ELUCIDATING THE BIOCHEMICAL BASIS OF INTERACTION BETWEEN TEA MOSQUITO BUG, *Helopeltis antonii* Signoret (HEMIPTERA: MIRIDAE) AND CASHEW (*Anacardium occidentale*)

By

NIMISHA T.

(2016-11-019)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

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Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY
COLLEGE OF HORTICULTURE
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KERALA, INDIA

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DECLARATION

I, Nimisha, T. hereby declare that the thesis entitled **"Elucidating the biochemical basis of interaction between tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) and cashew (*Anacardium occidentale*)"** is a bonafide record of research work done by me during the course of research and that this thesis has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara
4-09-2018

Nimisha T
(2016-11-019)

CERTIFICATE

Certified that the thesis entitled **"Elucidating the biochemical basis of interaction between tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) and cashew (*Anacardium occidentale*)"** is a bonafide record of research work done independently by Ms. Nimisha, T. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

Vellanikkara
4 -09-2018

Dr. Deepthy, K.B.
(Chairperson, Advisory Committee)
Assistant Professor
Agricultural Entomology
College of Horticulture, Vellanikkara

CERTIFICATE

We, the undersigned members of the Advisory Committee of Ms. Nimisha T., a candidate for the degree of **Master of Science in Agriculture** with major field in Agricultural Entomology, agree that the thesis entitled **"Elucidating the biochemical basis of interaction between tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) and cashew (*Anacardium occidentale*)"** may be submitted by Ms. Nimisha T., in partial fulfilment of the requirement for the degree.

04/9/18

Dr. Deepthy, K. B.
(Chairperson, Advisory Committee)
Assistant Professor
Department of Agricultural Entomology
College of Horticulture, Vellanikkara

4/9/18

Dr. Mani Chellappan
(Member, Advisory Committee)
Professor and Head
Department of Agricultural Entomology
College of Horticulture, Vellanikkara

04/09/18

Dr. Smitha, M. S.
(Member, Advisory Committee)
Assistant Professor
Department of Agricultural Entomology
College of Horticulture, Vellanikkara

04/09/18

Dr. Girija, T.
(Member, Advisory Committee)
Professor and Head
Department of Plant Physiology
College of Horticulture, Vellanikkara

04/09/2018

EXTERNAL EXAMINER

Dr. K. RAMEASH, Ph.D.,
Principal Scientist (Entomology)
ICAR - Central Institute for Cotton Research
Regional Station, Coimbatore - 641 003

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Introduction

1. INTRODUCTION

Cashew (*Anacardium occidentale* L.) is an important plantation crop in India and as an export oriented commodity, earns considerable foreign exchange for the country. India accounts for about 65 per cent of global cashew exports. The earnings from cashew and allied products during 2016-17 stood at Rs 5077 crores (US$ 780.41 million). The realization of its economic importance necessitated scientific investigation in production and protection technologies of cashew.

In spite of its economic significance, cashew in India suffers from low productivity, primarily due to the incidence of pests and diseases. Among the insect pests, tea mosquito bug (TMB), *Helopeltis antonii* Signoret (Hemiptera: Miridae) is the most important. It causes 30-50 per cent yield loss and during outbreak situations even up to 100 per cent loss has been reported (Devasahayam and Nair, 1986).

Tea mosquito bug attacks the cashew crop during critical growth stages such as flushing, flowering and fruit setting. The bug suck sap from tender plant parts leading to the formation of necrotic lesions around the feeding punctures. Severe infestation occurs during the emergence of new flushes and it leads to the scorched appearance of the entire tree (Abraham and Nair, 1981 ; Sundararaju and Sundarababu, 1999).

At present TMB is managed by two to three rounds of sprays with chemical insecticides but spraying operation is a tedious task in hilly terrains. The TMB management by chemical insecticides can only be a short term measure for increasing cashew productivity (Nambiar *et al.*, 1973). Sustainable long term strategies for management of TMB devoid of hazards such as pesticide residues in kernel, alone can offer permanent solution to the bug menace. Such technologies will have to be built on a foundation with plant resistance at its core.

Cashew varieties exhibit wide variation in response to TMB infestation. Hardly any variety has ever been recorded as resistant to TMB till date. However, a few

varieties are reported as capable of withstanding TMB infestation and hence have been grouped as less susceptible category (Ambika *et al.*, 1979; Sathiamma, 1977).

As the plant has got different adaptations to overcome herbivory, the insects also have different mechanisms to overcome plant defences. These counter defences, which often include enzymatic detoxification, physiological tolerance, and behavioral avoidance, protect insects from hazards of xenobiotic compounds (Schuler, 1996). Understanding detoxification enzymes levels in insect gives an indication of plasticity of the pest against host plant defense mechanism. Several biochemicals such as phenols, and oxidative enzymes reportedly play a major role in mediating cashew tea mosquito bug interactions. Equally, important could be the biochemical means employed by the bug to overcome the secondary metabolite mediated defences in cashew. Many studies regarding the susceptibility status of cashew accessions are documented (Beevi and Mahapatro, 2007). Elucidating above areas of interaction between the bug and the cashew can contribute to early detection of TMB resistance in cashew and can then lead to exploitation of the same for management of the bug.

Hence the present study has been proposed with the following objectives

I. To study the variation in secondary metabolites and defence enzymes in selected cashew varieties induced by tea mosquito bug infestation
II. To elucidate the secondary metabolite detoxification mechanisms in tea mosquito bug

Review of literature

17

2. REVIEW OF LITERATURE

Cashew (*Anarcadium occidentale* L), one of the leading tropical tree crop, was introduced to India by the Portuguese nearly five centuries ago. In India, cashew was first introduced to Goa, from where it spread to other parts of the country. The economic importance of this crop derives mainly by way of supplying cashew kernel for consumption, the employment opportunities it generates at the farm level and in agro based cashew processing industries and above all, it act as a promising source of the much needed foreign exchange reserves to the national economy.

Pest infestation is a major constraint in cashew production. The crop is attacked by a number of insect pests during different stages of its growth and development. The tea mosquito bug (TMB) is an important and most serious pest of cashew. Host plant and pest interaction studies are very much needed for formulating efficient management strategies and it contributes much for the resistance breeding programmes. Recently, works in relation with the biochemical interaction between insect pests and its host plants are gaining more importance as it reveal the host range of herbivorous insects.

2.1 DISTRIBUTION AND ECONOMIC IMPORTANCE

Stonedahl (1991) reported that the genus, *Helopeltis* contains 40 recognised species and has a Paleotropical distribution extending from West Africa to New Guinea and Northern Australia. Among the identified species, 26 are restricted to Africa, four are broadly distributed in the Oriental region, four are endemic to the Philippine island, two are distributed to the Malay Peninsula, Sumatra and Java and one species is endemic to each of the following areas *viz.*, South India and Sri Lanka, Pulo Laut island, Sulawesi, New Guinea and Northern Australia. *Helopeltis antonii* first discovered by Antoine Dohrn in Sri Lanka and later in 1858, the scientist Signoret named the species as *H. antonii* and reported

that TMB widely distributed in Indonesia, Vietnam, Sri Lanka. In India more occurred in Southern parts.

According to Devasahayam and Nair (1986), *H. antonii* is the most serious pest of cashew in India, TMB resulted in a yield loss up to 30-40 per cent. Field experiments on yield loss assessment of different plant parts of cashew conducted by Sathiamma (1977) revealed that panicles (48.5%) and fruits (32%) resulted in higher levels of infestation than the young shoots (14%).

2.2 HOST RANGE AND HOST SELECTION

Several authors reported that the TMB, *H. antonii* can fed on an around 39 species of both cultivated crop plants and weeds belonging to 25 families grown in the vicinity of cashew plantations (Table 1).

Table 1. Selected hosts of tea mosquito bug

Sl. No.	Alternate host plant	Major secondary metabolites	Family	Reference
1	Guava (*Psidium guajava L.*)	Pentacyclic triterpenoid, guajanoic acid, β sitosterol, uvaol, oleanoic acid, ursolic acid	Myrtaceae	Fasola *et al.*, 2012
2	Neem (*Azadirachta indica* A. Juss)	Azadirachtin, azadirone, gedunin, meliacarpin, nimbin, phenolic acids, salannin, vilasinin, phenolic acids, nimbidin and nimbidinin,salanno lactame I and II hydroxyazadiradio ne	Meliaceae	Hatti *et al.*, 2014

		alpha and beta, gedunin and diepoxy azadiradione, isomargosinolide & dihydrogedunin,		
3	Tea (*Camellia sinensis* (L.) Kuntze)	Polyphenols and catechin, theanine, caffeine	Theaceae	Li *et al.*, 2017
4	Avocado (*Persea Americana* Mill.)	Alkaloids, triterpenoids, tannins, flavonoids, saponins, polyphenols, avocadenols A-D (1-4) avocadoin, avocadenolA, avocadenol B, 1,2,4-trihydroxynonadecane, and (2R,4R)-1,2,4-trihydroxyheptadec-16-ene	Lauraceae	Lu *et al.*, 2012 and Abubakar *et al.*, 2017
5	Cocoa (*Theabromo cacao* L.)	Oligomeric flavan-3-ols epicatechin, catechin, proanthocyanidins, flavonols ,quercetin	Malvaceae	Damm *et al.*, 2016

		glycosides, methylxanthins theobromine, caffeine, N-phenylpropenoyl amino acids (NPAs), and phenolic acids		
6	Grape vine (*Vitis vinifera* L.)	Flavan-3-ols, anthocyanins, hydroxycinnamates, flavonols stilbenes, cyanidin, delphinidin, peonidin, petunidin, malvidin, catechin, epicatechin, kaempferol, quercetin, myricetin, rutin, galactosides, glucuronides, organic acids and sugars	Vitaceae	Rusjan *et al.*, 2008
7	Annatto (*Bixa orellana* L.)	Sesquiterpenes, monoterpenes, arenes, α-humulene, β mercene, cis ocemene, α-copaene, β caryophyllene	Bixaceae	Giorgi *et al.*, 2013

8	*Lawsonia alba* L.	Eugenol, hexadecanoic acid, phytol, α- terpeneo and eherphenylvinyl	Lythraceae	Manikanta *et al.*, 2013

TMB can thrive in a wide array of host plants having a broad range of secondary metabolites by its efficient detoxification mechanism. Many of these alternate hosts are having pesticidal properties, but TMB became adapted to live in these plants by detoxifying its toxic secondary metabolites. This give an indication that some physiological and biochemical changes occur in these bugs. From the table cited above (Table 1), it is very clear that TMB can survive in host plants of different nutritional composition and secondary metabolites. According to Johnson and Gregory (2006), a particular plant is selected as a host plant, based on the criteria, whether the particular insect is able to detoxify the plant's secondary metabolites.

2.3 NATURE OF DAMAGE

Both the nymphs and adults of TMB suck sap from tender leaves, new flushes, inflorescence and young developing nuts. Nymphs are seen congregating on the feeding sites while the adults were seen solitary. The main symptom of infestation is the development of discoloured necrotic lesion around the point of entry of the stylets within the plant tissues. Along with feeding, the insect injects toxic saliva and resulted in darker necrotic areas around the stylet insertion (Stonedahl, 1991). During first 2-3 h of feeding, a circular spot would be formed around the puncture, and within a period of 24 h, the infested area becomes wider, translucent, light brownish and later develop dark brown sunken spots and dries up. The affected leaves become curled, crinkled and deformed and can curl up. The damage caused by the insect with the help of the piercing and sucking mouth parts caused the tender shoots and nuts to exude resinous gummy secretion which on contact with

air gets hardened. The feeding habit of the insect was studied by Sana and Haq, (1974). According to them intense feeding activity of TMB is observed during night than day as evidenced from the number of feeding punctures/shoot. The most voracious feeder among the life stages TMB was fifth instar nymph and produce the most and the largest feeding lesions (Bhuyan and Bhattacharyya, 2006). However, single adult can make about 150 feeding lesions in a day (Hainsworth, 1952), and a female insect can produce feeding punctures over an area of 412 mm^2/day (Kalita *et al.*, 1995).

2.4 SUSCEPTIBILITY STATUS OF CASHEW VARIETIES TO TMB INFESTATION

Variation in host plant resistance dependent on the species and cultivars (Uthamasamy, 1996). The identification of promising cashew types having tolerance to TMB infestation would be one of the most desirable and eco-friendly non-chemical strategies to manage the pest and increase the productivity. The cashew varieties released from Kerala are grouped according to their susceptibility status (Mahapatro *et al.,* 2006). None of the varieties is resistant to the pest although some of the accessions show low incidence of the tea mosquito bug (Ambika *et al.*, 1979).

All the cashew accessions screened were susceptible to TMB. Because of their early flushing and flowering habit, the accessions, ME-4/4 and 1/64 – Madhuranthakam usually escape from the TMB infestation (DCR, 2008-09).

A modified method for susceptibility classes has been proposed by Beevi and Mahapatro (2007). They screened 68 cashew accessions for 5 consecutive years. The results revealed that none of the accessions found to be resistant to TMB infestation. However, based on degree of susceptibility screening methodology has been developed and cashew accessions classified in to 4 categories such as, less susceptible, moderately susceptible, susceptible and highly susceptible.

Sundararaju and John (1992) screened 32 varieties and 2 accessions, which were found relatively tolerant to TMB under field conditions and also in the laboratory. The highest damage occurred on shoots in most of the cashew accessions. Two accessions (G -11/6 located in an endemic area at Goa and VTH - 153/1 located in the germplasm at Vittal) are moderately susceptible while all the other accessions were highly susceptible.

In order to find out the suitable cashew types and their susceptibility levels against TMB, a study conducted at Cashew Research Station, Jhargram, during 1985 - 86 (Ghosh and Chattetjee, 1987). Among the 17 types, BLA -39-4 and TN-16 gave maximum yield with least susceptibility to TMB attack (10.22 kg/tree and 6 % damage). The highest susceptible level against the TMB was noticed in the types NLR-211 and H-3-17 (0.26 kg/tree and 52% damage) which gave minimum yield during the period of experiment.

Beevi *et al.* (2001) attempted in categorizing the cashew accessions based on damage score in to four groups; less susceptible (0-0.250), moderately susceptible (0.251-0.500), susceptible (0.501-0.750) and highly susceptible (0.751-1.000). Screening of 68 cashew accessions revealed that all the accessions were susceptible to TMB infestation with none resistant/tolerant to the target-insect pest. Accessions Amrutha, Damodar and Raghav were found to be least susceptible (LS), while Priyanka and Anagha were highly susceptible (HS). Damage analysis indicated that, none of the accessions exhibited damage below 10 per cent (very low) (Beevi and Mahapatro 2007). Even though a number of reports are available on susceptibility/tolerance of cashew accessions to *H. antonii* under field condition the exact mechanism of resistance has not been reported so far (Sundararaju, 1996).

2.5 HOST PEST INTERACTIONS

Insects deploy plants to quench their nutritional requirement for survival and fitness. However host plants have developed different defensive techniques *viz.,* morphological, behavioural or biochemical to deter polyphagous insects.

Biochemical defences in plants contribute to the selection process that has led to the diversity in insect pests.

The secondary metabolites or xenobiotics produced by plants negatively affect non adapted phytophagous insects by reducing food digestibility and induce toxicity. At the same time, herbivores have co-evolved by enhancing their detoxification mechanisms to reduce the effect of plant secondary metabolites. More studies have pointed out that insects greatly depend on their detoxification mechanism through defensive enzymes to overcome xenobiotics.

Host plant and pest interaction studies are essential to develop biochemical markers for early detection of resistance in crop plants especially perennial crops like cashew, hence very much useful for resistance breeding programme. Moreover, this kind of study will help to give an insight to the plasticity of host plant defence adaptation towards herbivory.

2.6 BIOCHEMICAL BASIS OF DEFENCE MECHANISM IN HOST PLANT

Plant secondary metabolites are accumulated for defence but are tolerated by adapted insects. Plants developed different modes of defence mechanisms like induction of defensive protein, volatiles that attract natural enemies, secondary metabolites and trichome density. In the same way insects also developed strategies such as detoxification of toxic compounds, avoidance mechanisms, sequestration of poison and alteration of gene expression.

Defensive proteins are also produced by plant in response to herbivore attack. One such study has been conducted by Haruta *et al.* (2001). According to them wound-induced accumulation of trypsin inhibitor protein observed in trembling aspen and the pattern as herbivore defence mechanism. This work established the presence of a functional protein-based inducible defence system in trembling aspen which is a widespread forest tree in North America and is susceptible to damage by a variety of lepidopteran and coleopteran pests. Clausen *et al.* (1989) found a 20 per cent increase in phenolic glycosides upon herbivory by the large aspen tortrix (*Choristoneura conflictana* Walker). In contrast, study conducted by

Shah *et al.* (2014) revealed that tea leaves when infested by *H. theivora* caused reduction in the total protein levels and total carbohydrates when compared to that of healthy plants.

The secondary metabolites *viz.,* phenol and tannin and defensive enzymes *viz.,* PPO and PAL play an important role in imparting resistance against herbivore.

2.6.1 Phenol

Plant phenolics are secondary metabolites involved in the defence mechanisms of plants against insect herbivores. Plants respond to diverse environmental stresses with an array of responses that would affect the susceptibility/resistance characteristics of host plant. According to the study conducted by Naik (2010) the highest content of total phenols recorded in the less susceptible cashew variety Ullal-1 (8.1 mg/g) where as highly susceptible V-4 has got least phenol content (0.3 mg/g fresh weight). This study revealed the importance of total phenols in imparting some degree of resistance to TMB attack.

The phenol content of less susceptible cashew accession (A-6-1) was high (7.522mg/g) upon infestation by tea mosquito bug, where as the phenol content ranged from 1.36 to 3.94 mg/g in the susceptible accessions (Bindu, 1996).

Thorough understanding of plant-herbivore interactions revealed the relevence of induced defence. According to study conducted by Simmonds (2003), salicylates in leaves of salix plant have a role in feeding behaviour of polyphagous larvae of *Operophthera brumeta* L. They observed that the level of salicylates correlated negatively with growth of larva exposed to leaves rich in these compounds.

Another study conducted by Rai and Naghraja (1988) observed phenols and O-dihydroxy phenols content of tender shoot tissue of least susceptible cashew accessions (VTH 2, 4,13 and 151) were significantly higher compared to highly susceptible accessions (VTH 5, 15 and 46). This study concluded that the total phenols and ortho-dihydroxy phenols play a significant role in delayed occurrence

of TMB on the least susceptible entries, probably due to least preference by the pest on these entries in the presence of other preferred varieties/accessions.

2.6.2 Tannin

Tannins are generally considered to be deleterious to phytophagous insects. Tannins could affect the growth of insects in three ways: they have an astringent taste, which affects palatability, and decrease feed consumption; they form complexes of reduced digestibility with proteins and act as enzyme inactivators (Lattanzio *et al*., 2006).

The plants may sense presence of insect herbivore through their salivary secretions. The secondary metabolite content in plants may also vary upon induction by herbivore. Schittko *et al*. (2001) identified the tobacco wound response eliciting jasmonic acid and ethylene production in response to attack by *Manduca sexta* L. This is the path way responsible for production of secondary metabolites like tannin.

According to Osipitan *et al*. (2015) tannin and saponin content in 15 cassava varieties shown negative correlation with population of larger grain borer (*Prostephanus truncates* Horn.), which is a major pest of farm stored maize cobs and dried cassava in Mexico. Chowdhury *et al*. (2016) studied the influence of TMB (*H. theivora*) infestation on the biochemical and physiological changes occur in tea leaves .The results revealed that physiological parameters like polyphenol, chlorophyll a, catechins, reducing sugar, and anti oxidant activity reduced when the shoots infested by TMB.

The study conducted by Klocke and Chan (1982) revealed that *Heliothis zea* Boddie provided with condensed tannin of cotton incorporated diet has got decreased activity of protease enzyme in midgut. The protein and sugar level of insect heamolymph lowered due to the decreased activity of enzyme. Presence of condensed tannin drastically affected efficiency of insect for converting digested matter in to insect biomass. The growth was also inhibited due to reduced food consumption. Another experiment conducted by Bialczyk *et al*. (1999) also

emphasized the importance of tannin in reducing the white fly incidence in tomato seedlings planted in glass house. The increased the tannin content in tomato plants planted inside the glass house by means of providing mechanical wounding, spraying with kinetin solutions, growth regulators, and the atmosphere enrichment to 680 µmol CO_2/mol air. The results suggest that tannins have a dosage-dependent effect on glasshouse whitefly. The glass house whitefly population can minimized by reducing the host plant quality by increasing tannin content.

Some insects will overcome tannin content to continue their feeding. The study conducted by Feeny, (1969) found that winter oak moth (*O. brumata*) has a modified physiological mechanism to overcome tannin content in its host plant. It has developed a high gut pH (9.2) which results in the dissociation of indigestible tannin- protein complexes.

2.6.3 Poly phenol oxidase

Polyphenol oxidase (PPO) is a widely distributed enzyme and probably present in all plants. This enzyme catalyzes oxidation of phenolic compounds results in browning of plants (Lee *et al*., 2007).

Surekha *et al*. (2014) reported that application of *Trichoderma viridae* Pers. in black gram against *Fusrium oxysporum* Schlecht and *Alternaria alternate* (Fr.) Keissl resulted in the induction of plant defensive enzymes peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase.

The infestation of tea leaves by *H. theivora* caused an increase in the oxidative enzymes *viz*; peroxidase (POX) and polyphenol oxidase (PPO) (Shah *et al*., 2014).

