SUSCEPTIBILITY OF *Tetranychus okinawanus* Ehara (PROSTIGMATA: TETRANYCHIDAE) INFESTING ORNAMENTAL PLANTS TO NOVEL ACARICIDES

By

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(2019-11-101)

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KERALA, INDIA

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2021

DECLARATION

I, Sreesha M. (2019-11-101) hereby declare that the thesis entitled **"Susceptibility of *Tetranychus okinawanus* Ehara (Prostigmata: Tetranychidae) infesting ornamental plants to novel acaricides"** is a bonafide record of research done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **"Susceptibility of *Tetranychus okinawanus* Ehara (Prostigmata: Tetranychidae) infesting ornamental plants to novel acaricides"** is a record of research work done independently by **Ms. Sreesha M.** (2019-11-101) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Introduction

1. INTRODUCTION

Floriculture has emerged as a viable economic option in the diversification of agriculture in India and it adds a significant share to the nation's total agricultural output. As per the National Horticulture Database, the area under floriculture in India is 3,05,000 hectares with a production of 23,01,000 tonnes of loose flowers and 7,62,000 tonnes of cut flowers (NHB, 2019-20). In Kerala, Thrissur district is considered the centre of the floriculture nursery business. However, the incidence of insect and mite pests is considered the main constraint in the production of ornamental plants in these nurseries.

Spider mites are a well-known agricultural and horticultural pest that feed on a wide variety of plants, including vegetables, fruit trees, and ornamentals (Jeppson *et al*., 1975). It is a serious threat to crop production throughout the world. Spider mites damage crop plants by feeding on the lower surface of leaves, removing leaf cell contents, and forms fine silken webs. Feeding injury leads to the loss of leaf chlorophyll and reduction in the photosynthetic rate resulting in necrotic spots, yellowing, leaf bronzing, and even death of plants, in case of severe infestation (Gorman *et al*., 2002).

The spider mite, *Tetranychus okinawanus* was recently recorded on the ornamental plant, *Adenium obesum*, in Thrissur district, Kerala for the first time in India (Zeity *et al*., 2016). The mite was first reported on *Pueraria lobata* from Okinawa Islands of Japan by Ehara (1995) and hitherto known from Japan, Taiwan and India on more than 94 host plants. These mites grow quicker and lay more eggs at an early age than other colonising spider mites, such as *Tetranychus kanzawai* and *Tetranychus urticae*. It has a short generation time and can complete its life cycle, from egg to adult, in about nine days under favourable temperature (25 °C). As a result, among tetranychid mites, *T. okinawanus* has the highest intrinsic rate of natural increase. The broad potential host range and high reproductive performance of *T. okinawanus* could make it a dangerous pest on agricultural crops, especially in controlled environments like greenhouses (Takafuji *et al*., 1996). Now *T. okinawanus* has emerged as the predominant species of spider mite infesting vegetables and

ornamental plants in Kerala. Recent studies on the diversity of spider mites in Thrissur district brought out the potential of *T. okinawanus* to turn invasive in Kerala's ecosystems (Jayalakshmi *et al.*, 2019 and Bhaskar, 2019).

For several decades, mite management has primarily relied on conventional insecticides and acaricides. However, 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 a few years of use (Vassiliou and Kitsis, 2013). There are over 800 cases of acaricide resistance development in phytophagous mites worldwide (APRD, 2017), with tetranychids accounting for 93 per cent of them (Ullah and Gotoh, 2013).

Acaricides currently being used to manage mite pest infesting ornamental plants in horticultural nurseries include fenazaquin, fenpyroximate, diafenthiuron and spiromesifen. Recently, several growers reported inefficacy of these commonly used novel acaricides against spider mites in many ornamental crops, suggesting that the mite species might have developed resistance to acaricides. Acaricide resistance monitoring is essential for efficiently managing mite pests and suppressing or delaying resistance development. Thus, acaricide resistance monitoring should form an integral part of chemical control to detect resistance as early as possible and take necessary corrective measures.

It is also essential to know the mechanisms of acaricide resistance of the mites for the implementation of a successful resistant management programme (Ay and Gurkan, 2005). Acaricide resistance can result from the selection of one or more mechanisms including behavioural modification, integument alterations, sequestrations, metabolic resistance and genetic mutations. Metabolic resistance, or enhanced detoxification of acaricides, is a mechanism of acaricide resistance, which involves the metabolism of the pesticide before it reaches the target site. This metabolism of acaricides is achieved by the quantitative or qualitative changes in major detoxification enzymes like esterases, glutathione-S-transferases (GST) and

cytochrome P450 monooxygenases (CYP450) (van Leeuwen *et al*., 2010). The increased activity of detoxification enzymes plays a major role in the acaricide resistance in mites (Stumpf and Nauen, 2002).

In this context, the present study entitled "Susceptibility of *Tetranychus okinawanus* Ehara (Prostigmata: Tetranychidae) infesting ornamental plants to novel acaricides" was undertaken with the following objectives.

- To investigate the susceptibility of *Tetranychus okinawanus* to novel acaricides
- To investigate the biochemical basis of acaricide resistance if any, in *T. okinawanus*

Review of literature

2. REVIEW OF LITERATURE

All mites are classified as Acari, the most diverse taxon within the subphylum Chelicerata, with more than 55,000 described species representing a wide range of life histories, from human and veterinary impact to agricultural damage (Skoracka *et al*., 2015). Tetranychidae, Tarsonemidae, Tenuipalpidae, and Eriophyidae are the main mite families injurious to agriculture (Hoy, 2011). Spider mites (Tetranychidae) are one of the major limiting factors in sustainable production of a number of crops including fruits, vegetables and ornamentals in both protected and open field conditions around the world. These cause damages to crop plants by feeding on the lower surface of leaves, removing leaf cell contents, and forming fine silken webs. Feeding injury leads to the loss of leaf chlorophyll and reduction in the photosynthetic rate resulting in necrotic spots, yellowing, leaf bronzing, and even plant death in severe infestation (Gorman *et al*., 2002).

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is the most commercially important plant-feeding mite pest in the world (van Leeuwen *et al*., 2010), out of more than 1,200 species of spider mites documented (Bolland *et al*., 1998; Milegeon and Dorkeld, 2021).

The spider mite, *Tetranychus okinawanus* was recently recorded on the ornamental plant, *Adenium obesum* in Thrissur district, Kerala for the first time in India (Zeity *et al*., 2016). The mite was first reported on *Pueraria lobata* from Okinawa Islands of Japan by Ehara, (1995) and hitherto known from Japan and Taiwan on more than 90 host plants. *Tetranychus okinawanus* is one of the spider mites having potential to become invasive in crop ecosystems. These mites grow quicker and lay more eggs at an early age than other colonising spider mites, such as *Tetranychus kanzawai* and *Tetranychus urticae*. As a result, among tetranychid mites, *T. okinawanus* has the highest intrinsic rate of natural increase. These mites do not have the ability to go into diapause and do not do well in low temperatures. Even though, the broad potential host range and high reproductive performance of *T. okinawanus* could make it a dangerous pest on agricultural crops, especially controlled environments like greenhouses (Takafuji *et al*., 1996).

The intensive use of pesticides was a common practice in the conventional crop management system. Many traditional insecticides and acaricides have been used to manage mites for several decades. The widespread usage of acaricides around the world has facilitated development of resistance in different mite species, making mite management challenging. As a result, many novel acaricides with distinct chemical structures and modes of action have been developed and marketed for mite control. However, after a few years of use, mite populations developed resistance to these newly introduced compounds too (Vassiliou and Kitsis, 2013). In the present study, the susceptibility of *T. okinawanus*, a recently recorded spider mite, which then turned invasive in Kerala's ecosystem to novel acaricides was evaluated. The biochemical mechanism of resistance in the mite was also assessed. The literature pertaining to the studies on acaricide resistance in spider mites are reviewed below.

2.1. RESISTANCE TO ACARICIDES IN MITES

A lot of research has been conducted on the development of resistance in spider mite species. High reproductive potential, inbreeding, arrhenotokous reproduction, short life cycle, several generations per year, and warmer circumstances all together contribute to the development of acaricide resistance in spider mites. (Van Leeuwen *et al.*, 2009). Of the more than 1,200 species of spider mites described (Bolland *et al.*, 1998; Milegeon and Dorkeld, 2021), *T. urticae* in particular has been documented to have evolved resistance to over 95 acaricidal/insecticidal active ingredients (van Leeuwen *et al*., 2010). It has got the dubious reputation to be the "most resistant species" when it comes to the overall number of pesticides to which populations have developed resistance (van Leeuwen *et al*., 2009). Furthermore, *T. urticae* can become fully resistant to new acaricides within two to four years, meaning that control of multi-acaricide resistant *T. urticae* has become increasingly difficult (Grbic *et al*., 2011).

2.1.1. Acaricide resistance - a global scenario

Natural enemies, diseases, and inadequate plant nutrition have historically held *T. urticae* populations at low to undetectable densities in nature. Development of resistance to chemicals is widespread in the mite population mainly due to an irrational use of these synthetic chemicals which indiscriminately affect both natural enemies and

mite itself. During and after World War II, modern agricultural systems, fertilisers, and an overuse of synthetic organic pesticides produced conditions that enabled *T. urticae* to reach extremely high densities and led to outbreaks. (Van de Vrie *et al*., 1972). Apart from the biological characteristics like, fast generation time and high fecundity, which contribute to potential pest status of *T. urticae* and improvements in agroecosystems, the main factor that contributing to the outbreaks of spider mites in the latter half of the twentieth century are their ability to evolve rapid acaricide resistance (Cranham and Helle, 1985). Spider mites have developed resistance to a variety of pesticides after being exposed to at least 85 distinct chemicals repeatedly in more than 40 countries, both in greenhouse and field crops (Nauen *et al*., 2001). Now evolution of resistance to synthetic chemicals has been reported from more than 80 countries worldwide (Motazedian *et al*., 2012) indicating that resistance to acaricides in spider mite is well-documented and has become a global phenomenon (Grbic *et al*., 2011). The resistance is predominantly caused by a less sensitive target site and enhanced detoxification (Marcic, 2012).

Tolerance to acaricides can develop in mites after a few applications (Sato *et al*., 2005). Furthermore, *T. urticae* can develop resistance to novel acaricide compounds during two to four years of continuous use, making multi-acaricide resistant *T. urticae* management more challenging (Grbic *et al*., 2011). *T. urticae* in particular having developed resistance to over 95 acaricidal/ insecticidal active ingredients (van Leeuwen *et al*., 2010). In 40 countries, *T. urticae* has evolved resistance to many organophosphate compounds in both greenhouses and open field crops (Tsagkarakou *et al*., 2002). Pyrethroid resistance has been found in *T. urticae* populations all over the world at high levels, with resistance levels scaling over 2000 times. Mitochondrial Electron Transport Inhibitors (METI) resistance in *T. urticae* has been reported in many different geographical regions and crops (van Leeuwen *et al*., 2009). There are several reports of abamectin resistance in *T. urticae* in a number of strains and field populations worldwide (Campos *et al*., 1995; Stumpf and Nauen, 2002), with resistance ratios mounting up to 668 (Sato *et al*., 2005).

In California, *T. urticae* infesting ornamental plants were tested for their susceptibility to abamectin. Resistance was correlated with the number of applications

and the total time of abamectin use. Resistance was not detected in nurseries that had used the product less than six times per year and for a total of less than 30 applications over a six-year period (Campos *et al*., 1995).

Acaricide resistance in *T. okinawanus* was reported for the first time in Japan against flufenoxuron (Goka *et al.,* 1998).

Devine *et al*. (2001) evaluated the acaricide resistance in *T. urticae* population collected from hops in England. The results showed that the population was resistant to four METI acaricides *viz*., tebufenpyrad, pyridaben, fenazaquin and fenpyroximate with resistance ratio of 46, 346, 168 and 77, respectively, and it was the first published incidence of METI acaricide resistance in Europe.

A marked resistance to fenbutatin oxide and tebufenpyrad was found in a single glasshouse populations of *T. urticae* during a survey of insecticide resistance in two problematic pests in UK glasshouse (Hewitt *et al*., 2001).

A laboratory study was conducted in Korea to determine the effects of six acaricides on eight field populations of *T. urticae,* collected from rose greenhouse. There were considerable differences in susceptibility and the local resistance ratios in the egg stage were lower than those of female adults (Kim *et al*., 2003).

Considerable increase in resistance to chlorpyrifos was detected in *T. urticae* population collected from greenhouses of Turkey. The resistance ratio ranged from 8 to 1774 (Ay, 2005).

In a population of *T. urticae* collected from a commercial strawberry field in the Brazilian state of Sao Paulo, selections for resistance and sensitivity to abamectin were made. Susceptible (S) and resistant (R) strains of *T. urticae* to abamectin were developed after five selections for resistance and five selections for susceptibility. Bioassay studies revealed a resistance ratio of up to 342. The stability of abamectin resistance was also studied under laboratory conditions. In the absence of selection pressure, abamectin resistance was unstable (Sato *et al*., 2005).

Resistance to acaricides was reported in *Panonychus ulmi* population collected from apple orchard to a number of acaricides *viz*., amitraz, dicofol, bromopropylate and fenpyroximate (Kumral and Kovanci, 2007).

Sokeli *et al*. (2007) conducted a study in *T. urticae* collected from apple orchards in Isparta Province to determine the development of acaricide resistance in mites. Mites were tested for resistance to three pesticides *viz*., propargite, chlorpyrifos and abamectin. The study showed a 2.3 to 40.2-fold resistance in field population to chlorpyrifos compared to the susceptible population.

In a study conducted to evaluate the effectiveness of seven acaricides against some *T. urticae* populations collected from greenhouse roses in Cyprus, the results showed a lower mortality suggesting resistance development in these populations (Stavrinides and Hadjistylli, 2009).

Van Leeuwen *et al*. (2010) conducted a study in California vineyards to determine the development of acaricide resistance in Pacific spider mite (*Tetranychus pacificus*). Mites are tested for resistance to three acaricides viz., bifenazate, propargite and pyridaben. The study showed a 11-fold resistance to pyridaben, 7-fold resistance to bifenazate and 4-fold resistance to propargite compared to the susceptible population.

Youssef *et al*. (2011) studied the resistance development in *T. urticae* collected from cotton plants against abamectin and etoxazole. Results showed a resistance ratio of 140 and 37.45 to abamectin and etoxazole, respectively.

In Jordan, studies were conducted on spiromesifen resistance development and observed moderate level of resistance (17.96-folds) in *T. urticae* population collected from cucumber (Al-Antary *et al*., 2012).

A study was conducted by Doker and Kazak (2012) on Turkish populations of *P. citri* using spirodiclofen, dicofol, tetradifon and fenbutatin oxide and the results revealed a medium level of resistance to each acaricide.

T. urticae infesting rose under protected cultivation in Italy was reported to show progressive loss of effectiveness to many newly introduced acaricides. Bioassays conducted to assess the response of some Italian strains of *T. urticae* to both

conventional and novel acaricides revealed high level of resistance in adult mites to fenpyroximate, tebufenpyrad and abamectin. Eggs exhibited high resistance to clofentezine, hexythiazox and flufenoxuron (Tirello *et al.*, 2012).

Demaeght *et al*. (2013) observed that two genetically different field collected strains of *T. urticae* exhibited a very strong resistance to spirodiclofen. The results showed up to 680-fold resistance against spirodiclofen.

According to the studies conducted by Nicastro *et al*., (2013) on stability, cross-resistance and resistance to chlorfenapyr in *T. urticae*, chlorfenapyr resistance was shown to be stable in the absence of selection pressure under laboratory conditions. The susceptibility study of *T. urticae* revealed that, the susceptibility of mites to chlorfenapyr was variable, with percentages of resistant mites ranging from 0.0 to 86 per cent. The ornamental plants had the highest resistance frequencies. chlorfenapyr resistance was also found in high levels in some cotton and papaya populations.

The level of resistance in *T. urticae* collected from hops in Pacific Northwestwas evaluated in the laboratory following leaf disc bioassay, in comparison to laboratory maintained susceptible reference strain. Resistance of *T. urticae* was detected to abamectin, bifenazate, and bifenthrin. The highest resistance ratio was recorded by abamectin followed by bifenazate, suggesting that the use of the two acaricides should be avoided or minimized for the control of *T. urticae* populations in hops (Piraneo, 2013).

Frequent control failures against *Tetranychus urticae* have been reported for some commonly used chemicals *viz*., abamectin, acrinathrin, fenazaquin, pirimiphos methyl, and bifenazate across the island of Cyprus. Standard leaf-disc spray application bioassay procedures were used to determine the LC_{50} for these five chemicals and resistance of *T. urticae* was detected to abamectin, acrinathrin, fenazaquin, and pirimiphos methyl (Vassiliou and Kitsis, 2013).

