INSECTICIDE RESISTANCE MANAGEMENT IN RICE WEEVIL, *Sitophilus oryzae* (L.) (COLEOPTERA: CURCULIONIDAE)

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by

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2021

DECLARATION

I, hereby declare that this thesis entitled "**INSECTICIDE RESISTANCE MANAGEMENT IN RICE WEEVIL, *Sitophilus oryzae* (L.) (COLEOPTERA: CURCULIONIDAE)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled "**INSECTICIDE RESISTANCE MANAGEMENT IN RICE WEEVIL, *Sitophilus oryzae* (L.) (COLEOPTERA: CURCULIONIDAE)**" is a record of research work done independently by Ms. Neethu P. (2019-11-011) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Neethu P

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LIST OF ABBREVIATIONS

%	Per cent
@	At the rate of
µg kg^{-1}	Microgram per kilogram
µg	Microgram
µL	Microlitre
Δ Abs	Change in absorbance
ai	Active ingredient
ANOVA	Analysis of Variance
APRD	Arthropod Pesticide Resistance Database
BSA	Bovine Serum Albumin
CD	Critical Difference
cm	Centimeter
CRD	Completely Randomized Design
CRM	Certified Reference Material
CWC	Central Warehousing Corporation
DCA	Dicarboxylic acid
EC	Emulsifiable Concentrate
ECD	Electron Capture Detector
et al	And others
FAO	Food and Agriculture Organization
FCI	Food Corporation of India
FCR	Folin Ciocalteu Reagent
Fig	Figure
FPD	Flame Photometric Detector
FSSAI	Food Safety and Standards Authority of India
g	Gram
GABA	Gamma Aminobutyric acid
GC	Gas Chromatography
GST	Glutathione S-transferases

h	Hour
HAT	Hours after treatment
HPLC DAD	High Performance Liquid Chromatography Diode Array Detection
i.e	That is
IARI	Indian Agricultural Research Institute
IGMRI	Indian Grain Storage Management and Research Institute
IRAC	Insecticide Resistance Action Committee
IRM	Insecticide Resistance Management
Kg	Kilogram
LC	Lethal Concentration
LC	Liquid Chromatography
LD	Lethal Dose
Ld p	Log dose probit
LOQ	Limit of Quantitation
M	Molar
MCA	Monocarboxylic acid
mg	Milligram
mg kg^{-1}	Milligram per kilogram
mL	Milliliter
MRL	Maximum Residue Limit
MS	Mass Spectrometer
MSPD	Matrix Solid Phase Dispersion
NPD	Nitrogen Photometric Detector
°C	Degree Celcius
OD	Optical Density
OP	Organophosphate
pmol	Picomole
ppm	Parts per million
PSA	Primary Secondary Amine
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe

rpm	Revolutions per minute
RR	Resistance Ratio
RSD	Relative Standard Deviation
S. E.	Standard Error
SC	Suspension Concentrate
SD	Standard Deviation
SG	Water soluble granules
SP	Synthetic pyrethroid
SWC	State Warehousing Corporation
Viz.	Namely
WP	Wettable Powder

Introduction

1. INTRODUCTION

Agriculture is a vital sector for ensuring food security in the world. The escalating human population, which is expected to reach 10 billion by 2050, is becoming a tremendous challenge for global agriculture in terms of food production. The seasonal nature of production and evenly spread demand for agricultural commodities throughout the year make storage a remarkable part of agriculture. Stored grains are ravaged by a variety of insect pests, bringing both qualitative and quantitative losses. Stored-product pests are estimated to cause up to 9 per cent post-harvest loss in developed countries and 20 per cent or more in developing countries (Philips and Throne, 2010).

Around 60-70 per cent of food grains produced in India are stored in domestic storage structures (Kanwar and Sharma, 2003) and the surplus food grains produced in the country are kept in warehouses controlled by the Food Corporation of India (FCI), the Central Warehousing Corporation (CWC), or the State Warehousing Corporations (SWCs) (Sharon *et al*., 2014). Storage losses of 14 million tonnes of food grain worth Rs. 7,000 crores are projected every year in India, with insects exclusively accounting for almost Rs. 1,300 crores (IGMRI, 2021).

The rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) is an important primary stored grain pest that attacks undamaged grain/ seed. It is cosmopolitan in nature and solely responsible for damage of 40 per cent of global stored grain (Mishra *et al*., 2013). They can also infest cereal crops at maturing stage in the field. In severe infestations, only the pericarp of the kernel is left behind, and both adults and larvae cause economic damage (Srivastava and Subramanian, 2016).

Among the various methods for controlling stored grain pests, chemical methods are so far reported as the primary measure for the management (White and Leesch, 1995). Pesticides have been used to preserve stored grains since 1945, beginning with lindane, progressing through malathion in 1958, to various organophosphates, and pyrethroids (Kljajic and Peric, 2007). Insecticide resistance and the presence of insecticide residues are two important risks associated with the use of insecticides. Repeated use of these insecticides against stored product pests leads to the

development of resistance against the insecticides, which makes the stored product pest management more difficult (Kljajic and Peric, 2009; Hagstrum and Phillips, 2017).

In a survey conducted by the Insecticide Resistance Action Committee (IRAC) in Philippines, *S. oryzae* has been reported to have high levels of resistance to organochlorines, organophosphates and carbamates, coupled with a high level of well-developed pattern of cross-resistance. Field resistance to phosphine and pyrethrins in *S. oryzae* has been reported by the Food and Agriculture Organization (FAO) (Badmin, 1990). During 1998, the resistance level of *S. oryzae* against phosphine in India was reported to have increased to 425 times that of the susceptible strain (Rajendran, 1999).

In India, malathion and deltamethrin are recommended for use in FCI godowns against stored grain pests. Many authors have reported resistance of *S. oryzae* against malathion and deltamethrin (Heather, 1986; Joia and Kumar, 1996). Insecticide resistance is due to changes in behavioural, physiological, and biochemical mechanisms in insects (Ishaaya, 2001). Target site mechanism and detoxification mechanism are the components of biochemical mechanism. The major families of detoxifying enzymes involve esterases, monooxygenases and glutathione S-transferases (GST) (Claudianos *et al*., 2006). Active participation of GSTs has been reported in the detoxification metabolism of organophosphates, organochlorines, and synthetic pyrethroids (Boyer *et al*., 2007; Fragoso *et al*., 2007; Che-Mendoza *et al*., 2009). The significance of esterases and cytochrome P450s in insecticide resistance has been documented in many insecticides, particularly in organophosphates (Zhu *et al*., 2010; Konus, 2015).

Pesticide residues in food products after harvesting are beyond the consumer's control and have a negative impact on human health (Bajwa and Sandhu, 2014). Several scientists have reported the presence of residues of deltamethrin (Savi *et al*., 2015; Yu *et al*., 2014) and malathion (Kong *et al*., 2016; Wang *et al*., 2021) in stored food products.

Non judicious use of conventional insecticides leads to insecticide resistance, secondary pest outbreaks, objectionable pesticide residues, direct hazard to users, and negative impacts on the environment and non-target organisms (Endersby and Morgan, 1991). Insecticide resistance makes chemicals incapable of controlling pests, resulting

in annual economic losses of food worth several billion dollars around the world (Elzen and Hardee, 2003). One way to combat the resistance and residue problem of insecticides is to use novel insecticide molecules having different modes of action and specific target sites. New generation insecticides are effective at low rates or dosages, have high selectivity, higher specificity for target pests, and minimal toxicity to non-target organisms and the environment (Kodandaram *et al*., 2010). Hence, it is very important to focus on the development of insecticide resistance in conventional insecticides commonly used in stored product pest management and to screen insecticides with newer modes of action as a possible solution to delay the development of insecticide resistance.

With the above background, the current study was carried out with the following objectives:

1. Assessment of resistance levels in rice weevil, *S. oryzae.*
2. Assessment of the biochemical basis of resistance.
3. Screening of new molecules for the management of *S. oryzae.*

Review of Literature

2. REVIEW OF LITERATURE

The rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) is a major internal feeder, causing significant damage to stored rice, wheat, millet, barley, maize, sorghum, and other cereals. The rice weevil infestation predisposes the grains to infection by microorganisms like fungi and bacteria, which reduce the quantity and quality parameters mainly by increasing the free fatty acid content of the grains. The non-judicious use of insecticides for grain protection has led to the development of resistance in *S. oryzae*. According to the Arthropod Pesticide Resistance Database (APRD), 39 resistance cases in *S. oryzae* have been documented up to date (APRD, 2021).

In this chapter, the literature pertaining to insecticide resistance, the biochemical basis of resistance, its management and residue problems in stored grains is addressed and presented under the following headings.

a. Malathion and deltamethrin resistance in *Sitophilus oryzae.*
b. Biochemical basis of resistance to malathion and deltamethrin in stored product pests.
c. Management of insecticide resistance using insecticides with newer modes of action.
d. Persistence of insecticide on stored grains.

2.1 MALATHION AND DELTAMETHRIN RESISTANCE IN *Sitophilus oryzae*

Malathion and deltamethrin are the recommended insecticides for pest control in FCI godowns in India. Malathion is an organophosphate (OP) insecticide that acts on the insect nervous system by inhibiting acetyl choline esterase. Deltamethrin is a synthetic pyrethroid (SP) that targets the voltage gated sodium channels (IRAC, 2021). Prolonged use of these insecticides for more than two decades in FCI godowns led to the development of insecticide resistance in stored product insect pests.

2.1.1 Resistance to Malathion in Rice Weevil, *Sitophilus oryzae*

Haliscak and Beeman (1983) investigated malathion resistance in more than 100 strains of important coleopteran storage pests by using dose discrimination technique. At LD_{95}, all the tested strains of *S. oryzae* showed above 95 per cent mortality, indicating a resistance factor of less than or equal to one.

Navarro *et al*. (1986) conducted a study to investigate the level of malathion resistance in field populations of insects collected from nine granaries in different parts of Israel. *T. castaneum* recorded a very high resistance factor of 538 followed by *R. dominica* (9), *Oryzaephilus sulrinamensis* (L.) (8), and *S. oryzae* (1.2).

In a study on insecticide resistance to malathion, de Villar *et al*. (1987) reported that out of the 17 species of stored grain insect from Argentina, *T. casteanum* and *T. confusum* showed greater resistance to malathion than *S. oryzae*.

Pacheco *et al*. (1990) reported that in the state of Sao Paulo, Brazil, about 79, 90 and 100 per cent populations of *S. oryzae*, *R. dominica*, and *T. castaneum* showed resistance to malathion. Cross resistance was detected in 15 per cent of the populations of *S. oryzae* and *R. dominica*. In another study from Sao Paulo state, Sartori *et al*. (1990) reported that out of 14 populations of *S. oryzae* collected, 11 showed malathion resistance.

A study conducted by Irshad and Gillani (1992) on 33 different strains of *S. oryzae* revealed that 13 strains were resistant to a discriminating dose (1.5 %) of malathion. According to Baker and Weaver (1993), field strains of *S. oryzae* were 1.6-fold more resistant to malathion compared to laboratory strain.

Joia and Kumar (1996) reported a resistance ratio ranging from 7 to 17.9 times against malathion in *S. oryzae* collected from 33 different locations of Punjab. About 60 per cent of the population studied showed a resistance ratio greater than 10.

Visalakshi and Gour (2006) evaluated different strains of *S. oryzae* collected from Hyderabad and observed 2.22-fold resistance to malathion in the Jeedimetla strain of *S. oryzae* compared to the Amberpet strain. The studies regarding the cross-

resistance spectrum of malathion-resistant strains of *S. oryzae* revealed the development of multiple resistance to deltamethrin and cross resistance to carbaryl.

Reddy and Sridevi (2017) evaluated malathion resistance in *S. oryzae* populations collected from three districts of Telangana. The highest resistance was reported in the Nizamabad population (10.8 fold), followed by Medak (8.3 fold) and the Hyderabad population (2.8 fold).

In a study on Egyptian populations of *T. castaneum* and *S. oryzae*, Attia *et al*. (2020) discovered that *S. oryzae* was more resistant to malathion than *T. castaneum*. When compared to the susceptible strain, *S. oryzae* was significantly resistant to malathion (199.6-fold) and had a low level of cypermethrin resistance (4.18-fold).

2.1.2 Resistance to Deltamethrin in Rice Weevil, *Sitophilus oryzae*

Heather (1986) conducted a study regarding sex linked resistance to pyrethroids in *S. oryzae.* Selection of *S. oryzae* in the laboratory over 25 generations using permethrin and deltamethrin resulted in 256-fold and 98-fold resistance, respectively. Ceruti and Lazzari (2003) determined deltamethrin resistance in stored product beetles by using an impregnated filter paper technique. *S. oryzae*, *S. zeamais,* and *R. dominica* were reported to have more than 9, 3, and 2-fold resistance compared to susceptible population of each species.

Toxicity of the contact insecticides dichlorvos, malathion, chlorpyrifos-methyl, pirimiphos methyl, deltamethrin, and bifenthrin on adults of granary weevil (*Sitophilus granaries* L.), rice weevil (*S. oryzae*) and maize weevil (*S. zeamais*) was investigated by Kljajic *et al*. (2006) using the filter paper impregnation method. Results revealed that deltamethrin was 5.5 times and 10.8 times less toxic to rice and maize weevils than to granary weevils at the LD_{50}.

Singh *et al*. (2007) reported that wheat grains treated with deltamethrin for 18 months showed more than 12-fold higher resistance compared to susceptible laboratory strains. Singh *et al*. (2021) reported 134-fold resistance in *S. oryzae* strain collected from grain storages across southern India against deltamethrin compared to susceptible strains. Both phenotype and molecular marker analyses demonstrated that deltamethrin

at 180 and 1000 ppm can be used to discriminate weakly and strongly resistant populations in *S. oryzae*, respectively.

2.1.3 Resistance Development in *Sitophilus oryzae* Against Other Insecticides

S. oryzae have developed a considerable level of resistance against various insecticides. Champ and Dyte (1976) showed presence of lindane and phosphine resistance in *S. oryzae* collected from various parts of India. Strong resistance to fumigant, phosphine was first recorded in *S. oryzae* in China during 1995-1997 survey (Zeng, 1999). In 1998, the resistance level of *S. oryzae* against phosphine in India was reported to have increased to 425 times that of the susceptible strain (Rajendran, 1999).

Collins and Wilson (1986) reported 2 to 4-fold resistance to fenitrothion in field populations of *S. oryzae* in comparison with the laboratory sample. Suleiman *et al*. (1994) found resistance to phosphine and methyl bromide in *S. oryzae*, *S. zeamais* (Motschulsky), *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (F.) collected from 30 locations in Malaysia, with resistance being more pronounced in *S. oryzae* and *T. castaneum* than in the other insects studied. Zafar *et al*. (2008) estimated the insecticide resistance in different strains of *S. oryzae* collected from various locations in Punjab. The Karachi and Lahore strains of *S. oryzae* were found more resistant to cypermethrin and phosphine in comparison with the susceptible strain.

Rajan *et al*. (2017) evaluated phosphine resistance in *S. oryzae* collected from various parts of Tamil Nadu. The results revealed that phosphine resistance was common in all the field populations of *S. oryzae* with resistance levels ranging from 21.21 to 93.38 per cent. In another study from Tamil Nadu, Yasodha *et al*. (2019) reported 66.67 to 90 per cent resistance to phosphine in *S. oryzae*.

Tingis *et al*. (2018) investigated phosphine resistance in *S. oryzae* populations collected from Turkey. The highest level of resistance was recorded in *S. oryzae* populations collected from Mersin provinces (102 to 104-fold), followed by Konya province (38 and 81-fold).

2.2 BIOCHEMICAL BASIS OF RESISTANCE TO MALATHION AND DELTAMETHRIN IN STORED PRODUCT PESTS

Metabolic resistance is an important resistance mechanism which relies mainly on enzymes such as cytochrome P450 monooxygenases, esterases, and glutathione S-transferases to detoxify insecticide molecules (Boyer *et al*., 2012). Enzymes can detoxify the insecticides into a nontoxic, rapidly excretable metabolite. Resistant insects quickly metabolise the insecticide due to the presence of enzymes with higher catalytic rates, or elevated levels of enzymes as a result of enhanced transcription or gene amplification (Panini *et al*., 2016). Detoxification consists of two stages *viz*., phase I or primary reactions and phase II or secondary reactions. Cytochrome P450 monooxygenases, carboxylesterases are involved in phase I reactions and glutathione S-transferases are associated with Phase II reactions. Oxidation or hydrolysis reactions are found associated with phase I detoxication and the structure of the toxin is enzymatically changed, rendering it incapable of interacting with lipophilic target sites. Phase II reactions involve conjugation of Phase I detoxification products for solubilization and transport (Hilliou *et al*., 2021).

2.2.1 Cytochrome P450 monooxygenases

Cytochrome P450s are important detoxifying enzymes in insects that help to build up resistance to insecticides. An essential feature of insect P450s involves their transcriptional overexpression in insecticide-resistant strains, which provide improved metabolic detoxification of insecticides (Zhu *et al*., 2008).

Collins *et al*. (1992) investigated a variety of enzymic systems linked with resistance in four strains of *Oryzaephilus surinamensis* (L.) with varying levels of resistance to malathion, fenitrothion, and chlorpyrifos-methyl. High monooxygenase activity was linked to fenitrothion resistance, while high esterase activity was linked to chlorpyrifos-methyl resistance. No such relationship was found in malathion resistance.

The biochemical analysis of insecticide resistance in various populations of *S. oryzae* by Konus (2015) indicated a two-fold increase in p-nitroanisole O-demethylation activity of cytochrome P450 monooxygenases in the malathion-resistant Ankara population, indicating the active role of cytochromeP450 monooxygenase in

malathion resistance. A cytochrome P450 gene, CYP6BQP, showing more than 200-fold greater expression in the deltamethrin-resistant QTC279 strain in comparison with the deltamethrin-susceptible lab-S strain was also identified.

Zhu *et al*. (2010) used functional genomics and reverse genetic techniques to identify and describe a P450 gene that is responsible for the majority of deltamethrin resistance in the *T. castaneum* QTC279 strain. A P450 gene, CYP6BQ9, was discovered in the deltamethrin-resistant QTC279 strain with 200-fold higher expression than the deltamethrin susceptible lab-S strain. CYP6BQ9 is predominantly found in a part of the central nervous system that contains voltage-gated sodium channels, targeted by deltamethrin.

2.2.2 Esterases

Carboxylesterases are members of the esterase family of enzymes that convert carboxylic esters into corresponding alcohol and free acid anion during hydrolysis. Esterase-mediated resistance could be produced by altering the quantity or quality of these enzymes, resulting in overexpression or structural modifications (Cui *et al*., 2015).

According to Guedes and Zhu (1998), the malathion-resistant *R. dominica* population has 1.3 and 3.7-fold higher esterase and phosphotriesterase activity, respectively, compared to the susceptible population. Malathion and fenitrothion resistance in a multi-organophosphorus (OP) resistant strain of the saw-toothed grain beetle *O. surinamensis* was found to be caused by higher esterase levels by Conyers *et al*. (1998). The Michaelis constant (Km) was the same for enzymes from resistant and susceptible insects, but the maximum rate of enzyme catalysed reaction (Vm) in the resistant strain was 7-fold higher than that of the susceptible strain. Although both enzymes were competitively inhibited by OPs, the enzymes of the resistant strain had a 3-fold lower affinity for malaoxon than the enzymes of the susceptible strain.

Shakoori *et al*. (2000) evaluated the relative activity of various esterases in six different strains of *R. dominica* collected from various locations in Pakistan. The carboxylesterase activity of the Lahore (352 %), Sailkot (198 %) and Chichawatni (214 %) strains was found to be significantly higher than that of the susceptible Karachi strain.

The total esterases of the Lahore, Sailkot, and Chichawatni strains showed 152 per cent, 63 per cent, and 73 per cent higher activity than the susceptible Karachi strain.

Lee and Lees (2001) observed elevated levels of carboxylesterase and cytochrome P450 monooxygenase activity in malathion resistant strains of *O. surinamensis*. Haubruge *et al*. (2002) reported that malathion specific resistance in a strain of *T. castaneum* is due to an increase in malathion carboxylesterase (MCE) activity (44-fold) compared to susceptible strains.

A study conducted by Lucena *et al*. (2012) regarding esterase analysis during the development stages of *S. oryzae* and its relation to malathion resistance revealed that esterase is related to resistance development in *S. oryzae*. Hafiz *et al.* (2018) found that deltamethrin-resistant populations of *T. granarium* had considerably higher levels of esterases in fourth, sixth instar larvae and adult beetles than susceptible populations.

