EVALUATION OF INSECTICIDE RESISTANCE PROFILE IN *Spodoptera litura* (FABRICIUS) POPULATIONS THROUGH BIOLOGICAL, BIOCHEMICAL AND MOLECULAR DIAGNOSIS

Dissertation

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
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in
ENTOMOLOGY
(Minor Subject: Biochemistry)

By

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(L-2009-A-13-D)

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CERTIFICATE I

This is to certify that the dissertation entitled, **"Evaluation of insecticide resistance profile in *Spodoptera litura* (Fabricius) populations through biological, biochemical and molecular diagnosis"** submitted for the degree of **Ph.D.**, in the subject of **Entomology** (Minor subject: **Biochemistry**) of the Punjab Agricultural University, Ludhiana is a bonafide research work carried out by **Harpreet Kaur Cheema (L-2009-A-13-D)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that the dissertation entitled, **"Evaluation of insecticide resistance profile in *Spodoptera litura* (Fabricius) populations through biological, biochemical and molecular diagnosis"** submitted by **Harpreet Kaur Cheema (L-2009-A-13-D)** to the Punjab Agricultural University, Ludhiana in partial fulfillment of the requirements for the degree of **Ph.D.** in the subject of **Entomology (**Minor Subject**: Biochemistry)** has been approved by the Student's Advisory committee after an oral examination on the same.

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ABSTRACT

Evaluation of insecticide resistance profile in *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) populations was done through biological, biochemical and molecular diagnosis. Population from Sangrur was found to be the least susceptible for most of the insecticides, except for chlorpyriphos, chlorantraniliprole and novaluron which were least toxic to population from Bathinda, and pyridalyl which was least toxic to population from Ludhiana. Population from Hoshiarpur was found to be the most susceptible for all insecticides except acephate and pyridalyl to which Bathinda population was most susceptible. Thiodicarb among the conventional group of insecticides and chlorantraniliprole among the newer insecticides were found to be the most toxic against all the test populations. The synergistic effect of TPP (6.16- fold), PBO (3.72- fold) and DEM (1.73- fold) in the least susceptible population suggested the involvement of esterases and mixed function oxidases in providing resistance to various insecticides. Synergistic effect of PBO (6.21- fold) on the fenvalerate- selected population followed by TPP (5.74- fold) and DEM (1.89- fold) indicated the possible role of mixed function oxidases, esterases and to some extent glutathione S- transferases in imparting resistance to pyrethroids in *S. litura*. Multiple detoxification enzymes and AChE insensitivity were found to be involved in imparting resistance in *S. litura* towards various insecticides. Selection with fenvalerate increased the activities of MFOs, esterases and GST by 1.84, 1.73 and 1.3 times in *S. litura* proving their role in pyrethroid-mediated resistance. Activities of MFO, esterases and insensitivity of AChE can be used as biochemical tools for monitoring insecticide resistance in *S. litura*. RAPD markers established the presence of inter and intra population variations that might be related to insecticide resistance. Alignment between COI sequences of fenvalerate- selected (SGR-Sel) and susceptible types (SGR) identified existence of a single base/ nucleotide polymorphism at position 421 represented by substitution of T in SGR-Sel with C in SGR type. Based on this polymorphism, COI based specific primers were developed, tested and validated for differentiating amongst these *S. litura* types.

KEY WORDS: *Spodoptera litura*, insecticide resistance, biochemical, detoxification enzymes, molecular

Signature of Major Advisor

Signature of the Student

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ਸਾਰ-ਅੰਸ਼

ਸਪੋਡਾਪਟਰਾ ਲਿਟੂਰਾ (ਫਾਬਰੀਸ਼ਿਅਸ) (ਲੈਪੀਡਾਪਟਰਾ: ਨਾਕਟੂਅੀਡੀ) ਸੰਖਿਆਵਾਂ ਵਿੱਚ ਕੀਟਨਾਸ਼ਕ ਪ੍ਰਤੀਰੋਧਕ ਪਰੋਫ਼ਾਈਲ ਦਾ ਮੁਲਾਂਕਣ ਜੀਵ-ਵਿਗਿਆਨਕ, ਜੀਵ-ਰਸਾਇਣਕ ਅਤੇ ਆਣਵਿਕ ਤਸ਼ਖ਼ੀਸ ਰਾਹੀਂ ਕੀਤਾ ਗਿਆ। ਸੰਗਰੂਰ ਦੀ ਅਬਾਦੀ ਤਕਰੀਬਨ ਸਾਰੇ ਕੀਟਨਾਸ਼ਕਾਂ ਲਈ ਘੱਟ ਤੋਂ ਘੱਟ ਪ੍ਰਭਾਵ-ਗ੍ਰਹੀ ਪਾਈ ਗਈ; ਸਿਵਾਇ ਕਲੋਰਪਾਰੀਫ਼ਾਸ; ਕਲੋਰਐਂਟ੍ਰਾਨੀਲੀਪ੍ਰੋਲ ਅਤੇ ਨੋਵਾਲੂਰਾਨ ਜੋ ਕੇ ਬਠਿੰਡੇ ਦੀ ਅਬਾਦੀ ਨੂੰ ਘੱਟ ਤੋਂ ਘੱਟ ਜ਼ਹਿਰੀਲੇ ਸਨ ਅਤੇ ਪੀਰੀਡਲਾਈਲ ਜੋ ਕੇ ਲੁਧਿਆਣੇ ਦੀ ਅਬਾਦੀ ਨੂੰ ਘੱਟ ਜ਼ਹਿਰੀਲੀ ਸੀ। ਸਾਰੇ ਕੀਟਨਾਸ਼ਕਾਂ ਲਈ ਹੁਸ਼ਿਆਰਪੁਰ ਦੀ ਅਬਾਦੀ ਵੱਧ ਤੋਂ ਵੱਧ ਪ੍ਰਭਾਵ-ਗ੍ਰਹੀ (ਸੰਵੇਦਨਸ਼ੀਲ) ਪਾਈ ਗਈ ਸਿਵਾਇ ਐਸੀਫ਼ੇਟ ਅਤੇ ਪੀਰੀਡਲਾਈਲ ਲਈ ਜਿਹਨਾਂ ਨੂੰ ਬਠਿੰਡੇ ਦੀ ਅਬਾਦੀ ਵੱਧ ਤੋਂ ਵੱਧ ਪ੍ਰਭਾਵ-ਗ੍ਰਹੀ ਸੀ।ਸਾਰੀਆਂ ਜਾਂਚ ਕੀਤੀਆਂ ਅਬਾਦੀਆਂ ਲਈ ਸਾਧਾਰਣ ਸ਼੍ਰੇਣੀ ਵਰਗ ਦੇ ਕੀਟਨਾਸ਼ਕਾਂ ਵਿੱਚੋਂ ਥਾਇਓਡੀਕਾਰਬ ਅਤੇ ਨਵੇਂ ਕੀਟਨਾਸ਼ਕਾਂ ਵਿੱਚੋਂ ਕਲੋਰਐਂਟ੍ਰਾਨੀਲੀਪਰੋਲ ਸਭ ਤੋਂ ਵੱਧ ਜ਼ਹਿਰੀਲੇ ਪਾਏ ਗਏ। ਘੱਟ ਤੋਂ ਘੱਟ ਪ੍ਰਭਾਵ-ਗ੍ਰਹੀ ਅਬਾਦੀ ਵਿੱਚ ਟੀ ਪੀ ਪੀ, ਪੀ ਬੀ ਓ ਅਤੇ ਡੀ ਈ ਐਮ ਦੇ ਯੁਕਤ-ਪ੍ਰਭਾਵੀ ਅਸਰ (ਕ੍ਰਮਵਾਰ 6.16, 3.72 ਅਤੇ 1.73- ਗੁਣਾ) ਤੋਂ ਐਸਟਰੇਜ਼ ਅਤੇ ਮਿਸ਼ਰਤ-ਕਾਰਜ ਔਕਸੀਡੇਜ਼ (ਐਮ ਐਫ਼ ਓ) ਦਾ ਵੱਖ-ਵੱਖ ਕੀਟਨਾਸ਼ਕਾਂ ਨੂੰ ਪ੍ਰਤੀਰੋਧਕਤਾ ਪ੍ਰਧਾਨ ਕਰਨ ਵਿੱਚ ਰੋਲ ਹੋਣ ਦਾ ਸੰਕੇਤ ਮਿਲਦਾ ਹੈ। ਫ਼ੈਨਵਲਰੇਟ-ਚੋਣਵੀਂ ਅਬਾਦੀ ਵਿੱਚ ਪੀ ਬੀ ਓ, ਟੀ ਪੀ ਪੀ ਅਤੇ ਡੀ ਈ ਐਮ ਦਾ ਯੁਕਤ-ਪ੍ਰਭਾਵੀ ਅਸਰ ਦਰਸਾਉਂਦਾ ਹੈ ਕਿ ਮਿਸ਼ਰਤ-ਕਾਰਜ ਔਕਸੀਡੇਜ਼, ਐਸਟਰੇਜ਼ ਅਤੇ ਕੁਝ ਹੱਦ ਤੱਕ ਗਲੂਟਾਥੀਆਨ ਐਸ-ਟਰਾਂਸਫ਼ਰੇਜ਼ (ਜੀ ਐਸ ਟੀ) ਦਾ *ਐਸ. ਲਿਟੂਰਾ* ਵਿੱਚ ਪਾਈਰੀਥਰਾਇਡ ਕੀਟਨਾਸ਼ਕਾਂ ਨੂੰ ਪ੍ਰਤੀਰੋਧਕਾ ਪ੍ਰਧਾਨ ਕਰਨ ਵਿੱਚ ਸੰਭਾਵੀ ਰੋਲ ਹੋ ਸਕਦਾ ਹੈ। *ਐਸ. ਲਿਟੂਰਾ* ਵਿੱਚ ਕਈ ਕੀਟਨਾਸ਼ਕਾਂ ਨੂੰ ਪ੍ਰਤੀਰੋਧਕਤਾ ਪ੍ਰਧਾਨ ਕਰਨ ਵਿੱਚ ਵਿਭਿੰਨ ਜ਼ਹਿਰੀਲਾਪਨ- ਘਟਾਉਣ ਵਾਲੇ ਐਨਜ਼ਾਈਮਾਂ ਅਤੇ ਏ ਸੀ ਐਚ ਈ ਦੀ ਅਸੰਵੇਦਨਸ਼ੀਲਤਾ ਦਾ ਰੋਲ ਹੈ। ਫ਼ੈਨਵਲਰੇਟ ਨਾਲ ਚੋਣ ਕਰਨ ਨਾਲ *ਐਸ. ਲਿਟੂਰਾ* ਵਿੱਚ ਐਮ ਐਫ ਓ, ਐਸਟਰੇਜ਼ ਅਤੇ ਜੀ ਐਸ ਟੀ ਦੀਆਂ ਕ੍ਰਿਆਵਾਂ ਦਾ 1.84, 1.73 ਅਤੇ 13- ਗੁਣਾ ਵਾਧਾ ਪਾਈਰੀਥਰਾਇਡ ਸੰਬਧਿਤ ਪ੍ਰਤੀਰੋਧਕਤਾ ਵਿੱਚ ਰੋਲ ਨੂੰ ਸਾਬਤ ਕਰਦਾ ਹੈ। ਐਮ ਐਫ ਓ ਅਤੇ ਐਸਟਰੇਜ਼ ਦੀਆਂ ਕ੍ਰਿਆਵਾਂ ਅਤੇ ਏ ਸੀ ਐਚ ਈ ਦੀ ਅਸੰਵੇਦਨਸ਼ੀਲਤਾ ਨੂੰ *ਐਸ. ਲਿਟੂਰਾ* ਵਿੱਚ ਕੀਟਨਾਸ਼ਕ-ਪ੍ਰਤੀਰੋਧਕਾ ਦਾ ਨਿਰੀਖਣ ਕਰਨ ਲਈ ਜੀਵ-ਰਸਾਇਣਕ ਜ਼ਰੀਏ ਦੇ ਤੌਰ ਦੇ ਵਰਤੋਂ ਕੀਤੀ ਜਾ ਸਕਦੀ ਹੈ। ਆਰ ਏ ਪੀ ਡੀ ਮਾਰਕਰਾਂ ਨੇ ਅੰਤਰ ਅਤੇ ਅੰਦਰ-ਅਬਾਦੀ ਵਿਭਿੰਨਤਾਵਾਂ ਨੂੰ ਸਥਾਪਿਤ ਕੀਤਾ ਜੋ ਕੇ ਕੀਟਨਾਸ਼ਕ ਪ੍ਰਤੀਰੋਧਕਤਾ ਨਾਲ ਸੰਬੰਧਿਤ ਹੋ ਸਕਦੀਆਂ ਹਨ। ਫ਼ੈਨਵਲਰੇਟ-ਚੁਣੀ ਅਤੇ ਸੰਵੇਦਨਸ਼ੀਲ ਕਿਸਮਾਂ ਦੇ ਸੀ ਓ 1 ਸੀਕੁਐਂਸ ਦੀ ਕਤਾਰਬੰਦੀ ਕਰਨ ਤੇ 421 ਸਥਾਨ ਤੇ ਇਕ ਬੇਸ/ਨਿਊਕਲੀਓਟਾਈਡ ਅਨੇਕਰੂਪਤਾ ਦੀ ਹੋਂਦ ਦਾ ਪਤਾ ਲਗਦਾ ਹੈ ਜੋ ਕੇ ਐਸਜੀਆਰ- ਸੈਲ ਦੇ 'ਟੀ' ਦੀ ਥਾਂ ਐਸਜੀਆਰ ਦੇ 'ਸੀ' ਹੋਣ ਨਾਲ ਜ਼ਾਹਿਰ ਹੁੰਦੀ ਹੈ। ਇਸ ਅਨੇਕਰੂਪਤਾ ਦੇ ਅਧਾਰ ਤੇ ਸੀ ਓ 1 ਅਧਾਰਿਤ ਵਿਲੱਖਣ ਪਰਾਈਮਰ ਬਣਾ ਕੇ ਟੈਸਟ ਅਤੇ ਪ੍ਰਮਾਣਿਤ ਕੀਤੇ ਗਏ ਜੋ ਕੇ *ਐਸ. ਲਿਟੂਰਾ* ਦੀਆਂ ਇਹਨਾਂ ਕਿਸਮਾਂ ਵਿੱਚ ਭੇਦ ਦੱਸ ਸਕਣ।

ਮੁੱਖ ਸ਼ਬਦ : *ਸਪੋਡਾਪਟਰਾ ਲਿਟੂਰਾ*, ਕੀਟਨਾਸ਼ਕ ਪ੍ਰਤੀਰੋਧਕਤਾ, ਜੀਵਰਸਾਇਣਕ, ਆਣਵਿਕ, ਐਨਜ਼ਾਈਮ

ਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖ਼ਰ

ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖ਼ਰ

CONTENTS

CHAPTER I

INTRODUCTION

Tobacco caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is an important sporadic, polyphagous and potentially destructive insect pest causing heavy foliage damage to more than 115 species of plants including pulses, cotton, cabbage, cauliflower, castor, groundnut, tuber and oilseed crops (Atwal and Dhaliwal 2009). It is emerging as a dominant pest on mungbean, urdbean and soybean in India and Bangladesh (Ahmed 2009, Cheema *et al* 2009, Patro *et al* 2009). The larval incidence of 27.36 /m^2 and an avoidable loss of 38.51per cent were recorded in soybean in Punjab (Taggar *et al* 2007). Recently, the pest outbreak was reported on soybean in Rajasthan and Maharashtra causing losses of several hundred crore rupees (Dhaliwal and Koul 2010). Krishnaiah *et al* (1983) recorded up to 70 per cent avoidable yield loss in urdbean. It was also found severely damaging cauliflower, cabbage between Aug- Nov (Battu *et al* 1998) and cotton (Arora *et al* 2007). The pest usually appears on summer mungbean during May-June and on *kharif* pulses (soybean, mungbean and urdbean crops) during August-September and continues up to maturity.

The presence of this pest on different crops throughout the year has widely exposed it to insecticides that resulted in rapid development of resistance. It was the first Lepidopteran pest and second agricultural pest reported to have developed resistance in India (Srivastava and Joshi, 1965). *Spodoptera* sp. ranks among the top 20 most resistant insect species (Whalon *et al* 2008). High resistance to various insecticides including organochlorines, organophosphates, carbamates and pyrethroids has been reported by various authors in India, Pakistan, Japan and China (Asakawa 1975, Murugesan and Dhingra 1995, Wu *et al* 1995, Armes *et al* 1997, Kranthi *et al* 2002, Huang and Han 2007 Ahmad *et al* 2008).

Several insecticides (including those with new chemistries) have been recommended for the control of this pest in many crops including cotton, pulses and vegetables (Anonymous 2009[a] and Anonymous 2009[b]). The development of resistance may be delayed but the possibility for development of resistance cannot be totally denied. Thus, the insecticide resistance must be continuously monitored as it gives information on changes in frequency and distribution of resistant insects (Rogers *et al* 1990). It must form an integral part of chemical control, to enable the detection of resistance as early as possible and to take necessary measures thereof (Regupathy 1996). Moreover, a base line data regarding the toxicity of the newer insecticides would help in understanding the level of resistance developed by the pest and any possible cross-resistance there in, could be assessed in advance. The ability to distinguish between the major mechanisms

of resistance in a monitoring programme would enhance the effectiveness of any strategy to manage any insect (McCaffery *et al* 1991).

Synergists increase the lethality of insecticides by inhibiting insecticide detoxifying enzymes. This makes synergists the tools for elucidating resistance mechanisms and overcoming metabolic resistance (Raffa and Priester, 1985). Moreover, the reversal of insecticide resistance by methylene dioxyphenyl synergists constitutes a useful *in vivo* indicator of the extent of involvement of detoxifying enzymes in insecticide resistance. Using this approach, mixed function oxidases (MFOs) have been shown of importance in causing resistance in various strains of insects. Synergists may also affect penetration of toxicants into insects (Brooks, 1989).

Biochemical analyses have been conducted to assess the mechanism of resistance in *S. litura* and other *Spodoptera* spp. (Huang and Han 2007, Yu 2006, Yu and McCord 2007). Insects have evolved a variety of physiological and behavioural responses to various toxins in natural and managed ecosystems (Li *et al* 2007). Insecticide resistance involves mainly three mechanisms viz., decreased penetration, enhanced detoxification and target site insensitivity. Decreased penetration, plays little role in most cases. The detoxification enhancement involves different enzymes viz., cytochrome P450 monooxygenase (also known as MFO), glutathione-S-transferase (GST) and esterase (Ishaaya 1993). It could also result in cross resistance among the insecticides. Knowledge of resistance mechanisms could serve as the basis for rational resistance management. The resistance could be overcome by use of synergists/ inhibitors of detoxification enzymes along with the insecticides (Huang and Han 2007) and the use of new chemistries with novel modes of action. Though biochemical analysis has helped to identify some of the resistance enzymes that are involved in resistance development, the same is limited by problems in handling of the samples and techniques, inactivation of specific enzymes and lack of quantitative accuracy.

Now available advanced techniques in molecular biology provide highly specific molecular diagnostic tools for identification of developed resistance in pest populations. These molecular diagnostic tools are based upon identified genetic elements that differentiate between susceptible and resistant genotypes as assessed by laboratory bioassays. Polymerase Chain Reaction (PCR) based specific amplification of such DNA regions has proved highly specific and useful tool to assess average resistance levels, estimate resistance potential and genetically monitor effects of insect control strategies (Cassanelli *et al* 2005). PCR has enabled molecular analysis to become an integral part of biosystematics, population biology and ecology (Saiki 1989, Mullis *et al* 1986). Although, a wide array of DNA marker techniques are available and are being exploited for genetic studies in insects, detection of insecticide resistance seems to be a multigenic character and needs investigation on individual insect type and insecticide in question.

Judicious use of insecticides, use of synergist, insect growth regulators, and new insecticides along with alteration of chemicals with unequal mode of action represent an exciting concept to counteract or delay the development of resistance in larvae, even though they are exposed to insecticide residues (Mehrotra, 1992).

So far, no systematic, comprehensive studies have been done on the status of insecticide resistance in *S. litura* populations in Punjab. Moreover, the biochemical studies have not been conducted to assess the basis/ mechanisms of insecticide resistance involved in case of *S. litura* populations of the region. Molecular markers have so far not been developed to enable quick detection of insecticide resistance in the *S. litura* populations. Keeping in view, the losses, failure of management, problem and prognosis of insecticide resistance in *S. litura*, it was felt necessary to carry out the study entitled 'Evaluation of insecticide resistance profile in *Spodoptera litura* (Fabricius) populations through biological, biochemical and molecular diagnosis' with the following objectives:

1. To establish the level of resistance in *S. litura* populations collected from diverse areas of Punjab towards recommended insecticides.
2. To investigate the mechanisms of insecticide resistance in *S. litura* through synergism and enzyme assays.
3. Molecular analysis of resistant and susceptible populations for molecular differences and development of molecular diagnostic markers for insecticide resistance.

CHAPTER II

REVIEW OF LITERATURE

Different species of *Spodoptera* are found viz. *Spodoptera litura* (F.), *Spodoptera exempta* (Walker), *Spodoptera exigua* (Hb.), *Spodoptera frugiperda* (J.E. Smith) and *Spodoptera littoralis* (Boisd.). Most species are migratory as adults within Africa, Asia and America (Hill, 1983). In addition to these, species that are distributed in India are *Spodoptera pectin* Guen., *Spodoptera abyssinia* Guen., *Spodoptera compta* (Moore.), *Spodoptera downsei* B. and *Spodoptera mauritia acronyctoides* Guen. (Nair 1995). Among all these species, *S. litura* is an important polyphagous insect pest on several crops such as cotton, tobacco, chilli, groundnut, castor, mulberry, cabbage, potato, sweet potato etc. (Lefroy, 1908). Fletcher (1919) reported cauliflower, jute, radish, lucerne, groundnut, cotton, rose, celery, poppy, apple, onion, tea and cotton as its host plants. Ramakrishna Ayyar (1942) reported damage to banana plantations in Malabar by this pest. A total of 112 species of plants belonging to 44 families are attacked by this pest (Moussa and Ketbey, 1960). Jotwani *et al* (1961) reported breeding of *Spodoptera* on tomato and cowpea. Nair (1995) reported that the pest has the ability to attack blackgram, redgram, greengram, gram, linseed, sunflower, jute, sugarcane, amaranthus, brinjal, colocassia, cauliflower, okra, onion, elephant foot yam, diascorea, peas, hemp, cocoa, coffee, tea, rose, lily and dhaincha. It has wide distribution throughout the tropical and temperate Asia, Australia and Pacific basin (Feakin, 1973).

The relevant literature has been reviewed under the following main headings:

2.1 Status of damage by *Spodoptera litura*.

2.2 Insecticide resistance in *Spodoptera litura* and other *Spodoptera* species to different insecticides.

2.3 Relative toxicity of different insecticides to *Spodoptera litura* and other *Spodoptera* species.

2.4 Mechanisms of resistance in *Spodoptera litura* and other related species.

2.5 Molecular studies for insecticide resistance

2.1 Status of damage by *Spodoptera litura*

Spodoptera litura (Fabricius) is a serious pest causing enormous losses to several important crops such as cotton, soybean, groundnut, tobacco and vegetables in India, China, Pakistan and Bangladesh (Dhaliwal and Koul 2010, Nair 1995, Qin *et al* 2004, Ahmad *et al*

2007). It is known to cause considerable damage to soybean, summer and *kharif* mungbean/urdbean, groundnut, cotton, oilseed and vegetable crops such as cabbage and cauliflower in India (Armes *et al* 1997, Kumar and Regupathy 2001, Rani *et al* 2002). The pest is known to cause 26-100 per cent losses in groundnut (Dhir *et al* 1992), 38.51 and 70 per cent avoidable losses in soybean and urdbean, respectively (Taggar *et al* 2007, Krishnaiah *et al* 1983). Incidence of tobacco caterpillar resulted in 60-90 per cent yield loss in groundnut and blackgram grown in Guntur and Prakasam districts (Azam, 1979). In Punjab, a severe outbreak of the pest was observed on cotton during *kharif* 2004 (Arora *et al* 2007). The summer and *kharif* mungbean crops recorded a very high incidence of the insect ranging from 12.44- 32.44 and 12.66- 21.00 larvae per square metre, respectively during 2005- 2008 and soybean had an incidence of 21.66-28.66 larvae per square metre (Taggar *et al* 2007). *S. litura* along with *Helicoverpa armigera* damaged 1-7 leaves per plant and caused 9.3- 30.7 per cent pod damage in Orissa (Patro *et al* 2009). Larval populations up to 12 per metre row length have been recorded on soybean at Pantnagar, Amravati, Pune, Dharwad and Ludhiana during 2009-10 (Anonymous 2010). An incidence of 3.9 larvae per metre row length and 38.65-46.40 per cent pod damage was reported from soybean by Patil and Hegde (2009). Yadav *et al* (2009) recorded an extremely high incidence of 20-70 individuals per metre causing 100 per cent pod damage in flood-ravaged areas of Bihar post flood. The pest is also known to cause 33.33-40.56, 77.86 and 90 per cent foliage damage to potato, groundnut and sunflower, respectively (Bhushan *et al* 2010, Jayanthi and Padmavathamma 2001, Sujatha and Lakshminarayana 2007).

2.2 Insecticide resistance in *Spodoptera litura* to different insecticides

In 2007, there were 553 species of insects and mites resistant to one or more pesticides in 7747 cases reported worldwide. The related species of *S. litura*, *Spodoptera littoralis* is the 16th most resistant insect pest species in the world (Whalon *et al* 2008). The Arthropod Pesticide Resistance Database has reported that *Spodoptera litura* has developed resistance to almost all groups of insecticides such as organochlorines, organophosphates, carbamates, pyrethroids and newer insecticides like indoxacarb, spinosad, emamectin benzoate, abamectin, etc. in various parts of the world mainly India, Pakistan, China, Japan and Taiwan (www.pesticideresistance.org).

S. litura was first reported to have acquired resistance to BHC from Rajasthan (Srivastava and Joshi 1965). In India, this pest was found resistant to endosulfan (Ramakrishnan *et al* 1984, Armes *et al* 1997, Kranthi *et al* 2002), chlorpyrifos (Kranthi *et al* 2002), quinalphos and monocrotophos (Armes *et al* 1997, Kranthi *et al* 2001^{b}, Ramegowda *et al* 2001), carbaryl

(Kranthi *et al* 2001[b]), methomyl (Armes *et al* 1997, Kranthi *et al* 2001[b]), cypermethrin (Armes *et al* 1997, Kranthi *et al* 2002) and fenvalerate (Armes *et al* 1997, Niranjan and Regupathy 2001). This suggested that South Asian populations of *S. litura* had the potential of developing resistance to a wide range of chemistries. The control failures by conventional insecticides and the concurrent outbreaks of this pest in India and Pakistan were therefore mostly associated with the development of *S. litura* resistance to various insecticides (Ahmad *et al* 2007).

Jotwani *et al* (1961) reported that out of 17 insecticides tested, only 6 were proved more toxic than p'-p' DDT. Isodrin, endrin, endosulfan, parathion, aldrin, dieldrin were about 3.2, 2.9, 2.8, 2.0, 1.6 and 1.1 times as toxic as p'-p' DDT. Thanite and nicotine sulphate were less effective to the larvae of *S. litura* as even 0.6 per cent thanite and 2 per cent nicotinic sulphate failed to give any kill of the caterpillar. *S. litura* was the second agricultural pest and the first lepidopteran pest in the country that developed resistance to BHC to the tune of seven fold (Srivastava and Joshi, 1965).