Mohammadzadeh *et al*. (2014) evaluated the efficacy of two acaricides, abamectin and propargite, against two populations of the spider mite collected from rose greenhouses in Iran. Results showed that resistance ratio for abamectin and propargite was 20285 and 130, respectively.

Low level of resistance to different acaricides (1.79 to 3.11for dicofol, 0.88 to 2.01 for lambda-cyhalothrin, 1.99 to 4.47 for amitraz, 0.92 to 2.26 for profenose, 1.37 to 1.67 for abamectin and 1.34 to 1.71 for endosulfan) were reported in field collected population of *T. urticae* in Ethiopia (Gutu *et al*., 2015).

A vial-leaf dipping bioassay was conducted to assess the resistance of six field populations of *T. urticae* to 10 acaricides; the results showed that the adult females of *T. urticae* populations had different resistance to each acaricide. All the six populations were highly resistant to abamectin and exhibited sensitivity to medium resistance for the other acaricides (Wang *et al*., 2015).

Sato *et al*. (2016) reported high percentage of spiromesifen resistant individuals in *T. urticae* population collected from open cultivated rose and chrysanthemum crops in Brazil.

Due to the fast activity and relatively low costs, abamectin has been extensively used for control of spider mite in cotton over the past decade in the Midsouth. Later, reduced efficacy and shortened residual control were reported indicating a possible issue with resistance development. Using a leaf-dip bioassay, studies were conducted to evaluate resistance levels to abamectin in 12 populations of *T. urticae* collected from the Midsouth. Two populations were highly resistant with resistance ratios of 630 and 1415-fold. In contrast, one population was slightly resistant with a resistance ratio of 11.1 compared with a susceptible control population. LC_{50} values for all colonies were significantly greater than the control population (Brown *et al*., 2017).

Yalcin *et al*. (2018) reported that a strain of *T. urticae* collected from strawberry fields in southern Turkey showed varied level of resistance to commonly used acaricides *viz*., abamectin, etoxazole, spiromesifen and tebufenpyrad (2.39-7.86, 6.80-15.39, 4.61-9.73, and 5.51-12.47-fold, respectively).

Low level of resistance (2.2 to 10.65-fold) to bifenazate was reported in field collected population of *T. urticae* in Switzerland (Chen *et al*., 2019).

Abamectin resistance was reported in *T. urticae* infesting cut rose in Mexico. Among the four field populations collected, all were resistant to abamectin with resistance ratio ranging from 2266 to 21141 (Diaz-Arias *et al*., 2019).

Inak *et al*. (2019) reported that ten strains of *T. urticae* collected from vegetable crops in Turkey showed varied level of resistance to commonly used acaricides. Nine out of ten population showed resistance to bifenthrin and half of the population showed resistance to abamectin and hexythiazox.

Resistance to abamectin was reported in European red mite, *Panonychus ulmi* from apple orchard of Iran. Among the 12 field populations collected, all were resistant to abamectin (RR ranged from 11 to 46-fold) (Rameshgar *et al*., 2019).

Alavijeh *et al*. (2020) observed that Iranian populations of the citrus red mite *Panonychus citri* exhibited a medium level of resistance to fenpyroximate. The results showed about 75-fold resistance to fenpyroximate and cross resistance was also observed against other METI- acaricides.

Badieinia *et al*. (2020) reported that, the populations collected from Urmia and Shahin Dej exhibited a moderate level of resistance (22 and 21-fold) to spiromesifen, respectively.

Population outbreaks and failures in controlling *T. urticae* were reported on ornamental plants in Florida. The susceptibility of two *T. urticae* field populations collected from hibiscus and croton plants from commercial nurseries were compared with a laboratory population to one new generation (cyflumetofen) and two conventional (abamectin and pyridaben) acaricides. Results of the study revealed high resistance levels to the two conventional acaricides (9.64 and 19.28-fold to abamectin, 12.34 and 34.08-fold to pyridaben), and low levels (1.88 and 2.39-fold) of cyflumetofen resistance in the hibiscus and the croton populations, respectively (Doker *et al*., 2020).

Field collected strains of *T. urticae* tested for acequinocyl and pyridaben resistance in Korea and these strains displayed resistance ratios of 1798.6 and 5555.6, respectively. These were screened for cross-resistance against several currently used acaricides. The acequinocyl resistant strain exhibited pyridaben cross-resistance, but the pyridaben resistant strain showed no cross-resistance (Kim *et al*., 2020).

A leaf dipping bioassay was conducted to assess the resistance of seven populations of citrus red mite, *Panonychus citri* to four acaricides; the results showed

that the mite populations had different levels of resistance to each acaricide. Some populations were highly resistant to abamectin (1,088-fold and 1,401-fold) and cyflumetofen (2,112-fold and 9,093-fold) (Pan *et al*., 2020).

Leaf dip bioassay was conducted to evaluate the status of acaricide resistance in *T. urticae* from Ethiopia. The six greenhouse and field collected populations were tested for resistance to various acaricides. All the collected populations showed resistance to fenbutatin oxide and fenpyroximate (Simma *et al*., 2020).

Jeffris *et al*. (2021) studied the status of acaricide resistance in South Carolina tomato populations of *T. urticae*. Low to moderate levels of resistance was reported in *T. urticae* to some of the tested acaricides *viz*., abamectin (2800-fold) and bifenthrin (250 to 350-fold).

Meshkov *et al*. (2021) conducted a study in protected ground of Russia to assess the status of acaricide resistance in commonly found mite species. The result of the study showed that the mites *T. urticae* and *T. cinnabarinus* were formed highly resistant populations against abamectin with a resistance ratio of up to 1060.

van Leeuwen *et al*. (2021) investigated the resistance levels of a *T. urticae* population collected from an ornamental greenhouse in Greece and found a striking case of multiple acaricide resistance in them with resistance ratios of 89-fold for abamectin, 1000-fold for clofentezine, 5000-fold for etoxazole, 27-fold for fenpyroximate and pyridaben, 20- and 36-fold for spirodiclofen and spirotetramat, respectively and 116 and 500-fold for cyenopyrafen and cyflumetofen, respectively.

2.1.2. Acaricide resistance - Indian scenario

Acaricide resistance development in spider mites has also been reported from different parts of India. Spider mite**s** have developed low to high level of resistance to all the chemical classes of acaricides.

In 2008, Kumar reported very high level of resistance to dicofol in *T. urticae* population from Bangalore (767 to 3690-folds) and Kolar districts (500 to 6491-folds) which were continuously exposed to dicofol.

Low to moderate level of resistance (5-32-fold) to fenazaquin was reported earlier from Bangalore district of Karnataka in *T. urticae* on tomato crop (Anonymous, 2009).

Bioassay studies conducted by Patil (2015) indicated that, *T. urticae* infesting grapes have developed resistance to sulphur, ethion, dicofol, fenpyroximate and fenazaquin with resistance ratios of 27.30, 12.54, 7.04, 6.75 and 4.45, respectively.

Sharma and Bhullar (2018) studied the status of acaricide resistance in field collected *T. urticae* from vegetable growing areas of Punjab, India. Low to moderate levels of resistance (3.19-24.65-fold) was reported in *T. urticae* to tested acaricides.

A study was conducted by Srinivasa and Khadri (2018) in Karnataka. In this study they sampled mites from tomato crop of four different districts and subjected to bioassay with four major acaricides namely, dicofol, fenazaquin, propargite and spiromesifen to determine the level of acaricide resistance. The level of resistance to dicofol was high in all the four populations (143 to 1038-folds). Resistance to propargite was moderate (15.65 to 32.83-folds), while resistance to spiromesifen was high in all the four districts.

A study was undertaken at College of Agriculture, Vellanikkara, KAU to investigate the status of acaricide resistance in *T. truncatus*, the predominant species of spider mite infesting vegetable crops of Thrissur district, Kerala. Susceptibility of different field strains of *T. truncatus* 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. Bioassay study revealed that *T. truncatus* strains collected from okra and amaranthus have developed 13 and 5.53-fold resistance to fenazaquin and 8 and 7-fold resistance to spiromesifen respectively (Bachhar *et al*., 2019).

A study on acaricide resistance in *T. urticae* on cucumber under protected cultivation was reported recently from Punjab, India. They tested the effects of four

acaricides on four different populations of *T. urticae.* The collected populations of *T. urticae* showed moderate to very high level of resistance to spiromesifen (3.76 to 32.10 folds), propargite (14.64 to 22.17 folds), fenpyroximate (17.10 to 32.10-folds) and fenazaquin (62.52 to 212.55 folds), respectively in Punjab (Kaur and Bhullar, 2019).

In a study conducted in Assam tea plantation of India, eight field populations of red spider mite (RSM), *Oligonychus coffeae*, were evaluated for resistance against five different acaricides (ethion, dicofol, propargite, fenazaquin, and fenpropathrin) using the leaf-dip method. The results showed that almost all the tested field populations had developed a high to very high level of resistance to ethion and dicofol. The highest level of resistance for RSM was found to ethion and dicofol with a resistance ratio of 134.27 and 65.38 (Roy *et al*., 2018).

Bioassays conducted to determine the response of six field collected population of *T. urticae* to three commonly used acaricides. All the populations were resistant to propargite (3.47 to 5.63), fenazaquin (3.62 to 4.26) and hexythiazox when compared to the susceptible strain (Titiksha, 2019).

Mohin (2020) recorded 41.73- fold resistance to diafenthiuron and 2231.8-fold resistance to dicofol in *T. urticae* population collected from tomato field of Chikkamagaluru and Shivamogga districts of Karnataka.

A study was conducted in Coimbatore, Tamil Nadu to monitor the acaricide resistance in *T. urticae* infesting carnation. The results of bioassay revealed that that LC_{50} values for fenazaquin was 418.3 ppm and 599.37 ppm and for propargite it was 373.33 ppm and 319.64 ppm, respectively in Kurkuthi and Kapati population. Resistance ratio of 217.86 and 312.17 for fenazaquin and 272.50 and 233.31 for propargite was recorded in Kurkuthi and Kapati population, respectively (Sumathi *et al*., 2020).

2.2. BIOCHEMICAL MECHANISM OF ACARICIDE RESISTANCE

The intensive use of insecticides and acaricides has led to resistance in many mite species all over the world. Strong reproductive capacity, inbreeding, arrhenotokous reproduction, and a very short life cycle resulting in several generations

per year hasten the development of resistance (van Leeuwen *et al*., 2010). More than 550 species of insects and mites have developed resistance to at least one class of insecticides/acaricides (van Leeuwen *et al*., 2010). Studies and research of the genetic, biochemical, and molecular mechanisms involved are expected to contribute to better resistance management programs. Organisms can become resistant to pesticides by reducing the effective dose at the target site, which can be attributed to mechanisms such as behavioural resistance, reduced penetration or absorption at the cuticle level, sequestration and metabolic detoxification.

Metabolic detoxification before it reaches the target site is influenced by quantitative or qualitative changes in major detoxification enzymes such as esterases, P450 monooxygenases, and glutathione-S-transferases. During detoxification, pests are able to shuttle out xenobiotic compounds, so that it never enters the cell of the target site (van Leeuwen *et al*., 2010).

Insects have evolved these enzymes to protect themselves from naturally occurring plant toxins (allelochemicals) such as alkaloids, terpenes, and phenols, in order to avoid the possible toxicity of the plants they eat (Gatehouse, 2002; War *et al*., 2012; Rane *et al*., 2016).

Metabolic resistance mechanisms seem to be most important in arthropod species exhibiting resistance to organophosphate and carbamate pesticides (Devonshire *et al*., 1982).

Pyrethroids, organophosphates, and carbamates are only a few of the chemical groups that esterase can act on (Hollingworth and Dong, 2008). Esterase can also act against neonicotinoids (Zhu and Luttrell, 2015) and even against *Bt* toxin (Gunning *et al*., 2005).

Cytochrome P450 monooxygenases (P450s) (or Mixed Function Oxidases (MFOs), or microsomal oxidases) can catalyse a variety of reactions, including epoxidation, hydroxylation, N-dealkylation, O-dealkylation, and desulphurization; as a result, they play an important role in the metabolism of a variety of insecticide groups, including carbamates, organophosphates, pyrethroids and neonicotinoids (Yu, 2008; Puinean, 2010; Alptekin *et al*., 2016).

Glutathione-S-transferases (GSTs) are involved in metabolism of different classes of insecticides, including organophosphates and pyrethroids. DDT resistance in houseflies and mosquitoes has also been linked to a DDT-dehydrochlorinase GST enzyme (Enayati *et al*., 2005).

Cross resistance among METIs from different chemical groups has been observed in most cases, suggesting a common resistance mechanism (Stumpf and Nauen, 2001; van Pottelberge *et al*., 2009).

Resistance in *T. urticae* to dicofol was found in New Zealand, USA, Japan and Europe and is possibly caused by increased degradation (Fergusson-Kolmes *et al*., 1991).

During the risk assessment of spirodiclofen resistance development in *T. urticae* by Rauch and Nauen (2002), combined synergistic, biochemical and metabolism evidence pointed towards an increased oxidative metabolism as the underlying cause.

The enzyme assay conducted in fenpyroximate resistant population of *T. urticae* showed that, both MFO and carboxyl esterase are involved in the resistance development in the mite. MFO activity was 2.5 times higher in the resistant strain than in the susceptible strain, while esterase hydrolysis activity towards α- and β-NA was 2.5 and 2.2 times greater in the resistant strain than in the susceptible strain, respectively (Kim *et al*., 2004).

When compared to a susceptible laboratory strain, a field-collected strain of *T. urticae* demonstrated high resistance to bifenthrin, dicofol, and fenbutatin oxide, as well as cross-resistance to a variety of acaricides. In the study to find out the mechanism of resistance, it was revealed that the observed resistance and cross-resistance was linked with detoxification due to increased mono-oxygenases and esterase activity (van Leeuwen *et al*., 2005).

Metabolic resistance mediated by carboxylesterases or P450s is well documented in pyrethroids (Khambay and Jewess, 2005). In the majority of pyrethroid resistance reports in *T. urticae*, various lines of evidence point to either enzymatic hydrolysis by carboxylesterases or oxidation by microsomal monooxygenases (Ay and Gurkan, 2005; van Leeuwen *et al*., 2005; van Leeuwen and Tirry, 2007).

According to the results of the study conducted by Ay and Gurkan (2005), general esterase plays a major in high bifenthrin resistance In *T. urticae* collected from Turkey.

In two Iranian strains of *T. urticae*, the resistance mechanisms to oxydemeton-methyl were investigated. Using the dipping process, a bioassay was performed on two strains and a resistance ratio of 20.47 was obtained for the resistant strain. The activity of esterase and glutathione S-transferase in resistant and susceptible strains revealed that esterase-based resistance and glutathione S-transferase were some of the resistance mechanisms to oxydemeton-methyl. The resistant strain's esterase activity was 2.5 and 2.14 times higher than the susceptible strains and there is 1.75- and 1.27-fold increase in glutathione S-transferase activity in the resistant strain (Ghadamyari and Sendi, 2008).

Susceptibility and carboxylesterase (CarE) activity to four selected acaricides (amitraz, propargite, azocyclotin, and diafenthiuron) were assayed for five field populations of *Panonychus citri* in China. Based on the detoxifying role of CarE and the sensitivity of mite populations to acaricides, the study suggested that CarE might be associated with lower sensitivity to acaricides (Ran *et al*., 2009).

Van Leeuwen *et al*. (2009) studied the biochemical mechanism of Mitochondrial Electron Transport Inhibitor acaricide (METI) resistance in a Belgian field strain of *T. urticae.* The METI resistant strains showed 23.5-fold increase in the activity of Cytochrome P450 compared to the susceptible strain.

Van Pottelberge *et al*. (2009) studied the biochemical mechanism of resistance in a laboratory-selected spirodiclofen-resistant strain of *T. urticae*. They inferred that the resistance was due to metabolic detoxification of spirodiclofen mainly by P450 monooxygenases, and also by esterases and glutathione-S-transferases.

Khajihali *et al*. (2011) conducted a study on the susceptibility of 15 strains of *T. urticae* collected from greenhouse rose culture in the Netherlands to several currently used acaricides, and resistance mechanisms were also investigated. Resistance levels to traditional acaricides such as bifenthrin and abamectin were prominent and resistance to more recently registered compounds was also detected in several populations. During

the enzyme assay, the activity of esterases, GSTs and MFOs in ten selected strains was evaluated in order to see whether a correlation exists between the observed resistance status and the general detoxifying activity. However, no direct link could be found between overall activity and resistance status. But resistance and an increase in MFO, GST and esterase activity were found in most field-collected strains compared to susceptible strain. A significant difference was observed in P450 monooxygenase activity between susceptible and resistant strains (1.33-fold to 7.29- fold).