The study conducted by Paul and Sarma (2005) revealed that, the increased levels of Peroxidase (PO), catalase, phenylalanine ammonia lyase (PAL) and poly phenol oxidase (PPO) were induced in black pepper treated with *Pseudomonas fluorescens* Flugge, which indicated the systemic protection offered to black pepper by the strains and helped to prevent foliar infection by the pathogen, *Phytophthora capsici* Leonian They also found that the increase in production of defence enzymes upon challenge

inoculation were higher in the non inoculated plants compared to the inoculated plants, indicating the lesser requirement of defence enzymes in the bacterized plants upon encounter with the pathogen.

Study conducted by Beegum, (2015) noticed that susceptible cow pea cultivars have got low PPO activity when compared to the varieties having low percentage damage.

2.6.4 Phenylalninine ammonia lyase (PAL)

Several authors reported that the, phenylalanine ammonia lyase (PAL) performs a major role in the biosynthesis of polyphenolic compounds. Sija *et al.* (2016) carried out to detect the activity of phenylalanine ammonia-lyase in different plant parts of *A. occidentale* such as flower, young leaves, shoot tip, mature leaves, raw cashew nut shell, shoot, root and cotyledons. These compounds are involved in the defense mechanism in harsh environments related to different stimuli. The results revealed that the highest activity rate of PAL was observed in cotyledon and least amount in root. Induction of such systemic resistance and associated biochemical responses is important in biological control.

Anita and Samiyappan (2012) conducted study on defense enzymes phenol, peroxidase (PO), polyphenol oxidase (PPO), phenyl ammonia lyase (PAL), super oxide dismutase (SOD) and chitinase in rice induced by *P. fluorescense* isolate Pf1, against challenge inoculation of *Meloidogyne graminicola* Golden. The results revealed that activity of phenol, PO, PPO, PAL and chitinase was higher in the bacterized rice plants and resulted in significant reduction in nematode infection. Similarly, study conducted by Neethu (2015) revealed that the stress indicator enzyme PAL was also found to be high in moderately resistant banana variety Big Ebanga when compared to highly susceptible Pisang Buntal.

Somegowda *et al.*, (2017) conducted a study to find out enzymatic changes and antioxidant potential of cucumber and chayote after infestation by melon fruit fly. They observed that phenolic content increased by 16.2 per cent as well as apparent doubling of PAL enzyme level in infested plants.

Another study conducted by Khorsheduzzaman et al. (2010) revealed that brinjal shoot and fruit borer (*Leucinodes orbonalis* L.) infestation results in increased level of PAL enzyme activity in brinjal (*Solannum melongena* L.) plant. Where it was observed that those brinjal varieties (TURBO, BLOO9) with higher level of PAL, PPO activity was having lower percentage infestation.

2.7 Role of defensive enzymes in insect

Insects attacking host plants have different mechanisms to overcome plant barriers and one of which includes enzymatic detoxification of plant allelochemicals (Castaneda *et al.*, 2009). Enhanced detoxification mechanisms could be seen in insects feeding on plants with higher level of allelochemicals. Induction of insect detoxifying enzyme activity in response to plant allelochemicals provided clear manifestation of biochemical changes occur inside the host plant and insect and has been documented in several studies. The major metabolic enzymes detoxifying xenobiotics in insects system include carboxylesterase (CarE), glutathione-S-tranferases (GST) and cytochrome P450 (Zhang *et al.*, 2010). The primary role of the numerous enzyme systems in insects is the conversion of lipophilic foreign compounds (xenobiotics) into hydrophilic products, thereby enhancing rates of solubilization and excretion (Ahmad *et al.*, 1986)

Enzymes involved in the detoxification mechanism of oriental tobacco bud worm *Helicoverpa assulta* Guenee the tobacco cutworm *Spodoptera litura* Fab., the beet armyworm *Spodoptera exigua* Hubner, and the tea mosquito bug *H. theivora* on different hosts have been studied (Xue *et al.*,2010; Zhang *et al.*, 2011).

The major defensive enzymes present in an insect that involved in the process of metabolic detoxification of insecticides are carboxyl esterases (CarE), glutathione-S-transferases (GSTs) and cytochrome P450 (Soderlund and Bloomquist, 1990) . According to the study conducted by Saha *et al.* (2012) general esterases (GEs), glutathione-S-transferases (GSTs) and cytochrome P450 mediated mono-oxygenases (CYPs) showed an increased activity in Terai and Dooars populations (*H. theivora*) compared with susceptible Darjeeling population of TMB.

Another interesting fact about detoxification mechanism in insect is that some endo symbionts associated within the insect capable of producing such enzymes. Study conducted by Dowd (1992) identified that fungi associated with bark beetles, ambrosia beetles, termites, leaf cutting ants, drug store beetle has got the capability to metabolize tannins, esters, and other toxins.

2.7.1 Carboxyl esterase

Carboxylesterases are one of the key components of insects xenobiotic defence system including insecticides (Oakeshott *et al.*, 2005). According to Li *et al.* (2007) esterase deserves detailed attention because they could be involved in resistance to the leading chemicals that are extensively used for vector and pest-control programs

Murthy *et al.* (2014) conducted a study on insecticide resistance in *Cotesia vestalis* Haliday, a braconid endolarval parasitoid of the diamondback moth and showed that resistant population with elevated esterase activity. Abamectin-resistant strains of Colorado potato beetle showed significantly higher carboxylesterase activity than in the susceptible strain (Argentine *et al.*, 1992). In insects the esterase are associated with pyrethroid and organophosphate detoxification (Liu *et al.*, 2011). The elevated activity of esterase in *Bemisia tabaci* Gennadius is reported to be a major resistance mechanism against pyrethroid (Young *et al.*, 2006).

Nehare *et al.* (2009) conducted a study with indoxacarb treated *Plutella xylostella* L. for 10 generations to develop a resistant strain and the biochemical analysis of indoxacarb resistance in different tissues of *P. xylostella* was carried out. Biochemical analysis revealed maximum esterase activity in the gut homogenates of indoxacarb resistant strains when compared to the susceptible strain. Detoxifying enzyme assay in *S. litura* collected from Korea Republic revealed that esterase activity varied from 2 to 6 fold among field populations treated with commonly used insecticides (Kim *et al.*, 1998). Another study conducted by Yu and McCord (2007) revealed that the activity of enzyme of general esterase was significantly higher in the field strain than in the susceptible strain of *Spodoptera frugiperda* Smith.

Abdel-Aal and Soderlund (1980) detected the metabolic detoxification of trans and cis isomers of permethrin mediated by esterase in different larval tissues such as cuticle, gut, fat body, head capsule, malpighian tubules and silk gland of southern army worm, *S. eridania*. Xie *et al.* (2011) observed that CarE activity of *B. tabaci* populations after a 3 year host induction on cabbage were the highest compared to the other hosts (poinsettia, cucumber, cotton and tomato) and significantly differed from populations on tomato, cotton and poinsettia.

Esterase based resistance due to qualitative changes in the enzyme has been recorded in *Musca domestica* L. (Diptera: Muscidae). It was noticed that esterase activity to α-naphthyl acetate in organophosphorous susceptible flies was mainly due to choline esterse and ali esterase enzymes. Both the enzymes were more active at higher pH (>8) (van Asperen , 1962).

Experiments conducted by Karuppaiah *et al.* (2017) noticed that the topical bioassay with *S. litura* population collected from Varanasi found to be more susceptible to pyrethroid insecticide when compared to Delhi and Sonepat populations and the highest esterase activity was also high in Delhi and Sonepat populations.

2.7.2 Cytochrome P 450

Cytochrome P450 is another important defensive enzyme having multiplicity and diversity for substrate recognition. It has got tremendous biochemical flexibility in the metabolic profiles of individual organisms. The modes of action of monoxygenase in detoxification mechanism include dealkylation, hydroxylation, deamination, and epoxidation (Schuler, 1996).

Resistance developed in insects by different mechanisms, and important one is increased detoxification mediated by cytochrome P450 monooxygenases (Oppenoorth., 1984). It is found to be important in pyrethroid detoxification in *S. litura* (Haung and Han, 2007). Abamectin resistance in Colorado potato beetles largely resulted from increased cytochrome P450 monooxygenase mediated detoxification in the resistant strains (Clark *et al.*, 1992).

Cytochrome P450 have role in biosynthesis of ecdysteroids and juvenile hormones in insects. It also involved in the metabolism and detoxification of insecticides. Since P450 used by plants (to produce toxins) as well as insects (as means of detoxification) it has importance in insect-plant warfare (Schuler, 1996). Cytochrome P450 play crucial roles in defence against secondary metabolites that insects have to overcome in order to feed plant parts. The ability of an insect cytochrome P450 to metabolize a specific natural product is often the key to the adaptation of insect herbivores to their host plants (Feyereisen, 1999).

Gram pod borer larvae metabolized pyrethroids using two enzyme systems, the monoxygenase and esterases leading to different levels of resistance in population (Gunning *et al*., 1993).

Frank and Fogleman (1992) reported that, four *Drosophila* species endemic to the Sonoran desert breed in alkaloid rich necrotic cactus tissue by overcoming the hazardous effect of the allelochemical. It was possible by the activities of the cytochrome P450 mediated reactions which was responsible for alkaloid metabolism and which induced in response to the presence of cactus alkaloid in diet of *Drosophila* larvae and adults. Many insects detoxify the xenobiotic compounds using cytchrome P450 monoxygenase and glutathione-S-transferases. In corn earworm exposure to xanthotoxin induces P450 expression (Li *et al*., 2000).

In the case of detoxification the study conducted by Krieger *et al*. (1971) revealed that aldrin epoxidation was measured in the gut homogenates of last instar larvae from 35 species of lepidoptera. Polyphagus species had an average fifteen times higher activity than monophagous species. Aldrin epoxidation represents P450 with broad substrate specificity is most abundant in insects that encounter a wide range of host plant metabolites (Berenbaum *et al*., 1992).

Xanthotoxin, phototoxic allomone found in many host plants could induce cytochrome P450 monoxygenase in black swallotail (*Papilio polyxenes* Fab.) when final instar larvae added to diet containing xanthotoxin (Cohen *et al*., 1986).

Recent research has indicated that the mixed-function oxidases of polyphagous insect, southern armyworm (*S. eridania* Stoll) was found to be induced by a diversity of secondary plant metabolites. Following induction, the larva was less susceptible to dietary poisoning. From this study it could argued that mixed-function oxidases play a major role in protecting herbivores against chemical stress from secondary plant substances (Brattsten *et al.*, 1977).

Prolonged ingestion of the same plant secondry metabolite compound induced increased detoxification activities against a particular plant poison in insect herbivores. For example, larval tobacco hornworms (*M. sexta*) experience a rapid increase in cytochrome P450 activity against nicotine after ingesting nicotine. While it is generally assumed that this induction process permited increased consumption of toxic plant tissues. When offered a nicotine diet, larvae failed to show a significant increase in consumption before 36 h, which was coincident with the time course of the induction of midgut P450 activities against aldrin and nicotine. They found that the increase in nicotine consumption following the induction of nicotine metabolism could be strongly inhibited by treatment with piperonyl butoxide, which by itself did not inhibit consumption. These results provide direct evidence for a causal connection between P450 mediated detoxification activity and consumption of a toxic plant compound (Snyder and Glendinning, 1996).

2.7.3 Glutathione- S- transferases

Glutathione-S-transferases, which are involved in the detoxification of wide range of xenobiotics and provides protection from oxidative damage, intracellular transport of hormones, endogenous metabolites and exogenous chemicals including insecticides (Enayati *et al.*, 2005).

Study conducted by Sanil *et al.* (2014) revealed that deltamethrin and cyfluthrin resistant strains of *Anopheles stephensi* Liston have significantly higher GST activity when compared to susceptible strain. They also noticed that larva and pupa of DDT resistant strain showed peak GST activity followed by propoxur resistant strain.

The activity of GST enzyme in *Myzus persicae* Sulzer (green peach aphid) was found to be increased upon infestation, in response to secondary metabolites from brassica plants and determined using different host plant species and confirmed using artificial diet with pure allelochemicals added (Francis *et al.*, 2005).

Kao *et al.* (1989) studied parathion and methyl parathion resistance mechanism in diamond back moth larvae. They found that among laboratory selected susceptible strain of diamondback moth, *P. xylostella*, with methyl parathion resulted in more than 2,600-fold resistance to this insecticide. Significantly higher GST activity towards 1, 2-dichloro-4-nitrobenzene was also detected in the strain selected in laboratory.

Detoxifying enzymes are induced in insects in response to the presence of allelochemicals in plants. The study on fall army worm (*S. frugiperda*) maintained on meridic diet containing various plant substances like sinigrin, glucosinolate and flavones found to have an elevated level of microsomal oxidase and glutathione-S-transferases activity (Yu, 1983).

Another study with diamond back moth, in which indoxacarb was treated to *P. xylostella* for 10 generations to develop a resistant strain and biochemical analysis of insect was carried out. Results of analysis revealed that GST activity was highest in the whole body homogenate and maximum increase was found in the gut homogenates of indoxacarb resistant strains over the unselected (Nehare *et al.*, 2009).

The activity of eleven enzymes involved in development, aging and metabolism of xenobiotics in insects, were investigated under the influence of 2,4-D (2,4 Dichlorophenoxyacetic acid), in the mustard aphid, *Lipaphis erysimi* Kaltenbach, after treating the second instar nymphs for 13, 25 and 37 h. It was observed that the increase in activity of esterases, glutathione-S-transferases, superoxide dismutase and catalase might be due to their involvement in the metabolism and degradation of 2,4-D (Sohal *et al.*, 2008).

Materials & Methods

2. MATERIALS AND METHODS

The present investigation on "Elucidating the biochemical basis of interaction between tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) and cashew *(Anacardium occidentale)*" was carried out at College of Horticulture, Vellanikkara. Facilities at the pesticide residue laboratory, All India Net work Project on Ornithology, Department of Agricultural Entomology, Cashew Research Station, Madakathara, Cocoa Research Centre, Vellanikkara , Centre for Plant Breeding and Molecular Biology Vellanikkara and College of Veterinary and Animal Sciences, Mannuthy were utilized at different stages of the study. The details of the materials used and methods followed are elaborated in this chapter.

3.1 VARIETIES SELECTED FOR THE STUDY

Three months old cashew grafts of four cashew varieties, namely Anagha and Madakkathara-1 which have been reported highly susceptible to TMB and Raghav, Damodar reported as less susceptible, were selected for the experiment. The grafts were maintained in the nursery under ambient conditions.

3.2 COLLECTION AND MAINTENANCE OF TEA MOSQUITO BUG

Stock culture of TMB was established from the field collected (CRS, Madkathara) tea mosquito bug adults (Plate 2a). Cashew seedlings kept inside a cage (60 x 60 x 90 cm) (Plate 2b) were used for rearing the insect (Plate 2c). The eggs were located by their chorionic hairs protruding from plant parts such as the leaf mid rib, shoots, leaf petiole, where the eggs were usually laid.

The technique developed by Sundararaju and John (1992) was followed for multiplication of TMB. Immediately after hatching, the nymphs (Plate 2d) were collected and transferred on to fresh healthy shoots of cashew placed in rectangular plastic boxes (41x 32.5x 23 cm) (Plate 2e). The cut end of shoots were

kept immersed in small glass vials (15 ml) containing water the shoots were held in position by plugging the mouth of vial with cotton. The box was then covered with muslin cloth and held tightly by clips. The tender shoots were replaced every day with new flushes as food.

On emergence as adults, 0-24 h old the female bugs were collected (plate 3a) in test tubes and prestarved for 3 h (plate 3b). Single prestarved adult female bug was released on test varieties (plate 3c) and covered with net cloth (plate 3d). Four plants (representing 6, 24, 48, 72 h) of each varieties used for release of single adult female TMB and plant as well as insect samples were taken before the release of TMB (0 h) as well as at corresponding intervals. The experiment was replicated thrice.

3.3 PREPARATION OF SAMPLES

Samples of plant and insect were collected at different time intervals (0, 6, 24, 48 and 72 h). Middle leaves were selected and weighed to 150 mg, wrapped with aluminium foil and kept in ice box. Liquid nitrogen was used for freeze drying by utilizing the facilities available at Cocoa Research Centre, KAU. Similarly insect samples were also taken at different time intervals (in eppendorf tubes) and freeze dried with liquid nitrogen. At the time of analysis, the samples were taken and homogenized in sodium phosphate buffer (pH 7.4). The supernatant obtained were used for further analysis.

3.4 BIOCHEMICAL ANALYSIS OF CASHEW LEAVES

Tea mosquito bug induced biochemical changes in cashew were studied by analysing variation in, the total protein, secondary metabolites *viz.,* tannin, phenol and defensive enzymes *viz.,* Polyphenol oxidase (PPO) and Phenyl alanine ammonia lyase (PAL). Cashew leaf samples were collected before as well as at 6, 24, 48 and 72 h after exposure to TMB and subjected for analysis by following standard protocols.

3.4.1 Estimation of protein

Total protein present in cashew leaf samples were estimated at 0, 6, 24, 48 and 72 h after release of TMB, as the procedure described by Lowry *et al.* (1951).

Standard bovine serum albumin (BSA) solution:

A stock BSA solution was prepared containing 50 mg BSA in 50 ml distilled water. Working standard was prepared by pipetting out 10 ml of stock solution and making up to 50 ml with distilled water so that 1ml of the solution contained 200µg of protein. From this working standard, different aliquots of 100µl, 200µl, 300µl, 400µl, 500µl, 600µl and 700µl were pipetted out in to different test tubes and made up to 1 ml with distilled water. A test tube with distilled water alone served as blank. The reagents used were detailed below.

Reagent A: 2% sodium carbonate in 0.1N

Reagent B: 0.5% copper sulphate solution in 1% sodium potassium tartarate solution prepared.

Reagent C: Mixture of 50 ml of solution A and 1 ml of solution B, prepared just prior to use.

Reagent D: Folin-ciocalteu reagent (FCR): The commercial FCR was diluted in 1:1 ratio with distilled water before use.

Sample preparation

Leaf sample (middle leaf) of each variety weighed (150 mg), cleaned and wrapped in aluminium foil and freeze dried using liquid nitrogen. The freeze dried samples were stored at -20 ^{0}C. Prior to analysis, each sample was ground in sodium phosphate buffer (7 ml, pH 6.5) with pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min at 4^{0}C. The supernatant obtained was used for protein estimation. To 1.5 ml supernatant, 3 ml sodium phosphate buffer (pH 6.5) as well as 5 ml of reagent C solution were added and allowed to stand at room temperature for 10 to 15 min. Then 0.5 ml of the reagent D was added, mixed well and incubated in dark for 30 min. The blue colour formed (Plate 4) was measured at 660 nm. Standard curve (Fig. 1) was prepared with micro gram of protein on X-axis and absorbance values on Y-axis and amount of protein present in a given unknown sample was calculated from the graph.

3.4.2 Total phenol estimation

The total phenols were determined by the method described by Malik and Singh (1980).

The homogenate was prepared by grinding 0.15g cashew leaf in 1.5ml of 80% ethanol. This homogenate was centrifuged at 10,000 rpm for 20 min and the supernatant was collected in a test tube and kept in hot water bath to evaporate the ethanol. The pellet obtained was dissolved in 1.5 ml distilled water. Folin – Ciocalteau reagent (0.5ml) was added into a test tube containing 0.1ml of sample solution and 2.9 ml distilled water. After heating the mixture for three minutes, 2 ml of Na_2CO_3 (20 %) was added to the test tube (Plate 5) and the absorbance was measured at 650 nm using spectrophotometer (Model-Cary 60 UV vis). The standard curve (Fig. 2) was prepared using known concentrations of catechol. The total phenol content in the test samples was calculated from the standard curve and expressed as mg. catechol equivalent of phenol/ g sample.

