpea [*Vigna unguiculata* (L.)Walp], ashgourd [*Benincasa hispida* (Thunb.)], brinjal [*Solanum melongena* L.], okra [*Abelmoschus esculentus* (L.) Moench], pumpkin [*Cucurbita pepo* L.], amaranthus [*Amaranthus tricolor* L.] and cucumber [*Cucumis sativus* L.] were collected during the survey. Spider mite infested leaf samples (Plate 3.2) were collected in polythene bag, label describing the locality, crop and date of collection were placed inside each bag and tied with rubber bands. The samples were brought to the laboratory for maintenance of isoline culture.

Information on currently used acaricides were also collected during the survey.

3.1.2 Maintenance of isoline culture

The mite infested leaves were observed under a stereo binocular microscope and a single gravid female mite (Plate 4) from an infested leaf was transferred to a fresh mulberry leaf using a fine camel hair brush. The mulberry leaf was then placed on a wet sponge surrounded by water in a tray. The population arising from each single gravid female was maintained as iso-line, assigning unique accession number. Isolines were maintained separately for mites collected from different crops and different localities on different dates surveyed.

3.1.3 Identification of mite specimens

Permanent slides of mite specimens from different isoline cultures were prepared by mounting separately adult female and male mites in a drop of Hoyer's medium (Annexure I) on the glass slide. The permanent slides were used for morphological characterization and identification of species using taxonomic keys. The shape of the aedeagus was used as the key character for species level identification (Henderson, 2001).

3.1.4 Maintenance of *Tetranychus truncatus* culture

The accessions identified as *T. truncatus* alone were maintained in the laboratory as field populations for further toxicological, biochemical and molecular studies, while other accessions were discarded. The laboratory culture of different accessions of *T. truncatus* were further maintained as monoculture as described in 3.1.2. The culture of *T. truncatus,* being maintained in the AINPAA for more than 150 generations without exposure

to any pesticides was used as the susceptible population/strain for the study (Plate 3.3, 3.4).

3.2 Evaluation of the status/level of acaricide resistance in *T. truncatus*

In order to identify the status of acaricide resistance in field populations of *T. truncatus*, susceptibility of different accessions to three different acaricides *viz*., spiromesifen 240 SC (Oberon), fenazaquin 10 EC (Magister) and diafenthiuron 50 WP (Pegasus) (Plate 3.5) (Table 1) was evaluated in the laboratory following leaf dip method (Aswin *et al.,* 2016). The laboratory maintained population of *T. truncatus* which was never exposed to insecticide/acaricide was used for comparison.

The concentrations of different treatments were prepared from stock solution through serial dilution. Stock solutions were prepared for each acaricide as shown in Table 1.

3.2.1 Laboratory bioassay

Bioassay studies were conducted in the laboratory to find out the susceptibility of different strains of *T. truncatus* to different acaricides. The effect of different treatments (Table 1) on adult mites was studied by the leaf-dip bioassay method (Plate 3.6). The required concentrations of the acaricides were prepared in water in beakers (Plate 3.7) and leaf bits of $8cm^2$ were dipped in the aqueous solution for 30 seconds and then air dried for one hour (Plate 3.8). Twenty five gravid females of uniform age taken from the monoculture were released on to the treated leaf bits kept on wet cotton pad in Petri plate by using moistened camel hair brush. To prevent the escape of mites from treated leaves, a thin layer of wet cotton was provided all around the leaf margin. Three replications were maintained for each treatment. Leaf

discs dipped in sterile water served as control. Observations on mortality of adult mites were recorded at 24 hours interval under the stereo binocular microscope and per cent mortality was calculated.

(a)

(b)

(c)

Plate 3.1. Surveys in different vegetable fields (a) Brinjal field ; (b) Pumpkin field (c) Okra field

Plate 3.2. Symptoms of spider mite infestation on green amaranthus

Plate 3. 3. Isoline culture of spider mite on mulberry leaf

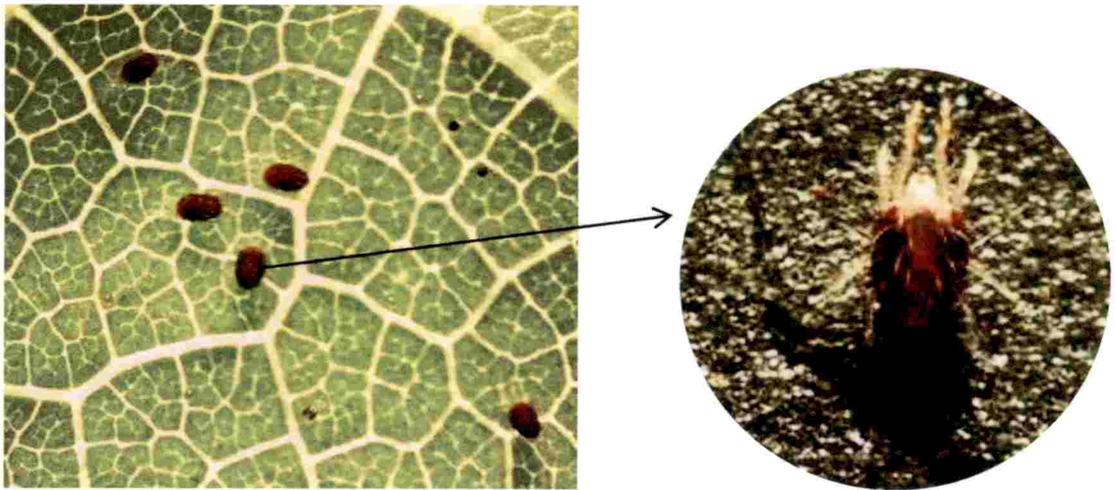

Plate 3. 4. Female *Tetranychus truncatus* in lab culture

3.2.2 Statistical analysis

The mortality count of *T. truncatus* in three replicates of each concentration for different acaricides was recorded and per cent mortality in each concentration was calculated. The LC_{50} values for each insecticide were calculated using probit analysis (Finney, 1971) by using the software Polo plus. The resistance level of different populations to different acaricides was calculated by taking the ratio of LC_{50} value of field collected populations to that of laboratory maintained susceptible population.

Table 1. Stock solution of acaricide for bioassay

Compound		Commercial formulation	Manufacturer	IRAC MoA classification	Stock solution (ml/250 ml) (g/250ml)
Spiromesifen	240 SC	Oberon	Bayer	23	10.91ml
Fenazaquin	10 EC	Magistar	Dupont	21A	25.00ml
Diafenthiuron	50 WP	Pegasus	Syngenta	12 A	5.00g

(a)

(b)

(c)

Plate 3.5. Commercial formulations of acaricides evaluated

(a) Spiromesifen (Oberon) (b) Fenazaquin (Magister) (c) Difenthiuron (Pegasus)

Plate3. 6. Leaf dip method

Plate 3.7. Treatments T1 to T8. (Concentrations prepared for leaf dip bioassay. T1 is the highest concentration; T7 is the lowest; T8 is control)

Plate 3.8. Layout of laboratory bioassay

3.3 Biochemical bases of acaricide resistance in *Tetranychus truncatus*

To identify the mechanism of resistance to acaricides in *T. truncatus*, the activity of detoxifying enzymes *viz*., carboxyl esterase and cytochrome P450 were estimated separately for the different field populations/strains and the susceptible strain of *T. truncatus* following the spectrophotometric method described by Hansen and Hodgson (1971) and Van Asperen (1962).

3.3.1 Preparation of buffers

3.3.1.1 Sodium phosphate buffer (0.1M)

Sodium phosphate buffer was prepared and stored at 4°C:

Sodium phosphate monobasic	119.98
Sodium phosphate dibasic	141.96

Solution A: 1.19 g of sodium phosphate monobasic (0.1M) was dissolved in 100 ml of distilled water.

Solution B: 1.41 g of sodium phosphate dibasic was dissolved in 100 ml of distilled water.

13 ml of solution A was mixed with 87 ml of solution B and the volume was made up to 200 ml and kept in 4°C for preservation. This solution was prepared fresh once in every fifteen days.

3.3.1.2 Homogenisation buffer

Homogenisation buffer for enzyme extraction from mites was prepared freshly using sodium phosphate buffer (0.1M) containing 1mM EDTA, 1mM DTT, 1mM PTU, and 1mM PMSF. The pH of the solution was maintained at 7.6.

3.3.2 Determination of Cytochrome P450 activity (Hansen and Hodgson, 1971)

3.3.2.1 Isolation of enzyme

Twenty mites (gravid female) were crushed in 40µl homogenisation buffer using pre-chilled micro pestle in pre-chilled eppendorf tubes (1.5ml). 60µl of homogenisation buffer was added and kept for centrifugation at 10,000 rpm for 25 minutes. The supernatant was collected in fresh tubes immediately and kept in ice and used for enzyme assay on the same day.

3.3.2.2 Enzyme assay

The assay mixture consisted of 50µl enzyme solution, 5 µl of 50.0 mM p-nitroanisole (prepared by adding 76.58 mg in 10 ml of 100% ethanol stored at 4°C) and 120µl of 0.1M sodium phosphate buffer (pH 7.6). The mixture was incubated for 3 min at 34°C. The reaction was initiated by adding 25 µl, 10.0 mM NADPH (was freshly prepared in 0.1 mM phosphate buffer 1 hour before assay). Three replications were used for each sample along with substrate blank and enzyme blank. The enzyme assay was done by using Vmax kinetic plate reader in 96 well plate (flat bottom) with the help of software Soft Max Pro. The readings were recorded (change in absorbance) in kinetics mode at 405nm for 5 minutes at 30 seconds interval. Total three replications were taken along with substrate blank and reagent blank. The MFO activity was expressed in nmols of p-nitrophenol formed minute^{-1} mg^{-1} of protein.

CytochromeP450 activity

$$= \frac{\text{Change In Absorbance} \times \text{Volume of Reaction Mixture} \times 1000}{\text{Time} \times \text{Volume of Sample} \times \varepsilon \times \text{Protien}}$$

- ε (extinction coefficient of p-nitroanisole)= 3.32mM^{-1} cm^{-1}
- Time is total time for which change is observed (5 mins.).

3.3.3 Determination of carboxyl esterase activity

The esterase activity was estimated by Van Asperen method (1962) with slight modifications

3.3.3.1 Preparation of stock solutions/reagents

3.3.3.1.1 Sodium phosphate buffer (4mM)

Solution A: 47.9mg sodium phosphate monobasic was dissolved in 100 ml of distilled water.

Solution B: 56.7mg of sodium phosphate dibasic was dissolved in 100 ml of distilled water.

Solution A and solution B was mixed in the ratio 51:49 and the final volume was made up to 200 ml. The pH of the solution was maintained at 6.8.

3.3.3.1.2 α napthyl acetate (1mM)

1.86 g α napthyl acetate was mixed with 10 ml of ethanol and stored at 4°C for further use.

3.3.3.1.3 α napthol

10mg of α napthol was dissolved in 10ml of 100 per cent ethanol and stored at 4°C for further use. This solution was prepared fresh once in every five days

3.3.3.1.4 Staining solution

5% sodium dodecyl sulphate (SDS) was prepared and kept at room temperature. 1% of fast blue BB salt was prepared by dissolving 0.1g of fast blue BB salt in 4mM phosphate buffer and stored at 4°C in the dark. The staining solution was made fresh each time by mixing 5 parts of 5% SDS and 2 parts of 1% fast blue BB salt.

3.3.3.2 Preparation of standard curve

Different concentrations of α napthol from 20µl to 200µl were prepared from the stock solution. The dilutions were done by adding 4mM sodium phosphate buffer. 100 µl of staining solution was added in each concentration and kept at 37°C for 30 minutes in dark. The readings were recorded at 550nm.

3.3.3.3 Isolation of enzyme

20 gravid females were crushed in 40µl of homogenisation buffer in pre-chilled eppendorf tubes using pre-chilled micro pestles. The final volume was made 100µl by adding 60µl of homogenisation buffer and centrifuged at 10,000 rpm for 25 minutes. Supernatant was collected in fresh pre-chilled eppendorf tubes and again centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected in fresh pre-chilled tube and used for enzyme assay on the same day within three hours.

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3.3.3.4. Preparation of substrate solution

1ml of α napthyl acetate stock solution was mixed with 1ml of 4mM phosphate buffer for preparing the substrate solution.

3.3.3.5 Enzyme assay

Enzyme -	10μl
Phosphate buffer (4mM) -	90μl
α-napthyl acetate -	50μl
Staining solution -	50μl

Enzyme, phosphate buffer and α napthyl acetate were added as mentioned above in flat bottom 96 well plate and kept in dark for incubation at 37°C for 30 minutes with occasional shaking at 10 minutes interval. Then, 50μl of staining solution was added to stop the reaction and develop colour. The OD value was measured at 550 nm in Vmax kinetic plate reader with the help of software Soft Max Pro.

$$\text{Esterase activity} = \frac{\text{concentration of standard} \times \text{OD of the test} \times \text{total volume}}{\text{OD of the standard} \times \text{incubation time} \times \text{volume of the enzyme} \times \text{protien(mg)}}$$

Incubation time : 30 minutes

3.3.4 Estimation of total protein

The quantification of protein was done as per Bradford (1976) method.

3.3.4.1 Preparation of Bradford standard curve

1000 ml of Bradford reagent (Annexure I) was prepared and stored at room temperature. Different concentrations of bovine serum albumin from 0.2mg/ml to 1mg/ml were made in 0.1N sodium hydroxide solution. 30 μl of the sample and 150 μl of Bradford reagent were mixed and added in 96 well

plate and kept for dark incubation at 37°C for 30 minutes. The OD value was measured at 595 nm in Versa max kinetic plate reader with the help of software Soft Max Pro. 0.1N sodium hydroxide served as blank.

3.3.4.2 Quantification of protein from samples

30μl of enzyme solution was mixed with 150μl of Bradford reagent and added in 96 well plate and kept in dark for incubation at 37°C for 30 minutes. The optical density (OD) of the mixture was measured at 595nm. 0.1M sodium phosphate buffer served as blank.

3.4 Molecular basis of acaricide resistance in *Tetranychus truncatus*

The mite accessions that showed higher enzyme activity were selected to study the molecular mechanism of resistance in them.

3.4.1 Isolation of DNA from spider mites

DNA was isolated from spider mites following CTAB method (Rogers and Bendich, 1994) with slight modifications.

Ten mites were crushed in 20μl of 2X CTAB (Cetyl Trimethyl Ammonium Bromide) buffer (Annexure II) in ice cold 1.5 ml eppendorf tube with pre-chilled micro pestles. 80μl of CTAB buffer was added and kept in dry bath at 60°C for one hour. Equal volume of 24:1 freshly prepared chloroform - isoamyl alcohol was added and invert mixed and kept for centrifugation at 10,000 rpm for 15 minutes. The supernatant (around 85μl) from middle transparent layer was collected in fresh ice cold eppendorf tube and 200μl of ice cold 96 per cent ethanol and 30μl of sodium acetate buffer were added and kept overnight for incubation at -20°C.

The sample was centrifuged at 13,000 rpm for 15 minutes and the supernatant was discarded. 100 μl of ice cold 70 per cent ethanol was then added and centrifuged at 13,000 rpm for 4 minutes. The supernatant was

discarded and the pellet was kept for drying at 37°C at dry bath for 45 minutes. The pellet was then dissolved in 10µl of autoclaved distilled water.

3.4.2 Quantification of DNA

The purity of DNA was checked in nanodrop (ND 1000) at 260 nm. The nanodrop readings were recorded at OD 260/280 and the samples showing ratios of OD 260/280 at 1.8 to 2.0 were considered as highly pure sample.

3.4.3 PCR primers

The PCR primers for enzyme specific genes were designed from the coding regions of the existing neucleotide sequences of cytochrome P450 and carboxylesterase retrieved from NCBI (Table. 2). The primers were then synthesised by Sigma-Aldrich.

3.4.4 DNA amplification

DNA amplification was done by using polymerase chain reaction in thermo cycler (Applied Biosystem) in 200µl PCR tubes. For standardization of annealing temperature, initially gradient PCR was run. The concentration of DNA was maintained at 80 ng μl^{-1} for amplification.