More than 3000-fold resistance to abamectin was detected in field collected population of *T. urticae*. Biochemical studies revealed that esterase activity in the resistant strain was 2.14-fold and 1.33-fold higher than that of the laboratory strain. GST and Cytochrome P450 were not responsible for the increased resistance (Memarizadeh *et al*., 2011).

Resistance mechanisms to fenazaquin were surveyed in three Iranian populations of *T. urticae*. Biochemical study revealed the involvement of the detoxifying enzymes in the resistance development. The esterase activities in the populations tested were 3.9, 1.8 and 1.5-fold higher than that in the susceptible population, when α-naphthyl acetate was used as the substrate and the GST activity in the three populations were 2.3, 2.4 and 1.4-fold higher than that in the susceptible (Moghadam *et al*., 2012)

Cytochrome P450 mediated detoxification was identified as one of the mechanisms of cross-resistance to cyflumetofen and cyenopyrafen in field collected strains of *T. urticae* (Khalighi *et al*., 2014).

The enzyme assay conducted in abamectin resistant Iranian population of *T. urticae* showed that three mechanisms of MFO, GST and esterase are involved in the abamectin resistance of the spider mite and the order of their involvement in the abamectin resistance was esterase > MFO > GST (Mohammadzadeh *et al*., 2014).

In a study of cross-resistance between cyenopyrafen and pyridaben in the two spotted spider mites *T. urticae*, it is found that the common detoxification mechanisms by cytochrome P450 was involved in resistance to these acaricides and carboxyl

esterases were also involved in cyenopyrafen resistance as a major factor (Sugimoto and Osakabe, 2014).

Studies conducted on resistance selection and biochemical mechanism of resistance against cyflumetofen in *Tetranychus cinnabarinus* showed that the activity of detoxifying enzymes GSTs, MFOs and carboxyl esterase were significantly increased in the final selected resistant strain of the experiment and the GST was the most important detoxifying enzyme conferring resistance against cyflumetofen in *T. cinnabarinus*as the level of GST increased more than 7-folds after selection (Wang *et al*., 2014).

Adesanya *et al*. (2017) conducted a synergistic assay in order to identify the role of detoxifying enzymes in mite growth inhibitor (MGI) (etoxazole and hexythiazox) resistance in hop yard-collected *T. urticae* populations. In this pre-treatment of the two ovicidal acaricide-selected populations with TPP and PBO (inhibitors of metabolic enzymes) was performed. TPP significantly increased the toxicity of hexythiazox and etoxazole, which indicated that resistance to these MGIs is mediated by carboxyl esterase. PBO showed a significant synergistic effect on hexythiazox. This suggests that cytochrome P450-mediated detoxification also plays an important role in resistance to MGIs by these populations.

The pyridaben-selected strain of *T. urticae* has showed a resistance ratio of more than 3000-fold when compared to the pre-selection strain. The results of synergism experiments revealed that cytochrome P450 enzyme detoxification was the primary mechanism of resistance to pyridaben in the spider mite population examined (Namin, 2017).

An elevated activity of detoxification enzymes was observed in cypermethrin-selected resistant strain of *T. urticae* compare to the susceptible strain. The specific activities of the esterases, GSTs and P450s were 1.58, 1.16, and 1.35-fold, respectively, in the resistant strain in comparison to the susceptible strain (Çağatay *et al*., 2018).

According to El-Dewy (2018), resistance in *T. urticae* against fenpyroximate and abamectin was associated with increased level of mixed function oxidase and carboxylesterase. The carboxylesterase was significantly increased in field strain

recording 1.9-fold higher than that of laboratory strain and mixed function oxidase activity was 1.16-fold higher in resistant strain than susceptible strain.

Twenty-two-fold increase in resistance to spirodiclofen was detected in field collected Iranian population of *T. urticae* compared to a susceptible population. Biochemical studies revealed that the cytochrome P450 monooxygenase activity was 3.02-fold higher in the resistant population and the GST had only a little role in detoxification (1.40-fold) (Farahani *et al*., 2018).

Roy *et al*., (2018) conducted a biochemical study in *Oligonychus coffeae*, collected from conventionally managed (synthetic acaricide usage) and an organically managed (no acaricide usage) tea plantations in Assam, India. The study concluded, a higher general esterases, GST and cytochrome P450 monooxygenase activity (2.83, 1.68, and 1.31 higher respectively) in mite population from the conventionally managed tea plantation as compared with the activity in mites from the organically managed tea plantation.

Studies conducted on biochemical mechanism of acaricide resistance in *T. truncatus* showed that the activity of detoxifying enzymes, MFO and carboxyl esterase were significantly higher in the field collected populations compared to the susceptible population. Among the different population, the okra strain and amaranthus strain recorded carboxylesterase of 2.7 and 1.24-fold respectively and cytochrome P450 activity of 2.59 and 1.18-fold respectively (Bachhar, 2019).

A significant increase in MFO (3.21-fold), GST (1.40- fold) and esterase (1.13 and 1.27-fold with α naphthyl acetate and β naphthyl acetate) activity was observed in a study conducted to assess the biochemical basis of resistance in laboratory selected fenazaquin resistant strain of *T. urticae*. The study suggested that enhanced metabolic detoxification might be the major mechanism responsible for imparting resistance to fenazaquin in *T. urticae* (Sharma *et al*., 2019).

Ay *et al*. (2020) conducted a study on mechanisms of abamectin resistance in *T. urticae* populations from cut flowers greenhouses in Turkey and found that the esterase, GST, and the P450 monooxygenase enzyme activities of the field populations

increased 0.77 to 1.56, 1.28 to 2.66, and 1.09 to 3.46-fold, respectively, compared to the susceptible population.

According to Alpkent *et al*. (2020), resistance in *T. urticae* against bifenthrin and hexythiazox was associated with increased activity of P450 and GST enzymes.

In a study conducted to investigate the mechanism of spiromesifen resistance in *P. ulmi*, the enzymatic assay indicated that esterases are likely to be involved in resistance (Badieinia *et al*., 2020).

A study was conducted by Khanjani *et al*. (2020) to determine the mechanism of resistance in *T. urticae* populations to fenpyroximate. The enzyme assay results revealed that, there was a significant increase in the activity of esterase and cytochrome P450 in the resistant strains.

According to Kim *et al*. (2020), resistance in *T. urticae* against acequinocyl was partially associated with increased levels of glutathione S-transferase activity. GST activity was 1.8 to 2.3-fold higher in resistant strain compared to the susceptible strain.

Materials and methods

3. MATERIAL AND METHODS

The research on "Susceptibility of *Tetranychus okinawanus* Ehara (Prostigmata: Tetranychidae) infesting ornamental plants to novel acaricides" was conducted at the College of Agriculture, Vellanikkara, Thrissur during 2019-21. The facilities at the All-India Network Project on Agricultural Acarology (AINPAA) and Pesticide Residue Testing Laboratory, Department of Agricultural Entomology were availed for the conduct of the research programme. The objectives of the study were to investigate the susceptibility of *Tetranychus okinawanus* to novel acaricides and to study the biochemical basis of acaricide resistance in *T. okinawanus.*

This chapter describes the materials, procedures and techniques used to perform different experiments in accordance with the objectives of the study.

3.1. COLLECTION AND REARING OF *Tetranychus okinawanus*

3.1.1. Field survey

Purposive surveys were conducted in commercial horticultural nurseries of Thrissur district (Table 1) (Plate 1). Leaves of the desert rose, *Adenium obesum* heavily infested with spider mites (Plate 2) from different nurseries were collected in polythene bags. Labels describing the locality and date of the collection were placed inside each bag and tied with rubber bands. The acaricide usage history was recorded by interviewing the growers and labourers. The mite infested samples were brought to the laboratory immediately after collection.

3.1.2. Maintenance of mite culture

Single gravid female mite from each sample was used to establish separate isoline cultures in the Acarology laboratory. The gravid female (Plate 3) was transferred to mulberry leaf placed upside down on a wet sponge in a plastic tray using a fine camel hair brush and allowed to multiply. Each isoline was assigned a unique accession number. The cultures were maintained in the laboratory (Plate 4) without further

pesticide selection, under constant light condition at 25± 3°C temperature and 70± 5% relative humidity (RH). Leaves were replaced with fresh ones in 3 - 4 days intervals.

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 on a glass slide. To establish the species identity, morphological characterisation of the slide mounted specimens was carried out. The shape of the aedeagus was used as the key character for species level identification (Plate 5) (Henderson, 2001). The accessions identified as *T. okinawanus* alone were used for toxicological and biochemical studies.

Table 1. Survey on *Adenium* plants in commercial nurseries in Thrissur

Sl. No.	Population	Accession code	Location of collection
1	Susceptible	SSP	10.548250°N, 76.282602°E
2	National Rose Garden, Therambam	NrAd1	10.568043°N, 76.268549°E
3	Mangadan Botanical Garden, Madakkathara	MgAd2	10.559274°N, 76.264941°E
4	Ayyappa Agri Farm, Mannuthy	AyAd3	10.537708°N, 76.259794°E
5	Saranamayyappa Nursery, Mannuthy	SyAd4	10.560810°N, 76.262221°E
6	Pooja Garden & Nursery, Mannuthy	PjAd5	10.536577°N, 76.260876°E
7	Adenium Gardens, Manalur	MnAd6	10.488395°N, 76.106569°E

(a)

(b)

(c)

(d)

(e)

(f)

Plate 1. Surveys in different horticultural nurseries (a) Mangadan Botanical Garden (b) Saranamayyappa Nursery (c) National Rose Garden (d) Pooja Gardens and Nursery (e) Adenium Gardens (f) Ayyappa Agri Farm

Plate 2. Mite infested leaves of *Adenium*

Plate 3. Adult gravid females of spider mite

Plate 4. Maintenance of mite culture

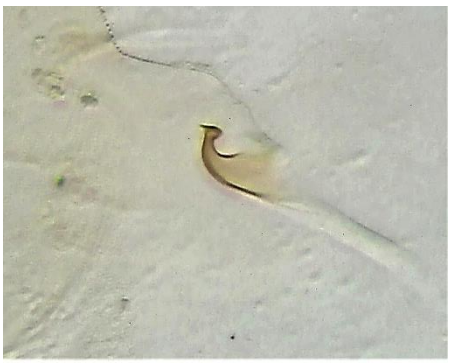

Plate 5. Aedeagus of *Tetranychus okinawanus*

3.1.4. Rearing of susceptible population of *Tetranychus okinawanus*

A laboratory population maintained in the acarology laboratory without any pesticide exposure for the past three years (around 90 generations) was designated as the susceptible population. It was reared and maintained on mulberry leaves.

3.1.5. Test acaricides

The acaricides used in this study were commercial formulations of two novel acaricides *viz*., fenazaquin and spiromesifen and a conventional acaricide, dicofol (Table 2) (Plate 6).

Table 2. Details of acaricides tested against *Tetranychus okinawanus*

Acaricide	Trade name	Mode of action	Source
Spiromesifen	Oberon 240 SC	Lipid biosynthesis inhibitor	Bayer
Fenazaquin	Magister 10 EC	Mitochondrial complex I electron transport inhibitor	Dupont
Dicofol	Hilfol 18.5 EC	Unknown	Hindustan Insecticides Ltd.

3.2. EVALUATION OF SUSCEPTIBILITY OF *Tetranychus okinawanus* TO ACARICIDES

Susceptibility of six different field collected populations of *T. okinawanus* to acaricides was tested in the laboratory against protonymph and adult mite (25 gravid female or protonymph/ replication). Adulticidal effect of the acaricides: fenazaquin and dicofol was evaluated following leaf dip bioassay (Plate 7) (Roy *et al*., 2010). The nymphicidal effect of spiromesifen was evaluated by topical application method (Plate 8) (van Leeuwen *et al*., 2004). The susceptible laboratory population maintained in the

acarology laboratory without any acaricide exposure was used for determining the susceptibility to these acaricides.

A stock solution of each acaricide was prepared and diluted serially to obtain required concentrations. A preliminary bioassay was conducted using a wide range of concentration of each acaricide to arrive at the actual concentration required for the bioassay for all the six populations, separately. The concentrations of each acaricide which recorded 20 to 80 per cent mortality of adult/protonymph in broad range bioassay were selected for evaluation of susceptibility in *T. okinawanus*. Bioassay involved a minimum of five concentrations for each population (fixed based on broad range bioassay) and a control, which were replicated thrice.

3.2.1. Susceptibility of *T. okinawanus* to fenazaquin

To evaluate the susceptibility of *T. okinawanus* to fenazaquin, bioassay was conducted on adult female mites following the leaf dip method of bioassay. Here also, concentrations of various acaricides were decided based on broad range assays that gave range of mortalities from 20 to 80 per cent. Mulberry leaf discs measuring 3 x 3 cm^2 were cut and dipped in the respective test chemical solutions for ten seconds. In the control treatment, the leaf disc was dipped in water. After shade drying at room temperature for 30 minutes, the treated disc was placed on a wet cotton pad kept in a Petri dish. The Petri dishes were moistened with distilled water to maintain the leaf disc turgidity. Twenty-five adult females of *T. okinawanus* were transferred to each leaf disc. A thin layer of wet cotton was placed along the perimeter of the leaf disc to create a barrier and prevent mites from walking off the disc, since mite walk-off is commonly reported in laboratory bioassays with *T. okinawanus* (Knight *et al*., 1990). Each concentration and the control treatment were replicated thrice. The number of live and dead mites in each replication was recorded at 24, 48 and 72h after treatment. Survival of individual mites was assessed by probing each mite with a fine brush, observing under a stereo binocular microscope. Mites that could move normally were labelled as alive, while mites that were moribund when touched with a fine brush were scored as dead. Individuals that escaped from leaf discs were excluded from data analyses.

(a) Spiromesifen **(b) Fenazaquin** **(c) Dicofol**

Plate 6. Commercial formulations of acaricides used

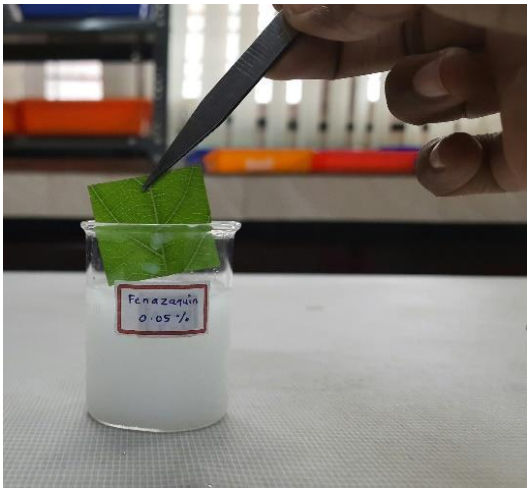

Plate 7. Leaf dip method for adulticidal bioassay

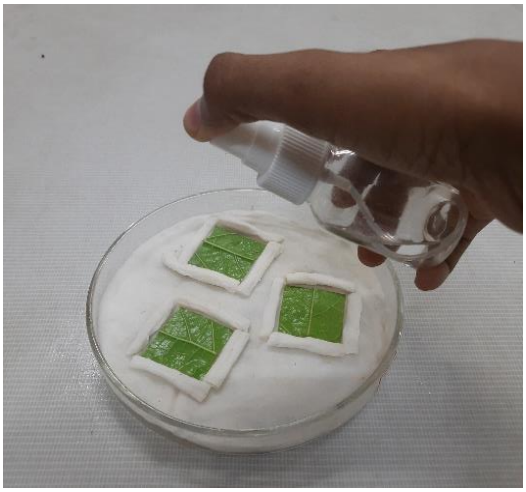

Plate 8. Topical application for nymphicidal bioassay

3.2.2. Susceptibility of *T. okinawanus* to spiromesifen

To evaluate the susceptibility of *T. okinawanus* to spiromesifen, bioassay was conducted on protonymph as adult mites are less susceptible to the acaricide (Marcic, 2012). The bioassay method described by van Leeuwen *et al*. (2004) was followed, with slight modifications. For the bioassay, four to six active female mites were released on a mulberry leaf bit measuring 3 cm x 3 cm, kept on a moist cotton pad in a Petri plate and allowed to oviposit for 24h. The edges of the leaf discs were covered with a thin layer of wet cotton to avoid the migration of the mites. After oviposition, adults were removed using a wet camel hair brush by observing under a stereo binocular microscope. The experimental set-up was kept in laboratory conditions until eggs hatched into larvae and then moulted into protonymphs (Plate 9). In each leaf disc, 30 protonymphs were maintained by removing the excess nymphs by pin pricking. The leaf disc was then sprayed with the treatment concentration using a hand atomizer (2ml/bit). Based on the broad range bioassay, five concentrations of spiromesifen were selected for evaluating the susceptibility. Distilled water spray served as control. Observations on mortality of protonymph (Plate 10) were recorded at 24 h, 48 h and 72 h after treatment.