Ramakrishnan *et al* (1984) recorded the acquisition of resistance by Tenali, Andhra Pradesh population of this pest to endosulfan (85.91 fold), chlorpyriphos (1 fold), pyrethrin (14.7 fold), lindane (16.3 fold) and malathion (5.7 fold).

Reddy and Reddy (1984) noted 4.86, 3.55 and 4.15 fold resistance to endosulfan, monocrotophos and carabaryl, respectively in Guntur strain as compared to Kurnool strain. There were 2.0, 8.2, 6.8, 4.4, 4.1, 3.8, 2.1 and 2.1 fold resistance for chlorpyriphos, HCH, DDT, malathion, fenitrothion, methyl parathion, quinalphos and phosalone.

Mayuravalli *et al* (1987) recorded 1.98 fold tolerance in *S. litura* to cypermethrin at LC_{50} and 3.4 fold level in Guntur population to BPMC during the period from 1981-87. Resistance was 1.6 fold for decamethrin, 1.6 fold for permethrin, 1.6 fold for fenvalerate and 1.5 fold for cyfloxylate.

Insecticide resistance in *S. litura* was assessed by Reddy and Devaprasad (1991) in two different locations of Andhra Pradesh *i.e.* Guntur and Srikakulam representing maximum and minimum insecticidal pressure. Resistance index in case of deltamethrin was maximum i.e. 20.5 followed by endosulfan, carbaryl, monocrotophos, cypermethrin and fenvalerate having resistance index values of 14.2, 12.5, 9.4, 6.0 and 2.6, respectively. Mehrotra (1993) suggested that *S. litura* around Delhi acquired high degree of tolerance to pyrethroids.

Murugesan and Dhingra (1995) reported the built-up of resistance in *S. litura* to various insecticides of different groups during three decades (1961-1995). An increase in the LC_{50} value of pyrethrum against *S. litura* during the period indicated development of resistance to the extent of 20.62-fold. The pest developed 9.71 and 14.71- fold resistance to endosulfan when compared

with baseline data given by Mukherjee and Srivastava (1970) and Jotwani *et al* (1961), respectively. There was 23-fold resistance to lindane during 1961-1995. *S. litura* developed resistance to all the organophosphates during the last quarter century with the degree of resistance development varying from 1.82-fold in monocrotophos to 39.14-fold in fenitrothion. Low level of resistance in monocrotophos could be due to the shift from using organophosphates to synthetic pyrethroids. Dimethoate, malathion, fenthion and methyl parathion recorded 9.39, 4.37, 4.01 and 3.59-fold resistance respectively, during 1970-1995. In the case of synthetic pyrethroids, *S. litura* developed 9.03, 1.45 and 98.00-fold resistance to cypermethrin, fenvalerate and deltamethrin within a short span of eight years (1987-1995).

The third instar larvae of *S. litura* (Fab.) obtained from Guntur district, Andhra Pradesh, were 4 and 5-fold resistant to cypermethrin and fenvalerate, respectively, when compared with the Delhi population (Rao and Dhingra 1996). Also, the third instar larvae were less susceptible to fenvalerate than cypermethrin from the same location, i.e., 4.5 and 6.2 times in respect of Delhi and Guntur populations, respectively. A comparison between Delhi population exposed to cypermethrin (LC_{50}, 0.000775) and Guntur population treated with fenvalerate (LC_{50}, 0.019601) revealed that the former was 25.3 times more susceptible. The LC_{50} values in respect of cypermethrin treated Guntur population (LC_{50}, 0.003155) and fenvalerate treated Delhi population (LC_{50}, 0.003481) were comparable. This suggested that the Delhi population of *S. litura* in North India appeared to be normal as compared to Guntur population in South India.

Resistance of *S. litura* to pyrethroids and organophosphate insecticides was monitored by Wu *et al* (1995) in the Shanghai region of China. The resistance ratios of the LC_{50} from 1979 (or 1982 for OPs) to 1994 were 43.9, 90.5, 171.9, 29.7 and 33.6 for cypermethrin, deltamethrin, fenvalerate, dichlorvos and acephate, respectively.

Armes *et al* (1997) recorded low level of resistance (1-6 fold) for endosulfan with an exception of one strain from Bapatla (13 fold), when different populations of *S. litura* from 8 locations in coastal Andhra Pradesh were compared with the susceptible Bangalore strain. For monocrotophos, the resistance levels were 2 to 362- fold with highest resistance levels in strains collected from insecticide treated groundnut crop in Guntur and Cuddapah districts. There was 0.2 to 197 fold resistance to cypermethrin in *S. litura* compared to the susceptible Bangalore strain. Furthermore, there was 8-121 fold resistance to fenvalerate, 1-29 fold resistance to quinalphos and 0.7 – 19 fold to methomyl among the twenty two strains of *S. litura*.

The resistance of *S. litura* (infesting cabbage, cauliflower and castor) collected from Hoshiarpur, Malerkotla and Mansa, Punjab, to endosulfan (2.0 µg/mL), profenofos (3.0 µg/mL), chlorpyrifos (0.15 µg/mL) and fenvalerate (0.25 µg/mL) was monitored using topical application

technique. The survival of all the populations when exposed to profenofos and chlorpyrifos ranged from 46.6 to 70% whereas survival for all exposed to endosulfan and fenvalerate ranged from 58.3 to 78.3 and 75.0 to 88.3%, respectively, showing development of resistance (Kaur *et al* 2006). Similarly, the third instar larvae of the Punjab population of *S. litura* showed 20.74, 15.46, 12.9, 9.59 and 7.05-fold and 16.74, 13.22, 21.18, 11.50 and 4.83-fold resistance to deltamethrin, alpha-cypermethrin, cypermethrin, beta-cyfluthrin and fenvalerate, respectively, in direct spray and leaf dip assays, respectively, compared with the Delhi population. Beta-cyfluthrin was the most effective against Delhi and Punjab population (Kodandaram and Dhingra 2006).

Organophosphate and carbamate resistance in five major pests of cotton collected from twenty two cotton growing districts across India was monitored by Kranthi *et al* (2001[b]). Among the eleven *S. litura* strains tested, only four were found to exhibit resistance factors of 10-30 folds to quinalphos and monocrotophos. The Bangalore (South India) and Nagpur (Central India) susceptible strains exhibited the lowest LD_{50} values and steep slopes of 2.2–3.1. All strains collected in South India, except from Karimnagar, exhibited high resistance levels of 61–148-fold to cypermethrin. Resistance was low at 1–9-fold in the majority of strains from Central India. Resistance levels to endosulfan were high at 27 and 92-fold in only two strains, collected from Bhatinda (North India) and Karimnagar (Central India) respectively. Otherwise resistance was low to moderate at 5–13-fold. All the strains collected from South India exhibited high levels of resistance to chlorpyriphos (45–129-fold). Low resistance levels (2-6 fold) were recorded in Central India except in the Warangal (RF 129) and Mahbubnagar (RF 127) strains, where it was high. The resistance was moderate, at 40-fold, in the single strain (Bhatinda) tested from North India (Kranthi *et al* 2002).

Ramegowda *et al* (2001) conducted laboratory experiments to know the insecticides resistance in *S. litura* and its dynamics to monocrotophos during 1998-1999. The resistance levels in comparison to a reference strain were of the order cypermthrin>quinalphos> monocrotophos> endosulfan. Monocrotophhos resistance was almost static from August 1998 to February 1999 (13.06 to 14.54 folds) excepting a hike during October 1998, from March onwards resistance started increasing and reached peak (37.16 folds) during April 1999, when it was last studied.

In South India, Kumar and Regupathy (2001) assayed resistance of *S. litura* to insecticides using the discriminating doses of endosulfan (2.0 µg), chlorpyrifos (0.15 µg), profenofos (3.0 µg) and fenvalerate (0.25 µg per larvae) in several locations in Tamil Nadu, India. High level of fenvalerate resistance was prevalent however; resistance to profenofos was negligible when compared to other insecticides monitored.

The highest levels of relative resistance exhibited by *S. litura* and beet army worm (*S. exigua*) since 1984 had considerably declined in both species for all the conventional groups of insecticides, i.e. cyclodienes (endosulfan), organophosphates (monocrotophos) and carbamates (methomyl), with the implementation of sustainable agricultural practices coupled with drastic reduction in insecticide consumption in the Guntur district of Andhra Pradesh (Kumar *et al* 2005). However, the author emphasized that the occurrence of moderate to high levels of resistance to pyrethroids (deltamethrin) in the *Spodoptera* spp. must be viewed with concern and caution should be exercised in usage of older chemistries of pyrethroids.

Radhika *et al* (2005[a]) reported that the Guntur population showed 19.50- and 49.81-fold resistance to carbaryl compared to Chittoor population at LD_{50} and LD_{90} levels, respectively, when applied topically.

Thiodicarb showed LD_{50} and LD_{90} values of 0.753 and 1.754 mg/larva by topical application and 0.682 and 1.552 µg/larva of *S. litura* by leaf sandwich method (Radhika *et al* 2007). This insecticide was found to be superior to methomyl, etofenprox, profenofos, cartap hydrochloride and diafenthiuron and the four conventional insecticides tested. Thiodicarb was not synergised by PBO even at higher synergist ratio of 1:10 in both topical and leaf sandwich methods of application of the mixtures, as the synergistic ratios varied between 1.0 and 1.38. The resistant population of *S. litura* can be managed with new molecule, thiodicarb and there is no necessity of mixing the synergist PBO with this new insecticide.

Field populations of *S. litura* from Pakistan were evaluated for their resistance to conventional insecticidal chemistries viz. organochlorine (endosulfan), organophosphates (chlorpyrifos, phoxim, quinalphos, profenofos), carbamates (methomyl, thiodicarb) and pyrethroids (bifenthrin, cyfluthrin) during 1997-2005 using a leaf-dip bioassay method (Ahmad *et al* 2007). Generally, resistance levels were very low to low to endosulfan, chlorpyrifos, phoxim, quinalphos, profenofos, bifenthrin and thiodicarb, and moderate to high to methomyl and cyfluthrin. Correlation analysis indicated that insecticides belonging to the same class such as organophosphate, carbamate or pyrethroid exhibited a positive cross-resistance in *S. litura*. Positive correlation was also found between endosulfan and carbamates. Except methomyl and bifenthrin, which were negatively correlated, there was no cross-resistance between organophosphate or carbamate or pyrethroid insecticides in the resistant populations of *S. litura*. The toxicity of representative newer insecticides, which are being used widely in Pakistan, were investigated against various populations of *S. litura* (Fabricius) collected from three different districts for 3 consecutive years. For spinosad, resistance ratio compared with Lab-PK were in the range of 7–122-fold, 3–95-fold for indoxacarb, 4–186-fold for abamectin, 2–77-fold for

emamectin and 13–224-fold for fipronil. The resistance ratio for insect growth regulator (IGR) tested was in the range of 2–66-fold for lufenuron, 8–56-fold for diflubenuron and 2–153 fold for methoxyfenozide. Pairwise comparisons of the log LC_{50}'s of insecticides tested for all the populations showed correlations among several insecticides, suggesting a cross-resistance mechanism. The most probable reason for low toxicity of these insecticides could also be the development of multiple resistance mechanisms. When these same products were tested against a susceptible laboratory population (Lab-PK), emamectin benzoate and indoxacarb were significantly more toxic than other compounds tested (Ahmad *et al* 2008).

Saleem *et al* (2008) reported that during 2004-2006, the resistance ratios in *S. litura* populations in Pakistan compared with Lab-PK were in the range of 10 to 92-fold for endosulfan, 5 to 111-fold for cypermethrin, 2 to 98-fold for deltamethrin, and 7 to 86-fold for ß-cyfluthrin. For organophosphates and carbamates, resistance ratios were in the range of 3- to 169-fold for profenofos, 18 to 421-fold for chlorpyrifos, 3 to 160-fold for quinalphos, 6 to 126-fold for phoxim, 7 to 463-fold for triazophos, and 10 to 389-fold for methomyl and 16 to 200-fold for thiodicarb. Resistance ratios were generally low to medium for deltamethrin and ß-cyfluthrin and high to very high for endosulfan, cypermethrin, profenofos, chlorpyrifos, quinalphos, phoxim, triazophos, methomyl, or thiodicarb. Pair wise comparisons of the log LC_{50} values of insecticides tested for all the populations showed correlations among several insecticides, suggesting a cross-resistance mechanism.

A population of *S. litura* collected from Dunyapur was reared for eleven generations under laboratory conditions without any insecticide exposure (Rehan *et al* 2011). The LC_{50} data was recorded through diet incorporation method against four insecticides such as emamectin benzoate, spinosad, imidacloprid and profenofos. For new chemistry insecticides the larval mortality data was taken after 72 hrs while in case of conventional insecticides the mortality data was taken after 48 hrs. Emamectin benzoate (1.59 ppm) was found to be most toxic on the basis of LC_{50} values followed by spinosad (7.77 ppm), profenofos (686.5 ppm) and imidacloprid (258.75 ppm) at generation 1. The decrease in the LC_{50} values after 11 generations as compared to the field population of *S. litura* was 4.81, 9.83, 9.30 and 13.82 folds against emamectin benzoate, spinosad, imidacloprid and profenofos, respectively. The estimated decrease in resistance was 11.36, 11.11, 16.67 and 9.61 for imidacloprid, spinosad, emamectin benzoate and profenofos, respectively. The results suggest that spinosad can be included in the control program of *S. litura*, due to its lower stability and higher reversion rate with insecticides bearing novel modes of action and this baseline susceptibility data could be very helpful in future monitoring of insecticide resistance in *S. litura*.

2.3 Relative toxicity of different insecticides to *Spodoptera litura* and other *Spodoptera* species

The relative toxicity serves as a ready reckoner for the selection of suitable insecticides for effective pest management under field conditions. Also, such baseline data would provide a record for detecting resistance level of *S. litura*, if any, to various insecticides at different periods.

Balasubramanian and Balasubramanian (1984) found that the LC_{50} values and index of susceptibility were in order of fenvalerate> chlorpyriphos> permethrin> methamidophos> cypermethrin> deltamethrin. Singh *et al* (1987) worked out the relative toxicity of different pyrethroids viz., cypermethrin, decamethrin, permethrin, fenvalerate and natural pyrethrins against *S. litura*. Decamethrin having LC_{50} value 0.00001 per cent was found to be most toxic followed by cypermethrin B, cypermethrin A, permethrin, fenvalerate and pyrethrins having LC_{50} values 0.00014, 0.00029, 0.00041, 0.000201, 0.02382 per cent, respectively.

Toxicity of different insecticides against *S. litura* collected from Coimbatore, Udumalpet and Madukarai was studied by Balasubramanian *et al* (1988) in Tamil Nadu. Based on LC_{50} values obtained, chlorpyriphos and monocrotophos were found to be most effective against the Coimbatore population having LC_{50} values 0.0002198 and 0.02105 per cent, respectively followed by Udumalpet (0.00263 and 0.03142%) and Madukarai (0.009831 and 0.04559%) population whereas carbaryl was most toxic against Udumalpet population having LC_{50} value 0.1150 per cent followed by Madukarai and Coimbatore population having LC_{50} values 0.1798 and 0.2292 per cent in the third instar stage, respectively.

Gupta and Singh (1988) conducted field trials with six granular insecticides on green gram in Uttar Pradesh. Results indicated that applications of mephosfolan @ 2 kg a.i. per ha at the time of sowing and thirty days after sowing reduced the population of leaf eating caterpillar to 5.33 per ten plants as compared to control (27.17). The decreasing order of efficacy of other insecticides at the same dose was aldicarb, phorate, carbofuran and disulfoton.

Kumar (1990) studied the efficacy of various insecticides against 9±1 day old larvae of *S. litura* and reported the order of toxicity based on their values as: fenvalerate > methyl paramethion > chlorpyriphos > lindane > malathion> pyrethrin > fenitrothion > fenthion.

Gupta *et al* (1992) tested the potency of some synthetic pyrethroids against *S. litura* in Rajasthan. On the basis of their LC_{50} values, deltamethrin, cypermethrin, fenvalerate and permethtrin were found to be 12, 5, 3 and 3 times more toxic than BHC.

Lohar *et al* (1995) carried out field and laboratory studies with cauliflower to determine

the efficacy of five insecticides against larvae of *S. litura* in Tandojam, Pakistan. Results from field studies showed >80 per cent mortality caused by Hostathion (triazophos), Karate (lambda cyhalothrin), Thiodan (endosulfan) and Azodrin (monocrotophos) whereas Raxion (dimethoate) could cause 70 per cent mortality. Under laboratory conditions, larvae collected from Hostathion and Karate treated fields of cauliflower showed 80 per cent mortality followed by Thiodan (70%), Azodrin (60%) and Raxion (50%).

Field trials for the evaluation of seven insecticides against *S. litura* on groundnut were conducted by Mohapatra *et al* (1995) during *rabi* season 1990-91. Carbofuran at 1 kg a.i. per ha was found to be most effective, resulting in the highest number of pods per 10 plants (226) and pod weight per 10 plants (148g) followed by phorate @ 1 kg q.i. per ha (213 pods and 140g per 10 plants) and quinalphos @ 0.65% a.i. per ha (207.3 pods and 133g per 10 plants).

Murugesan and Dhingra (1995) reported that among the eighteen insecticides tested against the third instar larvae of *S. litura,* deltamethrin was found to be the most toxic insecticide on the basis of LC_{50} values, followed by lambda cyhalothrin, cypermethrin, fenvalerate, fenpropathrin, methyl parathion, chlorpyriphos, phosphamidon, endosulfan, quinalphos, monocrotophos, malathion, pyrethrum, fenitrothion, lindane, fenthion and dimethoate. Fenthion and dimethoate were less toxic than lindane, being 0.76 and 0.75 times as toxic as lindane. Carbaryl (2%) gave only 10% larval mortality.

Bhanukiran *et al* (1997) studied the efficacy of two conventional insecticides viz., methomyl and triazophos alone or in combination with biopesticides viz., NPV, neem oil and diflubenzuron against S. *litura* on groundnut. Methomyl (0.025%)+ diflubenzuron (0.0125%) recorded significant reduction in *S. litura* population i.e. 53.23 per cent followed by triazophos (0.025%) and diflubenzuron (0.0125%) i.e. 50.38 per cent whereas combination with NPV @ 125 LE per ha and neem oil (0.5%) were less effective. Methomyl (0.05%) and triazophos (0.05%) alone recorded 45.20 and 43.32 per cent larval mortality, respectively.

Singh and Singh (1998) compared the relative susceptibility of 3rd instar with 2nd instar of *S. litura* against some pyrethroid and non-pyrethroid insecticides. The pest was found to be most susceptible to β cyfluthrin followed by bifenthrin, decamethrin, cypermethrin, lambda cyhalothrin, chlorpyriphos, fenvalerate, malathion, lindane and endosulfan. Second instar larvae were found to be more susceptible than those of third instar larvae to all the tested insecticides.

Pachori and Gargav (1998) conducted field trials for two years in Madhya Pradesh to evaluate the efficacy of different insecticides against *S. litura* on wheat and revealed that phorate @ 1kg a.i. per ha proved to be most effective followed by carbofuran @ 1 kg a.i. per ha, BHC @

2.5 kg a.i. per ha, endosulfan @ 0.5 kg a.i. per ha, quinalphos dust @ 0.40 kg a.i. per ha, quinalphos EC @ 0.20 kg a.i. per ha while carbaryl @ 1.25 kg a.i. per ha was at par with the untreated control.

Bioefficacy of three insecticides viz., monocrotophos, carbaryl and fenvalerate and two fungicides viz., carbendazim and mancozeb alone and in combination treatments was studied by Padmaja and Rao (2000) against *S. litura* in Andhra Pradesh. Based on the overall efficacy for two generations, the combination treatment fenvalerate (0.01%)+ mancozeb (0.1%) recorded highest per cent mortality (96.11), mancozeb (0.2 and 0.1%) and carbendazim (0.1 and 0.05%) at both concentrations showed slight insecticidal efficacy by recording 21.11, 13.89, 12.22 and 8.33 per cent mortality, respectively. Both the fungicides enhanced the bioefficacy of insecticides and all the three insecticides were more compatible with the fungicides giving significant reduction in the population of *S. litura*.

Johny and Muralirangan (2000) tested the susceptibility of *S. litura* populations collected from four different locations of Tamil Nadu to five commonly used insecticides. For all the populations, the order of toxicity from least toxic to most toxic was fenvalerate (369.01 ppm) followed by endosulfan (319.62), quinalphos (265.92 ppm), cypermethrin (136.17 ppm) and chlorpyriphos (28.87 ppm).

Kumar and Regupathy (2001) reported the LD_{50} of endosulfan, chlorpyriphos, profenophos, fenvalerate and deltamethrin were 0.7628, 0.0355, 0.3710, 0.0672 and 0.0647 ppm, respectively for third instar larvae of *S. litura* collected from the field in Madurai district of Tamil Nadu.

Ahmed *et al* (2001) studied ovicidal action of new insecticides against *S. litura* at Guntur (Andhra Pradesh). Thiodicarb recorded highest per cent mortality of 95.55 followed by indoxacarb, chlorpyriphos, spinosad, quinalphos and β cyfluthrin with 86.66, 75.55, 73.33, 71.10 and 66.66, respectively.

Efficacy of phorate, carbofuran, BHC, quinalphos, endosulfan and carbaryl against *S. litura* was evaluated by Pachori and Gargav (2001) in Hisar. Results revealed that phorate @ 1 kg per ha was found to be the most effective followed by carbofuran at the same dose and BHC at 2.5 kg per ha. Endosulfan @ 0.5 kg per ha, quinalphos dust @ 0.4 kg per ha and carbaryl @ 1.25 kg per ha were less effective.

Ramegowda and Basavanagoud (2001) tested the efficacy of new compounds, lambda cyhalothrin, carbosulfan, profenophos, spinosad and fipronil against *S. litura* and found that they

were highly effective against the susceptible strains of *S. litura*. Carbosulfan and profenophos were more effective against resistant strains.

Zeng *et al* (2001) tested the efficacy of chlorpyriphos (48%) to control 2nd and 3rd instar larvae of *Prodenia litura* at two locations in Fiji and China. Results were compared with reference pesticides deltamethrin (2.5%), phoxim (50%), dichlorvos (80%) and quinoxaline (25%). Significantly high control (91%) was obtained by chlorpyriphos after 1-3 days while it was 85 and 61 per cent after 7 and 10 days of application.

Ansari *et al* (2002) evaluated six insecticides viz. dichlorvos, malathion, fenvalerate, monocrotophos, endosulfan and cypermethrin against different instars of *S. litura* by topical application technique Fenvalerate was found to be highly toxic to 2nd, 4th and 5th instar larvae with LD_{50} values 0.00216, 0.00236 and 0.00247 per cent, respectively whereas cypermethrin was most toxic to 3rd instar larvae with LD_{50} value of 0.00178 per cent.

Synthetic pyrethroids like Nagata 45 EC (cypermethrin 5 + ethion 40), cypermethrin 25 EC, fenvalerate 20 EC, ethofenprox 10 EC and fenpropathrin 10 EC were evaluated against *S. litura* on sugarbeet by Mallikarjuna *et al* (2004) at Bangalore. Nagatta @ 675 g a.i./ha was found to be superior in reducing leaflet damage (0.33%) followed by ethofenprox and fenpropathrin @ 50 and 75 g a.i./ha reporting 15.80, 13.99, 22.03 and 20.45 per cent leaflet damage, respectively. Fenvalerate @ 100 g a.i./ha and cypermethrin @ 75 g a.i./ha recorded 28.94 and 19.50 per cent leaflet damage, respectively.

Ramesh Babu and Santharam (2002) reported lower LC_{50} values to 2nd instar larvae of *S. litura* when treated with betacyfluthrin by leaf disc method while to 3rd and 4th instar, higher LC_{50} values were required.

Gupta *et al* (2004) worked out the order of toxicity of different insecticides to *S. litura* with relative toxicity in parenthesis on the basis of LC_{50} value as emamectin benzoate (6.93) > fenvalerate (1.82) > indoxacarb (1.62) > cypermethrin (1.00) > abamectin (0.94) > quinalphos (0.67) > bifenthrin (0.51) > spinosad (0.44) > endosulfan (0.28) > betacyfluthrin (0.23) > lambda cyhalothrin (0.19).

Status of insecticide resistance in Kurnool (Andhra Pradesh) population of tobacco caterpillar, *S. litura* (Fab.) was estimated to choose right insecticide for management, to monitor and develop insecticide resistance management strategies (Rao 2008). The LC_{50} (µg per larva) values of lufenuron, emamectin benzoate, novaluron, indoxacarb, profenophos, endosulfan, chlorpyriphos, deltamethrin, quinalphos, spinosad, cypermethrin, dichlorvos, acephate and fenvalrate were 0.0068, 0.0069, 0.030, 0.21, 0.213, 0.645, 0.93, 0.95, 1.02, 1.14, 3.31, 5.1 2, 5.3

and 5.49. LC_{90} (µg per larva) values were also worked out. Taking LC_{50} value of cypermethrin as standard, relative toxicity of other chemicals was worked out. This kind of study would serve as ready-reckoner for the selection of insecticides for the management of field strains and also helpful in development of resistant management strategies for this polyphagous insect pest.

Dhawan *et al* (2009) assessed the toxicity of some new insecticides viz., emamectin benzoate, novaluron, chlorantraniliprole, pyridalyl and flubendiamide against *S. litura* on cotton and found them more toxic than conventional insecticides viz., endosulfan, thiodicarb and chlorpyriphos. Studies conducted by Kumar and Srivastava (2009) indicated that amongst synthetic pyrethroids deltamethrin was the most toxic insecticide and dimethoate was the least toxic to *S. litura*. At 0.01%, the earliest mortality response was observed at 2 hours after exposure (HAE) in deltamethrin (6.6%), at 3 HAE in each of the cypermethrin, lambda cyhalothrin and dimethoate (3.3 to 6.6%) and at 6 HAE in alphamethrin (3.3%). The LT_{50} value of deltamethrin, cypermethrin, alphamethrin, lambda cyhalothrin and dimethoate was 10.30, 10.93, 14.40, 22.54, 23.80 h, respectively at the concentration closer to LC_{50} at 24 HAE. Deltamethrin therefore was the fastest acting synthetic pyrethroid and lambda cyhalothrin was the slowest. Dimethoate was evidently slower than synthetic pyrthroids. At 24h exposure, dimethoate was almost 9 times less toxic than the most active synthetic pyrethroid deltamethrin and about 1.8 times less toxic than the least toxic alphamethrin at LC_{50} level. The order of toxicity was: deltamethrin (242) > cypermethrin (393) > lambda cyhalothrin (771) > alphamethrin (1166) > dimethoate (2200 ppm).

The susceptibility of the different larval stages of *S. litura* to 9 insecticides (chlorfenapyr, chlorfluazuron, chlorpyrifos, EPN, esfenvalerate, ethofenprox, lufenuron, tebufenozide and teflubenzuron was evaluated using the perilla leaf-dipping method. Median lethal concentration (LC_{50}) increased with larval development from 0.5 to 5.6 ppm, 9.9 to 27.9 ppm, 9.6 to 125.1 ppm and 24.3 to 546.6 ppm in the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar, respectively. The tolerance ratio (TR), which is the TR of 90% lethal concentration (LC_{90}) to the recommended concentration, was 0.04 to 0.8 in the 1^{st} instar, 0.2 to 7.5 in the 2nd instar, 0.7 to 115.3 in the 3rd instar and 1.2 to 485.4 in the 4th instar. Lower LC_{50} and DTR, which is the difference between the LC_{50} and the TR of 4th and other instars, respectively, were observed in chlorfenapyr, chlorpyrifos and EPN, while higher ones were observed in lufenuron, chlorfluazuron and teflubenzuron. These results indicate that insecticides with lower LC_{50} and DTR are effective in controlling larva of *S. litura* collected in Milyang, Korea Republic (Bae *et al* 2003).