For PCR amplification of enzyme specific genes, 50 µl of reaction mixture with the following composition was used

3.4.4.1 PCR reaction mixture

a) DNA(80 ng/ µl) -	2µl
b) Taq polymerases (3U) -	1.5µl
c) Taq buffer A -	6µl
d) DNTP -	3µl
e) Forward primer -	1.5µl
f) Reverse primer -	1.5µl
g) Sterile distilled water -	34.5µl
Total -	**50µl**

Thermal profile for PCR amplification of MFO gene (cytochrome P50) was carried out with the following program.

Initial denaturation	**94°C 3 minutes**	
Denaturation	**94°C 1 minute**	35 cycles
Primer annealing temperature	**58.9°C 1.30 minutes**	
Primer extension	**72°C 1.30 minutes**	
Final extension	**72°C 10 minutes**	
Storage	**4°C infinity**	

Thermal profile for PCR amplification of esterase gene (carboxylesterases) was carried out as follows.

Initial denaturation	**94°C 3 minutes**	
Denaturation	**94°C 1 minute**	35 cycles
Primer annealing tempareture	**58.5°C 1.30 minutes**	
Primer extension	**72°C 1.30 minutes**	
Final extension	**72°C 10 minutes**	
Storage	**4°C infinity**	

Table 2. PCR primers

Name	Primers	Sequences (5'3')	Annealing temperature
Cytochrome P450	Forward	ATTGGCGCTGACTATGAAGC	58.9°C
	Reverse	ATGCAAGGCGATCTTGTACC	
Caroxylesterase	Forward	TGACATGGCTTGTACCGTGT	58.5°C
	Reverse	CCCTGTTCACGATTCCACTT	

3.4.5. Gel documentation of PCR product

Assessment of proper amplification of the enzyme specific genes was done by agarose gel electrophoresis (AGE) on 2 per cent agarose gel (Annexure III). Agarose gel (2%) was prepared and submerged in 1% TAE buffer (Annexure III) in gel tank (Bio-Rad). 20µl of PCR product was mixed with 2µl of 6X gel loading dye (annexure IV). First well was loaded with 100bp ladder and the blank was loaded in last well. The gel was run at 80 V

voltage set at power pack (Bio-Rad) until the dye migrated to two - third length of the gel.

Gel documentation was done with BioRad gel documentation system and with the help of Quantity One software under auto exposure to UV radiation. The clear image of the ladder and the marker were documented.

3.4.6 Sequencing of PCR product

DNA was eluted from the marker and sequenced by out sourcing.

3.4.7. Insilco analysis of the sequences

4.4.7.1. Sequence alignment

The sequences obtained for different accessions were merged to form contigs using CAP3 sequence assembly program provided by prabi (http://doua.prabi.fr/software/cap).

3.4.7.2. BLASTn analysis of sequence

To assess the homology, the sequences were compared in Basic Local Alignment Search Tool (BLAST).The sequences from the database showing maximum identity and query coverage with least E value were identified and compared.

3.4.7.3. Identification of open reading frame (ORF)

ORFs were identified from the sequence using MEGA 5 software, an offline bioinformatics tool by using MUSCLE. The largest ORFs were identified and tabulated. In addition, Expasy Translate (https://web.expasy.org/translate/) tool was used.

3.4.7.4 Multiple sequence alignment

Multiple sequence alignment of the retrieved sequences was done by using the bioinformatics tools Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), Mega X (offline) and Matcher (https://www.ebi.ac.uk/Tools/psa/emboss matcher) for pairwise alignment.

3.4.7.5 Translation to amino acid sequence

The identified ORFs were translated in to amino acid using Expasy Translate.

3.4.7.6 BLAST P analysis of amino acid sequences

The retrieved amino acid sequences after translation of ORFs were used for homology search using BLAST P tool.

3.4.7.7 Pair wise sequence alignment of amino acid

The sequences were used for pair wise sequence alignment using needle (EBI) pairwise alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

3.4.7.8. Submission of sequences

The sequences were annotated and submitted to Genbank through BankIt tool (https://www.ncbi.nlm.nih.gov/BankIt/) provided by NCBI.

Results

4. Results

The results of the study entitled "Molecular bases of acaricide resistance in *Tetranychus truncatus* (Prostigmata: Tetranychidae) Ehara infesting vegetable crops" are presented hereunder.

4.1 Field populations of *Tetranychus truncatus*

A total of 13 strains of spider mites collected from different vegetable fields of College of Horticulture, Vellanikkra campus and KVK, Thrissur were maintained as isoline cultures in the Acarology laboratory (Table 3). Morphological characterisation of the mite specimens from the different isoline cultures showed that three different species of *Tetranychus* viz., *Tetranychus okinawanus* Ehara, *Tetranychus truncatus* Ehara and *Tetranychus mafarlanei* Pitchard represented the different accessions (Plate 4.1, 4.2 and 4.3) (Annexure V).

The accessions from Vellanikkra *viz*. VkOk1 (okra : Arka Anamika), VkAm3 (amaranthus: local variety) and VkPm3 (Pumpkin:Ambili) identified as *Tetranychus truncatus* were used for further studies.

4.2 Status/level of acaricide resistance in *Tetranychus truncatus*

. Information gathered during the field survey on the use of acaricide on vegetable crops showed that spiromesifen 240 SC was the most commonly used acaricde against spider mite, followed by fenazaquin 10 EC and diafenthiuron 50 WP at recommended dosage.

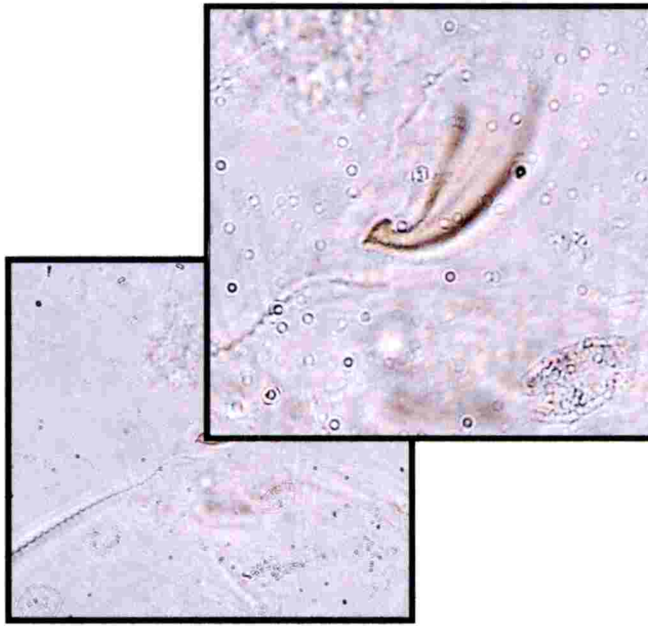

Plate 4.1. Male aedeagus of *Tetranychus truncatus*

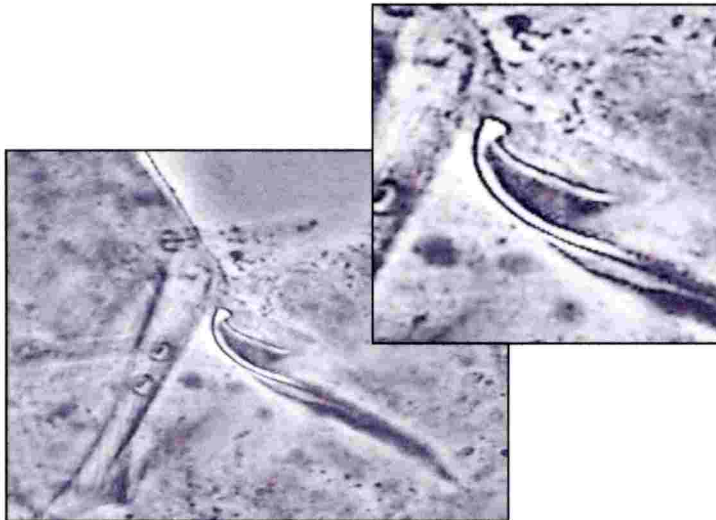

Plate 4.2. Male aedeagus of *Tetranychus macfarlanei*

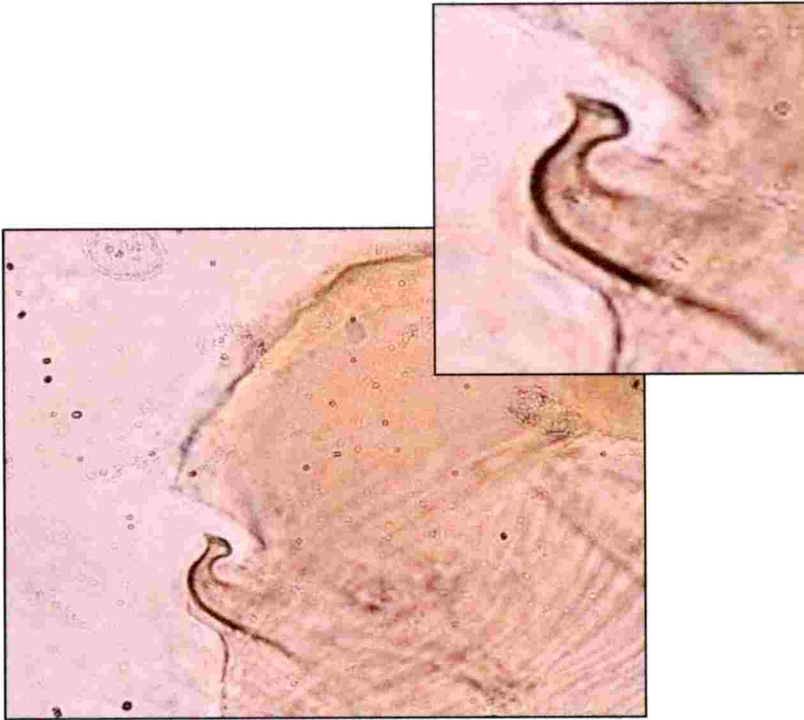

Plate 4.3. Male aedeagus of *Tetranychus okinawanus*

Table 3 Accessions of spider mites in isoline culture 58

Sl. No.	Date	Crop	locality	Accession No.	Species
1	1/04/17	Cow pea	Seed production plot, KAU	VkCp1	*T. okinawanus*
2	1/04/17	Cucumber	Seed production field, KAU	VkCu1	*T. okinawanus*
3	2/06/17	Brinjal	Olericulture field, KAU	VkBr1	*T. okinawanus*
4	8/07/17	Ash gourd	KVK, Thrissur	KvCr2	*T. okinawanus*
5	18/08/17	Brinjal	Seed production plot, KAU	VkBr2A	*T. macfarlanei*
6	26/08/17	Brinjal	Seed production plot, KAU	VkBr2B	*T. macfarlanei*
7	8/09/13	Cucumber	KVK, Thrissur	KCUC1	*T. okinawanus*
8	12/09/17	Cucumber	KVK, Thrissur	KCUC2	*T. okinawanus*
9	22/09/17	Cucumber	KVK, Thrissur	KCUC3	*T. okinawanus*
10	30/10/17	Okra	Experimental field, KAU	VkOk1	*T. truncatus*
11	6/12/17	Amaranthus green	Olericulture field, KAU	VkAm3	*T. truncatus*
12	6/12/17	Brinjal	Olericulture field, KAU	VkBr3	*T. macfarlanei*
13	22/01/18	pumpkin	Seed technology experimental plot, KAU	VkPm3	*T. truncatus*

4.2 Toxicological assay

Susceptibility of three field strains of *T. truncatus* namely okra (VkOk1), amaranthus (VkAm3) and pumpkin (VkPm3) to these currently used acaricides, *viz*. spiromesifen, fenazaquin and diafenthiuron was evaluated in the laboratory in comparison with a laboratory maintained strain (SS).

The LC_{50} value for the strains were calculated based on mortality with the help of Polo Plus software. The data are tabulated in Table 4 , 5 and 6.

4.2.1 Susceptibility of *Tetranychus truncatus* to spiromesifen

The results of the toxicity studies of spiromesifen to different field strains of *T. truncatus* showed that pumpkin strain recorded lowest LC_{50} value (302.743ppm) followed by amaranthus strain (1571.021ppm) and okra strain (1794.293ppm). However the susceptible strain recorded LC_{50} of only 224.48ppm.

On comparing the LC_{50} value of the laboratory susceptible strain, resistance ratio were worked out which showed that okra and amaranthus strains recorded significantly higher resistance of 8 and 7 fold respectively. The pumpkin strain recorded only 1.35 fold resistance ratio to spiromesifen (Table 4).

4.2.2 Susceptibility of *Tetranychus truncatus* to fenazaquin

The toxicity of fenazaquin to different strains evaluated in the laboratory is presented in Table 5. Among the different field strains, okra strain recorded the highest LC_{50} value (852.394 ppm) which was 13 fold higher compared to susceptible strain (65.548ppm). The amaranthus and pumpkin strains recorded LC_{50} values of 362.789 ppm and 73.604ppm, respectively. Amaranthus strain showed 5.53 fold resistance to fenazaquin

compared to laboratory susceptible strain, while pumpkin strain recorded a resistance ratio of 1.13 only.

4.2.3 Susceptibility of *Tetranychus truncatus* to diafenthiuron

Comparison of susceptibility of field collected strains to diafenthiuron showed that okra strain was least susceptible recording LC_{50} value of 1968.496ppm as against the LC50 of 197.459ppm in laboratory susceptible population. Amaranthus and pumpkin strains recorded LC_{50} values of 330.238ppm and 202.398ppm, respectively. Okra strain recorded a resistance ratio of 10 for diafenthiuron compared to 1.67 and 1.03 respectively, in VkAm3 and VkPm3 (Table 6).

Table 4. Relative toxicity of spiromesifen to field strains of *Tetranychus truncatus*

Sl.NO	Strain	Heterogeneity	Slope	LC_{50} (ppm)	Fiducial limit	Resistance ratio	LC_{90} (ppm)	Fiducial limit
1	SS	10.79	1.704	224.48	148.60-306.43	-	1268.992	818.44 - 2805.79
2	VkOk1	11.06	1.421	1794.293	1156.76-2596.81	8.00	14305.00	7924.60 - 46020.00
3	VkAm3	8.04	1.637	1571.021	1130.66-2076.98	7.00	9592.20	6272.70 - 18861.00
4	VkPm3	8.36	1.905	302.743	221.23-390.53	1.35	1424.56	998.27 - 671.37

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Table 5. Relative toxicity of fenazaquin to field strains of *Tetranychus truncatus*

Sl.NO	Strain	Heterogeneity	Slope	LC_{50}	Fiducial limit	Resistance ratio	LC_{90} (ppm)	Fiducial limit
1	SS	10.34	2.12	65.548	48.13-84.13	-	263.701	181.73 - 525.69
2	VkOk1	10.77	2.3	852.394	662.32-1090.22	13.00	3079.50	2127.60 - 5948.40
3	VkAm3	2.87	1.08	362.789	272.71-496.55	5.53	5570.80	2542.90 – 28177.00
4	VkPm3	20.07	2.00	73.604	54.45-95.30	1.13	322.229	216.14 - 671.37

Table 6. Relative toxicity of diafenthiuron to field strains of *Tetranychus truncatus*

Sl.NO	Strain	Heterogeneity	Slope	LC_{50}	Fiducial limit	Resistance ratio	LC_{90} (ppm)	Fiducial limit
1	SS	8.79	1.53	197.459	134.11-268.49	-	1364.38	848.68 – 3185.41
2	VkOk1	10.61	1.54	1968.496	1349.66-2767.84	9.97	13392.00	7839.50 - 36113.00
3	VkAm3	8.07	1.91	330.238	246.41-422.66	1.67	1543.97	1080.21 - 2748.53
4	VkPm3	9.17	1.70	202.398	142.40-269.86	1.03	1154.237	756.76 -671.37

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4.3 Biochemical basis of acaricide resistance

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The activity of the detoxifying enzymes *viz*., cytochrome P450 and carboxylesterase were determined in the field populations of okra (VkOk1), amaranthus (VkAm3) and pumpkin (VkPm3) accessions as well as in the susceptible population (SS).

4.3.1 Cytochrome P450

The activity of the enzyme, cytochrome P450 in three different field strains and the susceptible strain of *T. truncatus* is presented in Table 7. The activity of the enzyme is expressed in nmol of para nitrophenol $min^{-1}mg^{-1}$ of $protein^{-1}$.