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

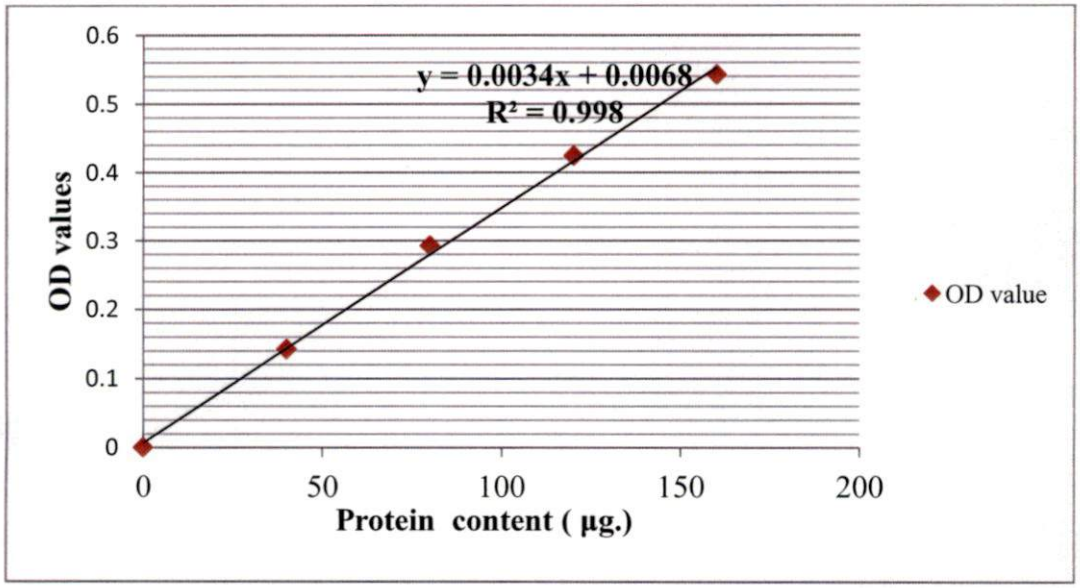
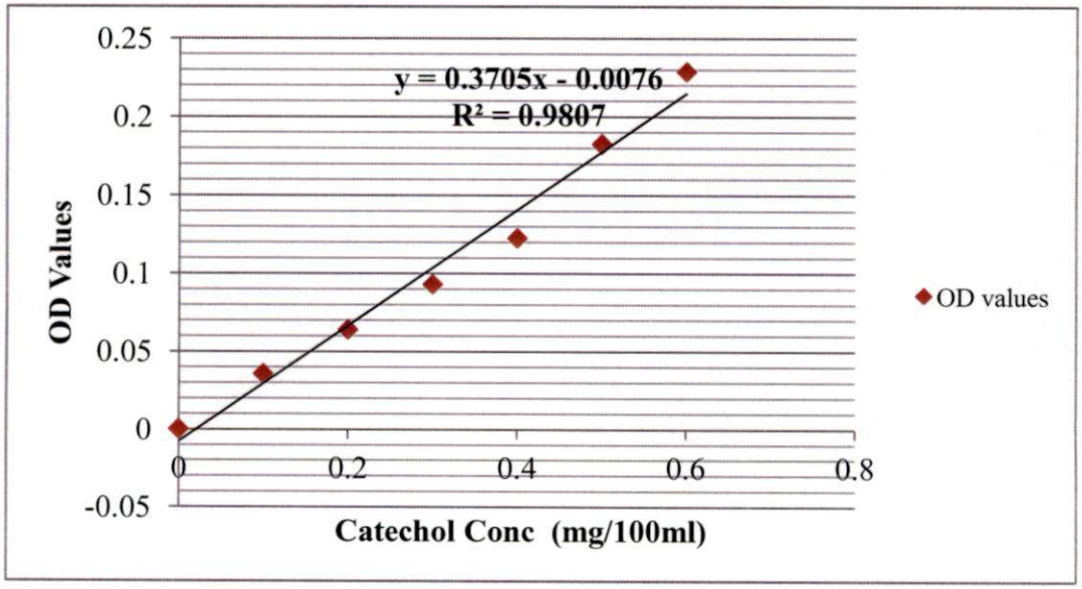


Fig. 2. Standard curve of catechol for phenol estimation



3.4.2 Estimation of tannins

Tannin was analysed by Vanillin Hydrochloride method as described by Sadasivam and Manikkam (1992).

Preparation of catechin standard

Stock solution of catechin was prepared by dissolving 5 mg catechin in 5ml methanol (which having 1000 µg/ml catechin). From the stock solution working standard was prepared with 10 times dilution (100 µg/ml catechin).

Preparation of sample

The homogenate was prepared by grinding 0.15 g cashew leaf in 7.5 ml of methanol and the homogenate was kept at 4 ^{0}C for 20-28 h. The sample was centrifuged at 10,000 rpm for 20 min at 4^{0}C and the supernatant was collected. To 1ml of supernatant 5 ml of vanillin hydrochloride reagent was added (equal volumes of 8% hydrochloric acid in methanol and 4 per cent vanillin in methanol)(plate 6). The solutions were mixed just before use. Readings were observed in a spectrophotometer (Model-Cary 60 UV vis) at 500 nm after 20 min. Standard graph was prepared (Fig 3) with 20–100 mg catechin using the diluted stock solution. From the standard graph, the amount of catechin (tannin) in the sample was calculated as per the absorbance values and expressed as catechin equivalents.

3.4.4 Estimation of poly phenol oxidase (PPO) activity

Polyphenol oxidase activity in cashew leaves was analysed as per the procedure by Esterbaner *et al.* (1977)

The enzyme was extracted by macerating 150 mg of cashew leaf with a pestle and mortar in 1.5 ml medium containing 0.9 ml 50Mm Tris-HCl (pH 7.2), 0.3 ml 10 mM NaCl and 0.3 ml 0.4 M sorbitol. The supernatant was obtained by centrifuging the homogenate at 10,000 rpm for 20 min. The enzyme extract of 0.2 ml was added to the cuvette containing 2.5 ml of 0.1M phosphate buffer (pH 6.5) and 0.3 ml of 0.01 M catechol solution and the readings were recorded using

spectrophotometer at 495 nm. The change in absorbance was recorded for every 30 seconds up to five minutes. Enzymatic unit was calculated by using the formula,

$$\text{Enzymatic unit in the test} = K \times (\Delta x / \text{min})$$

Where, K is 0.272 for catechol oxidase and Δx is the change in absorbance.

3.4.5 Estimation of phenyl alanine ammonia lyase (PAL)

Phenyl alanine ammonia lyase assay was carried out by the method described by Paul and Sharma, 2005.

Preparation of sample

For the extraction of the antioxidant enzyme PAL, 150 mg of cashew leaf sample was homogenized in 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) using pestle and mortar. The content was centrifuged at 10,000 rpm for 20 min at 4 ^{0}C. The supernatant was used for the PAL assay. To 500 µl of the enzyme extract, 500 µl of 0.5 M Tris HCl buffer was added followed by 500 µl of 0.15 M L-phenyl alanine. The mixture was incubated at 37^{0}C for 60 min. The reaction was stopped by adding 500 µl of 1M trichloro acetic acid and incubated at 40^{0}C for 5 min. The absorbance was read in the spectrophotometer at 270 nm. Blank containing buffer and L-phenyl alanine was added after TCA. The rate of the reaction was expressed as mg of trans cinnamic acid formed per gram fresh weight from trans cinnamic acid calibration curve (Fig. 4).

Fig. 3. Standard curve of catechin for tannin estimation

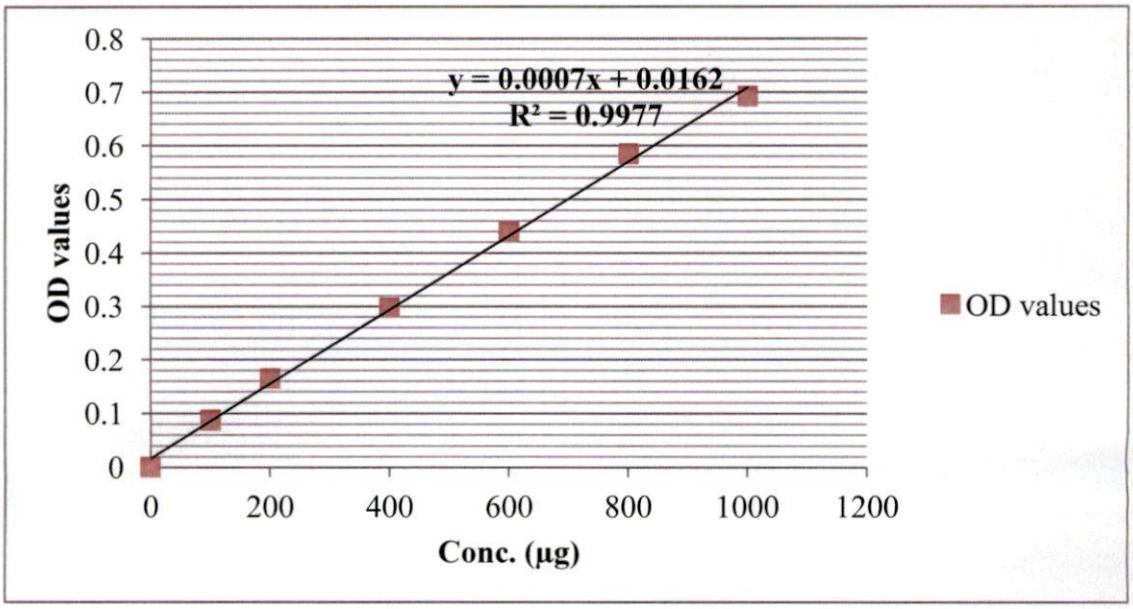
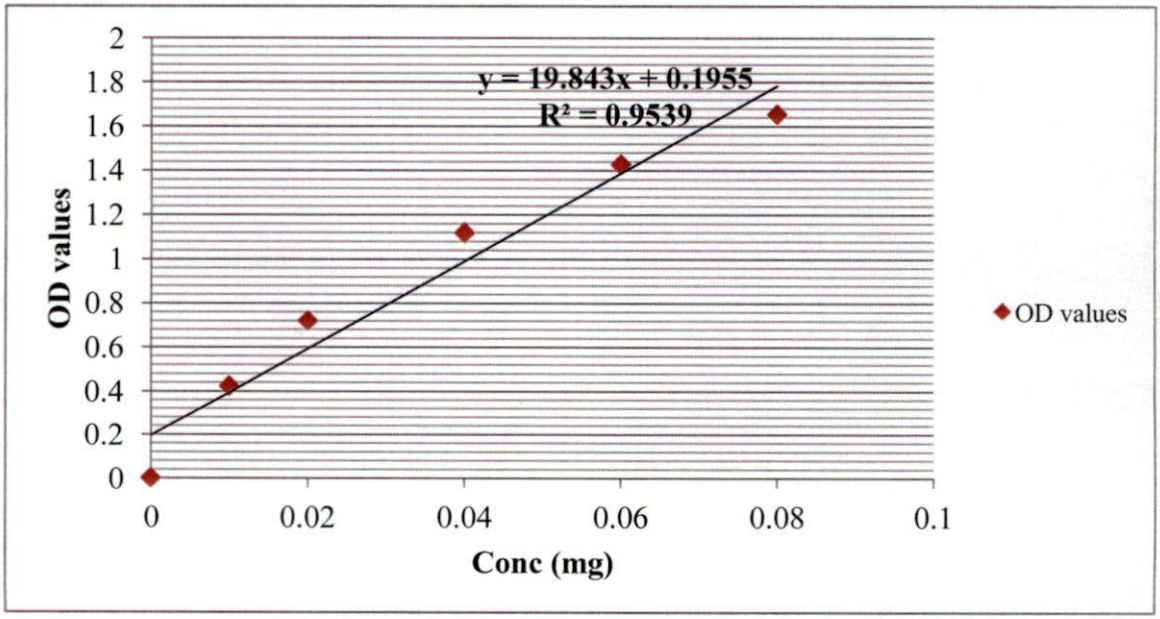


Fig. 4. Standard curve of trans cinnamic acid for phenyl alanine ammonia lyase estimation



3.5 Detoxification mechanism in tea mosquito bug

TMB adult females were collected before as well as at 6, 24, 48 and 72 h after feeding and analysis of protein, detoxifying enzymes like carboxyl esterase and cytochrome P-450 and glutathione -S- transferase were carried out as per the standard protocols.

3.5.1 Estimation of protein

Sample preparation

An adult female TMB was homogenized by pestle and mortar in 500µl of sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 20 min at 4^0C. The supernatant was collected and stored in deep freezer as 50 µl aliquots until estimation.

Protein estimation

Total insect protein was quantitatively estimated by following the method of Lowry *et al.* (1951). From the supernatant prepared 50 µl was taken and 2.5 ml reagent C was added. The reaction mixture was incubated for 10 min, following which 250 µl reagent D was added and again incubated for 30 min until readings were taken at 660 nm using spectrophotometer (Model: Carry -60 UV vis). The protein content was calculated from the standard graph prepared by using bovine serum albumin and expressed in mg /ml.

3.5.2 Protein profiling by sodium dodecylsulfate- poly acrylamide gel electrophoresis (SDS- PAGE)

The total protein analysed by SDS-PAGE by the protocol of Laemmli, (1970). The gel concentrations used were 4 per cent stacking gel and 8 per cent resolving gel.

Gel casting

The gel plates were thoroughly cleaned with water and wiped with tissue paper. Master plate (10.5×10.5) and base plate (10.5×10.5) were assembled

properly and fixed on casting unit. The leakage was checked by adding water in between the plates using a micro pipette. The water was removed and wiped with tissue paper. Resolving gel was prepared and suddenly poured into one corner of the glass mould to make a gel of 7cm height, without air bubble. For removing bubbles on the surface gently added a layer of water with micropipette. The overlay of water was decanted after polymerization (approximate 25-30 min) and pour stacking gel solution prepared as mentioned above and a well comb was introduced at the top of stacking gel and allowed to polymerize the gel for 30 min.

Sample loading

Sample protein (10µg/10µl) from crude insect homogenate was taken in eppendorf tube and boiled with treatment buffer/dye added 5 ml dye for 15 ml sample at 100 ^{0}C for 5 min in a water bath. After, the comb was removed the wells were cleaned with double distilled water. The casted gel was mounted on electrophoretic apparatus. The electrophoresis tank buffer was added to buffer chamber of the electrophoretic apparatus. The samples were gently loaded along with marker protein (4µl) in to each well using a micropipette.

Running the gel

The electrophoretic apparatus was connected to power supply unit. Initially, a voltage of 60 V was applied (for stacking gel) until the dye enters the resolving gel. Then the voltage was increased to 100 V. The unit was allowed to run until the tracking dye reaches bottom of the gel (7cm). Then the power supply was stopped. The plates were separated carefully and the stacking gel removed. Resolving gel was carefully transferred to staining box by rinsing with water.

Silver staining

The gels was immersed in fixing solution and kept under shaking for 1 h. The same step was repeated by changing the solution and fixed for 14 h. The gel was then washed in ethanol (30 %) for 10 sec and repeated two times. Then it was washed with deionised water. The gel was then transferred to the pretreatment solution and taken out within 1 min. It was again washed in deionized water thrice

for 30 sec, and impregnated in silver nitrate (0.2%) for 20 min in a shaker, followed by washing twice for 10 sec. It was dipped in developing solution for 5 - 10 min until clear band appeared with appropriate intensity. The gel was quickly transferred in to stop solution for 30 min. followed by washing twice in deionized water.

3.5.3 Carboxyl esterase assay

Esterase assay was carried out by the method described by van Asperen (1962).

Preparation of α-naphthol standard

Stock solution of α-naphthol (10 mM) was prepared by dissolving α-naphthol 0.03605 g in 25 ml methanol. From this, working standards of varying concentrations (400 µmol, 800 µmol, 1200 µmol, 1600 µmol, 2000 µmol) were prepared by pipetting out different aliquots (10µl, 20 µl, 30 µl, 40 µl, 50 µl and 60 µl) and made up to 1 ml with methanol. To this one ml of standard, 2 ml of extraction buffer (sodium phosphate buffer pH 7.4) was added. Phosphate buffer alone served as blank. The mixture was incubated at 30 ^{0}C for 30 min with constant stirring. Dye solution containing 22.5 mg fast blue RR salt in 2.25 ml distilled water and 5% SDS in distilled water (2:5 v/v) was added (0.05ml). The reaction mixture was incubated at 37^{0}C for 10 min for the colour development. The intensity of red colour was read at 600 nm absorbance in spectrophotometer. The calibration curve was prepared (Fig. 5) with OD values and corresponding concentration of alpha naphthol formed.

Sample preparation

Insect sample (3mg) was homogenized in sodium phosphate buffer (pH 7.4), and centrifuged at 10,000 rpm for 20 min at 4^{0}C to remove coarse materials. The supernatant was taken for enzyme assay. To 0.1 ml enzyme extract 1 ml 30 mM α- naphthyl acetate (enzyme substrate) dissolved in acetone (0.028 g α-naphthyl acetate in 5 ml acetone) was added. Other steps of enzyme analysis were carried out.

3.5.4 Glutathione-S-transferase (GST)

Glutathione- S- transferase quantification was carried using the method of Kao *et al.* (1989).

Preparation of sample

Insect sample (3mg) was homogenized in sodium phosphate buffer (pH 7.4), and centrifuged at 10,000 rpm for 20 minutes at 4^0C to remove coarse materials. The supernatant was taken for enzyme assay. One hundred and twenty microliters of 50 mM 2-4 1-Chloro-2,4- Dinitro Benzene (CDNB) and 375 µl of reduced glutathione (GSH) were added to 6.95 ml sodium phosphate buffer (100 mM, pH 6.0). Fifty microliters of enzyme stock were then added. The contents were gently shaken, incubated for 2–3 min at 20 °C and then transferred to a cuvette in the sample cuvette slot of a UV spectrophotometer. Reaction mixture without enzyme was placed in a cuvette in the reference slot. Absorbance at 340 nm was recorded for 5 min with 30 sec intervals. The GST activity was calculated using the formula

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

*9.6 mM/cm – extinction coefficient for CDNB-GSH conjugate.

3.5.5 Cytochrome P450

The cytochrome P450 assay was done according to the method of Brogdon *et al.* (1997) with slight modifications.

Preparation of cytochrome C standard

Pure cytochrome C from bovine heart type (Sigma®) 3.081 mg dissolved in 10 ml distilled water, this solutions had a concentration of 0.0025 mM. From the stock working standards of 0.025 nM to 0.2 nM were prepared.

Sample preparation

Insect sample (3mg) was homogenized in sodium phosphate buffer (pH 7.4), centrifuged at 10,000 rpm for 20 minutes at 4^0C to remove coarse materials. The supernatant was taken for enzyme assay. To the 50 µl of prepared enzyme extract, 500 µl of 0.05 % TMBZ solution (10 mg TMBZ dissolved in 5ml absolute methanol mixed with 15ml 0.25 nM sodium acetate buffer pH 5), 200 µl potassium phosphate buffer (pH 7.2), and 62.5 µl of 3% hydrogen peroxide were added. The reaction mixture incubated for 30 min. The absorbance was recorded with UV spectrophotometer (Model - Cary-60 UV vis) at 630 nm. The recorded absorbance was converted to end product formation from a standard curve (Fig 6) of cytochrome C (0.0025 nM to 0.02 nM) and total activity expressed as n mol equivalent cytochrome P450 mg $protein^{-1}$ min^{-1}

3.6 Statistical analysis

The data collected were subjected to statistical analysis using the statistical packages 'WASP' developed by ICAR- GOA.