A trial was conducted under laboratory conditions to assess the relative efficacy of some new chemicals viz. emamectin benzoate, indoxacarb, flubendiamide, fipronil and methoxyfenozide against third instar *S. litura* larvae under laboratory conditions (Ghosh *et al*

2007). The relative toxicity of insecticides was worked out, the acute toxicity i.e. LC_{50} value of flubendiamide was 4.75 ppm and was relatively more toxic to the 3rd instar larvae than the other insecticides tested. On the basis of LC_{50} values, the next best insecticides in order of descending effectiveness were emamectin benzoate (5.09 ppm), indoxacarb (7.86 ppm) and fipronil (136.48 ppm). Methoxyfenozide showed lowest acute toxic effect with highest LC_{50} value (738.41 ppm). The order of relative toxicity of different insecticides after 24 hr of exposure to *S. litura* was found as follows: flubendiamide (155.35) > emamectin benzoate (144.93) > indoxacarb (93.93) > fipronil (5.41) > methoxyfenozide (1.00). With the increase of exposure time all the chemicals showed steady decrease in LC_{50} values upto 48 hr. All the insecticides followed the same order of toxicity as 24 hr. Thus, flubendiamide closely followed by emamectin benzoate showed the best efficacy in controlling the test insect in laboratory conditions.

The efficacy of six new insecticides *viz.*, emamectin benzoate, indoxacarb, methoxyfenozide, novaluron, lufenuron and fipronil against chilli fruit borer *(S. litura* Fabr.) was evaluated during *rabi*, 2006–07 (Ghosh *et al* 2008). In the field experiment, highest mean reduction of fruit borer population was recorded in novaluron (95.75%) treated plots, followed by fipronil (91.95%), indoxacarb (90.35%), lufenuron (89.75%), emamectin benzoate (87.5%) and methoxyfenozide (86.4%). Relative toxicity of these six insecticides against the third instar larvae of *S. litura* was evaluated under laboratory conditions. The order of relative toxicity after 24 hours of exposure was emamectin benzoate (144.929) > indoxacarb (93.933) > fipronil (5.410) > novaluron (1.488) > lufenuron (1.037) > methoxyfenozide (1.000). With the increase of exposure time upto 48 hours, all the chemicals showed steady decrease in LC_{50} values.

To evaluate the resistance risk of *S. litura* to indoxacarb, resistance selection was conducted in the laboratory, and cross-resistance of indoxacarb-resistant *S. litura* population was investigated (Wang *et al* 2008). After selection with indoxacarb 6 times during 10 generations, a resistant population of *S. litura* was achieved with resistance ratio of 15.63 compared with unselected parent population, suggesting that *S. litura* had high potential to develop resistance to indoxacarb, as was confirmed by resistance risk assessment. Bioassay showed that the LC_{50} values of phoxim, beta cypermethrin and fipronil were 1.53, 2.42 and 1.53 folds higher in resistant population than that in unselected parent population, respectively, while the LC_{50} values of chlorfenapyr and methomyl were 0.78 and 0.96 folds lower in resistant population than that in unselected parent population, suggesting that indoxacarb-resistant *S. litura* had little cross-resistance to these tested insecticides.

The bioefficacy of five insecticides viz., indoxacarb, methomyl, fipronil, thiamethoxam and imidacloprid was determined against 7 d old larvae of *S. litura* by contact and leaf dip

methods (Ramanagouda and Srivastava 2009). Indoxacarb was the most toxic insecticide at 24 h exposure, at all the three concentrations, the values being 7.0 ppm (LC_{30}), 15 ppm (LC_{50}) and 126 ppm (LC_{90}) and imidacloprid was the least toxic with 281, 572 and 3313 ppm at respective LC levels. The order of toxicity by residue contact bioassay method at LC_{50} was: indoxacarb > methomyl > thiamethoxam > fipronil > imidacloprid. In leaf dip method also at 24 h after exposure, at all the three concentrations, indoxacarb was the most toxic insecticide (LC_{30}=1.0, LC_{50}=2.0 and LC_{90}=13 ppm) and imidacloprid was the least toxic (LC_{30}=2186, LC_{50}=3719 and LC_{90}=10377 ppm). The order of toxicity at all the three LC levels was: indoxacarb > methomyl > fipronil > thiamethoxam > imidacloprid. In residue contact bioassay, methomyl could cause 50% mortality in a shortest span of 5.38 h followed by imidacloprid (10.47 h) and indoxacarb (12.60 h). Thiamethoxam and fipronil were slowest in action causing 50% mortality in 33.37 and 23.93 h, respectively. In leaf dip method also methomyl was the fastest acting and thiamethoxam was the slowest acting insecticide with LT_{50} values 13.82 and 39.02 h, respectively. The LT_{50} values for the other three insecticides viz., fipronil, imidacloprid and indoxacarb were 15.72, 18.53 and 21.59 h, respectively. Fipronil proved better than imidacloprid and indoxacarb in leaf dip method. A comparative dose mortality response expressed in terms of relative toxicity (RT) indicated that at 48 hours after exposure (HAE), the RT values for indoxacarb and methomyl were 1.0 and 1.2, respectively. Thiamethoxam, imidacloprid and fipronil were 28.54, 37.45 and 44.36 times less toxic than indoxacarb. The RT values in the leaf dip method (stomach toxicity) were relatively higher owing to the higher LC values. Imidacloprid, fipronil and thiamethoxam, in particular, showed very high RT value indicating far less stomach toxicity to *S. litura* as compared to indoxacarb and methomyl. The data reflect a general increase in tolerance by *S. litura* in view of the field recommended doses of the insecticides.

Bioefficacy of novaluron (Rimon 10 EC) against *S. litura* (Fab.) (Noctuidae: Lepidoptera) attacking cotton was compared with other insecticides, namely, chlorpyrifos, thiodicarb, spinosad and endosulfan, in the laboratory during 2005 and at farmers' fields during 2006 (Saini *et al* 2010). For laboratory evaluation, initially the cotton plants at the research farm were sprayed with the requisite concentration of an insecticide; and afterwards batches of field collected larvae were also sprayed with the same concentration of the respective insecticide separately. Such larvae were provided with the leaves sprayed with the respective insecticide in the glass jars to record mortality. For field studies, different insecticides were evaluated at two locations keeping a plot size of 50 m^2 with three replications in a randomized block design. The results indicated that in the laboratory though initial mortality in novaluron at 0.005 and 0.01 per cent concentration was low, yet the larvae stopped feeding within two days and gradually all

larvae were dead when observed after nine days of treatment. Spinosad (Tracer 45 SC) 0.015 per cent had no toxicity against this pest. Under field conditions, novaluron at 37.5 and 50 g a. i. was as good as thiodicarb (Larvin 75 WP) 470 g a. i./ha after seven days of spray. Chlorpyrifos (Tricel 20 EC) 400 g a. i. and endosulfan (Endocel 35 EC) 650 g a. i./ha proved significantly inferior to the above insecticides, both in terms of population reduction and increase in seed cotton yield. Taking into account the bioefficacy as well as seed cotton yield, novaluron 50 g a. i./ha was considered a better option against *S. litura*.

The cutworm *S. litura* is a major insect pest of vegetables and cotton in China, and has been reported to develop resistance to various classes of insecticides. Chlorantraniliprole, an insecticide recently registered in China, provides a novel option for control of this pest. The susceptibilities of *S. litura* collected from Southeast China to chlorantraniliprole were determined by diet incorporation assay with neonates (Su *et al* 2012). The susceptibility variation among 12 field populations was low (<4-fold), with median lethal concentration (LC_{50}) values varying from 28.4 to 102.5µg/l. However, all the 12 field populations were less susceptible to chlorantraniliprole than a laboratory susceptible population. The most tolerant populations were sampled from Guangdong and Anhui Provinces where *S.litura* had been frequently challenged by insecticides. However, no correlation was found between LC_{50} values and the number of applications of this chemical. Against third instar LC_{50} values based on bioassay studies indicated that among the insect growth regulators, *viz.*, buprofezin, lufenuron, novaluron and the check diflubenzuron the highest relative toxicity was exhibited by novaluron at 76 hours and lufenuron at 96 and 120 h of exposure by topical application (Talikoti *et al* 2012). Novaluron exhibited greater toxicity irrespective of the time of exposure when sandwich method was used and lufenuron was intermediary in toxicity. Novaluron was highly toxic (LC_{50} = 0.0197%) at 72 h of exposure by topical application followed by lufenuron (LC_{50} = 0.0206%).

Pramanik and Chatterjee (2003) observed that abamectin had the lowest LC_{50} value of 271.416 ppm for *S. litura*, while novaluron had the highest LC_{50} of 1976.430 ppm. The order of relative toxicity was: abamectin > spinosad > acetamiprid > cartap hydrochloride > novaluron.

Beet armyworm, *S. exigua* (Hübner), and fall armyworm, *S. frugiperda* (J. E. Smith), are occasional pests of cotton, *Gossypium hirsutum* (L.), and soybean, *Glycine max* (L.) Merrill. These insects can be difficult to control due to insecticide resistance and larval behavior on plants. Indoxacarb, pyridalyl, spinosad, methoxyfenozide, and emamectin benzoate controlled beet armyworm infestations up to 10 days after treatment compared to the non-treated control. Thiodicarb reduced beet armyworm densities up to three d after treatment. The LC_{50} values of

indoxacarb and pyridalyl for beet armyworm and fall armyworm exceeded the highest concentrations tested (100-200 µg/vial) in the adult vial test (Cook *et al* 2004).

Efficiency of indoxacarb, spinetoram and methoxyfenozide on cotton leaf worm (*S. littoralis*) was determined through exposure of second and fourth instar larvae to dipped castor bean leaves (Hassan 2009). LC_{50} estimates of second instar larvae ranged from 0.004 to 0.006 ppm of indoxacarb, 0.022 to 0.033 ppm of spinetoram and 0.006 to 0.011ppm of methoxyfenozide. LC_{50} estimates of fourth instar larvae ranged from 0.36 to 0.54 ppm of indoxacarb, 1.78 to 2.64 ppm of spinetoram and 1.36 to 1.69 ppm of methoxyfenozide. Also, the ovicidal activity of these compounds was studied. Three day old eggs are more affected than that of one or two days old in case of indoxacarb and spinetoram while the reverse was in the case of methoxyfenozide. The histological examinations of 6th larval instar cuticle (after treatment of fourth larval instar by LC_{50} of methoxyfenozide) showed destruction in the cuticle layers, fissures in the endocuticle and irregular distribution of the hypodermal cells. While indoxacarb and spinetoram treatments showed slight effect in the cuticle layers as compared to methoxyfenozide. In addition, the deteriorative potentialities of aldehyde oxidase and α -glycerophosphate dehydrogenase pattern of *S. littoralis* were screened.

The LC_{50} values among insecticides ranged from 0.066 µg/mL for spinetoram to 5.27 µg/mL for lambda-cyhalothrin (Hardke *et al* 2011). The newer insecticides, chlorantraniliprole, cyantraniliprole, flubendiamide, and spinetoram had LC_{50} values of 0.068, 0.118, 0.930 and 0.066 µg/mL and were generally lower than those observed for the older traditional methoxyfenozide, novaluron, and spinosad) with LC_{50} values ranging from 0.166 µg/mL to 5.27 µg/mL. Fall armyworm larvae were significantly less susceptible to lambda-cyhalothrin than all other insecticides. Spinetoram (0.066 µg/mL) and chlorantraniliprole (0.068 µg/mL) were significantly more toxic to fall armyworm than all other insecticides.

The efficiency of the median lethal concentration (LC_{50} value) for the novel insecticide pyridalyl on 2nd, 4th, and 6th larval instars of cotton leafworm *S. littoralis* (Boisd.) was tested under laboratory conditions .The results showed that the pyridalyl is more effective on 4th instar larvae due to 78.0% larval mortality. Also fertility % was 0.0 in comparison to control and the number of eggs/female was the smallest one in comparison with other, estimated by 365.7 eggs. The tested LC_{50} value of pyridalyl showed highly histopathological disturbance in the epithelium of mid gut. The histochemical observation showed a conspicuous depletion in total protein content in both 4^{th} and 6^{th} treated larval instars (Dahi *et al* 2011).

The effects of conventional (profenofos) and nonconventional (emamectin benzoate, spinosad and chlorfluazuron) insecticides at their LC_{10}, LC_{25} and LC_{50} and their binary mixtures

were evaluated against 2nd instar larvae of cotton leaf worm, *S. littoralis* (Boisd.) under laboratory conditions (Korrat *et al* 2012). After 3 days of the treatment, emamectin benzoate was the most effective insecticide (LC_{50}=0.017 ppm) followed by chlorfluazuron (LC_{50}=0.42 ppm) and profenofos (LC_{50}=10.9 ppm) and finally spinosad which showed the lowest toxic effect (LC_{50}=19.9 ppm). After 12 days of the treatment, and at the LC_{25} level, spinosad showed the longest residual effect followed by chlorfluazuron, and then profenofos and emamectin benzoate. At the same concentration level, spinosad and chlorfluazuron had the higher effects on pupation, moth emergence, hatchability and sterility. Chlorfluazuron, especially at $LC_{50,}$ caused the highest effect on the percentages of deformed pupae and moths (14.86% and 32.76%, respectively). In general, all the tested mixtures increased mortality percentages of larvae. The highest potency was observed with the mixture of profenofos (at LC_{10}) and chlorfluazuron (at LC_{50}) which produced potentiation. However, the mixtures of both emamectin benzoate and spinosad with profenofos produced additive effects. Mixtures of chlorfluazuron and emamectin benzoate (at LC_{50}) with profenofos at LC_{10} gave the highest effect on biological parameters [there was no pupa comparing with the control (86.66%)]. Also, mixtures of spinosad (at LC_{50}) with profenofos at LC_{10} and LC_{25} gave the highest effect on egg production (393.9 egg/female) and hatchability (19.17%), comparing with the control (1151.6 egg/female and 96.36%). The obtained results indicated that mixtures of conventional–nonconventional insecticides had the combined advantages of quick speed of killing and a high level of safety.

A field population of *S. exigua* from Lodhran, Pakistan was found resistant to conventional insecticides (Ishtiaq *et al* 2012). It gave 65, 66, 92, 73, 34 and 29–fold resistance to deltamethrin, cypermethrin, chlorpyrifos, profenofos, abamectin and spinosad respectively compared with Lab-PK susceptible population. Field population was divided into two sub-populations. One was left unselected and the second (Del-SEL) selected for five generations at the dose equal to LC_{50}. Bioassays at G5 for Del-SEL strain gave resistance ratios of 976, 421, 118, 30, 15 and 17-fold for deltamethrin, cypermethrin, chlorpyrifos, profenofos, abamectin and spinosad, respectively compared with Lab-PK. Resistance was found stable for all the insecticides in the field population but it was more stable in Del-SEL strain for deltamethrin, cypermethrin and chlorpyrifos than profenofos, abamectin and spinosad when reared without exposing to deltamethrin (G5 - G10). It indicated that cross resistance occurred between deltamethrin, cypermethrin and chlorpyrifos. There was no effect of delta selection on the toxicity of three insecticides *i.e.*, profenofos, abamectin and spinosad, which indicated lack of cross resistance with deltamethrin.

2.4 Mechanisms of resistance in *Spodoptera litura* and other related species

The biochemical/physiological mechanisms of resistance can be categorized as target site insensitivity, increased metabolic detoxification and sequestration or lowered availability of the toxicant. These are achieved at the molecular level by: point mutations in the ion channel portion of a GABA receptor subunit (cyclodiene insecticides); point mutations in the vicinity of the acetylcholinesterase (AChE) active site (organophosphorus and carbamate insecticide resistance); amplification of esterase genes (organophosphorus and carbamate insecticides); mutations linked genetically to a sodium channel gene (DDT and pyrethroid insecticides); and mutations leading to the up-regulation of detoxification enzymes, such as cytochrome P450 and glutathione *S*-transferases (many classes of insecticides) (Feyereisen 1999[a]). The factors affecting the development of insecticide resistance include genetic, biological and operational factors. The biological factor is exemplified in terms of *S. litura* productivity, polyphagy, migration and activities.

2.4.1 Synergists

Synergists could play a useful role in combating resistant population of *S. litura* and reducing insecticide application rates with a consequent reduction in potential environmental contamination and improvement in the performance of IPM programmes (Shankarganesh *et al* 2009). Synergists are also of considerable help in indicating the type of the detoxification enzymes which are involved and thus suggesting the possible biochemical mechanisms of resistance (Metcalf 1967, Riskallah 1984). By combining various categories of synergists with insecticides in laboratory bioassays, most types of mechanisms of resistance can be identified based on differential mortalities (Prabhaker *et al* 1988).

The resistance of *S. litura* to pyrethroid and organophosphate insecticides was synergized with PBO and TPP when tested with cypermethrin, deltamethrin, fenvalerate, dichlorvos and acephate and showed that MFO and esterases played role in insecticide resistance of the pest (Wu *et al* 1995).

To understand the interaction of enzyme inhibitors with insecticides on *S. exigua* (Hübner) and *S. litura* (Fabricius), the laboratory toxicities of emamectin benzoate, chlorfenapyr, indoxacarb, chlorpyrifos, tebufenozide and methomyl on the 3-instars were determined using topical application (Liu *et al* 2011). The results indicated that the relative toxicities of emamectin benzoate, chlorfenapyr and indoxacarb were high on *S. exigua* and *S. litura* with the LC_{50} range between 0.69 µg/g and 16.32 µg/g, while only chlorpyrifos showed high toxicity to *S. litura* and

tebufenozide to *S. exigua* with LC_{50} 37.66 µg/g and 20.71 µg/g,respectively.The results of interaction of enzyme inhibitors with insecticides indicated that four different enzyme inhibitors of PBO, TPP, DEM, and SV1 showed significant restraint to chlorfenapyr and indoxacarb with the synergism ratio between 0.25 and 0.96, while showed synergism to emamectin benzoate, tebufenozide on *S. exigua* and emamectin benzoate, chlorpyrifos on *S. litura* with the synergism ratio between 1.22 and 16.2.

Endosulfan in combination with piperonyl butoxide (PBO) (1:10) showed synergistic factors of 4.90 and 16.71 by topical application and 5.99 and 8.45 by leaf sandwich ingestion. The same insecticide in combination with triphenyl phosphate (TPP) (1:10) showed synergistic ratios of 1.13 and 2.95 by topical application and 1.19 and 1.57 by leaf sandwich method at LD_{50} and LD_{90}, respectively. Thus, the resistance in *S. litura* to endosulfan was largely due to mixed function oxidases and slightly due to esterases (Radhika *et al* 2005[b]).

The role of esterases in the defence mechanism against intoxicant by fenitrothion in susceptible and filed tolerant strains of *S. littoralis* (El-Guindy *et al* 1982), the highest synergistic ratio obtained was that of fenitrothion+ DEF treatment on the field strain (3.28) followed by that of the same treatment in 'S' strain (2.0). Similar treatments with PBO on both strains did not produce any detectable levels of synergism. Therefore esterases could be considered as one of the most important mechanisms in fenitrothion resistance while mixed function oxidases were not.

El-Guindy *et al* (1983) demonstrated that against susceptible strain of cotton leafworm, *S. littoralis*, the toxicity of cypermethrin was not affected by PBO and TBP whereas fenvalerate exhibited additive action with TBP but its potency was not affected by PBO. However, apparent levels of antagonism were detected in the resistant strain to the action of pyrethroids when they are combined with either synergist in the ratio of 1:5.

While evaluating the effect of eight synergists on the toxicity of fenvalerate and deltamethrin against susceptible and resistant strains of the fourth instar larvae of *S. littoralis* by topical method, Nassar *et al* (1983) observed a slight level of synergistic effect (synergistic ratio 1.05- 2.65) in case of susceptible strain, while a marked increase in toxicity was achieved (synergistic ratio 1.14- 4.2) in case of resistant strain. Further, they stated that to achieve the highest degree of synergistic effect, the ratio of insecticide to the synergist should be 1:1.

In case of susceptible (S) strain, very slight synergism was recorded with fenvalerate synergist combinations, while antagonism occurred with decamethrin. With the resistant (R) strain, relatively high degree of synergism was brought about and antagonism was never detected. These findings confirm that synergist interfere with or delay penetration in *S. littoralis* larvae. The most accepted hypothesis to explain the mode of action of MDP synergists is that they act as

alternative substances for MFOs and thereby reduce the rate of metabolism and prolong the action of insecticides. DEF as a specific esterase inhibitor synergises different insecticides through inhibition of hydrolytic esterases. Based on these concepts, it was suggested that both hydrolytic and oxidative enzymes are involved, at least in part in the resistance of *S. littoralis* to pyrethroids. However, 'R' larvae still showed substantial resistance even when synergists were applied thereby indicating the possibility of the involvement of other mechanisms such as reduced sensitivity at the target site or reduced rate of cuticular penetration (Riskallah 1984).

Insecticide synergist ratios of 1:1 and 1:10 were more effective than 10:1 to *S. littoralis* when susceptible and permethrin resistant strains were tested with synergists viz., safrol, PBO, sulfoxide and DEF on the toxicity of fenvalerate and decamethrin (Riskallah *et al* 1984). Insecticides were used at the discriminating dose in combination with the synergists in 1:5 and 1:1 ratios, but the 1:1 ratio did not have any effect on tobacco budworm (Subramanyan *et al* 1989).

The physiological mechanism of resistance to carbaryl was investigated by McCord and Yu (1987) in a carbaryl resistant strain of *S. frugiperda*. PBO greatly reduced carbaryl resistance level from 90 to 6, indicating that microsomal P 450 dependent monooxygenases might have played a role in resistance. This finding was consistent with the metabolic data in which the oxidative metabolism of carbaryl by midgut homogenates was five times more active in the resistant than in the susceptible strain. In addition, the resistant strain showed increased activity of microsomal hydroxylation and epoxidation compared to the susceptible strain.

The studies carried out by Joyee *et al* (1988) clearly showed high synergistic factor (22.1) for *S. exigua* and *S. frugiperda* with 1:5 mixture of fenvalerate and PBO and 4.6 for S. *frugiperda* with 1:5 mixture of fenvalerate and MGK-264.

There was a marked synergism of cypermethrin toxicity with PBO resulting synergistic ratio of 2 to 121- fold at LD_{50} level in 3rd instar larvae of *S. litura* and completely restored susceptibility to cypermethrin in all strains tested. It indicated that enhanced detoxification by microsomal P450- dependent monooxygenases was the major mechanism of pyrethroid resistance in Andhra Pradesh. Pre-treatment with the synergist DEF, an inhibitor of esterases and the glutathione S-transferase system, resulted in a 2 to 3 fold synergism with monocrotophos indicating that esterases and possibly glutathione S-transferases were at least to some extent contributing to organophosphate resistance. The fact that full suppression of resistance was never achieved with DEF suggested that at least one or other mechanism was conferring OP resistance (Armes *et al* 1997).

Li (2006) studied the joint action and synergism of synergists (PBO, DEM, TPP, NT, T) with 3 insecticides (beta-cypermethrin, chlorpyrifos, methomyl) in *S. exigua*. Highest synergism (7.9 times) was observed between PBO and beta-cypermethrin within one hour after spotting, and the effect declined thereafter. Synergism increased with the dosage of PBO with the range of 10 to 20 μg/larva and decreased at the dosage of 40μg/larva. The synergism between NT and beta-cypermethrin increased with the dosage of NT, the maximum being 4.4 times. However, NT showed virtually no synergistic effect to chlorpyrifos and methmoyl. DEM showed synergistic effect to chlorpyrifos (2.5 times). NT had some synergistic effect to chlorpyrifos only (2.5 times).

The synergistic ratios of piperonyl butoxide (PBO), o, o-diethyl-o-phenyl-thiophosphate (SV1), triphenyl phosphate (TPP), and diethyl maleate (DEM) between fenvalerate-resistant strain (Fen-R) and susceptible strain of *S. exigua* were 10.2, 7.8, 12.5, and 1.1, and those between alpha-cypermethrin resistant strain (Cyp-R) and susceptible strain were 21.6, 15.5, 8.6, and 1.2, respectively. Significant synergisms of PBO, SV1, and TPP to fenvalerate and alpha-cypermethrin were observed, implying that multifunctional oxidase and carboxylesterase were involved in the resistance to fenvalerate and alpha-cypermethrin (Lan and Zhao 2010).

Toxicity of chlorfenapyr to beet armyworm larvae *S. exigua* (Hübner) and the synergism of TPP, PBO, and DEM to chlorfenapyr were detected by topical application method (Zhang 2009). The results indicated that the LD_{50} of chlorfenapyr to 3rd, 4th and 5th instar beet armyworm larvae were 7.9369 μg/g, 9.1730 μg/g and 8.1467 μg/g, respectively. For the susceptible strains, 24h after treatment, DEM and TPP had either synergism or antagonism and the synergism ratio were 1.15 and 0.92, while PBO had distinct synergism and the synergism ratio was 0.21. Compared to 24h after treated with chlorfenapyr, there were no changes on the effect of DEM and TPP, but the toxicity of chlorfenapyr to *S. exigua* went up. For the resistance strains, DEM had synergism and the synergism ratio was 1.43, while PBO and TPP had slight antagonism and the the synergism ratio were 0.94 and 0.83, respectively. After selection for 12 generation in the laboratory with chlorfenapyr at the dose of LD_{50}, *S. exigua* showed low level resistance (4.7-fold) to chlorfenapyr.

The cross-resistance and biochemical mechanism of the beet armyworm, *S. exigua* (Hübner), to spinosad was studied in the laboratory. The populations were collected from Shanghai suburb. After five generations of selection, the resistance of *S. exigua* to spinosad increased 345.4 times compared with the susceptible strain. There was no cross-resistance between spinosad and fenvalerate, phoxim, methomyl, abamectin, and cyfluthrin. When the inhibitors, PBO, TPP, DEF, and DEM were used as synergist in the susceptible strain and resistant strain, the synergistic ratio was 0.7-, 0.5-, 1.0-, and 0.6- fold for the susceptible strain,

and 9.8-, 1.5-, 2.6-, and 1.5-fold for the resistant strain, respectively. The results revealed that PBO had significant synergistic effect on the resistant strain (Wang *et al* 2006).

Deltamethrin resistance in Del-SEL strain of *S. exigua* from Pakistan was suppressed with the synergists such as piperonyl butoxide (PBO) and *S,S,S*-tributylphosphorotrithioate (DEF), suggesting the involvement of monooxygenase and esterase in the development of resistance in *S. exigua* (Ishtiaq *et al* 2012).

2.4.2 Detoxification enzymes

The metabolic resistance mechanisms are one of the most important mechanisms in which different enzyme systems are involved. Species with history of feeding on heavily chemically defended plants have elevated levels of enzymes that detoxify defensive chemicals. Thus they have enhanced ability to evolve resistance to synthetic toxins (Gordon 1961). Insect detoxification enzymes are distributed in different organs and tissues but the site of highest activity is midgut in most cases and also fat body along with malphigian tubules. These enzymes occur in multiple isoenzyme forms, so they have broad substrate specificity (Lindroth 1991). The major detoxification enzymes are hydrolases, mixed function oxidases and glutathione S-transferases.