The okra strain (VkOk1) showed significantly higher activity with 62.45 nmol of para nitrophenol $mg^{-1}min^{-1}$of protein followed by amaranthus strain (VkAm3) of 28.61 nmol. The pumpkin strain (VkPm3) showed an activity of 25.16 nmol, while the susceptible strain recorded the least activity of 23.16 nmol of para nitrophenol mg-1min-1of protein. The results indicated that the field collected *T. truncatus* strains of okra, amaranthus and pumpkin showed 2.7, 1.24 and 1.09 fold higher activity of cytochrome P450 respectively, compared to the laboratory maintained susceptible stain.

4.3.2 Carboxylesterase

The carboxylesterase activity in different strains of *T. truncatus* was determined following Van Asperen method (1961) and expessed in μMol of para α napthol min^{-1} mg^{-1} of protein (Table 8).

The field strain, VkOk1 showed greater activity of 80.102 μMol of para α napthol min^{-1} mg^{-1} of protein followed by VkAm3 strain (36.67 μMol of para α napthol min^{-1} mg^{-1} of protein) and VkPm3 strain (24.21μMol of para α napthol min^{-1} mg^{-1} of protein). The susceptible strain recorded an activity of 30.97 μMol of para α napthol min^{-1} mg^{-1} of protein. The activity

of carboxylesterase in different field strains when compared with the susceptible strain showed an increase in the enzyme activity in okra and amaranthus by 2.59 and 1.18 fold respectively. However, pumpkin strain recorded a decrease in activivity of carboxyleasterase compared to (0.78 fold) susceptible strain. (Standard curve: Annexure VI)

Table. 7. Activity of cytochrome P450 in susceptible and field strains of *Tetranychus truncatus*

Strain	**Activity(nMol of para nitro phenol min^{-1} mg^{-1} of protein)**	**Relative activity**
Susceptible strain	23.16	-
VkOk1	62.45	2.70
VkAm3	28.61	1.24
VkPm3	25.16	1.09

Table 8. Activity of carboxylesterase activity in susceptible and field strains of *Tetranychus truncatus*

Strain	**Activity (μMol of α napthol min^{-1} mg^{-1} of protein)**	**Relative activity**
Susceptible strain	30.97	-
VkOk1	80.102	2.59
VkAm3	36.67	1.18
VkPm3	24.21	0.78

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4.3.3 Total protein content

Total protein content in different samples ranged between 0.69mg/ml to 0.99mg/ml (Standard curve: Annexure II).

4.4 Molecular basis of acaricide resistance

In the present study, the genes encoding the detoxifying enzymes *viz.*, cytochrome P450 and carboxylesterase were analysed in two field strains of *T. truncatus* which showed comparatively higher activity of the enzymes, as well as one susceptible strain of.

4.4.1Quality of isolated DNA

Total genomic DNA was isolated from two field strains namely, VkOk1 and VkAm3 and one susceptible strain, SS maintained in the laboratory as isoline culture using the modified CTAB method. The purity of DNA was checked using nanodrop ND-1000 spectrophotometer. Nucleic acid showed absorption maximum at 260 nm whereas protein showed peak absorbance at 280nm.

The DNA concentration was obtained in the range of 78-566.2 ng/µL. The quantification and purity details of DNA based on nanodrop readings in ND 1000 software are presented in Fig. 4.1 and Table 9.

Table. 9: Quality and quantiy of DNA in different strains of *T. truncatus*

Si. No.	Accession	A260/280	A260/230	Quantity of DNA ng/µl
1	SS	1.97	1.15	109.7
2	SS	1.92	0.9	86.6
3	SS	1.85	1.00	96.1
4	SS	1.96	1.42	340.1
5	SS	1.8	0.96	78.0
6	SS	1.86	0.7	86.6
7	VkOk1	1.8	1.69	566.6
8	VkOk1	1.85	1.62	515.4
9	VkOk1	2.0	1.31	164.2
10	VkOk1	1.86	0.68	87.0
11	VkOk1	1.72	1.63	496.2
12	VkOk1	1.7	0.71	86.6
13	VkAm3	1.8	0.94	160
14	VkAm3	1.86	1.21	105.2
15	VkAm3	2.0	1.56	140
16	VkAm3	1.89	1.39	92

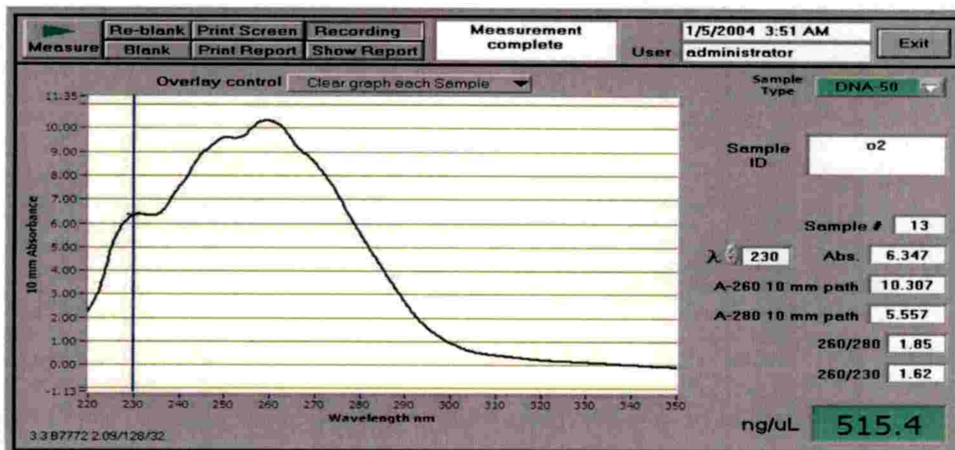

Figure 4.1. Quality and quantity of DNA in different strains of *T. truncatus* (Nanodrop software)

4.4.2 DNA amplification with Polymerase Chain Reaction (PCR)

With the help of gene specific primers for cytochrome P450 and carboxylesterase, (designed during the study), specific genes in the DNA were amplified for the resistant and susceptible strains of *T. truncatus*. After PCR amplification with gene specific primers, 20µl out of the product was used for agarose gel electrophoresis.

4.4.2.1 PCR product of cytochrome P450

PCR amplification of the gene cytochrome P450 showed that there was no amplification in the case of susceptible strain, whereas there were distinct markers in the case of strains, VkOk1 and VkAm3 1300 bp size. Gel picture for the amplification of cytochrome P450 is presented in Plate 4.4.

4.4.2.2 PCR product of carboxyl esterase

The PCR amplification showed distinct bands for carboxyl esterase in all the three strains. The strains VkOk1 and SS showed markers at 1500 bp and 1300bp size, respectively, while VkAm3 strain showed both markers (Plate 4.5)

4.4.3 Sequencing of PCR products

For sequencing cytochrome P450 amplicons, the PCR products of only VkOk1 and VkAm3 accessions were selected, as there was no amplification in the case of susceptible strain (SS). In the case of carboxylesterase gene, PCR products of SS and VkOk1 have been selected.

Sequences were obtained after the sequencing by Sanger dideoxy method carried out at AgriGenom Labs. Pvt. Ltd., Cochin and Eurofins genom India, Bangalore. The sequences of different accessions obtained after processing the sequences for cytochrome P450 and carboxylesterase are furnished in Annexure VII and VIII respectively in FASTA format.

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4.4.5. Alignment of sequences by using CAP 3

The forward and reverse sequences of carboxylesterase (okra, amaranthus and susceptible strain) and cytochrome P450 genes (okra and amaranthus strain) were merged with CAP3 sequence assembler to form contigs. Length of forward, reverse and contig sequences (in bp) obtained for the strains are presented in Table 10. Contigs were not formed in the case of okra and susceptible strains for carboxylesrterase gene. So in these cases, only forward sequences were used for sequence homology analysis.

Table 10. Length of forward, reverse and contig sequences

Sl.No.	Strain	Gene	Length of sequence		Contig length bp
			Forward bp	Reverse bp	
1.	VkAm3	Cytochrome P 450	913	871	1293
2	VkOk1	Cytochrome P 450	934	794	1184
3	SS	Carboxylesterase	960	1144	No contig
4	VkOK1	Carboxylesterase	993	980	No contig

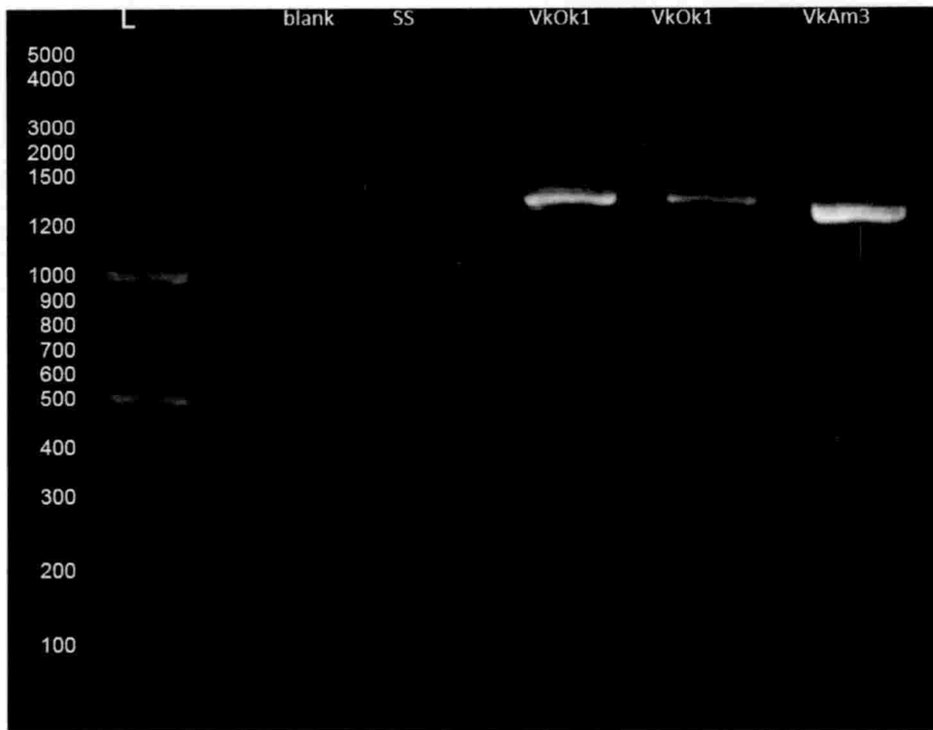

Plate. 4. 4. Gel documentation picture of PCR products of cytochrome P450 gene

L - ladder, Okra strain (VkOk1), Susceptible strain (SS) and Amaranthus strain (VkAm3)

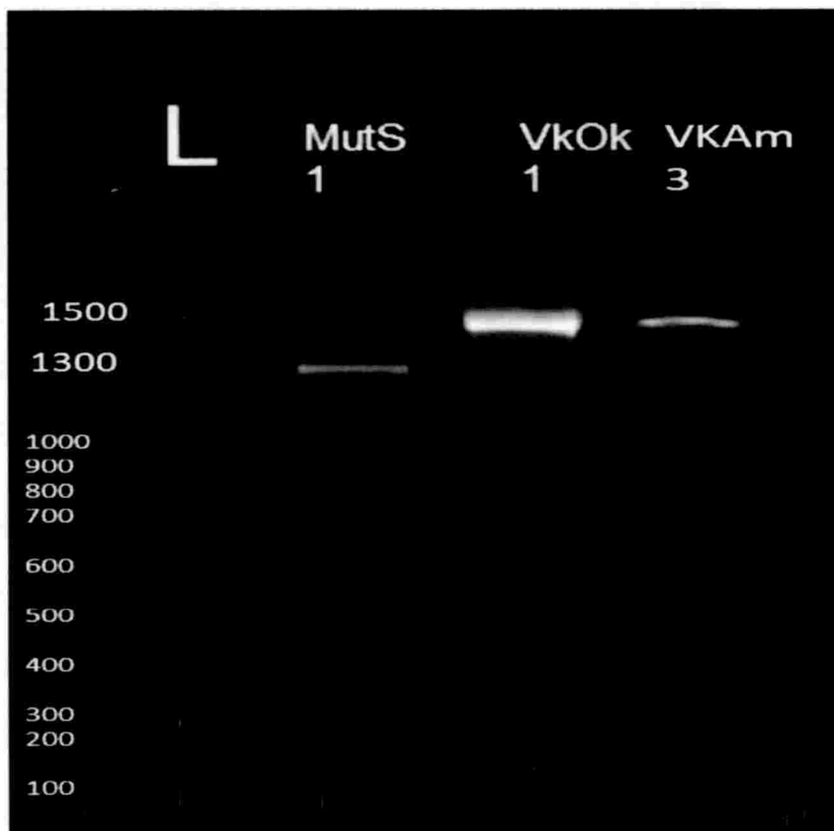

Plate. 4.5. Gel documentation picture of PCR products of carboxylesterase gene

L - ladder, Okra strain (VkOk1), Susceptible strain (SS) and Amaranthus strain (VkAm3)

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4.4.6 Insilco analysis of the sequences

Sequence homology was assessed using Mega BLASTn for all the retrieved sequences.

4.4.6.1 BLASTn analysis of cytochrome P450 sequence

The sequence of cytochrome P450 gene retrieved from the amplicon of VKAm3 and VkOk1 strain was used for homology search with the tool BLASTn. The results showed that the sequence of cytochrome P450 of *T. truncatus* has similarities with available cytochrome P450 sequences from different species of spider mites with an identity match ranging 82 to 97 per cent for VkAm3 strain and 81 to 96 per cent for VkOk1 strain. The highest similarity was found with an mRNA sequence of cytochrome P450 of *Tetranychus turkestani* (KX904518.1; KX904517.1). The next most similar sequence was that of *T. urticae* (NM 001323140.1). An mRNA sequence of cytochrome P450 gene from *Panonychus citri* strain showed similarity of 82 per cent (J69089.1). The results of BLASTn analysis are presented in Fig. 4.2 & 4.3 and Table. 11.

4.4.6.2 BLASTn analysis of carboxyl esterase sequence

BLASTn analysis of the sequences of SS and VkOk1 showed similarity with two mRNA sequences of carboxylesterase of two variants of *T. urticae* (XM015926774.2; XM 015926773.2). The sequences of susceptible strain (SS) and okra strain showed similarity of 96 per cent and 90 per cent, respectively. The BLAST analysed results are presented in Fig.4. 4, 4.5 and Table 12.

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4.4.5.2 Multiple sequence alignment

The sequences of okra and amaranthus strain for cytochrome P450 gene perfectly aligned with each other using Clustal Omega and having 99.3 per cent similarity with each other, proving that these are the same gene with 6 deletions in VkAm3 sequences and 1 deletion in VkOk1 sequences. There was one T to A transversion in VkOk1 sequence strand at position 273 bp. The data is furnished in Table 13 figure 4.6 and 4.7.

However, the sequences of carboxylesterase genes from the two accessions did not align together. Hence it can be concluded that there are two different genes controlling the resistance and susceptibility to acaricides in spider mites.

4.4.5.3 Open Reading Frames (ORF) gene

4.4.5.3.1. ORF for cytochrome P450

The sequences obtained after processing was used for identifying ORF using Expasy Translate. The results are furnished in Table 14.

4.4.5.3.2. ORF for carboxylesterase gene

Sequence analysis using MEGA 5 software identified a number of ORFs in the carboxylesterase genes of *T. truncatus*. The largest ORFs identified are furnished in Table 14.

4.4.6. Translation to amino acid

After translation of ORF, amino acid sequences were obtained for cytochrome P450 of 112 amino acid length in VkAm3 and 95 amino acid length in VkOk1. Sequence length of 48 amino acid and 55 amino acid were obtained respectively for caboxylesterase.

4.4.7 BLAST P Analysis

The BLASTP analysis showed that sequences of the VkAm3 and VkOk1 have similarity with cytochrome P450 amino acid sequences of different spider mites viz., *T. turkestani* (APG21196.1), and *T. urticae* (NP_001310069.1) at 99 per cent and 98 per cent respectively (Table 15; (Fig. 4.8 & 4.9)

The sequences of carboxylesterase amino acid obtained from SS and VkOk1 showed 93 per cent similarity with carboxylesterase amino acid sequences of *T. urticae* (XP_015782259.1; XP_015782260.1) (Table 16; Fig. 4.10 & 4.11).

4.4.8 Multiple sequence alignment of cytochrome P450 amino acid sequence

The sequences perfectly aligned together except one change in amino acid in position 5. (Fig. 4.12). The amino acid at position 5 is phenyl alnine in the case of VkOk1, while in VkAm3 it is tyrosine.

The amino acid sequence for carboxylesterase did not align with each other confirming they are different carboxylesterase amino acid.