3.2.3. Susceptibility of *T. okinawanus* to dicofol

To evaluate the susceptibility of *T. okinawanus* to dicofol, bioassay (Plate 11) was conducted on adult female mites following the leaf dip method of bioassay as described in section 3.2.1.

3.2.4. Statistical analysis

As the control treatment did not record any mortality in the bioassay for fenazaquin spiromesifen and dicofol, the mortality data were directly subjected to Probit Analysis (Finney, 1971) by using the software PoloPlus (LeOra Software, 2002) for calculation of median lethal concentration (LC_{50}). The level of resistance of different populations to different acaricides was determined by resistance ratio (RR). The resistance ratio was calculated as the ratio of LC_{50} of field collected population to

the LC_{50} of laboratory maintained susceptible population. The statistical comparison of LC_{50} was performed according to the values of 95% fiducial limit. The RR values of <10, 10-40, 40-60, and >60 indicate low, moderate, high and very high resistance levels, respectively (Fukami *et al*., 1983).

3.3. STUDIES ON BIOCHEMICAL BASIS OF ACARICIDE RESISTANCE IN *Tetranychus okinawanus*

The activity of the detoxifying enzymes *viz*., carboxyl esterase, glutathione S - transferase and cytochrome P450 were estimated in six different field populations (accessions) as well as the susceptible strain of *T. okinawanus* following the spectrophotometric method.

3.3.1. Sample preparation

Samples for analysis of protein and detoxifying enzymes were prepared by homogenizing 20 adult females of *T. okinawanus* in 100µl of sodium phosphate buffer (pH 7.4) in an ice bath and then centrifuged at 10,000 rpm for 20 minutes at 4°C to remove coarse particles. The supernatant was then stored at -20°C and utilized for protein and enzyme assay.

3.3.2. Estimation of protein

Total protein present in the populations of *T. okinawanus* was estimated as per the procedure given by Lowry *et al*. (1951).

3.3.2.1 Preparation of standard Bovine Serum Albumin solution

A stock bovine serum albumin (BSA) solution was prepared by dissolving 50 mg of BSA in 50 ml of double distilled water in a volumetric flask. The working standard was prepared by pipetting out 10 ml of stock solution and making up to 50 ml with double distilled water in a volumetric flask so that, 1 ml of solution contained 200 µg of protein. Different aliquots of 100, 200, 300, 400, 500, 600 and 700 µl were pipetted out in to different test tubes from the working standard and made up to 1 ml

Plate 9. Protonymph stage

Plate 10. Dead protonymphs

Plate 11. Treatment plates

with double distilled water to obtain the concentrations of 20, 40, 60, 80, 100, 120 and 140 ppm, respectively. A test tube with double distilled water alone served as blank. The reagents used are given in Table 3.

Table 3. Reagents for protein estimation

Sl. No.	Reagents	Particulars
1	Reagent A	2% sodium carbonate in 0.1 N sodium hydroxide
2	Reagent B	0.5% copper sulphate solution in 1% sodium potassium tartarate solution
3	Reagent C	50 ml of reagent A and 1 ml of reagent B, prepared just prior to the use
4	Reagent D	Folin-ciocalteu reagent (FCR) diluted in 1:1 ratio with double distilled water before use

Five millilitres of reagent C were added to all the test tubes, including Blank. The contents in the test tube were mixed well and allowed to stand for 10 minutes. Afterwards, Reagent D (0.5 ml) was added, mixed thoroughly and incubated at room temperature in dark condition for 30 minutes. The absorbance of the developed blue colour was recorded at 660 nm using spectrophotometer (Model: Agilent Cary 60 UV Vis®) (Plate 12). The standard graph was drawn by using the optical density (OD) values and the corresponding concentrations of BSA (Fig. 1).

3.3.2.2. Total protein estimation

Fifty microlitres of supernatant/ enzyme extract prepared (3.3.1.) were taken in a test tube and 2.5 ml of reagent C was added. After an incubation period of 10 min, 250 µl of reagent D was added. The reaction mixture was kept in the dark at room temperature for 30 min (Plate 13). Absorbance readings (OD) were measured at 660

nm in spectrophotometer. Protein content was calculated from the standard graph and expressed in mg/ ml.

3.3.3. Estimation of carboxylesterase

The activity level of general esterase was estimated using α- naphthyl acetate as substrate, following the method described by van Asperen (1962).

3.3.3.1. Preparation of α-naphthol standard

Stock solution of 10 millimolar (mM) α-naphthol (Merck) was prepared by dissolving 0.03605 g of α -naphthol in 25 ml methanol. Working standards of varying concentrations (100 µmol, 200 µmol, 300 µmol, 400 µmol, 500 µmol and 600 µmol) were prepared by pipetting out different aliquots (10 µl, 20 µl, 30 µl, 40 µl, 50µl and 60 µl) from the stock solution and made up to 1 ml with methanol. Two millilitres of extraction buffer (sodium phosphate buffer, pH 7.4) was added to working standards. A test tube with phosphate buffer alone served as blank. The reaction mixture was incubated for 10 min. at 30°C with constant stirring. The reaction was stopped by adding dye solution (50 µl) containing 22.5 mg fast blue RR salt (SRL, Mumbai) in 2.25 ml double-distilled water: 5 per cent sodium dodecyl sulphate (SDS) in double distilled water (2:5 v/v). The mixture was then kept for another 5 min. at 37°C for the red colour development. The intensity of red colour was measured in spectrophotometer at 600 nm. The standard curve was prepared with OD values and the corresponding concentrations (Fig. 2).

3.3.3.2. Estimation of Carboxylesterase Activity

Fifty micro litres of enzyme supernatant (3.3.1.) was incubated with the substrate, 1 ml of 30 mM α-naphthyl acetate dissolved in acetone (0.028 g α -naphthyl acetate in 5 ml acetone) for 15 min at 25°C. The procedure as described earlier (3.3.3.1**)** for the preparation of the standard curve was followed for the estimation of carboxylesterase. General esterase activity was expressed as micromoles per minute per milligram of protein.

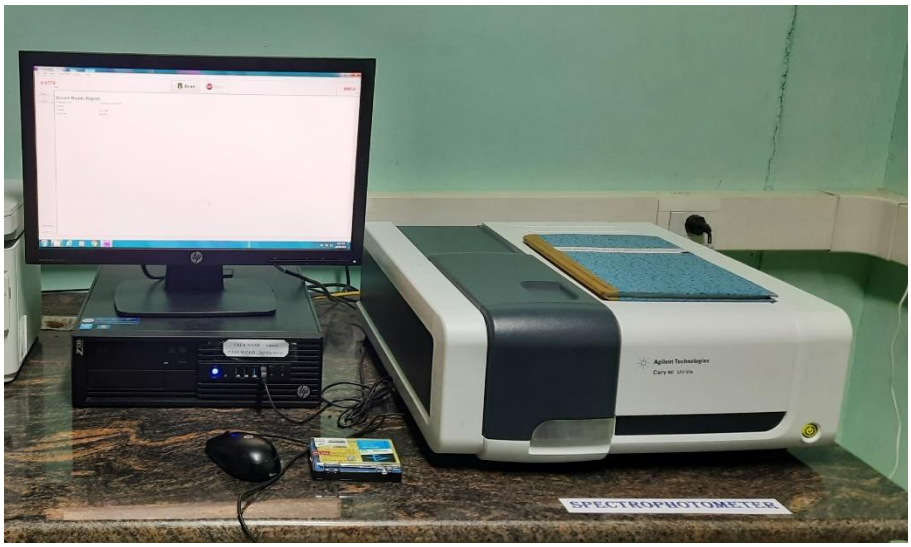

Plate 12. Spectrophotometer (Model: Agilent Cary 60 UV Vis®)

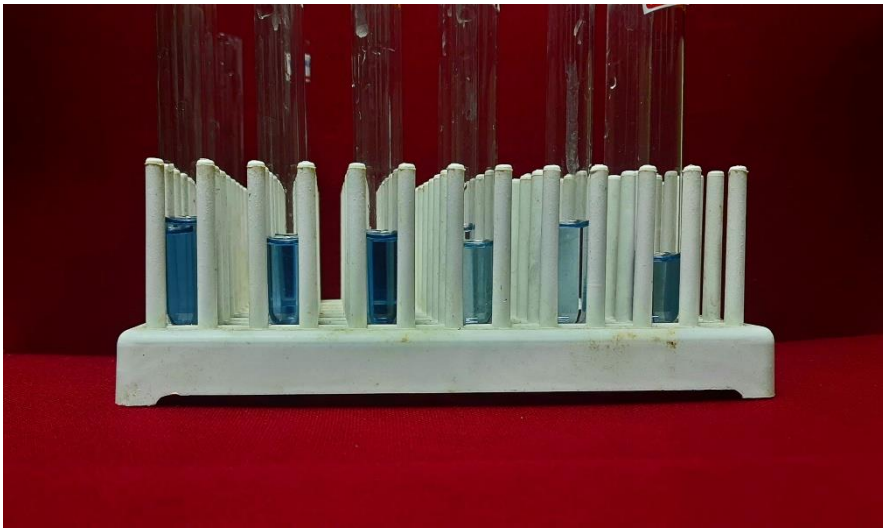

Plate 13. Estimation of total protein in different populations of *Tetranychus okinawanus*

3.3.4. Estimation of cytochrome P450

The level of cytochrome P450 was measured according to the method described by Brogdon *et al*. (1997) with slight modifications.

3.3.4.1. Preparation of cytochrome C standard

To make a stock solution of 0.025 mM concentration, pure cytochrome C from the bovine heart (Sigma Aldrich) (3.081 mg) was dissolved in 10 ml double distilled water. The stock solution prepared was used to make working standards ranging from 0.025 nanomolar (nM) to 0.2 nM. Hundred microliters of each working standard were taken separately in a test tube, and 1 ml of 0.05% TMBZ (SRL, Mumbai) (3, 3', 5, 5'-tetramethylbenzidine), 400 µl of potassium phosphate buffer (pH 7.2) and 125 µl of 3% hydrogen peroxide were added. TMBZ 0.05 per cent was prepared by dissolving 10 mg of TMBZ in 5 ml absolute methanol mixed with 15 ml 0.25 nm sodium acetate buffer (pH 5). The reaction mixture was incubated for 30 min. The absorbance was measured with UV spectrophotometer at 630 nm. Standard graph was drawn using the OD values and corresponding cytochrome C concentrations (Fig. 3).

3.3.4.2. Estimation of cytochrome P450 activity

To 50 µl of enzyme extract (3.3.1), 500 µl of TMBZ (0.05%), 200 µl potassium phosphate buffer (pH 7.2) and 62.5 µl of hydrogen peroxide (3%) were added. After 30 min. of incubation, absorbance was recorded using spectrophotometer at 630 nm. Cytochrome P450 activity was calculated from the standard graph and expressed in pmol mg protein^{-1} min^{-1}.

3.3.5. Estimation of glutathione-S- transferase (GST) activity

The activity level of glutathione-S- transferase was measured following the method described by Kao *et al*. (1989). A reaction mixture of 50µl of 50 mM 1-Chloro-2, 4-Dinitro Benzene (CDNB) (0.020 g in 2 ml ethanol) and 150 µl reduced glutathione (GSH) (0.077 g in 5 ml sodium phosphate buffer, pH 6.5) were mixed with 2.75 ml of sodium phosphate buffer (100 mM, pH 6.5). Fifty microliters of prepared enzyme extract were then added to the mixture and shaken well. After incubation for 2 - 3 min.,

3 ml of each reaction mixture were transferred to a cuvette for reading the absorbance in UV spectrophotometer. The reaction mixture without enzyme served as blank. Absorbance at 340 nm was recorded for 5 minutes at 30 second intervals employing kinetics (time scan) menu on the spectrophotometer. The GST activity was calculated using the formula

$$\text{CDNB-GSH conjugate (µmol mg protein}^{-1}\ \text{min}^{-1}) = \frac{\Delta \text{ Abs in min x 3 x 1000}}{\text{9.6* x 5 x mg of protein}}$$

(*9.6 mM/cm is the extinction coefficient for CDNB-GSH conjugate at 340 nm).

3.3.6. Statistical analysis

Data were statistically analysed by Analysis of variance (ANOVA) in SPSS 16.0 in CRD and means were separated by Tukey's test.

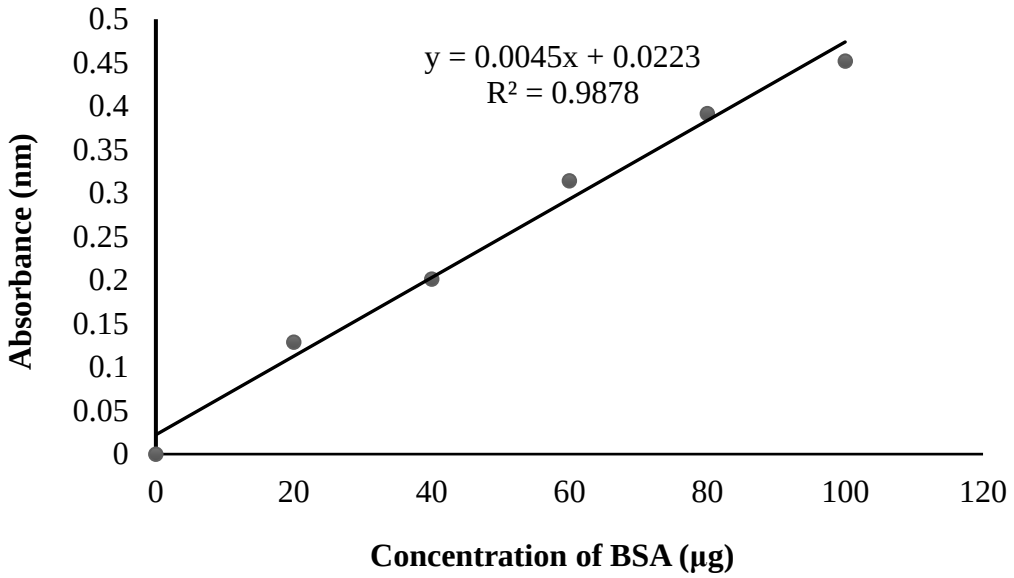

Fig. 1: Standard curve of bovine serum albumin (BSA) for protein estimation

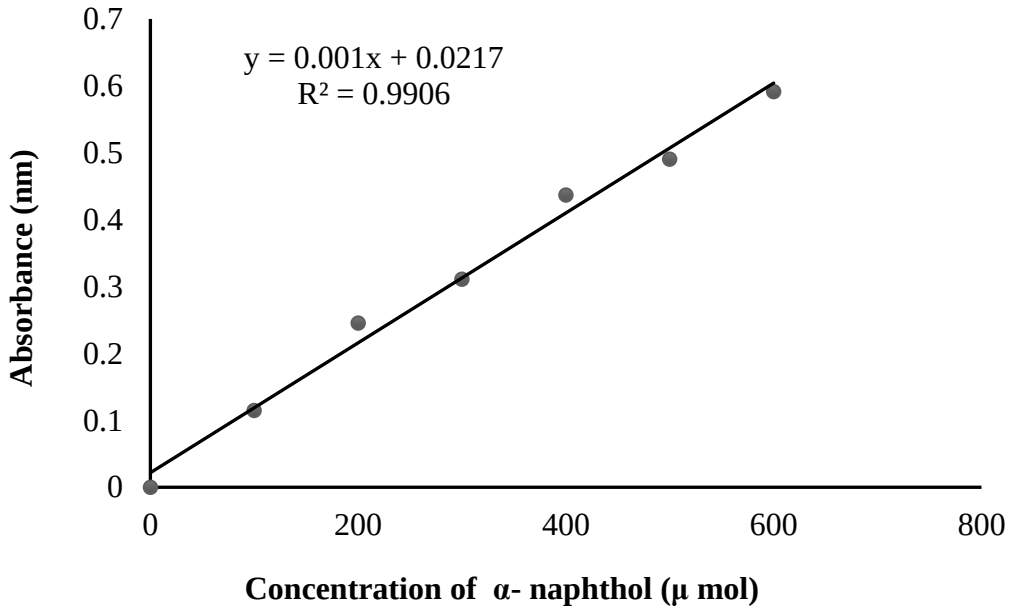

Fig. 2: Standard curve of α- naphthol for carboxyl esterase estimation

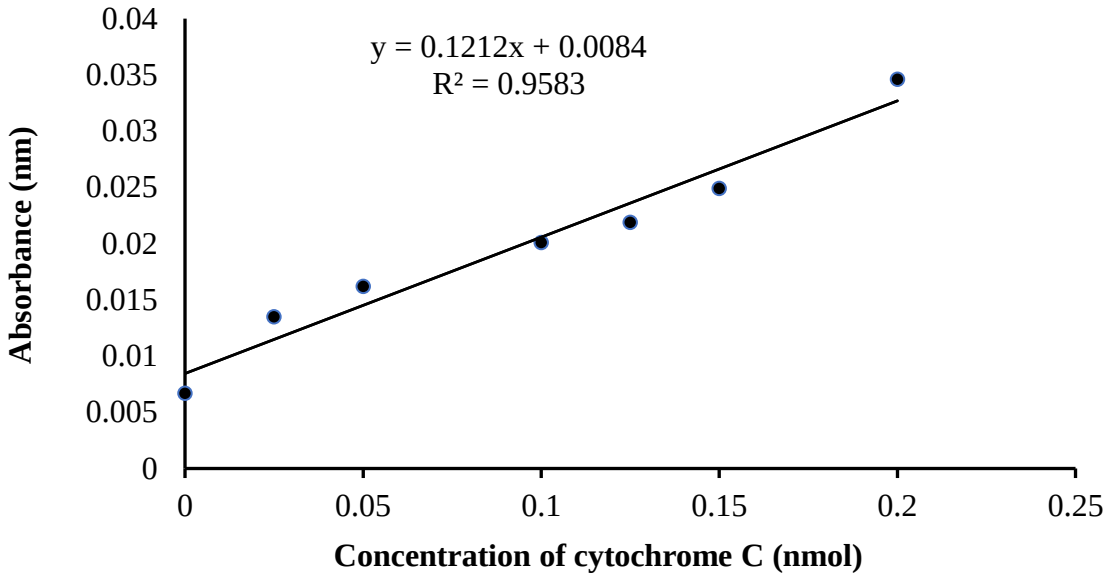

Fig. 3: Standard curve of cytochrome C for cytochrome P450 estimation

Results

4. RESULTS

The results of the study entitled "Susceptibility of *Tetranychus okinawanus* Ehara (Prostigmata: Tetranychidae) infesting ornamental plants to novel acaricides" are presented hereunder.