Wei *et al*. (2020) investigated the role of metabolic enzymes using RNAi in psocids and reported for the first time that esterase genes *via* up regulating their expression can mediate a decrease in malathion toxicity in *Liposcelis bostrychophila.* (Badonnel).

2.2.3 Glutathione S-transferases

Glutathione S-transferases are a broad family of multifunctional enzymes which metabolise pesticides by enabling reductive dehydrochlorination or conjugation processes with reduced glutathione, resulting in water-soluble metabolites that can be excreted more easily. They also help to remove hazardous oxygen free radical species created by pesticides (Enayati *et al*., 2005).

Fenitrothion, cyfluthrin, and malathion resistant strains of *T. castaneum* has 4 to 6-fold higher levels of glutathione S-transferase activity compared to susceptible strains (Reidy *et al*., 1990). Fragoso *et al*. (2003) recognised a greater than two-fold increase in glutathione S-transferase in pyrethroid (cypermethrin, deltamethrin, and permethrin) resistant strains of *S. zeamais* compared to susceptible strains. According to Fragoso *et al*. (2007), the enhanced activity of glutathione S-transferase is a reason for pyrethroid resistance in the *S. zeamais* population of Brazil.

2.3 MANAGEMENT OF INSECTICIDE RESISTANCE USING INSECTICIDES WITH NEWER MODES OF ACTION

Stored grain pests have developed resistance to majority of OP and SP insecticides. New generation insecticides with new modes of action are the best choice to substitute the conventional ones for managing them. This study examines the susceptibility of the rice weevil *S. oryzae* to indoxacarb, chlorantraniliprole, and fipronil. The oxadiazine compound indoxacarb blocks voltage-gated sodium channels. Chlorantraniliprole is a diamide insecticide that modulate *via* ryanodine receptor and inhibit normal muscle contraction. Fipronil, a phenyl pyrazole, is a nervous system toxin that acts as an antagonist to GABA (Gamma-aminobutyric acid) receptors, causing insects to die due to impaired function of the central nervous system (IRAC, 2021).

2.3.1 Susceptibility of Stored Product Pests to Fipronil

Kavallieratos *et al*. (2010) evaluated the efficacy of fipronil against *S. oryzae*, *T. confusum*, *R. dominica* and *Prostephanus truncates* (Horn). The results revealed that fipronil is an effective alternative to the existing insecticides in stored grain protection at doses equal to or higher than 1 mg kg^{-1} grain.

2.3.2 Susceptibility of Stored Product Pests to Indoxacarb

Hussain *et al*. (2005) determined the susceptibility of malathion resistant and organophosphate-susceptible strains of *T. castaneum* to six insecticides with new modes of action by using residual film bioassay. Abamectin was found to be the most toxic to *T. castaneum*, followed by indoxacarb, spinosad, buprofezin, polychlorinated petroleum hydrocarbons, and azadirachtin. In another study, a malathion resistant strain showed cross resistance to indoxacarb, concluding its least efficiency for the management of organophosphate resistant red flour beetles (Hussain and Ashfaq, 2009).

The potential of indoxacarb for controlling coleopteran storage pests was studied by Daglish and Nayak (2012), and they reported 100 per cent mortality of

S. oryzae adults at all doses of indoxacarb except 0.2 mg kg^{-1}, where mortality was 96.6 per cent.

Insecticidal efficacy of indoxacarb against adults of three major stored grain species, *S. oryzae*, *R. dominica,* and *T. confusum* in wheat and maize, was done by Miliordos *et al*. (2017). *R. dominica* was found to be more susceptible, followed by *S. oryzae* and *T. confusum*.

Khan (2020) investigated the effectiveness of indoxacarb and synergism by enzyme inhibitors in laboratory and field strains of *R. dominica*, *S. zeamais*, *S. oryzae*, *T. castaneum* and *O. surinamensis*, using dose mortality bioassays on wheat grains in Pakistan. Results of toxicity bioassays based on LD_{50} values revealed that field strains of *R. dominica* and *S. oryzae* were more susceptible to indoxacarb than those of the other stored grain pests tested.

2.3.3 Susceptibility of Stored Product Pests to Chlorantraniliprole

The efficacy of chlorantraniliprole against major stored product pests is dependent on the commodity type, dose rate, and exposure interval (Kavallieratos *et al*., 2013). Chlorantraniliprole at a rate of 1 mg kg^{-1} of grains causes low mortality in *S. oryzae* after 7 days of exposure. In whole rice, 100 per cent mortality was observed after 14 days of exposure.

Saglam *et al*. (2013) reported that spinetoram, thiamethoxam and chlorantraniliprole were potentially effective against adults and larvae of *T. confusum*. Among the insecticides, chlorantraniliprole and its combinations had an ovicidal effect. Toxicological studies conducted by Ahmed *et al*. (2017) reported that 0.00896% chlorantraniliprole induced a lethal effect in *S. zeamais*.

Babu *et al.* (2018) determined the toxicity of emamectin benzoate, lufenuron, chlorfenapyr, and chlorantraniliprole against *R. dominica* by the jute cloth disc impregnation method. Chlorfenapyr recorded highest relative toxicity against *R. dominica* which is 7.45 times greater than malathion and 7.34 times greater than deltamethrin. At LC_{50} level, the relative toxicity of chlorantraniliprole was 2.67 times higher than malathion and 4.25 times greater than deltamethrin.

Babu *et al*. (2020) evaluated the insecticidal activity of spinosad 45% SC, chlorantraniliprole 18.5% SC, emamectin benzoate 5 % SG, and chlorfenapyr 10 % SC against the pulse beetle, *Callosobruchus maculates* (Fabricius). At 24 hours after treatment, chlorantraniliprole and chlorfenapyr were found to be less toxic compared to spinosad.

2.4 PERSISTENCE OF INSECTICIDE ON STORED GRAINS

Post-harvest insecticide treatment for protection of raw grains and pulses against stored grain pests leads to the presence of residues in stored products. Pesticide residue analysis is critical for determining the amount of residue in a sample and ensuring the safety of the food.

George and Dikshit (1995) reported high deltamethrin persistence on stored blackgram, with 74-75 per cent of deltamethrin extractable residues after 6 months of storage. The dissipation of deltamethrin was biphasic, with a quick decline of residues up to 2 months followed by a slow and steady phase.

The persistence study of malathion residues in stored maize and beans by Lalah and Wandiga (1996) revealed that 34-60 per cent of the initial residues persisted in the grains after 51 weeks of storage. The penetration of malathion in maize was slightly higher than that of beans regardless of the method of storage.

Calumpang *et al*. (2001) established quick, semi-quantitative pesticide residue detection procedures for deltamethrin, cypermethrin (pyrethroids), malathion (organophosphate), and carbaryl (carbamate) used in stored farm products. In mung bean, corn, and rice grains, residues were reported as 2 mg kg^{-1}, 0.30 mg kg^{-1}, 8 mg kg^{-1}, and 5 mg kg^{-1} for deltamethrin, cypermethrin, malathion, and carbaryl respectively.

Dikshit (2002) reported high persistence and stability of deltamethrin residues on stored pulses, with 54-74 per cent deltamethrin on all the tested pulses after 6 months of storage. Rani *et al*. (2006) examined the persistence of deltamethrin in two wheat varieties by treating them with 40 mg kg^{-1} of deltamethrin in wheat grains and storing them for 150 days in jute and polythene bags. and reported that residues were below MRL in both the varieties within 150 days and that the degradation of residues was faster in grains stored in jute bags than in polythene bags.

Persistence and efficacy studies conducted by Pandey *et al.* (2016) reported that the residues of deltamethrin persisted for 90 days, while the persistence of spinosad and indoxacarb residues exceeded 120 days on stored wheat. Deltamethrin caused cent per cent mortality of *R. dominica, S. oryzae* and *T. granarium* up to 15 days of treatment.

Balinova *et al*. (2007) used gas chromatography to determine deltamethrin residues in wheat. When applied at 0.5 mg kg^{-1}, residues were in the range of 0.03 to 0.2 mg kg^{-1} at 180 days after treatment. At 270 days after treatment dosage of 4 mg kg^{-1}, the residues were in the range of 0.4-1.5 mg kg^{-1}.

Detection and quantification of insecticide residues in grain extracts was conducted by Aldana-Madrid *et al*. (2008) using gas chromatography with an electron capture detector. Recovery of pyrethroids, organochlorines, and organophosphate insecticides was 84.22 per cent, 81.37 per cent, and 74.58 per cent, respectively.

Tsochatzis *et al.* (2010) devised a multi-residue approach to determine the presence of eight pesticides in rice grains. Pesticide extraction and clean-up were carried out on neutral alumina using an improved matrix solid-phase dispersion (MSPD) methodology and acetonitrile as the elution solvent. A high-performance liquid chromatography-diode array detection (HPLC-DAD) system was used to evaluate the samples. The limits of detection and quantification ranged from 0.002 to 0.200 mg kg^{-1} and 0.006 to 0.600 mg kg^{-1}, respectively. Acceptable recoveries (74-127 %) were obtained with RSD less than 12 per cent.

Daba *et al*. (2011) investigated pesticide residues in wheat and khat samples collected from different locations of Ethiopia. p,p'-DDT concentrations in khat samples from Galemso and Aseno varied from 141.2 to 973.0 µg kg^{-1} and 194.3-999.0 µg kg^{-1} respectively. Diazinon was found in all the khat samples (173.9-686.9 µg kg^{-1}) and all wheat samples from BadaBuna, Arsi and Bale (125.8 and 125.6 µg Kg^{-1}). Aldrin levels were below the quantitative limit in all samples.

Using gas chromatography and a nitrogen phosphorus detector, Uygun *et al*. (2005) studied residue levels of malathion, its metabolites, and fenitrothion, in wheat

during storage, milling, and baking. Malathion residues were reduced to around 95 per cent in wheat flour, and 82 per cent in baked white bread.

Ogah and Coker (2012) determined organophosphate and carbamate residue in maize samples using gas chromatograph with mass spectrometric detector (GC-MS). More than one OPs, and carbamate were found in all of the maize samples, with mean values ranging from 12.0 to 1565.4 µg kg^{-1}.

Jean *et al*. (2013) used gas chromatography with a nitrogen photometric detector (GC-NPD) and GC-MS to analyse nine organophosphate insecticides in stored cowpea. The most common insecticides detected were malathion, methyl-parathion, and dichlorvos. Lozowicka *et al*. (2014) reported that chlorpyrifos methyl and pirimiphos methyl were the most frequently identified residues in 80 samples of barley, oat, rye, and wheat collected from Kazakhstan. The efficiency of ozone gas in the removal of bifenthrin (91.9%) and deltamethrin (92.7%) residues in rice grains was reported by de Avila *et al.* (2017) using GC-ECD.

Zelelew *et al*. (2018) evaluated the concentration levels of eight pesticides in wheat samples collected from farms and storage. The RSD for method validation was below 20 per cent and recoveries were between 80 and 110 per cent. Four analytes (2,4-D, aldrin, endosulfan, p,p-DDT) were discovered in both field and stored samples, while the rest of the analytes were not detected at all.

Mebdoua and Ounane (2019) used GCMS to assess the presence of pesticide residues in wheat grains and related products by QuEchERS method. Detectable residues were discovered in 62.5 per cent of the wheat samples (80) that were examined. In 46 samples, pirimiphos methyl was the most common pesticide identified.

Materials and Methods

3. MATERIALS AND METHODS

The present study on "Insecticide resistance management in rice weevil *Sitophilus oryzae*" was carried out during 2019-2021 to assess the resistance levels in rice weevil, biochemical basis of resistance and screening of new molecules for the management of *S. oryzae*. The experiments were conducted by utilising the facilities available at the Department of Agricultural Entomology and Pesticide Residue Research and Analytical Laboratory under the All-India Network Project on Pesticide Residues of the College of Agriculture, Vellayani, Thiruvananthapuram. This chapter specifies the materials used and the procedures followed in this research.

3.1 COLLECTION OF RICE WEEVIL, *Sitophilus oryzae* (L.)

Three different populations of rice weevil, *S. oryzae*, were collected from Food Corporation of India (FCI) godowns situated at the different geographical locations in Kerala, *viz*., Thikkodi (Kozhikode district; 11°29'35"N, 75°37'32"E), Kollam (Kollam district; 9°2′40″N, 76°49′46″E) and Valiyathura (Thiruvananthapuram district; 8°28'13"N, 76°55'30"E). A susceptible sample of *S. oryzae* (Plate 1) was obtained from the Division of Entomology, IARI, New Delhi, which was maintained without pesticide exposure for 4 years (48^{th} generation).

3.2 REARING OF RICE WEEVIL, *Sitophilus oryzae* (L.)

The different populations of rice weevils were further reared in the laboratory at a temperature of 30 ± 2^0C and a relative humidity of 70 ± 5%. Rice grains (250 g) were taken in clear plastic bottles (12 cm x 9 cm) and 50 to 100 insects were released for feeding and oviposition in each bottle (Plate 2). Rice grains in each bottle were replaced by new grains before caking. Bioassays were carried out by using homogeneous F_2 populations of rice weevil.

3.3 EVALUATION OF RESISTANCE OF *Sitophilus oryzae* TO INSECTICIDES.

The film method of bioassay (Paramasivam and Selvi, 2017) was adopted to evaluate the resistance of *S. oryzae* populations to insecticides (Plate 3). The details of the insecticides chosen for the study are given in Table 1. Commercial formulations of

Plate 1. Adult *Sitophilus oryzae*

Plate 2. Rearing of different sample populations of *Sitophilus oryzae*

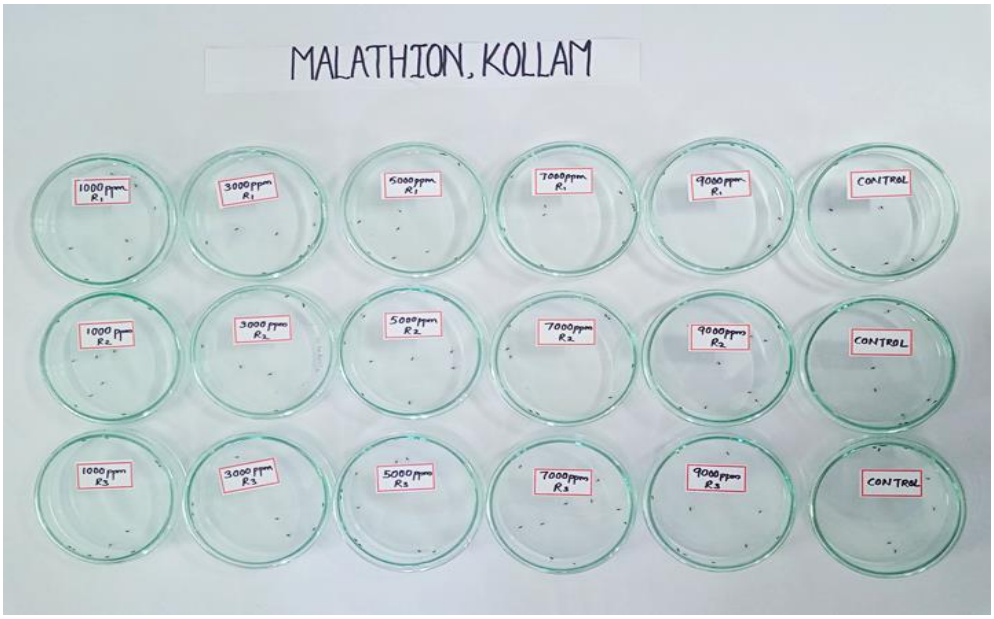

Plate 3. Bioassay experiment of *Sitophilus oryzae* against malathion

Table 1. Details of insecticides used for resistance study

Sl. No.	Chemical Name	Trade Name	Toxicity label	Manufacturer	Chemical group	Mode of Action as per IRAC, 2021
1	Malathion 50 EC	Killers	Blue	Jaya Krishna Pesticides (P) Ltd.	Organophosphates	Acetylcholine esterase (AChE) inhibitors
2	Deltamethrin 2.8 EC	Decis	Yellow	Bayer Crop Science Ltd.	Synthetic pyrethroids	Sodium channel modulators

malathion 50 EC and deltamethrin 2.8 EC dissolved in double distilled water were used for bioassay. Concentrations for bioassays were fixed based on preliminary range fixing studies. Six concentrations, including control (double distilled water) replicated thrice, were taken for the present study. The required concentrations for bioassays were prepared by serial dilution of the stock solution in volumetric flasks. One millilitre of insecticide solution was then pipetted into 100 mm ×20 mm glass Petri dishes, swirled gently to ensure uniform distribution in the dish, and then air dried at room temperature. Ten adults of *S. oryzae* were released onto the insecticide film in each Petri dish. After 24 h of exposure, the number of dead insects was counted and LC_{50} was calculated using probit analysis (Finney, 1971) for each insecticide. The susceptible sample of *S. oryzae* was used to compare the development of resistance in the FCI collected populations. The resistance ratio for each population against each insecticide was worked out using the formula.

$$\text{Resistance ratio} = \frac{LC_{50/90} \text{ of collected sample}}{LC_{50/90} \text{ of susceptible sample}}$$

The probit analysis was done using IBM SPSS software version 28.0.0.0

3.4 STUDY ON BIOCHEMICAL BASIS OF INSECTICIDE RESISTANCE

The most resistant population of *S. oryzae* was selected based on the results of the previous experiment and the biochemical basis of resistance was further studied using enzymatic assays. The total proteins and level of detoxifying enzymes like mixed function oxidases, carboxylesterases, and glutathione S-transferases were estimated in the most resistant as well as susceptible sample using the methods described below.

3.4.1 Sample Preparation

An insect sample (16 mg) was homogenised in 700 µL of sodium phosphate buffer (pH 7.4) and centrifuged at 10000 rpm for 20 minutes at 4°C to remove coarse particles. Protein and enzyme assays were performed on the supernatant. The details of materials used for protein estimation and enzyme bioassay are given in Table 2.

Table 2. Reagents used for total protein estimation and enzyme assay

Reagents	Manufacturer
Total protein estimation	
Bovine Serum Albumin (BSA)	Sisco Research Laboratories Pvt. Ltd. (SRL)
Sodium carbonate	Nice chemicals
Sodium hydroxide.	Nice chemicals
Copper sulphate	Nice chemicals
Sodium potassium tartarate	SRL
Folin-Ciocalteu reagent (FCR)	SRL
Caboxylesterase estimation	
α-naphthol	SRL
Methanol	Nice chemicals
Fast blue RR salt	SRL
Sodium dodecyl sulphate	Nice chemicals
Naphthyl acetate	SRL
Acetone	Nice chemicals
Glutathione S-transferase estimation	
1-Chloro-2, 4-Dinitro Benzene (CDNB)	SRL
Reduced glutathione (GSH)	SRL
Sodium phosphate buffer	Nice chemicals
Cytochrome P450 estimation	
Pure cytochrome C from bovine heart	Merck
3, 3', 5, 5'-Tetramethylbenzidine	SRL
Sodium acetate buffer	SRL
Potassium phosphate buffer	SRL
Hydrogen peroxide	Nice chemicals

3.4.2 Estimation of Protein

The total protein present in the most resistant and susceptible population of *S. oryzae* were estimated as per the procedure given by Lowry *et al*. (1951).

3.4.2.1 Preparation of Standard Bovine Serum Albumin (BSA) Solution

A stock solution of BSA was prepared by dissolving 50 mg of accurately weighed BSA in 50 mL of double distilled water in a volumetric flask. A working standard containing 200 g of protein mL^{-1} was prepared by pipetting out 10 mL of stock solution into a volumetric flask and diluting it with double distilled water up to a final volume of 50 mL. From the working standard, different aliquots of 100, 200, 300, 400, 500, 600, and 700 µL were pipetted into different test tubes and made up to 1 mL with double distilled water. A test tube containing double distilled water alone was used as a blank. The reagents used are given below.

Reagent A: 2 % sodium carbonate in 0.1 N sodium hydroxide.

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

Reagent C: Mixture of 50 mL of reagent A and 1 mL of reagent B, prepared just prior to the use.

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

5 mL of reagent C were added to all the test tubes including blank. The contents in the test tube were mixed thoroughly and allowed to stand for 10 min. Subsequently, reagent D (0.5 mL) was added, mixed well, and incubated at room temperature in the dark for 30 min. The absorbance of the developed blue colour was measured at 660 nm using a spectrophotometer (Plate 4). The standard graph was drawn by using the OD (Optical Density) values obtained and the corresponding concentrations of BSA (Fig. 1).