Table 11. BLASTn results for homology of cytochrome P450 gene

Sl.No	Accession No.	Gene	Gene Bank sequence and Accession No.	Query coverage	E value	Identity %
1	VkAm3	Cytochrome P450 *T. truncatus* variant A2 partial CDS	*Tetranychus turkestani* cytochrome P450 D2 mRNA, partial cds KX904518.1	28	8e-172	97
			Tetranychus urticae cytochrome P450 4C1-like (LOC107365376), Mrna NM 001323140.1	55	2e-178	97
			Tetranychus turkestani cytochrome P450 D1 mRNA, partial cds KX904517.1	16	9e-92	97
			Panonychus citri cytochrome P450 monooxygenase cyp4 (P450) gene 1, complete cds J69089.1	26	2e-74	82
2	VkOk1	Cytochrome P450 *T. truncatus* variant A1 partial CDS	*Tetranychus turkestani* cytochrome P450 D2 mRNA, partial cds KX904518.1	30	2e-142	96
			Tetranychus urticae cytochrome P450 4C1-like (LOC107365376), Mrna NM 001323140.1	50	1e-140	96
			Tetranychus turkestani cytochrome P450 D1 mRNA, partial cds KX904517.1	13	5e-64	96
			Panonychus citri cytochrome P450 monooxygenase cyp4 (P450) gene 1, complete cds J69089.1	26	7e-63	81

Table. 12 BLASTn result for homology of carboxylesterase gene

Sl.No	Accession No.	Gene	GenBank sequence name and Accession No.	Query coverage (%)	E value	Identity (%)
1	SS	Carboxylesterase gene AB 2	*Tetranychus urticae* carboxylesterase 4A (LOC107360148), transcript variant X2, mRNA XM015926774.2	27	2e-62	96
			Tetranychus urticae carboxylesterase 4A (LOC107360148), transcript variant X1, Mrna XM 015926773.2	27	2e-62	96
2	VkOk1	Carboxylesterase gene AB 1	*Tetranychus urticae* carboxylesterase 4A (LOC107360148), transcript variant X2, mRNA XM015926774.2	60	1e-59	90
			Tetranychus urticae carboxylesterase 4A (LOC107360148), transcript variant X1, mRNA XM 015926773.2	60	1e-59	90

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Table. 13 Positions of deletion and SNPs in cytochrome P450 gene sequence

Strain	**Gene name**	**Type of variation**	**Position**
VkAm3	Cytochrome P450 *T. truncatus* variant A2 partial CDS	Deletion	363 bp
			435bp
			529bp
			683bp
			708bp
			709bp
VkOk1	Cytochrome P450 *T. truncatus* variant A1 partial CDS	Deletion	451bp
		Transversion	273bp

Table. 14. ORFs and their positions on cytochrome P450 and carboxylesterase genes of *Tetranychus truncatus*

Strain	Gene	Size of the sequence (bp)	Number of ORFs	Strand	Frame	Nucleotide position	
						Start	End
VkAm3	**Cytochrome P450 variant A2** *T. truncatus* partial CDS	1293	1	-	3	60	440
VkOk1	**Cytochrome P450 variant A1** *T. truncatus* partial CDS	1184	1	-	3	60	403
SS	**Carboxylesterase gene AB 2** (Carboxylesterase gene *Tetranychus trancatus* variant ab2 partial genomic DNA)	960	2	+	2	400	583
				+	2	694	889
VkOk1	**Carboxylesterase gene AB 1** (Carboxylesterase gene *Tetranychus trancatus* variant ab1 partial genomic DNA)	993	3	+	2	122	353
				+	2	380	596
				+	2	698	866

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Table 15. BLAST P result for Cytochrome P450 amino acid sequence

Strain	Amino acid	Length of amino acid	Query coverage	Identity coverage	E value	Sequence
VkAm3	*T. truncatus* CyP450 variant A2	112	100	99	2e-75	Cytochrome P450 D2, partial [*Tetranychus turkestani*] APG 21196.1
			100	99	4e-73	Cytochrome P450 4C1-like [*Tetranychus urticae*] NP_001310069.1
VkOk1	*T. truncatus* CyP450 variant A1	95	100	98	4e-61	Cytochrome P450 D2, partial [*Tetranychus turkestani*] APG 21196.1
			100	98	5e-59	Cytochrome P450 4C1-like [*Tetranychus urticae*] NP_001310069.1

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Table 16. BLAST P result for carboxylesterase amino acid sequence

Strain	Amino acid	Length of amino acid	Query coverage	Identity coverage	E value	Sequence
SS	Carboxylesterase *T. truncatus* variant A2	48	91	93	5e-19	Carboxylesterase 4A isoform X2 [*Tetranychus urticae*] XP_015782259.1
			91	93	7e-19	Carboxylesterase 4A isoform X1 [*Tetranychus urticae*] XP_015782260.1
VkOk1	Carboxylesterase *T. truncatus* variant A1	55	78	93	8e-18	Carboxylesterase 4A isoform X2 [*Tetranychus urticae*] XP_015782259.1
			78	93	8e-18	carboxylesterase 4A isoform X1 [*Tetranychus urticae*] XP_015782260.1

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4.4.5.4 Submission of sequences to NCBI GenBank

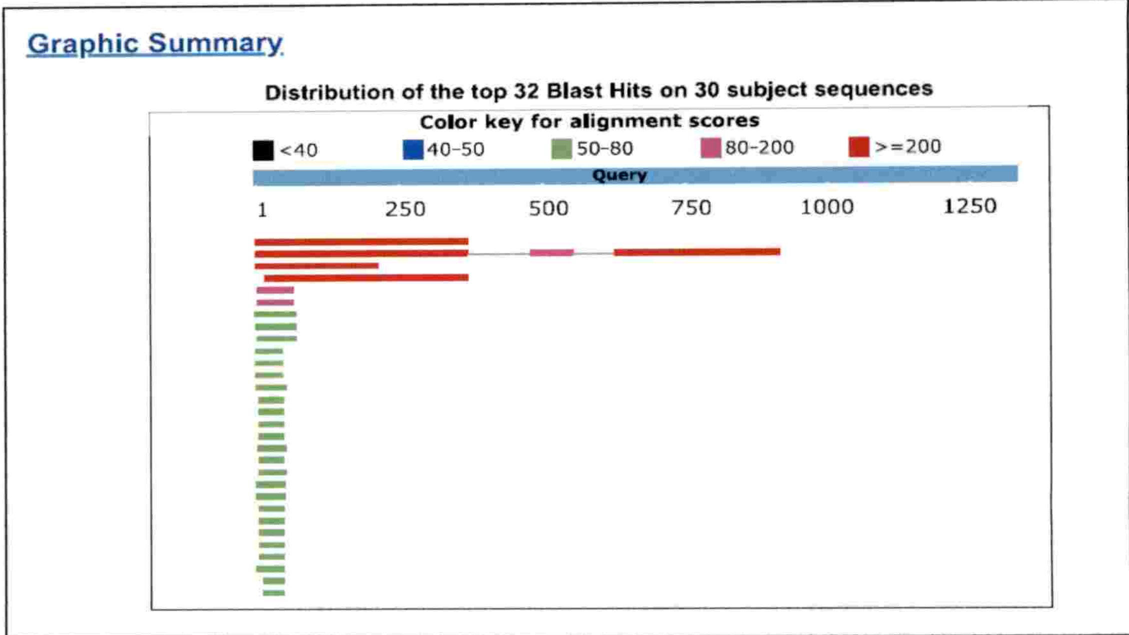
Two sequences of carboxylesterase gene (okra and susceptible strain) were submitted to GenBank through the submission tool Bankit and the accession numbers were obtained which are presented in Table. 17 and 18.

Table 17. GenBank Accession number of cytochrome P450 gene of *T. truncatus*

Sl.No.	Strain	Gene name	GenBank Accession No.
1	VkAm3	Cytochrome P450 variant A2 *T. truncatus* partial CDS	MH665975
2	VkOk1	Cytochrome P450 variant A1 *T. truncatus* partial CDS	MH665974

Table 18. GenBank Accession number of carboxylesterase gene of *T. truncatus*

Sl.No.	Strain	Gene name	GenBank Accession No.
1	SS	**Carboxylesterase gene AB 2** (Carboxylesterase gene *Tetranychus trancatus* variant ab2 partial genomic DNA)	MH603568
2	VkOk1	**Carboxylesterase gene AB 1** (Carboxylesterase gene *Tetranychus trancatus* variant ab2 partial genomic DNA)	MH603567

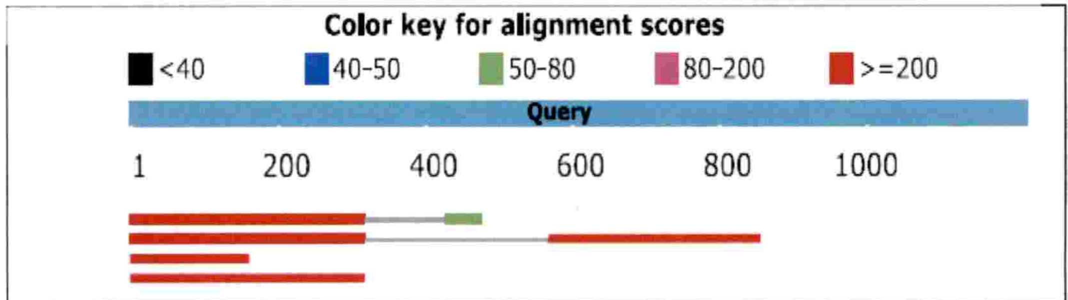


Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus turkestani cytochrome P450 D2 mRNA, partial cds	616	616	28%	8e-172	97%	KX904518.1
Tetranychus urticae cytochrome P450 4C1-like (LOC107365376), mRNA	604	1172	55%	2e-168	97%	NM_001323140.1
Tetranychus turkestani cytochrome P450 D1 mRNA, partial cds	350	350	16%	9e-92	97%	KX904517.1
Panonychus citri cytochrome P450 monooxygenase cyp4 (P450) gene 1, complete cds	292	292	26%	2e-74	82%	JQ690089.1

Figure 4.2. BLASTn result for the sequence of amaranthus (VkAm3) strain of *T. truncatus* (cytochrome P450 gene)



Descriptions

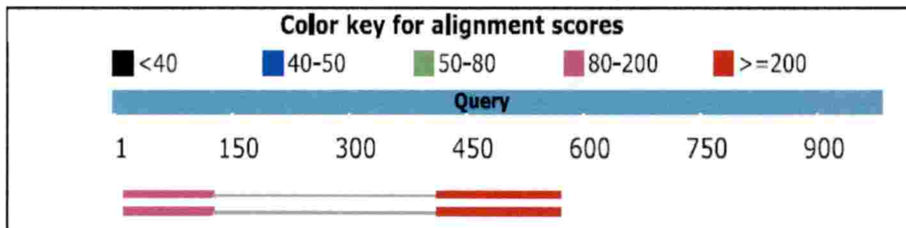
Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus turkestani cytochrome P450 D2 mRNA, partial cds	518	589	30%	2e-142	96%	KX904518.1
Tetranychus urticae cytochrome P450 4C1-like (LOC107365376), mRNA	512	955	50%	1e-140	96%	NM_001323140.1
Tetranychus turkestani cytochrome P450 D1 mRNA, partial cds	257	257	13%	5e-64	96%	KX904517.1
Panonychus citri cytochrome P450 monooxygenase cyp4 (P450) gene 1, complete cds	254	254	26%	7e-63	81%	JQ690089.1

Figure 4.3. BLASTn result for the sequence of okra (VkOk1) strain of *T. truncatus* (cytochrome P450 gene)

Graphic Summary

Distribution of the top 4 Blast Hits on 2 subject sequences



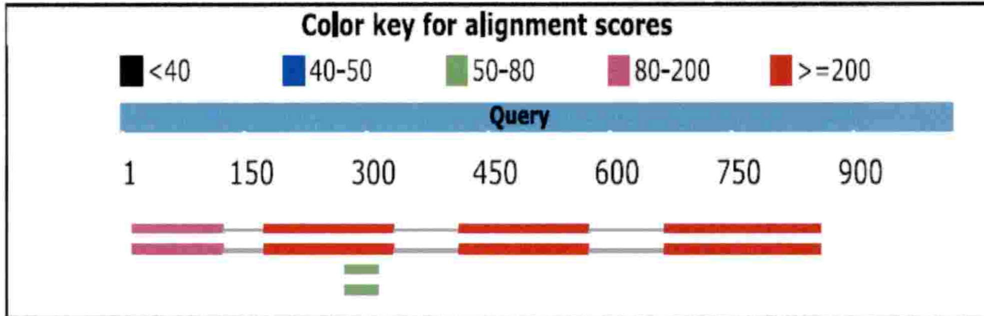
Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Tetranychus urticae carboxylesterase 4A (LOC107360148), transcript variant X2, mRNA	252	386	27%	2e-62	96%	XM_015926774.2
PREDICTED: Tetranychus urticae carboxylesterase 4A (LOC107360148), transcript variant X1, mRNA	252	386	27%	2e-62	96%	XM_015926773.2

Figure 4.4. BLASTn result for the sequence of susceptible (SS) strain of *T. truncatus* (carboxylesterase gene)

Graphic Summary



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Tetranychus urticae carboxylesterase 4A (LOC107360148), transcript variant X2, mRNA	243	896	60%	1e-59	90%	XM_015926774.2
PREDICTED: Tetranychus urticae carboxylesterase 4A (LOC107360148), transcript variant X1, mRNA	243	896	60%	1e-59	90%	XM_015926773.2
PREDICTED: Apis dorsata esterase FE4-like (LOC102674083), transcript variant X2, mRNA	62.1	62.1	3%	4e-05	97%	XM_006619019.1
PREDICTED: Apis dorsata esterase FE4-like (LOC102674083), transcript variant X1, mRNA	62.1	62.1	3%	4e-05	97%	XM_006619018.1

Figure 4.5. BLASTn result for the sequence of VkOk1 strain of *T. truncatus* (carboxylesterase gene)

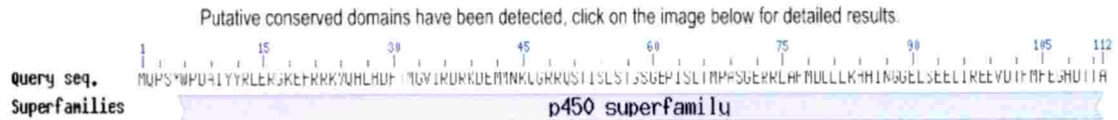
```
Okra_450_tetr      301 ACATTCTCCGACTCTGAAAAATTAATTTAAAGGTAAAATTAGAGAATGAT   350
                       ||||||||||||||||||||| ||||||||||||||||||||||||||||
amaranthus_co      353 ACATTCTCCGACTCTGAAAA-TTAATTTAAAGGTAAAATTAGAGAATGAT   401

Okra_450_tetr      351 TAATTGTTTTGAGAAAACTTAGGTTACTCTGAAACATTCCGTATCATTTG   400
                       ||||||||||||||||||||||||||||||||| ||||||||||||||||
amaranthus_co      402 TAATTGTTTTGAGAAAACTTAGGTTACTCTGAA-CATTCCGTATCATTTG   450

Okra_450_tetr      401 ATGTAATTTATACTTACTCATAGACAGCTCGACAATAGCTATTGTTAGGG   450
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
amaranthus_co      451 ATGTAATTTATACTTACTCATAGACAGCTCGACAATAGCTATTGTTAGGG   500

Okra_450_tetr      451 -TTGTTTTGAGCACCAATCGATACTCCCCATTATGGTTTCTAAAATTTAG   499
                        ||||||||||||||||||||||||||| ||||||||||||||||||||||
amaranthus_co      501 GTTGTTTTGAGCACCAATCGATACTCCC-ATTATGGTTTCTAAAATTTAG  549


Okra_450_tetr      600 CGATTGCTTCACCAGATCTTGCAATTTCTCTTAATTTTTGAAACCATAAT   649
                       ||||||||||||||||||||||||||||||||||||||| ||||||||||
amaranthus_co      650 CGATTGCTTCACCAGATCTTGCAATTTCTCTTAATTTT-GAAACCATAAT   698

Okra_450_tetr      650 GCTACCTTGGTTTCATTGAAAACAGGCACAAAATCGTGAAGAATCTTGAA   699
                       |||||||||  |||||||||||||||||||||||||||||||||||||||
amaranthus_co      699 GCTACCTTG  TTCATTGAAAACAGGCACAAAATCGTGAAGAATCTTGAA   746
```