4.1. COLLECTION AND REARING OF *Tetranychus okinawanus*

Surveys were conducted in six commercial horticultural nurseries in Thrissur districts and the spider mites collected from *Adenium* from each nursery was maintained as isoline culture with a unique accession number. Morphological characterisation of the slide mounted mite specimens from the six isoline cultures showed that all the six accessions were *Tetranychus okinawanus* Ehara (Plate). The history of acaricide usage collected from different nurseries are shown in the Table 4.

Table 4. History of acaricide usage in different nurseries

Sl. No.	Name of the nursery	Acaricide usage
1	National Rose Garden, Therambam	Spiromesifen & fenazaquin frequently
2	Mangadan Botanical Garden, Madakkathara	Not using any chemicals
3	Ayyappa Agri Farm, Mannuthy	Diafenthiuron and fenazaquin occasionally
4	Saranamayyappa Nursery, Mannuthy	Not using any chemicals
5	Pooja Garden & Nursery, Mannuthy	Not revealed history of acaricide usage
6	Adenium Gardens, Manalur	Rational rotation of acaricides, botanicals and Horticultural mineral oil

4.2. SUSCEPTIBILITY OF FIELD POPULATIONS OF *Tetranychus okinawanus* TO ACARICIDES

Susceptibility of *T. okinawanus* population collected from different commercial horticultural nurseries of Thrissur district to three commonly used acaricides like spiromesifen, fenazaquin and dicofol were evaluated in the laboratory in comparison with the reference susceptible strain maintained in the laboratory following toxicological bioassay. LC_{50} values were calculated by probit analysis with the help of PoloPlus software. All field-collected populations showed different levels of susceptibility to selected acaricides.

4.2.1. Susceptibility of *Tetranychus okinawanus* to fenazaquin

Adulticidal assay was carried out to assess the susceptibility of different populations of *Tetranychus okinawanus* to fenazaquin. The results of the toxicity studies of fenazaquin to different strains of *T. okinawanus* are showed that, the LC_{50} value in different populations ranged from 1.937 to 27.856 ppm (Table 5). Among the different field populations, the accession NrAd1 recorded the highest LC_{50} value (27.856 ppm), followed by the accessions PjAd5 (18.792 ppm), AyAd3 (7.878 ppm), MnAd6 (7.341 ppm), SyAd4 (6.254 ppm) and MgAd2 (3.223 ppm). The susceptible population (SS) recorded an LC_{50} value of 1.937 ppm.

The resistance ratio was estimated by comparing the LC_{50} values of the field populations with that of the susceptible population. Among the different populations, the accession NrAd1 recorded a significantly higher resistance of 14. 38-fold followed by PjAd5 (9.70-fold), AyAd3 (4.06-fold), MnAd6 (3.78-fold), SyAd4 (3.23-fold) and MgAd2 (1.66-fold).

Differences in toxicity were considered significant when 95% fiducial limit (FL) did not overlap (Adams *et al*., 1990). However, the data showed that there is overlap between individual values of fiducial limit in the case of SS and MgAd2. Thus, there are no significant differences in susceptibility between these two populations. Fiducial limits of all other strains at LC_{50} and LC_{90} was significantly higher over the

susceptible strain. The chi-square (χ^2) value was below the table value, which indicated that the used mite population was homogenous.

4.2.2. Susceptibility of *Tetranychus okinawanus* to spiromesifen

Nymphicidal assay was carried out to assess the susceptibility of different populations of *Tetranychus okinawanus* to spiromesifen. The LC_{50} value in different populations ranged from 0.258 to 7.046 ppm (Table 6). Among the different field populations, the accession NrAd1 recorded the highest LC_{50} value (7.046 ppm) followed by the accessions PjAd5 (1.852 ppm), MnAd6 (1.017 ppm), AyAd3 (0.459 ppm), MgAd2 (0.367 ppm) and SyAd4 (0.274 ppm). The susceptible population (SS) recorded an LC_{50} value of 0.258 ppm.

On comparing the LC_{50} values of the field populations with that of the susceptible population, the accession NrAd1 recorded significantly higher resistance of 27.31-fold followed by PjAd5 (7.18-fold), MnAd6 (3.94-fold), AyAd3 (1.78-fold), MgAd2 (1.42-fold) and SyAd4 (1.06-fold).

Since individual values of the fiducial limit of two populations, SyAd4, and MgAd2, overlap with that of susceptible strain, there are no substantial differences in the toxicity of spiromesifen between these populations and the susceptible population. Fiducial limits of all other strains at LC_{50} were significantly higher over the susceptible strain. The chi-square values of all the bioassays were lower than the table value. So, the populations were homogenous.

4.2.3. Susceptibility of *Tetranychus okinawanus* to dicofol

The data revealed that, all the field collected populations of *T. okinawanus* have a low level of resistance to dicofol compared to other tested acaricides. LC_{50} value for various populations ranged from 28.20 to 84.673 ppm. The highest LC_{50} value for dicofol was recorded in the accession NrAd1 (84.673 ppm) followed by PjAd5 (37.533 ppm), MnAd6 (36.587 ppm), AyAd3 (30.414 ppm), SyAd4 (28.992 ppm) and MgAd2 (28.200 ppm), respectively. The susceptible population recorded an LC_{50} of 23.170 ppm (Table 7).

Based on the LC_{50} values, the accession NrAd1 had the highest resistance ratio of 3.65 followed by PjAd5 (1.62-fold), MnAd6 (1.58-fold), AyAd3 (1.31-fold), SyAd4 (1.25-fold) and MgAd2 (1.22-fold).

Since individual values of the fiducial limit of some populations, such as MnAd6, SyAd4, and MgAd2, overlap with that of susceptible strain, there are no substantial differences in the toxicity of dicofol between these populations. Fiducial limit of all other strains at LC_{50} were significantly higher over the susceptible strain. The chi-square values of all the bioassays were lower than the table value. So, the populations were homogenous.

Table 5. Susceptibility of different populations of *Tetranychus okinawanus* to fenazaquin

Population	LC_{50} (ppm) (95% fiducial limit)	LC_{90} (ppm) (95% fiducial limit)	Slope	Heterogeneity		Resistance ratio (RR)
				d. f.	χ^2	
SS	1.937 (1.60-2.24)	7.642 (5.87-11.68)	2.150	3	0.54	_
NrAd1	27.856 (23.76-33.49)	148.041 (99.22-283.99)	1.767	4	2.46	14.38
MgAd2	3.223 (1.94-4.65)	15.397 (8.99-62.33)	1.887	3	5.29	1.66
AyAd3	7.878 (7.42-8.41)	11.587 (10.41-13.85)	7.650	4	4.55	4.06
SyAd4	6.254 (5.95-6.52)	9.187 (8.67-9.91)	7.673	4	1.23	3.23
PjAd5	18.792 (15.31-21.98)	79.731 (60.19-127.03)	2.042	3	1.12	9.70
MnAd6	7.341 (7.04-7.67)	10.657 (9.82-12.02)	7.916	3	1.64	3.78

LC_{50} = Concentration (ppm) calculated to give 50 per cent mortality; LC_{90} = Concentration (ppm) calculated to give 90 per cent mortality

Table 6. Susceptibility of different population of *Tetranychus okinawanus* to spiromesifen

Population	LC_{50} (ppm) (95% fiducial limit)	LC_{90}(ppm) (95% fiducial limit)	Slope	Heterogeneity		Resistance ratio (RR)
				d. f.	χ^2	
SS	0.258 (0.19-0.34)	0.651 (0.45-1.67)	3.185	3	3.12	_
NrAd1	7.046 (6.33-7.92)	10.366 (8.86-15.78)	7.642	3	6.15	27.31
MgAd2	0.367 (0.33-0.40)	0.940 (0.76-1.32)	3.144	3	2.43	1.42
AyAd3	0.459 (0.35-0.56)	0.915 (0.68-2.65)	4.278	3	2.76	1.78
SyAd4	0.274 (0.18-0.40)	0.973 (0.57-5.91)	2.327	3	6.12	1.06
PjAd5	1.852 (1.64-2.09)	5.549 (4.46-7.63)	2.689	3	2.85	7.18
MnAd6	1.017 (0.81-1.20)	2.566 (1.97-4.39)	3.188	3	3.26	3.94

LC_{50} = Concentration (ppm) calculated to give 50 per cent mortality; LC_{90} = Concentration (ppm) calculated to give 90 per cent mortality

Table 7. Susceptibility of different populations of *Tetranychus okinawanus* to dicofol

Population	LC_{50} (ppm) (95%fiducial limit)	LC_{90} (ppm) (95% fiducial limit)	Slope	Heterogeneity		Resistance ratio (RR)
				d. f.	χ^2	
SSP	23.170 (17.06-29.53)	70.376 (48.86-165.75)	2.656	3	4.53	_
NrAd1	84.673 (81.43-88.52)	121.836 (111.88-139.03)	8.110	3	2.53	3.65
MgAd2	28.200 (24.98-31.89)	86.116 (67.84-124.17)	2.643	3	2.39	1.22
AyAd3	30.414 (25.46-36.62)	69.990 (53.22-120.49)	3.541	3	3.73	1.31
SyAd4	28.992 (24.01-34.95)	65.647 (50.25-111.09)	3.611	3	4.10	1.25
PjAd5	37.533 (33.51-42.52)	119.491 (93.10-173.93)	2.548	4	3.06	1.62
MnAd6	36.587 (30.65-43.09)	79.401 (61.39-142.10)	3.808	3	3.95	1.58

LC_{50} = Concentration (ppm) calculated to give 50 per cent mortality; LC_{90} = Concentration (ppm) calculated to give 90 per cent mortality

4.3. BIOCHEMICAL BASIS OF ACARICIDE RESISTANCE IN *Tetranychus okinawanus*

To determine the biochemical mechanism of resistance, the level of detoxifying enzymes *viz*., carboxyl esterase, glutathione S-transferase and cytochrome P450 in different populations of *T. okinawanus* were determined in the field collected populations as well as in the susceptible population.

4.3.1. Estimation of protein

Lowry's method was followed to determine the total protein content in various populations of *T. okinawanus*. Among the various populations, the accession NrAd1 recorded the highest protein content of 0.964 mg/ml followed by PjAd5 (0.886 mg/ml), MnAd6 (0.859 mg/ml), SyAd4 (0.829 mg/ml), AyAd3 (0.799 mg/ml), and MgAd2 (0.707 mg/ml). The lowest protein content of 0.705 mg/ml was recorded in the susceptible population (Table 8).

Table 8. Total protein (mg/ml) in the populations of *Tetranychus okinawanus*

Sl. No.	Population	Total protein (mg ml^{-1})
1	SS	0.705 ^{e}
2	NrAd1	0.964 ^{a}
3	MgAd2	0.707 ^{e}
4	AyAd3	0.799 ^{d}
5	SyAd4	0.829 ^{cd}
6	PjAd5	0.886 ^{b}
7	MnAd6	0.860 ^{bc}

Figures followed by same letters are not significantly different at 0.05% level of significance.

4.3.2. Estimation of carboxylesterase

The activity of the enzyme, carboxyl-esterase in different field populations and susceptible population of *T. okinawanus* was determined following the method described by van Asperen (1962). General esterase activity was expressed as micromoles per minute per milligram of protein (Table 9). The accession NrAd1 showed significantly higher activity of 2.805 µMol min^{-1} mg $protein^{-1}$ followed by PjAd5 (2.101 µMol min^{-1} mg $protein^{-1}$), AyAd3 (1.507 µMol min^{-1} mg $protein^{-1}$), MnAd6 (1.253 µMol min^{-1} mg $protein^{-1}$), SyAd4 (0.833µMol min^{-1} mg $protein^{-1}$) and MgAd2 (0.823 µMol min^{-1} mg $protein^{-1}$). The susceptible population (SS) maintained in the laboratory exhibited the lowest carboxyl esterase activity of 0.795 µMol min^{-1} mg $protein^{-1}$.

Carboxyl-esterase activity was significantly higher in NrAd1, PjAd5, AyAd3 and MnAd6 compared to the susceptible strain. The accessions, SyAd4 and MgAd2 exhibited comparable carboxyl esterase activity, which was not significantly different from that of susceptible strain.

Table 9. Carboxyl-esterase activity in the populations of *Tetranychus okinawanus*

Sl. No.	Population	Carboxyl esterase activity (µMol min^{-1} mg $protein^{-1}$)
1	SS	0.795 [e]
2	NrAd1	2.805[a]
3	MgAd2	0.823 [e]
4	AyAd3	1.507 [c]
5	SyAd4	0.833[e]
6	PjAd5	2.101[b]
7	MnAd6	1.253[d]

Figures followed by same letters are not significantly different at 0.05% level of significance.

4.3.3. Estimation of cytochrome P450

The accession NrAd1 recorded the highest cytochrome P450 of activity of 2.757 pMol min^{-1} mg $protein^{-1}$ followed by PjAd5 (2.270 pMol min^{-1} mg $protein^{-1}$). Cytochrome P450 activity in MnAd6, AyAd3, SyAd4 and MgAd2 were 1.880, 1.640, 1.435 and 1.340 pMol min^{-1} mg $protein^{-1}$, respectively. The lowest cytochrome P450 activity was recorded in the susceptible strain (1.320 pMol min^{-1} mg $protein^{-1}$) (Table 10).

The activity of cytochrome P450 was significantly higher in NrAd1 when compared to all other populations. The accessions PjAd5, MnAd6 and AyAd3 also recorded significantly higher cytochrome P450 activity in comparison to the susceptible population. But the accessions, SyAd4 and MgAd2 exhibited comparable cytochrome P450 activity, which were not significantly different from that of susceptible strain.

Table 10. Cytochrome P450 activity in the populations of *Tetranychus okinawanus*

Sl. No.	Population	Cytochrome P450 activity (pMol min^{-1} mg $protein^{-1}$)
1	SS	1.320 [e]
2	NrAd1	2.757[a]
3	MgAd2	1.340 [e]
4	AyAd3	1.640[d]
5	SyAd4	1.435[e]
6	PjAd5	2.270[b]
7	MnAd6	1.880[c]

Figures followed by same letters are not significantly different at 0.05% level of significance.

4.3.4. Estimation of glutathione-S- transferase (GST) activity

The glutathione S- transferase activity in different populations of *T. okinawanus* was determined by the method described by Kao *et al*. (1989).

Glutathione S-transferase activity in all the field strains was at par with that of the susceptible strain (Table 11). The highest GST activity was recorded in the accession, PjAd5 (1.460 µMol min^{-1} mg $protein^{-1}$) followed by NrAd1 (1.400 µMol min^{-1} mg $protein^{-1}$), AyAd3 (1.340 µMol min^{-1} mg $protein^{-1}$), MgAd2 (1.330 µMol min^{-1} mg $protein^{-1}$), SyAd4 (1.276 µMol min^{-1} mg $protein^{-1}$) and MnAd6 (1.239 µMol min^{-1} mg $protein^{-1}$). The lowest cytochrome P450 activity was recorded in the susceptible strain (1.099 µMol min^{-1} mg $protein^{-1}$). However, the enzyme activity in different field populations did not differ significantly from the susceptible population.