3.4.2.2 Total Protein Estimation in Sitophilus oryzae

Fifty micro litres of supernatant/ enzyme extract prepared was taken and 2.5 mL of reagent C was added. 250 µL of reagent D was added after an incubation period of

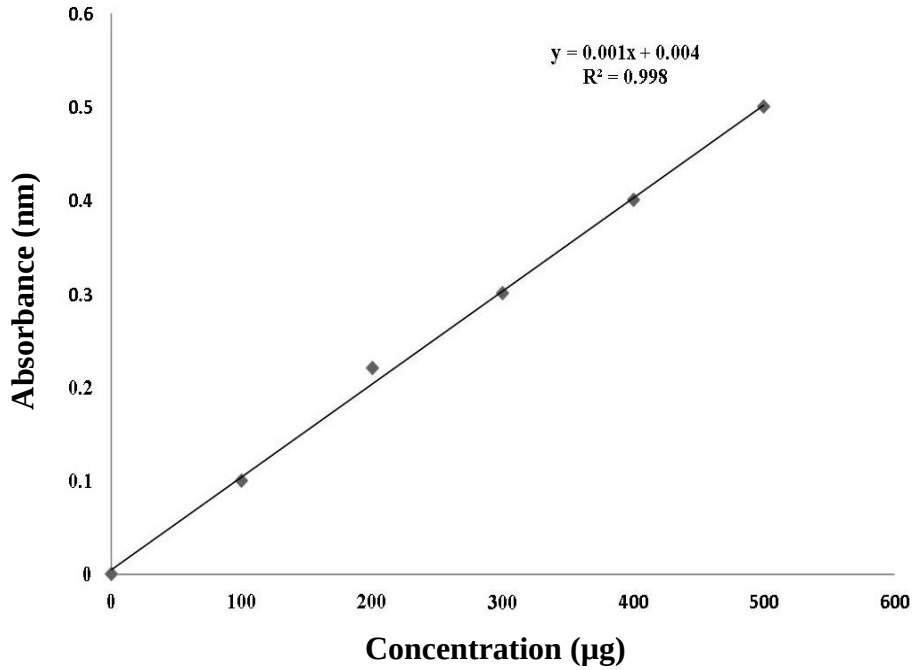

Fig. 1. Standard curve of bovine serum albumin (BSA) for protein estimation

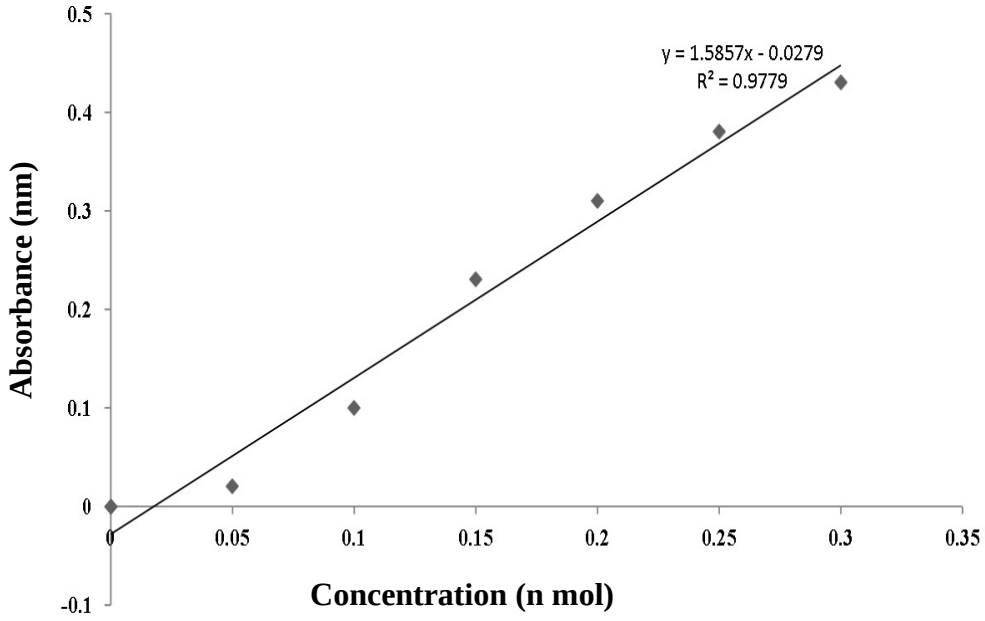

Fig. 2. Standard curve of cytochrome C for cytochrome P450 estimation

120 min. The reaction mixture was kept in the dark at room temperature for 30 min. Absorbance readings were taken at 660 nm in a spectrophotometer. Protein content was calculated from the standard graph and expressed in mg mL^{-1}.

3.4.3 Estimation of Cytochrome P450

The cytochrome P450 assay was carried out according to the method of Brogdon *et al*. (1997) with slight modifications as per Anusree (2019).

3.4.3.1 Preparation of Cytochrome C Standard

A stock solution of 0.0025 mM concentration was prepared by dissolving 3.081 mg pure cytochrome C from bovine heart in 10 mL double distilled water. Working standards ranging from 0.025 to 0.2 nM were prepared from the stock solution. Each test tube was filled with 100 microliters of working standard. 1 mL of 0.05 % TMBZ (3, 3', 5, 5'-Tetramethylbenzidine) (10 mg TMBZ dissolved in 5 mL absolute methanol mixed with 15 mL 0.25 nm sodium acetate buffer, pH 5), was added to the working standards, along with 400 µL of potassium phosphate buffer (pH 7.2) and 125 µL of hydrogen peroxide (3 %). After 30 minutes of incubation, the reaction mixture was analysed. A UV spectrophotometer was used to measure the absorbance at 630 nm. A standard graph was drawn from the OD values and associated cytochrome C concentrations (Fig. 2).

3.4.3.2 Estimation of Cytochrome P450 Activity in Sitophilus oryzae

500 µL TMBZ (0.05 %), 200 µL potassium phosphate buffer (pH 7.2), and 62.5 µL hydrogen peroxide (3 %) were added to 50 µL enzyme extract and incubated for 30 min. The absorbance was measured at 630 nm using a spectrophotometer. The standard graph was used to compute cytochrome P450 activity and represented in pmol mg $protein^{-1}$ min^{-1}.

3.4.4 Estimation of Carboxylesterase

The esterase assay was performed by using the method described by van Asperen (1962).

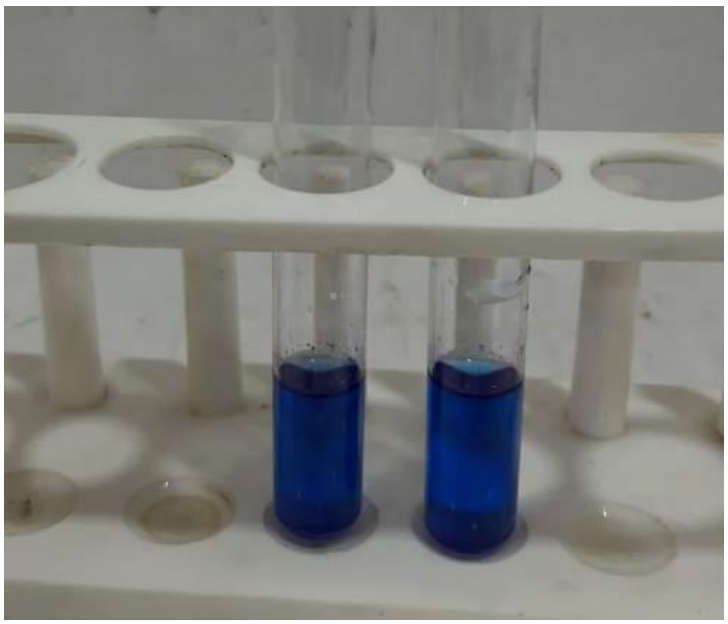

Plate 4. Laboratory procedure for the estimation of protein in *Sitophilus oryzae*

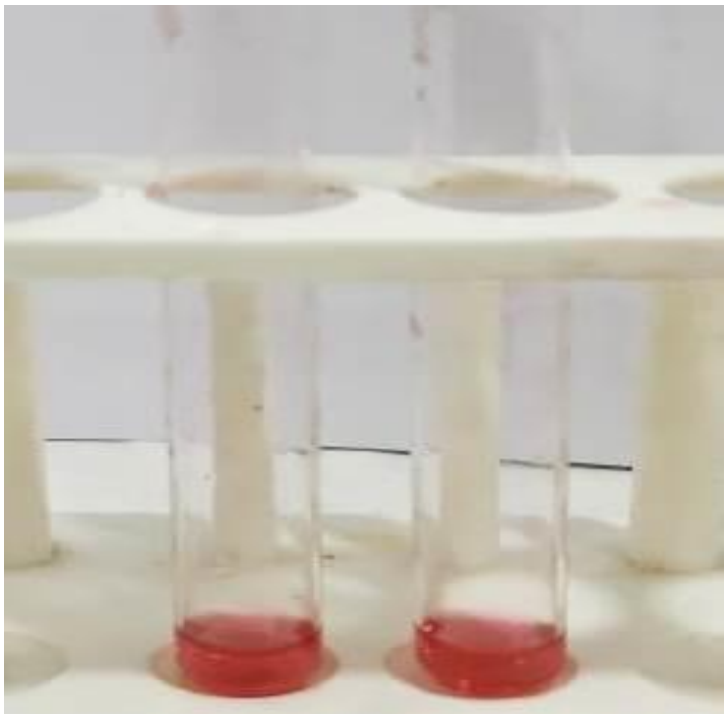

Plate 5. Laboratory procedure for the estimation of carboxylesterase in *Sitophilus oryzae*

3.4.4.1 Preparation of α-naphthol Standard

A stock solution of 10 mM α-naphthol was prepared by dissolving 0.03605 g of α-naphthol in 25 mL of methanol. Working standards of varying concentrations (100 µmol, 200 µmol, 300 µmol, 400 µmol, 500 µmol and 600 µmol) were prepared by pipetting out different aliquots (10 µL, 20 µL, 30 µL, 40 µL, 50 µL, and 60 µL) from the stock solution and made up to 1 mL with methanol. 2 mL extraction buffer (sodium phosphate buffer, pH 7.4) was added to working standards. Phosphate buffer alone was kept as a blank. The reaction mixture was incubated for 10 min. at 30°C with constant stirring. 50 µL of dye solution containing 22.5 mg of fast blue RR salt in 2.25 mL of double distilled water and 5 % sodium dodecyl sulphate in double distilled water (2:5 v/v) was added to the reaction mixture. The mixture was again incubated for 5 min. at 37°C for colour development. A spectrophotometer was used to measure the intensity of the red colour at 600 nm (Plate 5). A standard curve was prepared with the OD values obtained and the corresponding concentrations (Fig. 3).

3.4.4.2 Estimation of Carboxylesterase Activity in Sitophilus oryzae

The enzyme assay was performed using 50 µL of enzyme extract. 1 mL 30 mM -naphthyl acetate diluted in acetone (0.028 g of naphthyl acetate in 5 mL acetone) was added to this extract as an enzyme substrate. Absorbance reading and preparation of standard curve were done as same as the procedures for total protein estimation.

3.4.5 Estimation of Glutathione S-transferase (GST)

Glutathione S-transferase estimation was done by the method described by Kao *et al*. (1989).

3.4.5.1 Estimation of Glutathione S-transferase Activity in Sitophilus oryzae

50 mM 1-Chloro-2, 4-Dinitro Benzene (CDNB) (50µL) and 150 µL reduced glutathione (GSH) were added to 2.75 mL of sodium phosphate buffer (pH 6.5). Fifty microliters of prepared enzyme extract were added to this mixture. The contents were gently shaken, incubated for 2-3 min, and then transferred to a cuvette for reading in UV spectrophotometer. Reaction mixture without enzyme was used as blank.

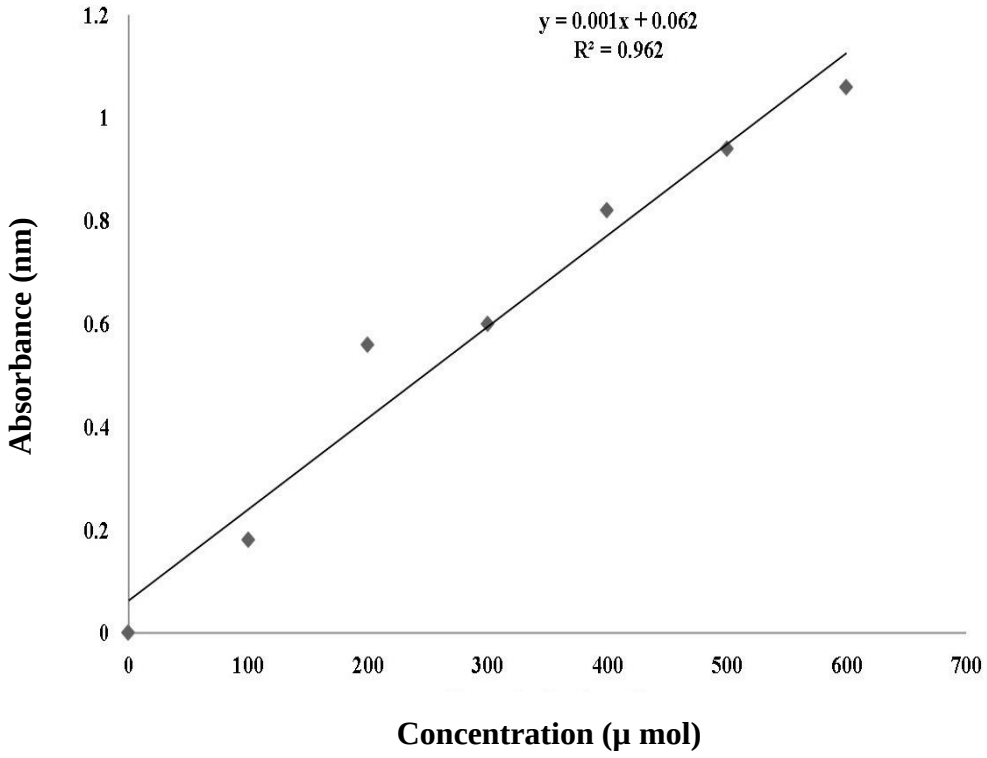

Fig. 3. Standard curve of α-naphthol for carboxylesterase estimation

Absorbance at 340 nm was recorded for 5 min at 30 sec intervals. The GST activity was calculated using the formula,

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

Extinction coefficient for CDNB-GSH conjugate - 9.6 mM cm^{-1}.

3.4.6 Statistical Analysis

Data was analysed by Analysis of variance (ANOVA) in GRAPES software in Completely Randomized Design (CRD) and means were separated by Tukey's test (Gopinath *et al*., 2021).

3.5 LABORATORY EVALUATION OF NEW INSECTICIDE MOLECULES AGAINST RESISTANT POPULATION OF *Sitophilus oryzae*

3.5.1 Susceptibility of the Resistant Population to Newer Insecticide Molecules

The newer molecules selected for the present study included chlorantraniliprole, fipronil and indoxacarb and the details are given in Table 3. The most resistant population of *S. oryzae* identified from 3.3 were bioassayed for their susceptibility to the new molecules and compared it with the highest resistant insecticide to find out the relative toxicity (Plate 6). Relative toxicity of each insecticide was calculated by the formula given below.

$$\text{Relative toxicity} = \frac{LC_{50} \text{ of malathion}}{LC_{50} \text{ of test insecticide}}$$

3.6 COMPARATIVE EVALUATION OF NEWER INSECTICIDES AGAINST *Sitophilus oryae* INFESTED GRAINS IN JUTE BAGS

For comparative evaluation of newer insecticides, jute bags containing 1 kg of rice grains were taken and 50 adults of *S. oryzae* introduced in each bag (Plate 7). The population having maximum resistance from experiment 3.3 was taken for this study.

Table 3. Details of new generation insecticides used for the management of *Sitophilus oryzae*

Sl. No.	Chemical Name	Trade Name	Toxicity label	Manufacturer	Chemical group	Mode of Action as per IRAC, 2021
1	Chlorantraniliprole 18.5 SC	Coragen	Green	FMC	Diamides	Ryanodine receptor modulators
2	Fipronil 5 SC	Regent	Yellow	Bayer Crop Science Ltd.	Phenyl pyrazoles	GABA gated chloride channel blockers
3	Indoxacarb 14.5 SC	Kingdoxa	Yellow	Gharda Chemicals Limited	Oxadiazines	Voltage dependent sodium channel blockers

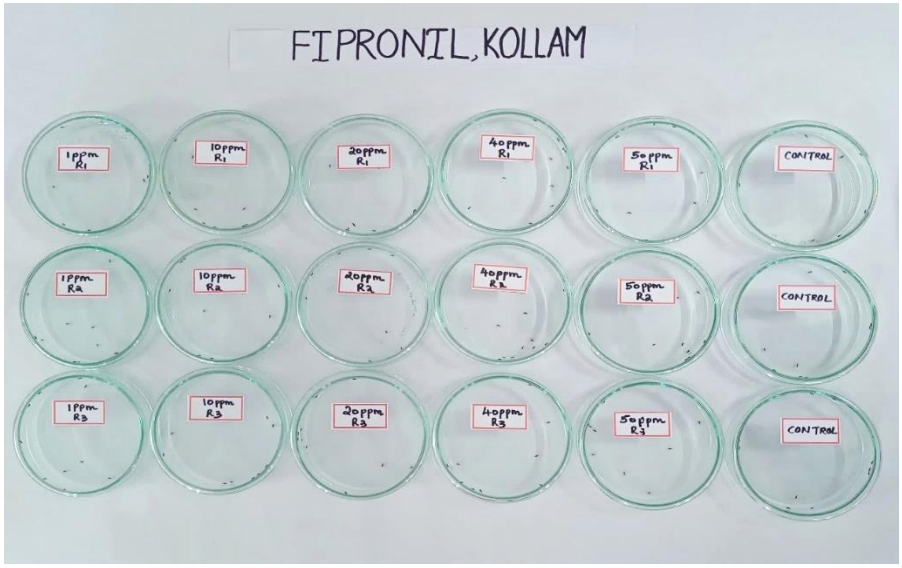

Plate 6. Bioassay of *Sitophilus oryzae* against fipronil

Plate 7. Experiment for comparative evaluation of fipronil against *Sitophilus oryzae*

These bags were then sprayed with commercial formulations of newer molecules like chlorantraniliprole, fipronil and indoxacarb. Deltamethrin and malathion were taken as positive control treatments along with an untreated control. The dosage of newer insecticides was fixed at 10 times the LC_{50} value (Anusree, 2019) obtained in experiment 3.5, and for positive checks, the FCI dose was taken, which is 10mL L^{-1} for malathion 50 EC and 40 g L^{-1} for deltamethrin 2.5 WP. The experiment was conducted in completely randomized design and 4 replications were maintained for each treatment.

Deltamethrin 2.8 EC was utilised in this experiment, and the concentration was estimated based on the active ingredient. The quantity of spray was taken as per FCI guidelines of 30mL m^{-2}. Mortality of *S. oryzae* was recorded at 24 h and 48 h after spraying. Statistical analysis was carried out using GRAPES software (Gopinath *et al*., 2021).

Table 4. Treatment details of the insecticides used for comparative evaluation against *Sitophilus oryzae*

Treatment	Insecticide	Dose (%)
T_1	Fipronil 5% SC	0.006
T_2	Indoxacarb 14.5% SC	0.09
T_3	Chlorantraniliprole 18.5% SC	4.04
T_4	Deltamethrin 2.8% EC	3.57
T_5	Malathion 50% EC	1
T_6	Untreated control	Water alone

3.7 ESTIMATION OF RESIDUES OF INSECTICIDES IN GRAIN SAMPLES

The persistence of the most effective newer insecticide against *S. oryzae* from experiment 3.6 was further studied along with the FCI recommended insecticides (positive check).

Small jute bags containing 1 kg of rice were arranged in a single layer in an area of 1 m^2 for each insecticide. Commercial formulations of insecticides were sprayed on these bags, simulating the pesticide spray in FCI godowns. The insecticides were sprayed on jute bags and the samples were taken at 2h, 1, 3, 5, 7, 10, 15, 20, and 30 day intervals. 3 replications were maintained for each interval, and a total of 27 jute bags with grains were taken for a single insecticide. The dosage of the newer insecticide and the positive checks (malathion and deltamethrin) were taken the same as in experiment 3.6. All the insecticide solutions were sprayed as per FCI recommendation (30 mL m^{-2}).