Figure.4.6 Positions of deletions in cytochrome P450 gene sequences

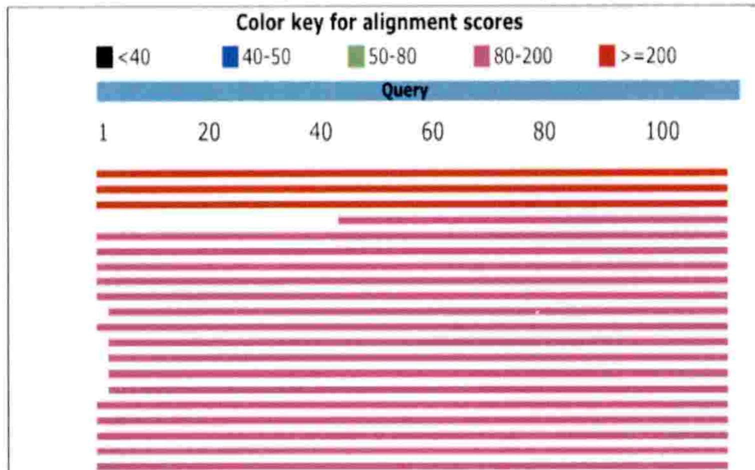
```
Okra_450_tetr    251 TAGTAAATTGCATCTGGCCAAAAACTAGGTTGCATCAATCTTTCAAGGAA    300
                     ||||||    |  |||||||| . ||     ||||||||||||| | | |
Amaranthus_co    333 TAGTAAATTGCATCTGGCCAATAACTAGGTTGCATCAATCTTTCAAGGAA    382
```

Figure 4.7. Positions of SNPs in cytochrome P450 gene sequence

Graphic Summary



Distribution of the top 107 Blast Hits on 100 subject sequences



Descriptions

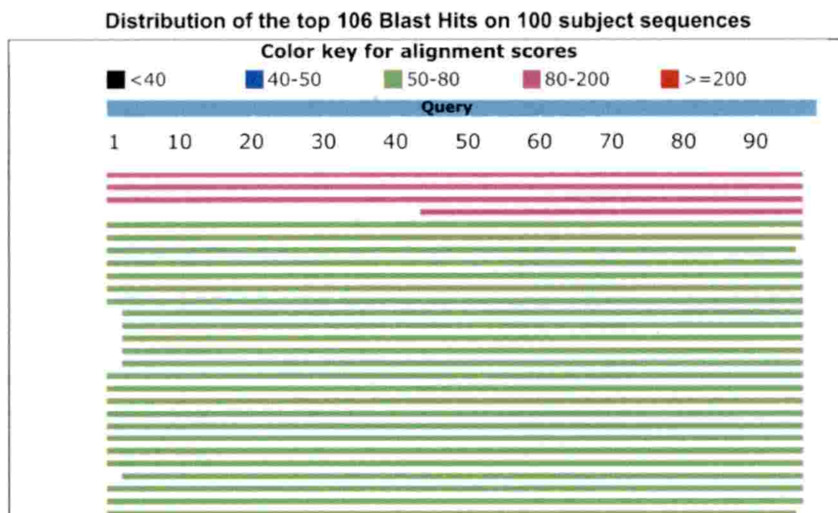
Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
cytochrome P450 D2 [Tetranychus turkestani]	234	234	100%	2e-75	99%	APG21196.1
cytochrome P450 4C1-like [Tetranychus urticae]	236	236	100%	4e-73	99%	NP_001310069.1
cytochrome P450 monooxygenase [Panonychus citri]	223	223	100%	6e-68	96%	AFJ05092.1
cytochrome P450 D1 [Tetranychus turkestani]	140	140	61%	2e-39	99%	APG21195.1
PREDICTED: cytochrome P450 4c3-like [Rhagoletis zephyria]	116	179	100%	9e-28	56%	XP_017488151.1
cytochrome P450-like protein [Euroglyphus maynei]	109	109	100%	4e-26	50%	OTF75649.1
cytochrome P450 4c3-like isoform X3 [Varroa destructor]	98.6	98.6	100%	1e-21	46%	XP_022651607.1

Figure. 4.8 BLAST P result for the sequence of VkAm3 strain of *T. truncatus* (cytochrome P450)

Graphic Summary

No putative conserved domains have been detected



Descriptions

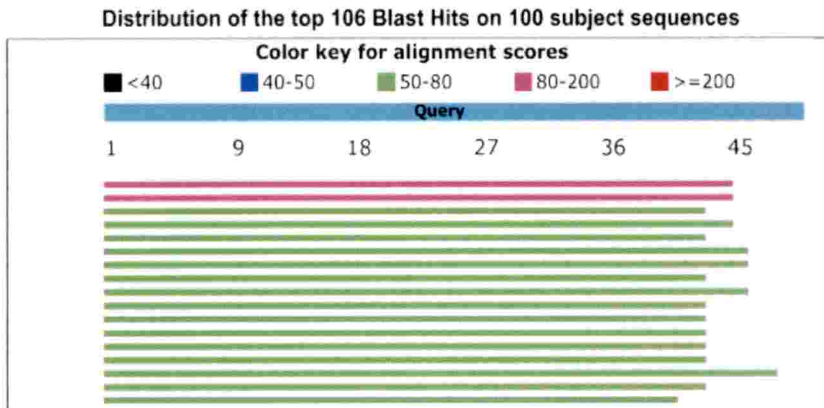
Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
cytochrome P450 D2 [Tetranychus turkestani]	197	197	100%	4e-61	98%	APG21196.1
cytochrome P450 4C1-like [Tetranychus urticae]	198	198	100%	5e-59	98%	NP_001310069.1
cytochrome P450 monooxygenase [Panonychus citri]	189	189	100%	4e-55	95%	AFJ05092.1
cytochrome P450 D1 [Tetranychus turkestani]	105	105	54%	5e-26	98%	APG21195.1

Figure. 4.9 BLAST P result for the sequence of VkAm3 strain of *T. truncatus* (cytochrome P450)

Graphic Summary

Putative conserved domains have been detected, click on the image below for detailed results.



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
carboxylesterase 4A isoform X1 [Tetranychus urticae]	87.8	87.8	91%	5e-19	93%	XP_015782259.1
carboxylesterase 4A isoform X2 [Tetranychus urticae]	87.4	87.4	91%	7e-19	93%	XP_015782260.1
acetylcholinesterase-like [Parasteatoda tepidariorum]	63.2	63.2	87%	2e-10	60%	XP_015917339.2
Esterase FE4 [Stegodyphus mimosarum]	60.5	60.5	91%	1e-09	57%	KFM78571.1
esterase E4 [Parasteatoda tepidariorum]	60.5	60.5	87%	2e-09	60%	XP_021002433.1
esterase E4 isoform X1 [Tetranychus urticae]	58.2	58.2	93%	1e-08	51%	XP_015790984.1
venom carboxylesterase-6 isoform X2 [Tetranychus urticae]	58.2	58.2	93%	1e-08	51%	XP_025017769.1

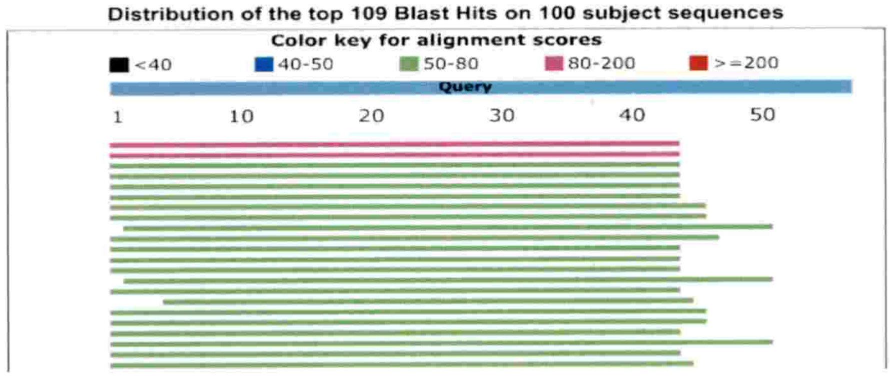
Figure. 4.10 BLAST P result for the sequence of SS strain of *T. truncatus* (carboxylesterase)

Graphic Summary

Putative conserved domains have been detected, click on the image below for detailed results.

Query seq. M V F I G H G T F E T G D S S L Y S P Q K I M D R G I V _ V T F N H R I G L V S N L S L Q K L L R G _ R I S S

Superfamilies: Abhydrolase superfamily



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
carboxylesterase 4A isoform X2 [Tetranychus urticae]	84.7	84.7	78%	8e-18	93%	XP_015782260.1
carboxylesterase 4A isoform X1 [Tetranychus urticae]	84.7	84.7	78%	8e-18	93%	XP_015782259.1
acetylcholinesterase-like [Parasteatoda tepidariorum]	62.4	62.4	78%	6e-10	60%	XP_015917339.2
Esterase FE4 [Stegodyphus mimosarum]	61.2	61.2	78%	7e-10	58%	KFM78571.1
esterase E4 [Parasteatoda tepidariorum]	62.0	62.0	78%	8e-10	60%	XP_021002433.1
esterase-like protein 3 [Sarcoptes scabiei]	58.9	58.9	78%	1e-08	53%	KPM06717.1
venom carboxylesterase-6 isoform X2 [Tetranychus urticae]	58.5	58.5	81%	1e-08	51%	XP_025017769.1

Figure. 4.11 BLAST P result for the sequence of VkOk1 strain of *T. truncatus* (carboxylesterase)

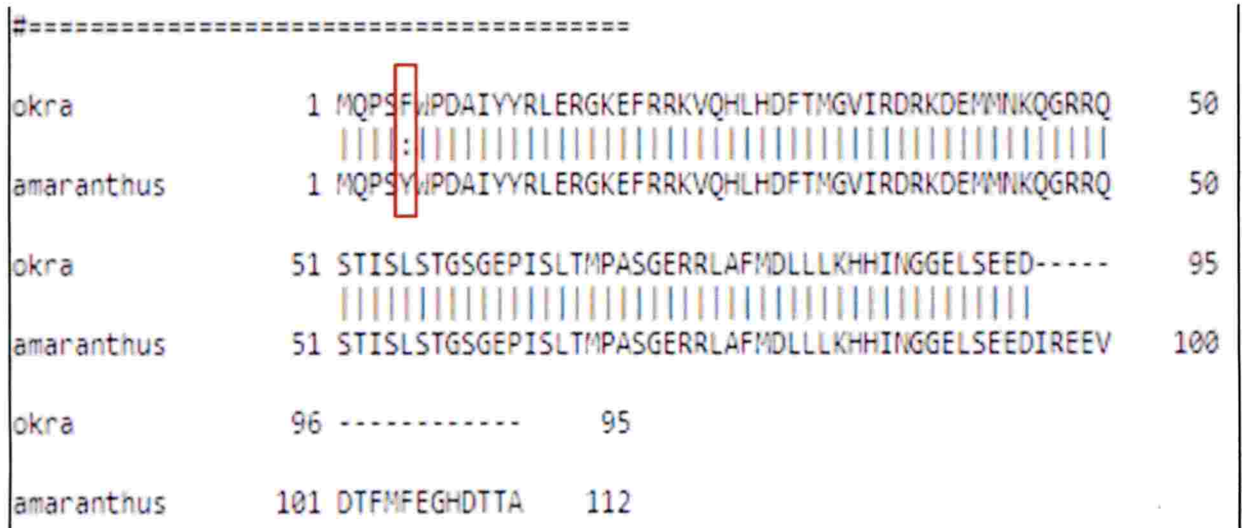


Figure 4. 12. Pair wise alignment of amino acid (Cytochrome P 450) sequences of *T. truncatus* strains

Discussion

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5. Discussion

The results of present study "Molecular basis of acaricide resistance in *Tetranychus truncatus* (Prostigmata: Tetranychidae) Ehara infesting vegetable crops"are discussed in the light of available literature.

5.1 Evaluation of the status/level of acaricide resistance in *T. truncatus*

The laboratory bioassay revealed that the susceptibility of different field strains of *T.truncatus* varied for different acaricides evaluated viz., spiromesifen, fenazaquin and diafenthiuron (Fig. 5.1). The field populations were found to have developed resistance to the three acaricides belonging to three different chemical classes of pesticides which are commonly in use for mite management. However, the level of resistance varied among the strains. The mode of action of these acaricides differ from one another. Spiromesifen, a tetraonic acid derivative is a lipid biosynthesis inhibitor that acts on acetyl coA carboxylase, a key enzyme in fatty acid biosynthesis. Fenazaquin is an acaricide which belongs to quinazoline class of chemicals which inhibits mitochondrial electron transport (MET) at complex 1. Diafenthiuron is a thiourea derivative which is basically a nerve poison, and also a potent inhibitor of mitochondrial ATP synthesis (Marcic *et al.*, 2011). Inefficiency of acaricides in control of spider mites caused by resistance development were reported for compounds, viz., organophosphates, dicofol, organotins, hexythiazox, clofentezine and abamectin after few years of their introduction (Cranham and Helle 1985, Herron *et al.*, 1993). Recently, Ullah and Gotoh (2013) reported development of resistance in *T. truncatus* to two acaricides, spirodiclofen and fenpyroximate from Bangladesh. Spirodiclofen is a lipid biosynthesis inhibitor, of the same IRAC MoA class as spromesifen, while fenpyroximate belong to the class of fenazaquin, an inhibitor of MET.

Though development of resistance in *T. truncatus* to acaricides has not been reported earlier from India, there are a number of studies on resistance to pesticides in the two spotted spider mite, *T. urticae* (AINPAA, 2009; Aji *et al.*, 2007; Kumari, *et al.*, 2015; Sharma and Bhullar, 2018). *T. urticae* is notorious for its ability to rapid development resistance to acaricides (Van Leeuwen *et al.*, 2008). Selection for resistance is accelerated by its high fecundity, inbreeding, arrhenotokous reproduction and very short life cycle resulting in many generations per year, especially in warmer conditions (Van Leeuwen *et al.*, 2009). A large number of synthetic pesticides with unique modes of action have been used for many years to control *T. urticae* and consequently this species is currently considered as 'most resistant' in term of the total number of pesticides to which it has developed resistance (Van Leeuwen *et al.*, 2010).

In the present study, VkOk1 and VkAm3 strains of *T. truncatus* recorded 8 and 7 fold resistance to spiromesifen respectively. Resistance to the novel acaricide, spiromesifen has been reported in *T. urticae* from different parts of the world including India. Laboratory bioassays conducted in Kangra, Himachal Pradesh showed 32.13 fold resistance to spiromesifen in laboratory reared *T. urticae* population (Kumari *et al.*, 2015). Development of resistance in four different field populations of *T. urticae* on brinjal has been reported recently from Punjab. The resistance ratio in different populations ranged from 11.14 to 21.40 (Sharma and Bhullar, 2018). Sato *et al.* (2016) reported spiromesifen resistance in populations of *T. urticae* collected from different crops at various locations in Brazil, where spiromsifen was commonly used for the management of mites and whiteflies on beans, cotton, melon, soybean, and tomatoes. Spiromesifen resistance in *T. urticae* was also reported from Jordan on cucumber which showed 17.96-fold resistance (Mohamed *et al.*, 2012).

VkOk1 and VkAm3 strains also recorded significantly higher level of resistance against the acaricide fenazaquin, which was 13 and 5.53 fold higher compared to the susceptible strain. Low to moderate level of resistance to fenazaquin was reported earlier from Bangalore district (5 -32 fold) and Kolar district (8-25 fold) of Karnataka in *T. urticae* on tomato crop (AINPAA, 2009). Sharma and Bhullar (2018) evaluated the status of acaricidal resistance in *T. urticae* collected from vegetable fields of four districts of Punjab. In their study, Patiala population recorded 24.65 fold resistance to fenazaquin, while populations collected from Malerkotla, Hoshiarpur and Amritsar exhibited 17.12, 15.18 and 6.67 fold resistance, respectively. A Belgian field strain of *T. urticae* was reported to have developed 35 fold resistance against fenazaquin (Van Pottelberge *et al.*, 2009).