Table 11. Glutathione S-transferase activity in the populations of *Tetranychus okinawanus*

Sl. No.	Population	Glutathione S- transferase (µMol min^{-1} mg $protein^{-1}$)
1	SS	1.099[a]
2	NrAd1	1.400[a]
3	MgAd2	1.330[a]
4	AyAd3	1.340[a]
5	SyAd4	1.276[a]
6	PjAd5	1.460[a]
7	MnAd6	1.239[a]

Figures followed by same letters are not significantly different at 0.05% level of significance.

Discussion

5. DISCUSSION

The present study entitled “Susceptibility of *Tetranychus okinawanus* Ehara (Prostigmata, Tetranychidae) infesting ornamental plants to novel acaricides”, was undertaken to determine the susceptibility of different populations of *T. okinawanus* collected from horticultural nurseries of Thrissur district to some of the recommended acaricides like spiromesifen, fenazaquin and dicofol and to investigate the biochemical mechanism of acaricide resistance. The data obtained through these experiments are discussed below in the light of available literature.

5.1. COLLECTION AND REARING OF *Tetranychus okinawanus*

Surveys in different horticultural nurseries of Thrissur district revealed that spider mite is a major limiting factor in the production of *Adenium* plants. The study showed that the mite species collected from Adenium in all the six nurseries surveyed was *Tetranychus okinawanus* Ehara. The spider mite, *Tetranychus okinawanus* was first reported in India on the ornamental plant, *Adenium obesum*, in a horticultural nursery in Thrissur district, Kerala (Zeity *et al*., 2016). Now, *T. okinawanus* has widened its host range and emerged as the predominant species of spider mite infesting vegetables, banana and ornamental plants in Kerala (Lenin *et al*., 2015; Arunima *et al*., 2018; Jayalakshmi *et al*., 2019; Bhaskar, 2019).

Surveys in the nursery, National Rose Garden revealed that mite management on Adenium plants in the nursery relies on the two novel acaricides, spiromesifen and fenazaquin, which were used continuously, as the mite problem persisted in the nursery for several years. This nursery has a well-established propagation unit where large-scale propagation of Adenium plants is being taken up. It also maintains a large number of different varieties of Adenium and supplies Adenium saplings to many other nurseries in Kerala.

The nursery, Adenium Gardens at Manalur is an exclusive nursery for Adenium plants, and the owner of the nursery maintains a good rapport with the College of Agriculture, Vellanikkara, seeking advice on plant propagation and protection

practices. The problem of mite pests in the nursery was brought to the notice of AINP on Agricultural Acarology, KAU Centre, Department of Agricultural Entomology during December, 2017 and since then, was being monitored by the centre. Mite management in the nursery is being undertaken as per the advice of AINPAA which includes rational rotation of acaricides, neem-based botanicals, wettable sulphur and horticultural mineral oil (HMO).

The other horticultural nurseries surveyed maintained only few plants of Adenium. In Pooja Gardens and Nursery, though mite infestation could be noticed on a majority of the plants maintained, the owner and workers did not reveal on the acaricides being used for mite management. In Ayyappa Agri Farm surveyed, mite population could be found only a few plants. Fenazaquin and diafenthiuron were being used here for mite management, whenever mite pest infestation was noticed. In the nurseries, Mangadan Botanical Garden and Saranamayyappa nursery mites were not a problem pest, and hence, acaricides were not used.

5.2. SUSCEPTIBILITY OF DIFFERENT POPULATIONS OF *Tetranychus okinawanus* TO COMMONLY USED ACARICIDES

The present study, presents a comprehensive resistance profile of *T. okinawanus* populations collected from commercial horticultural nurseries of Thrissur district, suggesting that a low to moderate level of resistance has been developed in mite populations against recommended novel acaricides. The laboratory bioassay revealed that the susceptibility of different field populations of *T. okinawanus* varied for each acaricides tested. The field populations were found to have developed resistance to three acaricides like fenazaquin, spiromesifen and dicofol which belong to different classes of acaricides and having different modes of action. This is the first report of the development of resistance in *T. okinawanus* to acaricides from India and the first record of resistance to the acaricides fenazaquin, spiromesifen and dicofol from the world. Earlier, resistance in *T. okinawanus* was reported against flufenoxuron from Japan (Goka *et al.*, 1998).

The high reproductive potential and short duration of the lifecycle of *T. okinawanus* (Takafuji *et al*., 1996) combined with frequent application of acaricides and the possibility of induction of multiple resistance might have driven the observed development of resistance and tolerance to these compounds. The results of this study show that the level of resistance to different acaricides varied among the mite populations collected from different nurseries, which is in agreement with the history of exposure of the mite pest to a particular acaricide.

Though development of resistance in *T. okinawanus* to acaricides has not been widely reported earlier, there are a number of studies on resistance development in other spider mite species. The development of acaricide resistance is accelerated by high reproductive potential, inbreeding, short life cycle, numerous generations in a year and warmer conditions (van Leeuwen *et al.,* 2009). Spider mite resistance to acaricides is a well-documented event (Grbic *et al*., 2011). *Tetranychus urticae* in particular has been documented to have evolved resistance to over 95 acaricidal/insecticidal active ingredients (van Leeuwen *et al*., 2010). It has got a dubious reputation to be the "most resistant species" in terms of the total number of pesticides to which populations have become resistant (van Leeuwen *et al*., 2009). Furthermore, *T. urticae* can become fully resistant to new acaricides within two to four years, making it increasingly difficult to manage multi-acaricide resistant *T. urticae* (Grbic *et al*., 2011).

5.2.1. Susceptibility of *Tetranychus okinawanus* to fenazaquin

Fenazaquin belongs to the chemical family quinazolines and it targets the electron transport in the mitochondrial respiratory chain which provides the driving force for ATP synthesis in mitochondria (Hollingworth and Ahammadsahib, 1995). Four METI acaricides including fenazaquin were developed and launched in close succession in 1990's. From then, these gained popularity among growers rapidly due to their quick knockdown effect, long residual activity and their high effectiveness against all life stages of several important mite pest species (Konno *et al*., 1990; Stumpf and Nauen, 2001). However, the possibility of the resistance development in mites to these acaricides also has become a matter of concern from the outset.

METI resistance was first reported in *T. kanzawai* (Kishida) which was collected from tea fields in Japan (Ozawa, 1994), within a few years of launching the acaricide. Rapid evolution of resistance to METIs in phytophagous tetranychids is a serious and increasing problem that has been reported in several geographical regions and many crops worldwide (Croft and van de Baan 1988, van Leeuwen *et al*., 2009).

The mite population collected from the nursery, National Rose Garden at Therambam, (NrAd1) developed a moderate level of resistance, recording 14.38-fold resistance to fenazaquin, which is the highest resistance ratio for fenazaquin in the study. The mite population in Pooja Gardens and Nursery also recorded a significant level of resistance (9.70-fold) to fenazaquin. However, populations from other nurseries were found to have developed a low level of resistance to fenazaquin (Fig. 6).

Log dose-probit (ld-p) lines could be used as a tool to compare the susceptibility of different populations of the mite to a particular acaricide. When the ld-p lines of field collected, populations were compared to those of susceptible populations in terms of fenazaquin, a significant rightward shift was detected, indicating the development of fenazaquin resistance in field collected populations (Fig.7).

The history of acaricide usage collected from different nurseries revealed that, fenazaquin was one of the commonly used acaricides for the management of mites infesting ornamental plants. The results of this study clearly demonstrated that *T. okinawanus* collected from different horticultural nurseries have developed moderate to low level of resistance to fenazaquin. This is the first report on the resistance development of *T. okinawanus* to fenazaquin.

Fenazaquin resistance in two spotted spider mite, *T. urticae* was reported way back in 2001 by Devine *et al*. in a population collected from hops, which showed 77-fold resistance to fenazaquin. Resistance of European red mite, *P. ulmi*, to fenazaquin was studied under laboratory conditions and resistance ratios ranged from 19.8 to 28.8 when compared with the susceptible reference populations (Auger *et al*., 2003). Later, a low level of resistance (7.2-fold) to fenazaquin in *T. urticae* was reported by Kim *et al*. (2004). A Belgian field strain of *T. urticae* showed 35-fold resistance to fenazaquin compared to the susceptible strain (van Pottelberge *et al*., 2009). The studies conducted

in Iran, revealed that the populations of *T. urticae* from Isfahan and Yazd regions recorded very high level of resistance of 3109 and 439.5 folds, respectively, against fenazaquin, while a population from Rasht recorded only 10.53 folds resistance, in comparison to susceptible population (Moghadam *et al*. 2012). Fenazaquin resistance in *T. urticae* was also reported from Cyprus on tomato (310-fold) and rose (189-fold) (Vassiliou and Kitsis 2013).

Resistance to fenazaquin has also been reported in spider mites from different parts of India. Low to moderate level of resistance (5 to 32-fold) to fenazaquin was reported earlier from Bangalore district of Karnataka in *T. urticae* on tomato (Anonymous, 2009). Sharma and Bhullar (2018) studied the status of acaricide resistance in field collected *T. urticae* from vegetable growing areas of Punjab and reported low to moderate levels of resistance (6.67 to 24.65-fold) to fenazaquin. The study conducted by Srinivasa and Khadri (2018) in Karnataka revealed that the levels of resistance in *T. urticae* to fenazaquin remained moderate to high at different locations. A similar report of resistance in *T. urticae* against fenazaquin was obtained in a study conducted recently from Punjab on *T. urticae* infesting cucumber under protected cultivation, where the population developed high level of resistance (62.52 to 212.55 folds) (Kaur and Bhullar, 2019). Very low level of resistance (1.7-fold) to fenazaquin was observed in *Oligonychus coffeae*, collected from conventionally managed Assam tea plantations (Roy *et al*., 2018). Fenazaquin resistance (3.62 to 4.26-fold) in *T. urticae* was also reported from Himachal Pradesh by Titiksha (2019).

Recently, Bachhar *et al*. (2019) reported the development of resistance in *T. truncatus* on okra (13- fold) and amaranthus (5.53-fold) from Thrissur district, Kerala, which showed low to moderate level of resistance to fenazaquin.

5.2.2. Susceptibility of *Tetranychus okinawanus* to spiromesifen

Ketoenols form a novel class of insecticides/acaricides that chemically belong to the spirocyclic tetronic acid derivatives. Spiromesifen is a compound derived from spirocyclic tetronic acids, and it has been commercialised for the control of mites (Rauch and Nauen, 2002) acting on fecundity, fertility and development of mites,

possibly *via* inhibiting lipid biosynthesis, through selective and potent inhibition of acetyl-CoA carboxylase (Bretschneider *et al*., 2007; Kontsedalov *et al*., 2009).

In this study, nymphicidal assay was followed for assessing the susceptibility of the populations *T. okinawanus* to spiromesifen. Studies have demonstrated that, immature developmental stages of spider mites were more susceptible to spiromesifen compared to adult females. The activity of spiromesifen against adult females was reported to be slower and it took several days for the adult to die after treatment (Çobanoğlu and Kandiltas, 2019).

The findings of this investigation showed that *T. okinawanus* populations from different horticultural nurseries have evolved low to moderate levels of resistance to spiromesifen. (Fig. 6). This is the first report on the resistance development of *T. okinawanus* to spiromesifen. The mite population collected from the nursery, National Rose Garden at Therambam, (NrAd1) developed a moderate level of resistance, recording 27.31-fold resistance to spiromesifen, which is the highest resistance ratio for spiromesifen in this study. The mite population in Pooja Gardens and Nursery also recorded a significant level of resistance (7.18-fold) to spiromesifen. However, populations from other nurseries were found to have developed only a low level of resistance to spiromesifen. The ld-p lines for spiromesifen showed the same trend as that of fenazaquin. There was a significant shift in the ld-p lines of all the field collected populations towards right compared to the susceptible population, indicating the development of resistance to spiromesifen (Fig. 8).

There are a number of reports on resistance to keto-enols (spiromesifen and spirodiclofen) in spider mites, especially in *T. urticae* (Demaeght *et al*., 2013; Ferreira *et al*., 2015; Herron *et al*., 2018; Kramer and Nauen, 2011; Lee *et al*., 2004; Van Leeuwen and Dermauw, 2016; Wu *et al*., 2019). In Jordan, studies were conducted on spiromesifen resistance development and observed a moderate level of resistance (17.96-folds) in *T. urticae* population collected from cucumber (Al-Antary *et al*., 2012). A similar study conducted by Doker and Kazak (2012) on Turkish populations of *P. citri* using spirodiclofen and reported a moderate level of resistance to spiromesifen. Sharma and Bhullar (2018) reported a moderate level of resistance (11.14 to 21.40

folds) to spiromesifen in *T. urticae*. Yalcin *et al*. (2018) reported that a strain of *T. urticae* collected from strawberry fields in southern Turkey showed low level of resistance to spiromesifen (4.61 to 9.73). Recently, Bachhar *et al*. (2019) reported the development of resistance in *T. truncatus* on okra (8-fold) and amaranthus (7-fold) collected from Thrissur district, Kerala, which showed a low level of resistance to spiromesifen. Badieinia *et al*. (2020) reported that, the populations collected from Urmia and Shahin Dej exhibited a moderate level of resistance (22 and 21-fold) to spiromesifen, respectively. Kaur and Bhullar (2019), observed low to moderate level of spiromesifen resistance in *T. urticae* on cucumber under protected cultivation from Punjab. Recently, Van Leeuwen *et al*. (2021) reported the development of resistance in *T. urticae* population collected from an ornamental greenhouse in Greece.

High level of resistance to spirodiclofen has been reported earlier in some laboratory selected strains of *T. urticae* and *P. ulmi* (Demaeght *et al*., 2013; Rauch and Nauen, 2002; Van Pottelberge *et al*., 2009). Sato *et al*. (2016) reported a high percentage of spiromesifen resistant individuals in *T. urticae* population collected from open cultivated rose and chrysanthemum crops in Brazil. Populations of *T. urticae* occurring on tomato in Karnataka showed an extremely high resistance (431 to 969-fold) to spiromesifen (Srinivasa and Khadri, 2018).

5.2.3. Susceptibility of *Tetranychus okinawanus* to dicofol

Dicofol is an organochlorine pesticide that is chemically related to DDT. It is a nerve poison and has excellent acaricidal action and residual effect against spider mites. The exact mode of action of dicofol is not known (Roy *et al*., 2018).

The study revealed that, all the field collected populations of *T. okinawanus* has developed low level of resistance to dicofol as compared to other tested acaricides. The mite population collected from the nursery, National Rose Garden (NrAd1) developed a significantly higher resistance ratio of 3.65, which is the highest resistance ratio for dicofol in this study. Populations from other nurseries recorded resistance ratios ranging from 1.22 to 1.62 (Fig. 6). The ld-p lines of all the populations with dicofol except that of the accession, NrAd1, were parallel to each other and were almost cluttered together, confirming uniform susceptibility of these populations to dicofol (Fig. 9).

The history of acaricide usage collected from surveyed nurseries revealed that dicofol is not being used against mite pests on ornamental crops in Kerala for long. The results of the bioassay study are also in agreement with this. METI-acaricides usually confers multiple resistance to dicofol (Stumpf and Nauen, 2001). In this study the mite populations collected from different horticultural nurseries have developed a low level of resistance to dicofol even in the absence of exposure to this molecule. A strong correlation of 0.891 was found between fenazaquin resistance and resistance to dicofol in *T. okinawanus* populations, suggesting multiple resistance between these compounds. So, the low level of resistance to dicofol observed in *T. okinawanus* populations in the nurseries might be due to multiple-resistance with fenazaquin.

Korean population of *T. urticae* showed high level of resistance to dicofol with a resistance ratio of 82 (Cho *et al*., 1995). Resistance to dicofol was reported earlier in *P. ulmi* population collected from apple orchards (Kumral and Kovanci, 2007). In 2008, Kumar reported a very high level of resistance to dicofol in*T. urticae* population from Bangalore (767 to 3690-folds) and Kolar districts (500 to 6491-folds) which were continuously exposed to dicofol. A medium level of resistance to dicofol was reported in Turkish populations of *P. citri* in a study conducted by Doker and Kazak (2012)

Low level of resistance to dicofol (1.79 to 3.11-fold) was reported in field collected population of *T. urticae* in Ethiopia (Gutu *et al*., 2015). Bioassay studies conducted by Patil (2015) indicated that, *T. urticae* infesting grapes had developed a low level of resistance to dicofol (7.04-fold). Srinivasa and Khadri (2018) monitored the acaricide resistance in *T. urticae* in four different districts of Karnataka and reported a high level of resistance to dicofol in the mites collected from tomato crop in all four districts (143 to 1038 -folds). A high level of resistance to dicofol (65.38-fold) was also reported in *Oligonychus coffeae* collected from conventionally managed Assam tea plantations (Roy *et al*., 2018). Mohin (2020) recorded a very high level of resistance to dicofol (2231.8-fold) in *T. urticae* population collected from the tomato fields of Chikkamagaluru and Shivamogga districts of Karnataka.