3.7.1 Method Validation for Pesticide Residue Analysis in Rice

3.7.1.1 Chemicals and Reagents

Certified reference materials (CRM) of known purity of malathion (99.46 %), deltamethrin (99.7 %) and fipronil (97.3 %) were purchased from Sigma-Aldrich Pvt. Ltd. Acetonitrile, methanol (HPLC grade), sodium chloride and anhydrous sodium sulphate were procured from Merck, Germany. Primary secondary amine (PSA) was purchased from Agilent Technologies, USA. Sodium chloride, anhydrous sodium sulphate and magnesium sulphate were activated in a muffle furnace at a temperature of 40^0C for 4 h and kept in desiccators.

3.7.1. 2 Preparation of Standards

Standard stock solutions of malathion, deltamethrin, and fipronil were prepared from the CRM. The CRM stored in the freezer was taken out and brought to room temperature. An accurately weighed out quantity of CRM of malathion and deltamethrin was dissolved in 1:1 n-hexane: toluene solvent and fipronil in 1:1 methanol: toluene solvent in a standard flask and made up to the mark. From the stock solution of 400 ppm, a working standard (10 ppm) was prepared. Required concentrations of each pesticide were prepared by serial dilution of working standards. In order to plot the calibration curve, different concentrations (0.01, 0.05, 0.25, 0.1, and 1 mg kg^{-1}) of standard solutions of each pesticide were injected into the gas chromatograph.

3.7.1.3 Recovery Experiments

Recovery studies were conducted by spiking 0.05, 0.1 and 0.25 mg kg^{-1} concentrations of analytical standards of malathion, deltamethrin and 0.01, 0.02 and 0.05 $mgkg^{-1}$ concentrations of fipronil. Three replicates were analysed at each spiking level and the accuracy of the analytical methods was determined based on repeatability and relative standard deviation, which is mandatory for residue validation.

3.7.2 Estimation of Residues of Insecticide

QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) is a solid phase extraction method for extraction of pesticide residues in food (Anastassiades *et al*., 2003). A modified QuEChERS method as described below was employed for the current study. The residue estimation of malathion and deltamethrin was carried out in GC-FPD (Gas Chromatography-Flame Photometric Detector) and GC-ECD (Gas Chromatography-Electron Capture Detector) and fipronil in LC-MS/MS (Liquid Chromatography-Mass Spectrometer).

3.7.2.1 Extraction and Clean up

A whole rice sample (1 kg) was ground in a mixer and 10 g of the ground sample was weighed and transferred to a 50 mL centrifuge tube. 10 mL of double distilled water and 20 mL of acetonitrile were added to the tube and mixed thoroughly for 30 min. 4.8 g of sodium chloride was added again and shaken well. These centrifuge tubes are closed tightly and centrifuged at 8000 rpm for 8 min at 8^0C. Then 12 mL of supernatant was transferred to a 50 mL centrifuge tube containing 1.5 g of magnesium sulphate and 1.5 g of sodium sulphate. The contents were vortexed for 30 seconds and centrifuged at 8000 rpm for 8 min at 8^0C. After centrifugation, 8 ml of supernatant was transferred to separate 15 mL centrifuge tubes containing 0.07 g of primary secondary amine (PSA) and 0.5 g of magnesium sulphate. Again, the contents were vortexed for 30 sec. and centrifuged at 4500 rpm for 5 min. at 8°C. 4 mL of supernatant was transferred to a turbo tube for GC- ECD and GC- FPD analysis, and 3 mL was pipetted for LC-MS/MS analysis. These tubes were evaporated using turbovap, which uses a gentle steam of nitrogen at 40^0C and a 7.5 psi nitrogen flow. The residue was reconstituted in 1.5 mL of methanol and filtered through a 0.2 µ PVDF filter and added

Table 5. Operating parameters of GC-FPD

Parameter	Details
Injector temperature	250^0C
Detector temperature	300^0C
Column flow rate	1.0 mL min^{-1}
Injection volume	2 µL
Hydrogen flow	95 mL min^{-1}
Zero air flow	120 mL min^{-1}
Carrier gas	Nitrogen
Run time	29.57 min

Table 6. Operating parameters of GC-ECD

Parameter	Details
Injector temperature	250^0C
Detector temperature	300^0C
Carrier gas	Nitrogen (99.99 %)
Flow rate	0.79 mL min^{-1}
Injection volume	2 µL
Run time	70.33 min

Table 7. Operating parameters of LC-MS/MS

Parameter	Details
System	Acquity UPLC (Waters) +API-3200 LC- MS/MS system
Column	Atlantis dC18 (5 µm, 2.1 × 100 mm)
Column oven temperature	40^0C
Mobile phase A	10 % Methanol in water + 5 milli molar Ammonium acetate
Mobile phase B	Water in Methanol + 5 milli molar Ammonium acetate
Flow rate	0.8 mL min^{-1}

to labelled vials prior to estimation in LC-MS/MS. The residue was reconstituted in 1 mL of n- hexane for GC-ECD and GC-FPD analysis.

3.7.2.2 Instrumentation

Operating parameters of the GC-FPD, GC-ECD and LC-MS/MS are provided in Table 5-7.

3.7.2.3 Residue Quantification

Based on the peak area of the chromatogram obtained for various insecticides, the quantity of residue was determined as given below.

Pesticide residue (mg kg^{-1}) = Concentration of the analyte obtained from the instrument × Dilution factor

$$\text{Dilution factor} = \frac{\text{Volume of the solvent added} \times \text{Final volume of extract}}{\text{Weight of the sample} \times \text{Volume of extract taken for concentration}}$$

Half-life ($t_{1/2}$), which is the time taken for the disappearance of pesticide to 50 per cent of its initial concentration was calculated by using dissipation data (Hoskin, 1961).

$t_{1/2} = \log 2 / k_1$

Where k_1 is the slope of regression line.

Results

4. RESULTS

The rice weevil, *Sitophilus oryzae,* is an important primary pest infesting whole grains of stored rice, wheat, millet, barley, maize, sorghum, and other cereals. In the present study, resistance levels in three different FCI populations of *S. oryzae viz*., Kollam, Valiyathura, and Thikkodi against malathion and deltamethrin were assessed and compared with susceptible populations from IARI. The biochemical basis of resistance was also investigated further. For resistance management, *in vitro* evaluation of newer insecticides was carried out against *S. oryzae,* and further comparative evaluation was done by simulating the godown conditions. The persistence of residues of the promising newer insecticide along with recommended insecticides were further studied. The results obtained from these investigations are presented below.

4.1 EVALUATION OF RESISTANCE OF *Sitophilus oryzae* TO INSECTICIDES

The results showing the lethal concentrations to kill 50 per cent (LC_{50}) and 90 per cent (LC_{90}) of the different populations of *S. oryzae* as well as the resistance ratio with reference to the susceptible population are given in Table 8-11.

4.1.1 Malathion

The *S. oryzae* population from Kollam showed the least susceptibility with the highest LC_{50} for malathion (3634.48 ppm). The fiducial limits for this population ranged from 2881.07 to 4437.12 ppm. The Valiyathura population had the second highest LC_{50} (2769.50 ppm) and the fiducial limits within 2214.11 to 3374.78 ppm, followed by the Thikkodi population having LC_{50} (2126.83 ppm) with fiducial limits ranging from 1603.36 to 2607.92 ppm. The susceptibility was found to be maximum in the IARI lab sample of *S. oryzae*, recording the lowest LC_{50} value of 243.23 ppm with lower and upper fiducial limits of 185.37 ppm and 303.80 ppm, respectively (Table 8).

Similarly, LC_{90} of malathion was also found higher in the Kollam population (11092.18 ppm) and lowest in the lab culture from IARI (832.37 ppm) with fiducial limits ranging from 8347.10 to 17875.82 ppm and 619.93 to 1334.05 ppm, respectively.

Table 8. Susceptibility of different sample populations of *Sitophilus oryzae* to malathion

Sample population	χ^2	d.f	LC_{50} (ppm) (95 % fiducial limit)	LC_{90} (ppm) (95 % fiducial limit)	Slope ± SE
Kollam	2.83	3	3634.48 2881.07- 4437.12	11092.18 8347.10- 17875.82	2.64 ± 0.41
Valiyathura	2.94	3	2769.50 2214.11- 3374.78	8269.53 6278.91- 12896.65	2.70 ± 0.39
Thikkodi	3.52	3	2126.83 1603.36-2607.92	6253.74 4895.44- 9300.29	2.74 ± 0.43
IARI lab culture	4.83	3	243.23 185.37- 303.80	832.37 619.93- 1334.05	2.40 ± 0.35

Table value of χ^2 at 3 df= 7.815**,** χ^2 is non-significant at: $p< 0.05$

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

Table 9. Resistance ratio of different samples of *Sitophilus oryzae* to malathion

Sample population	Resistance ratio	
	LC_{50}	LC_{90}
Kollam	14.94	13.33
Valiyathura	11.39	9.93
Thikkodi	8.74	7.51

LC_{90} values for Valiyathura (8269.53 ppm) and Thikkodi (6253.74ppm) populations were found to have fiducial limits in the range of 6278.91 to 12896.65 ppm and 4895.44 to 9300.29 ppm, respectively (Table 8). All these probit regression analyses with three degrees of freedom had chi-square values lower than the table value (7.815), indicating that the populations were homogeneous.

The resistance ratio of the Kollam, Valiyathura and Thikkodi population based on their LC_{50}/LC_{90} value in comparison with the susceptible lab population is presented in Table 9. Kollam population had highest resistance ratio (14.94) followed by Valiyathura (11.39) and Thikkodi (8.74). The resistance ratio based on the LC_{90} value also followed the same pattern with Kollam samples having highest resistance ratio (13.33) followed by Valiyathura (9.93) and Thikkodi (7.51).

4.1.2 Deltamethrin

The *S. oryzae* population collected from Kollam FCI recorded the highest LC_{50} for deltamethrin (937.68 ppm), followed by Valiyathura (809.17 ppm) and Thikkodi (565.62 ppm), whereas the lab population of *S. oryzae* showed the lowest LC_{50} (103.82 ppm). The fiducial limits of Kollam, Valiyathura, Thikkodi and lab samples were 812.91 to 1054.38 ppm, 734.79 to 888.29 ppm, 471.55 to 655.00 ppm and 68.18 to 141.33 ppm, respectively (Table 10).

The LC_{90} values of deltamethrin against the different samples of *S. oryzae* also followed the same trend, with Kollam (1739.41 ppm) > Valiyathura (1317.79 ppm) > Thikkodi (1216.02 ppm) > lab (577.38 ppm) having fiducial limits within 1462.33 to 2420.16 ppm, 1140.39 to 1730.09 ppm, 1007.27 to 1646.05 ppm and 384.37 to 1162.00 ppm, respectively (Table 10). The Chi square values for the probit analyses of different populations were below the table value (7.815) indicating it as a homogeneous population.

The resistance ratios obtained for the three populations of *S. oryzae* are given in Table 11. Maximum resistance was shown by Kollam (9.03), followed by Valiyathura (7.79) and Thikkodi (5.45).

Table 10. Susceptibility of different sample populations of *Sitophilus oryzae* to deltamethrin

Sample population	χ^2	d.f	LC_{50} (ppm) (95 % fiducial limit)	LC_{90} (ppm) (95 % fiducial limit)	Slope ± SE
Kollam	5.22	3	937.68 812.91-1054.38	1739.41 1462.33- 2420.16	4.78 ± 0.87
Valiyathura	4.79	3	809.17 734.79- 888.29	1317.79 1140.39- 1730.09	6.05 ± 1.06
Thikkodi	2.11	3	565.62 471.55- 655.00	1216.02 1007.27- 1646.05	3.85 ± 0.59
IARI lab culture	4.12	3	103.82 68.18-141.33	577.38 384.37- 1162.00	1.72 ± 0.28

Table value of χ^2 at 3 df= 7.815**,** χ^2 is non-significant at: $p< 0.05$.

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

Table 11. Resistance ratio of different sample populations of *Sitophilus oryzae* to deltamethrin

Sample population	Resistance ratio	
	LC_{50}	LC_{90}
Kollam	9.03	3.01
Valiyathura	7.79	2.28
Thikkodi	5.45	2.11

4.2 STUDY ON BIOCHEMICAL BASIS OF INSECTICIDE RESISTANCE

The population that showed maximum resistance to malathion and deltamethrin were further assayed for the biochemical basis of resistance and compared with the lab population. The different parameters selected for the biochemical study were total protein and activity of enzymes *viz*., carboxylesterase, glutathione S-transferase, and cytochrome P450.The results are presented in Table 12.

4.2.1 Total Protein Estimation in Different Populations of *Sitophilus oryzae*

The Kollam sample showed significantly higher protein content (6.00 mg mL^{-1}) than the lab sample (4.80 mg mL^{-1}).

4.2.2 Estimation of Enzyme Activity *viz*., Cytochrome P450, Carboxylesterase, and Glutathione S-transferase in the Resistant Kollam and Lab Populations of *Sitophilus oryzae*

a) Cytochrome P450

Cytochrome P450 activity in the *S. oryzae* population from Kollam (0.74 µmol min^{-1} mg $protein^{-1)}$ was significantly higher than that of the lab sample (0.40 µmol min^{-1} mg $protein^{-1}$).

b) Carboxylesterase

Carboxylesterase activity in the *S. oryzae* population from Kollam (0.65 µmol min^{-1} mg $protein^{-1)}$ was significantly higher than that of the lab sample (0.42 µmol min^{-1} mg $protein^{-1}$).

c) Glutathione S-transferase (GST)

Glutathione S-transferase activity was significantly higher in the *S. oryzae* collected from the Kollam FCI than in the lab sample. The observed glutathione S-transferase activities in the Kollam and the lab samples were 0.41 µmol min^{-1} mg $protein^{-1}$ and 0.26 µmol min^{-1} mg $protein^{-1}$, respectively.

Table 12. Total protein and enzyme activity in the most resistant and lab populations of *Sitophilus oryzae*

Sample population	Total protein (mg mL^{-1})	Enzymes		
		Cytochrome P450(p mol min^{-1}mg $protein^{-1}$)	Carboxylesterase (µmol min^{-1} mg $protein^{-1}$)	Glutathione S-transferase (µmol min^{-1} mg $protein^{-1}$)
Kollam	6.00^{a}	0.74^{a}	0.65^{a}	0.41^{a}
Lab	4.80^{b}	0.40^{b}	0.42^{b}	0.26^{b}
CD (0.05)	(0.540)	(0.079)	(0.031)	(0.025)

4.3 LABORATORY EVALUATION OF NEW INSECTICIDE MOLECULES AGAINST RESISTANT POPULATION OF *Sitophilus oryzae*

LC_{50} and LC_{90} of the selected newer insecticides are tabulated in Table 13-15. The relative toxicity of the tested insecticide is provided in Table 16.

The calculated LC_{50} of fipronil against the Kollam sample of *S. oryzae* was 5.86 ppm with fiducial limits ranging between 3.26 to 9.05 ppm. For the lab sample, LC_{50} was recorded at 4.36 ppm with lower and upper fiducial limits of 2.93 ppm and 5.98 ppm, respectively. LC_{90} of the Kollam and lab samples were 62.99 ppm and 23.45 ppm, with fiducial limits of 36.25 to 158.20 ppm and 15.81 to 43.34 ppm, respectively (Table 13).

The LC_{50} of indoxacarb in the Kollam sample was found to be 90.57 ppm with lower and upper fiducial limits as 73.32 ppm and 105.92 ppm. LC_{50} for the lab sample was 72.82 ppm, with fiducial limits of 57.48 to 87.61 ppm. Similarly, LC_{90} of the Kollam and lab samples were 214.22 ppm and 208.31 ppm, with fiducial limits of 175.14 to 300.01 ppm and 160.66- 327.16 ppm, respectively (Table 14).

In the case of chlorantraniliprole, LC_{50} in the Kollam and lab samples were 4041.43 ppm and 1397.56 ppm, with lower and higher fiducial limits of 3330.66 to 4724.50 ppm, and 1156.91 to 1642.12 ppm, respectively. The Kollam and lab samples recorded 9550.72 ppm and 3412.43 ppm in the case of LC_{90}, with fiducial limits of 7765.36 to 13355.97 ppm and 2727.48 to 4932.47 ppm, respectively (Table 15). The chi square values obtained on probit analysis for Kollam and the lab samples were below the table value, indicating that the populations were homogenous.

Among the different populations of *S. oryzae* studied, the Kollam population turned out to be more resistant, and the resistance development was highest in malathion than deltamethrin. Hence, the relative toxicity of the newer molecules was calculated by comparing their LC_{50} with the LC_{50} of malathion in the Kollam sample. The relative toxicity of fipronil, indoxacarb, and chlorantraniliprole against *S. oryzae* populations from Kollam and the lab samples are given in Table 16. Fipronil showed the highest relative toxicity (619.80), followed by indoxacarb (40.12), whereas chlorantraniliprole (0.90) had the lowest calculated relative toxicity.

Table 13. Susceptibility of the most resistant and the susceptible sample populations of *Sitophilus oryzae* to fipronil

Sample population	χ^2	d.f	LC_{50} (ppm) (95 % fiducial limit)	LC_{90} (ppm) (95 % fiducial limit)	Slope ± SE
Kollam	6.24	3	5.86 3.26- 9.05	62.99 36.25- 158.20	1.24 ± 0.20
IARI lab culture	1.69	3	4.36 2.93- 5.98	23.45 15.81- 43.34	1.75 ± 0.26

Table value of χ^2 at 3 df= 7.815, χ^2 is non-significant at: p< 0.05.

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

Table 14. Susceptibility of the most resistant and the susceptible sample populations of *Sitophilus oryzae* to indoxacarb

Sample population	χ^2	d.f	LC_{50} (ppm) (95 % fiducial limit)	LC_{90} (ppm) (95 % fiducial limit)	Slope ± SE
Kollam	6.31	3	90.57 73.32-105.92	214.22 175.14- 300.01	3.43 ± 0.55
IARI lab culture	4.36	3	72.82 57.48- 87.61	208.31 160.66 - 327.16	2.81 ± 0.46

Table value of χ^2 at 3 df= 7.815**,** χ^2 is non-significant at: p< 0.05.

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

Table 15. Susceptibility of the most resistant and the susceptible sample populations of *Sitophilus oryzae* to chlorantraniliprole

Sample population	χ^2	d.f	LC_{50} (ppm) (95 % fiducial limit)	LC_{90} (ppm) (95 % fiducial limit)	Slope ± SE
Kollam	3.51	3	4041.43 3330.66 -4724.50	9550.72 7765.36 -13355.97	3.43 ± 0.52
IARI lab culture	4.39	3	1397.56 1156.91-1642.12	3412.43 2727.48 -4932.47	3.31 ± 0.50

Table value of χ^2at 3 df= 7.815, χ^2 is non-significant at: $p< 0.05$.

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

Table 16. Relative toxicity of new generation insecticides with respect to malathion against *Sitophilus oryzae*

Insecticide	Kollam sample population	IARI lab culture
Fipronil	619.80	55.79
Indoxacarb	40.13	3.34
Chlorantraniliprole	0.90	0.17

4.4 COMPARATIVE EVALUATION OF NEWER INSECTICIDES AGAINST *Sitophilus oryae* INFESTED GRAINS IN JUTE BAGS

Comparative evaluation of fipronil, indoxacarb, chlorantraniliprole, deltamethrin, and malathion was carried out by spraying them on jute bags containing 1 kg of rice grains infested with 50 adults of *S. oryzae*. The results on per cent mortality among the different treatments are presented in Table 17.

At 24 h after treatment, the per cent mortality was in order of fipronil (86.5 %) > indoxacarb (72 %) > deltamethrin (53 %) > malathion (50.5 %)> chlorantraniliprole (46.5 %). The insecticidal treatments fipronil and indoxacarb were significantly different from the other insecticides in their efficacy, whereas deltamethrin, malathion, and chlorantraniliprole were on par in their efficiency. No dead adult beetles were found in untreated bags.

The same trend was followed after 48 h of treatment. Highest mortality was noted in fipronil (89.5 %) followed by indoxacarb (76 %), deltamethrin (59 %), malathion (58 %) and chlorantraniliprole (49.5 %). Fipronil, indoxacarb and chlorantraniliprole were found significantly different from other insecticides, whereas malathion and deltamethrin were found on par.