In this study, VkOk1 strain was found to have developed 10 fold resistance to diafenthiuron. However, VkAm3 and VkPm3 did not show resistance to the compound. Resistance level of 79 fold was reported in a strain of *T. urticae* on tomato crop to diafenthiuron from Kolar district of Karnataka (Aji *et al.*, 2007; AINPAA, 2009).

5.2 Biochemical basis of acaricide resistance

The change in the activity of metabolic enzymes was considered to be one of the most important biochemical mechanisms of resistance development in Tetranychidae (Stumpf *et al.*, 2001; Yang *et al.*, 2002; Van Leeuwen and Tirry, 2007). Resistance mechanisms involve enhanced detoxification of acaricides through the enzymatic activity of esterases, glutathione-S-transferases and P450 monooxygenases, as well as modification of the acaricide target site (Van Leeuwen *et al.*, 2010). In the present study, to elucidate the biochemical bases of

resistance in *T. truncatus* to different acaricides, the activity of two major detoxifying enzymes *viz.*, cytochrome P450 and carboxylesterase were determined in the field populations and compared with those of the susceptible strain maintained in the laboratory without exposure to any pesticides for nearly 150 generations.

The results of the enzyme assay showed a 2.7 fold and 2.59 fold increase in activities of the enzymes, cytochrome P450 and carboxylesterase, respectively in VkOk1 (okra strain) compared to the susceptible strain. Toxicological bioassay with okra strain had also showed significantly higher level of resistance against all the three acaricides tested *viz.*, spiromesifen, fenazaquin and diafenthiuron (Table 19). This indicates the role of the enzymes, cytochrome P450 and carboxylesterase in detoxification and development of resistance in *T. tuncatus* to these acaricides.

Carboxylesterases is reported to efficiently catalyze the hydrolysis of esters and classified as the serine hydrolase superfamily. They are involved in the detoxification or metabolic activation of various drugs, environmental toxins, and carcinogens, and play an important physiological role in lipid metabolism (Ran *et al.*, 2009). In the present study, the activity of carboxylesterase was found to be higher in the resistant strains. Spiromesifen is a lipid biosynthesis inhibitor. As carboxylesterase play a significant role in lipid metabolism, the enhanced activity of the enzyme in resistant strains might have interrupted the inhibition of lipid biosynthesis by spiromesifen.

The MFO, cytochrome P450 is found in mitochondrion and inherited maternally. Cytochrome P450 was reported to be linked with resistance to mitochondrial electron transport chain inhibitor (METI) compounds resistance (Kim *et al.*, 2004, 2006; Van Pottelberge *et al.*, 2009). In the present study, okra(VkOk1) and amaranthus (VkAm3) strains which were found resistant to fenazaquin, an METI compound, recorded enhanced activity of cytochrome

P450 confirming the role of the enzyme in development of resistance to METI compound.

Khajehali *et al.*, (2011) evaluated the activity of enzymes viz, esterases, glutathione-S-transferases (GSTs) and cytochrome P450 monooxygenases (MFOs) in nine resistant field strains of *T. urticae* on rose which were selected based on toxicological assay with acaricides bifenazate, spiromesifen, etoxazole and acequinocyl. They found that P450 monooxygenase activity in the field strains increased 1.33-fold to 7.29-fold compared with the susceptible strain. Also, significant variation in esterase activity was found in five out of nine strains, while increased GST activity was found in six out of nine strains. Similarly, studies on susceptibility and carboxylesterase activity of five field populations of *Panonychus citri* (Mcgregor) (Acari: Tetranychidae) to four acaricides in southwestern China clearly indicated the role of carboxylesterase in lower sensitivity to diafenthiuron (Ran *et al.*, 2009).

Tirello *et al.* (2012) reported 2.66 and 1.95-fold increased activity of MFOs in METI resistant strains of *T. urticae* collected from rose in green houses of Italy compared to the susceptible strain. The resistant strains also reported 1.30 and 1.69 folds increase in activity of carboxylesterase. Stumpf and Nauen (2001) also have reported increased activity of cytochrome P450 in METI compound resistant strains of *T urticae*. The METI resistant strains collected from ornamental plants and hop garden near Worcester, England, showed 2.4 and 1.7 fold enhanced cytochrome P450 activity. Stumpf and Nauen (2002) also reported elevated levels of P450 and glutathione S transferase activity to abamectin resistance in *T. urticae* strains collected in the Netherlands, Brazil and Colombia.

Riga *et al*. (2014) demonstrated the functional link between cytochrome P450-mediated metabolism and abamectin resistance in *T. urticae*. They recombinantly expressed three cytochrome P450s (CYP392A16, CYP392D8 and CYP392D10) which were associated with high levels of abamectin resistance in a resistant *T. urticae* strain collected from Greece. CYP392A16 was expressed predominately in its P450 form.

The amaranthus strain (VkAm3) showed 1.21 and 1.18 fold higher activity of the enzymes, cytochrome P450 and carboxylesterase, respectively which also recorded significantly higher level of resistance to spiromesifen and fenazaquin. Though development of resistance was recorded in amaranthus strain in the laboratory bioassay, the increase in the activity of the enzymes was not as high as in okra strain. There are also other enzymes which take part in detoxification mechanisms such as GST (khajihali *et al*., 2011) and acetyl choline esterase (Ghadamyari and Sendi, 2008) which were not determined in the present study.

The pumpkin strain (VkPm3) recorded 1.09 fold enhanced activity of cytochrome P450, while the activity of carboxylesterase was found to have decreased by 0.78 fold compared to the susceptible strain. Toxicological studies also did not record significant level of resistance in pumpkin strain to any of the acaricides evaluated.

In the present study, the different field strains of *T. truncatus* showed varied level of activity of detoxifying enzymes as well as varied level of resistance to the three different insecticides evaluated. Though the different strains were collected from different host plants, all of them were collected from the same locality where, pesticide use pattern was similar against both insect and

100

mite pests. Hence it was expected that *T. truncatus* population in the locality would have developed same level of resistance to the commonly used acaricides. However, the population collected from different host plants varied in their level of resistance to spiromesifen, fenazaquin and diafenthiuron in comparison to the laboratory reared susceptible strain. This clearly indicates the role of host plants on susceptibility to acaricides in the spider mite.

Host plants can modify the susceptibility of herbivorous arthropods to pesticides (Yu, 1986; Brattsten, 1988). This has been well documented in the case of insect pests. For example, the populations of *Aphis gossypii* from cotton and melon plants showed different susceptibilities to pirimicarb (Furk *et al.*, 1980). Similarly, the susceptibility of the aphid, *Myzus persicae* to organophosphate insecticides was reported to be greatly influenced by the host plants (Ambrose and Regupathy, 1992). Larvae fed on climbing nightshade showed the highest resistance factor for fenvalerate, but when fed on other host plants, *viz*., potato, tomato and buffalo bur, increased the LD_{50} showing significant difference in susceptibility to fenvalerate (Mahdavi *et al.,* 1991).

Studies on influence of host plant on the susceptibility of acaricides to spider mites are few. Neiswander *et al*., (1950) reported that populations of two spotted spider mite responded differently to the same acaricide after feeding on different host plants. A population feeding on rose was more resistant to acaricides than the one on beans, and a population on beans was more resistant than the one on tomato.

Yang *et al*., (2001) studied the influence of host plant on the susceptibility of two spotted spider mite, *T. urticae* to pesticides. Synthetic pyrethroids were more toxic to mites reared on cucumber than mites reared on lima bean and maize. Susceptibility of mites on lima bean and cucumber did not change from 24 h to 7 days, but mites reared on maize developed more

susceptiblility to bifenthrin and l - cyhalothrin after 7days of exposure than after 24 h. Conversely, mites on maize were more susceptible to dimethoate at 24 h than at 7 days.

A study from Brazil reported varied levels of the susceptibility to spiromesifen in 23 *T. urticae* strains collected from several crops (States of Goias, Mato Grosso and Sao Paulo) with percentages of resistant mites ranging from 0.0 to 81.5 per cent. The highest resistance frequencies were found in ornamental plants (roses and chrysanthemum). Populations of *T. urticae* with up to 29.8 per cent resistance were also detected in strawberry fields, in the same state (Sato *et al.*, 2016).

Different levels of susceptibility of insect pests maintained on specific plants have been related to different levels of metabolizing enzymes, probably induced by the plants (Yu, 1982 and 1983; Ambrose and Regupathy 1992; Tan and Guo 1996). Many enzymes involved in detoxification mechanisms act on a broad range of substrates, including both naturally occurring plant allelochemicals and artifcial pesticides (Gordon, 1961). Therefore, physiological response of herbivores to host plants may lead to enhanced metabolism of pesticides because; mechanisms that function in detoxification of plant allelochemicals in their diets may also be effective at detoxifying pesticides.

Table 19. Relative resistance and enzyme activity in field Populations of *T. truncatus*

Strain	Acaricide	Relative Resistance	Relative enzyme activity	
			Cytochrome P450	Carboxylesterase
VkOk1	Spiromesifen	8.00	2.69	2.58
	Fenazaquin	13.00		
	Diafenthiuron	10.00		
VkAm3	Spiromesifen	7.00	1.21	1.18
	Fenazaquin	5.53		
	Diafenthiuron	1.67		
VkPm3	Spiromesifen	1.35	1.09	0.78
	Fenazaquin	1.13		
	Diafenthiuron	1.03		

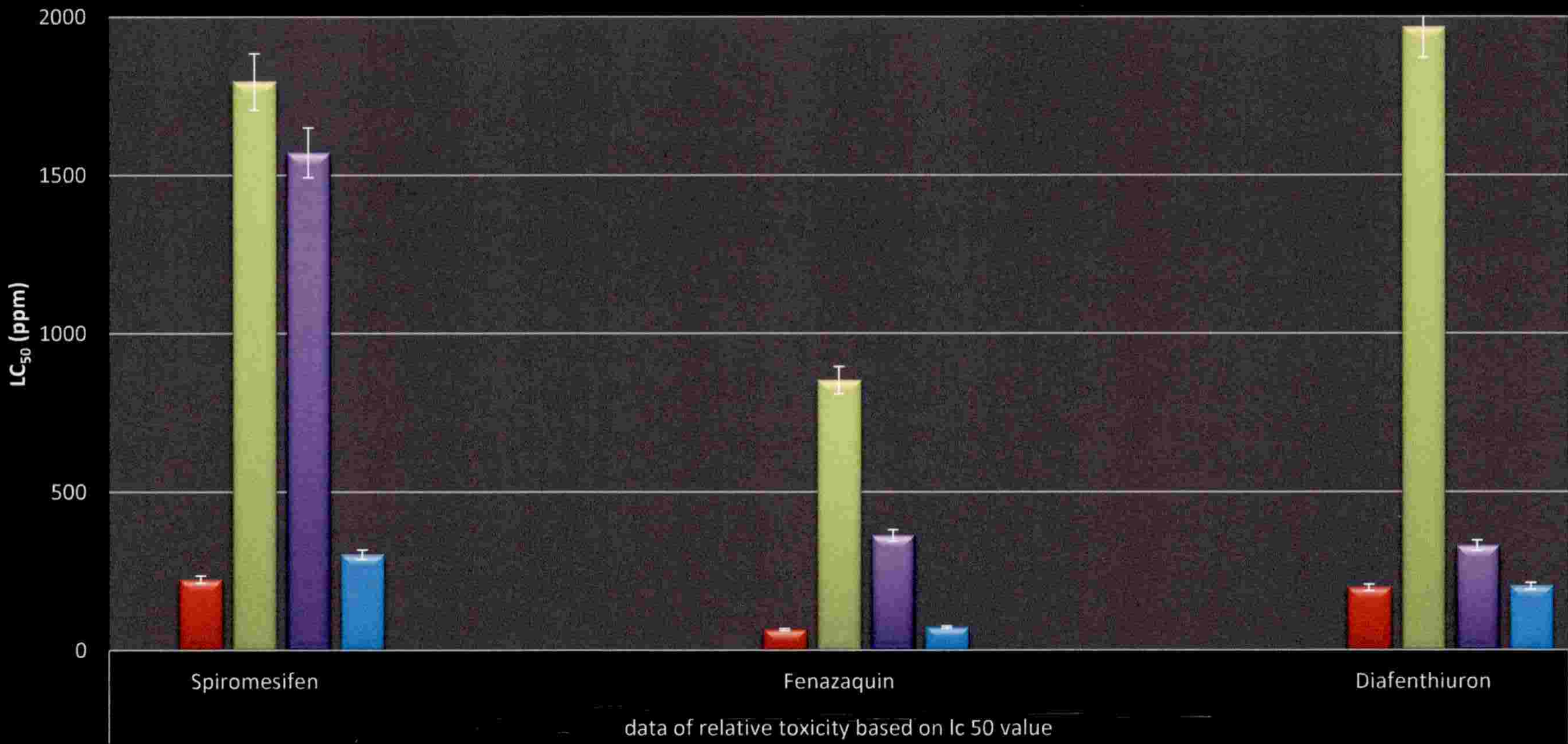

Figure 5.1. Relative susceptibility of different strains of of *T. truncatus* based on LC_{50} value