Fig. 5. Standard curve of α-naphthol for carboxyl esterase estimation

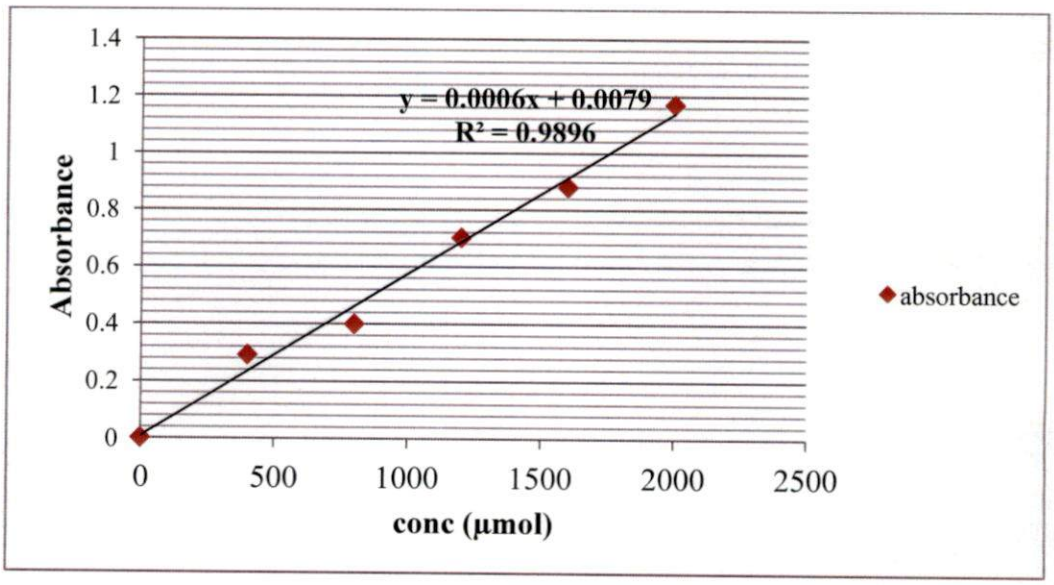


Fig. 6. Standard curve of cytochrome C for cytochrome P450 estimation

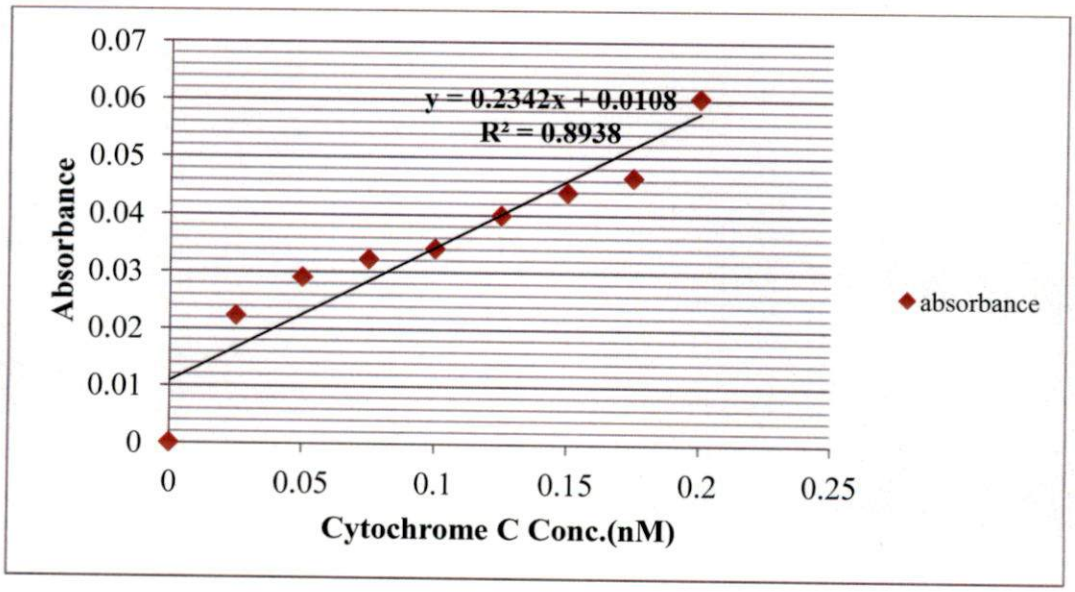


Plate 1. Cashew grafts of four varieties

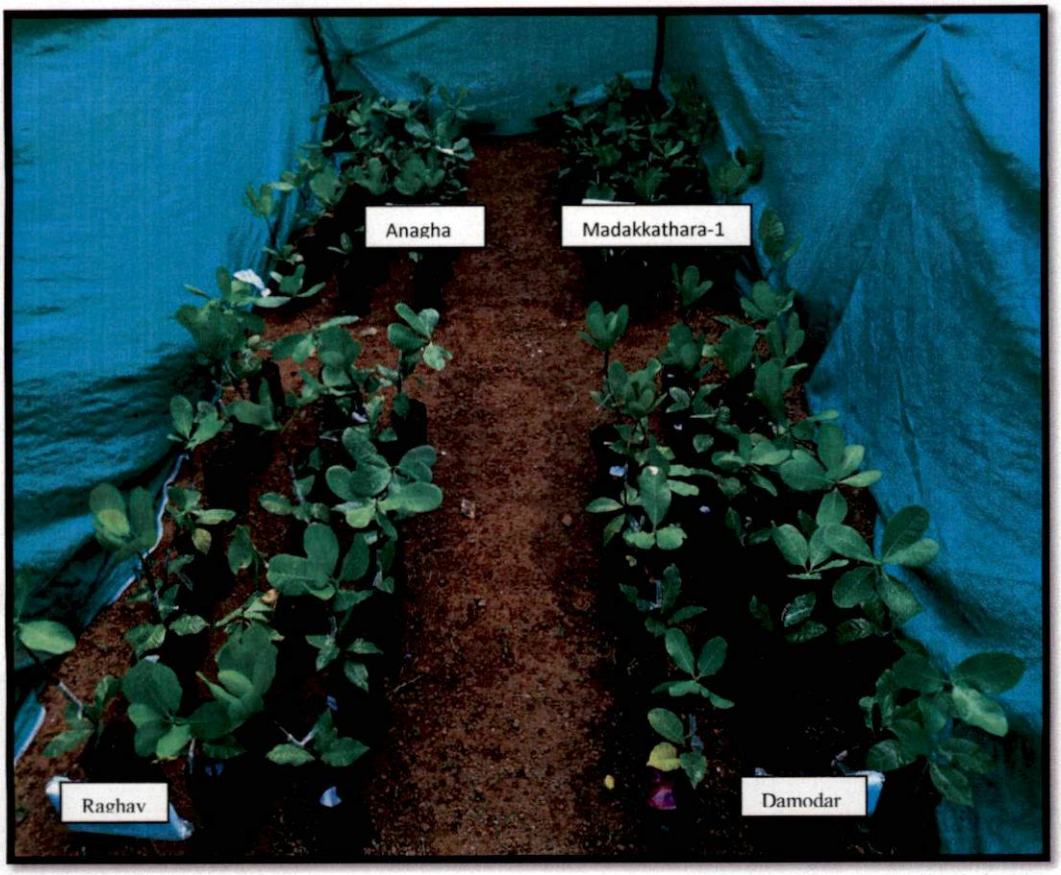


Plate 2. Field collection and maintenance of tea mosquito bug population in the laboratory

Plate 2a. Field collection of tea mosquito bug

Plate 2b. Rearing cage

Plate 2c. TMB adult

Plate 2d. TMB nymph

Plate 2e. Rearing box for nymphs

Plate 3. Release of first generation adult TMB in selected cashew grafts

Plate 3a. Collecting 0-24 h old female TMB adult

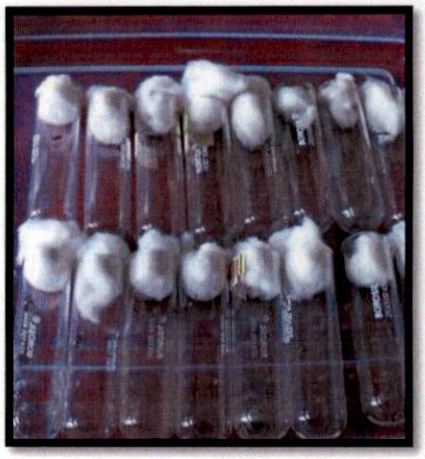
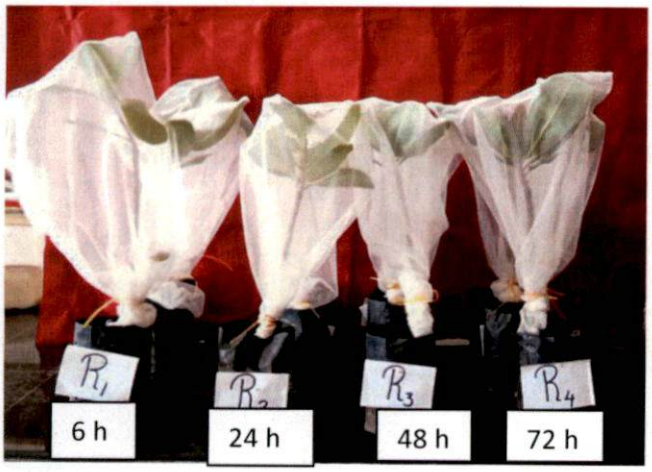


Plate 3b. Prestarvation of 0-24 h old adult female

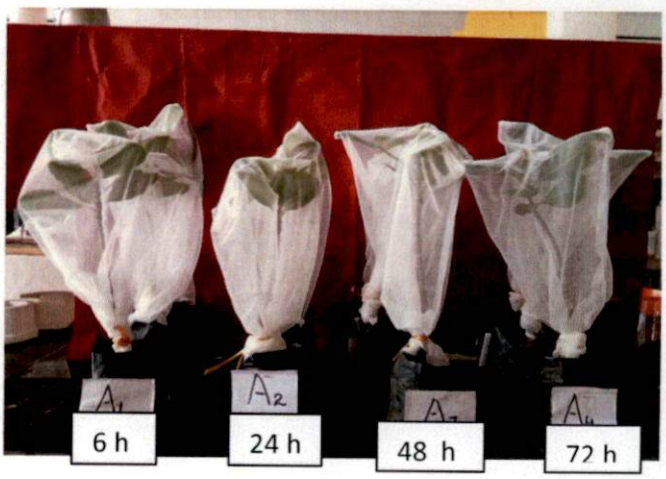
Plate 3c. Release of prestarved bug (Adult female)



Raghav

Damodar

Madakkathara-1



Anagha

Plate 3d. Cashew grafts covered with net cloth after the release of TMB

Results

4. RESULTS

The results of the experiment entitled "Elucidating the biochemical basis of interaction between tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) and cashew (*Anacardium occidentale*)" conducted at Department of Agrl. Entomology, College of Horticulture, Vellanikkara, Cashew Research Station, Madakathara, Pesticide residue laboratory, All India Network Project on Ornithology, Cocoa Research Centre, Centre for Plant Biotechnology and Molecular Biology, Vellanikkara and College of Veterinary and Animal Sciences, Mannuthy during the period of 2016-18 are described in this chapter

4.1 BIOCHEMICAL ANALYSIS OF THE SELECTED CASHEW VARIETIES

Adult 0-24 h old female bug was allowed to feed for 0, 6, 24, 48 and 72 h duration on three month old grafts of four selected varieties. Physiologically mature leaves from the middle portion of grafts were collected as already described at specific intervals. Leaf protein, total phenols, tannins, polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) were estimated as per the standard protocols.

4.1.1. Protein

Prior to TMB exposure, the highest protein content of 1.852 mg/g was observed in the less susceptible variety Damodar, which was significantly superior to the remaining varieties. The least protein content of 1.094 mg/g was recorded by the highly susceptible variety Anagha (Table 2).

Variation in protein levels were manifested as early as 6 h after release of TMB. There was a drastic decline in the protein content of both highly susceptible and less susceptible varieties, compared to that of uninfested plants. All the four varieties registered decline in protein content by 43.27 per cent (Raghav) to 62.53 per cent (Damodar). The greatest reduction observed was in Damodar while Anagha registered 54.03 per cent reduction.

After 6 h, both Raghav and Damodar had high leaf protein content than the highly susceptible Madakkathara-1 and Anagha, both being on par with each other.

The leaf protein content continued to decrease in less susceptible Raghav (0.683 mg/g) and Damodar (0.415 mg/g) after 24 h of exposure to TMB. In contrast highly susceptible varieties Anagha and Madakathara-1 showed significant increase in protein content (0.720 mg/g and 0.604 mg/g) compared to the corresponding values after 6 h.

After 48 h, highly susceptible Anagha again recorded appreciable increase in protein content (1.028 mg/g). Damodar also registered significant increase in protein content over that at 24 h while Madakkathara-1 recorded only a marginal increase.

The highest protein content of 1.063 mg/g after 72 h of exposure to TMB was recorded by Damodar, while Raghav recorded the lowest protein content of 0.406 mg/g.

All the four varieties recorded decrease in leaf protein content after exposure to TMB, when compared to the infestation levels. The extent of reduction at the end of the study period was greatest in Anagha with 75.68 per cent reduction while Raghav recorded the lowest reduction by 32.17 per cent.

Without considering time intervals the highest mean protein content was observed in less susceptible Damodar (0.993 mg/g) followed by highly susceptible Anagha (0.834 mg/g). Lowest was observed in highly susceptible Madakkathara-1 (0.673 mg/g).

Irrespective of the varieties highest mean protein content was recorded before TMB release (1.349 mg/g) and lowest was at 6 h of release (0.603 mg/g).

Table 2. Effect of TMB infestation on total protein (mg/g) content of selected cashew varieties

	Mean protein content (mg/g) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible varieties						
Raghav	1.262	0.716	0.683	0.602	0.406	0.734
Damodar	1.852	0.694	0.415	0.938	1.063	0.993
Highly susceptible varieties						
Anagha	1.094	0.503	0.720	1.028	0.828	0.834
Madakathara-1	1.185	0.498	0.604	0.605	0.473	0.673
Mean	1.349	0.603	0.606	0.793	0.692	

CD for varieties : 0.0181
CD for period of infestation : 0.0203
CD for variety x period of infestation : 0.0406

4.1.2 Phenol

Damodar has higher phenol content of 63.960 mg/g prior to infestation by TMB and was followed by Raghav (61.641 mg/g) where, both varieties were on par with each other. Similarly the highly susceptible Anagha and Madakkathara-1 were on par with phenol content of 42.027mg/g and 45.055 mg/g respectively (Table 3).

After 6 h, Raghav, Damodar and Madakkathara-1 registered significantly high phenol content of 72.831 mg/g, 71.014 mg/g and 68.747 mg/g respectively, where as Anagha showed a reduction by 49 per cent. All the varieties recorded higher phenol content immediately after infestation except Anagha. Twenty four hour after exposure the highly susceptible variety Madakkathara-1, Raghav and Damodar had comparable values for phenol content. All the four varieties,

however recorded lower phenol content as compared to the corresponding values after 6 h, through the reduction was non significant.

There was no significant variation in phenol content of either Raghav or Damodar after 48 h of exposure to TMB. However, Madakkathara-1 showed a reduction to 54.844 mg/g, where as Anagha showed a significant increase to 31.422 mg/g, but which was the lowest value among all the varieties.

After 72 h, both Damodar (79.273mg/g) and Madakkathara-1 (67.847 mg/g) showed significant increase in phenol content compared to previous values. However, with Raghav and Anagha registered no significant variation from previous values.

Both the less susceptible varieties Raghav and Damodar were on par with each other with mean phenol contents of 67.207 mg/g and 69.834 mg/g respectively. Irrespective of varieties, the highest phenol content was observed at 72 h of feeding by TMB.

Table 3. Effect of TMB infestation on total phenol (mg/g) content of selected cashew varieties

Varieties	Mean phenol content (mg/g) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible						
Raghav	61.641	72.831	67.223	67.745	66.593	67.207
Damodar	63.960	71.014	67.379	67.541	79.273	69.834
Highly susceptible						
Anagha	42.027	20.578	23.662	31.422	30.435	29.625
Madakathara-1	45.055	68.747	67.709	54.844	67.847	60.840
Mean	53.171	58.293	56.493	55.388	61.037	

CD for varieties : 3.087
CD for period of infestation : 3.451
CD for variety x period of infestation : 6.902

4.1.3 Tannin

Prior to infestation, the less susceptible variety , Raghav recorded significantly highest tannin content of 10.335 mg/g and was followed by Damodar with tannin content of 6.657 mg/g.

There was significant reduction in tannin content of all the four varieties after 6 h of TMB infestation. Raghav (2.077 mg/g) and Anagha (0.887 mg/g) registered reduction of 79.90 and 81.01 per cent respectively. Anagha recorded the lowest tannin content of 0.887 mg/g after 6 h (Table 4).

The variation in tannin content continued to be proceeded after 24 h as well. Raghav recorded the highest value of 5.436 mg/g, which was 26 per cent higher than the corresponding value after 6 h. Madakkathara-1, which had recorded the highest tannin content of 3.464 mg/g after 6 h, and the lowest tannin content of 0.7844 mg/g after 24 h. Anagha, with 3.801mg/g showed a 4 fold increase over the corresponding values after 6 h.

Tannin content showed wide fluctuation after 48 h. Damodar with a 4 fold increase over previous value, recorded the highest tannin content of 6.029 mg/g and was followed by Madakkathara-1, with 2.215 mg/g. Raghav with the lowest tannin content of 1.033 mg/g and Anagha (1.569 mg/g) registered significant reduction over previous values.

Seventy two hours after exposure to TMB, Damodar continued to record the highest tannin content of 5.725 mg/g. which was significantly higher than all the varieties. The highly susceptible Anagha had the least tannin content of 2.820 mg/g. There was significant reduction in tannin content of all the four varieties following infestation. The reduction after 72 h was most pronounced in Raghav (69%), but was less so in case of Damodar (15%). The highly susceptible varieties Anagha and Madakkathara-1 recorded less tannin content of 4.682 mg/g and 4.975 mg/g respectively and were on par with each other.

Without considering time intervals both less susceptible varieties Raghav and Damodar were on par with each other with regard to the mean tannin content of 4.42 mg/g and 4.276 mg/g respectively. Irrespective of the varieties the highest tannin content was observed before release of TMB.

Table 4. Effect of TMB infestation o total tannin (mg/g) content of selected cashew varieties

Varieties	Mean tannin content (mg/g) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible varieties						
Raghav	10.335	2.077	5.436	1.033	3.223	4.420
Damodar	6.657	1.566	1.403	6.029	5.725	4.276
Highly susceptible varieties						
Anagha	4.682	0.887	3.801	1.569	2.820	2.752
Madakathara-1	4.975	3.464	0.7844	2.215	3.506	2.988
Mean	6.662	1.998	2.856	2.711	3.818	

CD for varieties : 0.294
CD for period of infestation : 0.329
CD for variety x period of infestation : 0.658

4.1.4. Poly phenol oxidase (PPO)

All the varieties except Anagha had comparable levels of PPO activity prior to infestation in common. Anagha had a significantly lowest PPO activity of 0.00036 EU/g/min (Table 5).

Six hours of post infestation, all the four varieties had elevated levels of PPO activity when compared to the uninfested plants. Damodar registered the highest value of 0.00317 EU/g/min and was followed by Raghav with a value of 0.0014

EU/g/min. The highly susceptible Anagha (0.00083 EU/g/min) recorded least PPO specific activity. Madakkathara-1, highly susceptible variety, however had a PPO value of 0.00131 EU/g/min which was on par with previous infestation level.

Significant increase in PPO activity was observed in all the varieties except in case of Damodar, after 24 h of exposure to TMB. Madakkathara-1 (0.00309 EU/g/min) followed by Damodar (0.00298 EU/g/min) and Anagha (0.00279 EU/g/min) recorded higher PPO specific activity and were on par with each other. Raghav recorded a significantly lower value of 0.00242 EU/g/min when compared to other varieties.

Plant samples taken after TMB exposure to 48 h revealed that the highest PPO specific activity was recorded by the less susceptible variety Raghav (0.00804 EU/g/min) and was immediately followed by Damodar (0.00511 EU/g/min). The highly susceptible Anagha recorded least specific activity of PPO (0.0018 EU/g/min).

All the four accessions recorded lower PPO activity at 72 h after TMB infestation, as compared to corresponding values after 48 h. Damodar recorded the highest value of 0.00339 EU/g/min and was significantly superior to other varieties. It was followed by Madakkathara-1 and Raghav, with 0.00261 EU/g/min and 0.00232 EU/g/min values for PPO activity respectively. Anagha recorded the lowest enzymatic activity of 0.00124 EU/g/min which was significantly lower than other varieties.

Regardless of the time intervals less susceptible varieties Raghav and Damodar were on par with each other with regard to the mean PPO specific activity (0.003063 EU/g/min and 0.003158 EU/g/min respectively) and the lowest being recorded by the highly susceptible Anagha (0.001406 EU/g/min).

Irrespective of varieties the highest PPO activity observed after 48 h of TMB release.

Table 5. Effect of TMB infestation on total PPO specific activity (*EU/g/min) of selected cashew varieties

Varieties	Mean PPO specific activity (*EU/g/min) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible varieties						
Raghav	0.00114	0.00140	0.00242	0.00804	0.00232	0.003063
Damodar	0.00114	0.00317	0.00298	0.00511	0.00339	0.003158
Highly susceptible varieties						
Anagha	0.00036	0.00083	0.00279	0.0018	0.00124	0.001406
Madakathara-1	0.00141	0.00131	0.00309	0.00367	0.00261	0.002419
Mean	0.00101	0.0016	0.0028	0.0046	0.0023	

*EU - Enzyme unit

CD for varieties : 0.00022
CD for period of infestation : 0.00025
CD for variety x period of infestation : 0.000507

4.1.5. Phenyl alanine ammonia lyase (PAL)

Prior to TMB exposure, the highest PAL activity of 0.438 µg/g/min was recorded by the less susceptible variety Damodar and the lowest was found in the highly susceptible variety, Madakathara-1 (0.318 µg/g/min). Irrespective of susceptibility status, the PAL activity is increasing in all the four varieties after TMB infestation (Table 6).

After 6 h following TMB release, both less susceptible as well as highly susceptible varieties showed increased PAL activity. All the varieties were on par with respect to the PAL activity at 6 h after TMB infestation.

As the TMB exposure exceeded 24 h, there observed reduction in PAL activity in the varieties Raghav, Damodar and Anagha when compared to previous values.

Forty eight hour after following release of TMB, both Raghav (0.558 µg/g/min) and Anagha (0.609µg/g/min) showed an increased PAL activity, over that at 24 h. Damodar and Madakkathara-1 with PAL values of 0.513 µg/g/min and 0.637 µg/g/min on the other hand recorded a reduction in enzyme activity .

At 72 h after release, there was a significant decline in PAL activity in all the varieties. Madakkathara-1 recorded the highest PAL activity of 0.591 µg/g/min, which was significantly superior to other varieties. The lowest PAL activity was recorded by the highly susceptible variety, Anagha (0.317 µg/g/min). Two varieties namely Raghav and Madakkathara-1 showed significantly higher PAL activity following exposure to TMB for 72 h. However, with Damodar and highly susceptible Anagha registered enzymatic activity on par with the infestation levels.

Irrespective of time intervals the highest mean PAL specific activity of 0.575 µg/g/min was observed for highly susceptible Madakkathara-1,whereas Raghav (0.494 µg/g/min), Damodar (0.505 µg/g/min) and Anagha (0.481 µg/g/min) were on par with each other.

With out consideration of the varieties the PAL specific activity reached peak after 6 h of TMB release.

Table 6. Effect of TMB infestation on total PAL (µg/ g fresh weight/min) specific activity of selected cashew varieties

Varieties	Mean PAL specific activity (µg/ g fresh weight/ min) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible varieties						
Raghav	0.325	0.640	0.470	0.558	0.475	0.494
Damodar	0.438	0.588	0.587	0.513	0.385	0.505
Highly susceptible varieties						
Anagha	0.359	0.587	0.530	0.609	0.317	0.481
Madakathara-1	0.318	0.624	0.704	0.637	0.591	0.575
Mean	0.360	0.610	0.573	0.579	0.442	

CD for varieties : 0.064
CD for period of infestation : 0.072
CD for variety x period of infestation : 0.144

4.2 DETOXIFICATION MECHANISM IN TEA MOSQUITO BUG

Single 0-24 h old adult female bugs were released on individual three month old grafts of selected varieties of cashew for 6, 24, 48 and 72 h, after which the bugs were collected for biochemical analysis. In order to understand detoxification mechanism in tea mosquito bugs, variation in total protein, total protein profiling by SDS PAGE, defensive enzymes *viz.*, carboxyl esterase, cytochrome P450 and glutathione-S-transferase were estimated as per the standard protocol.

4.2.1 Protein

Six hour after release insect samples from the less susceptible Raghav, showed a significant increase in protein content to 1.046 mg/ml, while there was no significant difference in case of other varieties (Table 7).

Significant variation was observed in the protein content of bugs released on both Raghav and Anagha after 24 h. The highest protein content of 1.659 mg/ml was recorded in case of bug fed on Anagha, followed by those fed on Madakkathara-1(0.836 mg/ml), both showing increase over values after 6 h. On the other hand, Raghav, a less susceptible variety showed significant reduction in protein content, with a value of 0.152 mg/ml.