2.4.2.1 Hydrolases

Hydrolases catalyse reactions that split ester, amide or phosphate linkages in insecticides by the addition of water to yield an acid and alcohol. The enzymes are ubiquitous and are present in all living organisms including plants, insects and higher animals. The hydrolase activity towards insecticides is generally high only in insecticide resistant insects (Kranthi 2005). Insecticides such as pyrethroids, carbamates and organophosphates have ester, amide and phosphate linkages and hence are readily attacked by hydrolases.

Esterase is the one of important detoxification enzyme in phase I. In insect, esterase is found in cytosol, microsome as well as mitochondria and nuclei. It is classified into main two groups; A- type esterase including arylesterases that are not inhibited by organophosphate. Another one is B-type esterase such as carboxylesterase and cholinesterase. B-type esterase is inhibited by organophosphate due to irreversible phosphorylation of the active serine site (Dauterman, 1985). Carboxylesterase (EC 3.1.1.1) is a multigene family and occurs in animals, plants, insects, and microbes (Satoh and Hosokawa 1998, Marshall *et al* 2003, Ranson *et al* 2002, Bornscheuer *et al* 2002). They are implicated in the resistance of insects to organophosphates, carbamates, and pyrethroids through gene amplification, upregulation and coding sequence

mutations (Li *et al* 2007, Hemingway and Karunaratne 1998). Elevated esterase activities were shown to be responsible for cross resistance to OPs, carbamates and pyrethroids (Zhao *et al* 1996).

Cholinesterases (EC 3.1.1.7) are capable of splitting acetylcholine to yield acetate and choline. The properties of cholinesterase in insects are similar to mammals. It is therefore called either acetylcholinesterase or cholinesterase. These enzymes are very sensitive to organophosphate inhibition (Visetson, 1991).

Resistance to organophosphates was mainly due to high esterase activity and insensitive acetyl choline esterase, while, preliminary evidence indicated that certain specific esterase isozymes contribute to resistance against endosulfan (Kranthi, 1998).

Marked biochemical changes however, being recognized in *S. littoralis* as marked SDS-polyacrylamide gel electrophoresis representing molecular weights in protein showed there are three, two and one bands were found to be specific to pyridalyl treated 4^{th}, 2^{nd}, and 6^{th} larval instars, respectively. Also the activity of both α and ß esterase enzymes analysis showed differences in esterase pattern in the treated 4th instars than control (Dahi *et al* 2011).

The synergism of transcypermethrin in resistant strains of *H. zea*, *S. frugiperda* and *Agrotis ipsilon* by DEF was reported to be due to the inhibition of one or more of the esterases responsible for detoxification of pyrethroids. Electrophoretic studies confirmed the presence of one or more DEF sensitive esterases that hydrolyzed α- and β- naphthyl acetate and were capable of hydrolyzing trans-cypermethrin (Usmani and Knowles 2001).

Saratchandrudu *et al* (1994) studied the development of insecticide resistance in *S. litura* by determining percentage inhibition of activity of acetyl cholinesterase (AChE) in 3^{rd} instar larvae collected from three districts of Andhra Pradesh, India. Lowest inhibition of AChE was generally observed for dimethoate followed by phosphamidon, parathion methyl and monocrotophos in Guntur district.

2.4.2.2 Mixed function oxidases

Mixed function oxidases also referred as mono-oxygenases, are a group of oxidative enzymes that are localised in microsomes of the endoplasmic reticulum and require NADPH as a co-factor. They are abundant in fat bodies, Malpighian tubules and the midgut of insects. The mono-oxygenases constitute a number of components of which cytochrome P450 constitutes the terminal oxidases of the system. The P450 monooxygenases are known to be extremely versatile in their enzymatic properties. Apart from being involved in endogenous metabolism, they play an important role in detoxification of dietary toxins and exogenously applied insecticides. They are

involved in conferring resistance to a wide range of insecticides in various insects (Kranthi 2005). Kranthi *et al* (2001[a]) reported that enhanced synergism by PBO was positively correlated with high levels of cytochrome P450. The mechanism of pyrethroid resistance in *S. litura* was accompanied by enhanced metabolic detoxification of multifunctional oxygenase (MFO). Mechanism of organophosphorus resistance was correlated with an increased MFO and esterase activities (Zhou and Huang 2002). Clarke *et al* (1990) showed that pyrethroid resistance in *H. virescens* was largely due to a PBO-synergisable monooxygenase and that the resistant strains possessed a six-fold higher quantity of total cytochrome P450 than the susceptible strain.

2.4.2.3 Glutathione S- transferases

Glutathione S- transferases (GST) (EC 2.5.1.18) are a group of multifunctional detoxification enzymes catalyzing the conjugation of reduced glutathione (GSH) with electrophilic substrate (Chasseaud 1979). The conjugates are then eliminated from the cell via the glutathione S- conjugate export pump (phase III detoxification system) and subsequently transformed in animals to give excretable mercapturic acids (Dykstra and Dautermann 1978). Thus glutathione dependent conjugation is regarded as an important detoxification mechanism in insects. Glutathione S- transferases have received considerable attention due to their role in insecticide metabolism and resistance in insects.

2.4.2.4 Involvement of multiple detoxification enzymes in resistance

Biochemical assays were performed to determine the potential mechanisms involved in the tolerance to chlorantranoiliprole observed in field populations. Most field populations showed significantly enhanced activities of mixed function oxidase enzymes compared with the susceptible strain. Only a few populations had higher activities of esterase and glutathione-S-transferase than the susceptible strain. No correlations were observed between activities of metabolic enzymes and chlorantraniliprole toxicity, suggesting that these detoxification enzymes were not the main cause of the field tolerance observed in this study, and there might be other mechanisms conferring the tolerance variation to chlorantraniliprole in *S. litura* (Su *et al* 2012). Oxidases and esterases were found to be important mechanisms mediating pyrethroid resistance in *H. armigera* in India (Kranthi *et al* 1997) and Australia (Gunning 1994).

The carboxylesterase activities in the fourth instar larvae of cypermethrinr resistant strain and fenvalerate resistant strains were 1.9 and 2.2 folds of the corresponding susceptible strains, respectively, but no differences were found in the glutathione-S-transferase activities between the resistant and susceptible strains, which indicated that carboxylesterase played an important role in

the resistance of *S. exigua* to fenvalerate and alpha-cypermethrin, while glutathione-S-transferase contributed little to the resistance. There were no significant differences in the Na-K-ATPase activities between the resistant and susceptible strains, but the inhibition of fenvalerate and alpha-cypermethrin on Na-K-ATPase was higher in the susceptible strains than in the resistant strains, indicating the decreased sensitivity of Na-K-ATPase in resistant strains (Lan and Zhao 2010).

The activity of GST was induced significantly from 6h to 24h after treating beet armyworm larvae with chlorfenapyr. The content of cytochrome P450 was also induced by chlorfenapyr. The specific activity of GST and superoxide dismutase, content of P450 were higher in the resistant strain than those in the susceptible strain, the ratio of enzymatic activity of GST, P450 and superoxide dismutase to susceptible strain was 2.01, 1.36 and 1.1 respectively, and the difference was significant; the ratio of enzymatic of CarE to susceptible was 0.6; the ratio of enzymatic of peroxidase and catalase to susceptible was 0.98 and 0.95, respectively, there were no significant differences (Zhang 2009).

Two field strains of the fall armyworm, *S. frugiperda* (J E Smith), collected from corn in north Florida showed high resistance to carbaryl (626 and 1159-fold) and moderate resistance to parathion-methyl (30 and 39-fold) as compared with a laboratory susceptible strain. No cross-resistance to indoxacarb, a novel oxidiazine insecticide, was observed. Biochemical studies revealed that activities of detoxification enzymes (microsomal oxidase, glutathione *S*-transferase and general esterase) were significantly higher in the field strains than in the susceptible strain, indicating that these detoxification enzymes were not actively involved in the resistance to indoxacarb (Yu and McCord 2007).

The *in vitro* activity of microsomal-*O*-demethylase and glutathione *S*-transferase in the resistant strain of *S. exigua* in Shanghai was 5.2- and 1.0-fold of the susceptible strain, respectively. The results implied that microsomal-*O*-demethylase might be important in conferring spinosad resistance in the *S. exigua* population (Wang *et al* 2006).

Huang and Han (2007) studied the mechanisms for multiple resistances in China. Bioassay revealed that the two field strains of *S. litura* were both with high resistance to pyrethroids (RR: 63–530), low to medium resistance to organophosphates and carbamates, AChE targeted insecticides (RR: 5.7–26), and no resistance to fipronil (RR: 2.0–2.2). Selection with deltamethrin in laboratory could obviously enhance the resistance of this pest to both pyrethroids and AChE targeted insecticides. Synergism test, enzyme analysis and target comparison proved that the pyrethroid resistance in this pest associated only with the enhanced activity of cytochrome P450 monooxygenase (MFO) and esterase. However the resistance to the AChE targeted insecticides depended on the target insensitivity and also the enhanced activity of MFO

and esterase. Thus, the cross resistance between pyrethroids and the AChE targeted insecticides was thought to be resulted from the enhanced activity of MFO and esterase. In another set of biochemical studies in Korea Republic with laboratory selected strains showed that esterase was associated with both pyrethroid and phosphate resistance in *S. litura* (Cho *et al* 1999).

A strain of the fall armyworm, *S. frugiperda* (J.E. Smith), collected from corn in Citra, Florida, showed high resistance to carbaryl (562-fold) and methyl parathion (354-fold) (Yu *et al* 2003). Biochemical studies revealed that various detoxification enzyme activities were higher in the field strain than in the susceptible strain. In larval midguts, activities of microsomal oxidases (epoxidases, hydroxylase, sulfoxidase, N-demethylase, and O-demethylase) and hydrolases (general esterase, carboxylesterase, β- glucosidase) were 1.2- to 1.9- fold higher in the field strain than in the susceptible strain. In larval fat bodies, various activities of microsomal oxidases (epoxidases, hydroxylase, N-demethylase, O-demethylases, and S-demethylase), glutathione S-transferases (CDNB, DCNB, and p-nitrophenyl acetate conjugation), hydrolases (general esterase, carboxylesterase, β- glucosidase, and carboxylamidase) and reductases (juglone reductase and cytochrome c reductase) were 1.3- to 7.7-fold higher in the field strain than in the susceptible strain. Cytochrome P450 level was 2.5-fold higher in the field strain than in the susceptible strain. In adult abdomens, their detoxification enzyme activities were generally lower than those in larval midguts or fat bodies; this is especially true when microsomal oxidases are considered. However, activities of microsomal oxidases (S-demethylase), hydrolases (general esterase and permethrin esterase) and reductases (juglone reductase and cytochrome c reductase) were 1.5 to 3.0-fold higher in the field strain than in the susceptible strain. Levels of cytochrome P450 and cytochrome b5 were 2.1 and 1.9-fold higher, respectively, in the field strain than in the susceptible strain. In addition, acetylcholinesterase from the field strain was 2- to 85-fold less sensitive than that from the susceptible strain to inhibition by carbamates (carbaryl, propoxur, carbofuran, bendiocarb, thiodicarb) and organophosphates (methyl paraoxon, paraoxon, dichlorvos), insensitivity being highest toward carbaryl. Kinetics studies showed that the apparent Km value for acetylcholinesterase from the field strain was 56% of that from the susceptible strain. The results indicated that the insecticide resistance observed in the field strain was due to multiple resistance mechanisms, including increased detoxification of these insecticides by microsomal oxidases, glutathione S- transferases, hydrolases and reductases, and target site insensitivity such as insensitive acetylcholinesterase.

Janarthanan *et al* (2003) compared the esterase activity of various populations of *S. litura*, collected from cotton fields of different regions of Tamil Nadu, India between October and

December 2001 using α - and β- naphthyl acetate as substrates for enzyme reaction. Among the ten populations analysed, five populations showed elevated activities for esterases.

To screen insecticides with high effect against *S. litura* and safe to *Bombyx mori* and guide scientific and rational application of pesticides in mulberry field, the selective toxicity of 14 insecticides between *S. litura* and *B. mori* was investigated by leaf dipping method in the laboratory (Lan and Huang 2009). The results showed that the toxicity order of 14 insecticides to *S. litura* was arranged as follows: Indoxacarb emamectin benzoate chlorfenapyr methoxyfenozide spinosad deltamethrin lambda-cyhalothrin fipronil chlorpyrifos tebufenozide abamectin profenofos beta-cypermethrin methomyl. The toxicity order to *B. mori* was arranged as follows: emamectin benzoate abamectin deltamethrin spinosad beta-cypermethrin indoxacarb lambda-cyhalothrin chlorpyrifos tebufenozide methomyl fipronil profenofos methoxyfenozide chlorfenapyr.The selective toxicity of chlorfenapyr was the highest among 14 insecticides tested, and its selective toxicity ratio (STR, *B. mori* LC_{50} / *S. litura* LC_{50}) reached 36.26. Indoxacarb showed rather high selective toxicity, and STR was 5.56. The seletctive toxicity of abamectin was the lowest.The results suggest that chlorfenapyr is an insecticide suitable to control *S. litura* in mulberry field and safe to *B. mori*.

2.5 Molecular studies for insecticide resistance

Conventional detection of resistance is based on insecticide susceptibility tests that consist of dose mortality experiments usually performed in the laboratory. Resistance management requires more effective techniques for detecting resistance in its early stages of development (Shah *et al* 2002). Molecular diagnostics have been postulated to increase accuracy and reduce the variability associated with insecticide bioassays that result from both intrinsic (genetic structure) and extrinsic (bioassay conditions, sample size, etc.) factors (Brown and Brogdon 1987, Ffrench-Constant and Roush 1990).

Molecular markers developed after the discovery of restriction endonucleases and the PCR have been proving as powerful tools in analysing the phylogeny, evolution, ecology and population dynamics of insects (Symondson and Lidell 1996). A molecular marker is a specific gene/ DNA sequence on a chromosome that is associated with a particular gene or trait. The molecular variation in this sequence due to mutation (natural during adaptation/ induced) result in alteration in the nucleotide sequence that can be detected with molecular marker that has been developed based upon this altered sequence. A genetic marker may be a short DNA sequence such as SNP, or a long one like minisatellites. Some commonly used types of genetic markers are RFLP, AFLP, RAPD, microsatellite, SNP, SFP and DArT. RAPD -a dominant PCR based marker

has proved a powerful tool in organisms with previously unknown molecular characteristics for analysis of genetic variation, taxonomic studies, biotype identification and geographical distribution (Lima *et al* 2002).

Heckel *et al* (1995) showed that RAPD technique generated a sufficient number of DNA markers to allow a linkage analysis of the gene responsible for the difference in resistance among the the resistant and susceptible strain of *Plutella xylostella*.

Lima *et al* (2002) used RAPD markers in Brazil to estimate the genetic relatedness in different populations of *Bemisia tabaci*. A total of 72 markers were generated by five RAPD primers. All the primers produced patterns that clearly distinguished the *Bemisia* biotypes and two other whitefly species.

Among the 40 random primers screened to reveal the existence of polymorphism between 6 different ecotypes of *S. litura*, eight showed scorable banding patterns and three primers (OPA-01, OPA-05, OPM-01) exhibited distinguishable banding patterns. However, Chengalpattu and Chennai populations revealed their closed relatedness and Coimbatore population stood distantly from others (Janarthanan *et al* 2002). Furthermore, specific amplification for esterase gene using custom made primers produced amplified product in resistant populations (Janarthanan *et al* 2003).

Ferguson and Pineda (2010) used RAPD- PCR to identify polymorphic genomic DNA that would discriminate among cyromazine-resistant, abamectin-resistant, and susceptible *Liriomyza trifolii* leaf miners (Burgess) (Diptera: Agromyzidae). Using a reference strain that was susceptible to both cyromazine and abamectin, and a cyromazine-resistant strain and an abamectin-resistant strain, 400 oligonucleotides were assayed using RAPD-PCR. Two oligonucleotides, B10 and G16, amplified unique bands in the cyromazine-resistant strain but not in the reference or abamectin-resistant strains. Three oligonucleotides, K04, J13, and I02, showed polymorphisms unique to the abamectin-resistant strain but not in the reference or cyromazine-resistant strain.

Shah *et al* (2002) had used RAPD markers for investigating its role in pesticide resistance monitoring in susceptible and propargite- resistant strains of *Tetranychus urticae* Koch. The DNA products obtained with the primer B8 were reproducible but inconsistent causing heterogenneity of PCR banding patterns. Sharma *et al* (2006) showed that RAPD-PCR analysis of imidacloprid treated whiteflies produced amplicons that were exclusively present in the selected population of whiteflies.

Andreev *et al* (1994) developed a PCR diagnostic for cyclodiene insecticide resistance in the red flour beetle, *Tribolium castaneum.* They cloned and sequenced a partial cDNA of the

cyclodiene insecticide resistance gene *Rdl*, a γ-aminobutyric-acid-gated chloride-ion channel. This cDNA spanned exon 7, the region containing the resistance-associated mutation, and part of exon 8. An 'allele-specific' oligonucleotide primer, carrying the resistance-associated mutation at its 3′ end, was used in combination with a flanking 'allele-independent' primer in the polymerase chain reaction to selectively amplify a single resistance-associated mutation from all seven strains collected worldwide. A similar molecular diagnostic based upon PCR-mediated amplification of specific alleles (PASA) was capable of detecting endosulfan-resistant in coffee berry borer *Hypothenemus hampei* (Ferrari) using total DNA isolated from adults, larvae or eggs of the insect pest (Ffrench-Constant *et al* 1994).

Rosario-Cruz *et al* (2009) evaluated susceptibility of Mexican *Rhipicephalus microplus* tick populations to synthetic pyrethroids (SP's) and the two major resistance mechanisms whereas esterase activity did not correlate with SP's resistance, the same correlated significantly with presence of the sodium channel mutation and this resistance could be measured by PASA and Larval Packet Test (LPT). Similarly Qui *et al* (2007) reported that 64 fold resistance in house fly *Musca domestica* to deltamethrin was synergized by PBO. The 5' flanking sequence of the cytochrome P450 gene CYP6D1 in the resistant strain had a 15-bp insert.

Several workers have used PCR-RFLP based diagnostic test to identify resistance alleles at the amplified esterase locus in *Culex pipiens* (Berticat *et al* 2000), modified AChE or knockdown resistance (*kdr*) in *Myzus persicae* (Cassanelli *et al* 2005) and pyrethroid and organophosphate resistance in Q biotype of *Bemisia tabaci* (Tsagkarakou *et al* 2009). Chou *et al* (2010) used a PCR-RFLP based diagnostic test for detecting the presence of an OP-resistance allele of the *ace* gene in populations of *Bactrocera dorsalis* in Hawaii. Thus simple molecular diagnostics serve as accurate and robust and their exploitation with classical resistance bioassays can be useful both for preventing ineffective insecticide applications and for early identification of resistant populations (Tsagkarakou *et al* 2009).

P450s and their associated P450 reductases comprise the only metabolic system that can mediate resistance to all classes of insecticides because of their genetic diversity, broad substrate specificity, and catalytic versatility (Feyereisen 2005). More than 660 insect P450 genes, distributed in *CYP4*, *CYP6*, *CYP9*, *CYP12*, *CYP15A*, *CYP18A*, *CYP28A*, *CYP29A*, *CYP48*, *CYP49*, *CYP301-CYP318*, *CYP319A*, *CYP321A*, *CYP324*, *CYP325*, *CYP329*, and *CYP332-CYP343* families and subfamilies, have been characterized in genome sequencing projects and smaller resistance research projects (Li *et al* 2007, Ranson *et al* 2002, Tijet *et al* 2001).

Various molecular mechanisms for P450- mediated resistance might be involved and several studies have corroborated on the changes in insecticide resistance in different insects with

changes in nucleotide sequences in this region. Increased expression is achieved through mutations and insertions/deletions (indels) in *cis*- acting promoter sequences and/or trans- acting regulatory loci (Li *et al* 2007).

In *M. domestica*, alleles of *CYP6A1* (on chromosome 5) and *CYP6D1* (on chromosome 1) in insecticide-resistant Rutgers and Learn pyrethroid-resistant (LPR) strains are downregulated by factors derived from chromosome 2 of susceptible strains, as evidenced by reduction of transcription of both resistance genes to the level in susceptible strain when both copies of chromosome 2 in crosses were from susceptible strains (Cariño *et al* 1994, Liu and Scott 1996). These observations suggest that overexpression of *CYP6A1* and *CYP6D1* alleles is due at least in part to loss-of-function mutations in negative regulatory loci on chromosome 2 of the Rutgers and LPR strains (Li *et al* 2007). In the case of the house fly *CYP6A1* and *CYP6D1*, hypertranscription of *CYP6A2* and *CYP6A8* in the DDT and malathion-resistant 91-R and MHIIID23 strains is due at least in part to loss-of function mutations in the repressor loci (Maitra *et al* 2000). Genomic changes in the cis acting element, the 5'-promoter region of the overexpressed CYP6G1 alleles in *Drosophila melanogaster* and its sister species *D. simulans* appear to contribute to resistance to DDT (Catania *et al* 2004). Point mutations have been reported to play a secondary role in P450-mediated resistance. Varying number of amino acid substitutions exist in the *CYP6X1, CYP6D1, CYP6D3,* and *CYP6A2* sequences of resistant strains of *D. melanogaster* (Feyereisen 2005, Kasai and Scott 2001, Scott 1999, Zhu and Snodgrass 2003).

Chapter – III

MATERIAL AND METHODS

The present studies on "Evaluation of insecticide resistance profile in *S. litura* (Fabricius) populations through biological, biochemical and molecular diagnosis" were conducted in Toxicology Laboratory and Insect Molecular Biology Laboratory, Department of Entomology and Department of Biochemistry at Punjab Agricultural University, Ludhiana.

3.1 Test insect

Tobacco caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae)

3.2 Raising of food plants for the test insect

Castor plants were sown in the field as well as collected from the nearby areas. Insect free leaves were used for rearing *S. litura* and for conducting bioassay experiments.

3.3 Collection of test insect

The populations comprising of egg masses and larvae of *S. litura* were collected from infested plants in various pulse growing areas of Punjab (Plate 1) viz; Jagraon Ludhiana (30° 47' N, 75° 29' E), Sangrur (30° 52' N, 75° 88' E), Bathinda (30.11°N, 74.56°E) and Hoshiarpur (31° 32' N, 75° 57' E) during the period from August to October of 2010 and May and October 2011 and brought to the laboratory in perforated polythene bags and/ or plastic containers covered with muslin along with infested leaves.

3.4 Rearing of test insect

Populations of *S. litura* were reared on fresh leaves of castor (*Ricinus communis*) leaves in an B.O.D. incubator (Mfd. by M/S Macro Instruments, Ambala) maintained at 26 ± 1˚C and 65 ± 5 per cent relative humidity (Plate 2). The larvae were placed in glass jars (10 x15 cm) and covered with a piece of muslin fastened with rubber bands around its rim. Leaves were changed daily. The mature larvae were transferred to battery jars containing sieved and sterilized sand layer of about 10 cm for pupation. The pupae were then collected and transferred into separate glass jars covered with muslin and secured with rubber bands and kept till the moths emerge. The emerging adults were sexed and transferred into separate battery jars. A cotton swab dipped in 10 per cent honey solution was hung from top of the muslin covering the mouth of the jars. The

honey solution was renewed every 24 hours. Paper strips were hanged alongside the walls of the jars for oviposition. The deposited egg clusters were collected daily and kept on castor leaves in glass jars. Fresh castor leaves were provided to the growing larvae every day. Under these conditions, the life cycle of *S. litura* was recorded to be around 30 days (Plate 3).

3.5 Test insecticides

Commercially available insecticides viz; chlorpyriphos, acephate, thiodicarb, fenvalerate, novaluron, indoxacarb, pyridalyl and chlorantraniliprole were used for bioassay of test insect (Table 1).

3.6 Monitoring of insecticide resistance in *S. litura* populations from different locations in Punjab to selected insecticides.

3.6.1 Bioassay by leaf dip method

Tender succulent castor leaves were cleaned and used to cut out 10 cm leaf discs. Serial dilutions of various formulations viz., chlorpyriphos, acephate, thiodicarb, fenvalerate, novaluron, indoxacarb, pyridalyl and chlorantraniliprole were prepared in water. The discs were dipped in test concentrations for 20 sec and then air dried at room temperature (Plate 2). Castor leaves dipped in water alone were used as control. Ten third instar larvae (~30 mg) were placed in glass jars containing 3 leaf discs treated with required concentration of an insecticide. There were three replications, in each treatment.

3.6.2 Observations

The larvae were allowed to feed and larval mortality was recorded for 24, 48 and 72 hours. A larva was considered dead if it failed to move in coordinated manner, when prodded with a blunt needle.

Insect mortality in control was rare, but where necessary, corrections were made by using Abbott's formula (1925).

$$\text{Corrected mortality (\%)} = \frac{\text{Mortality in treatment (\%)} - \text{Mortality in control(\%)}}{100 - \text{Mortality in control (\%)}} \times 100$$

Table 1. Insecticides used against *S. litura*

Insecticides	Formulation	Brand	Chemical name	Insecticide group	Source
Fenvalerate	25 EC	Sumicidin	(RS)-*alpha*-Cyano-3-phenoxybenzyl (RS)-2-(4-chlorophenyl)-3-methylbutyrate	Synthetic pyrethroid	New Chemical Industries Ltd, Gujarat
Chlorpyriphos	20 EC	Dursban	*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate)	Organo-phosphate	Dow Agro Sciences India Pvt Ltd
Acephate	75 SP	Asataf	*N*-(Methoxy-methylsulfanylphosphoryl)acetamide	Carbamate	Rallis India Ltd
Thiodicarb	75WP	Larvin	dimethyl n, n'-thiobis(methylimino) carbonyloxy bis ethanimidothioate	Carbamate	Bayer Crop Science Ltd
Novaluron	10 EC	Rimon	(*RS*)-1-[3-chloro-4-(1,1,2-trifluoro-2-trifluoromethoxyethoxy)phenyl]-3- (2,6-difluorobenzoyl)urea	Benzophenyl urea	Makhtheshim Chemical Works Ltd Israel
Indoxacarb	15.8EC	Avaunt	(S)-7-chloro-2-[methoxycarbonyl-(4-trifluoromethoxyphenyl)-carbamoyl]-2,5-dihydroindeno[1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylic acid, methyl ester	Oxadiazine	EI Dupont India Pvt Ltd
Pyridalyl	10 EC	Sumipleo	2,6-dichloro-4-(3,3-dichloro-2-allyloxy)phenyl-3-[5-(trifluoromethyl)-. 2-pyridyloxy]propyl ether.	Unclassified	Somitomo Industries Pvt. Ltd.
Chlorantranili-prole	20SC	Coragen	3-bromo-4'-chloro-1-(3-chloro-2-pyridyl)-2'-methyl-6'-(methylcarbamoyl)pyrazole-5-carboxanilide	Anthranilic diamide	Sigma Aldrich Ltd.

3.6.2 Statistical Analysis

The log concentration-mortality regression was estimated by probit analysis using the POLO programme based on calculations given by Finney (1971). The values of LC_{50}, heterogeneity (χ^2), intercept (a), slope of the regression line (b) and fiducial limits were calculated. The toxicity ratios were worked out by dividing the LC_{50} value of the least toxic insecticide with the LC_{50} value of the insecticide in question. The degree of resistance acquired by *S. litura* (Resistance ratio) was calculated by dividing the higher LC_{50} value of a population with a lower LC_{50} value of another population for each insecticide.