5.3. BIOCHEMICAL BASIS OF ACARICIDE RESISTANCE IN *Tetranychus okinawanus*

Today the major emphasis in resistance research lies on unravelling the underlying mechanisms of resistance development in an attempt to use this knowledge to control the development and spread of resistant populations. There are many possible adaptations that permit a mite to survive lethal doses of an acaricide. The majority of cases involve changes in the sensitivity of the target site due to point mutations, or metabolism of the insecticide before it reaches the target site due to quantitative or qualitative changes in major detoxification enzymes (esterases, P450 monooxygenases and glutathione-S-transferases) (van Leeuwen *et al*., 2010).

In the present study, to elucidate the biochemical basis of resistance in *T. okinawanus* to different acaricides, the activity of detoxifying enzymes like carboxyl esterases, cytochrome P450 and glutathione-S-transferases were determined in the field collected populations and compared with that of the laboratory maintained susceptible population. The enzymes, carboxyl-esterases and cytochrome P450 monooxygenases had shown increased activity in the populations of *T. okinawanus* collected on adenium from different horticultural nurseries, clearly indicating its role in the development of resistance to fenazaquin and spiromesifen. The involvement of these detoxifying enzymes in acaricide resistance in mite populations has been well documented (van Leeuwen *et al*., 2010).

5.3.1. Activity of carboxylesterase

Carboxylesterases are members of a superfamily of serine hydrolases that hydrolyse chemicals containing functional groups such as carboxylic acid ester, amide and thioesters. These are widely distributed in microbes, plants and animals (Satoh and Hosokawa, 2006). Most pesticides in use today are esters of substituted phosphoric, carbamic or cyclopropane carboxylic acids, and are consequently subjected to degradation by esterases (Devonshire, 1991). Esterase-mediated insecticide resistance has been reported in more than 30 pest species (Bass and Field, 2011).

In this study, the results of the enzyme assay showed that the level of carboxylesterase enzyme was 1.03 to 3.52-fold higher in field populations of *T. okinawanus* compared to the susceptible population. The mite population collected from the National Rose Garden recorded a highest esterase activity of 3.52-fold. In bioassay studies, this population recorded a significantly higher level of resistance of 14.38 and 27.31-fold to fenazaquin and spiromesifen, respectively. The population from Pooja Gardens and Nursery also recorded an increased enzyme activity (2.6-fold) and the population showed 9.7-fold resistance to fenazaquin. It also showed 7.18-fold resistance to spiromesifen. A significant increase in carboxylesterase activity was also found in the mite populations collected from Ayyappa Agri Farm (1.89-fold) and Manalur Adenium Gardens (1.57-fold), where 4.06 and 3.78-fold resistance was recorded against fenazaquin and 1.78 and 3.94-fold against spiromesifen.

The two populations from Saranamayyappa Nursery and Mangadan Botanical Garden, showed esterase activity comparable to the susceptible population (1.04 and 1.03-fold, respectively) which recorded a resistance ratio of 3.23 and 1.66, respectively in the case of fenazaquin and 1.06 and 1.42 in the case of spiromesifen (Fig. 10).

Carboxyl-esterases hydrolyse chemicals containing carboxyl esters to the corresponding component alcohols and acids (Satoh and Hosokawa, 2006). From the chemical structure of spiromesifen, it would be evident that the carboxyl ester bond is present in the acaricide molecule (Fig. 4), where carboxyl-esterase would act and hydrolyse the molecule to less toxic components. Hence, development of resistance in the populations of *T. okinawanus* in the present study to spiromesifen could be due to the higher esterase activity.

Metabolic resistance *via* carboxyl-esterase activity is reported to be one of the main mechanisms of resistance to cyclic keto-enols, including spiromesifen and spirodiclofen (Demaeght *et al*., 2013; Wei *et al*., 2019; Pan *et al*., 2018). Synergism and enzymatic assays also have previously pointed out the involvement of carboxylesterase in spirodiclofen resistance in spider mites. Spirodiclofen is a lipid biosynthesis inhibitor, of the same IRAC MoA class as spiromesifen (van Pottelberge *et al*., 2009). In a study conducted to investigate the mechanism of spiromesifen

resistance in *P. ulmi*, the enzymatic assay indicated that esterases are likely to be involved in resistance (Badieinia *et al*., 2020).

Elevated levels of esterases also contribute to fenazaquin resistance as increased carboxyl-esterase activity has also been reported for METI resistance in *T. urticae* by van Pottelberge *et al*. (2009). Kim *et al*. (2004) showed a correlation between esterase activities and fenpyroximate (METI acaricide) resistance in *T. urticae*. Sharma *et al*. (2019) observed that esterase enzyme activity plays an important role in fenazaquin resistance in *T. urticae*. Moghadam *et al*. (2012) studied resistance mechanisms of three Iranian populations of two spotted spider mite to fenazaquin and found that esterase activities in the Isfahan, Yazd and Rasht populations were 3.9, 1.8 and 1.5-fold more than that in the susceptible population. Bachhar (2019) examined the mechanism of fenazaquin and spiromesifen resistance in *T. truncatus* and observed 2.59-fold esterase activity in the resistant strain, compared to laboratory maintained susceptible strain. Roy *et al*. (2018) also reported that general esterases exhibited higher activity in mite populations from the conventionally managed tea plantation in Assam.

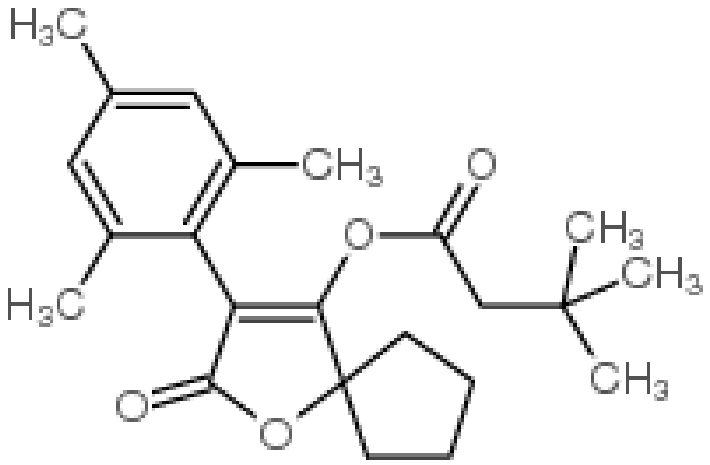

Fig. 4: Structure of spiromesifen

5.3.2. Activity of cytochrome P450

Cytochrome P450 monooxygenases (CYPs) (or Mixed function oxidases (MFOs), or microsomal oxidases) belong to a protein superfamily of heme-containing multifunctional enzymes that catalyze the mono-oxygenation of many xenobiotics and endogenous compounds such as drugs, plant toxins and pesticides. CYPs have been implicated in the insecticide resistance of many pests (Feyereisen, 2005). These are found to catalyse a variety of reactions, including epoxidation, hydroxylation, N-dealkylation, O-dealkylation, and desulphurization. As a result, they play an important role in the metabolism of several classes of insecticides/acaricides, including carbamates, organophosphates, pyrethroids, METI acaricides and neonicotinoids (Stumpf and Nauen 2001; Yu, 2008; Puinean *et al*., 2010; Alptekin *et al*., 2016).

The results of the enzyme assay conducted in field populations showed that the activity of cytochrome P450 was significantly higher in the mite population collected from National Rose Garden (2.08-fold) when compared to all other populations. In bioassay studies, this population has recorded a significantly higher level of resistance of 14.38 and 27.31-fold to fenazaquin and spiromesifen, respectively. The population from Pooja Gardens and Nursery recorded the second-highest cytochrome P450 activity (1.72-fold) and the population showed 9.7-fold resistance to fenazaquin. It also showed 7.18-fold resistance to spiromesifen. The increase in cytochrome P450 activity over susceptible strain was also found significant in mite populations collected from Adenium Gardens (1.40-fold) and Ayyappa Agri Farm (1.24-fold). But the mite populations from Saranamayyappa Nursery and Mangadan Botanical Garden exhibited comparable cytochrome P450 activity (1.08-fold and 1.01-fold respectively), which were not significantly different from that of susceptible strain (Fig. 10). The increase in cytochrome P450 activity in the field populations collected in the present study was significant, indicating that it plays an important role in the resistance to fenazaquin and spiromesifen in *T. okinawanus*.

5.3.2.1. Role of cytochrome P450 in the detoxification of fenazaquin

Cytochrome P450 has been reported to be important in imparting resistance to METI acaricides. The result of the present study also suggests that metabolism by

cytochrome P450 is involved in the fenazaquin resistance in *T. okinawanus*, and confirms to previous studies regarding the correlation of increased P450 activity to METIs complex I resistance in spider mites (Kim *et al.*, 2004; Stumpf and Nauen, 2001; van Pottelberge *et al.*, 2009).

Despite their overall different chemical structure, all METIs including fenazaquin contain heterocyclic rings with at least one tertiary butyl group. The hydroxylation of this group could be a common mechanism of oxidative degradations (Stumpf and Nauen, 2001) (Fig. 5). Reports of cross-resistance among METIs also suggest a common resistance mechanism in this group (Stumpf and Nauen, 2001; van Leeuwen *et al*., 2009).

Kim *et al*. (2004) observed that cytochrome P450 enzyme activity play an important role in fenpyroximate (METI acaricide) resistance in *T. urticae* strains. van Leeuwen *et al*. (2009) studied the biochemical mechanism of METI - acaricide resistance in a Belgian field strain of *T. urticae* and the METI resistant strains showed 23.5-fold increase in the activity of cytochrome P450 compared to the susceptible strain. Cytochrome P450 mediated detoxification was identified as one of the mechanisms of cross-resistance to cyflumetofen and cyenopyrafen (METI-acaricides) in field-collected strains of *T. urticae* (Khalighi *et al*., 2014). Cytochrome P450 enzyme detoxification was found to be the primary mechanism of resistance to pyridaben (METI-acaricide) in the spider mite population examined (Namin, 2017). According to El-Dewy (2018), resistance in *T. urticae* against fenpyroximate was associated with increased level of mixed function oxidase. A significant increase in MFO activity (3.21-fold) was observed in a study conducted to assess the biochemical basis of resistance in laboratory selected fenazaquin resistant strain of *T. urticae* in Punjab, India (Sharma *et al*., 2019). Khanjani *et al*. (2020) observed that cytochrome P450 enzyme play an important role in fenpyroximate resistance in *T. urticae* strains.

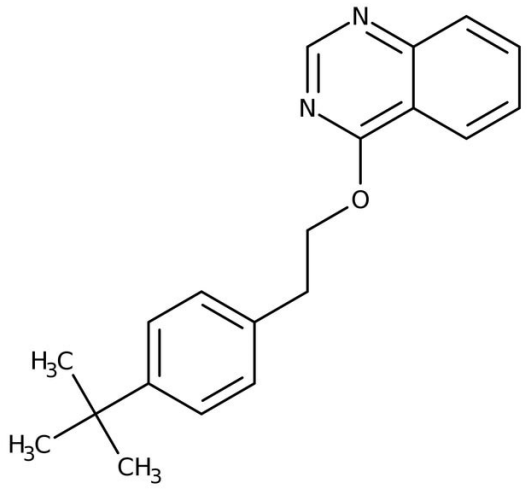

Fig. 5: Structure of fenazaquin

5.3.2.2. Role of cytochrome P450 in the detoxification of spiromesifen

Detoxification by cytochrome P450 has also been correlated to spiromesifen resistance in spider mites by several authors. Spirodiclofen resistance development was studied in *T. urticae* by Rauch and Nauen (2002), where the combined synergistic, biochemical, and metabolism evidence pointed towards an increased oxidative metabolism as the underlying cause. The activity of P450 was higher in spirodiclofen resistant strain of *T. urticae*, indicating that this enzyme played an important role in spirodiclofen resistance (Van Pottelberge *et al*., 2009). Biochemical studies conducted by Farahani *et al*. (2018) revealed that the cytochrome P450 monooxygenase activity was 3.02-fold higher in the spirodiclofen resistant strain of *T. urticae*. Bachhar (2019) examined the mechanism of spiromesifen resistance in *T. truncatus* and observed 2.7-fold esterase activity in the resistant strain, compared to laboratory maintained susceptible strain. Roy *et al.* (2018) examined the resistance mechanism of acaricide resistance in *Oligonychus coffeae*, and they observed fairly high P450 activity in the resistant strain compared with the susceptible strain.

5.3.3. Activity of glutathione-S- transferase

Glutathione-S-transferases (GSTs) are a group of enzymes that are important in the detoxication of many different xenobiotics in insects. The enzymes protect cells against toxicants by conjugating the thiol group of the glutathione to electrophilic xenobiotics. GST conjugates polar products with various endogenous compounds, such as sugars, sulphate, phosphate, amino acids, or glutathione (Yu, 2008) which will aid development of pesticide resistance especially to organochlorine, organophosphate, and pyrethroid groups (Hemingway *et al*., 2004; Das *et al*., 2017).

The present study revealed that the activity of GST did not differ significantly in field collected populations of *T. okinawanus* compared to the susceptible population indicating that, GST is not a contributing factor in the development of resistance in *T. okinawanus* against spiromesifen, fenazaquin and dicofol (Fig. 10).

Biochemical studies conducted in cypermethrin-selected resistant strain of *T. urticae* revealed that the increase in GST activity (1.16-fold) was not significant compared to the susceptible population (Çağatay *et al*., 2018). An increase in resistance to spirodiclofen was detected in field collected Iranian population of *T. urticae* compared to a susceptible population. Biochemical studies revealed that GST had only a minor role in detoxification (1.40-fold) in resistant population of spider mite (Farahani *et al*., 2018).

However, the role of glutathione S-transferase in detoxification of acaricides leading to development of resistance in spider mite populations has been reported by a few workers. Moghadam *et al*. (2012) monitored the resistance mechanisms to fenazaquin in Iranian populations of the two-spotted spider mite, and showed that glutathione S-transferase (GST) activity in the Isfahan, Yazd and Rasht populations was 2.3, 2.4 and 1.4 times more than the susceptible population collected from Rasht, Iran, respectively. The activity of GST was significantly higher (1.81 to 2.3-fold) in the field population of *T. urticae* than in the susceptible strain (Kim *et al*., 2020).