4.5 ESTIMATION OF RESIDUES OF INSECTICIDES IN GRAIN SAMPLES

4.5.1 Method Validation for Pesticide Residue Analysis in Rice

The results of the method validation study for the estimation of malathion, deltamethrin and fipronil residues in rice samples showed satisfactory recovery for the compounds at the different levels of fortification.

The method was validated with satisfactory linearity and recovery that was within the acceptable range of 70-120 per cent. The instrument calibration showed good linearity within a range of 0.01-1 mg kg^{-1} (Fig. 4-6). Repeatability of the recovery results as shown by relative standard deviations (RSD) were below 20 per cent, indicating that the method was sufficiently trustworthy for pesticide residue analysis and the results are shown in Tables 18-20.

Table 17. Comparative evaluation of insecticides against *Sitophilus oryzae*

SL. No.	Treatments	Mortality (%)	
		24 HAT	48 HAT
1	Fipronil 5 % SC @ 0.006 %	86.5 (68.76)[a]	89.5 (71.28)[a]
2	Indoxacarb 14.5% SC @ 0.09 %	72 (58.09)[b]	76 (60.73)[b]
3	Chlorantraniliprole 18.5 % SC @ 4.04 %	46.5 (42.99)[c]	49.5 (44.71)[d]
4	Deltamethrin 2.8 % EC @ 3.57 % (Positive control)	53.0 (46.73)[c]	59 (50.22)[c]
5	Malathion 50 % EC @ 1 % (Positive control)	50.5 (45.29)[c]	58 (49.63)[c]
6	Untreated control	0 (0.41)[d]	0 (0.41)[e]
	C.D (0.05)	(4.647)	(4.519)

Figures in parenthesis are angular transformed values; HAT- Hours after treatment

Treatments with same letters are not significantly different

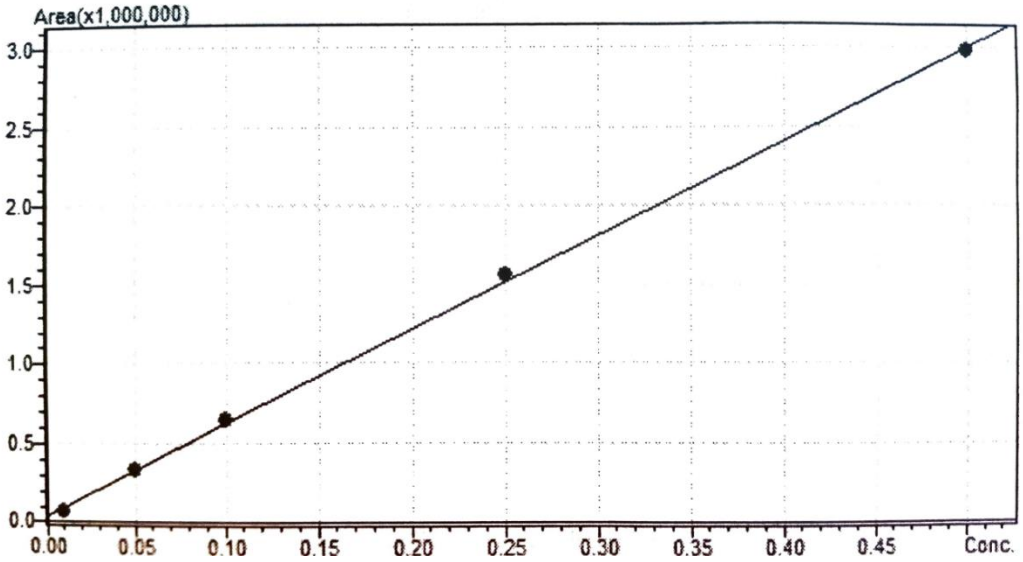

Fig. 4. Calibration curve of malathion

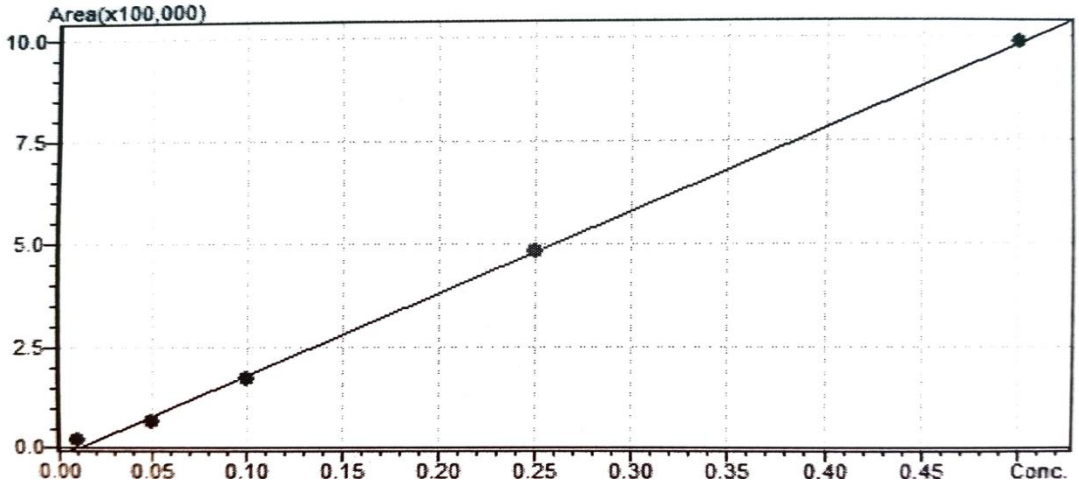

Fig. 5. Calibration curve of deltamethrin

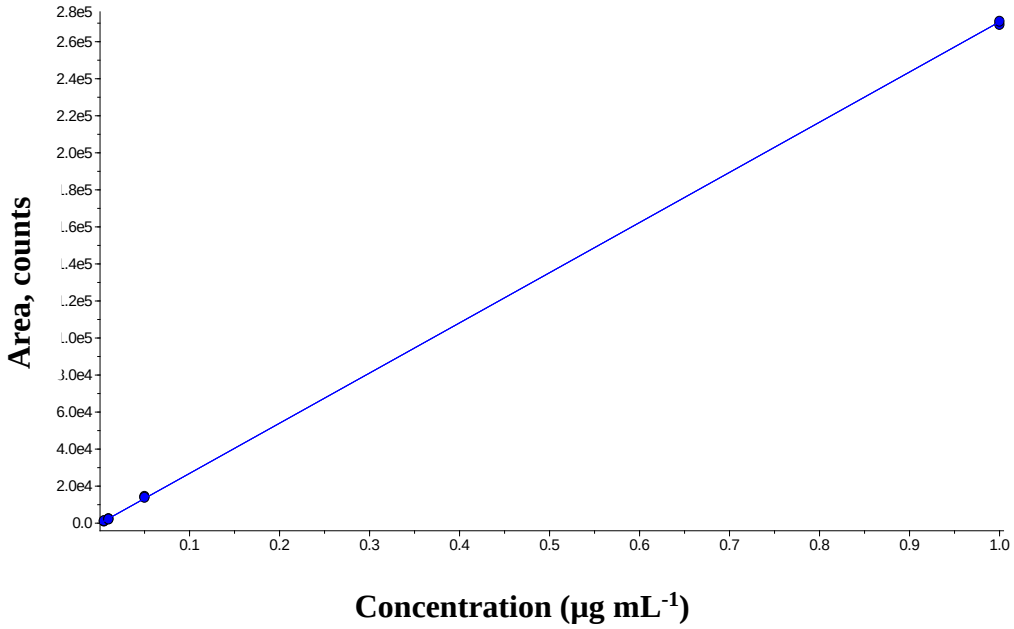

Fig. 6. Calibration curve of fipronil

Table 18. Per cent recovery of malathion fortified at different levels

LOQ (mg kg^{-1})	Mean Recovery (%) ± SD	RSD (%)
0.05	72 ± 2	2.78
0.1	102.33 ± 3.05	2.98
0.25	75.2 ± 3.82	5.07

LOQ- Limit of quantification; SD- Standard deviation; RSD- Relative standard deviation

Table 19. Per cent recovery of deltamethrin fortified at different levels

LOQ (mg kg^{-1})	Mean Recovery (%) ± SD	RSD (%)
0.05	83.33 ± 3.05	3.67
0.1	86 ± 4.58	5.33
0.25	92.93 ± 3.40	3.66

Table 20. Per cent recovery of fipronil fortified at different levels

LOQ (mg kg^{-1})	Mean Recovery (%) ± SD	RSD (%)
0.01	90 ± 10	0.11
0.02	100	0
0.05	100.67 ± 5.03	0.05

The average recovery obtained for malathion was 72, 102.33 and 75.2 per cent with relative standard deviation of 2.78, 2.98 and 5.07 per cent at 0.05, 0.1 and 0.25 mg kg^{-1} fortification levels, respectively. At the same levels of fortification, the average recovery of deltamethrin was in the range of 83.33 to 92.93 per cent. When fortified at the lowest level of 0.05 mg kg^{-1}, the mean recovery per cent was 83.33 and the RSD worked out was 3.67. At 0.1 mg kg^{-1} level, an average of 86 per cent residue was recovered with the RSD of 5.33 per cent. At higher level of fortification (0.25 mg kg^{-1}), 92.93 per cent of residues were recovered and the RSD obtained was 3.66 per cent.

The selected method gives 90-100.67 per cent recovery for fipronil residues at 0.01,0.02 and 0.05 mg kg^{-1} levels of fortification. At 0.01 mg kg^{-1} fortification levels, the mean recovery of fipronil was 90 per cent with an RSD of 0.11 per cent. When fortified at 0.02 mg kg^{-1} 100 per cent recovery of fipronil residues was obtained. At a high concentration of 0.05 mg kg^{-1}, the mean recovery per cent was 100.67 with an RSD of 0.05 per cent.

4.5.2 Persistence and Dissipation of Insecticides in rice

The persistence of the most effective insecticide, fipronil, based on the previous experiment was further studied and compared with the recommended insecticides malathion and deltamethrin. After spraying on jute bags containing rice grains with the commercial formulations, residues of malathion, deltamethrin and fipronil were analysed at 0 (2 h after spray), 1, 3, 5, 7, 10, 15, 20 and 30 day intervals. Pesticide residues were detected in rice and dissipation per cent at different sampling intervals, along with calculated half- life are given in Table 21.

At two hours after the spraying of malathion on rice grains, a mean initial deposit of 0.13 mg kg^{-1} of residues was detected. The malathion residues got dissipated and on the first day it was found to be 0.09 mg kg^{-1}, which was 31 per cent lesser than the initial residue. On the third day, the residue dissipated by 54 per cent and reached 0.06 mg kg^{-1}. No residue was detected above the limit of quantification (LOQ) from the fifth day of spraying. The calculated half-life of malathion on rice grains was 2.71 days.

Table 21. Persistence and dissipation of insecticides in rice grains

Days after spraying (DAS)	Malathion 50 % EC @ 1 %		Deltamethrin 2.8 % EC @ 3.57 %		Fipronil 5 % SC @ 0.006 %	
	Mean residue (mg kg^{-1})	Dissipation (%)	Mean residue (mg kg^{-1})	Dissipation (%)	Mean residue (mg kg^{-1})	Dissipation (%)
0 (2 h after spraying)	0.13	-	0.09	-	LOQ	
1	0.09	31	0.07	22.22	-	
3	0.06	54	LOQ		-	
5	LOQ		-		-	
7	-		-		-	
10	-		-		-	
15	-		-		-	
20	-		-		-	
30	-		-		-	
Half- life (Days)	2.71		2.48		-	

LOQ-Limit of quantification; LOQ for malathion and deltamethrin= 0.05 mg kg^{-1}, LOQ for fipronil= 0.01mg kg^{-1}

The mean initial deposit of deltamethrin in rice samples was found to be 0.09 mg kg^{-1} at two hours of spraying. On the first day of spraying, it got dissipated to 0.07 mg kg^{-1} with a dissipation percentage of 22.22. At three days after spraying, deltamethrin residues were below LOQ (0.05mg kg^{-1}) and the half-life calculated was 2.48 days.

Fipronil residues were below the LOQ (0.01 mg kg^{-1}) from the initial sampling interval itself. All the insecticides studied were below the detection limit at 5 days after spraying. The residue levels of all the tested insecticides were below the MRL (Maximum Residue Limit) fixed by the Food Safety and Standards Authority of India (FSSAI) at all the sampling intervals.

Discussion

5. DISCUSSION

Post-harvest storage and protection of food grains are inevitable to ensure food security. Insect pests are a major threat to stored grains, causing significant losses in both quality and quantity of grains. Rice weevil *S. oryzae* is an important primary pest capable of causing 12 to 20 per cent grain loss which may extend to 80 per cent under favourable conditions (Keba and Sori, 2013; Tefera *et al*., 2013). Chemical control is the most feasible method that can be employed in storage godowns and warehouses and, so far, it is recognised as the best control measure for stored product pests. Development of resistance is acknowledged as a challenging factor in the use of insecticides, as their increased application may eventually lead to economic as well as ecological damage *via* the deposition of residues and may threaten human health. The occurrence of insecticide resistance in insects is mainly through behavioural, physiological, and biochemical mechanisms. Biochemical mechanisms involving various metabolic enzymes which detoxify toxic chemicals are the primary and common mechanism behind insecticide resistance development.

Monitoring the extent and frequency of insecticide resistance is important for the development and implementation of an effective resistance management strategy (Stadler *et al*., 2003). Rotation of insecticides with different modes of action is an important strategy to overcome the development of insecticide resistance (Sparks and Nauen, 2015), and new generation insecticides with different modes of action are the best substitutes to combat resistance. The application of insecticides leads to deposition of residues on food grains. Hence, pesticide residue analysis is important to determine the residue levels in food grains and to ensure the safety of consumers.

The present study evaluated the resistance levels of the commonly used insecticides in FCI godowns like malathion and deltamethrin in rice weevil *S. oryzae* collected from three FCI godowns of Kerala and compared it with a laboratory sample of *S. oryzae* obtained from IARI. Further, the biochemical mechanism behind the resistance was assessed and for managing resistance, selected newer molecules were screened against *S. oryzae* and the persistence of the effective new generation insecticide on rice grains was further evaluated along with malathion and deltamethrin. The results obtained are discussed in this chapter.

5.1 EVALUATION OF RESISTANCE OF *Sitophilus oryzae* TO INSECTICIDES

5.1.1 Malathion

In the present investigation, the LC_{50}/LC_{90} of the FCI and lab populations against malathion were found by probit analysis, and the $LC_{50/90}$ of the three FCI sample populations collected from Kollam, Valiyathura, and Thikkodi were higher compared to the IARI lab sample (Table 8). This indicates the development of resistance and the highest LC_{50} was recorded in the Kollam population of *S. oryzae*, followed by Valiyathura and Thikkodi. Log dose- probit (ld-p) lines can be used to compare the susceptibility of different populations of test insects to a particular insecticide. When the ld-p lines of malathion were analysed, a considerable rightward shift in the FCI gathered population from the lab sample was detected, indicating resistance development in *S. oryzae* towards malathion (Fig. 7). The slopes of the probit lines of FCI collected populations were found similar, and cluttered together, indicating their uniform response towards malathion.

The resistance ratio, expressed as RR_{50}/RR_{90}, can be computed by comparing the LC_{50}/LC_{90} of the FCI population with that of the lab population, and it illustrates the extent of resistant development in *S. oryzae*. While analysing the RR_{50} values, the Kollam population was 14.94-fold more resistant to malathion than the susceptible sample, followed by Valiyathura (11.39) and Thikkodi (8.74) samples. RR_{90} values followed the same trend for Kollam (13.33), Valiyathura (9.93) and Thikkodi (7.51) samples (Table 9).

Rajak *et al*. (1973) confirmed 5.8-fold resistance to malathion in *S. oryzae* samples collected from Uttar Pradesh, which is regarded as the first reported case of malathion resistance in *S. oryzae* from India. During the 1970s, a FAO global survey revealed a large-scale prevalence of malathion resistance in *S. oryzae* all over the world (Champ and Dyte, 1976). Later, many scientists have reported malathion resistance in *S. oryzae* (Navarro *et al*., 1986; de Villar *et al*., 1987; Pacheco *et al*., 1990; Sartori *et al.*, 1990; Irshad and Gillani, 1992; Visalakshi and Gour, 2006).

Joia and Kumar (1996) reported resistance ratio ranging from 7 to 17.9 against malathion in *S. oryzae* collected from 33 different locations of Punjab, India. The level

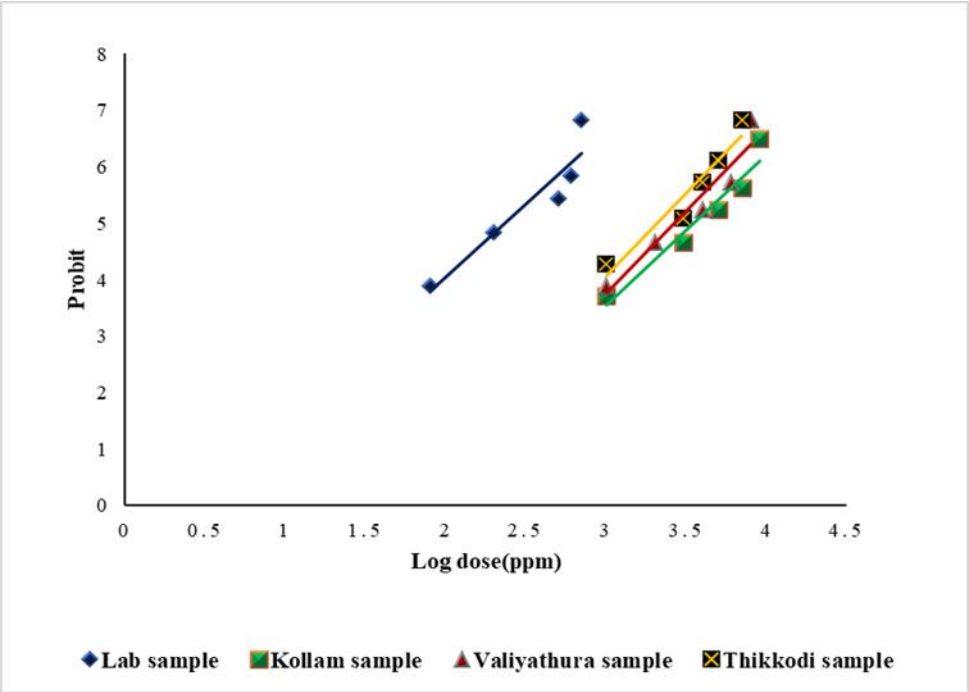

Fig. 7. Log dose -Probit line of malathion

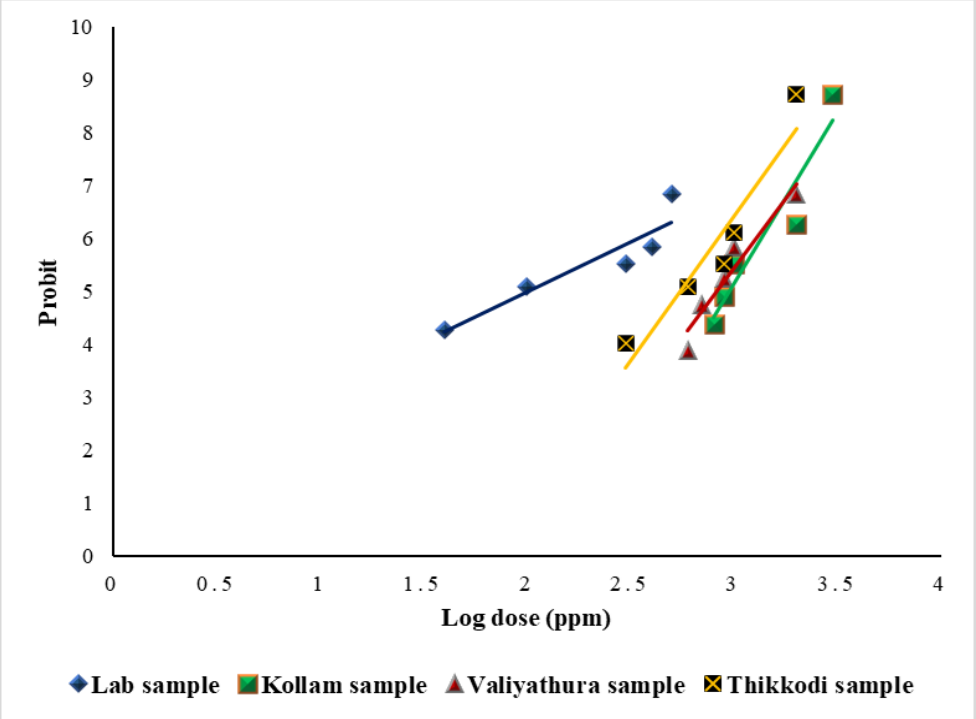

Fig. 8. Log dose – Probit line of deltamethrin

of resistance obtained in malathion against *S. oryzae* in the present study is congruent with this report.