3.7 Investigation of the mechanisms of insecticide resistance in the *S. litura* population through synergism assays

3.7.1 Generation of susceptible population (SUS)

The susceptible population (SUS) was reared on castor leaves in an insecticide free environment for 11 generations. The population was initially collected from Hoshiapur area (HSP) that was found to be most susceptible to fenvalerate among the four populations of *S. litura.*

3.7.2 Selection of *S. litura* population with fenvalerate

S. litura population collected from Sangrur (SGR) that was found to be the least susceptible, was selected with fenvalerate for 14 generations. The progeny of field- collected SGR population was exposed to a series of concentrations and LC_{50} was determined. The individuals surviving from the treated population at concentrations $\geq$ LC_{50} were collected and reared on a fresh leaves to obtain a batch of first selected generation that was designated as F_1. The larvae from F_1 generation were also exposed to single selective concentration equivalent to LC_{50} value. The 3rd instar larvae of F_2 were treated with different concentrations to calculate LC_{50} for that generation and individuals surviving at $\geq LC_{50}$ were reared further. This LC_{50} value generated was used to select two subsequent generations. Similar procedure was followed for the F_5, F_8 and F_{11} generations where LC_{50} values were worked out and subsequently used for selection of two succeeding generations. The number of larvae subjected to selection in each generation varied depending upon the number and vigour of the preceding generation. Finally, the LC_{50} values were calculated for the F_{14} generation, designated as SGR-Sel.

The insecticide resistant and susceptible populations of *S. litura* were used to assess the mechanism of resistance through synergism studies.

3.7.3 Test chemicals

Synergists used in the study are as follows:

i) Piperonil butoxide (3,4-methylenedioxy-6-propylbenzyl-n-butyl diethyleneglycolether) or PBO from Sigma Aldrich Co.

ii) Diethyl maleate (DEM) from Himedia Laboratories Pvt Ltd

iii) Tri phenyl phosphate (TPP) from Loba Chemie Lab Reagents and Fine Chemicals

3.7.4 Methodology

S. litura populations identified as susceptible (SUS), resistant (SGR) and fenvalerate selected population (SGR-Sel) were tested to assess the degree of PBO/ DEM/ TPP mediated resistance acquired. The insecticide was tested alone and in combination with synergists (1:10). The dilutions required were prepared from commercial formulations. The 3rd instar larvae were treated using leaf dip bioassays. There were three replications with 10 larvae each. The mortality data were subjected to probit analysis and LC_{50} and LC_{90} values were calculated.

3.7.5 Observations to be recorded

The mortality data was recorded after 48 hrs of treatment. A larva was considered dead if it failed to move in coordinated manner, when probed with a blunt needle.

3.7.6 Statistical analysis

The mortality data was subjected to Abbott's correction (Abbott, 1925) before computing LC_{50} values by Probit Analysis (Finney, 1971). Synergistic factor was calculated by comparing the LC_{50} values of an insecticide with that of LC_{50} value of an insecticide+ PBO/DEM/TPP mixture. On the basis of resistance and synergistic factors, the degree of metabolic mediated i.e., PBO, DEM and GST suppressible resistance was determined.

3.8 Investigation of the mechanisms of insecticide resistance in the *S. litura* population through enzyme assays

3.8.1 Test insect

The field collected populations from Sangrur and Hoshiarpur (SGR, HSP) and SGR-Sel and SUS were used to estimate the detoxification enzymes associated with resistance.

3.8.2 Reagents and chemicals

1. Mono sodium dihydrogen phosphate anhydrous (S. d. fine-chem Ltd, Mumbai)
2. Di sodium hydrogen phosphate anhydrous (S. d. fine-chem Ltd, Mumbai)
3. EDTA (S. d. fine-chem Ltd, Mumbai)
4. Dithiothreitol (DTT) (Sisco Res Lab Pvt Ltd)
5. 1-phenyl, 2- thiorea (PTU) (Sigma Aldrich Co.)
6. Phenyl methane sulfonyl fluoride (S. d. fine-chem Ltd, Mumbai)
7. Triton X 100 (S. d. fine-chem Ltd, Mumbai)
8. p-nitro anisole (Sigma Aldrich Co.)
9. Nicotinamide adenine dinucleotide phosphate reduced tetra sodium salt (NADPH) (Sisco Res Lab Pvt Ltd)
10. p-nitro phenol (S. d. fine-chem Ltd, Mumbai)
11. 1-chloro 2, 4- dinitro benxene (CDNB) (S. d. fine-chem Ltd, Mumbai)
12. Glutathione reduced (GSH) (Sisco Res Lab Pvt Ltd)
13. Acetyl thiocholine iodide (ATChI) (Himedia Labs Pvt Ltd)
14. 5,5'-dithiobis dinitro benzene Ellman's Reagent (DTNB) (S. d. fine-chem Ltd, Mumbai)
15. Chlorpyriphos (Sigma Aldrich Ltd India)
16. α- naphthyl acetate extra pure AR (Sisco Res Lab Pvt Ltd)
17. α- naphthol (S. d. fine-chem Ltd, Mumbai)
18. Acetone (S. d. fine-chem Ltd, Mumbai)
19. Fast Blue RR (Sigma Aldrich Co.)
20. Sodium dodocyl sulphate (SDS) (S. d. fine-chem Ltd, Mumbai)
21. Sodium hydroxide (S. d. fine-chem Ltd, Mumbai)
22. Copper sulphate (S. d. fine-chem Ltd, Mumbai)
23. Sodium potassium tartarate (S. d. fine-chem Ltd, Mumbai)
24. FC Reagent (S. d. fine-chem Ltd, Mumbai)
25. Bovine Serum Albumin (Sigma Aldrich Co.)

3.8.3 Estimation of cytochrome P450 monooxygenase/ MFO activities

MFO activity / p-nitroanisole o-demethylase (ODM) assay was done by modifying methods given by Hansen and Hodgson (1971) and Kranthi (2005).

a) Reagents: The following reagents were used.

i) 0.1 M sodium phosphate buffer, pH 7.6

ii) 50.0 mM *p*-nitroanisole (38.28 mg dissolved in 5 mL ethanol)

iii) 10 mM NADPH in sodium phosphate buffer

b) Enzyme preparation: The midgut from five fifth instar larvae were homogenized with 5 mL homogenization buffer (0.1 M sodium phosphate buffer, pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PTU, and 1 mM PMSF). After centrifugation at 10,000 rpm for 30 min at 4˚C, the supernatant was collected and used as enzyme resource for analysis of the activity of MFO (Kranthi 2005).

c) Enzyme assay: The assay mixture consisted of 1 mL enzyme solution, 40 µL of 50.0 mM *p*-nitroanisole and 760 µL of 0.1M sodium phosphate buffer (pH 7.6). The mixture was incubated for 2 min at 34˚C and then the reaction was initiated by adding 200 µL, 10.0 mM NADPH. Change in absorbance was recorded at 405 nm at 15 sec interval for 20 min. The activity of MFO was expressed in terms of nM of p-nitrophenol formed min^{-1} mg^{-1} protein).

$$\text{MFO activity} = \frac{\text{Change in absorbance} \times \text{volume of reaction mixture} \times 1000}{\text{time} \times \text{Ɛ} \times \text{protein (mg)}}$$

where

Ɛ is the extinction coefficient = 3.32 $mM^{-1}cm^{-1}$

time is total time for which change was observed (min)

3.8.4 Estimation of glutathione S-tranferases

Glutathione transferases catalyse the conjugation of 1-chloro, 2, 4-dinitrobenzene (CDNB) with reduced glutathione (GSH) to produce a yellow product that has an absorbance maxima at 340 nm and the rate of product formation that indicates the enzyme activity can be calculated by following the increase in absorbance at 340 nm. Extraction and estimation of glutathione S- transferase was done using methodology by Chien and Dauterman (1991).

a) Reagents: The following reagents were used:

i) Ethanolic CDNB (1-chloro-2, 4-dinitro benzene): 10mM CDNB was prepared in 95% ethanol as enzyme substrate.

ii) Reduced glutathione (GSH): 50mM GSH was prepared in distilled water.

iii) Sodium phosphate buffer: 0.1 M pH7.6 containing 1mM phenylthiourea.

b) Extraction of enzyme: The 2% w/v homogenate was prepared in buffer and centrifuged at 10,000 rpm for 30 min. The pellet was discarded and the supernatant was recentrifuged at 10,000 rpm for 20 min. The pellet was discarded again and the supernatant obtained was used for assay.

c) Assay method: The assay mixture consisted of 30 µL of ethanolic CDNB solution, 100 µL GSH solution and 50 µL of crude enzyme solution with 0.1M sodium phosphate buffer (pH 7.6) containing PTU in a total volume of 2 mL. Absorbance was recorded at 340 nm at intervals of 1 min for 5 min. The activity of GST was expressed in terms of µM of CDNB conjugated min^{-1} mg^{-1} protein.

$$\text{GST activity} = \frac{\text{Change in absorbance} \times \text{volume of reaction mixture} \times 1000}{\text{time} \times \varepsilon \times \text{protein (mg)}}$$

where

Ɛ is extinction coefficient of CDNB= 9.6 $mM^{-1}cm^{-1}$

time is total time for which change was observed (min)

3.8.5 Estimation of Acetyl choline esterase (Ellman *et al* 1961)

Acetyl choline esterase (AChE) activity was examined by the method of Ellman *et al* (1961) using acetyl thiocholine as substrate with slight modification. Enzyme inhibition by organophosphate compound and their mixtures was determined in the presence of substrate (Zhu *et al* 1996).

a) Reagents: The following reagents were used:

i) 0.1 M sodium phosphate buffer (pH 8.0)

ii) 0.1 M acetyl thiocholine iodide (ATChI) in sodium phosphate buffer

iii) 0.01 M 5, 5'- dithiobis (2-nitrobenzenzoate) (DTNB) in sodium phosphate buffer (0.1M, pH 8.0 containing sodium carbonate)

b) Enzyme preparation: Whole body of 3rd instar larvae were used for enzyme preparation. Insects were homogenized with pre chilled teflon glass homogenizer using ice cold 0.1 M sodium phosphate buffer (@500µl/ insect), pH 7.6, containing 0.05% Triton X-100 to assist solubilising membrane bound enzyme. The crude homogenate was centrifuged at 10,000 rpm for 20 min at 4˚C. The pellet was discarded and aliquots of the supernatant were taken for determination of esterase and acetylcholine esterase activity and protein content as well (modified from Huang and Han 2007).

c) Enzyme assay: To the 4 mL sample cuvette, 2.86 mL of sodium phosphate buffer and 100 µl of the enzyme was added and this was incubated at room temperature for 5 minutes. To this, 10 µL of DTNB solution and 30 µL ATChI were added. In the reference cuvette, 10 µL DTNB and 30 µL ATChI were added to 2.96 mL sodium phosphate buffer. The rate of colour production was measured at 412 nm for 30 minutes against the blank.

The enzyme activity was measured by following the increase of yellow colour produced from thiocholine when it reacts with dithiobisnitrobenzoate ion. It is based on the coupling of these reactions:

Acetylthiocholine + Enzyme → thiocholine + acetate

Thiocholine + dithiobisnitrobenzoate → yellow coloured complex

The AChE activity was expressed as µmoles of acetyl choline hydrolysed min^{-1} mg^{-1} protein

$$\text{AChE activity} = \frac{\text{Change in absorbance} \times \text{volume of reaction mixture} \times 1000}{\text{time} \times \varepsilon \times \text{protein (mg)}}$$

where

ε is the extinction coefficient of the chromophore at 412 nm= 1.36 X 10^4 $mM^{-1}cm^{-1}$

time is time is total time for which change was observed (min)

3.8.6 Determination of I_{50} for acetylcholine esterase

Inhibition studies were carried out by adding 0.1 mL of variable concentrations of insecticide to the reaction mixture along with the enzyme extract as given in 3.8.5. The activity of acetylcholine esterase was determined at different concentrations of organophosphate chlorpyriphos. The activity and concentrations were plotted on a graph and the concentration of insecticide at which the activity reduced to 50 per cent was determined.

3.8.7 General esterases

Determination of general esterase activity was done using α naphthyl acetate as substrate (Van Aspersen (1962).

a) Reagents: The following reagents were used.

i) 0.04M phosphate buffer pH 6.8

A= mono sodium dihydrogen phosphate 0.2 M (15.6 g in 100 mL double distilled water)

B= di sodium hydrogen phosphate (17.798 g in 100 mL of double distilled water).

Solutions A and B were mixed in ratio of 51:49 and made the volume upto 200 mL to get 0.2 M phosphate buffer and then dilute it to make 0.04M.

ii) α- naphthyl acetate (0.03 mM) in 0.04 M sodium phosphate buffer.

Stock solution of α- naphthyl acetate (0.3 mM) was prepared by adding 0.9188 mg in 20 mL acetone and 1 mL of stock solution was diluted with 0.04M phosphate buffer to make 100 mL susbstrate solution.

iii) Staining solution was prepared fresh by mixing 2 parts of 1 % Fast Blue RR salt w/v (in 0.04M phosphate buffer pH 6.8) and 5 parts of 5 % sodium dodecyl sulphate (SDS) w/v in double distilled water.

b) Enzyme preparation: As given in 3.8.5

c) Enzyme assay: The assay mixture was prepared with 1mL of enzyme (10µL of enzyme stock + 990 µL phosphate buffer 40 mM, pH 6.8) and 5 mL substrate solution. The tube was incubated for 30 min at 37˚C in dark with occasional shaking at 10 min interval. The reaction was stopped and colour developed by adding 1 mL of staining solution. The absorbance of the sample was recorded against reagent blank at 590 nm. The enzyme activity was calibrated from the α-naphthol standard curve. It was prepared as follows:

A stock solution of 50 µg/mL α-naphthol was prepared using acetone. The subsequent dilutions were made in buffer. Absorbance of each of the diluted standard concentrations was read and plotted against concentration.

Esterases activity was expressed in terms of µg of α-naphthol produced min^{-1} mg^{-1} protein.

$$\text{Esterase activity} = \frac{\text{Concentration of standard} \times \text{OD of test} \times \text{total volume}}{\text{OD of test} \times \text{volume of enzyme} \times \text{protein (mg)}}$$

3.8.8 Estimation of total proteins (Lowry *et al* 1951)

a) Reagents: The following reagents were used.

Reagent A: 2 per cent sodium carbonate in 0.1N sodium hydroxide

Reagent B: 0.5 per cent copper sulphate in 1 per cent solution of sodium potassium tartarate.

Reagent C: Freshly prepared by mixing 50 mL of reagents A and 1 mL of reagent B.

Reagent D: Folin- Ciocaltaeu phenol reagent diluted 2- fold with distilled water (1:1) to make it 1N.

b) Estimation: To 0.1 mL of enzyme extract (extraction procedure as discussed in Section 3.8.3, 3.8.4 and 3.8.5) taken in duplicates, 5 mL of reagent C was added. The contents were mixed well and allowed to stand for 10 min at room temperature. To this 0.5 mL of reagent D was added very rapidly and mixed well. The intensity of blue colour so developed was read after 30 min at 520 nm using Beckmann Spectrophotometer taking only water and reagents as blank. The total protein content was calculated from the standard curve prepared simultaneously by using Bovine Serum Albumin (BSA) as standard 20-100 µg.

3.8.9 Statistical analysis: All the enzyme assays had three replications. The data obtained was subjected to Completely Randomized Design (CRD) and analysis of variance using the statistical software CPCS1.

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3.9 Molecular analysis of *S. litura* populations for genetic relatedness

The genetic relatedness amongst different *S. litura* populations was assessed through comparative RAPD- PCR (Random amplified polymorphic DNA- polymerase chain reaction) and analysis of its data for genetic similarity by UPGMA (NTSYS PC version 2.02) (Rohlf 1998). Various steps are described below:

3.9.1 DNA extraction from *S. litura* populations

About 10-15 larvae of each *S. litura* population were preserved in 90 per cent ethanol and stored in a deep freezer (-20°C) until these were required to be processed for DNA isolation.

3.9.1.1 DNA Isolation

Total DNA from the individual larva was isolated using a modified CTAB method of Cubero *et al* (1999). CTAB was two per cent solution of cetyl trimethyl ammonium bromide

(CTAB) in 100mM Tris.Cl (pH 8.0), which additionally contained 20mM of Na_2EDTA (pH 8.0) and 1.4M NaCl. Immediately before use, CTAB solution was amended with mercaptoethanol to provide a concentration of 0.1 per cent.

For DNA extraction, the individual larvae from different populations were individually crushed in a 1.5 mL microcentrifuge tube with the help of a sterilized micropestle containing 200 µL CTAB solution. After adding more CTAB along the walls (300 µl), the tubes were incubated at 65°C for 30 minutes with intermittent mixing of tube contents. Thereafter, the lysate was extracted with 500 µL of chloroform: isoamyl alcohol (24:1) by mixing the contents by vortexing to form an emulsion followed by centrifugation at 10,000 rpm for 1 min. Using a disposable pipette tip, upper aqueous layer containing DNA was transferred to a clean microcentrifuge tube and total DNA precipitated by adding 50 µL of 3M sodium acetate and 500 µL of isopropanol. The precipitated DNA was collected in pellet by centrifugation at 10,000 rpm for 5 min. The DNA pellet was washed once with 70 per cent ethanol and allowed to air dry at room temperature. The dried DNA pellet was dissolved in 250 µL of TE buffer containing DNAse free pancreatic RNAse A (10µg per mL) and stored at -20°C until used.

3.9.1.2. Gel Electrophoresis

The quality of DNA isolated from the larva was determined by horizontal agarose (0.7 per cent in 1X TAE containing ethidium bromide @1µg per mL) gel electrophoresis in 1X TAE buffer at 75V for 1 hr. The DNA bands were visualized on a UV transilluminator and photographed using 'Ultra Lum Gel Documentation System'. The composition of 1X TAE buffer is as under:

Component	**Quantity per 1,000 mL**	**Working solution**
Tris base	242.00 g	0.04 M
Glacial acetic acid	57.10 mL	1.00 M
EDTA (0.5M, pH 8.0)	100.00 mL	1.00 mM
Sterile water	-	to make 1,000 mL

Additionally the quality and concentration of DNA solution was determined by A_{260}/A_{280} ratio of diluted DNA solution in a UV spectrophotometer. The concentration of DNA was estimated by following formula:

1 A_{260} unit of double stranded DNA = 50µg/ mL

Absorbance ratio of A_{260}/A_{280} nearing 2.0 (1.9-2.1) indicates high purity DNA

3.9.2 Comparative RAPD-PCR analysis for the identification of genetic variants amongst different populations of *S. litura*

In order to identify possible molecular differences amongst individuals of different *S. litura* populations, total DNA from five individual larvae from each population was PCR amplified using four RAPD primers from Operon Technologies Inc., Almada, USA, in a comparative manner. PCR amplified DNA fragments were resolved on 1.5 per cent agarose gel in TAE buffer by horizontal gel electrophoresis. The sequence of used RAPD primers is given below:

Primer	Sequence
OPB-07	GGTGACGCAG
OPB-10	CTGCTGGGAC
OPG-03	GAGCCCTCCA
OPL-08	AGCAGGTGGA

Comparative RAPD-PCR analysis of *S. litura* from different populations was performed using total DNA as template and a specific RAPD primer for PCR amplification. PCR reactions were performed in 25 µL reaction mixtures, each containing ~20 ng template DNA solution (2 µL), 1 mM dNTPs mix (5 µL), 10 µM RAPD primer (5 µL), 2.0 U Taq Polymerase (MBI, Fermentas) and 1.5 mM $MgCl_2$ in 1X Taq reaction buffer. The PCR amplification programme consisted of 95°C for 5 min (initial denaturation), 95°C for 1 min (denaturation), 38°C for 1.5 min (primer annealing), 72°C for 2 min (extension) (38 cycles), 72°C for 10 min (final extension) and stored at 4°C until used. The amplified DNA products were separated by electrophoresis along with a MW marker (100 bp ladder plus, MBI Fermentas) using 1.5 per cent agarose gel in TAE. The gel was stained with ethidium bromide and the banding profiles visualized and photographed using UV Gel Documentation System (UltraLum).

Composition of different solutions

0.5 M EDTA	:	186.12 g of EDTA. Disodium salt was dissolved in 700 mL of ddH_2O. pH was adjusted to 8.0 by adding NaOH pellets (~16-18 g). Thereafter total volume was adjusted to 1 litre with ddH_2O.
5 M NaCl	:	292.2 g of NaCl dissolved in 700 mL ddH_2O and total volume was adjusted to 1 litre with ddH_2O.
TE Buffer	:	10 mL of 1 M Tris HCl pH 8.0 was mixed with 2 mL of 0.5 M EDTA and diluted to 1 litre with ddH_2O.

CTAB solution : 2g of cetyl trimethyl ammonium bromide, 28 mL of 5M NaCl, 4 mL of 0.5M EDTA and 10 mL of 0.1M Tris HCl were dissolved in dd H_2O to make 100 mL. The solution was steam sterilized (15psi of steam) and amended with β-mercaptoethanol (1% level) just before use.

TAE Buffer : A 50X stock TAE buffer solution was prepared by dissolving 121g of Tris base, 28.6 mL acetic acid and 50 mL of 0.5M EDTA, pH 8.0 in ddH_2O to make 500 mL. when needed the stock solution was 50 fold diluted with ddH_2O.

Gel loading buffer : 0.25 per cent bromophenol blue, 0.25 per cent xylene cyanol and 30 per cent glycerol in water

3.9.3 Analysis of RAPD-PCR profiles for genetic relatedness

DNA isolated from four different populations of *S. litura* was PCR- amplified with RAPD primers and the amplified DNA fragments were analyzed through agarose gel electrophoresis and banding patterns photographed in a gel documentation system. All the individual bands in RAPD banding profile of different *S. litura* populations were scored for presence (1) or absence (0) of all the individual bands. The data matrix was used to construct a genetic relatedness dendrogram using 'SimQual' function of the UPGMA program of the software NTSYS pc version 2.02 as per Rohlf (1998).

3.9.4 Molecular analysis of *S. litura* field population and insecticide- selected population

The molecular differences between field population of *S. litura* (SGR) and one selected with synthetic pyrethroid insecticide (SGR-Sel) were assessed through comparative RAPD- PCR using six different primers, their sequences are given below:

Primer	Sequence
OPB-07	GGTGACGCAG
OPB-10	CTGCTGGGAC
OPG-03	GAGCCCTCCA
OPL-08	AGCAGGTGGA
OPN-10	ACAACTGGGG
OPP-04	GTGTCTCAGG

PCR reactions were performed in 25 µL reaction mixtures, each containing 20 ng template DNA solution (2 µL), 1 mM dNTPs mix (5 µL), 10 µM RAPD primer (5 µL), 2.0 U Taq Polymerase (MBI, Fermentas) and 1.5 mM $MgCl_2$ in 1X Taq reaction buffer. The PCR amplification programme consisted of 95°C for 5 min (initial denaturation), 95°C for 1 min (denaturation),38°C for 1.5 min (primer annealing), 72°C for 2 min (extension) (38 cycles), 72°C for 10 min (final extension) and stored at 4°C until used. The amplified DNA products were separated by electrophoresis along with a MW marker (100 bp ladder plus, MBI Fermentas) using 1.5 per cent agarose gel in TAE. The gel was stained with ethidium bromide and the banding profiles visualized and photographed using UV Gel Documentation System (UltraLum).

3.9.5. Molecular analysis of mtCOI region of *S. litura* populations

Based upon banding pattern of the amplified DNA, DNA preparations from field and insecticide selected *S. litura* individuals, which showed DNA polymorphism were selected for further detailed studies. The two genetic variants were investigated for molecular differences using mitochondrial cytochrome oxidase I (mtCOI) gene region that has been universally accepted taxonomically important 'DNA barcode' region (Fig. 1).

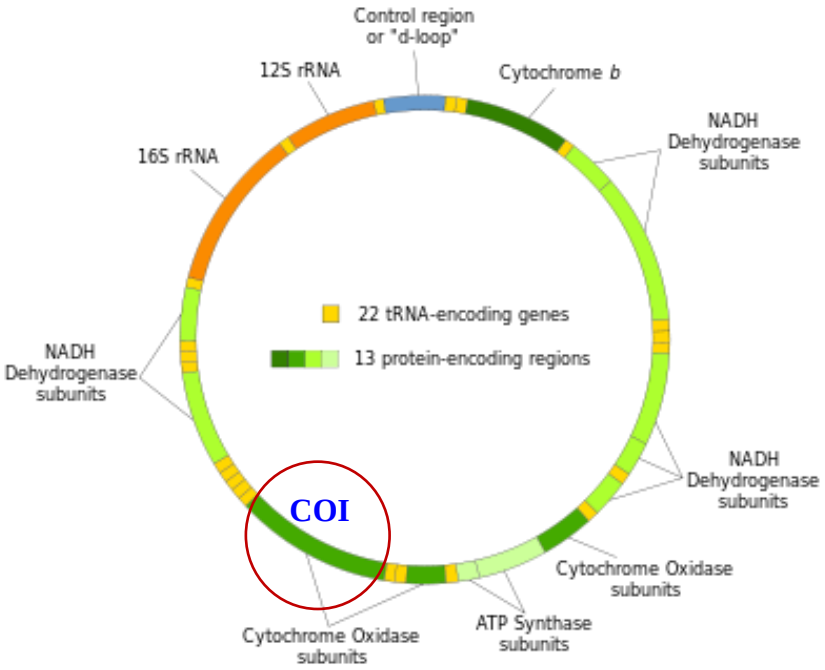


Fig. 1. Mitochondrial DNA map

3.9.5.1. Custom synthesis of primers

The region specific primers i.e., mtCOI were custom synthesized through facility of Integrated DNA Technologies, Inc, Coralville, IA, USA. Each primer was dissolved in 1/10 TE buffer to form '100 μM of stock primer solution'. When needed, stock primer solutions were further diluted to provide '10 μM of working primer solution' before use.

For this purpose, sequences for region specific PCR primers nucleotide sequence of primers used for amplification of mtCOI region complete DNA from *S. litura* were as given by Hajiababaei *et al* (2006)

Lep-F: ATTCAACCAATCATAAAGATATTGG

Lep-R: TAAACTTCTGGATGTCCAAAAAATCA

3.9.5.2. PCR amplification of mtCOI DNA

PCR amplification of mtCOI from *S. litura* total DNA was carried out in a reaction volume of 20 μL, which contained:

Component	**Stock concentration**	**Volume (μL)**
Insect DNA	~20.0 ng/μl	2.0
Lep-F primer	10.0 μM	1.0
Lep-R primer	10.0 μM	1.0
dNTPs mix	1.0 mM	5.0
Taq polymerase	5.0 units/μL	0.6
Taq buffer (with 1.5mM $MgCl_2$)	10X	2.0
Sterile Milli-Q H_2O to make 20 μl	-	8.4

All PCR- amplifications were accomplished in a programmable DNA thermalcycler (Mastercycler Gradient- eppendorf™) using the following PCR program:

Step 1 : Initial denaturation at 95°C for 5 min

Step 2 : Denaturation at 95°C for 1 min

Step 3 : Primer annealing at 52°C for 1 min

Step 4 : Extension at 72°C for 1 min

Step 5 : Repeated steps 2 to 4 (35 cycles)

Step 6 : Final extension at 72°C for 10 min and

Step 7 : Store at 4°C

PCR amplified products were resolved by horizontal agarose gel electrophoresis using 0.7 per cent (w/v) agarose gel (supplemented with ethidium bromide @ 1.0 mg/l) in 1X TAE buffer. 20µL of PCR product was mixed with 3µL of 6X 'gel loading buffer' and loaded into the wells of agarose gel. The gel was subjected to electrophoresis at constant voltage (75 V) for 1 hour. The resolved DNA bands of the amplified products in the gel were visualized under UV transilluminator (using low intensity of UV light) and recorded with a Gel Documentation System (Ultra Cam). Size of amplified bands was ascertained by co-running a molecular weight standard (100 bp DNA ladder plus, Fermentas Life Sciences) along with the samples in the gel.