Forty eight hour after release, bug fed on the less susceptible varieties Raghav (1.294 mg/ml) and Damodar (0.621 mg/ml) had significantly higher protein content compared to corresponding values 24 h after release. Bugs released on both the highly susceptible, Anagha and Madakkathara-1 recorded significantly lower protein content of 0.352 and 0.417 mg/ml respectively, compared to that after 24 h.

Bugs released on Raghav continued to register highest protein content 72 h after release as well, with a value of 1.149 mg/ml. The TMB fed on the less susceptible Damodar and highly susceptible Anagha had significantly lower values of 0.382 mg/ml and 0.075 mg/ml as compared to the same after 48 h of exposure. Insect samples from the highly susceptible Madakkathara-1 also registered appreciable increase in protein content after 72 h compared to corresponding value after 48 h.

The bugs released on less susceptible Raghav consistently recorded higher protein content compared to unfed TMB throughout the study except at 24 h after release. The less susceptible Damodar also recorded 54 per cent reduction in protein content when compared to the protein content of unfed TMB, while insect samples from Anagha registered significant reduction in protein content after 72 h of exposure.

Irrespective of time intervals the highest mean protein content of 0.895 mg/ml was observed in bug released on less susceptible variety Raghav and lowest was in TMB released on less susceptible Damodar (0.604 mg/ml).

Without considering varieties the mean protein content at 0 h, 6 h and 24 h of TMB exposure (0.833 mg/ml, 0.827 mg/ml and 0.785 mg/ml respectively) were on par with each other and lowest value observed at 72 h of release (0.617 mg/ml).

Table 7. Variation in total protein content (mg/ml) of TMB on exposure to selected cashew varieties

	Mean protein content (mg/ml) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible varieties						
Raghav	0.833	1.046	0.152	1.294	1.149	0.895
Damodar	0.833	0.689	0.491	0.621	0.382	0.604
Highly susceptible varieties						
Anagha	0.833	0.784	1.659	0.352	0.075	0.790
Madakathara-1	0.833	0.790	0.836	0.417	0.863	0.748
Mean	0.833	0.827	0.785	0.671	0.617	

CD for varieties : 0.0933
CD for period of infestation : 0.1044
CD for variety x period of infestation : 0.208

4.2.2 Protein profiling by SDS PAGE

SDS-PAGE analysis of total crude protein from 0-24 h old adult female TMB exposed to different time intervals (0, 6, 24, 48, and 72 h) was done by following standard protocol of Laemmli (1970). The analysis showed the distinct expression of about 3-5 stained protein bands of varying molecular weight

ranging from 17- 75 kDa (plate 7).The analysis also revealed that variation in level of expression was noticed in different samples at different intervals of exposure.

Tea mosquito bug fed on Raghav, Damodar, Anagha and Madakathara-1 showed distinct protein bands majority ranging from 11-17 kDa, 17-20 kDa, and 25-35 kDa were found to be over expressed for TMB fed on varieties compared to control (0 h of exposure), which is evident from the gel image as black coloured thick bands during all the exposure intervals (6, 24, 48, and 72 h).

In TMB fed on Raghav, additional band of 48-63 kDa were expressed after 48 and 72 h of feeding. In case of TMB fed on Anagha, one additional band of 75-100 kDa was expressed during 72 h of exposure. The bands of same molecular weight were also expressed in TMB fed on Damodar but at 6 and 24 h of exposure.

Protein band ranging from 35-48 kDa was found to be over expressed in TMB fed on Madakathara-1 after 6 h and one additional band of 48-63 kDa was expressed after 72 h of exposure.

4.2.3 Carboxyl esterase

Damodar showed six fold increase over that of unfed bugs (278.683 µmol/min/mg protein) (Table 8), while no significant difference were observed in the carboxyl esterase specific activity levels of bugs released on Raghav as well as the highly susceptible Anagha and Madakkathara-1 after 6 h of release.

Twenty four hour after release, bugs from both Raghav and Damodar, with esterase specific activity of 859.683 and 500.464 µmol/min/mg protein respectively, showed significant increase over corresponding values at 6 h after release. However Anagha, which had the lowest enzyme activity of 32.674 µmol/min/mg protein as well as Madakkathara-1 with 55.463 µmol/min/mg protein, did not show any significant difference in enzyme activity for the corresponding period.

The bugs exposed to the less susceptible Raghav again recorded the highest value for esterase specific activity (189.803 µmol/min/mg protein) at 48 h after exposure. This was significantly superior to the corresponding values of other three varieties, which were on par with each other. Bugs on both Raghav and Damodar exhibited significant reduction in enzyme activity in comparison with the values after 24 h. However the enzymatic activity showed no significant variation in case of bug released either on Anagha or Madakkathara-1.

Seventy two hours after release, bugs exposed to all varieties except Anagha showed comparable esterase specific activity, and was significantly higher than that of Anagha (43.050 µmol/min/mg protein).

The mean esterase activity recorded highest for TMB released on less susceptible variety Raghav (258.117 µmol/min/mg protein) and observed lowest for TMB released on highly susceptible variety Anagha (53.377 µmol/min/mg protein).

Irrespective of varieties the highest mean enzyme activity observed at 24 h of TMB release and lowest observed for unfed TMB.

Table 8. Variation in carboxyl esterase specific activity (µmol/min/mg protein) TMB on exposure to selected cashew varieties

	Mean carboxyl esterase specific activity (µmol/min/mg protein) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible varieties						
Raghav	47.319	60.244	859.683	189.803	133.522	258.117
Damodar	47.319	278.683	500.464	53.920	164.194	208.916
Highly susceptible varieties						
Anagha	47.319	55.178	32.674	88.666	43.050	53.377
Madakathara-1	47.319	55.981	55.463	88.526	141.728	77.804
Mean	47.319	112.521	362.071	105.229	120.624	

CD for varieties : 40.025
CD for period of infestation : 44.749
CD for variety x period of infestation : 89.498

4.2.4 Cytochrome P450

Six hours after exposure, TMB collected from Damodar showed the highest cytochrome P450 specific activity value of 0.545 nmol/min/mg protein, which was significantly higher than that of bugs exposed to other varieties and also to that of unfed bugs (Table 9). The cytochrome P450 specific activity values of insect samples from other three varieties showed no significant difference among themselves as also with the corresponding values of samples prior to exposure.

After 24 h of exposure insect samples from Raghav showed highest value of 0.430 nmol/min/mg protein, followed by Damodar (0.186 nmol/min/mg protein). Bugs from both the above less susceptible varieties had significantly higher enzyme activity as compared to the highly susceptible Anagha (0.047 nmol/min/mg protein), which however was on par with bugs fed on Madakkathara-1 (0.096 nmol/min/mg protein).

There was significant variation in the cytochrome P450 activity of insect samples from different varieties 48 h after exposure. Insects collected from Damodar had the highest enzyme activity of 0.749 nmol/min/mg protein followed by the highly susceptible Anagha (0.339 nmol/min/mg protein), Madakkatahra-1 (0.122 nmol/min/mg protein) and Raghav (0.083 nmol/min/mg protein), and the last two being on par with each other. While bugs infesting Raghav showed a significant reduction in enzyme activity over that at 24 h. Insect samples from both Damodar and Anagha exhibited significant increase in the enzyme activity over that at 24 h.

Tea mosquito bug fed on Damodar exhibited significantly higher enzyme activity of 0.379 nmol/min/mg protein at 72 h after release as well. Insect samples from Raghav (0.109 nmol/min/mg protein) as well as the highly susceptible Anagha (0.083 nmol/min/mg protein) and Madakkathara-1 (0.074 nmol/min/mg protein) showed comparable enzyme activity. Bugs from the less susceptible varieties showed increased enzyme activity over that at 48 h. while those exposed from highly susceptible varieties had lower enzymatic activity over that at 48 h, however the values were significant only in case of Damodar as well as Madakkathara-1.

Without considering time intervals the mean specific activity of cytochrome P450 was observed highest for TMB released on less susceptible variety Damodar (0.372 nmol/mim/mg protein) and observed least for bug released on highly susceptible variety Madakkathara-1 (0.074 nmol/mim/mg protein).

Irrespective of varieties the mean specific activity reached highest value at 48 h following release of TMB (0.323 nmol/mim/mg protein) and lowest mean specific activity was observed for unfed bug (0.004 nmol/mim/mg protein).

Table 9. Variation in cytochrome P450 activity (nmol/min/mg protein) of TMB after exposure to selected cashew varieties

Varieties	Mean cytochrome P450 activity (nmol/min/mg protein) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible varieties						
Raghav	0.004 (0.064)*	0.091 (0.301)	0.430 (0.638)	0.083 (0.286)	0.109 (0.329)	0.143 (0.324)
Damodar	0.004 (0.064)	0.545 (0.665)	0.186 (0.429)	0.749 (0.856)	0.379 (0.613)	0.372 (0.526)
Highly susceptible varieties						
Anagha	0.004 (0.064)	0.091 (0.301)	0.047 (0.213)	0.339 (0.580)	0.083 (0.286)	0.113 (0.289)
Madakkathara-1	0.004 (0.064)	0.073 (0.270)	0.096 (0.309)	0.122 (0.349)	0.074 (0.271)	0.074 (0.253)
Mean	0.004 (0.064)	0.200 (0.384)	0.190 (0.397)	0.323 (0.518)	0.161 (0.375)	

* $\sqrt{x}$ +0.5 transformed values in parentheses.

CD for varieties : 0.078
CD for period of infestation : 0.087
CD for variety x period of infestation : 0.175

4.2.5 Glutathione -S- transferases

The glutathione-S-transferases specific activity in TMB showed no significant variation after 6 h of release in any of the varieties (Table 10).

As the exposure time reached 24 h there was a rapid and significant increase in GST activity of TMB released on less susceptible Raghav (1128.547 µmol/min/mg protein), whereas all the bugs released on other varieties *viz.*, Damodar, Anagha and Madakkathara-1 showed enzyme activity on par with each other as well as with the corresponding values after 6 h.

After 48 h of exposure, peak enzyme activity was observed in TMB released on less susceptible Damodar (1559.604 µmol/min/mg protein), followed by insect samples from highly susceptible Anagha (481.211 µmol/min/mg protein). Bugs from Madakkathara-1 (252.222 µmol/min/mg protein) and Raghav (161.904 µmol/min/mg protein) were got enzyme activity on par with each other. While Damodar, Anagha and Madakkathara-1 showed significantly higher enzyme activity over that after 24 h. TMB from Raghav showed a steep reduction (161.904 µmol/min/mg protein).

The GST activity was found to be decreasing in all the bugs after 72 h of exposure except in case of that released on Madakkathara-1 (398.005 µmol/min/mg protein), which however were on par with Damodar (375.119 µmol/min/mg protein). The reduction from the enzyme activity at 48 h was significant in case of bugs fed on all varieties except Raghav.

Irrespective of time intervals the mean specific activity of GST in TMB recorded highest value of 501.879 µmol/min/mg protein in bug released on less susceptible variety Damoadr. The lowest mean specific activity was observed for TMB released on highly susceptible variety Madakkathara-1 (198.268 µmol/min/mg protein).

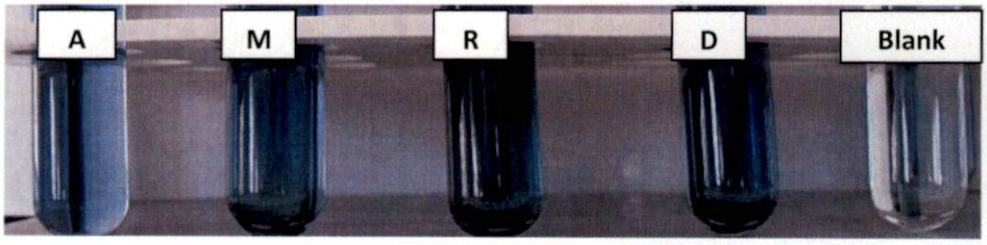
Irrespective of the varieties the mean specific activity of GST observed highest value at 48 h of release (613.735 µmol/min/mg protein) and observed least value at 6 h of release (172.501 µmol/min/mg protein).

Table 10. Variation in glutathione-S-transferase activity (µmol/min/mg protein) of TMB after exposure to selected cashew varieties

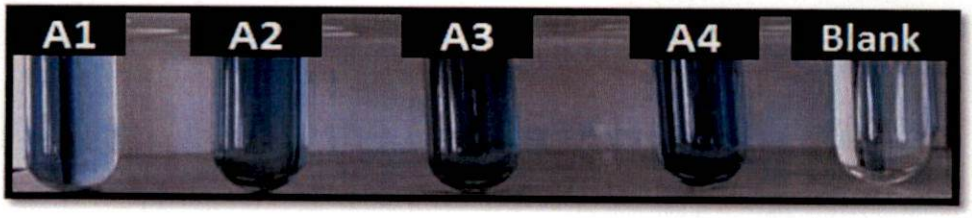
Varieties	Mean GST activity (µmol/min/mg protein) after different hours of feeding					Mean
	0 h	6 h	24 h	48 h	72 h	
Less susceptible varieties						
Raghav	211.464	178.297	1128.547	161.904	146.102	365.262
Damodar	211.421	224.105	139.147	1559.604	375.119	501.879
Highly susceptible varieties						
Anagha	211.335	171.522	102.619	481.211	205.267	234.391
Madakathara-1	211.464	116.081	13.569	252.222	398.005	198.268
Mean	211.421	172.501	345.971	613.735	281.123	

CD for varieties : 50.894
CD for period of infestation : 56.901
CD for variety x period of infestation : 113.802

Plate 4. Analysis of protein content in selected cashew varieties before and after TMB infestation

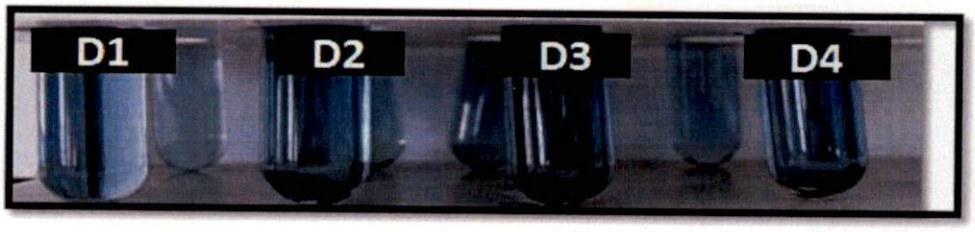


Control

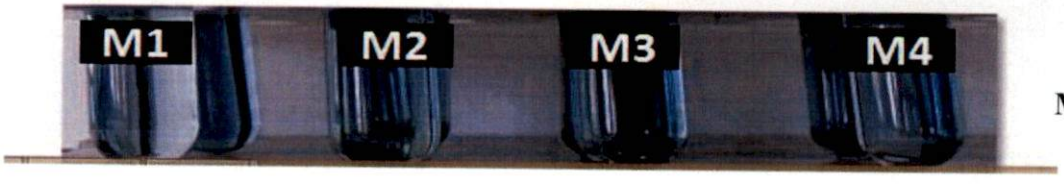


Anagha

Raghav



Damodar



Madakkathara-1

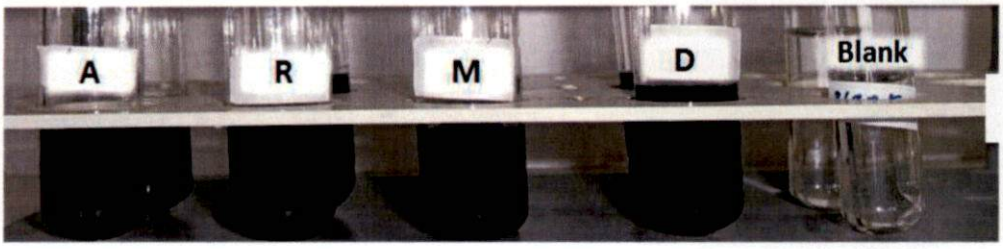
A – Anagha (0 h) , A1 - 6 h, A2 – 24 h, A3- 48 h, A4 – 72 h

R – Raghav (0 h) , R1 – 6h, R2 – 24 h, R3 – 48 h, R4 – 72 h

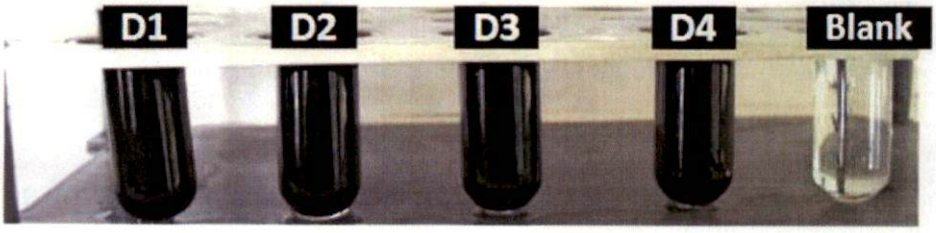
D – Damodar (0h) , D1 – 6h, D2 – 24 h, D3- 48 h, D4 – 72 h

M – Madakkathara-1, M1 – 6 h, M2 – 24 h, M3 – 48 h, M4 – 72 h

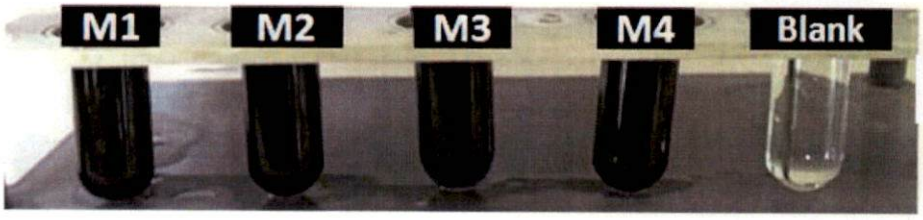
Plate 5. Analysis of total phenol content in selected cashew varieties before and after TMB infestation



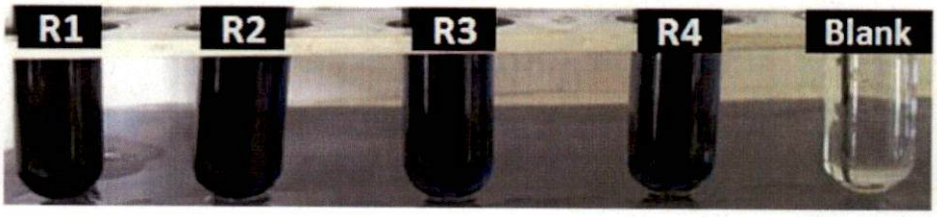
Control



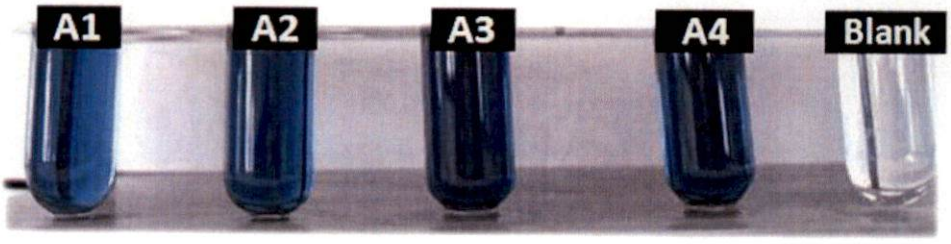
Damodar



Madakkathara-1



Raghav



Anagha

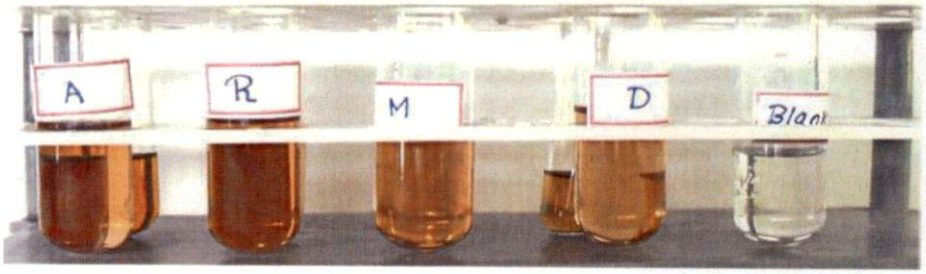
A – Anagha (0 h) , A1 - 6 h, A2 – 24 h, A3- 48 h, A4 – 72 h

R – Raghav (0 h) , R1 – 6h, R2 – 24 h, R3 – 48 h, R4 – 72 h

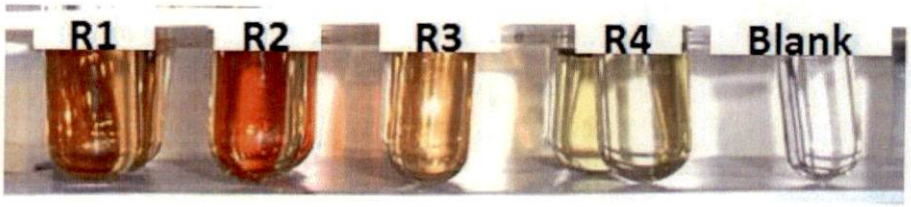
D – Damodar (0h) , D1 – 6h, D2 – 24 h, D3- 48 h, D4 – 72 h

M – Madakkathara-1, M1 – 6 h, M2 – 24 h, M3 – 48 h, M4 – 72 h

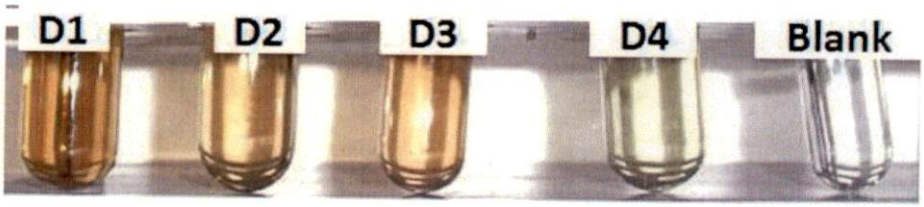
Plate 6. Analysis of total tannin content of selected cashew varieties before and after TMB infestation