The fiducial limits determined for various FCI collected populations were found to overlap each other, indicating the uniform development of resistance in *S. oryzae* (Table 8). It is probably due to the uniform exposure of insects in FCI godowns to malathion as they follow the same protocol for spraying insecticides.

5.1.2 Deltamethrin

The LC_{50}/LC_{90} of the three FCI sample populations of *S. oryzae* against deltamethrin were higher than the IARI lab samples (Table 10), and the ld-p lines of deltamethrin were identical to those of malathion, with a significant rightward shift in the regression lines of all the FCI collected samples from the laboratory sample, indicating the development of resistance to deltamethrin (Fig. 8). Compared to malathion, the rightward shift was less here, indicating its lower resistance than malathion.

The resistance ratios showed the same trend as for malathion. Kollam samples showed the maximum resistance ratio (RR_{50}) of 9.03 followed by Valiyathura (7.79) and Thikkodi (5.45) (Table 11). The resistant ratio (RR_{90}) at LC_{90} showed a significant reduction in comparison with RR_{50}. The RR_{90} values for the Kollam, Valiyathura, and Thikkodi populations were 3.01, 2.28, and 2.11, respectively, which were 3, 3.42, and 2.59-fold lower than the corresponding RR_{50} values, and this result is in accordance with the findings of Attia *et al*. (2017).

The first report of deltamethrin resistance in *S. oryzae* was from Australia, where insect populations had 98-fold more resistance than susceptible strains (Heather, 1986). Later, deltamethrin resistance in *S. oryzae* has been reported in different parts of the world (Singh *et al*., 2007; Attia *et al*., 2017; Haddi *et al*., 2018). Ceruti and Lazzari (2003) and Attia *et al.* (2017) reported less than 10-fold resistance in *S. oryzae* to deltamethrin, and it corroborates the findings of the present investigation, where the resistance ratio of deltamethrin ranged from 2.11 to 9.03. The toxicity studies conducted by Singh *et al*. (2021) in *S. oryzae* populations collected from various grain storages in southern India identified four weakly resistant populations to deltamethrin, out of which

three were from Kerala. The current investigation also revealed a weak resistance to deltamethrin with a resistance ratio of less than 10.

From the present study, it is revealed that *S. oryzae* collected from Kollam, Valiyathura, and Thikkodi were resistant to the commonly used insecticides, *viz*., malathion and deltamethrin, possessing different modes of action, indicating multiple resistance, which corroborates the findings of Visalakshi and Gour (2006).

In the current investigation, malathion resistance was higher in all the three FCI collected populations than deltamethrin resistance. Organophosphates, carbamates, and chlorinated hydrocarbons were introduced between 1940 and the late 1960s and regarded as the "second-generation insecticides". Synthetic pyrethroids became a part of this category during the 1970s, when chlorinated hydrocarbons were being phased out (Hummel, 1983). Hence, synthetic pyrethroids are comparatively newer molecules than organophosphates. Before the introduction of deltamethrin, malathion was used for controlling stored pests. Therefore, the chances of development of resistance against malathion is more compared to deltamethrin. In 1971, 37.76-fold resistance to malathion was reported in *T. castaneum* in India by Bhatia *et al*. (1986). This substantiates the development of higher resistance in the FCI collected population of *S. oryzae* to malathion than deltamethrin in the present study.

5.2 BIOCHEMICAL BASIS OF INSECTICIDE RESISTANCE

Insecticide resistance occurs as a consequence of three major mechanisms, *viz*., behavioural, physiological, and biochemical mechanisms. The behavioural mechanism involves alterations in insect behaviour to avoid insecticide exposure. The physiological mechanism entails alterations in penetration, excretion and transportation of the pesticide into the insect body (Scott, 1999). Changes in target-site binding activity and improved detoxification by various metabolic enzymes are part of the biochemical mechanism of resistance (Gao *et al.*, 2006; Konus, 2015).

The detoxifying enzymes imparting metabolic resistance include cytochrome P450, esterases, and glutathione S-transferases (GST). Detoxification of insecticides consists of two stages *viz.*, phase I or primary reactions and phase II or secondary reactions. Cytochrome P450 monooxygenases, carboxylesterases are involved in phase I

reactions and glutathione S-transferases are associated with phase II reactions. Oxidation or hydrolysis reactions are found associated with phase 1 detoxication and phase II reactions involve conjugation of Phase I detoxification products for solubilization and transport (Berenbaum and Johnson, 2015). Elevated levels of enzymes reduce the availability of insecticide doses at the target site, which is necessary to cause a lethal effect on an insect. Target site mutation hinder the binding between insecticide molecule and target site (Panini *et al.*, 2016).

The toxicological assays revealed the Kollam population of *S. oryzae* as the most resistant one among the three FCI collected samples. To confirm the results, the biochemical basis of resistance was further studied in the Kollam and lab samples. The activity of detoxifying enzymes *viz*., cytochrome P450, carboxylesterases and glutathione S-transferase and the total protein content of the Kollam population were significantly higher than in the lab sample. The highest enzyme activity was of cytochrome P450, which was 1.85-fold higher than in the laboratory sample. The estimated values of carboxylesterase and GST were 1.55 and 1.58-fold higher than the lab sample (Fig. 9). The higher levels of the enzymes contribute to the higher total protein content in the resistant population of *S. oryzae* compared to the laboratory sample.

5.2.1 Malathion

The detoxifying enzymes like cytochrome P450, carboxylesterases, and glutathione S-transferases have a role in the metabolism of malathion. Cytochrome P450 monooxygenases oxidise malathion to malaoxon by the conversion of the P=S bond in malathion to a P=O bond *via* oxidative desulphuration (Reed and Rubin, 2014). Malaoxon is more toxic than malathion and is responsible for most of the cholinergic toxicity. The most common method of malathion detoxification is through carboxylesterase. The esterase reaction, defined as the hydrolysis of an ester to its component alcohol and acid, includes carboxylic, thio, phospho, and other ester substrates (Yan *et al*., 2009; Hatfield *et al*., 2016). Carboxylesterases were found to target the carboxyl ester component (Fig. 10) of malathion (Matsumura and Brown, 1960) and malaoxon and hydrolyse to form malathion or malaoxon monocarboxylic acid (MCA) and dicarboxylic acid (DCA), also known as the specific esterase

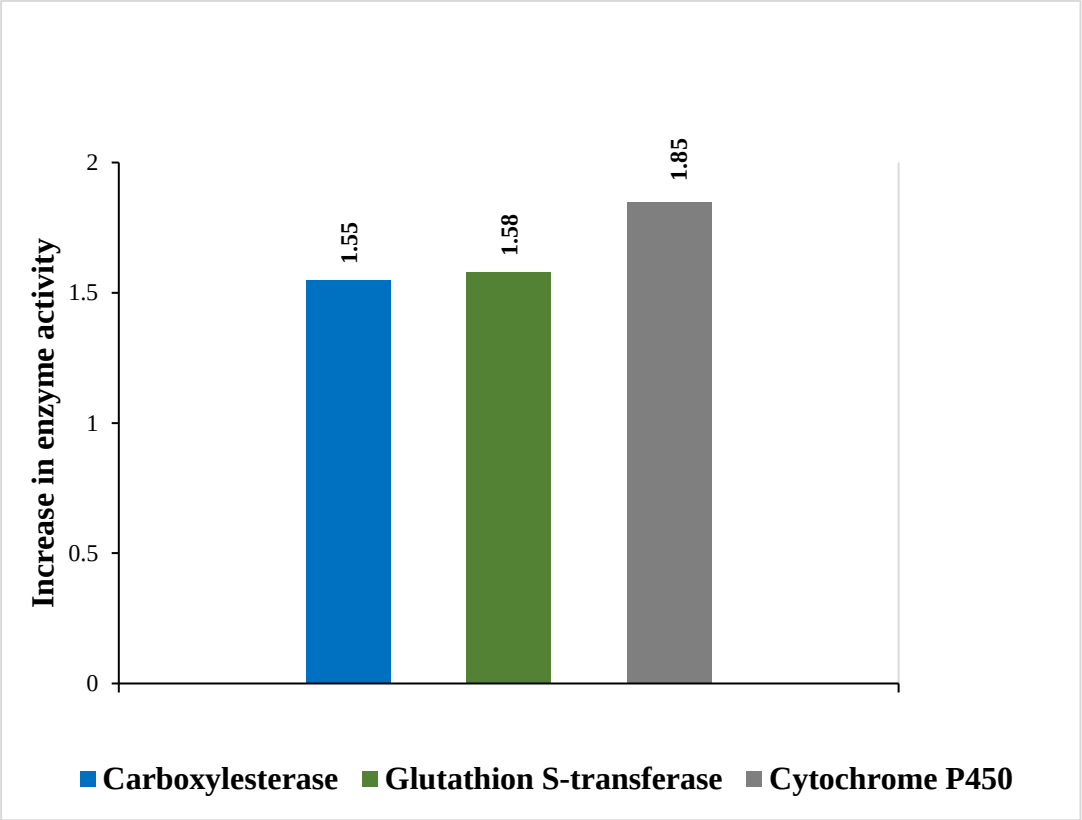

Fig. 9. Increase in enzymatic activity in the Kollam sample of *Sitophilus oryzae* over susceptible sample

mechanism, which was confirmed in most cases of malathion resistance in *T. castaneum* strains (Julio *et al.*, 2017). GSTs act by catalysing the conjugation of a wide range of compounds having an electrophilic site with reduced glutathione, resulting in reduced toxicity and easy excretion from the cells (Meister and Anderson, 1983). A minor metabolic pathway of malathion and malaoxon is demethylation through glutathione S-transferase (Reed and Rubin, 2014).

Cytochrome P450 oxidises insecticides to make them more water soluble or creates complexes with pesticides to make them more easily excreted and transported from the body (Lee and Lees, 2001). However, in some cases, such as with many organophosphates, monooxygenase-mediated bioactivation produces a more toxic metabolite than the parent insecticide, as like malaoxon (Wheelock and Scott, 1990), which potentially increases toxicity rather than resistance. But it is also possible that resistance could be achieved through decreased activation of organophosphates to more toxic compounds (Scott, 1999). In the current study, even though elevated levels of cytochrome P450 was observed, malathion resistance observed in all the FCI collected populations, was probably due to reduced activation of malathion to malaoxon or due to the multiple resistance to deltamethrin.

The biochemical analysis for insecticide resistance in various populations of *S. oryzae* by Konus (2015), indicated a 2-fold increase in p-nitroanisole O-demethylation activity of cytochrome P450 monooxygenases in the malathion resistant Ankara population, indicating the active role of cytochrome P450 monooxygenase in malathion resistance, which is comparable with the 1.85-fold cytochrome P450 estimated in this study.

According to Guedes and Zhu (1998), the malathion-resistant *R. dominica* population has 1.3-fold higher esterase activity compared to the susceptible population, which is in par with the results of the present study where samples collected from Kollam FCI were found to have 1.55-fold higher content of carboxylesterase. Anusree (2019) reported 1.15-1.49-fold higher esterses in *T. castaneum* collected from five FCI godowns in Kerala compared to a susceptible strain. The result of the current study is consistent with this result.

GSTs are frequently linked to organophosphate resistance (Soderlund and Bloomquist, 1990; Yu, 1996). In insects, GST mediated organophosphorus resistance is assumed to be caused by enhanced protein expression or the emergence of a novel isozyme involved in insecticide metabolism in the resistant strain (Ottea and Plapp, 1984).

5.2.2 Deltamethrin

In general, metabolism of deltamethrin involves oxidation by cytochrome P450, further hydrolysis of the central ester bond by carboxylesterases followed by conjugation reactions (Anand *et al*., 2006; Dalefield, 2017).

The cytochrome P450s are confirmed to be responsible for pyrethroid and organophosphate resistance (Schenkman and Jansson, 2003; Scott, 1999). Zhu *et al*. (2010) found 200-fold higher expression of cytochrome P450 in the deltamethrin resistant strain of *T. castaneum* than in the susceptible strain. The significant higher level of cytochrome P450 obtained in the present study could have a role in multiple resistance imparting both malathion and deltamethrin resistance in *S. oryzae*.

In many stored product insects, metabolic resistance to organophosphate (Guedes and Zhu, 1998; Lucena *et al.,* 2012) and pyrethroid pesticides (Dyte and Rowlands, 1968; Wool and Front, 2002; Hafiz *et al*., 2018) is mediated by carboxylesterases. Since malathion and deltamethrin have ester bonds in their structure (Fig. 10 and 11), esterase can hydrolyse both malathion and deltamethrin insecticides. Hence, the high activity of esterase found in the Kollam population of *S. oryzae* suggests that esterase could be involved in malathion and deltamethrin resistance mechanisms in this population. This result is also in accordance with the findings of esterase mediated resistance against malathion and deltamethrin in *S. oryzae* by Attia *et al.* (2017).

GSTs have been found to be associated with pyrethroid resistance in insects (Grant and Matsumura, 1989; Reidy *et al*., 1990; Legacid *et al*., 1993). Fragoso *et al*. (2003) recognised more than two-fold increase in glutathione S-transferase in pyrethroid (Cypermethrin, deltamethrin and permethrin) resistant strains of *S. zeamais*

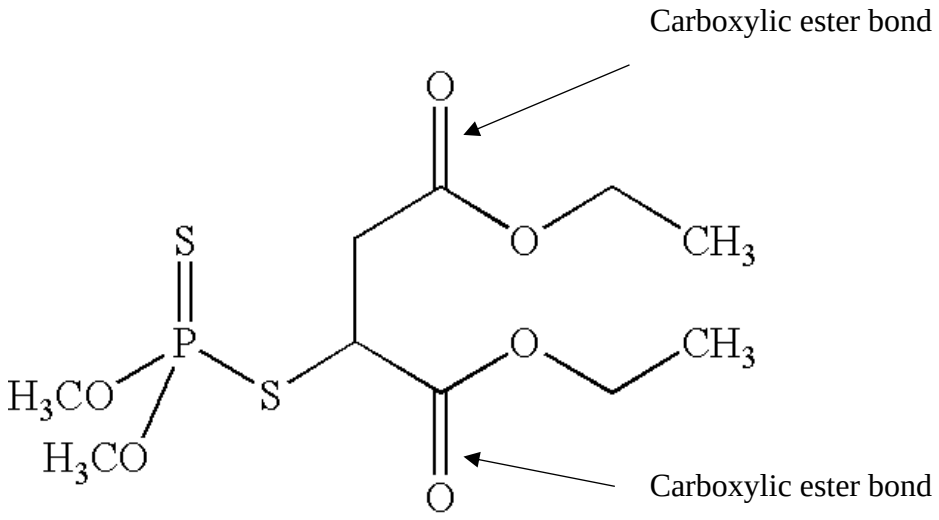

Fig. 10. Structure of malathion

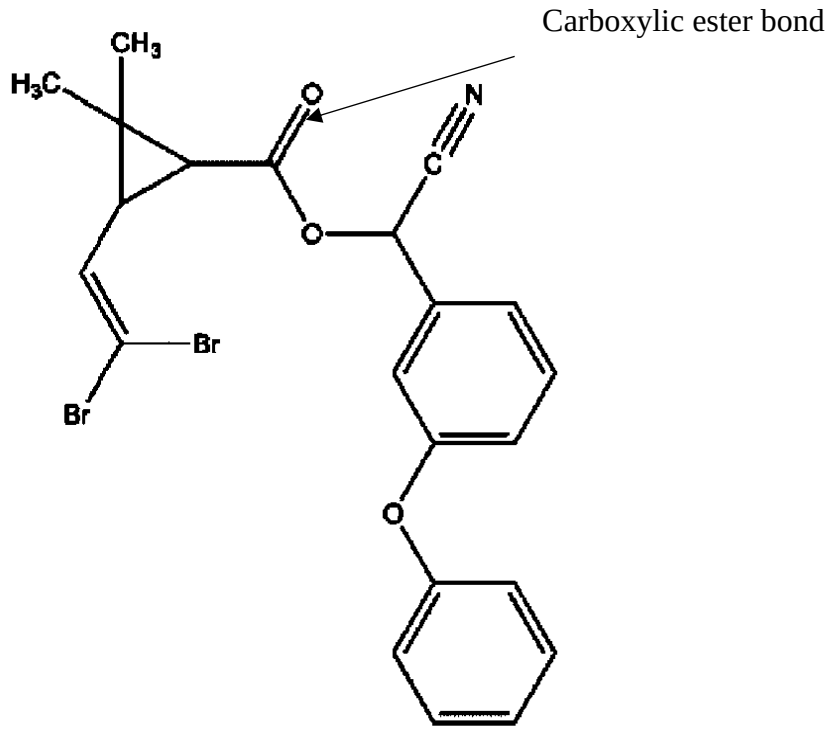

Fig. 11. Structure of deltamethrin

compared to susceptible strains. According to Fragoso *et al*. (2007), the enhanced activity of glutathione S-transferase is a reason for pyrethroid resistance in the *S. zeamais* population of Brazil. The present study also found elevated GST activity in the resistant population of *S. oryzae*, which could be a reason behind the resistance developed against both malathion and deltamethrin.

5.3 LABORATORY EVALUATION OF NEW INSECTICIDE MOLECULES AGAINST RESISTANT POPULATION OF *Sitophilus oryzae*

Results of the present study revealed that the three FCI collected samples from Kollam, Valiyathura, and Thikkodi were resistant to malathion and deltamethrin, and among them, malathion showed more resistance. When LC_{50} values of deltamethrin were compared with those of malathion, the relative toxicity of deltamethrin was found to be 3.88, 3.42, 3.76, and 2.34 times more in the sample populations of Kollam, Valiyathura, Thikkodi, and IARI lab cultures, respectively (Fig. 12).

In the present study, *S. oryzae* has developed resistance to malathion and deltamethrin. Malathion is an organophosphate insecticide that acts on the insect nervous system by inhibiting acetylcholine esterase, which leads to acetylcholine accumulation and consequently disrupted neurotransmission. Deltamethrin is a synthetic pyrethroid which affects the nervous system of insects by modulating sodium channels and interfering with the closing dynamics of these channels (Sparks and Nauen, 2015). For resistant management, one of the main principles is choosing insecticides with different modes of action. Hence, new generation insecticides with different modes of action such as fipronil, indoxacarb, and chlorantraniliprole were evaluated against resistant population of *S. oryzae* to investigate their efficacy for the management of *S. oryzae*.