3.9.5.3 Purification of PCR products of variants of *S. litura*

The agarose block containing the specific amplified DNA band was excised from the agarose gel with a clean, sharp scalpel blade and transferred to a 1.5 µL microcentrifuge tube. From this gel band, the DNA fragments were purified using 'QIAquick Gel Extraction Kit' (Qiagen) as per manufacturer's protocol. In this protocol, the cut agarose block was weighed (mg) and incubated with 3 volumes (µL) of buffer QX1 at 55°C for 5 minutes (or until the gel slice was completely dissolved). After adding 10 µL of QIAEX II suspension, the solution was mixed thoroughly to allow binding of DNA fragments to QIAEX II particles. QIAEX II particles mass (now with bound DNA) was recovered in pellet by centrifugation (10,000 rpm, 1 min) and pellet washed with 500µL of ice-cold PE Buffer (two times). The ultimate pellet was air-dried and the bound DNA fragments eluted in 30 µL of TE buffer by incubation at 55°C for 5 minutes. DNA elute was recovered by centrifugation (10,000 rpm, 1 min) and transferred into a fresh tube. The size and concentration of the eluted DNA was ascertained by running 10 µL of DNA solution in 0.7 per cent (w/v) agarose gel in comparison with a DNA size marker (100 bp DNA ladder plus).

3.9.5.4 Cloning of purified DNA fragments in PCR cloning vector pTZ57R/T

The purified DNA fragments were cloned into a 'PCR product cloning plasmid vector pTZ57R/T'(Fig. 2) and the ligation reaction product transformed into *Escherichia coli* DH5-alpha host cells using 'INST/ACLONE™ PCR Product Cloning Kit' (Fermentas Life Sciences) using manufacturer's protocol.

i) Ligation of purified PCR amplified fragments

The ligation of purified PCR amplified fragments into cloning vector was performed in a 15 µL of ligation reaction in a 500 µL microfuge tube as follows:

Components of ligation mixture		Volume (µL)
PCR cloning vector pTZ57RT/A DNA	:	1.0
Purified PCR fragment (Insert)	:	5.0
5X ligation buffer	:	3.0
T_4 DNA ligase (5 units/ µL)	:	1.0
Milli-Q H_2O	:	5.0

The ligation mixture was incubated at 22°C for 2 hour in a 'programmable temperature controlled thermostat' (Eppendorf).

ii) Transformation of ligation product into cells of cloning host *E. coli* for isolation of recombinant clones

Transformation of ligated product into *E. coli* DH5- α host cells involved transformation of competent *E. coli* DH5α host cells with ligated PCR fragments in cloning vector pTZ57RT/A followed by selective growth of transformants on LB-Amp-X-GAL-IPTG agar (LB agar supplemented with ampicillin @ 100 µg. mL^{-1}in Petri plates and surface coated with X-GAL and IPTG) after overnight incubation at 37°C and selection of the white recombinant clones from individual bacterial isolates (Fig. 3).

a) Preparation of Luria broth/ Agar medium: To make the medium, all components were dissolved in 800 mL of water and pH adjusted to for LB agar medium, LB broth was supplemented with bacteriological agar at 1.6 per cent level. Both the LB broth and LB agar were autoclaved at 15 psi of steam for 45 min. before use. The composition of LB agar medium is mentioned below:

Component	Amount (g/l)
Bacto Tryptone	10.00
Bacto Yeast extract	5.00
NaCl	10.00
Agar	16.00
Distilled water to make	1000 mL
pH adjusted to	~ 6.95- 7.05

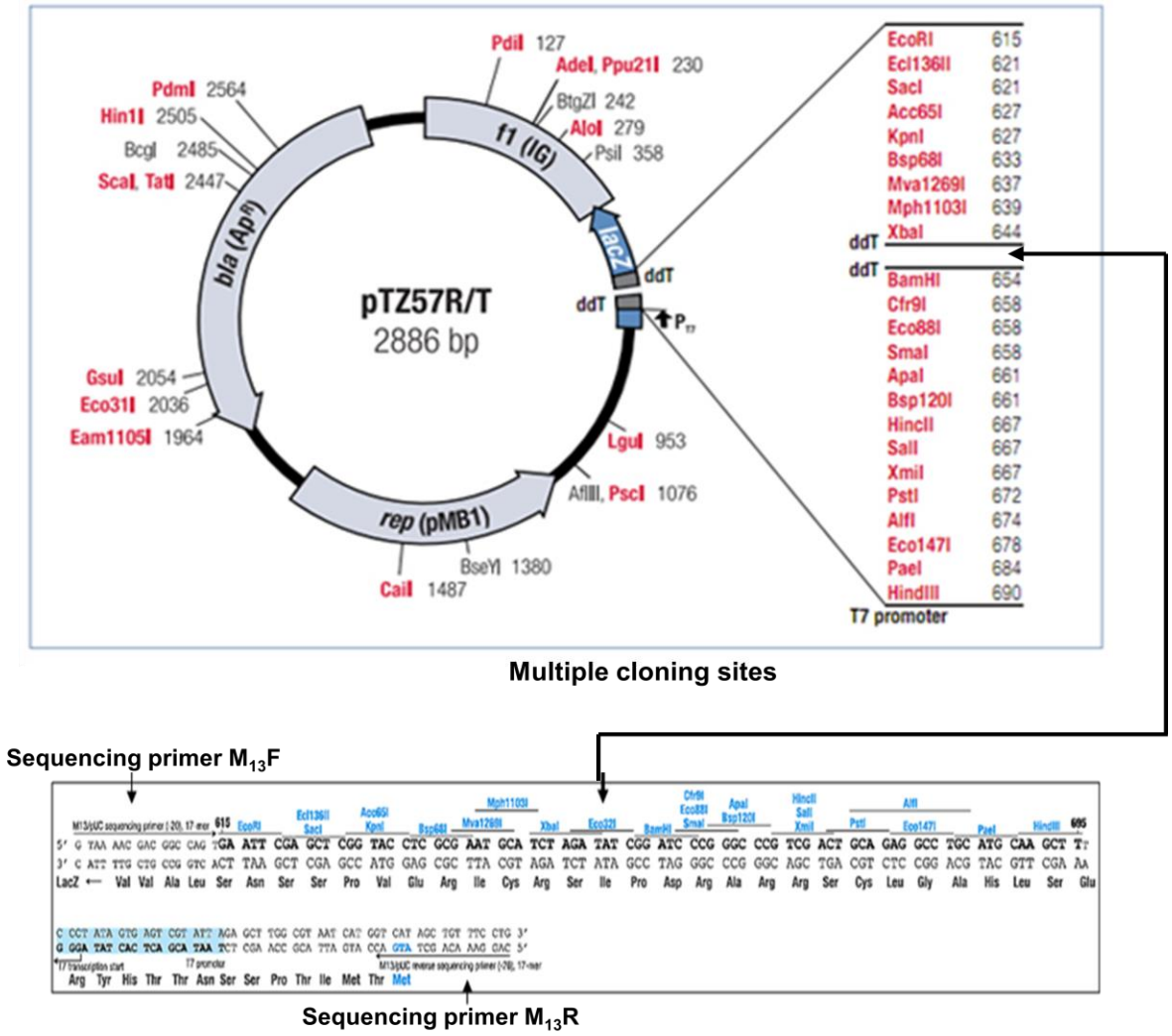
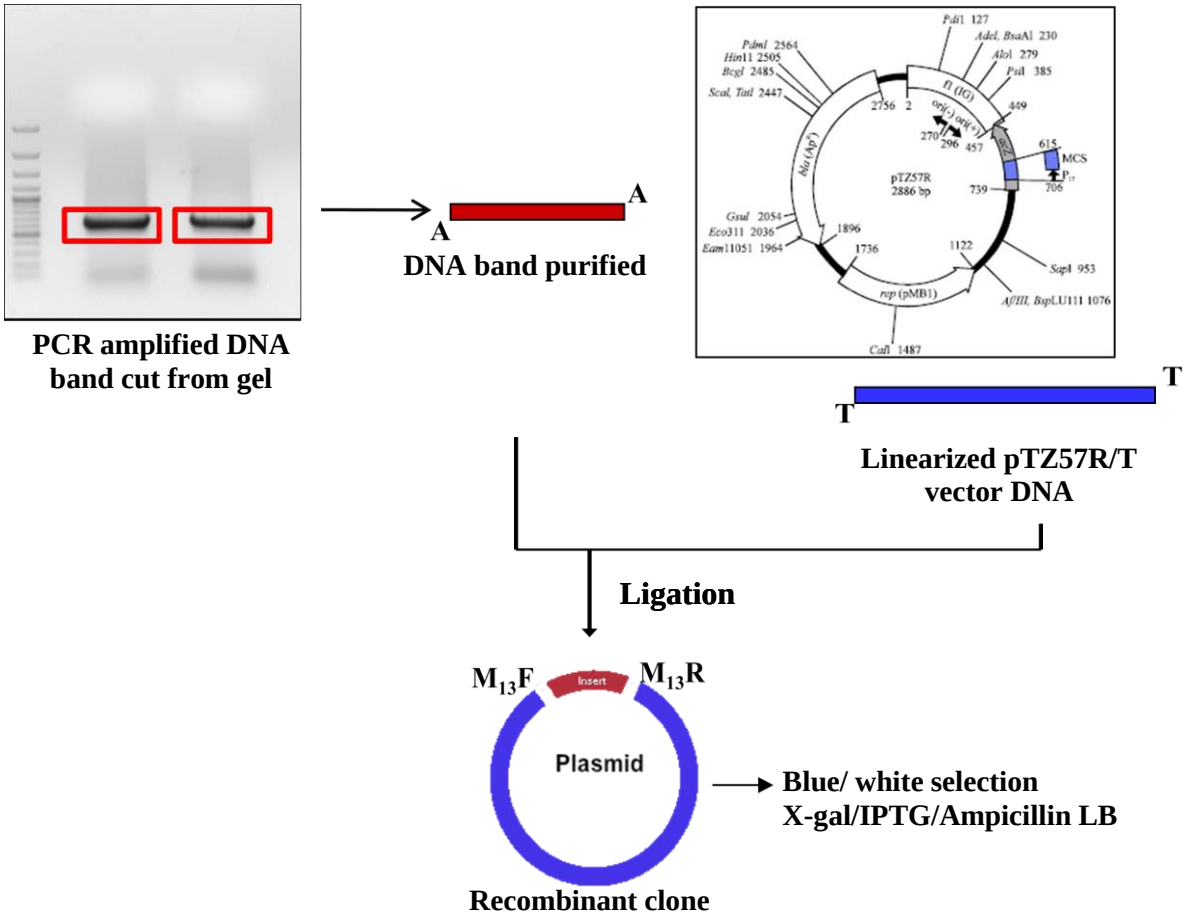


Fig. 2. pTZ57RT: Description and restriction map of cloning vector (2886 bp)



- **Sequencing grade plasmid purification**
- **Custom nucleotide sequencing of cloned DNA (mt COI) (Xcleris Labs, Ahmedabad)**
- **Sequence analysis (DNA Club software)-nucleotide**
- **Sequence alignment with reported sequences (CLC Sequence Viewer)**

Fig. 3. Summary of methods used in amplification, cloning, sequencing and analysis of mtCOI sequences of *S. litura* DNA

b) Use of Ampicillin in LB broth and preparation of LB Agar-Ampicillin plates: The cloning plasmid vector pTZ57R contained Ampicillin resistance gene (AmpR) as a positive selectable marker gene. Therefore, for selective growth of *E. coli* cells, which harboured the cloning vector (recombinant or non-recombinant) ampicillin was consistently included in LB broth and LB agar at a level of 100 µg/mL. For use in liquid media, 100 mL of LB broth was supplemented with 100 µl of ampicillin stock solution (100 mg ampicillin-sodium salt/ mL sterilized Milli-Q water).

Similarly, for use in solidified media, 100 mL of LB-agar medium was melted in a microwave oven, allowed to cool to ~55°C, and mixed with 100 µl of ampicillin stock solution. The medium was distributed in sterile petri dishes (90 mm dia) and allowed to solidify at room temperature.

c) Preparation of LB-ampicillin agar plates for Blue/White selection of recombinant clones: The multiple cloning site (MCS) in the plasmid vector pTZ57R was located in the β- galactosidase gene under the transcriptional control of LacZ promoter, to serve as screenable marker gene for recombinant plasmids in the presence of its chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and inducer IPTG (isopropyl-β-D-thiogalactopyranoside) in the medium. Therefore, the solidified LB-ampicillin agar medium in plates was uniformly smeared upon with 40 µL of X-gal solution (20mg/mL in N, N-dimethyl formamide) and 4 µL of IPTG solution (0.1M in Milli-Q water).

d) Preparation of competent cells of *E. coli* strain DH5α: Three mL of LB broth in a 12 mL culture tube was inoculated with a single colony of *E. coli* strain DH5α from a fresh overnight grown culture on LB agar plate. The culture was allowed to grow overnight under shaking conditions (150 rpm) at 37°C and 150 µL of this culture was inoculated into 1.5 mL (for each of 2 transformations) of fresh 'Transform Aid^{TM} C-Medium' and allowed to grow at 37°C under shaking conditions as above for two hours. Thereafter, the cells were harvested by centrifugation (at 10,000 rpm for one min) and resuspended in 300 µL of ice-cold mixture (1:1) of T-solution (A) and T-solution (B). The suspension was allowed to stand in ice for five min and cells recovered in pellet by centrifugation as above, and resuspended in 120 µL of ice-cold T-solution mixture to serve as competent cells for transformation of ligation reaction product.

e) Transformation and selection of recombinant clones: For transformation, 60 µL of competent cells were mixed with five µL of ligation reaction product, the mixture was allowed to stand in ice for five min and then uniformly spread and plated on a LB-ampicillin-X-gal/IPTG-

agar plate. The plates were incubated overnight at 37°C and observed for growth of blue coloured (non recombinant) and white (recombinant) clones (Fig.4).

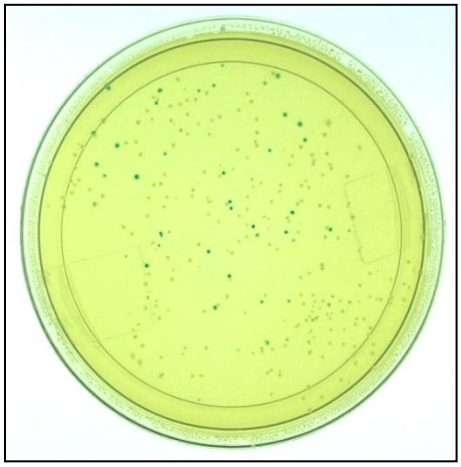


Fig. 4. Selection of white recombinant clones

f) Isolation of recombinant plasmid DNA from recombinant clones of *E. coli:* Three individual recombinant (white) clones from individual plates were picked up with a sterile tooth pick, inoculated into culture tubes containing 3 mL of LB-Ampicillin broth and the tubes were incubated overnight at 37°C under shaking conditions (180 rpm). Using this broth culture, miniprep plasmids were isolated using 'alkaline lysis method' of Birnboim and Doly (1979). Under this method, bacterial cell pellet from 1.5 mL of the overnight grown culture in a 1.5 mL micro centrifuge tube (at 13,000 rpm for one min) was resuspended in 100 µL of an ice-cold solution P-I by vortexing. After adding 200 µL of solution P-II, the tube contents were mixed by inverting the tube two to three times. The tubes were then stored on ice (five min), 150 µL of solution P-III was added to the tube, tube contents mixed by inversion and allowed to stand in ice for 5 min. After centrifugation (13,000 rpm for 5 min), the supernatant containing the plasmids was transferred to a fresh tube and plasmid DNA was precipitated by addition of 450 µL of isopropanol. The tube contents were centrifuged (13,000 rpm for 5 min) and the plasmid DNA recovered as pellet. The supernatant was removed by vacuum and the DNA pellet washed with 1 mL of 70 per cent ethanol. Thereafter, the DNA pellet was allowed to dry at room temperature and dissolved in 50 µL of TE (pH 8.0) containing DNAase-free pancreatic RNAase (20µg/mL). Subsequently all plasmid DNA preparations were stored at -20°C, until used.

Composition of different solutions:

P-I: Cell suspension solution-50mM Tris Cl pH 8.0-1mM EDTA

P-II: Cell lysis solution-0.2 N NaOH-1per cent (w/v) Sodium Lauryl Sulphate

P-III: Neutralizing solution-this solution was prepared by mixing 60 mL of 5M potassium acetate with 11.5 mL of glacial acetic acid and 28.5 mL of H_2O. These yielded a 3 M solution of Sodium acetate having a pH of 4.8, TE Buffer: 10 mM Tris.Cl, pH 8.0; 1mM EDTA, pH 8.0

g) Analysis of recombinant plasmid DNA for cloned insert DNA: The size of insert DNA, in different recombinant plasmids was determined by PCR amplification using insert specific primer sets (CO I) and universal M_{13} primers. The amplification products were analyzed as per method given earlier except that the amplification reactions contained 10 ng of plasmid DNA as template DNA in these reactions. Besides above, the recombinant plasmid was double-restricted with restriction enzymes EcoR1 and Pst1 (Fermentas Life Sciences) in the following restriction mixture:

Component	**Volume (µL)**
Plasmid DNA (~100 ng/µl)	5.0
Buffer-O^+ (10X)	1.5
EcoR1 (10 units/ µL)	1.0
Pst1 (10 units/ µL)	1.0
H_2O	6.5
Total volume	15

The restriction reaction mixture in a 1.5 mL microcentrifuge tube was incubated overnight at 37°C and restricted products resolved by gel electrophoresis on 0.7 per cent (w/v) agarose gel in TAE as described earlier. The restriction pattern was observed and recorded on a Gel Documentation system (UltraLum).

3.9.5.5 Nucleotide sequencing of cloned DNA

i) Purification of sequence grade plasmid DNA

The information on nucleotide sequence of the targeted insert DNA was obtained through Custom Sequencing Services of 'M/S Xcelris, Ahmedabad India'. For this purpose, sequencing grade plasmid DNA was purified from the respective recombinant clone using 'Gene Elute ™

Miniprep Plasmid Kit' of 'Sigma' as per manufacturer's protocol. In this protocol, bacterial cell pellet from 5 mL of overnight grown culture was suspended in 100 µL of 'Cell suspension solution', the cells lyzed with 200 µL of 'Alkaline lysis solutions' for 3 min and the lyzate neutralized with 150 µL of 'Neutralization/ binding buffer'. After centrifugation (10,000 rpm, 5 min), the supernatant was transferred into a 'Gene Elute Miniprep' binding column positioned in an outer 1.5 mL micro centrifuge tube and the assembly again subjected to centrifugation. The plasmid DNA bound to the membrane of the binding column was washed (2 times) with wash buffer and ultimately eluted in 100 µL of TE Buffer.

ii) Analysis of sequence data

The sequence of respective insert in the custom sequence of respective clone was identified using available information on the nucleotide sequence of cloning site in the cloning vector pTZ57R and sequence of forward and reverse primers of respective primer set (COI). The natural orientation of each sequence was determined by aligning of each sequence among them selves using 'Gene Align function' of the DNA software program 'CLC Free Workbench ver 6.0.2' of CLC bio A/S. Besides above, protein sequence of the amplified COI region was also derived using 'Translate to Protein' function of the above program.

Chapter - IV

RESULTS AND DISCUSSION

The present studies were undertaken to determine the relative toxicity and susceptibility status of different insecticides against *S. litura* populations collected from different locations of Punjab and to generate insecticide resistance data for this pest, to investigate the mechanisms of insecticide resistance through synergism and enzyme assays and molecular analysis of resistant and susceptible populations to identify molecular differences and develop molecular diagnostic markers for insecticide resistance. The results of these studies are presented under the following main headings:

4.1 Estimation of insecticide resistance levels in different *S. litura* populations of Punjab

4.2 The mechanisms of insecticide resistance in *S. litura*

4.3 Molecular analysis of resistant and susceptible populations of *S. litura*

4.4 Development of resistance specific molecular diagnostic markers for monitoring of insecticide resistance in *S. litura*, their evaluation and validation

4.1 Estimation of insecticide resistance levels in different *S. litura* populations of Punjab

The results showing the susceptibility status of *S. litura* populations, collected from different locations of Punjab to recommended insecticides with different concentrations of fenvalerate, chlorpyriphos, acephate, thiodicarb, indoxacarb, pyridalyl, chlorantraniliprole and novaluron are summarized in Tables 2 to 9.

4.1.1 Susceptibility of *S. litura* to fenvalerate

The LC_{50} values and related parameters of fenvalerate to *S. litura*, collected from diverse areas of Punjab are given in Table 2. The lowest LC_{50} value (0.04%) was obtained against the population collected from Hoshiarpur, which is comparable to the LC_{50} value (0.0369%) obtained by Johny and Muralirangan (2000), while the highest LC_{50} value (0.197%) was found against population collected from Sangrur. Thus, *S. litura* population from Sangrur was 4.925 times resistant than the most susceptible population. The levels of resistance for Ludhiana and Bhatinda populations were 2.70 and 1.175 times for fenvalerate as compared to Hoshiarpur populations. The LC_{50} value of fenvalerate for *S. litura* from Hoshiarpur has earlier been reported to be 0.014 per cent (Kaur *et al* 2007). The LC_{50} value for the population from Hoshiarpur obtained during present studies was 2.86 times higher (0.04%). Populations of *S. litura* collected from Ludhiana, Sangrur and Bhatinda areas have been found to be about 7.71, 14.03 and 3.36 times more tolerant

as compared to baseline LC_{50} value (0.014%) obtained from Hoshiarpur population. The results indicate that the population of *S. litura* collected from various parts of Punjab have picked up tolerance to fenvalerate. Mayuravalli *et al* (1987) recorded 1.6 fold tolerance in *S. litura* to fenvalerate at LC_{50} in Guntur population during the period from 1981-87, while Reddy and Devaprasad (1991) observed a resistance index of 2.6 for fenvalerate. Armes *et al* (1997) reported the resistance levels of 0.2 to 197- fold for cypermethrin and 8 to 121- fold for fenvalerate in *S. litura* populations from eight locations in Andhra Pradesh, between 1991 and 1996. Kodandaram and Dhingra (2006) showed 16.74, 13.22, 21.18, 11.50 and 4.83-fold resistance to deltamethrin, alpha-cypermethrin, cypermethrin, beta-cyfluthrin and fenvalerate, respectively, in Punjab population (Phagwara) compared with the Delhi population.

On comparing the results obtained with LC_{50} value of 0.000341 for fenvalerate by Gupta *et al* (1992), the resistance ratio (RR) calculated under the present studies ranged from 117 to 578 in *S. litura* collected from the four locations of Punjab. Murugesan and Dhingra (1995) also reported the built-up of resistance in *S. litura* during the period 1961-1995 with an increase in the LC_{50} value of pyrethrin and resistance to the extent of 20.62-fold. The insect developed 9.03, 1.45 and 98.00-fold resistance to cypermethrin, fenvalerate and deltamethrin within a short span of eight years (1987-1995). The resistance level was 4- fold in Guntur population of *S. litura* as reported by Rao and Dhingra (1996). In China, resistance of *S. litura* to pyrethroids was monitored by Wu *et al* (1995) and the resistance ratios worked out for cypermethrin, deltamethrin and fenvalerate were 43.9, 90.5 and 171.9, respectively.

Table 2. Susceptibility of *S. litura* populations from different locations of Punjab to fenvalerate

Locations	LC_{50} (%)	Slope	FL at 95%CL	Heterogeneity $\chi 2$ (d.f.)	RR	
					*	**
LDH	0.108	1.907 ± 0.236	0.085 – 0.144	1.678 (7)	7.71	316.7
SGR	0.197	1.460 ± 0.217	0.137 – 0.343	1.968 (6)	14.07	577.7
BTH	0.047	2.674 ± 0.323	0.040 – 0.056	0.581 (6)	3.36	137.8
HSP	0.040	2.455 ± 0.287	0.034 – 0.049	1.512 (6)	2.86	117.3

LDH Ludhiana; SGR Sangrur; BTH Bhatinda; HSP Hoshiarpur
RR Resistance ratio
Baseline value of fenvalerate (%) = *0.014 (Kaur *et al* 2007) and **0.000341 (Gupta *et al* 1992)

4.1.2 Susceptibility of *S. litura* to chlorpyriphos

The data in Table 3 revealed that the LC_{50} values and related parameters of chlorpyriphos for *S. litura* population collected from diverse areas of Punjab ranged from 0.005 to 0.033 per cent. The lowest LC_{50} value (0.005%) was obtained against the population of *S. litura* from Hoshiarpur and Ludhiana, while the highest LC_{50} value (0.033%) was found against population collected from Bhatinda. The results are in agreement with the findings of Kodandaram (2003) who reported LC_{50} values for Punjab and Delhi populations as 0.00505 and 0.0082 against seven day old *S. litura*. LC_{50} values for Malerkotla (Sangrur) and Hoshiarpur were observed to be 0.002 and 0.004 per cent, respectively as reported by Kaur *et al* (2007). Reddy and Reddy (1984) noted two fold resistance to chlorpyriphos in Guntur strain as compared to Kurnool strain. Balasubramanian *et al* (1988) in Tamil Nadu found chlorpyriphos to be most effective against the Coimbatore population having LD_{50} values of 0.000219 followed by Udumalpet (0.00263) and Madukarai (0.00983 µg/larva) population. Similarly, Johny and Muralirangan (2000) found chlorpyriphos as the most toxic among five conventional insecticides with an LC_{50} of 0.00288 per cent. Considering the LC_{50} value by Sahoo *et al* (2007) for Ludhiana population as the baseline, the resistance ratio worked out in the present study was 5.00 fold for Ludhiana and Hoshiarpur populations, 29 and 33- fold for Sangrur and Bhatinda populations, respectively. Forty fold resistance in the single strain (Bhatinda) tested from North India was reported by Kranthi *et al* (2002). In South India, all the strains exhibited high levels of resistance to chlorpyriphos (45–129 fold). The highest resistance levels in Central India were recorded in the Warangal (RF 129) and Mahbubnagar (RF 127) strains. It appears that over the years, there is not much significant level of resistance in *S. litura* with respect to chlorpyriphos in Punjab.