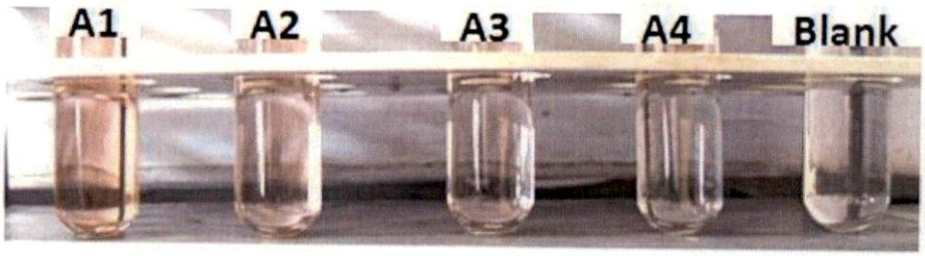
Control



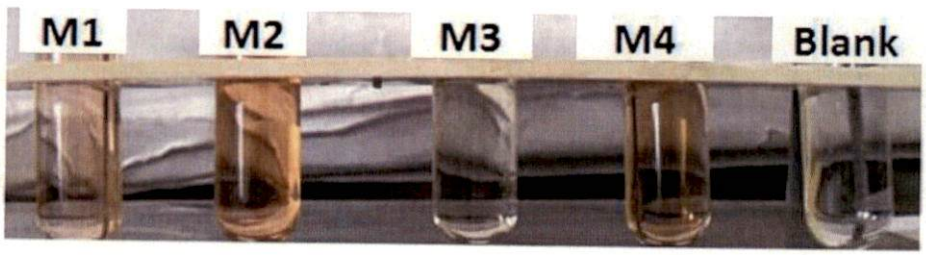
Raghav



Damodar



Anagha



Madakkathara-1

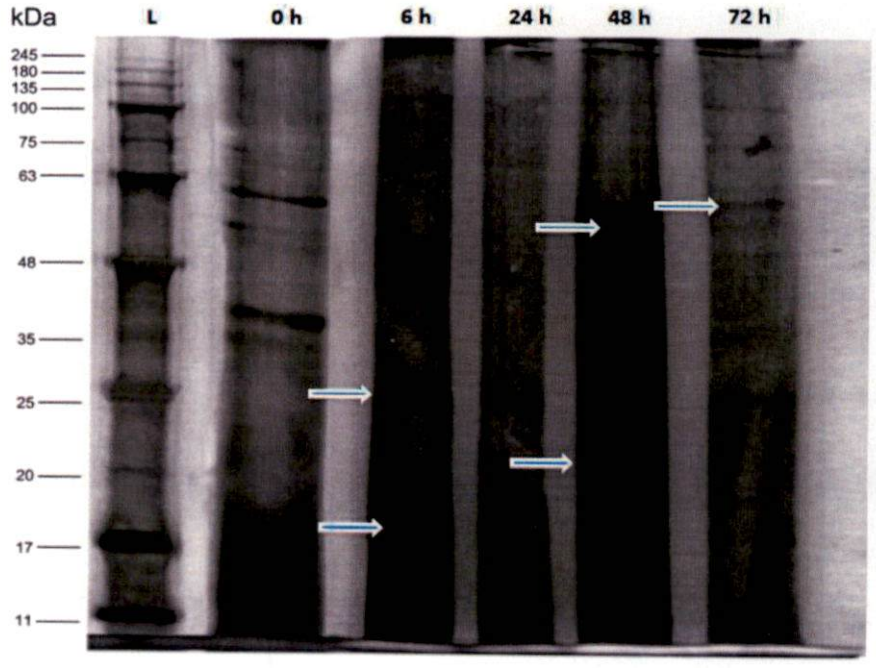
A – Anagha (0 h) , A1 - 6 h, A2 – 24 h, A3- 48 h, A4 – 72 h

R – Raghav (0 h) , R1 – 6h, R2 – 24 h, R3 – 48 h, R4 – 72 h

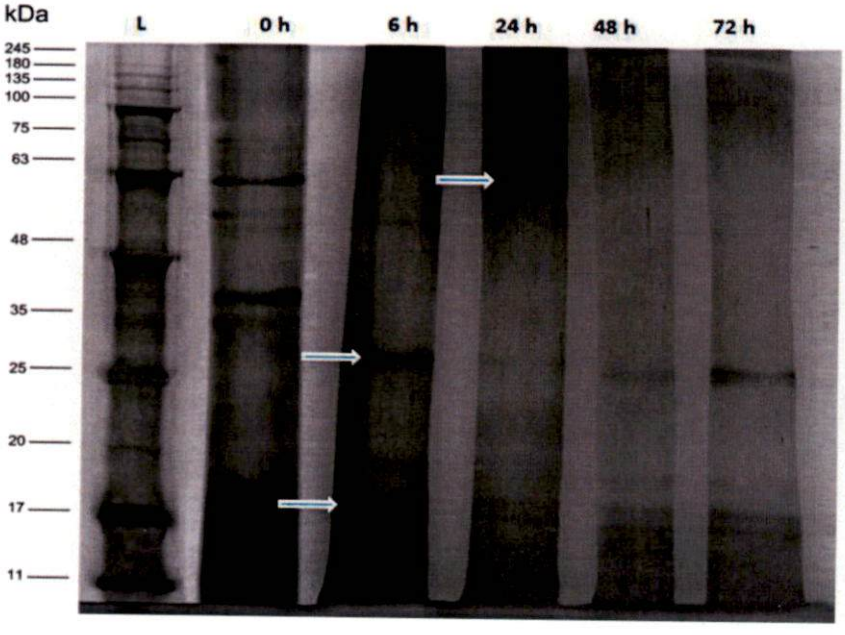
D – Damodar (0h) , D1 – 6h, D2 – 24 h, D3- 48 h, D4 – 72 h

M – Madakkathara-1, M1 – 6 h, M2 – 24 h, M3 – 48 h, M4 – 72 h

Plate 7 SDS PAGE for crude protein of TMB after feeding cashew varieties for different intervals

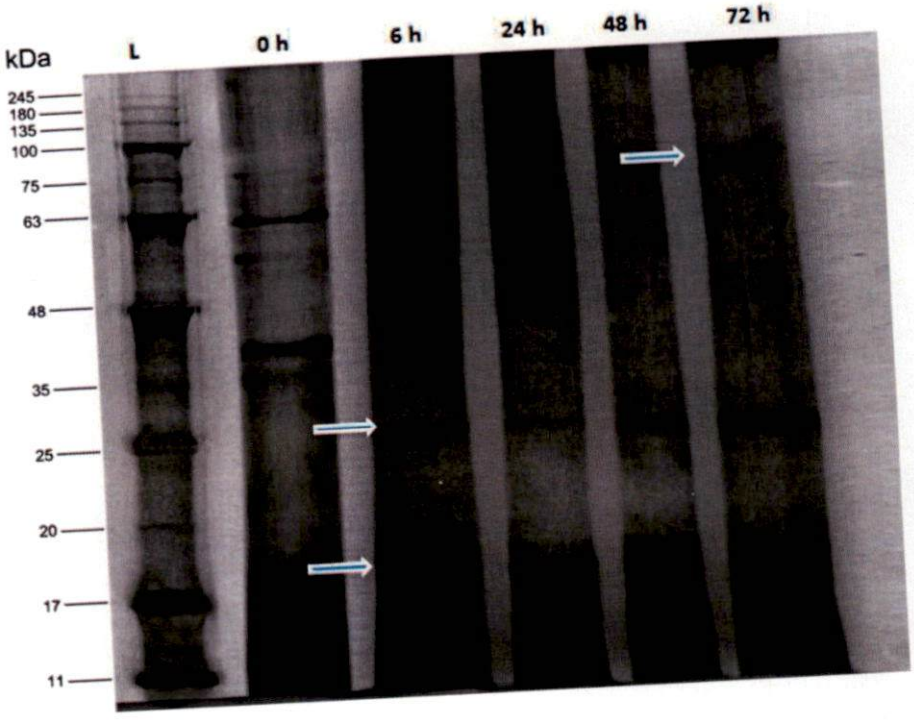


7a. Protein profile of TMB released on Raghav

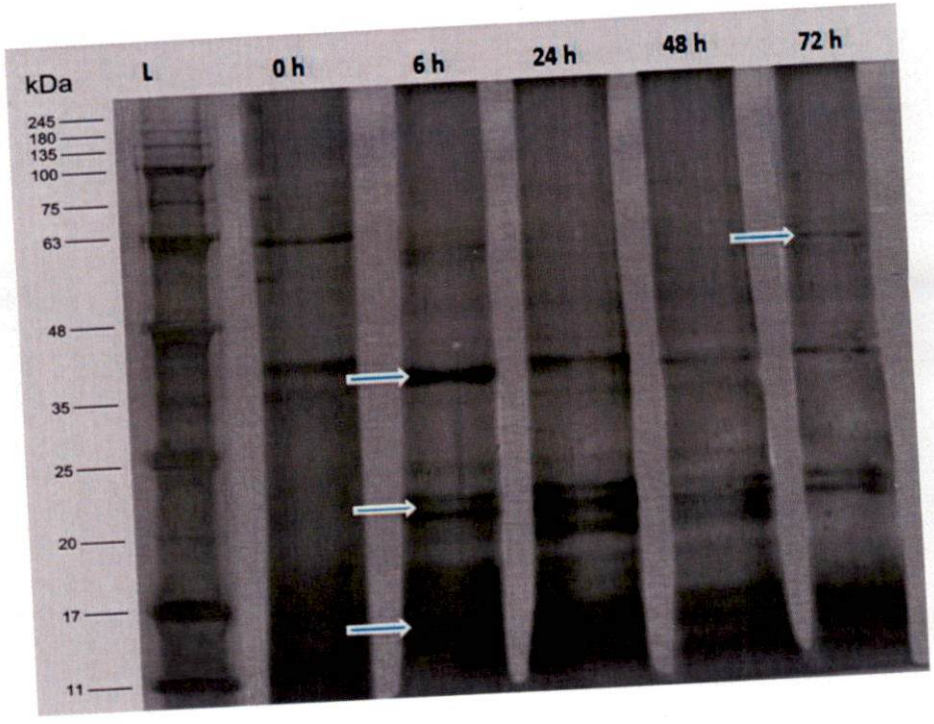


7b. Protein profile of TMB released on Damodar

Lane L: Prestained protein ladder(5µl) Lane 1: control; Lane 2-5: protein isolated at different time intervals (6 h, 24 h, 48 h and 72 h)(10µl)



7c. Protein profile of TMB released on Anagha



7d. Protein profile of TMB released on Madakkathara-1

Lane L: Prestained protein ladder(5µl) Lane 1: control; Lane 2-5: protein isolated at different time intervals (6 h, 24 h, 48 h and 72 h)(10µl)

Discussion

5. DISCUSSION

The experiment entitled "Elucidating the biochemical basis of interaction between tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) and cashew (*Anacardium occidentale*)" conducted at the Department of Agricultural Entomology, College of Horticulture Vellanikkara during 2016-18. The results obtained from the study presented in the previous chapter are discussed below.

5.1 BIOCHEMICAL ANALYSIS OF CASHEW VARIETIES

Introduction of large genetic variability through hybridization and varietal improvement programmes in cashew has induced pest population build up in highly susceptible varieties (Beevi *et al.*, 2001). Even though there is no evidence on the occurrence of a completely resistant variety against tea mosquito bug (TMB), wide variation exists in the response of cashew varieties to TMB infestation (Ambika *et al.*, 1979; Sathiamma, 1977). The biochemical basis of interaction between selected cashew varieties and tea mosquito bugs were investigated in the present study which would be very useful in resistance breeding programmes. Unraveling defence mechanism of tea mosquito bug to different xenobiotics will also open up new avenues for developing alternative strategies for pest management.

Tea mosquito bug induced biochemical changes in selected cashew varieties were stated in the present study and the variations in protein, secondary metabolites *viz*, phenol, tannin and the defence enzymes like polyphenol oxidase (PPO) and phenyl alanine ammonia lyase (PAL) were worked out.

5.1.1 Protein

There was a significant variation in the protein content of selected cashew varieties (Fig. 7). The highest protein content was observed in the uninfested plants of less susceptible variety Damodar and was followed by Raghav, which also belong to less susceptible category. Raghav showed a declining trend in protein content throughout the period of TMB infestation, whereas the variety Damodar recorded decrease in total protein content for up to 24 h of feeding and thereafter it showed an increasing trend of protein content up to 72 h of TMB attack, with a significantly superior protein content of 1.063 mg/g. Irrespective of the susceptibility status, protein content decreased in all the four varieties compared to that of uninfested plants, and this was in confirmation with the finding by Shah *et al.* (2014). The study revealed that infestation of tea leaves by *H. theivora* caused reduction in the level of total protein and total carbohydrate compared to healthy plants. The mean protein content was found to be highest in less susceptible Damodar followed by highly susceptible Anagha. Even though Damodar had highest protein content, it was categorized as less susceptible group possibly due to presence of high level of secondary metabolites *viz.*, phenol and tannin, which could make the plant protein unavailable to the insect.

Many plant proteins acts as digestive inhibitors in herbivores. Through jasmonic acid pathway some defensive proteins are induced in plant parts, which interfere with herbivore digestion. For example, some legumes synthesize α-amylase inhibitors that hinder the action of starch digesting enzyme α-amylase. Other plant species produce lectins, which are defensive proteins that bind to carbohydrate containing proteins. Lectin woluld bind to epithelial cells lining the digestive tract and interfere with nutrient absorption (Taiz and Zeiger, 2010).

5.1.2 Phenol

TMB infestation resulted in a significant increase in the total phenol content of all the test varieties (Fig. 8) except in case of highly susceptible variety Anagha. Before infestation, the total phenol content was highest in the less susceptible varieties Damodar and Raghav (63.960 mg/g and 61.641 mg/g respectively) which were on par with each other. The highly susceptible varieties Anagha and Madakathara-1 had lowest phenol content (42.027 mg/g and 45.055 mg/g respectively). Phenol content ranged from 61.641 mg/g to 79.273 mg/g in the less susceptible varieties, while it ranged from 20.578 mg/g to 68.747 mg/g in highly susceptible varieties at different time intervals following TMB infestation. The less susceptible variety Damodar showed an increasing trend across all the time intervals and the highest phenol content was observed at 72 h of TMB feeding (79.273 mg/g). The variety Raghav which also belonged to the less susceptible type recorded higher phenol content for up to 48 h of feeding and then showed a slight decrease in phenol content (67.745 mg/g).

The highly susceptible variety Anagha reported a drastic decline in phenol content after 6 h of feeding and from 24 h of TMB exposure, phenol content gradually increased up to 48 h. These findings are in agreement with the observations of Nagaraja *et al.* (1994) where they observed an increase in phenol content during the first 24 h of TMB feeding. Similar results were also obtained by Bindu (1996) where she reported that the highest phenol content (17.522 mg/g) was recorded in a less susceptible accession (A-6-1) and the lowest phenol content was observed in the highly susceptible accessions H-8-7 and BLA 256-4 (1.099 mg/g each) at 12 h after feeding by TMB. Several authors have confirmed the importance of phenolics in plant resistance against insect pests (Chelliah and Sambandam, 1971). Rai and Naghraja (1988) reported higher content of phenols and O-dihydroxy phenols in cashew accessions less susceptible to TMB. According to a study conducted by Naik (2010) the least susceptible variety Ullal-1 recorded the highest phenol content (8.1 mg/g) where as the highly susceptible

variety V-4 had recorded the lowest phenol content (0.3 mg/g). All these works indicate the importance of phenols in imparting resistance to TMB in cashew.

According to van Sumere *et al.* (1975) most of the poly phenols bind nonspecifically with protein, consequently reducing the dietary protein availability to insects or inhibiting the enzyme activity. Catechol based phenolic compounds oxidize rapidly to ortho quinones in the presence of oxygen. The highly reactive ortho quinone has a higher affinity for lysine, which forms covalent bonds resulting in the reduction in the dietary lysine thereby decreasing the host nutritional value (Hurrel *et al.*, 1982).

Biochemical induction of secondary metabolites and defence enzymes in different wheat genotypes against aphid infestation was done by Kaur *et al.*, (2017). They had reported enhanced phenol content in all the infested plants of different wheat genotypes, compared to the uninfested plants. Phenol compounds inhibit larval development and growth by acting as feeding deterrents. A study conducted by Helmi and Mohamed (2016) stated that the phenolic compounds induced in plants have different roles in plants. They also reported that, these compounds become either directly toxic to insects or otherwise mediate various signaling and transduction pathways which resulting in the formation of various toxic secondary metabolites and enhanced activity of various defence enzymes.

Fig.7. Variation in total protein (mg/g) content of selected cashew varieties before and after TMB infestation

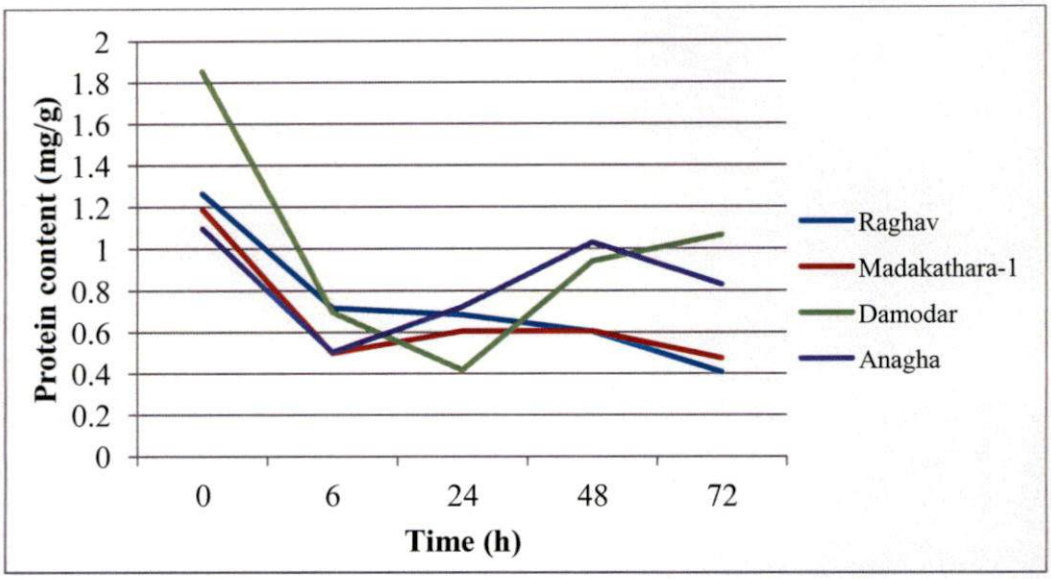
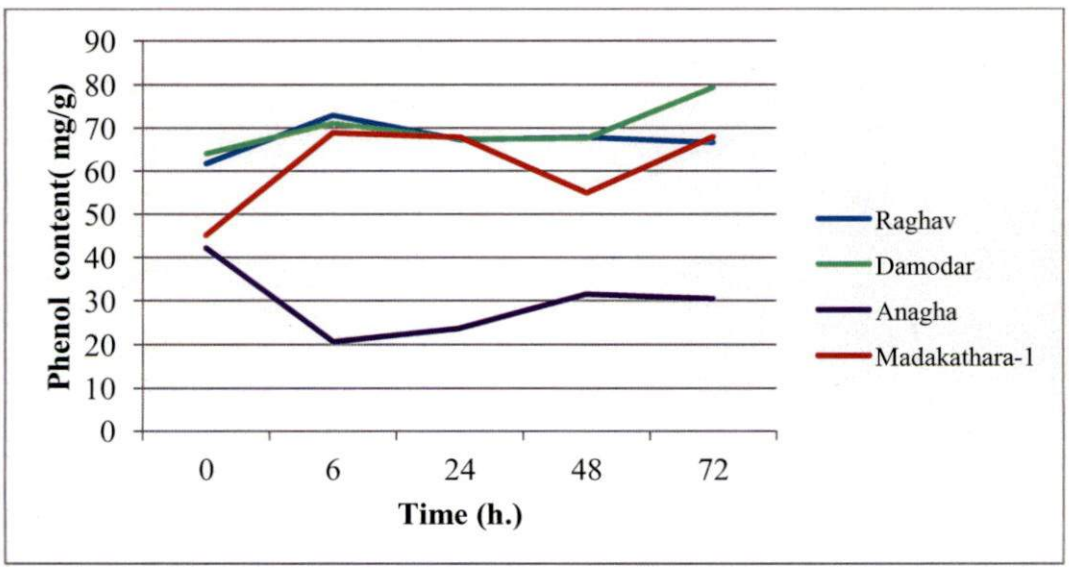


Fig.8. Variation in total phenol content (mg/g) content of selected cashew varieties before and after TMB infestation



5.1.3 Tannins

The variation in tannin content after different periods of TMB release are presented in Fig.9. Prior to TMB infestation the less susceptible variety Raghav had the highest tannin content (10.335 mg/g). Similarly Damodar, which also belonged to the same susceptibility group, also had a relatively higher tannin content of 6.657 mg/g. Both these varieties were found to be superior over highly susceptible Madakathara-1 and Anagha. This is in conformity with the findings of War *et al.* (2012) who reported that the amount of total phenols and condensed tannin were higher in the less susceptible varieties of ground nut against *Helicoverpa armigera,* as compared to the highly susceptible varieties.