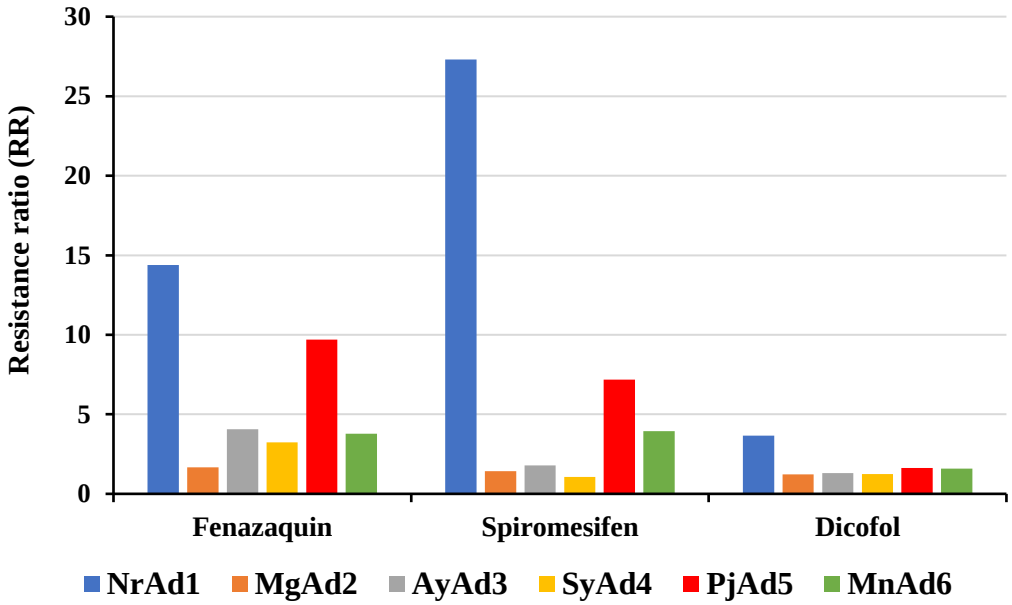

Fig. 6: Relative susceptibility of different populations of *Tetranychus okinawanus*
(Based on resistance ratio)

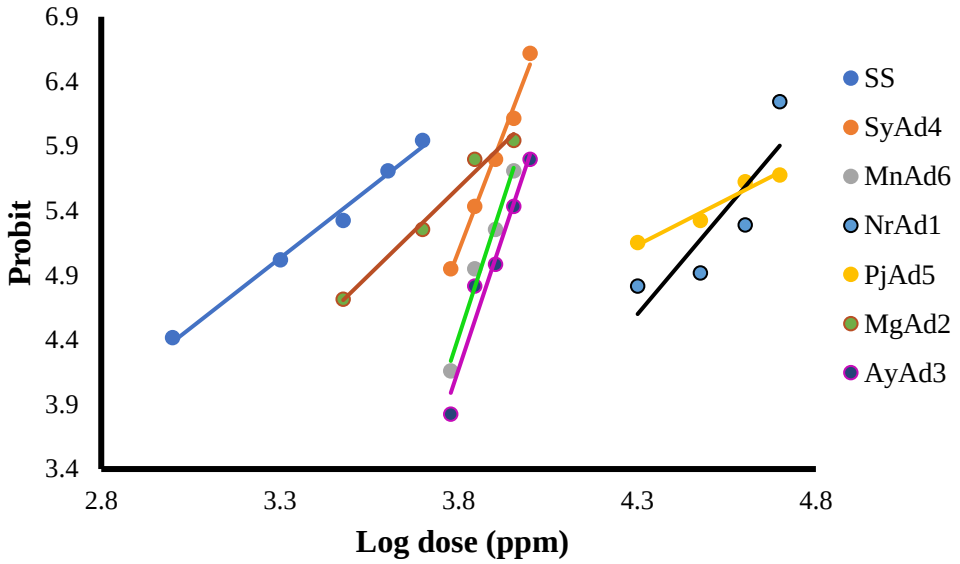

Fig. 7: Log dose - Probit line of fenazaquin

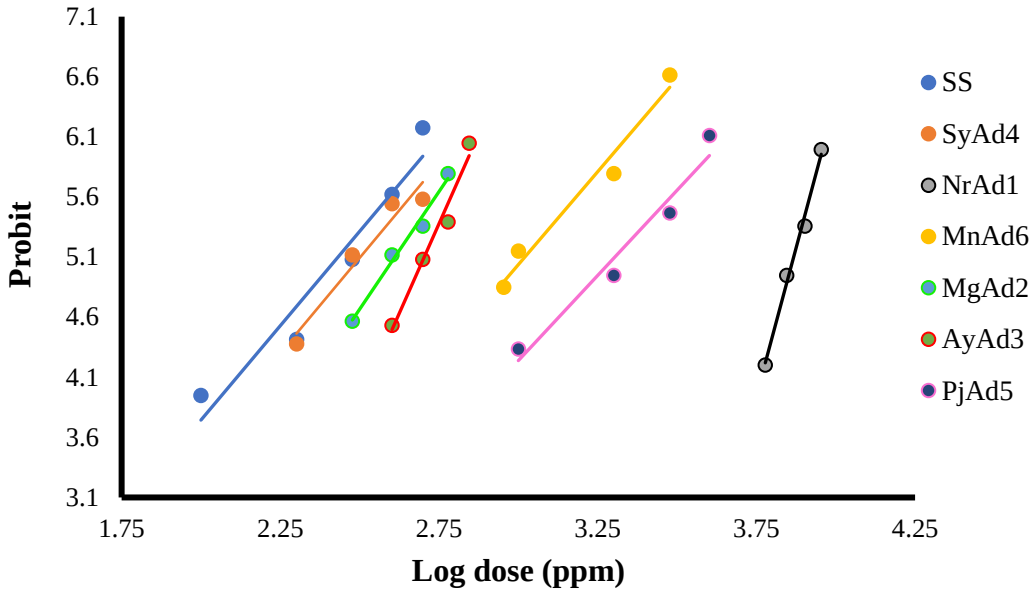

Fig. 8: Log dose - Probit line of spiromesifen

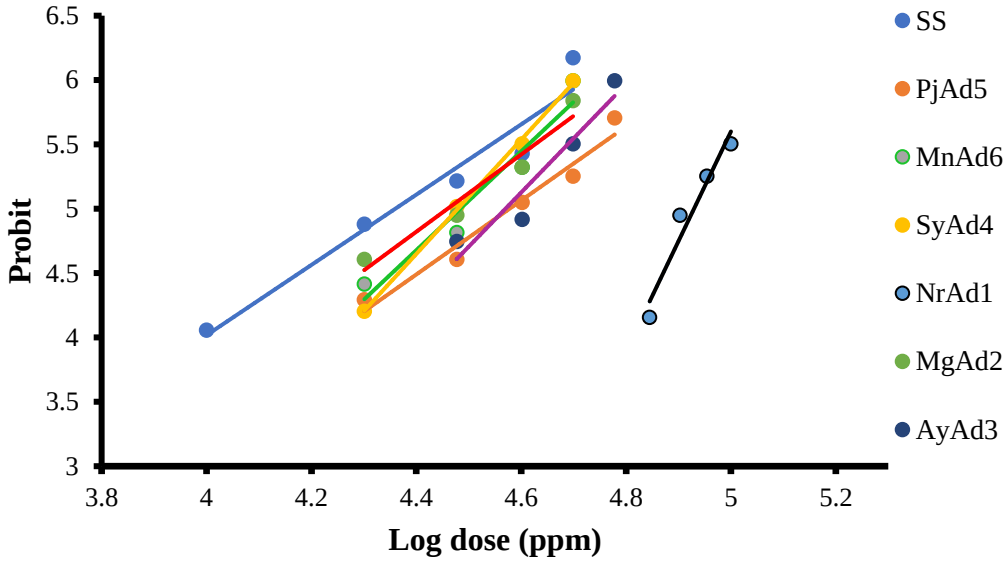

Fig. 9: Log dose - Probit line of dicofol

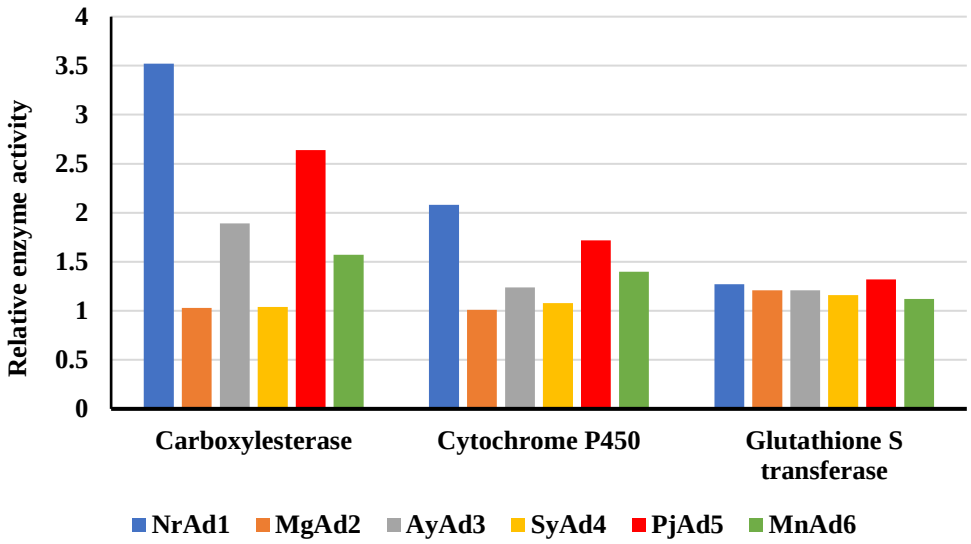

Fig. 10: Relative enzyme activity in different populations of *Tetranychus okinawanus*

Summary

6. SUMMARY

The study on "Susceptibility of *Tetranychus okinawanus* Ehara (Prostigmata: Tetranychidae) infesting ornamental plants to novel acaricides" was carried out at Acarology laboratory and Pesticide Residue Testing Laboratory, Department of Agricultural Entomology, College of Agriculture, Vellanikkara during 2019-21. The study involved evaluation of the susceptibility of populations of *Tetranychus okinawanus* on Adenium collected from different horticultural nurseries in Thrissur district to acaricides through laboratory bioassay and investigation on the biochemical mechanism of acaricide resistance by estimating the activity of detoxifying enzymes in different populations of *T. okinawanus.*

The salient findings of the study are summarised hereunder.

- Susceptibility of six different field collected populations of *T. okinawanus* to acaricides *viz*., fenazaquin, spiromesifen and dicofol was evaluated in the laboratory. The susceptible population maintained in the Acarology laboratory without exposure to any acaricides was used for determining base-line values for susceptibility to these acaricides. The field populations showed varying levels of susceptibility to the selected acaricides.
- The results of the toxicity studies of fenazaquin to different populations of *T. okinawanus* showed that, the accession NrAd1 recorded the highest LC_{50} value (27.856 ppm) followed by the accessions PjAd5 (18.792 ppm), AyAd3 (7.878 ppm), MnAd6 (7.341 ppm), SyAd4 (6.254 ppm) and MgAd2 (3.223 ppm). The susceptible population (SS) recorded an LC_{50} value of 1.937 ppm.
- The populations exhibited resistance ratios for fenazaquin in the range of 1.66 to 14.38. The accession NrAd1 recorded significantly higher resistance of 14. 38-fold followed by PjAd5 (9.70-fold), AyAd3 (4.06-fold), MnAd6 (3.78-fold), SyAd4 (3.23-fold) and MgAd2 (1.66-fold).
- Susceptibility studies of spiromesifen to different populations of *T. okinawanus* also showed that the accession NrAd1 recorded the highest LC_{50} value (7.046 ppm) followed by the accessions PjAd5 (1.852 ppm), MnAd6 (1.017 ppm),

AyAd3 (0.459 ppm), MgAd2 (0.367 ppm) and SyAd4 (0.274 ppm). The susceptible population (SS) recorded an LC_{50} value of 0.258 ppm.

- The populations exhibited resistance ratios for spiromesifen in the range of 1.06 to 27.31. The accession NrAd1 recorded significantly higher resistance of 27.31-fold followed by PjAd5 (7.18-fold), MnAd6 (3.94-fold), AyAd3 (1.78-fold), MgAd2 (1.42-fold) and SyAd4 (1.06-fold).
- Susceptibility studies on dicofol recorded the highest LC_{50} value in the accession NrAd1 (84.673 ppm) followed by PjAd5 (37.533 ppm), MnAd6 (36.587 ppm), AyAd3 (30.414 ppm), SyAd4 (28.992 ppm) and MgAd2 (28.200 ppm) respectively. The susceptible population recorded an LC_{50} of 23.170 ppm.
- The accession NrAd1 had the highest resistance ratio of 3.65 followed by PjAd5 (1.62-fold) in the case of dicofol.
- Activities of detoxifying enzyme *viz*., carboxylesterase and cytochrome P450 were higher in all the six field populations of *T. okinawanus* compared to that of susceptible population.
- The accession NrAd1 showed significantly higher carboxyl esterase activity of 2.805 μMol min^{-1} mg $protein^{-1}$ followed by PjAd5 (2.101 μMol min^{-1} mg $protein^{-1}$). The accessions AyAd3, MnAd6, SyAd4 and MgAd2 recorded esterase activity of 1.507, 1.253, 0.833, and 0.823 respectively. Susceptible population (SS) exhibited the lowest carboxyl esterase activity of 0.795 μMol min^{-1} mg $protein^{-1}$.
- The accession NrAd1 recorded the highest cytochrome P450 activity of 2.757 pMol min^{-1} mg $protein^{-1}$ followed by PjAd5 (2.270 pMol min^{-1} mg $protein^{-1}$). The lowest activity was recorded in the susceptible population (1.32 pMol min^{-1} mg $protein^{-1}$).
- Activity of glutathione S-transferase (GST) did not differ significantly in field collected populations of *T. okinawanus* compared to the susceptible population indicating that, GST is not a contributing factor in the development of resistance in *T. okinawanus* against spiromesifen, fenazaquin and dicofol.
- The study recorded low to moderate level of resistance in different field collected populations of the spider mite, *T. okinawanus* to spiromesifen,

fenazaquin and dicofol, the acaricides belonging to three different chemical groups, for the first time in the world.

- The increased activity of the detoxifying enzymes, carboxylesterases and cytochrome P450 monooxygenases in the field populations of *T. okinawanus* on Adenium indicates the role of these enzymes in development of resistance in the mite species to two commonly used novel acaricides, fenazaquin and spiromesifen.

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SUSCEPTIBILITY OF *Tetranychus okinawanus* Ehara (PROSTIGMATA: TETRANYCHIDAE) INFESTING ORNAMENTAL PLANTS TO NOVEL ACARICIDES

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ABSTRACT OF THE THESIS

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ABSTRACT

Spider mites of the family Tetranychidae are well-known agricultural and horticultural pests that feed on a wide variety of plants, including vegetables, fruit trees, and ornamentals. Many traditional insecticides and acaricides have been used to manage mite pests for several decades. The widespread usage of acaricides around the world has facilitated development of resistance in different mite species, making mite management challenging. As a result, many novel acaricides with distinct chemical structures and modes of action have been developed and marketed for mite control. However, after a few years of use, mite populations developed resistance to these newly introduced compounds too.

The spider mite species, *Tetranychus okinawanus*, recently recorded on an ornamental plant *Adenium* in Thrissur district, for the first time in India, has now emerged as the predominant species of mite infesting ornamental plants in Kerala. Recently, several growers reported inefficacy of the commonly used novel acaricides against mite pests in many ornamental crops, suggesting that the mite populations might have developed resistance to acaricides. Hence a study was carried out to investigate the status of acaricide resistance in *Tetranychus okinawanus* infesting *Adenium* in horticultural nurseries and also to elucidate the biochemical mechanism involved in development of resistance.

Purposive surveys were conducted in six commercial horticultural nurseries in Thrissur district *viz*., National Rose Garden, Mangadan Botanical Garden, Ayyappa Nursery, Saranamayyappa Nursery, Pooja Gardens and Nursery, and Manalur Adenium Garden and samples of spider mite infesting *Adenium* were collected. Mites were maintained as separate isoline cultures assigning unique accession numbers as NrAd1, MgAd2, AyAd3, SyAd4, PjAd5 and MnAd6. Morphological characterisation of the slide mounted mite specimens from the isoline cultures was carried out to confirm the species identity as *T. okinawanus.* Susceptibility of the six field populations to three acaricides *viz*., spiromesifen, fenazaquin and dicofol was evaluated in the laboratory, in comparison with the reference susceptible population maintained without exposure to any acaricides in the laboratory, following toxicological bioassay.

Susceptibility studies with fenazaquin revealed that the accession NrAd1 recorded the highest LC_{50} value (27.85 ppm) and has developed moderate level of resistance (14.38-fold) to fenazaquin. This was followed by PjAd5 (9.70-fold), AyAd3 (4.06-fold), MnAd6 (3.78-fold), and SyAd4 (3.23-fold). The lowest resistance ratio was recorded by the accession MgAd2 (1.66). The toxicity studies of spiromesifen also recorded low to moderate levels of resistance in different populations of *T. okinawanus*. The accession NrAd1 recorded highest resistance ratio of 27.31 followed by PjAd5 (7.18), MnAd6 (3.94), AyAd3 (1.78), MgAd2 (1.42) and SyAd4 (1.06). However, the mite populations showed only low level of resistance to dicofol, recording resistance ratios in the range of 3.65 to 1.22.

Biochemical basis of acaricide resistance in different populations of *T. okinawanus* was investigated by estimating the activity of detoxifying enzymes such as carboxyl-esterase, cytochrome P450 and glutathione S- transferase. Carboxyl-esterase enzyme showed an enhanced activity of 1.03 to 3.52-fold, while cytochrome P450 monooxygenases recorded 1.01 to 2.08-fold higher activity in the field collected populations, compared to the susceptible population. The level of these detoxifying enzymes was found to be higher in the accession NrAd1, which also recorded the highest resistance ratio in the study. However, the activity of glutathione S-transferase (GST) did not differ significantly among the field populations and also with susceptible population, indicating that GST is not a contributing factor in the development of resistance in *T. okinawanus* against spiromesifen, fenazaquin and dicofol.

The study recorded development of resistance in the spider mite, *T. okinawanus* on *Adenium* to spiromesifen and fenazaquin, in the horticultural nurseries in Thrissur district, Kerala for the first time in the world. The significant role of the detoxifying enzymes, carboxyl-esterases and cytochrome P450 monooxygenases in imparting resistance in *T. okinawanus* to the two novel acaricides, fenazaquin and spiromesifen was also confirmed in the study. The study demands formulation of a suitable resistance management strategy in horticultural nurseries in the state for suppressing or delaying resistance development in mite populations.