Table 3. Susceptibility of *S. litura* populations from different locations of Punjab to chlorpyriphos

Locations	LC_{50} (%)	Slope	FL at 95% CL	Heterogeneity $\chi 2$ (d.f.)	RR
LDH	0.005	0.985 ± 0.136	0.003 – 0.008	1.083(4)	5.00
SGR	0.029	1.540 ± 0.153	0.021 – 0.038	2.305 (7)	29.00
BTH	0.033	1.315 ± 0.139	0.024– 0.047	2.603 (8)	33.00
HSP	0.005	1.120 ± 0.149	0.003 – 0.008	1.495 (4)	5.00

Baseline value of chlorpyriphos (%) = 0.001 Source: Sahoo *et al* (2007)

4.1.3 Susceptibility of *S. litura* to acephate

The LC_{50} values of acephate for *S. litura* populations collected from diverse areas of Punjab ranged between 0.011 and 0.076 per cent (Table 4). The lowest LC_{50} value (0.011%) was obtained against the population from Bhatinda, while its highest LC_{50} value (0.076%) was found against population from Sangrur. The results indicate that the difference between the baseline LC_{50} value and the highest LC_{50} value is not much apparent (7-fold). The population of *S. litura* collected from Ludhiana and Hoshiarpur showed the LC_{50} values 0.035 and 0.018 per cent, respectively. The LC_{50} values of acephate for *S. litura* from different locations of Punjab have earlier been reported to be ranging from 0.037 to 0.046 per cent (Kaur *et al* 2007) and 0.0502 per cent (Kodandaram 2003). Thus, the susceptibility status of *S. litura* towards acephate seems to remain unchanged over the years, with no apparent indications of resistance development.

Table 4.Susceptibility of *S. litura* populations from different locations of Punjab to acephate

Locations	LC_{50} (%)	Slope	FL at 95% CL	Heterogeneity $\chi 2$ (d.f.)	RR
LDH	0.035	1.067 ± 0.138	0.023 – 0.57	2.090 (5)	3.18
SGR	0.076	1.260 ± 0.150	0.054 – 0.108	0.498 (6)	6.91
BTH	0.011	0.976 ± 0.099	0.007 – 0.017	1.264 (7)	1.00
HSP	0.018	1.157 ± 0.135	0.012 – 0.027	1.122 (6)	1.64

4.1.4 Susceptibility of *S. litura* to thiodicarb

The data presented in Table 5 revealed that thiodicarb was the most toxic insecticide (among all the conventional insecticides) to all tested populations of *S. litura* with LC_{50} values of 0.001 - 0.006 per cent. The results of the present studies are in conformity with that of Radhika *et al* (2007) who reported the highest efficacy of thiodicarb with LD_{50} value (1.552µg/ larva by leaf sandwich method) than methomyl, etofenprox, profenofos, cartap hydrochloride and diafenthiuron and the four conventional insecticides (endosulfan, monocrotophos, carbaryl and cypermethrin) tested. Thiodicarb was reported to have good results against several lepidopteron insect pests such as *S. exempta* (Nyirenda 1983), *S. litura* (Kuwazawa 1999) and *Helicoverpa armigera* (Attique *et al* 1998, Kranthi *et al* 2001[b] and Rao *et al* 2001). The results for Hoshiarpur

populations are in agreement with the findings of Kaur *et al* (2007) who reported LC_{50} value of 0.001 per cent. However, the LC_{50} values for Malerkotla (Sangrur) and Bhatinda populations were reported to be 0.001 and 0.00039 per cent, respectively. This indicates a 6 and 15.38 fold increase in the resistance level in the Malerkotla and Bhatinda populations, respectively within the period from 2003-04 to 2010-11 in the Punjab state. Dhawan *et al* (2009) also reported higher LC_{50} values ranging from 0.045 to 0.064 per cent for thiodicarb while evaluating its toxicity against four different populations of *S. litura* from Punjab. However, in Pakistan, moderate to high levels of resistance (16- to 200- fold) were reported in *S. litura* populations, with LC_{50} values ranging from 0.00406 to 0.0507 per cent (Saleem *et al* 2008). Similarly, in another study, Ahmad *et al* (2007) reported LC_{50} value of thiodicarb as 0.00652 – 0.0584 per cent.

Table 5. Susceptibility of *S. litura* populations from different locations of Punjab to thiodicarb

Locations	LC_{50} (%)	Slope	FL at 95% CL	Heterogeneity $\chi 2$ (d.f.)	RR	
					*	**
LDH	0.002	0.811 ± 0.101	0.001 – 0.003	2.626 (7)	5.13	19.61
SGR	0.006	1.260 ± 0.137	0.004 – 0.009	1.632 (6)	15.38	58.82
BTH	0.005	1.026 ± 0.140	0.004 – 0.008	1.174 (6)	12.82	49.02
HSP	0.001	0.909 ± 0.102	0.0002 – 0.001	2.188 (6)	2.56	9.80

Baseline value of thiodicarb (%) = 0.00039* (Kaur *et al* 2007) and 0.000102** (Shad *et al* 2012)

4.1.5 Susceptibility of *S. litura* to indoxacarb

The oxadiazine compound, indoxacarb was also tested against all the populations of *S. litura.* The data showing the toxicity to different populations are presented in Table 6. This reduced risk pesticide showed more effectiveness than other organophosphorus insecticides. The LC_{50} values of indoxacarb for all the tested populations of *S. litura* were found to vary between 0.0000125 and 0.0001582 per cent. The LC_{50} values of indoxacarb for Ludhiana and Hoshiarpur populations were found to be almost equal (0.0000125 and 0.0000132 per cent, respectively) and these populations were more susceptible than the other populations. Earlier Kaur *et al* (2007) reported LC_{50} values ranging from 0.00001 to 0.0003 per cent against various populations of *S.*

litura in Punjab and in the present study, no appreciable difference has been observed in the baseline LC_{50} values. So, by considering the LC_{50} value (0.00001%), the resistance ratios of Ludhiana, Hoshiarpur, Bhatinda and Sangrur populations were found to be 1.25, 1.32, 7.92 and 15.82, respectively. Wang *et al* (2008) reported that after selection with indoxacarb 6 times during 10 generations, a resistant population of *S. litura* was achieved with resistance ratio of 15.63 compared with unselected parent population, suggesting that *S. litura* had high potential to develop resistance to indoxacarb. Development of resistance in *S. litura* to indoxacarb was also reported from Pakistan where the LC_{50} values of 0.0884 per cent had increased by 95 fold (Ahmad *et al* 2008).

Table 6. Susceptibility of *S. litura* populations from different locations of Punjab to indoxacarb

Locations	LC_{50} (%)	Slope	FL at 95% CL	Heterogenity $\chi 2$ (d.f.)	RR
LDH	0.0000125	0.611 ± 0.069	0.0000055-0.0000257	0.888 (6)	1.25
SGR	0.0001582	0.852 ± 0.081	0.0001037-0.0002375	0.477 (10)	15.82
BTH	0.0000792	0.791 ± 0.089	0.0000490-0.0001226	2.253 (9)	7.92
HSP	0.0000132	0.592 ± 0.068	0.0000056-0.0000277	0.491 (5)	1.32

Baseline value of indoxacarb (%) = 0.00001% Source: Kaur *et al* (2007)

4.1.6 Susceptibility of *S. litura* to pyridalyl

Pyridalyl, another novel insecticide, that has been successfully introduced for the control of various lepidopterous and thysanopterous pests (Sakamoto *et al* 2003). The LC_{50} values and related parameters of pyridalyl for *S. litura* populations collected from different regions of Punjab are illustrated in Table 7. The lowest LC_{50} value (0.0007132%) was obtained against the population from Sangrur, while its highest LC_{50} value (0.0009809%) was found against population from Ludhiana. The results indicate that the difference between these values is not much apparent (1.37 fold). Earlier Dhawan *et al* (2007) had reported LC_{50} value of 0.00004 per cent and considering this as the baseline value, the resistance ratios worked out in the present study for Ludhiana, Sangrur, Bhatinda and Hoshiarpur populations were 24.52, 17.83, 18.42 and

23.21 folds, respectively. Similar values (LC_{50} of 0.0008926%) were reported by Dahi *et al* (2011) against fourth instar larvae of *S. littoralis.* High insecticidal activity of pyridalyl was reported by Sakamoto *et al* (2003) with LC_{50} values against bollworm, *H. armigera* as 0.000136 per cent. Baseline responses of *H. zea* against pyridalyl with LC_{50} and LC_{90} values of 0.000155 and 0.000791 per cent, respectively were worked out by Cook *et al* (2005).

Table 7. Susceptibility of *S. litura* populations from different locations of Punjab to pyridalyl

Locations	LC_{50} (%)	Slope	FL at 95% CL	Heterogenity $\chi 2$ (d.f.)	RR
LDH	0.0009809	0.783 ± 0.080	0.0006202-0.0015929	5.116 (6)	24.52
SGR	0.0007132	0.889 ± 0.078	0.0004863-0.0010508	0.477 (11)	17.83
BTH	0.0007367	1.091 ± 0.091	0.0005283-0.0010333	5.410 (11)	18.42
HSP	0.0009286	0.727 ± 0.077	0.0005707-0.0015472	0.322 (11)	23.21

Baseline value of pyridalyl (%) = 0. 00004 Source: Dhawan *et al* (2007)

4.1.7 Susceptibility of *S. litura* to chlorantraniliprole

In general, chlorantraniliprole, a novel insecticide in the anthranilic diamide class and effective against several lepidopteron insects was found to be an excellent option for insecticides resistance management (IRM) strategies as an additional class of chemistry and mode of action. The LC_{50} values and related parameters of chlorantraniliprole are given in Table 8. The insecticide was found to be toxic to all the tested populations of *S. litura* from Punjab with LC_{50} values (0.0000005-0.0000100%). The results are in agreement with the findings of Hardke *et al* (2011) who recorded LC_{50} of 0.0000068 per cent against *S. frugiperda*. Insecticidal activity of chlorantraniliprole was similar to that reported by Lahm *et al* (2007) who worked out the LC_{50} values as 0.0000002 for *Plutella xylostella* and *S. frugiperda*, and 0.000004 per cent for *Heliothis virescens*. Temple *et al* (2009) also reported high toxicity of chlorantraniliprole against bollworm, *Helicoverpa zea* (LC_{50} 0.000004 to 0.000009%). Considering the LC_{50} value (0.0000002%) by Chowdary *et al* (2011) as baseline, the resistance ratios were worked out to be 2.5 to 50 for *S. litura* populations collected from different regions. The susceptibilities of *S. litura* collected from Southeast China to chlorantraniliprole determined by diet incorporation assay with neonates of 12

field populations was low (< 4-fold), with LC_{50} values varying from 0.00000284 to 0.00001025 per cent (Su *et al* 2012). However, the results are contradictory with the findings of Dhawan *et al* (2009) who reported LC_{50} values of chlorantraniliprole as 0.004 to 0.007 per cent against *S. litura.*

Table 8. Susceptibility of *S. litura* populations from different locations of Punjab to chlorantraniliprole

Locations	LC_{50} (%)	Slope	FL at 95% CL	Heterogenity $\chi 2$ (d.f.)	RR
LDH	0.0000009	0.458 ± 0.086	0.0000001-0.0000030	0.169 (5)	4.50
SGR	0.0000035	0.491 ± 0.076	0.0000009-0.000009	0.810 (5)	17.50
BTH	0.000010	0.684 ± 0.074	0.0000062-0.0000164	1.289 (10)	50.00
HSP	0.0000005	0.561 ± 0.094	0.0000001-0.0000014	0.491 (5)	2.50

Baseline value of chlorantraniliprole (%) = 0. 0000002 Source: Chowdary *et al* (2011)

4.1.8 Susceptibility of *S. litura* to novaluron

The LC_{50} values of novaluron for *S. litura* populations collected from different areas of Punjab ranged from 0.000441 to 0.00208 per cent (Table 9). The results are in agreement with the findings of Dhawan *et al* (2007) who reported LC_{50} value of 0.002 per cent, and Dhawan *et al* (2009) who reported LC_{50} values as 0.004- 0.007 per cent. Talikoti *et al* (2012) reported higher toxicity (LC_{50} = 0.013%) at 72 h of exposure by leaf sandwich method application than other insecticides, though the value was higher than those recorded in the present study. Hardke *et al* (2011) recorded lower LC_{50} (0.0000166%) for novaluron against *S. frugiperda*. Rao (2008) reported LD_{50} of 0.030 µg per larva for novaluron against Kurnool (Andhra Pradesh) population. Ishaaya *et al* (2003) observed no appreciable resistance to novaluron in a field strain of *S. littoralis* collected from cucumber field in the central part of Israel.

Table 9. Susceptibility of *S. litura* populations from different locations of Punjab to novaluron

Locations	LC_{50} (%)	Slope	FL at 95% CL	Heterogenity $\chi 2$ (d.f.)	RR
LDH	0.000793	0.626 ± 0.062	0.000457-0.001341	3.413 (13)	1.79
SGR	0.001176	0.570 ± 0.060	0.000658-0.0002080	5.110 (13)	2.60
BTH	0.002080	0.688 ± 0.059	0.001332-0.003283	3.283 (13)	4.71
HSP	0.000441	0.666 ± 0.059	0.000267-0.000699	3.688 (13)	1.00

4.1.9 Estimation of insecticide resistance

The commercial formulations of recommended insecticides viz., fenvalerate (Sumicidin 20EC), chlorpyriphos (Dursban 20 EC), acephate (Asataf 75SP) and thiodicarb (Larvin 75 WP), with different concentrations giving mortality rate between 10 to 90 per cent were tested against four populations of *S. litura*. The Hoshiarpur population showed more susceptibility against fenvalerate, chlorpyriphos and thiodicarb and the population from Bhatinda showed more susceptibility against acephate. Among different populations, Sangrur population showed least susceptibility against fenvalerate, acephate and thiodicarb. The Bhatinda population was found to be least susceptible to chlorpyriphos among all the *S. litura* populations. These results confirmed that thiodicarb was the most effective insecticide as reported earlier by Radhika *et al* (2007), Kaur *et al* (2007) and Kuwazawa (1999). In the present study, fenvalerate was observed to be the least effective insecticide (Table 10). Earlier, works by Rao (2008), Kaur *et al* (2007) and Kodandaram and Dhingra (2003) also reported that fenvalerate caused low mortality of *S. litura*.

As far as the new chemistries are concerned, Ludhiana and Hoshiarpur populations showed more susceptibility to indoxacarb. Hoshiarpur population was also found to be most susceptible to chlorantraniliprole and novaluron. The susceptibility level against pyridalyl was almost same for all the tested populations. Chlorantraniliprole was found to be the most effective insecticide against all populations (Table11).

Table 10. Susceptibility of *S. litura* populations from different locations to conventional insecticides

Insecticide	Fenvalerate		Chlorpyriphos		Acephate		Thiodicarb	
Locations	**LC_{50} (%)**	**Slope**	**LC_{50} (%)**	**Slope**	**LC_{50} (%)**	**Slope**	**LC_{50} (%)**	**Slope**
LDH	0.108 (2.70)	1.907 ± 0.236	0.005 (1.00)	0.985 ± 0.136	0.035 (3.18)	1.067 ± 0.138	0.002 (2.00)	0.811 ± 0.101
SGR	0.197 (4.92)	1.460 ± 0.217	0.029 (5.80)	1.540 ± 0.153	0.076 (6.91)	1.260 ± 0.150	0.006 (6.00)	1.260 ± 0.137
BTH	0.047 (1.18)	2.674 ± 0.323	0.033 (6.60)	1.315 ± 0.139	0.011 (1.00)	0.976 ± 0.099	0.005 (5.00)	1.026 ± 0.140
HSP	0.040 (1.00)	2.455 ± 0.287	0.005 (1.00)	1.120 ± 0.149	0.018 (1.64)	1.157 ± 0.135	0.001 (1.00)	0.909 ± 0.102

Figures in parentheses are the resistance factors

Table 11. Susceptibility of *S. litura* from different places to newer insecticides

Insecticide	Indoxacarb		Pyridalyl		Chlorantraniliprole		Novaluron	
Locations	**LC_{50} (%)**	**Slope**	**LC_{50} (%)**	**Slope**	**LC_{50} (%)**	**Slope**	**LC_{50} (%)**	**Slope**
LDH	0.0000125 (1.00)	0.611 ± 0.069	0.0009809 (1.37)	0.783 ± 0.080	0.0000009 (1.80)	0.458 ± 0.086	0.000793 (1.79)	0.626 ± 0.062
SGR	0.0001582 (12.66)	0.852 ± 0.081	0.0007132 (1.00)	0.889 ± 0.078	0.0000035 (7.00)	0.491 ± 0.076	0.001176 (2.60)	0.570 ± 0.060
BTH	0.0000792 (6.34)	0.791 ± 0.089	0.0007367 (1.03)	1.091 ± 0.091	0.000010 (20.00)	0.684 ± 0.074	0.002080 (4.71)	0.688 ± 0.059
HSP	0.0000132 (1.06)	0.592 ± 0.068	0.0009286 (1.30)	0.727 ± 0.077	0.0000005 (1.00)	0.561 ± 0.094	0.000441 (1.00)	0.666 ± 0.059

Figures in parentheses are the resistance factors

4.1.10 Relative toxicity of different insecticides to *S. litura* populations from different locations

The relative toxicities of different insecticides against *S. litura* were calculated and are presented in Tables 12-13 and Fig 5. It was observed that thiodicarb was 54 times more toxic than fenvalerate in the Ludhiana population, while chlorpyriphos and acephate showed 21.60 and 3.08 times more toxicity than fenvalerate. In Sangrur population, the toxicities of thiodicarb, chlorpyriphos and acephate were 32.83, 6.79 and 2.59 times that of fenvalerate. The more toxic insecticide thiodicarb showed 9.40 and 40 times more toxicity than fenvalerate in Bhatinda and Hoshiarpur populations, respectively. Based on LC_{50} values obtained, the order of toxicity of conventional insecticides was found to be thiodicarb > chlorpyriphos > acephate > fenvalerate for Ludhiana, Sangrur and Hoshiarpur populations. However, for *S. litura* population from Bhatinda, the trend of toxicity was thiodicarb > acephate > chlorpyriphos > fenvalerate.

The data presented in Table 13 revealed that the order of toxicity of the new insecticides against pest populations of Ludhiana and Hoshiarpur was found to be chlorantraniliprole> indoxacarb> novaluron> pyridalyl whereas it was chlorantraniliprole> indoxacarb> pyridalyl> novaluron for *S. litura* populations from Sangrur and Bhatinda. The insecticides chlorantraniliprole demonstrated higher toxicity against all the four populations of *S. litura*. Chlorantraniliprole is a novel anthranilic diamide insecticide that targets the ryanodine receptors in muscle cells (Lahm *et al* 2005). It is reported to be highly effective against several lepidopteron species at relatively low application rates (Cordova *et al* 2006; Lahm *et al* 2007). The new chemistries are highly effective against *S. litura* owing to their new modes of action. These insecticides may therefore, prove to be promising substitutes for the effective control of resistant populations of this pest.

Table 12. Relative toxicity of newer insecticides against *S. litura* of different locations

Locations	LC_{50} (%)			
	Fenvalerate	**Chlorpyriphos**	**Acephate**	**Thiodicarb**
Ludhiana	0.108 (1.00)	0.005 (21.60)	0.035 (3.08)	0.002 (54.00)
Sangrur	0.197 (1.00)	0.029 (6.79)	0.076 (2.59)	0.006 (32.83)
Bhatinda	0.047 (1.00)	0.033 (1.42)	0.011 (4.27)	0.005 (9.40)
Hoshiarpur	0.040 (1.00)	0.005 (8.00)	0.018 (2.22)	0.001 (40.00)

Figures in parentheses are the toxicity ratios

Table 13. Relative toxicity of newer insecticides against *S. litura* populations from different locations

Locations	LC_{50} (%)			
	Indoxacarb	**Pyridalyl**	**Chlorantraniliprole**	**Novaluron**
Ludhiana	0.0000125 (78.47)	0.0009809 (1.00)	0.0000009 (1089.88)	0.000793 (1.24)
Sangrur	0.0001582 (7.43)	0.0007132 (1.65)	0.0000035 (336.00)	0.001176 (1.00)
Bhatinda	0.0000792 (26.26)	0.0007367 (2.82)	0.000010 (208.00)	0.002080 (1.00)
Hoshiarpur	0.0000132 (70.35)	0.0009286 (1.00)	0.0000005 (1857.2)	0.000441 (2.10)

Figures in parentheses are the toxicity ratios

4.2 The mechanisms of insecticide resistance in *S. litura*

S. litura possesses a versatile and multiple resistance mechanism to overcome toxicity of insecticides. The faster degradation of insecticides by metabolic enzymes is one such mechanism commonly associated with insecticide resistance. The synergist and biochemical experiments conducted in the present study suggested that metabolic resistance was the major mechanism responsible for imparting resistance in the insect pest under study. The findings are presented under the following headings:

4.2.1 Generation of susceptible population (SUS) and selection of field population with fenvalerate

The susceptible population (SUS) was reared on castor leaves in an insecticide free environment for 11 generations. The population was initially collected from Hoshiapur area (HSP) that was found to be most susceptible to fenvalerate among the four populations of *S. litura* (as reported in Table 2). The dose – mortality relationship was worked out for the SUS strain and is presented in Table 14.

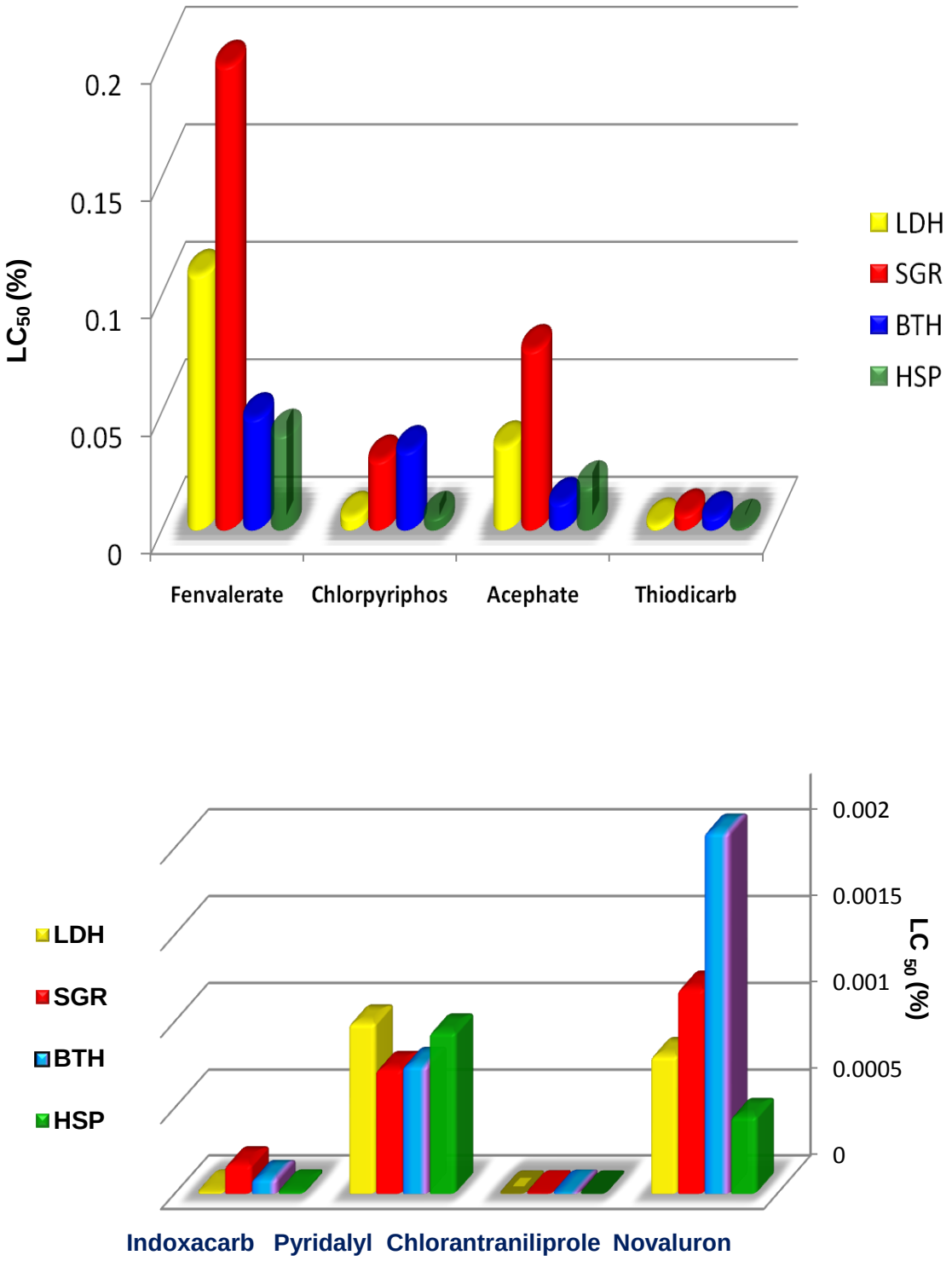


Fig 5. Toxicity of various insecticides against *Spodoptera litura*

Table 14. Toxicity of fenvalerate to SUS strain of *S. litura*

Strain	LC_{50} (%)	FL at 95% CL	Slope	Heterogenity $\chi 2$ (d.f.)	Ratio
HSP	0.040	0.034 – 0.049	2.455 ± 0.287	1.512 (6)	1.00
SUS	0.016	0.012- 0.020	1.798 ± 0.210	1.170 (6)	2.50

S. litura collected from Sangrur (SGR) was selected with fenvalerate for 14 generations. Dose- mortality relationships were worked (Table 15). The LC_{50} in the initial population SGR was 0.197 per cent (highest among the populations). Selection pressure up to 14 generations resulted in an increase in the LC_{50} of fenvalerate to 0.826 per cent in the selected strain (SGR-Sel). This indicates a 4.19 fold increase in the resistance level. The 95 per cent confidence limits did not overlap in the two populations, hence these can be considered as significantly different. There was decrease in slope value from 1.460 to 0.851, which indicates a considerable heterogeneity in the response of the strain, suggesting a greater potential for the development of higher levels of resistance.

Table 15. Toxicity of fenvalerate to SGR- Sel strain of *S. litura*

Strain	LC_{50} (%)	FL at 95% CL	Slope	Heterogenity $\chi 2$ (d.f.)	Ratio
F_0 SGR	0.197	0.137– 0.343	1.460 ± 0.217	1.968 (6)	1.00
F_2	0.210	0.097-0.314	1.820 ± 0.235	0.363 (6)	1.07
F_5	0.268	0.176- 0.356	1.648 ± 0.248	0.765 (6)	1.36
F_8	0.384	0.276-0.499	1.526 ± 0.242	0.422 (6)	1.95
F_{11}	0.580	0.427- 0.742	1.161± 0.562	0.360 (6)	2.94
F_{14} SGR-Sel	0.826	0.462– 2.166	0.851 ± 0.136	1.957 (8)	4.19

4.2.2 Mechanism of resistance through synergism studies

Synergists are of considerable help in indicating the type of the detoxification enzymes which are involved and thus suggesting the possible biochemical mechanisms of resistance (Metcalf 1967, Riskallah 1984). *S. litura* populations collected from Sangrur (SGR) along with the susceptible strain (SUS) and fenvalerate- selected strain (SGR-Sel) were tested to assess the

degree of resistance acquired by using different synergists viz., piperonil butoxide (PBO), diethyl maleate (DEM) and triphenyl phosphate (TPP) (Table 16). The synergists PBO, TPP and DEM are normally considered as the inhibitor of mixed function oxidases (MFOs), esterases and glutathione S-transferase (GST), respectively.

When PBO, TPP and DEM were used as synergist in the susceptible strain and resistant strain, the synergistic ratio was 1.07, 0.94 and 0.89 for the susceptible strain (SUS), and 6.21, 1.89 and 5.74 for the fenvalerate-selected strain (SGR-Sel), respectively (Table 21). The greater synergism observed in the resistant strains could be ascribed to the widely accepted hypothesis that resistant insects contain more of the detoxification mechanisms than the susceptible insects (Oppenoorth and Welling 1976).