There was a decline in tannin content for up to 24 h following infestation in all the varieties. After 24 h, the tannin content elevated back but was significantly lower than pre infestation level even after 72 h. This is in contrast with the study conducted by Chowdhury *et al.* (2016), where they revealed that tea mosquito bug infested tea leaves has got higher tannin content (21.3 mg/g) when compared to un infested leaves (18.69 mg/g).

As early as in 1976, Feeny reported that the tannins are important compounds having protective function and they act by reducing the availability of dietary protein. But the results obtained in a study conducted by McColl and Noble (1992) on cotton against *H. armigera* revealed that there was no correlation between larval biomass gain and condensed tannin content of inflorescence.

According to Swain (1977), tannins mainly act by blocking the digestive process in the arthropod gut. Later Bernays *et al.* (1981) reported that tannins primarily act as feeding deterrants. Herbivores preference towards host plant depends on many factors like nutritional quality of plant tissue, relative abundance of food types and the risk of feeding on the kind of food. Tannin would form hydrogen bonding with protein and make it unavailable to herbivore. It is also involved in chelation of metal ions which are essential for activation of enzymes

in insects; hence render bioavailability to phytophagous insects (Barbehenn and Constabel, 2011)

Rather than acting as a hindering agent towards the plant protein availability to insects, the presence of tannin may also adversely affect insect physiology and anatomy. One such example for adverse effect on insect was reported by Sharma *et al.* (2009). They found that in *H. armigera* midgut lesions were formed after fed on pegion pea which was rich in tannin.

5.1.4 Poly phenol oxidase (PPO)

Irrespective of the varieties, specific activity of PPO increased (Fig 10) upon TMB infestation across the varieties studied, though the level of expression varied with varieties. Before release of the bug, PPO specific activity was higher in the highly susceptible variety Madakkathara-1 (0.00141 EU/g/min).

After 6 h of TMB infestation the highest PPO activity was reported by the less susceptible variety Damodar (0.00317 EU/g/min) and was immediately followed by Raghav (0.0014 EU/g/min) which is also a less susceptible variety. Lowest PPO activity was recorded (0.00083 EU/g/min) by the highly susceptible Anagha variety. The less susceptible varieties Raghav and Damodar recorded an increasing trend in PPO activity up to 48 h after feeding by TMB (0.00804 EU/g/ min and 0.00511 EU/g/ min respectively). Similar findings were observed in a study conducted by Kaur *et al.* (2017). While evaluating biochemical response of different wheat genotypes against aphid attack, it was observed that the infested wheat plants showed significantly higher PPO activity when compared to the uninfested wheat plants. PPO activity was maximum at 28 days after the emergence of flag leaf in the wheat genotype HD 2967.

Raj *et al.* (2006) reported that the main function of PPO was to catalyse the oxidation of phenols to quinones and these quinones are highly toxic to pathogen. They have reported that PPO activity is negatively correlated with the disease occurrence.

Helmi and Mohamed (2016) stated that the quinones formed by PPO caused alkylation of essential amino acids and thereby decreased the plant nutritional quality. Quinones also producing oxidative stress in the gut lumen of herbivores.

A study conducted by Ishaaya and Sternlicht (1971) reported that polyphenols will be converted in to quinones due to increased activity of polyphenol oxidase in eriophyid infested citrus leaves. Melanin and tannins were formed as a result of reaction between quinones and proteins in plant tissue there by resulted in discolouration of damaged leaves.

The nutritional value of plant tissue is reduced by alkylation of aminoacid as incited by PPO. This in turn adversely affected growth and development of *H. armigera* and *S. litura* (Bhonwong *et al*., 2009).

Fig.9. Variation in total tannin (mg/g) content of selected cashew varieties before and after TMB infestation

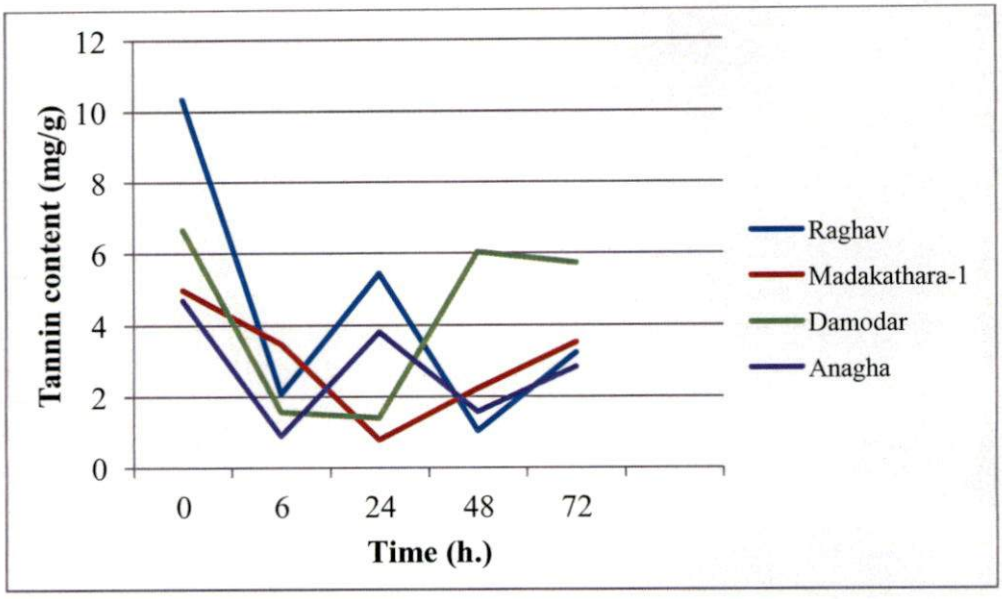
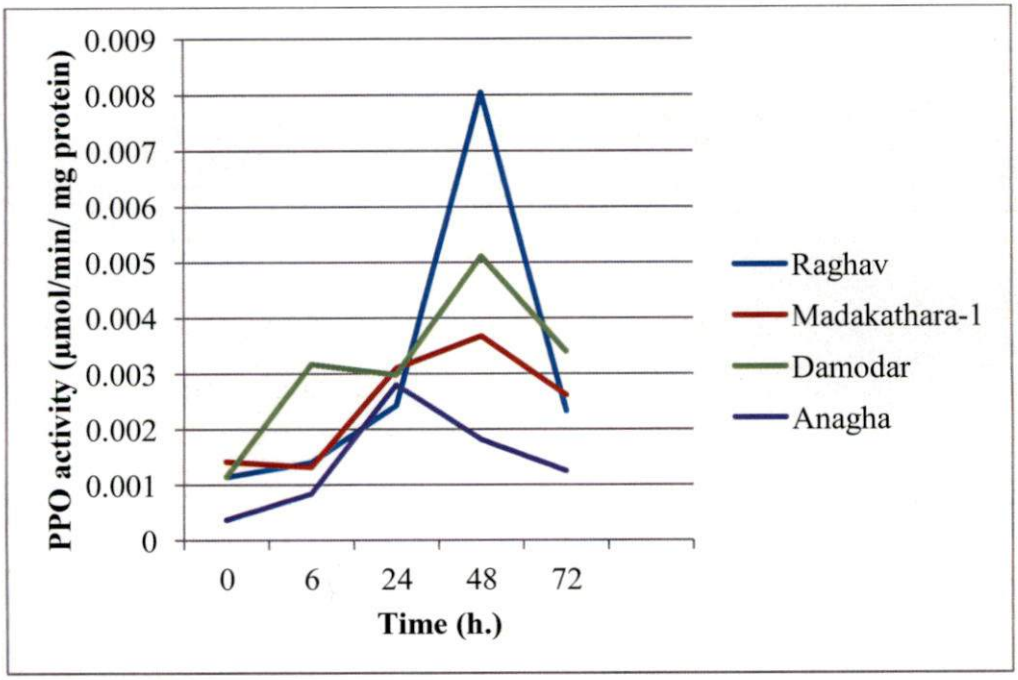


Fig.10. Variation in total PPO activity (µmol/min/mg protein) of selected cashew varieties before and after TMB infestation



5.1.5 Phenyl alanine ammonia lyase (PAL)

The hyper sensitive reaction in plants upon induced by stress condition resulted in increased specific activity of PAL enzyme. The role of PAL as defense enzyme was reported in many studies.

Irrespective of the tolerance level, specific activity of PAL was found to be increasing in all the four cashew varieties after TMB infestation (Fig. 11). In a study conducted by Suganthi *et al.* (2018), TMB infestation in tea resulted in higher PAL activity compared to uninfested samples, irrespective of the susceptibility status of varieties. The reason for increased PAL activity might be due to production of new PAL enzyme as a result of hypersensitive reaction. Tobacco infected with tobacco mosaic virus also showed an increased PAL activity triggered by hyper sensitive reaction (Duchesne *et al.*, 1977).

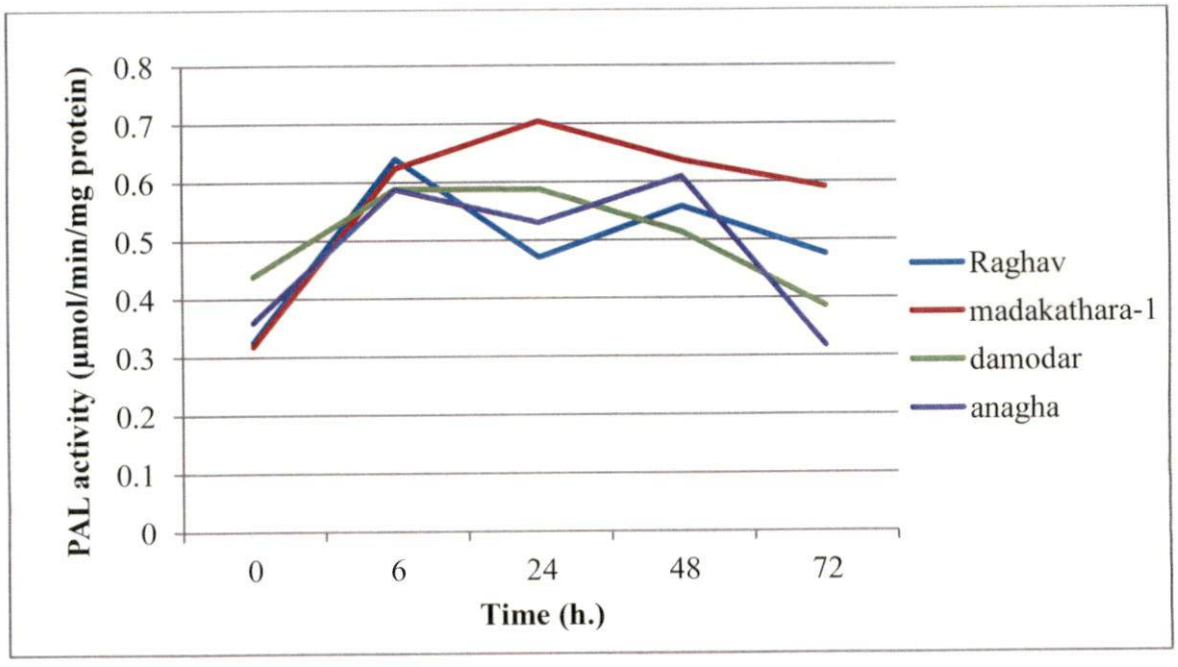
The possibility for increase in PAL activity could be, the inactive form of PAL already present in the plant being converted to active form. An experiment conducted by Blondel *et al.* (1973) found that the PAL converted from inactive form to its active form in radish cotyledon when exposed to light of wavelengths 710-720 nm.

Specific activity of PAL was showing increasing as well as decreasing trend at different time intervals after TMB infestation. The decrease in enzyme activity could be due to utilization of the formed enzyme for coversion of phenyl alanine to salicyilic acid. The PAL enzyme catalyse the formation of cinnamic acid from phenyl alanine, and converted finally to salicylic acid, which has got an important role in plant defence (Raskin, 1992).

At 72 h after feeding there was a drastic decline in PAL activity in all the varieties infested with TMB, and was confirmity with the results obatained in an experiment conducted by Brueske (1980), where he found that tomato root infested with root knot nematode showed lower PAL activity after 108 h of infestation, that incresed after 120 h of infestation.

Increased amount of PAL resulted in the synthesis of phenolic compounds as it calyse the formation of phenolic secondary compounds from phenyl alanine, which is a product of shikimic acid pathway. This path way will convert simple carbohydrate precursors in to three aromatic amino acids; phenyl alanine, tyrosine, and tryptophan (Herrmann and Weaver, 1999), which is very essential for animal nutrition since animals and insects have no way to synthesize aromatic amino acids (Taiz and Zeiger, 2010). Increased specific activity of PAL in the highly susceptible variety Madakkathara-1 could have contributed to the increased production of the heterocyclic aromatic aminoacids, that are providing the essential nutrients to feeding by TMB on this variety.

Fig.11. Variation in total PAL activity (µmol/min/mg protein) of selected cashew varieties before and after TMB infestation



5.2 DETOXIFICATION MECHANISM IN TEA MOSQUITO BUG

The role of insect detoxification enzymes in overcoming plant defence is well established. Analysing defensive enzymes is one of method to study the reason for tolerance in insects.

Over production of detoxification enzymes as well as change in insect protein content in response to plant secondary metabolites (tannin and phenol) and defensive enzymes *viz.,* PAL and PPO were analysed in this study. Carboxylesterase (CarE), glutathione-S-tranferases (GST) and cytochrome P450 were found to be enhanced upon TMB feeding and showed variation with respect to the susceptibility status of the varieties.

5.2.1 Insect protein

Insect protein content was showing wide variation upon exposure to different cashew varieties during different time intervals (Fig. 12).

After 6 h of release insect samples from Raghav showed a significant increase in protein content, this could be due to increased feeding.

The highest protein content was observed in bug fed on Anagha after 24 h and in Madakkthara-1 after 72 h. This could be due to enhanced detoxification enzyme activity.

After 48 h the protein content was high in bug fed on less susceptible varieties Raghav and Damodar. This could be due to increased specific activity of detoxification enzymes.

Since crude protein of insect included metabolic protein as well as detoxification enzymes the exact role of insect protein towards over coming plant defence mechanisms could not able to reveal from this study.

5.2.2 Protein profiling by SDS PAGE in TMB

SDS- PAGE was employed to analyse the molecular weight and banding pattern of proteins in TMB after different intervals of exposure on cashew grafts belonging to less susceptible and highly susceptible categories.

The electro phoretic pattern of total TMB protein at different time intervals revealed a different pattern with notable variations in the appearance of bands. The whole body homogenate of single adult un fed female TMB, the protein bands of~ 63 kDa and 35-48 kDa were appeared, as the exposure time proceeded, protein bands ranging from 17-75 kDa appeared. This is in conformity with the study conducted by Ayyangar and Rao (1990), where they observed that *S. litura* when exposed to azadirachtin showed disappearance of few protein bands. The reason behind protein decline in insect may be due to influence of plant products on endocrine glands of insect, which will cause changes in level of protein.

In TMB fed on Raghav, additional band of 48-63 kDa were expressed after 48 and 72 h of feeding. In case of TMB fed on Anagha, one additional band of 75-100 kDa was expressed during 72 h of exposure. The bands of same molecular weight were also expressed in TMB fed on Damodar but at 6 and 24 h of exposure.

Certain extra bands appeared after exposure of insect to host plant. Chen and Levenbook (1966) observed appearance and disappearance of protein bands during insect development. SDS PAGE results of three cereal aphid species suggest that three different protein bands might be corresponding to three subunits. The molecular weight of subunits ranged from 24,000 to 28,500 Da. As GST is made of several subunits it may be able to detoxify several allelochemicals (Leszczynski *et al.*, 1994). All these studies are pointing towards the fact that protein profile in insects will vary depending on exposure of insects to certain xenobiotics.

5.2.3 Carboxyl esterase (CarE)

The carboxyl esterase specific activity was increased in TMB after feeding (Fig.13) on all the four cashew varieties. The CarE specific activity was recorded maximum in TMB fed on less susceptible Raghav (859.683 µmol/min/mg protein) and was followed by Damodar (500.464 µmol/min/mg), which was having highest phenol content of 79.273 mg/g was recorded at 24 h of feeding. From this, it could be inferred that the insect utilizes most of its energy for detoxification of secondary metabolites present in plants. This is in agreement with the previous findings of Saha *et al*, (2012), where they found that the general esterase activity of *H. theivora* increased when it was reared on secondary metabolite rich *Mikania micrantaha* Kunth was higher when compared to that reared on tea. Another reason for increased specific activity of CarE is due to the induction of inactive forms present in insect upon exposure to secondary metabolites. Esterase present in insects has got different forms that will get induced in the presence of allelochemicals leading to changes in susceptibility of insect towards insecticides (Yang *et al*, 2001).

The mean specific activity of CarE in TMB infested on less susceptible varieties *viz.,* Raghav and Damodar was found to be high when compared to TMB fed on highly susceptible Anagha and Madakathara-1. This could be due to the fact that less susceptible varieties are richer in tannins and phenols. The insect has to produce biochemical armours to overcome plant defence. Any kind of stress *viz*., allelochemicals or insecticides may result in over production of esterase enzymes. These results are in agreement with the findings of Saha *et al*. (2012), where they reported that enhanced esterase activity in *H. theivora* exposed to insecticidal spray when compared to bugs collected from non sprayed area.

Fig.12. Variation in total protein (mg/g) content of TMB before and after infestation

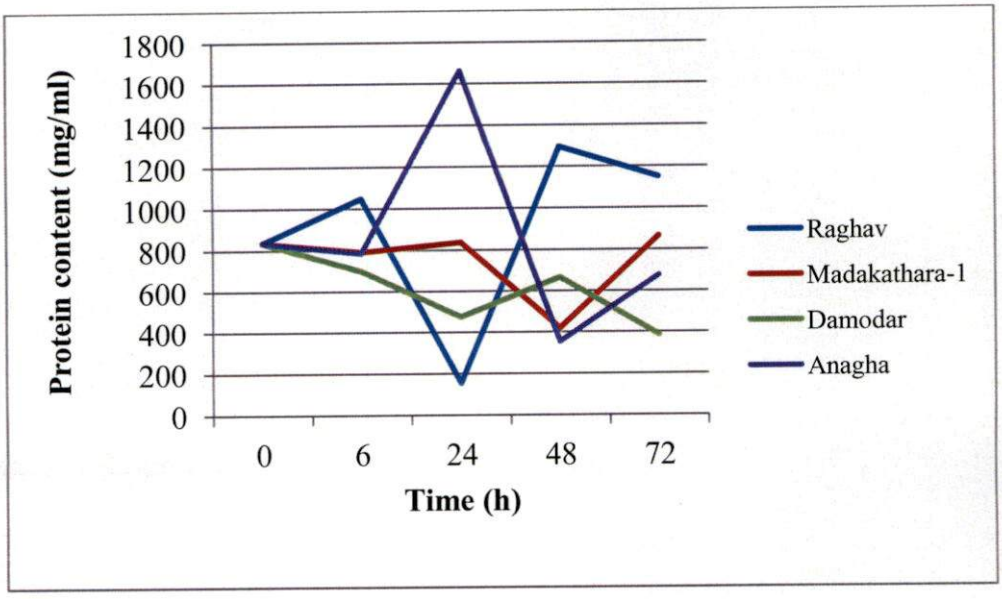
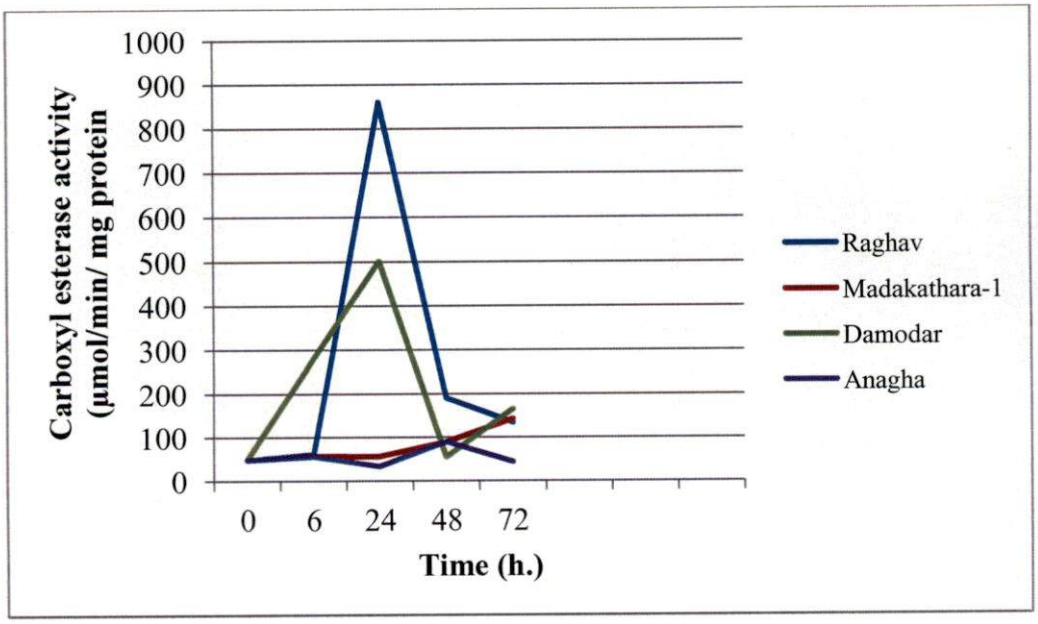


Fig.13. Variation in carboxyl esterase specific activity (µmol/min/ mg protein) of TMB before and after infestation



5.2.4 Cytochrome P450

TMB released on less susceptible variety Raghav showed highest cytochrome P450 activity after 24 h of feeding, whereas, Damodar showed higher activity after 48 h of release when compared to highly susceptible Anagha and Madakathara-1 (Fig. 14).