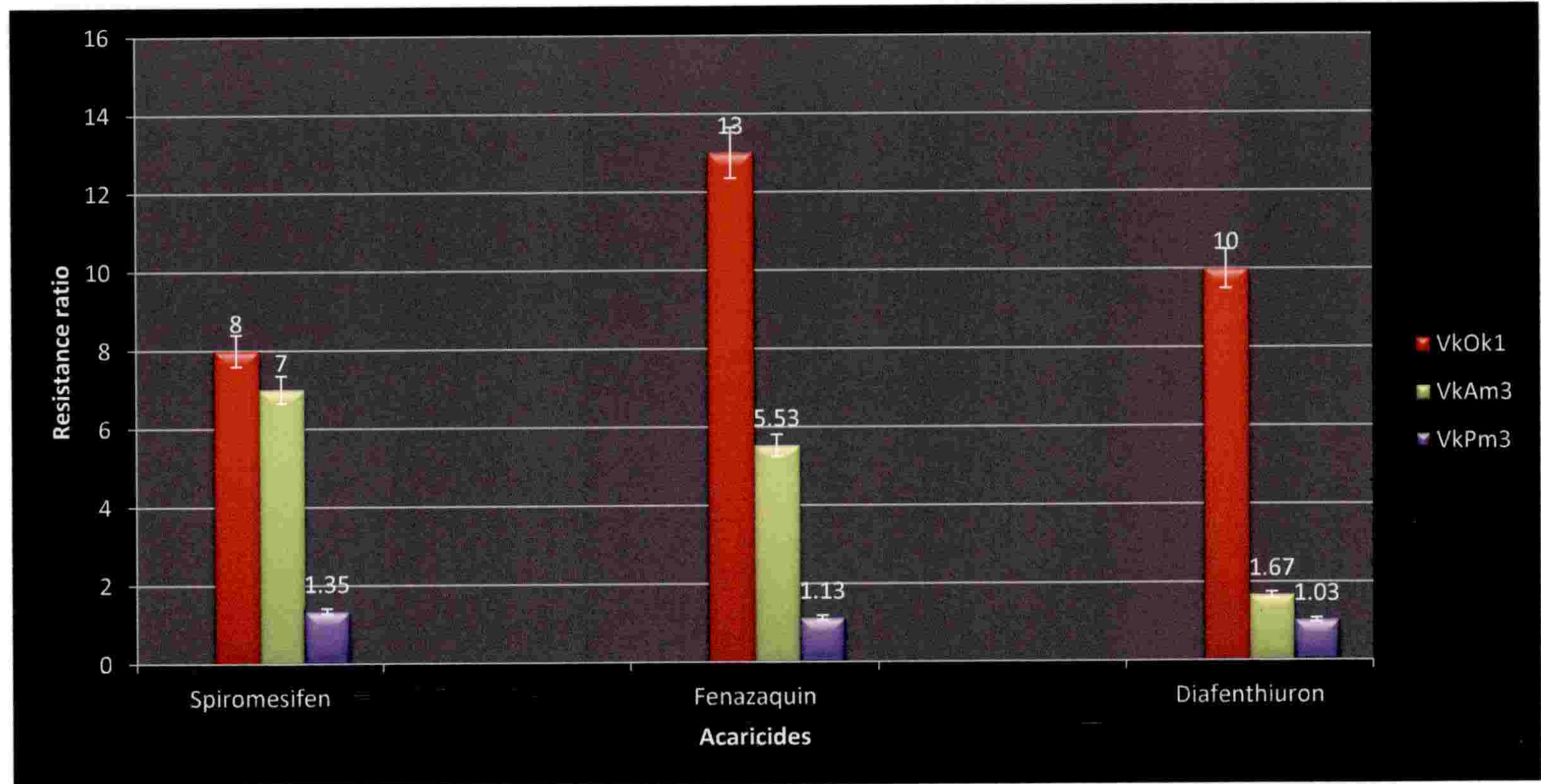

Figure 5.2. Relative resistance of different strains of *T. truncatus*

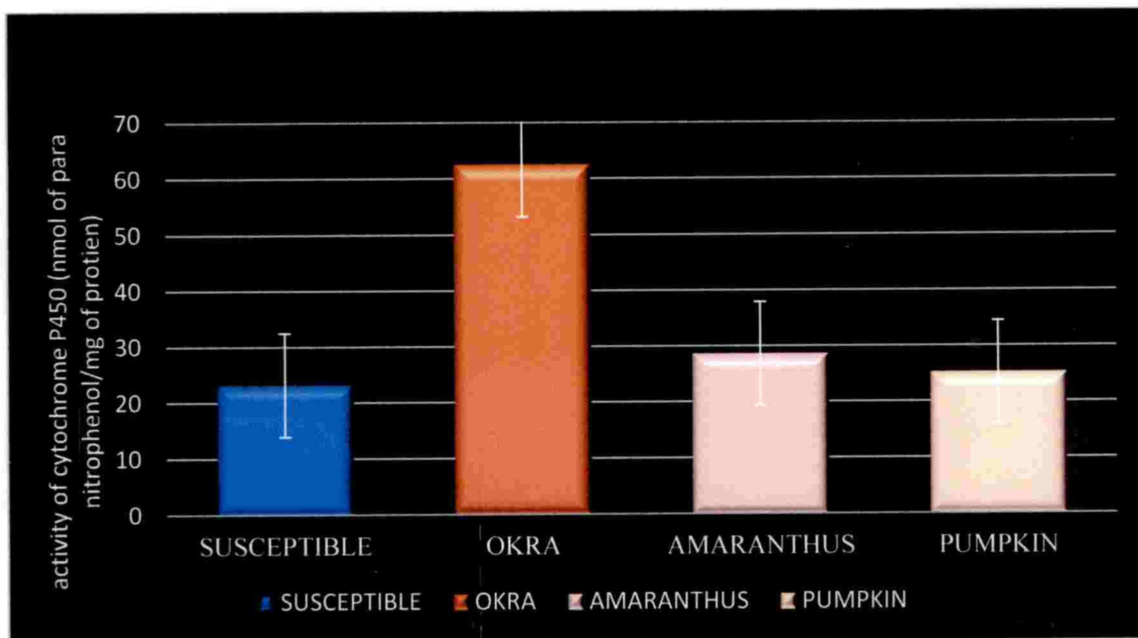

Figure 5.3. Activity of cytochrome P450 in different strains of *Tetranychus truncatus*

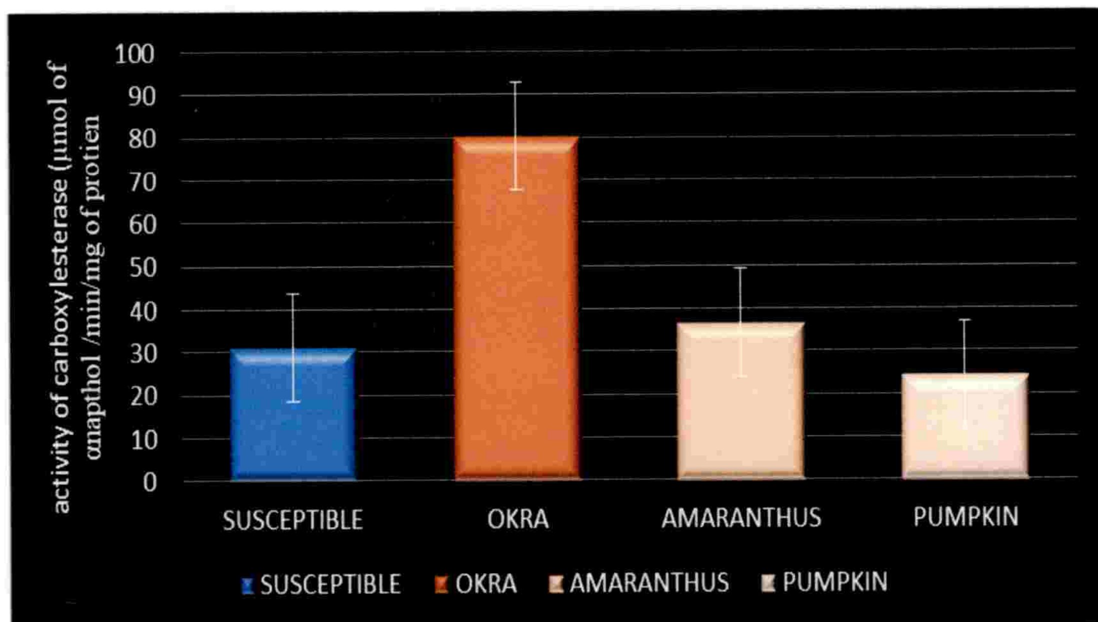

Figure 5.4. Activity of carboxylesterase in different strains of *Tetranychus truncatus*

5.3 Genetic basis of acaricide resistance

Using the BLASTn tool provided by NCBI, the sequences of carboxylesterase and cytochrome P450 were compared with the existing sequences in GenBank and sequence homology was ascertained.

The homology search for the two sequences (okra strain and susceptible strain) of carboxylesterase showed similarity with the carboxyleserase gene of *T. urticae* (Grbic *et al.*, 2011). However, no sequence of *T. tuncatus* is available in the NCBI data base for carboxylesterase gene. On sequence alignments using Clustal Omega, it was found that the carboxylesterase sequences of susceptible and resistant strain are not getting aligned. This shows that there are two genes deciding the resistance and susceptibility to acaricides in *T. truncatus*.

Carboxylesterase is understood to be a multigene family. Proteins of carboxylesterase family share a significant level of dissimilarity in their primary DNA sequences and have widely differing substrate specificities. Nevertheless, due to their structural resemblance and the conserved arrangement of residues in the catalytic site, esterases are thought to have originated from a common ancestor (Nardini & Dijkstra 1999, Oakeshott *et al.* 2005).

In the case of resistant amaranthus strain, PCR amplification of DNA with carboxylesterase specific primers produced two markers (amplicons), one marker matching with that of resistant okra and the other with that of susceptible strain. This shows heterogeneity in the carboxylesterase gene. The resistant strains of okra and amaranthus are progenies of single female *T. truncatus* collected from field on the respective host plants and maintained in the laboratory as isoline cultures. Hence the resultant population/strain is of homogenous and homozygous in nature. However, the PCR amplification result showed presence of both the markers for carboxyleserase gene in

VkAm3 strain. Studies were conducted by Grigoraki *et al*., (2017) on carboxylesterase gene amplification associated with insecticide resistance in mosquito from Greece and Florida, USA. It was found that two variants of carboxylesterase *viz*., *CCEae3a* and *CCEae6a* were two independent gene variants of carboxylesterase and they are co-amplifying in some strains, while amplified individually in some strains, controlling resistance to organophosphates.

In insects, carboxylesterase have versatile biological functions, such as metabolism of specific hormones and detoxification of dietary and environmental xenobiotics (Kontogiannatos *et al*., 2011). In several studies it was found that the elevation of esterase activity through amplification or up-regulation of gene transcription accounts for some degree of resistance to insecticides in insects (Heminway, 2000). It was also found that, enhanced gene amplification due to an exposure to xenobiotic compounds enhance the expression levels of metabolic enzymes that, in turn, trigger resistance (Yu, 2004).

The synergistic action of several CarEs in conferring resistance against acaricides is known in some mites and ticks. It has been well established that CarEs contribute in resistance development against bifenthrin, fenpyroximate, and spirodiclofen in *Tetranychus urticae.* (Kim, *et al*., 2004; Rauch and Nauen, 2002; Van Leeuwen, and Tirry, 2007) Resistance to phoxim in *Panonychus citri* is also enhanced by caboxylesterase (Chen *et al*., 2009). Elevation of esterase activity through up-regulated esterase transcription and point mutations within esterase genes were found in mechanisms involved in esterase-mediated insecticide resistance in the resistant populations of the cattle tick, *Rhipicephalus bursa* (Pan *et al*., 2009; Sun *et al*., 2005). Significant increase in activity of esterases was also reported in resistant strains of *Rhipicephalus bursa* (Enayati, *et al*., 2010). Baffi *et al*. (2008) reported involvement of esterases

group enzymes in pyrethroid and organophosphate resistance in *Riphicephalus microplus*.

Shi *et al.* (2016) first reported a successful heterologous expression of an esterase gene from the carmine spider mite, *Tetranychus cinnabarinus*. The study identified an over expression of esterase gene (*TCE2*) in resistant mites. To identify this gene's role in resistance development, the expression levels of *TCE2* in susceptible line and also in abamectin-, fenpropathrin-, and cyflumetofen-resistant line were knocked down (65.02%, 63.14%, 57.82%, and 63.99%, respectively) via RNA interference study. The bioassay data showed that the resistance level against three acaricides in mite population were significantly reduced followed by a down-regulation of *TCE2*, indicating a correlation between the expression of *TCE2* and the acaricide-resistance in *T. cinnabarinus*. *TCE2* gene was then re-engineered for heterologous expression in *Escherichia coli*. The recombinant *TCE2* showed α-naphthyl acetate activity (483.3 ± 71.8 nmol/mg pro. Min^{-1}), and the activity of this enzyme could be subpressed by abamectin, fenpropathrin, and cyflumetofen, respectively. HPLC and GC results showed that 10 μg of the recombinant *TCE2* could effectively decompose 21.23% fenpropathrin and 49.70% cyflumetofen within 2 hours. The study reported that *TCE2* is a functional gene involved in acaricide resistance in *T. cinnabarinus*.

Many workers have reported that acaricide resistance is mainly under monogenic control (Martinson *et al.*, 1991; Herron and Rophail, 1993; Goka, 1998), while some reports suggest polygenic control of acaricide resistance (Clark *et al.*, 1995).

Ashahara *et al.* (2008) reported involvement of more than one locus in hexythiazox resistance in *T. urticae* from Japan. However, Herron and Rophail (1993) reported earlier that hexythiazox resistance is under monogenic control in an Australian population of *T. urticae*. In a Belgian population of *T. urticae*, chlorfenapyr resistance was reported to be under

polygenic control (Van Leeuwen *et al.*, 2004; 2006), while a Japanese population of spider mite showed the resistance under monogenic control(Uesugi *et al.*, 2002).

Several authors have suggested that monogenic resistance is favored under field selection regimes (Roush and McKenzie 1987; McKenzie, and Batterham, 1992).

The present study reports development of resistance in *T. truncatus* to novel acaricide molecules for the first time in India. The field populations collected on okra from Vellanikkara developed significant level of resistance to acaricides belonging to three major Insecticide Resistance Action Committee (IRAC) MoA class, while the mite population on amaranthus developed resistance to acaricides in two different classes. This makes mite management challenging and warrants alternative strategies replacing the three molecules with acaricides of different modes of action for mite management in vegetable crops.

This study reports for the first time, cytochrome P450 and carboxylesterase genes conferring resistance to acaricides in *T. truncatus*. The study also developed standard markers for discriminating the resistant and susceptible population in *T. truncatus*.

Summary

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In order to identify the status of resistance in *Tetranychus truncatus* infesting vegetable crops to commonly used acaricides, and to understand the molecular mechanism involved in the acaricide resistance, the current study was undertaken at Centre for Plant Biotechnology and Molecular Biology and All India Network Project on Agricultural Acarology, College of Horticulture, Kerala Agricultural University,Vellanikkara.

The Salient findings of the study are summarised hereunder.

- Susceptibility of three field strains of *T. truncatus* namely okra (VkOk1), amaranthus (VkAm3) and pumpkin (VkPm3) to three commonly used acaricides, *viz.* spiromesifen, fenazaquin and diafenthiuron was evaluated in the laboratory in comparison with a laboratory maintained strain (SS). The field populations were found to have developed resistance to the three acaricides which were in use for mite management. However, the level of resistance varied among the strains
- The toxicity studies of spiromesifen showed that pumpkin strain recorded lowest LC_{50} value (302.743ppm) followed by amaranthus strain (1571.021ppm) and okra strain (1794.293ppm). However the susceptible strain recorded LC50 of only 224.48ppm. Spiromesifen was found to be the least toxic to okra strain recording 8 fold resistance, while amaranthus strain recorded 7 fold resistance to the compound. However, spiromesifen showed significantly higher toxicity to pumpkin strain.
- Susceptibility studies on fenazaquin showed that okra strain recorded the highest LC50 value (852.394 ppm) which was 13 fold higher compared to the susceptible strain (65.548ppm). The amaranthus strain recorded LC50 of 362.789 ppm and showed 5.53 fold

resistance to the acaicide. However the susceptibility of pumpkin strain (LC50 - 73.604ppm) was on par with the susceptible strain.

- Okra strain was also found to be the least susceptible to diafenthiuron (LC50 - 1968.496ppm), followed by amaranthus (330.238ppm) and pumpkin (LC50-202.398ppm). Okra strain recorded 10 fold resistance to diafenthiuron. But amaranthus and pumpkin strains did not show resistance to dafenthiuron.
- The activity of the detoxifying enzymes *viz.*, cytochrome P450 and carboxylesterase when determined in the field populations, showed that okra strain recorded significantly higher activity of both cytochrome P450 and carboxylesterase followed by amaranthus strain.
- Okra, amaranthus and pumpkin strains showed 2.7, 1.24 and 1.09 fold higher activity of Cytochrome P450 respectively, compared to the laboratory maintained susceptible strain.
- The activity of carboxylesterase in okra and amaranthus showed an increase by 2.59 and 1.18 fold, respectively. However, pumpkin strain recorded a decrease in activity of carboxylesterase compared to the susceptible strain.
- With the help of gene specific primers for cytochrome P450 and carboxylesterase (designed during the study), specific genes in the DNA were amplified for the resistant and susceptible strains of *T. truncatus*
- PCR amplification of the gene, cytochrome P450 showed that there was no amplification in the case of susceptible strain, whereas there were distinct marker in the strains, okra and amaranthus at 1300bp size.
- The PCR amplification showed distinct bands for carboxylesterase in all the three strains. The okra and susceptible strains showed markers

at 1500bp and 1300bp size respectively, while amaranthus strain showed double bands at 1300bp and 1500bp.

- The sequence homology search by BLASTn analysis showed that the sequences of cytochrome P450 of *T. truncatus* has similarities with available cytochrome P450 sequences from different species of spider mites with an identity match ranging 85 to 96 per cent. The highest similarity was found with an mRNA sequence of cytochrome P450 of *Tetranychus turkestani*.
- BLASTn analysis of the carboxylesterase sequences of okra and susceptible strains showed similarity with two mRNA sequences of carboxylesterase of *T. urticae*. The sequences of okra and susceptible strains showed similarity of 96 per cent and 90 per cent, respectively.
- The sequences of carboxylesterase genes from the two accessions did not align together. Hence it is concluded that there are two different genes controlling the resistance and susceptibility to acaricides in spider mites.
- BLAST P analysis of translated cytochrome P450 amino acid sequence showed 99 per cent to 98 per cent similarity with *T. turkestani*, and *T. urticae* cytochrome P450 amino acid sequence respectively.
- BLAST P analysis of carboxylesterase amino acid sequences from okra and susceptible strains showed 93 per cent similarity with carboxylesterase amino acid sequences of *T. urticae*.
- The study recorded resistance in *T. truncatus* to three novel acaricide molecules, belonging to three major Insecticide Resistance Action Committee (IRAC) MoA class, for the first time from India. This is the first report of cytochrome P450 and carboxylesterase genes conferring resistance to acaricides in *T. truncatus*. The study also developed standard markers for discriminating the resistant and susceptible population in *T. truncatus*.