Fipronil is a phenyl pyrazole compound which blocks GABA-gated chloride channels in the central nervous system, resulting in overstimulation of the nervous system. Indoxacarb is an oxadiazine compound that acts by blocking the voltage-dependent sodium channels in the nervous system of insects. Chlorantraniliprole is a ryanodine receptor modulator that causes uncontrolled activation of ryanodine receptor

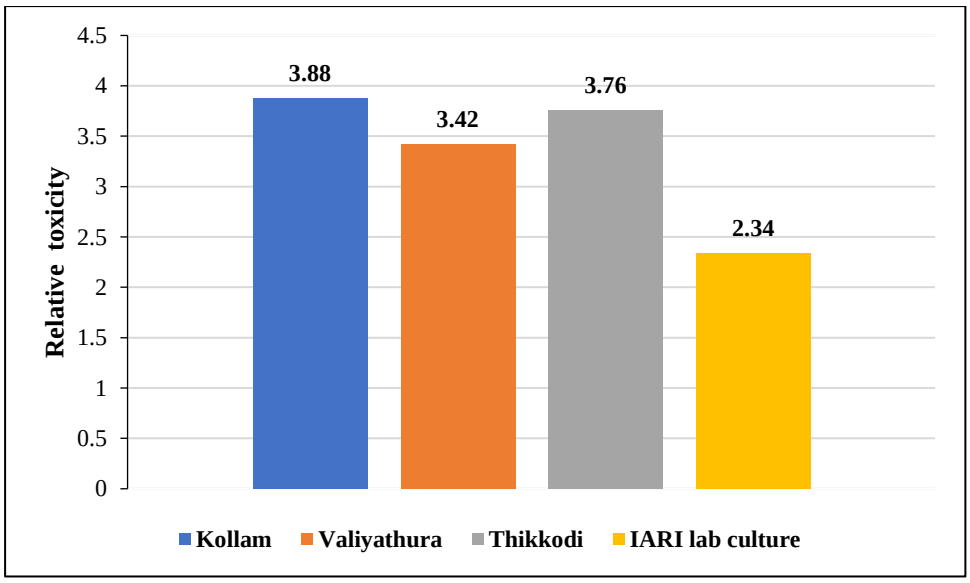

Fig. 12. Relative toxicity of deltamethrin to different population of *Sitophilus oryzae* with respect to malathion

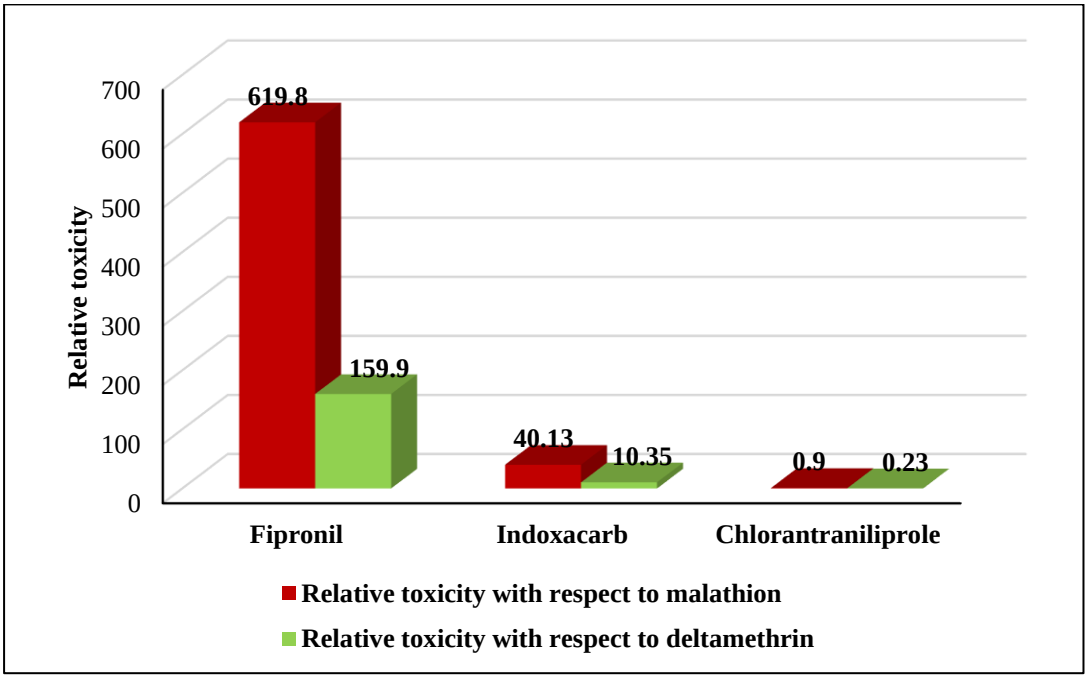

Fig. 13. Relative toxicity of new generation insecticides against resistant population of *Sitophilus oryzae* with respect to malathion and deltamethrin.

channels, resulting in depletion of internal calcium stores and impairment of the regulation of muscle contraction (Bentley *et al*., 2010).

Bioassays done to find out their toxicity towards the resistant *S. oryzae* sample population revealed fipronil as most toxic with lower LC_{50} than indoxacarb and chlorantraniliprole (Table 13-15). When their toxicity was compared with respect to malathion and deltamethrin, fipronil showed highest relative toxicity compared to indoxacarb and chlorantraniliprole (Fig. 13).

Fipronil is the first commercially used member of the phenyl pyrazole insecticides that interact antagonistically with the GABA-gated chloride channel (Wang *et al*., 2009). Even though the use of fipronil against crop pests is well documented (Sibin, 2002; Gupta *et al.,* 2009; Mann *et al.,* 2009; Shi *et al.,* 2012; Morales-Rodriguez and Wanner, 2015), the studies on the efficacy of fipronil for the management of stored product pests are scanty.

Fig. 13 shows that fipronil is 619.8 and 159.9 times more toxic to resistant *S. oryzae* samples from Kollam FCI when compared to toxicity of malathion and deltamethrin, respectively. The ld-p lines of fipronil against Kollam and lab samples showed overlapping lines (Fig. 14). Both the samples had similar slopes and overlapping fiducial limits, indicating that the resistant and susceptible lab population had a similar response towards fipronil.

Kavallieratos *et al*. (2010) evaluated the efficacy of fipronil as a grain protectant against *S. oryzae, T. confusum, R. dominica* and *Prostephanus truncates* (Horn). Fipronil appeared to be very effective for controlling all the test insects at doses equal to or higher than 1ppm and also suppressed progeny production. The present study also finds fipronil as an effective insecticide for the management of *S. oryzae* even at a low concentration of 5.86 ppm.

Indoxacarb is a low risk oxadiazine compound having low mammalian toxicity and its broad-spectrum efficacy is well recognised for the management of a wide range of crop pests (Rao *et al*., 2007; Saimandir and Gopal, 2009; Bird, 2015; da Silva *et al*., 2021; Gesraha and Ebeid, 2021). The efficacy of indoxacarb for the management of stored grain pests has not been explored much.

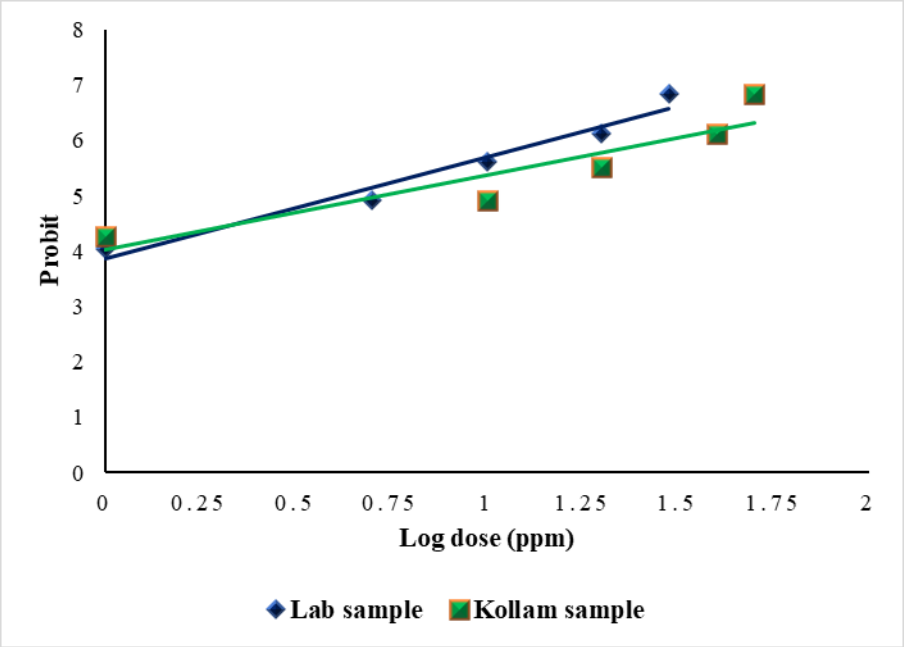

Fig. 14. Log dose – Probit line of fipronil

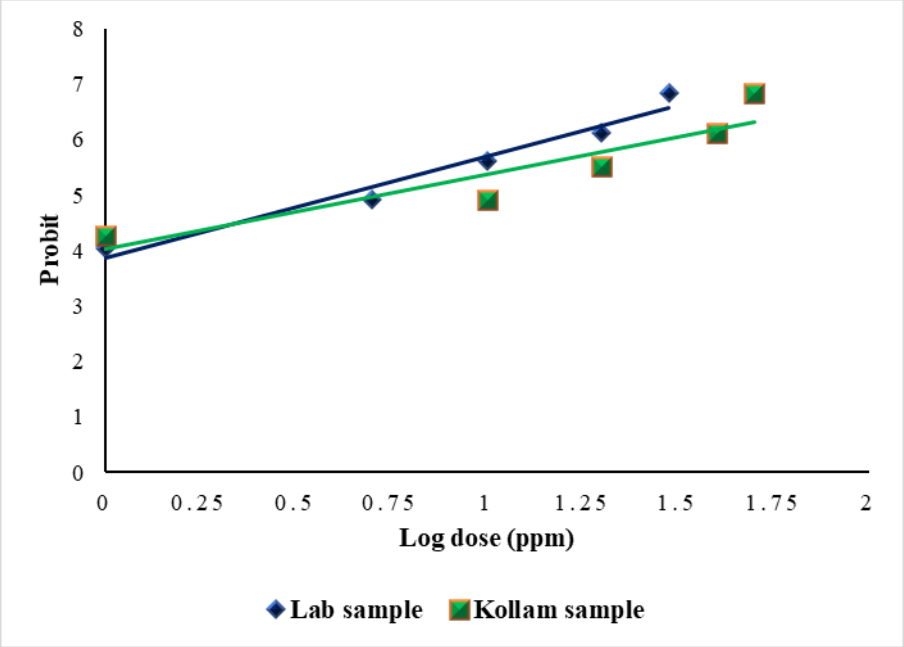

Fig. 15. Log dose – Probit line of indoxacarb

Indoxacarb was found to be 40.13-fold toxic to the resistant population of *S. oryzae* with respect to malathion. The overlapping ld-p lines of indoxacarb for resistant (Kollam) and lab samples indicate the similar susceptibility of the populations towards indoxacarb (Fig. 15).

Daglish and Nayak (2012) studied the efficacy of imidacloprid and indoxacarb in the control of stored grain beetles. Indoxacarb was found very effective against *R. dominica* and *S. oryzae*, but not against *T. confusum*. Indoxacarb was significantly more effective than imidacloprid against *S. oryzae* adults, causing substantial mortality ranging from 96.6 to 100 per cent in all doses ranging from 0 to 5 mg kg^{-1}. Indoxacarb caused cent per cent mortality of *S. oryzae* at all the test doses except at 0.2 mg kg^{-1} where mortality was 96.6 per cent. Progeny production of *S. oryzae* was significantly affected by indoxacarb, with a total suppression at 5 mg kg^{-1}.

According to Miliordos *et al*. (2017), indoxacarb was found to be effective as a grain protectant, but its effectiveness varied depending on the commodity and species. In their study, they reported that indoxacarb was effective against *R. dominica* and *S. oryzae*, but not against *T. confusum*. Similar results were also reported by Daglish and Nayak (2012). These studies corroborate the findings of the present investigation.

Chlorantraniliprole is an anthranilic diamide, and a green labelled new generation insecticide with low mammalian toxicity. It has been proven to be effective for the management of a wide variety of crop pests (Han *et al*., 2012; Su *et al.,* 2014; Lutz *et al*., 2018; Zhang *et al.,* 2020), but the data available on its efficacy for the management of stored grain pests is very limited.

The LC_{50} values obtained for chlorantraniliprole in the toxicity studies were much higher than fipronil, indoxacarb, and the FCI used insecticides *viz.,* malathion and deltamethrin, indicating its inefficiency in managing the pest. The parallel ld-p lines obtained for the FCI collected sample and the lab sample indicate their uniform susceptibility towards chlorantraniliprole (Fig. 16).

The stored product pest management by chlorantraniliprole varied depending on the pest, product, dose rate, and exposure period (Kavallieratos *et al*., 2013). Chlorantraniliprole proved to be less effective than deltamethrin for the management

of *Callosobruchus maculatus* (F.) (Babu *et al*., 2020) which is on par with the findings of the current study.

5.4 COMPARATIVE EVALUATION OF NEWER INSECTICIDES AGAINST *Sitophilus oryzae* INFESTED GRAINS IN JUTE BAGS

After the laboratory bioassays, the efficacy of fipronil, indoxacarb, chlorantraniliprole along with deltamethrin, and malathion were evaluated by spraying on jute bags containing *S. oryzae* infested rice grains. Ten times of LC_{50} value was the dose taken for newer insecticides and FCI recommended dose for malathion and deltamethrin.

The results of comparative evaluation revealed fipronil@ 0.006 % as the most effective among the tested insecticides, causing 86.5 per cent and 89.5 per cent mortality of *S. oryzae* at 24 and 48 h of treatment, respectively. Indoxacarb @ 0.09 % proved to be the next best treatment with 72 per cent and 76 per cent mortality at 24 and 48 h of treatment, respectively. Chlorantraniliprole @ 4.04 % showed 46.5 and 49.5 per cent mortality at 24 and 48 h which was lower than that of malathion @ 1% and deltamethrin @ 3.57 %. The efficacy of malathion and deltamethrin was on par at 24 and 48 h after spraying (Table 17).

Mortality depends on exposure interval in which higher mortality was recorded after 48 h of exposure than 24 h. As fipronil showed significantly higher mortality against resistant *S. oryzae* population than other insecticides, it can be considered as an alternative to malathion and deltamethrin.

5.5 ESTIMATION OF RESIDUES OF INSECTICIDES IN GRAIN SAMPLES

Residues are one of the important consequences of indiscriminate use of pesticides. These residues in food, which include active ingredients of pesticides, their metabolites, and breakdown products, have a potential detrimental effect on human health. Pesticide residue analysis is necessary, not only for ensuring human health protection but also for international trade and regulatory compliance. The most effective newer insecticide from the comparative evaluation was further evaluated for its persistence and degradation on rice grains along with deltamethrin and malathion.

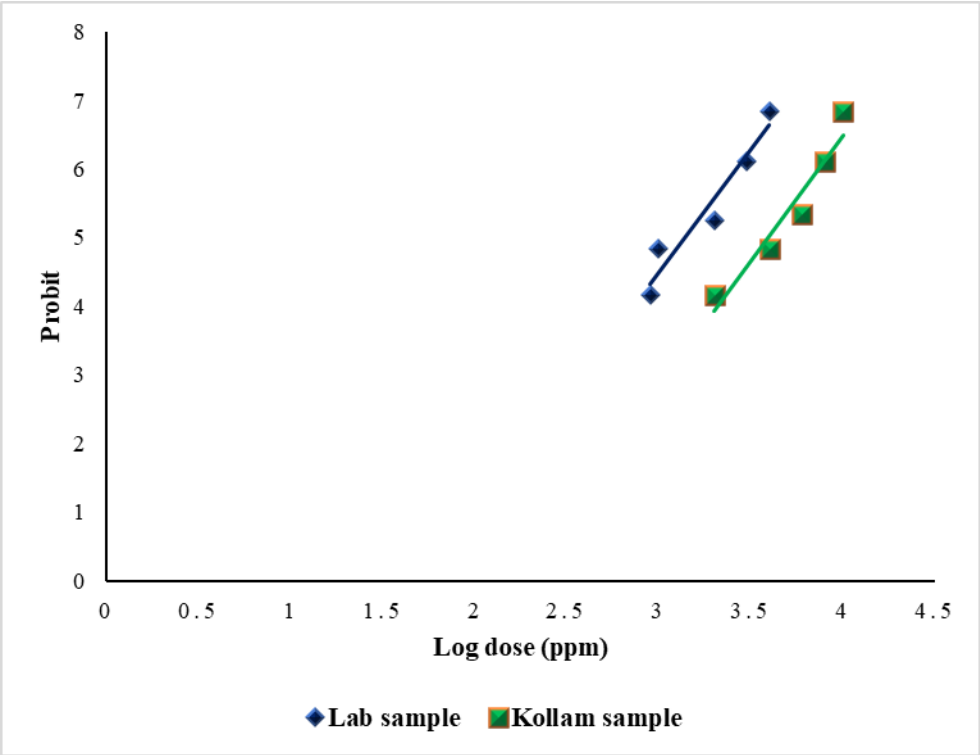

Fig.16. Log dose – Probit line of chlorantraniliprole

Analytical techniques for the detection and quantification of pesticide residues should be sensitive and validated to assess residue at very low quantities, thereby ensuring the quality of food materials (Tsochatzis *et al*., 2010). Prior to residue estimation in rice grains, recovery experiments were carried out to assess the efficiency of extraction and clean up procedures adopted and to standardise the procedure for residue estimation from rice. Calibration curves plotted for each insecticide for validating the analytical instruments showed good linearity (Fig. 4-6).

In the present study it was found that the residues of deltamethrin and malathion persisted up to 3 and 5 days after spraying and no residues of fipronil were detected on the first sampling interval itself, which is in contrast with the findings of Balinova *et al*. (2007). They used gas chromatography to determine deltamethrin residues in wheat. When applied at 0.5 mg kg^{-1}, residues were in the range of 0.03 to 0.2 mg kg^{-1}at 180 days after treatment. When applied at 4mg kg^{-1}, the residues persisted for up to 275 days. Storage conditions and pesticide treatments play a significant effect in determining the rate and amount of insecticide breakdown on the grain (Uygun *et al*., 2007). In the present study, spraying on the surface of jute bags containing rice grains, showed a lower persistence of residues on grains as the chances of direct contact between rice grains and insecticides were less. However, a smaller amount of residue was determined in rice grains in the case of malathion and deltamethrin during the initial days is probably due to the slight penetration of insecticides into the grain. The residue levels of all the tested insecticides were below the MRL (Maximum Residue Limit) fixed by the Food Safety and Standards Authority of India (FSSAI) at all the sampling intervals.

Summary

6. SUMMARY

The rice weevil, *Sitophilus oryzae* (L.), is an important primary stored grain pest that attacks undamaged grain seeds and is responsible for significant losses of stored grains. Chemical control is the most effective management measure for the stored grain pests in warehouses and godowns. However, the irrational and frequent use of insecticides eventually leads to the development of insecticide resistance, which makes it difficult to control the pest in the recommended dosages of insecticides. The present study includes the evaluation of the resistance levels in *S. oryzae* collected from the FCI godowns at three different locations in Kerala against the commonly used insecticides in FCI godowns *viz*., malathion and deltamethrin followed by its comparison with the laboratory sample procured from IARI, assessment of the biochemical basis of resistance in the most resistant population of *S. oryzae* along with the laboratory sample, screening of new molecules for the management of resistant population of *S. oryzae*, comparative evaluation of the selected new generation insecticides by simulating the FCI conditions, and study on the persistence of the most effective new generation insecticide along with the FCI recommended insecticides on grains.

The findings of the study are summarized here.

- Sample populations of *S. oryzae* collected from the three FCI godowns of Kerala located at Kollam, Valiyathura and Thikkodi were resistant to commonly used insecticides in FCI godowns *viz*., malathion and deltamethrin. The Kollam population showed the highest resistance to both malathion and deltamethrin with resistance ratios of 14.94 and 9.03, respectively, followed by Valiyathura with resistance ratios of 11.39 and 7.79, respectively. The Thikkodi population showed the least resistance to malathion and deltamethrin, with resistance ratios of 8.74 and 5.48, respectively.

- The resistance development was more towards malathion than deltamethrin.

- Biochemical assays of the most resistant Kollam sample and the susceptible IARI lab culture revealed that the Kollam sample population have significantly higher total protein (6 mg ml^{-1}) and activity of detoxifying enzymes *viz*., carboxylesterases (0.65µmol min^{-1} mg $protein^{-1}$), glutathione S-transferases

(0.41µmol min^{-1} mg $protein^{-1}$) and cytochrome P450 (0.74 p mol min^{-1}mg $protein^{-1}$) than the lab sample.

- Toxicity bioassays of newer insecticide molecules on the resistant S. *oryzae* sample *i.e.,* the Kollam sample population revealed that fipronil was extremely toxic with an LC_{50} value of 5.86 ppm, followed by indoxacarb (90.57 ppm). When compared to malathion, fipronil and indoxacarb were 619.8 and 40.13-fold toxic to resistant population of *S. oryzae*.

- Among the newer insecticides, chlorantraniliprole recorded the highest LC_{50}.

- When the newer and FCI recommended insecticides were sprayed on gunny bags for a comparative evaluation, fipronil 5% SC@ 0.006% recorded significantly higher mortality of 86.5 and 89.5 per cent at 24 and 48 h of treatment followed by indoxacarb 14.5% SC@ 0.09%. Chlorantraniliprole 18.5% SC@ 4.04% recorded significantly lower mortality compared to deltamethrin 2.8 % EC@ 3.57% and malathion 50% EC@ 1% at both the observation intervals.