The increased monoxygenase activity in *H. theivora* female fed on *Mikania micrantha* and *Psidium guajava* than on *Cammelia sinensis*. This difference was due to variation in the levels of xenobiotics among these plants. This enzyme has got wide substrate range and which includes an array of allelochemicals (Terriere, 1984). Cytochrome P-450 has got a role in metabolism of wide variety of xenobiotics and secondary metabolites in host plants (Cohen *et al*, 1986). This enzyme is involved in transfer of oxygen from molecular oxygen to a substrate, and also they involved in reduction of other oxygen atom into water. Since these enzymes are involved in both oxidation and reduction reaction they are known as mixed function oxidase (Rose and Hodgson, 2004)

There was increased enzyme activity in TMB released on Damodar following 72 h of release. The phenol content in Damodar also found to be high at 72 h (79.273 mg/g). This finding is in agreement with study conducted by Feyereisen, (1999). Where it was observed that, enhanced activity of detoxifying enzyme would help to increase the solubility of plant secondary metabolites and there by aid in the elimination of such toxic metabolites entered inside the body of insect. Another study conducted by Chandra *et al*. (2016) found that expression levels of cytochrome P450s positively correlate with concentration of gossypol in *Helicoverpa armigera*.

The four species of *Drosophila* endemic to the Sonoran desert colonized in alkaloid rich cactus tissue by overcoming the hazardous effect of allelochemicals. It was possible by the activities of the cytochrome P450 mediated reactions which were responsible for alkaloid metabolism. This was evident from the induced

response of cytochrome P450 to the presence of cactus alkaloid in the artificial diet of *Drosophila* sp. maggots and adults (Frank and Fogleman, 1992).

Many insects detoxify the xenobiotic compounds using cytchrome P450 monoxygenase and glutathione S-transferases. In corn earworm, exposure to xanthotoxin induced cytochrome P450 expression (Li *et al.*, 2007).

According to a study conducted by Snyder and Glendinnig, (1996) the P450 inhibitor pipernoyl butoxide when provided with diet resulted in decreased consumption of tobacco hornworm. From this it is very clear that P450 has an important role in continuing the consumption by overcoming the barriers like xenobiotics.

5.2.5 Glutathione-S- transferases (GSTs)

GSTs are a group of multifunctional enzymes, which are involved in conjugation of glutathione and xenobiotic substances for detoxification and protection from oxidative damage (Board and Menon, 2013). It would catalyse the conjugation of electrophilic compound with reduced glutathione (GSH).

In this experiment, the GST activity in TMB was increased (Fig. 15) after feedig on different cashew varieties, when compared to the enzyme activity before releasing them on their host plants. This result was in conformity with the findings of Francis *et al.* (2005). They observed that the activity of GST enzyme in *M. persicae* (green peach aphid) increased in response to secondary metabolites from brassica plants.

The highest GST activity was recorded in less susceptible Damodar which has got higher secondary metabolites (Fig 15). Another study coinciding with this result has been conducted by Yu (1983), where GST inductions in response to the presence of plant allelochemicals in artificial diets of lepidopteran species *Spodoptera frugiperda* were observed.

As the feeding time reached to 24 h there was a sharp increase in GST specific activity in TMB fed on Raghav which belongs to less susceptible

category. The enzyme activity recorded was lowest in TMB fed on highly susceptible Madakathara-1 (13.569 µmol/min/mg protein). This variation in enzyme activity might be due to variation in the level of secondary metabolites, which play important role in enhancing detoxification enzyme level. This is in agreement with the study conducted by Yu (1983), where gossypol was found to enhance the activity of GST in fall armyworm.

After 48 h of exposure, TMB fed on Damodar, Anagha and Madakkathara-1 showed significantly higher enzyme activity over that after 24 h. TMB from Raghav showed a steep reduction (161.904 µmol/min/mg protein).

The specific activity of GST was significantly reduced when feeding time reached up to 72 h except in case of those that fed on highly susceptible Madakathara-1. These findings are in conformity with previous study conducted by Arora *et al.* (2008), where they observed that the second instar nymphs of *Lipaphis erysimi* when exposed to coumarin in mustard has shown significant induction of GST activity during initial period of feeding. As the period of feeding increased, the GST activity notably decreased. It was also observed that, there was induction of GST activity after 24 h of feeding in aphid when host plant changed from winter wheat to winter triticale (less susceptible cultivar), but as the feeding time increased to 24 h the GST activity reduced (Lukasik *et al.*, 2015).

Essence of the study is that, the plant secondary metabolites like tannins, total phenols and the defence enzyme PPO have a definite role in imparting resistance to TMB in cashew. This would help to develop biochemical markers in identifying resistant varieties in perennial crops especially cashew and resistance screening can be done in the graftling stage itself.

The enhanced enzyme activity in TMB revealed its polyphagous nature and the reason for the lack of a completely resistant variety in cashew. Understanding the detoxification mechanism of TMB to variations in secondary metabolites would help to develop alternate pest management strategies.

Further studies with more varieties are needed for the development of biochemical markers and the survival and fitness of TMB reared on each variety should be studied in order to confirm that whether the energy costs for detoxification sacrificed the survival and fecundity of TMB.

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Fig.14. Variation in cytochrome P450 specific activity (nmol/min/mg protein) of TMB before and after infestation

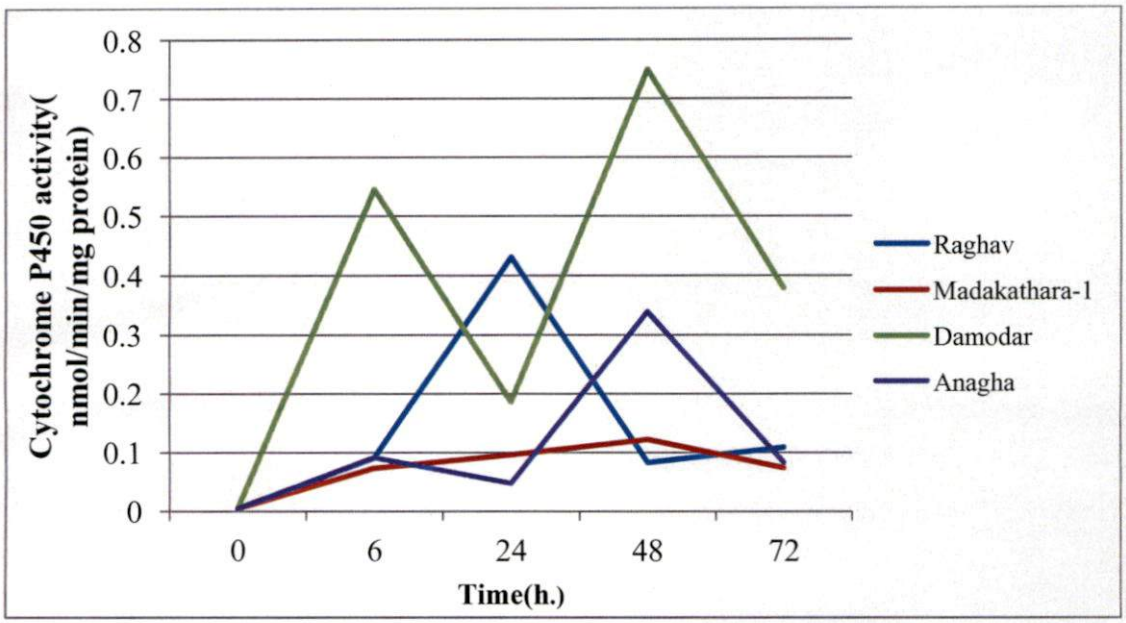
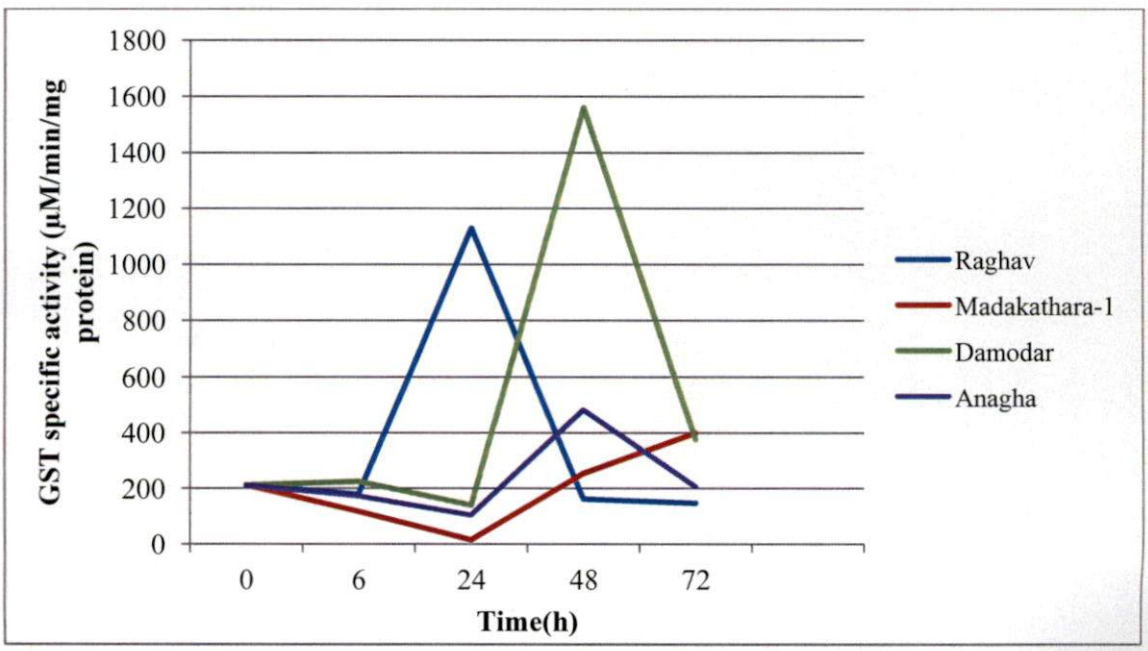


Fig.15. Variation in GST specific activity (µmol/min/mg protein) of TMB before and after infestation



Summary

6. SUMMARY

The present work entiled on "Elucidating the biochemical basis of interaction between tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) and cashew *(Anacardium occidentale)*" was carried out at the pesticide residue laboratory, AINP on Ornithology, Department of Agricultural Entomology COH,Vellanikkara, CRS, Madakathara, Cocoa Research Centre,Vellanikkara ,CPBMB Vellanikkara and College of Veterinary and Animal Sciences, Mannuthy during 2016-18. It consists of the study of variation in total protein, secondary metabolites like phenol, tannin and defensive enzymes like PAL, PPO of selected cashew varieties belongs to highly susceptible and less susceptible categories, upon induced by TMB infestation. It also include variation in total protein, SDS PAGE profiling of TMB protein at different intervals of feeding, analysis of detoxifying enzymes like carboxyl esterase, cytochrome P450, and GST after feeding on highly susceptible and less susceptible cashew types.

The important findings of the study are summarized here under

- Biochemical parameters of two highly susceptible varieties namely, Madakkathara-1 and Anagha as well as two less susceptible varieties such as Raghav and Damodar were assessed before and after infestation to understand varietal response to TMB attack.

- Biochemical analysis of the tea mosquito bug before and after feeding on the less susceptible as well as highly susceptible cashew varieties were assayed to study the response of TMB to biochemical defences of selected cashew varieties.

- There was no significant correlation between the plant protein content and susceptibility levels of varieties. Total protein was high in less susceptible Damodar and in highly susceptible Anagha, while it was least in the highly susceptible Madakkathara-1.

- Total phenol content was high in less susceptible varieties Damodar and Raghav. The highly susceptible Anagha recorded lowest phenol content
- High tannin content was recorded in less susceptible varieties Raghav and Damodar while Anagha recorded lowest tannin content.
- PPO specific activity observed highest in Damodar, followed by Raghav and observed least in Anagha
- PAL specific activity was highest in Madakathara-1 and other three varieties recorded lower activity
- Insect crude protein after 72 h of exposure was highest in TMB fed on less susceptible variety Raghav and lowest in TMB fed on Damodar
- Changes were observed in the protein profile of the bugs following exposure to selected varieties with proteins of varying molecular weight being synthesized after feeding. Further studies are required to identify specific proteins.
- Carboxylesterase activity was maximum in TMB fed on less susceptible Raghav and least in TMB fed on highly susceptible varieties
- Cytochrome P450 activity was highest in TMB fed on less susceptible variety Damodar and observed least in TMB fed on highly susceptible varieties
- GST activity was highest in TMB fed on Damodar and least in bug fed on highly susceptible Madakathara-1
- The study revealed that, biochemicals play a significant role in mediating interaction between cashew and its herbivore, the tea mosquito bug. The secondary metabolites in cashew, such as phenol, tannin and PPO could have a role in imparting resistance to TMB attack and the conformation of such a correlation can be useful in the early detection of resistance against TMB. The enhanced detoxification enzymes levels in TMB gave an

indication of the plasticity of the bug against host plant defense mechanisms as well as synthetic insecticides.

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Appendix

APPENDIX-1

Sodium phosphate buffer

Stock solutions

A : 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml)

B : 0.2 M solution of dibasic sodium phosphate (53.65 g of Na_2HPO_4. $7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000ml)

x ml of A and y ml of B diluted to a total of 200ml.

x	y	pH
68.5	31.5	6.5
56.5	43.5	6.7
45.0	55.0	6.9
33.0	67.0	7.1
23.0	77.0	7.3
16.0	84.0	7.5

APPENDIX -2

Tris. HCl Buffer

Stock solutions

A : 0.2 M solution of Tris. HCl (24.2 g in 1000ml)

B : 0.2 N HCl

50 ml 0f A , x ml of B, diluted to a total of 200ml

x	pH
44.2	7.2
41.4	7.4
38.4	7.6
32.5	7.8
26.8	8.0
21.9	8.2

APPENDIX -3

Preparation of reagents for SDS-PAGE

Acrylamide stock solution (40%)

The stock solution was prepared by dissolving 38g acrylamide and 2g bis acylamide in 100 ml double distilled water. It was stored at 4^0C in an amber coloured bottle.

Resolving gel buffer (1.5M Tris buffer, pH 6.8)

The buffer was prepared by dissolving 18.5 g tris base (MW 121.1) in 50-60 ml double distilled water. The pH was adjusted to 8.8 with 1N HCl and made up the volume to 100 ml.

Stacking gel buffer (0.5 M Tris buffer, pH 6.8)

Prepared by dissolving 6g tris base in 50-60 ml double distilled water, the pH was adjusted to 6.8 with 1N HCl and then the volume made up the to 100 ml.

Electrophoresis tank buffer (0.025 M Tris buffer, 0.192 M glycine, 0.1% SDS, pH 8.3). The tank buffer was prepared by dissolving 3.05 g tris buffer, 14.4 g glycine and 1g SDS in distilled water and made up the volume to 1000 ml.

SDS (10%) Prepared by dissolving 10 g SDS in 100 ml double distilled water.

Treatment buffer (0.125 M tris buffer)

Prepared by mixing 2.5 ml stacking gel buffer, 2 ml glycerol, 0.2 ml mercapto ethanol, 0.2 g bromophenol blue, 4 ml of 10% SDS and 10 ml of double distilled water.

Initiator/ammonim per sulphate (APS) 10%

It was prepared freshly by dissolving 0.1 g APS taken in eppendorf tube covered with aluminium foil (to prevent contact with light) and mixed 1 ml of distilled water.

TEMED (N, N,N,'N,' Tetra Methyl Ethylene Diamide) was used as catalyst. It was added directly from reagent bottle.

Resolving gel (8%)

Double distilled water 8.5 ml, acrylamide stock solution (40%) 3.2 ml , 4ml of 1.5 M resolving gel buffer, 160µl of 10% SDS, 160 µl APS and 16 µl TEMED were mixed together for preparation of resolving gel .

Stacking gel (4%)

For stacking gel preparation 6.3 ml double distilled water, 1ml of 40% acrylamide stock solution, 2.5 ml of stacking gel buffer, 100 µl of 19 % SDS, 100 µl of APS and 10 µl TEMED mixed properly and used for casting the gel.

Fixing solution

The fixing solution was prepared by mixing 30% ethanol and 10% acetic acid in 1:1 ratio.

Pretreatment solution

Sodium thiosulphate(0.02%) was prepared by dissolving 0.02g sodium thiosulphate in 100 ml of distilled water.

Developing solution

Formaldehyde (250 µl) was mixed with 3 g of sodium carbonate and 0.5 mg of sodium thiosulphate. The volume was made up to 100 ml with distilled water.

Stop solution

4% tris buffer was prepared and added with 2 % acetic acid.

ELUCIDATING THE BIOCHEMICAL BASIS OF INTERACTION BETWEEN TEA MOSQUITO BUG, *Helopeltis antonii* Signoret (HEMIPTERA: MIRIDAE) AND CASHEW (*Anacardium occidentale*)

By

NIMISHA T.

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ABSTRACT OF THE THESIS

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Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR – 680 656
KERALA, INDIA
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ABSTRACT

Cashew is one of the important foreign exchange earning crops of India with an export value of Rs. 5,077 crores during 2016-17.Cashew production is not in tune with increase in area under its cultivation. Low productivity in cashew is mainly due to the incidence of pests and diseases. Among the insect pests, tea mosquito bug (TMB), *Helopeltis antonii* Signoret (Hemiptera: Miridae) is the most important. It causes 30-50 per cent yield loss and during outbreak situations even up to 100 per cent loss has been reported. Cashew varieties exhibit wide variation in response to TMB infestation. Hardly any variety has ever been recorded as resistant to TMB till date. However, a few accessions are reported to be capable of withstanding TMB infestation and hence have been grouped as less susceptible category. Understanding the basis of interaction between the bug and the cashew is a prerequisite in developing varieties resistant to TMB.

The present study entitled "Elucidating the biochemical basis of interaction between tea mosquito bug, *Helopeltis antonii* Signoret (Hemiptera: Miridae) and cashew (*Anacardium occidentale*)" was undertaken at the Department of Agricultural Entomology, College of Horticulture, Vellanikkara during September 2017 to July 2018. The objective of the study was to understand the variation in secondary metabolites and defense enzymes in selected cashew varieties induced by tea mosquito bug infestation and to elucidate the secondary metabolite detoxification mechanisms in the pest.

Three months old grafts of four cashew varieties *viz.*, two from the highly susceptible category (Anagha, Madakkathara-1) and two from less susceptible category (Damodar, Raghav) were used to conduct the experiment. Female adult bug (0-24 h old) was allowed to feed on each variety for different time intervals of 6, 24, 48 and 72 h. The biochemical parameters such as protein, phenol, tannin and defense enzymes *viz.*, polyphenol oxidase (PPO) and phenyl alanine ammonia lyase (PAL) in leaves of TMB infested and non infested cashew grafts were analysed before release (0 h) as well as at different intervals of release. Biochemical analysis of the released TMB (crude homogenate of whole insect) *viz.*, variation in total protein, SDS PAGE profiling of TMB crude protein,

detoxifying enzymes *viz.*, carboxyl esterase, cytochrome P450, and glutathione-s-transferases (GST) were carried out before releasing on the plant and at different intervals after release.

The total leaf protein was found to be highest in the less susceptible variety Damodar (0.9925 mg g^{-1}) and the lowest in the highly susceptible Madakkathara-1 (0.6729 mg g^{-1}). Total phenol content was highest in the less susceptible Damodar (69.834 mg g^{-1}) and Raghav (67.207 mg g^{-1}) and the lowest was recorded in Anagha (29.625 mg g^{-1}). Regardless of the varieties, highest phenol content was recorded in samples taken after 72 h of TMB infestation. Tannin content was also high in the less susceptible varieties Raghav (4.420 mg g^{-1}) and Damodar (4.276 mg g^{-1}) while highly susceptible varieties Anagha and Madakkathara-1 recorded lower values. Irrespective of the varieties, the highest tannin content was recorded in samples before the release of TMB (6.662 mg g^{-1}).

Results of the present study revealed that, detoxifying enzyme specific activity of PPO was highest in Damodar (0.003158 EU $g^{-1}min^{-1}$) and observed lowest value in Anagha (0.001406 EU $g^{-1}min^{-1}$). Irrespective of the varieties, enzyme activity reached highest at 48 h of release (0.00367 EU $g^{1}min^{-1}$). PAL specific activity was highest in Madakkathara-1 (0.575 µg g^{-1} min^{-1}) and lowest in Anagha and was on par with Raghav and Damodar.

In SDS PAGE, the whole body homogenate of adult female TMB revealed presence of bands in the range of~ 63 kDa and 35-48 kDa in the early stage of infestation. However, as the exposure time increased, protein bands ranging from 17-75 kDa appeared. Defensive enzymes *viz.,* carboxyl esterase and GST expressed elevated activity in TMB that fed on less susceptible Raghav (258.117 µmol $min^{-1}mg^{-1}$ protein, and 365.262 µmol $min^{-1}mg^{-1}$ protein respectively) and Damodar (208.916 µmol $min^{-1}mg^{-1}$protein, and 501.879 µmol $min^{-1}mg^{-1}$protein respectively) when compared to the highly susceptible varieties. Cytochrome P450 showed highest activity in TMB fed on Damodar (0.372 nmol $min^{-1}mg^{-1}$ protein) and it was having lowest activity in insect fed on other three varieties.

The study revealed that, the secondary metabolites *viz.*, phenol, tannin and PPO have a definite role in imparting resistance in cashew to TMB attack. These

metabolites have potential use in early detection of resistance in cashew against TMB. The enhanced levels of detoxification enzymes in TMB indicate plasticity of the pest against host plant defense and chance of resistance development against synthetic insecticides.