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Annexure

Annexure I

Composition of Hoyer's medium

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Sl. No.	Content	Quantity
1	Chloral Hydrate	200g
2	Gum Arabic	30g
3	Glycerol	20ml
4	Distilled water	50ml

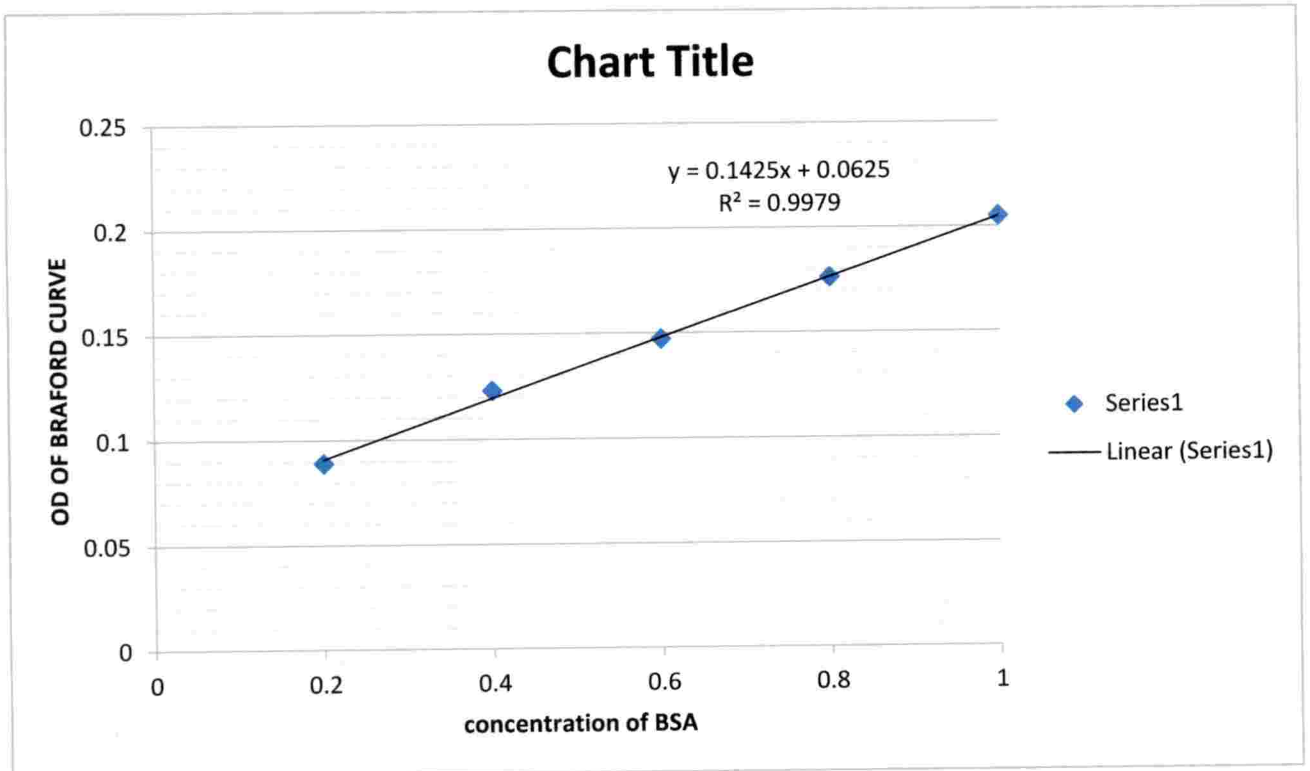
Annexure II

Bradford reagent (Bradford *et al.*, 1976)

1. Dissolve 100mg Coomassie Brilliant Blue G-250 in 50ml 95% ethanol, add 100ml 85% (w/v) phosphoric acid.

2. Once the dye has completely dissolved, dilute to 1 litre with distilled water.

3. Filter through Whatman #1 paper just before use.



Bradford method: BSA standard curve

Annexure III

Reagents: For DNA isolation

1. 2X CTAB extraction buffer (100 ml) (Cetyl trimethyl ammonium bromide)

CTAB -	2g
Distilled water -	54ml
Tris HCL (1M) pH=8 -	10ml
EDTA (0.5M) pH=8 -	2ml
NaCl (5M) -	30ml

2. Chloroform-Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

Ethanol (96%)

96 ml of ethanol and 4ml of sterile distilled water mixed together and stored.

3. Sodium acetate (3M)

40.8g of Sodium acetate (Molecular weight = 136.08) in 100ml distilled water was mixed and stored in refrigerator at $9^{o}C$ and was used for study.

4. Ethanol (70%)

To the 70 ml ethanol (100%), 30 ml of sterile distilled water was added to make 70 per cent ethanol.

Annexure IV

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

Tris base	: 242 g
Glacial acetic acid	: 57.1ml
0.5 M EDTA (pH=8)	: 100ml

2. Loading Dye (6X)

0.25% Bromophenol blue
0.25% Xylene cyanol
30% Glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of Ethidium bromide (stock 10 mg/ml; working concentration 0.5 μg/ml (SRL) and was stored at room temperature in a dark bottle.

4. **Agarose**
-2 per cent (for PCR samples)
1.2g Agarose in 60 ml of1X TAE buffer

5. **Electrophoresis unit**-BioRad power PAC 1000, gel casting tray, comb.

Annexure V

Identification of species of Genus *Tetranychus* Defour, 1832

Hysterosoma having 4th pair of dorso ventral setae in normal dorsal position, with one pair of para anal setae, empodium distally spilit.

1.Taxonomic characters to identify *Tetranychus truncatus* Ehara (1956).

Female:

- Empodia with 6 proximoventral hairs.
- Tarsus I with sockets of 4 tactile setae proximal to proximal pair of duplex setae.
- Tarsus III with 1 proximal tactile seta, peritreme hook 22-25μm long, ventral striae without lobes, pregenital striae entire.

Male:

- Empodia I-II each with an obvious dorsal spur, I-II 3-4 μm long, III-IV 2 μm long.
- Empodium I claw like (uncinate), empodium II-IV with proximoventral hairs free and long.
- Aedeagus with small knob, anterior projection rounded, short, posterior projection pointed, short, dorsal surface flat to slightly, convex, with medial indentation.

2. Taxonomic characters to identify *Tetranychus okinawanus* (Ehara, 1995)

Female:

- Body, including rostrum, 476μm long, 266"μm wide, red in color.
- Dorsal setae on idiosoma slender, pubescent, much longer than distance between consecutive setae.

- Hysterosoma with longitudinal striae between pair of setae C3 and between pair of setae C4, forming a diamond shaped figure between these setae, lobes on dorsal striae very variable in shape, mostly rounded.
- Peritremes strongly hooked distally.
- Pregenetal area with longitudinal striae, the striae broken medially, solid laterally.
- Genital flap with longitudinal striae on anterior part, with transverse striae on posterior part.
- Palpus with spinneret slightly longer than broad, dorsal sensillum fusiform, approximately as long as spinneret.
- The number of setae on leg segments: Tarsus I with 3 tactile setae proximal to proximal set of duplex setae, with 1 tactile setae and 1 solenidion at or near the level of proximal duplex to duplex set. Tarsi III and IV each with solenidion proximal, extending almost to mediodistal tactile setae. Each empodium composed of 3 pairs of hairs and 1 pair of somewhat shorter, proximoventral filaments, with the strong mediodorsal spur.

Male:

- Body, including rostrum, 434µm long, 213µm wide.
- Aedeagus upturned distally, terminal knob 3.5µm long, much longer than the width of neck, approximately one half as long as dorsal margin of shaft, the axis of knob sub parallel with dorsal margin of shaft, anterior projection of knob broadly rounded, the posterior projection very narrow, acute.
- Palpus with spinneret about twice as long as broad, dorsal sensillum slender, fusiform.

- The number of setae on leg segments: Tarsus I with 3 tactile setae and 2 solenidia proximal to proximal set of duplex setae with 1 tactile seta and 1 solenidion at or near the level of proximal to proximal duplex set, tarsus II with 3 tactile setae and 1 solenidia proximal to proximal set of duplex setae.

- Empodium I with 1 pair of claw-like divisions and 1 pair of somewhat shorter, proximoventral filaments, and with the strong mediodorsal spur, empodia II-IV each consisting of 3 pairs of hair and 1 pair of proximoventral filaments, with the strong mediodorsal spur.

3. Taxonomic characters to identify *Tetranychus macfarlanei* (Baker and Pritchard, 1960).

Female:

- Empodia with 6 proximoventral hairs, empodia I-IV each with dorsal spur 2-3 μm long.

- Tarsus I with the sockets of 3 tactile setae overlapping with the socket of the proximal duplex setae, socket of 1 tactile seta proximal to socket of proximal duplex setae.

- Tarsus III with 1 proximal tactile setae, dorsal striae with lobes, ventral striae between genital region and setae with lobes.

- Pregenital striae almost entire but weak and/or broken medially.

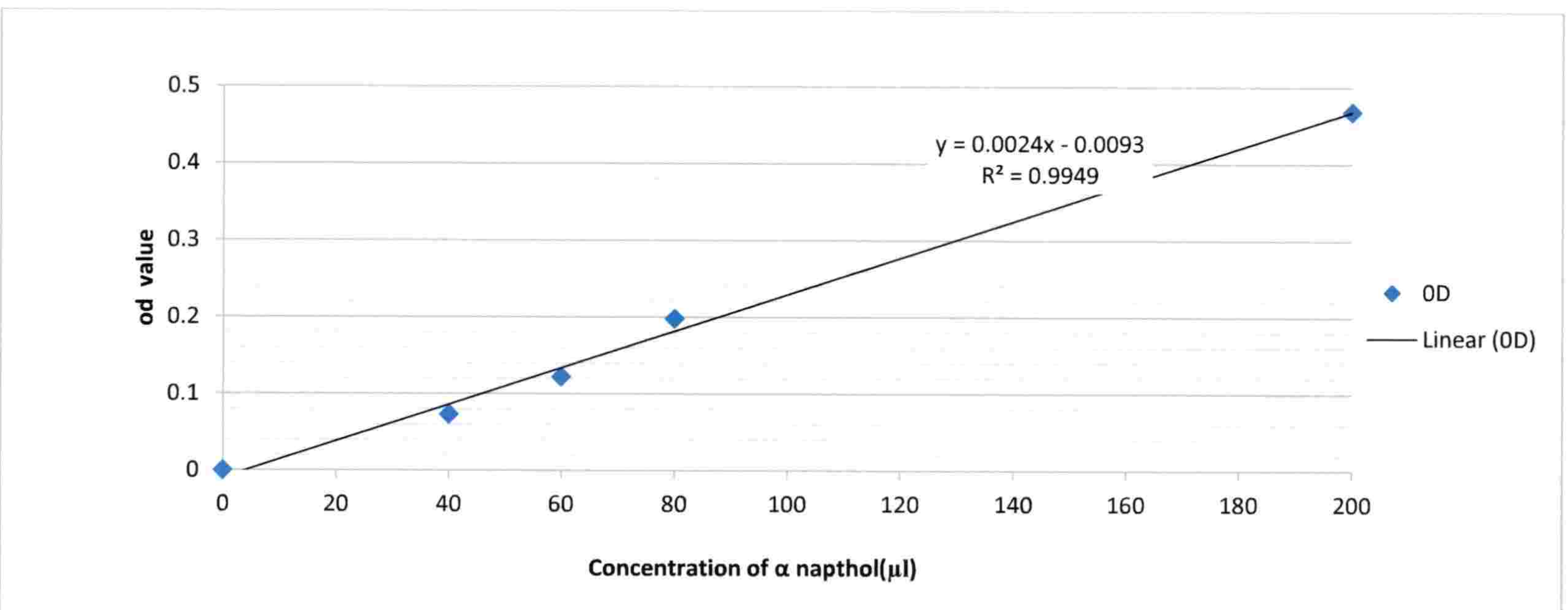
Male:

- Empodia I claw-like (uncinate), empodia II-IV each with proximoventral hairs long and free.

  Aedeagus with small anvil-shaped knob, anterior and posterior projections tiny, dorsal surface flat to slightly convex

Annexure VI

α Napthol Standard Curve



Annexure VII

Cytochrome P450 DNA sequences

>CYP450_*T.truncatus*_VkAm3_forward

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>CYP450_*T.truncatus*_VkAm3_reverse

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>CYP450_*T.truncatus*_VkOk1_forward

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>CYP450_*T.truncatus*_VkOk1_reverse

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Annexure VIII

Carboxylesterase DNA sequnce

>carboxylesterase_*T.truncatus*_SS_forward

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>carboxylesterase_*T.truncatus*_SS_reverse

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>Carboxylesterase_*T.truncatus*_VkOk1_Forward

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>Carboxylesterase_*T.truncatus*_VkOk1_reverse

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AAAAAGCAACAAAATTTGGTTTTTAATCAGATGGATTAGATTACC
TTTAGACCGAGGCGATATAAGGTGGAAA

Molecular basis of acaricide resistance in *Tetranychus truncatus* Ehara (Prostigmata: Tetranychidae) infesting vegetable crops

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ABSTRACT OF THESIS

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Abstract

Spider mites of the family Tetranychidae are considered as one of the most serious sucking pests of vegetable crops worldwide. Intensive use of conventional acaricides had lead to the development of resistance in many mite species around the globe. In view of this, several novel acaricides with unique chemical structure and mode of action were introduced and commercialized for mite management.

In Kerala, mite management in vegetable crops solely depends on the use of novel acaricides. Of late, several farmers have raised concern over their poor efficacy against mite pests. Spider mites have the ability to develop resistance quickly on continuous exposure to a pesticide. In this context, the present study was undertaken to investigate the status, biochemical and molecular bases of acaricide resistance in *Tetranychus truncatus* Ehara, the predominant species of spider mite infesting vegetable crops of Kerala.

Purposive surveys were conducted in the vegetable fields of Vellanikkara, Thrissur and spider mites were collected and reared in the laboratory by assigning accession numbers. Three accessions/strains *viz*., VkOk1 (okra), VkAm3 (amaranthus) and VkPm3 (pumpkin) which were identified as *T. truncatus* were used for the study.

Susceptibility of the three field strains to three commonly used acaricides, *viz*., spiromesifen, fenazaquin and diafenthiuron was evaluated in the laboratory following leaf dip bioassay in comparison with a laboratory maintained susceptible strain (SS). Bioassay study revealed that the strain VkOk1 recorded highest LC_{50} value and has developed 8, 13 and 10 fold resistance to spiromesifen, fenazaquin and diafenthiuron, respectively. This was followed by VkAm3 which showed 7.0, 5.53 and 1.67 fold resistance, while VkPm3 recorded 1.35, 1.13 and 1.03 fold resistance.

The activity of the detoxifying enzymes *viz.*, cytochrome P450 and carboxylesterase, was significantly higher in VkOk1strain followed by VkAm3. The strains VkOk1, VkAm3 and VkPm3 showed 2.69, 1.24 and 1.09 fold enhanced activity of Cytochrome P450, respectively compared to SS, while carboxylesterase in VkOk1 and VkAm3 showed an increased activity by 2.59 and 1.18 fold. However, the strain VkPm3 recorded a decrease in activity of carboxyleasterase by 0.78 fold compared to the susceptible strain.

DNA isolated from the two resistant strains (VkOk1 and VkAm3) and the susceptible strain (SS) was amplified with the help of gene specific primers for cytochrome P450 and carboxylesterase. The results of PCR for cytochrome P450 gene showed that there was no amplification in the case of SS, whereas there were distinct markers in the resistant strains, okra and amaranthus at 1300 bp size. However, PCR amplification showed distinct markers for carboxyl esterase in all the three strains. The strains VkOk1 and SS showed markers at 1500 bp and 1300bp size respectively, while VkAm3 strain showed both the markers.

The sequence homology search by BLASTn analysis showed that the sequences of cytochrome P450 of *T. truncatus* has similarities with cytochrome P450 sequences from different species of spider mites with an identity match ranging from 81 to 97 per cent, while carboxylesterase sequences showed similarity with two mRNA sequences of carboxylesterase of *T. urticae*. Further, the translated sequences of cytochrome P450 and carboxylesterase aminoacids from *T. truncatus* when analysed by BLAST P showed similarity with the amino acid sequences from other spider mites. The sequences of carboxylesterase genes from the resistant and susceptible strains did not align together, showing that there are two different caboxylesterase genes controlling the resistance to acaricides in *T. truncatus*.

The study recorded resistance in *T. truncatus* to three novel acaricide molecules, for the first time from India. This is the first report of cytochrome P450 and carboxylesterase genes conferring resistance to acaricides in *T. truncatus*. The study also developed standard markers for discriminating the resistant and susceptible population in *T. truncatus.*

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