- The persistence of fipronil along with malathion and deltamethrin, on rice grains was estimated using a validated method. The analytical method used for the estimation of residues gave good per cent recovery for malathion residues ranging from 72-102.33 per cent with a relative standard deviation of 2.78-5.07 per cent when fortified at 0.05, 0.1, and 0.25 mg kg^{-1}. At the same levels of fortification, the average recovery of deltamethrin was in the range of 83.33 to 92.93 per cent. The selected method gives 90-100.67 per cent recovery for fipronil residues at 0.01, 0.02 and 0.05 mg kg^{-1} levels of fortification. Good linearity for the calibration curve was obtained within the range of 0.01-1 mg kg^{-1}.

- Fipronil 5 % SC @ 0.006% residues were below the limit of quantification at 2 h after spraying. The residues of deltamethrin 2.8% EC@ 3.57% and malathion 50% EC@ 1% persisted up to 3 and 5 days after spraying, with a half-life of 2.48 and 2.71 days, respectively.

- The present study confirmed the resistance development in *S. oryzae* against commonly used insecticides in the FCI godowns of India, *viz.,* malathion and deltamethrin and it could be due to the presence of higher levels of detoxifying enzymes in the resistant population. Fipronil 5% SC @ 0.006% is highly effective and less persistent compared to malathion and deltamethrin for the management of *S. oryzae*.

- The continuous use of malathion and deltamethrin in FCI godowns will necessitate a higher dosage and more frequent applications of these chemicals for the management of *S. oryzae*, which in turn leads to the deposition of pesticide residues. Rotation of insecticides with different modes of action is an effective IRM (Insecticide Resistance Management) strategy to combat insecticide resistance. As *S. oryzae* has developed resistance to OPs and SPs, trials with newer insecticide molecules with different modes of action must be carried out for the management of *S. oryzae*, so as to obtain a new insecticide recommendation to ensure proper chemical control in FCI. Similar studies can be done on other stored product pests for their successful management.

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7. REFERENCES

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Appendices

APPENDIX- I

Per cent mortality of *Sitophilus oryzae* in bioassay with malathion

Dose(ppm)	Per cent mortality			
	Kollam	Valiyathura	Thikkodi	IARI lab culture
80	-	-	-	20
200	-	-	-	40
500	-	-	-	60
600	-	-	-	76.67
700	-	-	-	96.67
1000	13.33	13.33	23.33	-
2000	-	36.67	-	-
3000	36.67	-	53.33	-
4000	-	56.67	76.67	-
5000	46.67	-	86.67	-
6000	-	76.67	-	-
7000	76.67	-	100	-
8000	-	100	-	-
9000	96.67	-	-	-

APPENDIX- II

Per cent mortality of *Sitophilus oryzae* in bioassay with deltamethrin

Dose(ppm)	Per cent mortality			
	Kollam	Valiyathura	Thikkodi	IARI lab culture
40	-	-	-	23.33
100	-	-	-	53.33
300	-	-	16.67	70
400	-	-	-	80
500	-	-	-	96.67
600	-	13.33	53.33	-
700	-	40	-	-
800	20	-	-	-
900	50	60	70	-
1000	73.33	80	86.67	-
2000	83.33	96.67	100	-
3000	100	-	-	-

APPENDIX- III

Per cent mortality of *Sitophilus oryzae* in bioassay with fipronil

Dose (ppm)	Per cent mortality	
	Kollam	IARI lab culture
1	23.33	16.67
5	-	46.67
10	46.67	73.33
20	70	86.67
30	-	96.67
40	86.67	-
50	96.67	-

APPENDIX- IV

Per cent mortality of *Sitophilus oryzae* in bioassay with indoxacarb

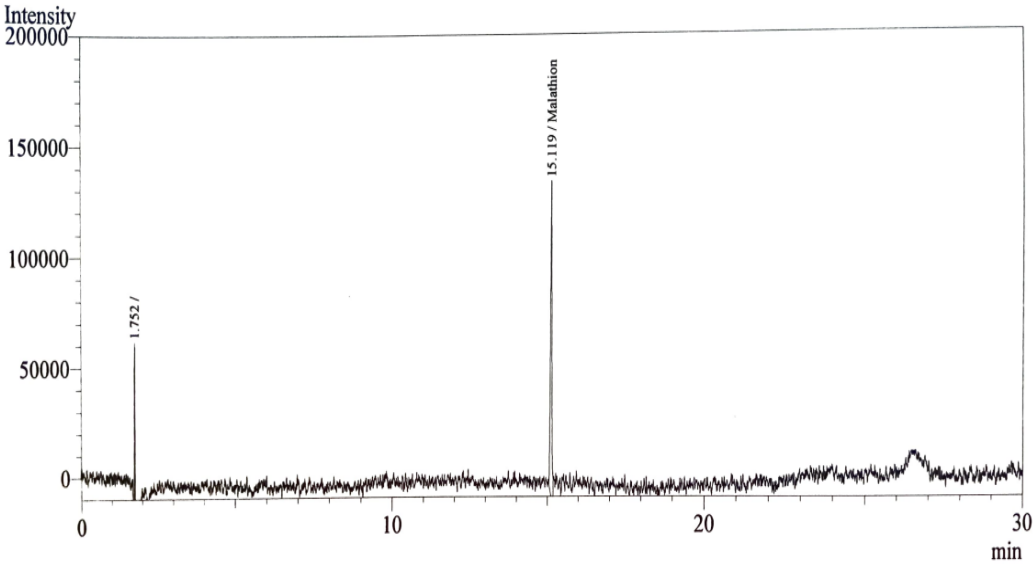
Dose (ppm)	Per cent mortality	
	Kollam	IARI lab culture
30	-	20
50	23.33	-
80	-	50
100	50	60
150	70	73.33
180	80	100
200	100	-

APPENDIX- V

Per cent mortality of *Sitophilus oryzae* in bioassay with chlorantraniliprole

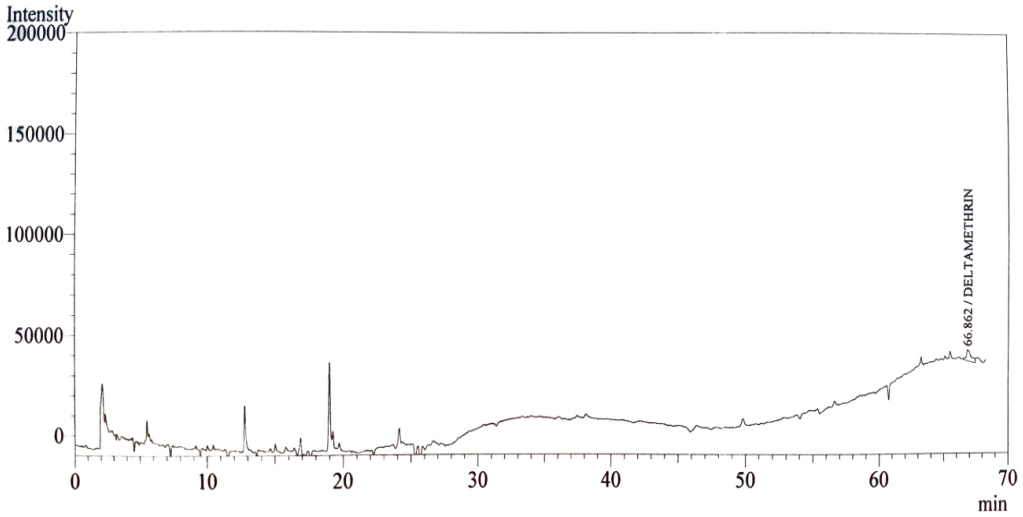
Dose (ppm)	Per cent mortality	
	Kollam	IARI lab culture
900	-	20
1000	-	43.33
2000	20	60
3000	-	86.67
4000	43.33	100
6000	63.33	-
8000	86.67	-
10000	100	-

APPENDIX- VI



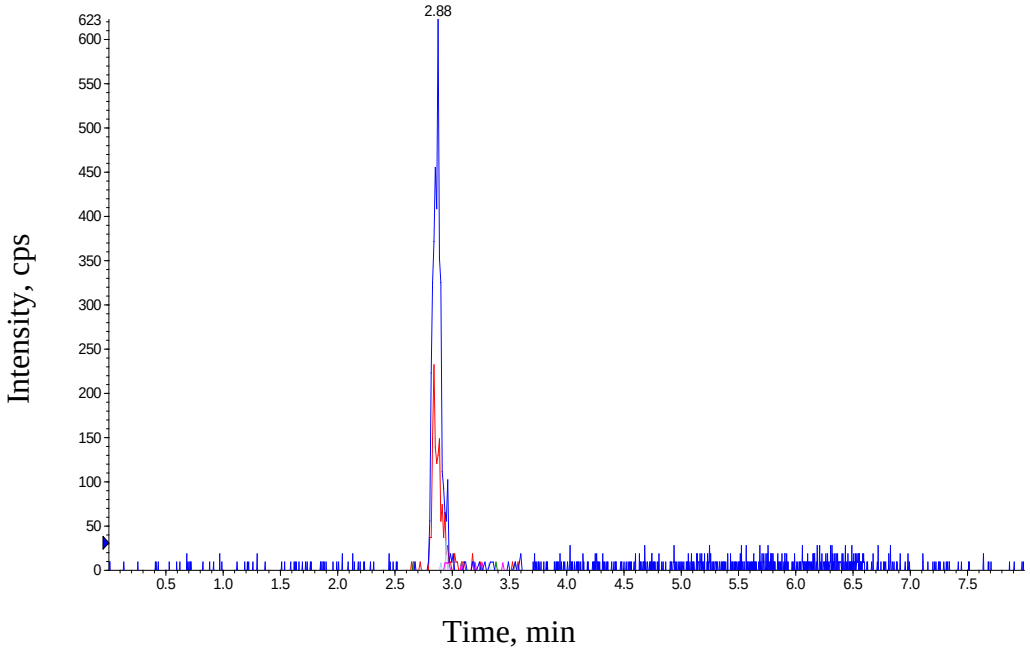
Chromatogram of malathion

APPENDIX-VII



Chromatogram of deltamethrin

APPENDIX-VIII



Chromatogram of fipronil

INSECTICIDE RESISTANCE MANAGEMENT IN RICE WEEVIL, *Sitophilus oryzae* (L.) (COLEOPTERA: CURCULIONIDAE)

by

NEETHU P.

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ABSTRACT

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Kerala Agricultural University

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COLLEGE OF AGRICULTURE

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Abstract

The research work entitled "Insecticide resistance management in rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae)" was done at College of Agriculture, Vellayani during 2019 to 2021.The objectives of the study were to assess the resistance levels in rice weevil, biochemical basis of resistance and screening of new molecules for the management of *S. oryzae.*

Different sample populations of *S. oryzae* were collected from the three godowns of Food Corporation of India (FCI) *viz.*, Kollam, Valiyathura, and Thikkodi, as well as a susceptible lab culture of *S. oryzae* maintained without pesticide exposure, was obtained from the Division of Entomology, Indian Agricultural Research Institute (IARI). Resistance levels of the commonly used insecticides in FCI *viz*., malathion and deltamethrin were evaluated in the three populations collected from Kollam, Valiyathura and Thikkodi by film method of bioassay and compared it with the susceptible lab population from IARI. Kollam population showed highest resistance to both malathion and deltamethrin with resistance ratios of 14.94 and 9.03 followed by Valiyathura with resistance ratios of 11.39 and 7.79, respectively. Thikkodi population showed the least resistance to malathion and deltamethrin, with resistance ratios of 8.74 and 5.48, respectively. Malathion resistance was higher in all the three populations than the deltamethrin resistance.

The most resistant population selected from the previous experiment *i.e.,* the Kollam population was further assayed for its biochemical basis along with the susceptible IARI lab culture. The Kollam population was found significantly higher in total protein (6 mg ml^{-1}) and activity of detoxifying enzymes *viz.*, carboxylesterases (0.65 µmol min^{-1} mg $protein^{-1}$), glutathioneS-transferases (0.41 µmol min^{-1} mg $protein^{-1}$) and cytochrome P450 (0.74 p mol min^{-1}mg $protein^{-1}$) than the lab sample.

The sample population collected from Kollam was again screened for their susceptibility to new generation insecticides like fipronil, indoxacarb and chlorantraniliprole by film method of bioassay. Fipronil was found to be more toxic with LC_{50} value of 5.86 ppm followed by indoxacarb (90.57 ppm) and chlorantraniliprole

(4041.43 ppm). Fipronil and indoxacarb were 619.8- and 40.13-fold toxic to resistant population of *S. oryzae* with respect to malathion.

The newer insecticides in the previous experiment along with malathion and deltamethrin were further screened simulating the conditions of FCI godown. The insecticides were sprayed on jute bags containing 1 kg of rice grains and 50 adult beetles of Kollam population of *S. oryzae*. The dosages of newer insecticides were taken 10 times more the LC_{50} value obtained in the laboratory bioassay and FCI recommended dosage was taken for malathion and deltamethrin. Fipronil 5% SC @ 0.006% recorded significantly higher mortality of 86.5 and 89.5 per cent at 24 and 48 h of treatment followed by indoxacarb 14.5% SC @ 0.09% and chlorantraniliprole 18.5% SC @ 4.04%.

The persistence of the most effective insecticide fipronil along with malathion and deltamethrin on rice grains were further studied by recording the residue levels at different time intervals after spraying. Dosages were same as the previous experiment. Insecticides were sprayed on jute bags containing rice grains and residues were analysed at 0 (2 h after spray), 1, 3, 5, 7,10, 15, 20 and 30days intervals using the methods validated prior to residue estimation. Fipronil residues were below the limit of quantification at 2 h after spraying. The residues of deltamethrin and malathion persisted up to 3 and 5 days after spraying with a half-life of 2.48 and 2.71 days, respectively.

From the present study it is revealed that *S. oryzae* collected from Kollam, Valiyathura and Thikkodi were resistant to the commonly used insecticides *viz*., malathion and deltamethrin and it is confirmed here by the presence of higher levels of detoxifying enzymes in the resistant population. Further screening of newer insecticide molecules against the resistant population of *S. oryzae* suggested that fipronil 5% SC @ 0.006% is highly effective and less persistent on grains when compared to malathion and deltamethrin.

സംഗ്രഹം

"അരിച്ചെള്ളിലെ (സിറ്റോഫിലസ് ഒറൈസേ, കോളിയോപ്ടെറ: കുർക്കുലിയോനിടെ) കീടനാശിനി പ്രതിരോധ നിയന്ത്രണം" എന്ന പഠനം വെള്ളായണി കാർഷിക കോളേജിലെ കീടശാസ്ത്ര വിഭാഗത്തിൽ 2019-2021 കാലയളവിൽ നടത്തുകയുണ്ടായി. അരിച്ചെള്ളിലെ കീടനാശിനി പ്രതിരോധത്തിന്റെ അളവ്, പ്രതിരോധത്തിന്റെ ബയോകെമിക്കൽ അടിസ്ഥാനം, അരിച്ചെള്ളിന്റെ നിയന്ത്രണത്തിനായി പുതിയ കീടനാശിനി തന്മാത്രകളുടെ സ്ക്രീനിംഗ് എന്നിവ വിലയിരുത്തുക എന്നിവയായിരുന്നു ഗവേഷണത്തിന്റെ പ്രധാന ലക്ഷ്യങ്ങൾ.

ഫുഡ് കോർപറേഷൻ ഓഫ് ഇന്ത്യയുടെ (എഫ്സിഐ) കൊല്ലം, വലിയതുറ, തിക്കോടി എന്നീ മൂന്ന് ഗോഡൗണുകളിൽ നിന്നും ഇന്ത്യൻ അഗ്രികൾച്ചറൽ റിസർച്ച് ഇൻസ്റ്റിട്യൂട്ടിൽ (ഐഎആർഐ) നിന്നുമാണ് ഗവേഷണത്തിനാവശ്യമായ അരിച്ചെള്ള് ശേഖരിച്ചത്. എഫ്സിഐയിൽ നിന്നും ശേഖരിച്ച അരിച്ചെള്ളിൽ എഫ്സിഐയിൽ സാധാരണയായി ഉപയോഗിക്കുന്ന മാലത്തിയോൺ, ഡെൽറ്റാമെത്രിൻ എന്നീ കീടനാശിനികൾക്കെതിരെയുള്ള പ്രതിരോധശേഷി ഫിലിം ബയോഎസ്സെയിലൂടെ കണ്ടുപിടിച്ച് ഐഎആർഐയിൽ നിന്നും ശേഖരിച്ച കീടനാശിനി സമ്പർക്കമില്ലാതെ പരിപാലിച്ചിരുന്ന അരിച്ചെള്ളിന്റെ പ്രതിരോധശേഷിയുമായി താരതമ്യം ചെയ്തു. മാലത്തിയോൺ, ഡെൽറ്റാമെത്രിൻ എന്നീ കീടനാശിനികൾക്ക് എതിരിരെയുള്ള അരിച്ചെള്ളിന്റെ പ്രതിരോധത്തിന്റെ അനുപാതം ശേഖരിച്ച സ്ഥലത്തിന്റെ അടിസ്ഥാനത്തിൽ യഥാക്രമം കൊല്ലം (14.94, 9.03), വലിയതുറ (11.39, 7.79), തിക്കോടി (8.74, 5.48) എന്നിങ്ങനെ ആണെന്ന് കണ്ടെത്തി.

മുൻ പരീക്ഷണത്തിൽ നിന്ന് തിരഞ്ഞെടുത്ത കീടനാശിനി പ്രതിരോധശേഷി കൂടുതലുള്ള കൊല്ലം എഫ്സിഐ സാമ്പിളുകളിൽ ഐഎആർഐ സാമ്പിളുകളേക്കാൾ കൂടുതൽ അളവിൽ പ്രോട്ടീൻ, കീടനാശിനികളുടെ വിഷാംശം ഇല്ലാതാക്കുന്ന എൻസൈമുകളായ, സൈറ്റോക്രോം പി450, കാർബോക്സിലെസ്റ്ററേസ്, ഗ്ലൂട്ടാത്തയോൺ എസ്-ട്രാൻസ്ഫെറേസ് എന്നിവ കണ്ടെത്തി.

ലബോറട്ടറിയിൽ സ്ക്രീൻ ചെയ്ത പ്രകാരം പുതിയ കീടനാശിനികളായ ഫിപ്രോനിൽ, ഇൻഡോക്സാകാർബ് എന്നിവയ്ക്കു അരിച്ചെള്ളിന്റെ നിയന്ത്രണത്തിനായി മാലത്തിയോണിനെക്കാൾ യഥാക്രമം 619.8, 40.13 മടങ്ങു കൂടുതൽ വിഷവീര്യം ഉള്ളതായി കണ്ടെത്തി.

മേൽപറഞ്ഞ പരീക്ഷണത്തിൽ നിന്നും പുതിയ കീടനാശിനികളിൽ ഏറ്റവും ഫലപ്രദമാണെന്നു കണ്ടെത്തിയ ഫിപ്രോനിലിന്റേയും എഫ്സിഐയിൽ ഉപയോഗിക്കുന്ന മാലത്തിയോൺ, ഡെൽറ്റാമെത്രിൻ എന്നീ കീടനാശിനികളുടെയും അരിയിലെ വിഷ അവശിഷ്ട പരീക്ഷണങ്ങളും ഈ പഠനത്തിന്റെ ഭാഗമായി നടത്തി. അതിൻപ്രകാരം മേൽപറഞ്ഞ കീടനാശിനികൾ അരിനിറച്ച ചാക്കുകളിൽ തളിച്ച് വ്യത്യസ്ത ഇടവേളകളിലായി അരിയിലെ കീടനാശിനികളുടെ അവശിഷ്ടങ്ങൾ ലബോറട്ടറിയിൽ പരിശോധിച്ചു. തളിച്ചതിന് ശേഷം രണ്ടു മണിക്കൂറിനുള്ളിൽ ഫിപ്രോണിൽ അവശിഷ്ടങ്ങളുടെ അളവ് പരിധിയ്ക്കു (ലിമിറ്റ് ഓഫ് ഡിറ്റക്ഷൻ) താഴെയായിരുന്നു. ഡെൽറ്റാമെത്രിൻ, മാലത്തിയോൺ എന്നിവയുടെ അവശിഷ്ടങ്ങൾ യഥാക്രമം 2.48, 2.71 ദിവസങ്ങൾ അർദ്ധയുസ്സോടുകൂടി 3, 5 ദിവസങ്ങൾ വരെ കാണപ്പെട്ടു.