The results obtained in the present study revealed that PBO had highest synergistic effect on the selected strain (6.21- fold) followed by TPP (5.74- fold). In the SGR and SGR-Sel, PBO showed 3.72 and 6.21- fold synergism. PBO had stronger synergism for fenvalerate than the other two synergists. No obvious synergism was observed with DEM in the SUS strain. In the SGR and SGR-Sel populations, DEM showed slight synergism (1.73 and 1.89- fold, respectively). The synergism of TPP was 6.16 and 5.74- fold in SGR and SGR-Sel, respectively, which suggests the involvement of esterases in imparting resistance to various insecticides like organophosphates, synthetic pyrethroids and other insecticides like indoxacarb. These results are consistent with that obtained by Gunning *et al* (1999), who attributed resistance to pyrethroids to an overproduction of esterases, as implied by synergism studies in *H. armigera*. The differences in the resistant and susceptible populations noticed in this study are similar to those reported in *S. littoralis* (Radwan *et al* 1979, Riskallah 1984).

It is likely that enhanced detoxification by MFOs is the major mechanism of pyrethroid resistance in the fenvalerate- selected population of *S. litura* (SGR-Sel) followed by esterase. Huang and Han (2007) reported similar results wherein higher PBO synergism was found to be associated with deltamethrin resistance. The results are also in agreement with Armes *et al* (1997) who reported that pre-treatment with the metabolic inhibitor, PBO resulted in complete suppression of cypermethrin resistance (2 to 121- fold synergism) in nearly all strains of *S. litura*, indicating that enhanced detoxification by MFOs was probably the major mechanism of pyrethroid resistance. Pre-treatment with the synergist DEF, an inhibitor of esterases and the GST system, resulted in a 2 to 3- fold synergism with monocrotophos indicating that esterases and possibly glutathione S-transferases were at least to some extent contributing to organophosphate resistance (Armes *et al* 1997). Similar results were obtained in the present study using DEM as a synergist, where a low level of synergism implied that GST might be less important in conferring

resistance in the *S. litura* populations. The studies carried out by Joyee *et al* (1988) clearly showed high synergistic factor (22.1) for *S. exigua* and *S. frugiperda* with 1:5 mixture of fenvalerate and PBO and a value of 4.6 for *S. frugiperda* with 1:5 mixture of fenvalerate and MGK-264. Studies on *S. litura* in Pakistan have shown that the synergism by PBO and DEF implied that both monoxygenases and esterases might be involved in imparting resistance to indoxacarb, pyrethroids and emamectin benzoate (Ahmad *et al* 2007, Shad *et al* 2010). The resistance of *S. litura* to pyrethroid and organophosphate insecticides was synergized with PBO and TPP when tested with cypermethrin, deltamethrin, fenvalerate, dichlorvos and acephate and showed that MFO and esterases played role in insecticide resistance of the pest (Wu *et al* 1995).

In both SGR and SGR-Sel populations, resistance to fenvalerate dropped from 12.3 and 51.6-fold to 3.3 and 8.3- fold, respectively with the use of PBO, implying that resistance was highly suppressed (Table 17).

4.2.3 Mechanism of resistance through biochemical studies

Insecticide resistance is mainly based on mutation of the target protein resulting in decreased affinity to the respective insecticide and increased detoxification by hydrolases, cytochrome P450 dependant monooxygenases/ mixed function oxidases (MFOs) and glutathione S- transferase (GST). Populations from Sangrur (SGR), Hoshiarpur (HSP), Sangrur (fenvalerate selection) and susceptible (SUS) populations were subjected to biochemical analysis for the following parameters:

4.2.3.1 Hydrolases

4.2.3.2 Mixed function oxidases

4.2.3.3 Glutathione-S transferases

4.2.3.4 Correlation between enzyme activities and LC_{50} values

4.2.3.5 Enzyme inhibition studies

4.2.3.1 Hydrolases

4.2.3.1.1 Esterases

The data presented in Table 18 indicated that all the populations of *S. litura* (SGR-Sel, SGR and HSP) had significantly higher esterase activity than in the SUS population (1.02 µg of naphthol min^{-1} mg^{-1}). The field populations (HSP and SGR) had 1.40 and 2.14- fold enzyme activity as compared to the SUS strain. The results are in conformity with data obtained by Huang and Han (2007) who reported five-fold esterase activity in the deltamethrin resistant strain as

Table 16. Synergism of fenvalerate by PBO, DEM and TPP in different populations of *S. litura*

Insecticide	LC_{50} (%)	Fiducial limits	LC_{90} (%)	Fiducial limits	Slope ± S.E.	χ^2	df	SR
SUS								
Fenvalerate	0.016	0.012-0.020	0.081	0.057-0.134	1.798 ± 0.210	1.170	6	1.00
Fenvalerate + PBO (1:10)	0.015	0.011-0.019	0.077	0.055-0.126	1.795± 0.212	0.542	6	1.07
Fenvalerate + DEM (1:10	0.017	0.014-0.022	0.087	0.062-0.145	1.824 ± 0.214	1.305	6	0.94
Fenvalerate + TPP (1:10)	0.018	0.014-0.023	0.088	0.063-0.146	1.861 ± 0.218	1.066	6	0.89
SGR								
Fenvalerate	0.197	0.137–0.343	1.489	0.715-5.469	1.460 ± 0.217	1.968	6	1.00
Fenvalerate + PBO (1:10)	0.053	0.045–0.065	0.169	0.125-0.268	2.563 ± 0.318	0.421	6	3.72
Fenvalerate + DEM (1:10)	0.115	0.087-0.16	0.727	0.419-1.770	1.597 ± 0.207	1.119	7	1.71
Fenvalerate + TPP (1:10)	0.032	0.026-0.038	0.109	0.083-0.163	2.375 ± 0.275	0.314	6	6.16
SGR-Sel								
Fenvalerate	0.826	0.462–2.166	26.464	7.167-303.110	0.851 ± 0.136	1.9567	8	1.00
Fenvalerate + PBO (1:10)	0.133	0101–0.194	0.797	0.457-1.980	1.651 ± 0.218	1.304	7	6.21
Fenvalerate + DEM (1:10	0.438	0.266-1.022	7.618	2.505-64.209	1.034 ± 0.171	1.516	7	1.89
Fenvalerate + TPP (1:10)	0.144	0.106-0.219	0.991	0.531-2.826	1.528 ± 0.210	1.685	7	5.74

SR- Synergistic ratio= LC_{50} insecticide alone/ LC_{50} of insecticide + synergist

Table 17. Synergism of PBO, DEM and TPP on fenvalerate in different populations of *S. litura*

Population	LC_{50} (%)			
	Control	PBO	DEM	TPP
Sus	0.016 (1.00) [1.00]	0.015 (1.07) [0.94]	0.017 (0.94) [1.06]	0.018 (0.89) [1.12]
SGR	0.197 (1.00) [12.31]	0.053 (3.72) [3.31]	0.114 (1.73) [7.13]	0.032 (6.16) [2.00]
SGR-Sel	0.826 (1.00) [51.62]	0.133 (6.21) [8.31]	0.438 (1.89) [27.38]	0.144 (5.74) [9.00]

Figures in () parentheses are the synergistic ratios
Figures in [ ] parentheses are the resistance ratios

compared to susceptible strain. Similarly, the esterase activity of the deltamethrin resistant strain was significantly different from the activity in the field strain of *H. armigera*, since there was R/S ratio of 2.6 fold (Buès *et al* 2005).

Shankarganesh *et al* (2012) reported increased esterase activity in the resistant Guntur population of *S. litura* by 26 per cent. Moreover, its suppression by about 55 per cent to insecticide synergist dihydrodillapiole suggested the involvement of esterases in hydrolysis of cypermethrin. Riskallah (1983) reported that the resistant strains of *S. littoralis* had 3 to 6.5- fold higher pyrethroid hydrolytic activity than susceptible strain. Higher levels of general esterases in resistant strain (1.4- fold) were reported by Gujar and Sohal (2010) in resistant strains of diamondback moth *Plutella xylostella* and 3.98- fold in *H. armigera* by Sangha (2007) under Punjab conditions. Delorme *et al* (1988) showed that the resistance in *S. exigua* was due to increased esterasic metabolism of deltamethrin. Gunning *et al* (1996) reported that pyrethroids resistant *H. armigera* had higher esterase activity up to 50- fold.

4.2.3.1.2 Acetyl choline esterase (AChE)

AChE is one of the most conserved enzymes of higher eukaryotes (Rusell *et al* 2004). The specific activity of this enzyme in whole body homogenates of *S. litura* was significantly higher in the SGR-Sel, SGR and HSP as compared to susceptible (SUS) strain (Table 18). It was 3.78 and 3.54 nmoles min^{-1} mg^{-1}, respectively for populations collected from field (SGR and HSP) and the values were statistically on par. The fenvalerate- selected population (SGR-Sel) had 1.61 times higher enzyme activity than the SUS population. Yu (2006) also reported 1.25- fold

higher specific activity of AChE in field strain of *S. frugiperda* (0.34) than the susceptible strain (0.27 µmol/min/mg protein). However, Sangha (2006) reported a negligible increase (1.09 times) in the specific activity of AChE in the insects of relatively resistant Abohar population of *H. armigera* (8.61) as compared to that of Ludhiana region (7.89 nmoles min^{-1} mg^{-1}). Since resistant populations in the present study exhibit higher specific activity, the enzyme assay can be used as a biochemical tool for monitoring insecticide- resistance in *S. litura*.

4.2.3.2 Mixed function oxidases (MFOs)

Cytochrome P450 dependant monooxygenases/ mixed function oxidases (MFOs) mediated detoxification is one of the most important mechanism of insecticide resistance. Due to the broad substrate spectra of MFOs, this mechanism may potentially affect several classes of insecticides (Agosin 1985, Oppenoorth 1971, Feyereisen 1999[b], Scott 1999). The activity of MFO was estimated using the midgut of the fifth instar larvae. The specific activity of the enzyme was higher in SGR-Sel (460.73) and SGR (250.81nmoles) compared to that of susceptible population (SUS) which exhibited specific activity of 108.24 nmoles min^{-1} mg^{-1} protein, which was significantly lower than all other populations (Table 18). MFO activity of the resistant populations SGR-Sel, SGR and HSP were 4.26, 2.32 and 1.41- fold, respectively compared with SUS population. Yang *et al* (2004) highlighted that the elevated cytochrome P450 monooxygenases are a major metabolic mechanism responsible for pyrethroid resistance in *H. armigera* from China, India and Pakistan with combined evidence from synergism experiments, monooxygenase activity assays with multiple substrates, and *in vitro* metabolism study. The authors reported MFO activity in *H. armigera* as 0.072 and 0.4 nmol min^{-1} mg^{-1} protein in the field and 14 generations fenvalerate- selected resistant strain, which was 2.1 and 11.7 times higher than the susceptible strain (0.034 nmol min^{-1} mg^{-1} protein). With the resistance ratios to fenvalerate ranging from 2.5- to ~ 20- fold in the three resistant populations, the MFO activities were accordingly 1.4- to ~ 4- fold higher than in susceptible population. This further indicated that enhanced monooxygenase activity is a major mechanism of fenvalerate resistance in *S. litura* populations of Punjab. These studies are in conformity with those conducted by Huang and Han (2007) who had observed higher activities of MFO in the resistant strains in comparison to susceptible strains of *S. litura*. They further reported that selection with deltamethrin further increased the MFO activity. Su *et al* (2012) also reported significantly higher MFO activity in most field strains than susceptible strain of *S. litura*. The MFO activity can therefore, be used as a biochemical marker for MFO- mediated pyrethroid resistance in field populations of *S. litura.*

Table 18. Specific activity of detoxification enzymes in different populations of *S. litura*

Population	Hydrolases				MFO[3]		GST[4]	
	Esterase[1] Specific activity	Ratio	AChE[2] Specific activity	Ratio	Specific activity	Ratio	Specific activity	Ratio
SUS	1.016± 0.08	1	2.42± 0.21	1	108.237± 11.24	1	0.341± 0.02	1
HSP	1.422± 0.09	1.40	3.54± 0.17	1.46	153.187± 12.85	1.41	0.321± 0.02	0.94
SGR	2.180± 0.08	2.14	3.78± 0.24	1.56	250.807± 11.51	2.32	0.447± 0.01	1.31
SGR-Sel	3.764± 0.14	3.70	3.90± 0.36	1.61	460.733± 6.61	4.26	0.585± 0.012	1.72
CD (p=0.05)	0.33		0.84		35.28		0.055	

Mean of three replications ± SE

SUS- Susceptible population, HSP – field population from Hoshiarpur, SGR– field population from Sangrur, SGR-Sel- fenvalerate selected Sangrur population
MFO- mixed function oxidase, GST- Glutathione-S- transferase, AChE- Acetylcholine esterase

[1] µmoles of α- naphthol formed min^{-1} mg^{-1} of protein
[2] nmoles of free thiol formed min^{-1} mg^{-1} of protein
[3] nmoles of *p*- nitrophenol formed min^{-1} mg^{-1} of protein
[4] µmoles of 1-chloro 2, 4- dinitro benzene conjugated min^{-1} mg^{-1} of protein

4.2.3.3 Glutathione-S transferases (GST)

The activity of glutathione-S-transferases in whole body homogenates of *S. litura*, as inferred from CDNB assay, was higher in the SGR and SGR-Sel populations as compared to the SUS strain (Table 22). SGR-Sel recorded 1.72- fold enzyme activity (0.585) and SGR had 1.31-fold higher GST activity (0.447) than the SUS strain (0.341 µmoles min^{-1} mg^{-1} protein). The data presented in Table 17 showed some synergism of fenvalerate with DEM (SR 1.89 in SGR-Sel). Thus, from the synergist experiments and enzyme assay studies, it can be inferred that there may be some involvement of GSTs in pyrethroid resistance in *S. litura*. Similar results have been reported in *H. armigera* (Yang *et al* 2004), in *Nilaparvata lugens* (Vontas *et al* 2001), and in *Sitophilus zeami*s (Fragoso *et al* 2003) indicating the role of GSTs in detoxification of insecticides in the resistant populations.

4.2.3.4 Correlation between enzyme activities and LC_{50} values

Activities of different enzymes in four different populations of *S. litura* showed a positive correlation with LC_{50} values of fenvalerate (Table 19). A positive correlation was observed in all the cases. Significant positive correlation was observed between esterase and LC_{50} value (r= 0.98), MFO and LC_{50} value (r= 0.98) and GST and LC_{50} value (r= 0.96). AChE showed a non-significant positive correlation with LC_{50} values of fenvalerate (r= 0.61). From coefficient of determination R^2, it is clear that MFO was the major contributing factors in imparting metabolic resistance followed by esterase and GST.

These results are in accordance with the studies conducted by Kranthi *et al* (1997) who also observed a positive correlation between LC_{50} value of cypermethrin and general esterase activity in *P. xylostella.* Gunning *et al* (1996) reported that resistance levels in *H. armigera* were positively correlated with esterase titres and that increasing resistance was accompanied by increasing esterase activity. Owing to a significantly high correlation, the activity of esterases can be used as a biochemical tool for monitoring of insecticide resistance in the pest. Gujar and Sohal (2010) also suggested the use of general esterases and acetylcholine esterases as a biochemical tool for monitoring resistance in *P. xylostella* in different areas of Punjab. Similar studies conducted for pyrethroid resistance in *H. armigera* also showed higher MFO activity in the selected strains and a strong positive correlation between MFO activity and pyrethroid resistance (Buès *et al* 2005, Chen *et al* 2005). So these studies also corroborate the results obtained from the present investigations.

Table 19. Correlation between specific activity of enzyme and LC_{50} values of fenvalerate against *S. litura*

Enzyme	r	R^2
Esterase	0.98*	0.957
AChE	0.61	0.445
MFO	0.98*	0.966
GST	0.96*	0.926

r is correlation coefficient, R^2 is coefficient of determination
* significant at 5% level of significances

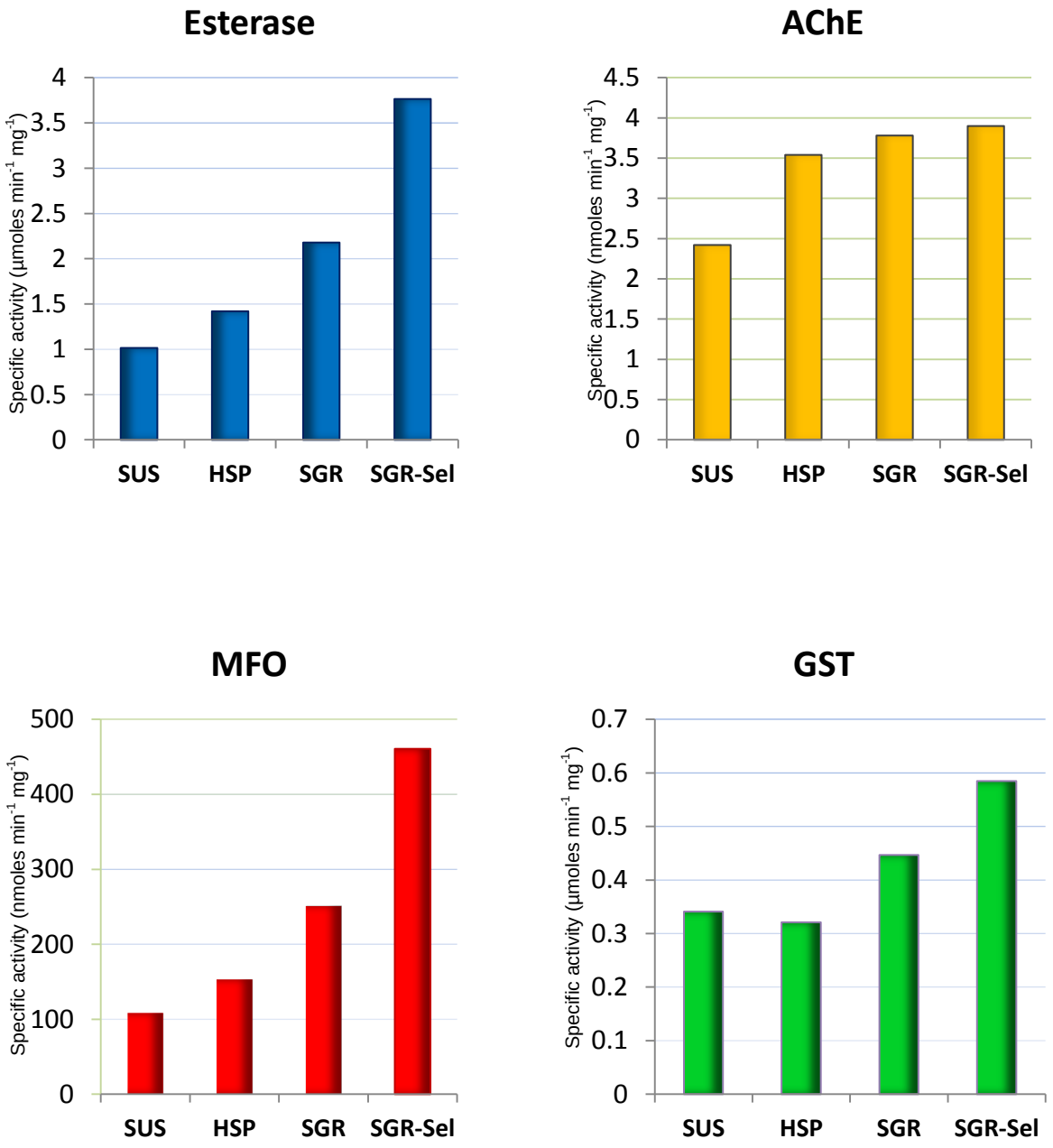


Fig. 6 Specific activity of detoxification enzymes in different populations of *S. litura*

AChE Acetylcholine esterase, MFO mixed function oxidase, GST glutathione S- transferase,

SUS- Susceptible population, HSP – field population from Hoshiarpur, SGR– field population from Sangrur, SGR-Sel- fenvalerate selected Sangrur population

4.2.3.5 Enzyme inhibition- *In vitro* effect of chlorpyriphos on activity of AChE of *S. litura*

Decreased sensitivity of AChE is recognized as one of the common mechanisms of resistance to organophosphates in many insects and mites (Zhu and Gao 1999, Yang *et al* 2009, Kang *et al* 2006). In the present studies, the resistant populations of *S. litura* had a higher tolerance towards chlorpyriphos than the SUS population. Variation in the activity of enzyme with increasing concentration of chlorpyriphos is presented in Fig 7. The I_{50} with chlorpyriphos in the individuals of SGR was observed to be 12.8 µg/mL, about 1.16- fold higher than that of HSP (11 µg/mL) and 1.72- fold higher than SUS population (7.45 µg/mL) (Table 20). It was 1.96 fold higher in the fenvalerate-selected strain (SGR-Sel). All the populations except SUS had insensitive AChE to chlorpyriphos. Higher concentrations of the insecticide were required to inhibit 50 per cent of the enzyme in case of resistant populations than in the susceptible one implying that the insensitive AChE played an important role in insecticide resistance. Similar observations were made by Sangha *et al* (2007) who determined I_{50} with chlorpyriphos as 1.37 fold higher in the resistant Abohar population of *H. armigera* (0.17µg) as compared to the relatively susceptible population collected from Ludhiana (0.124 µg). The present findings are also in conformity with the results of Haung and Han (2007) who reported that the enzyme in pyrethroid- selected and field strains of *S. litura* was inhibited 1.7- 3.8 fold by organophosphate insecticides. Organophosphates are poor substrates for this enzyme. They phosphorylate the enzyme readily but the dephosphorylation of the same is a very slow process that is considered irreversible. The active site of the enzyme remains occupied and therefore incapable of hydrolysing its normal substrate Ach. The upregulation of AChE is known to play only a modest role in resistance (Charpentier and Fornier 2001). High level effects are due to point mutations that decrease inhibitor efficiency. These mutations involve steric effects that may hinder inhibitor access to, or binding at, the active site (Hollingworth and Dong 2008). Several workers have identified, using gene sequences, the molecular nature of alteration leading to the lowered sensitivity to inhibitor (Rusell *et al* 2004). Thus, a decrease in the sensitivity of AChE to inhibition by some organophosphates results in increased resistance in the insects, as is the case of *S. litura* resistant population in the present study. Insensitive AChE thus appears to play a role in the resistance and acts in conjunction with metabolic detoxification to confer overall resistance to, organophosphates and carbamates. Hence, it is evident from the biochemical experiments, both increased AChE activity and insensitive AChE can contribute to resistance against organophosphates as reported for greenbug, housefly and *Spodoptera* species (Shi *et al* 2001, Li and Han 2002, Byrne and Toscano 2001, Voss 1980).

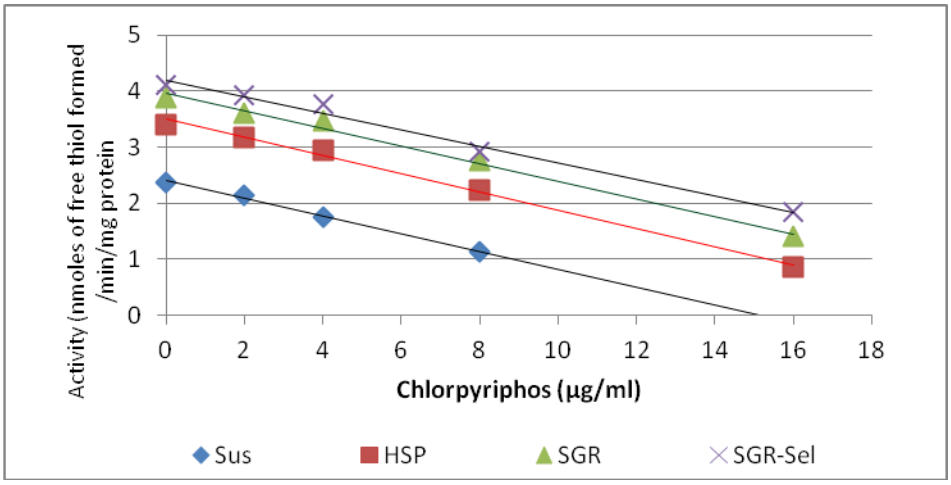


Fig. 7 Variation in specific activity of AChE with increase in concentration of chlorpyriphos

Table 20. I_{50} values of chlorpyriphos for different populations of *S. litura*

Population	LC_{50} (%)	Resistance Ratio	Specific activity of AChE (nmoles min^{-1} mg^{-1} of protein)	I_{50} (µg / mL)	Inhibition Ratio
SUS	0.00098	1.00	2.38	7.45	1.00
HSP	0.005	5.10	3.40	11.00	1.48
SGR	0.029	29.59	3.88	12.80	1.72
SGR-Sel	0.042	42.86	4.11	14.60	1.96

4.3 Molecular analysis *S. litura* populations for genetic relatedness

In order to differentiate amongst closely related species and strains of different organisms, molecular analytical methods have proved to be significant, specific and advantage. These are based upon identification of a species (both inter- and intra-) specific DNA fragment (molecular markers) by specific PCR amplification of the same from total DNA only from the target species.

4.3.1. DNA isolation from different populations of *S. litura*

The DNA isolated from *S .litura* larva each from all populations, and analyzed on 0.7 per cent agarose gel represented a high molecular weight of DNA. It was seen as a single condensed band that was free from any major degradation products as depicted below for the ten individual

larvae from Sangrur population (Fig 8). Total DNA isolated from individuals from all other populations also had similar quality of isolated DNA. The DNA concentration as determined by A_{260} varied between 20- 30 ng μL^{-1} in 250 µL DNA solutions obtained from a single larva for each sample. This represented a good quality of DNA preparation and was considered adequately suited for subsequent molecular analysis of *S .litura* larva samples from different populations.

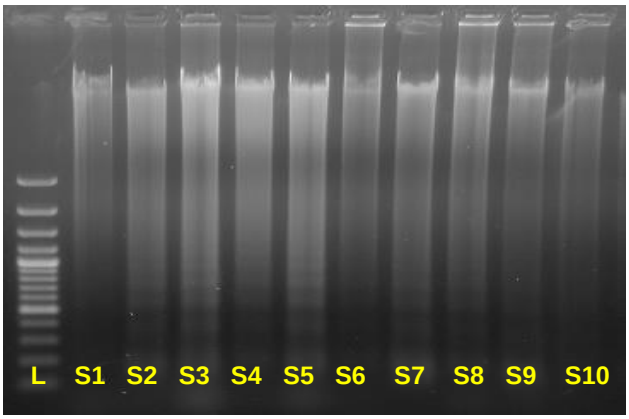


Fig. 8 Total DNA isolated from ten individual *S. litura* larvae (Sangrur population)

4.3.2. Comparative RAPD-PCR analysis for the identification of molecular differences amongst different individuals of *S .litura* of different populations

Total DNA from five larvae of four populations was PCR amplified using four RAPD primers, OPB-07, OPB-10, OPG-03 and OPL-08. The comparative RAPD banding pattern of these amplified PCR products from five individuals belonging to four different populations obtained with these primers is presented in Fig. 9.

All the primers amplified numerous products from individual larvae resulting in identification of 72 products in comparative RAPD- profile from different larvae from various populations (Table 21). The products obtained from each primer ranged from 16 (Primer OPB-10, OPG-03 and OPL-08) to 24 (Primer OPB-07).

Existence of such a high level of polymorphism suggested that all the populations were heterogeneous mixed genotype populations. The individuals with differing banding pattern represented different genotypes within the individual population of *S. litura*. Genetic relatedness dendrogram as developed using RAPD data by UPGMA was used to measure the quantitative description of these genetic differences (Fig. 10). The similarity coefficients based on the frequency of bands shared among the populations varied from 0.55 to 1.00.

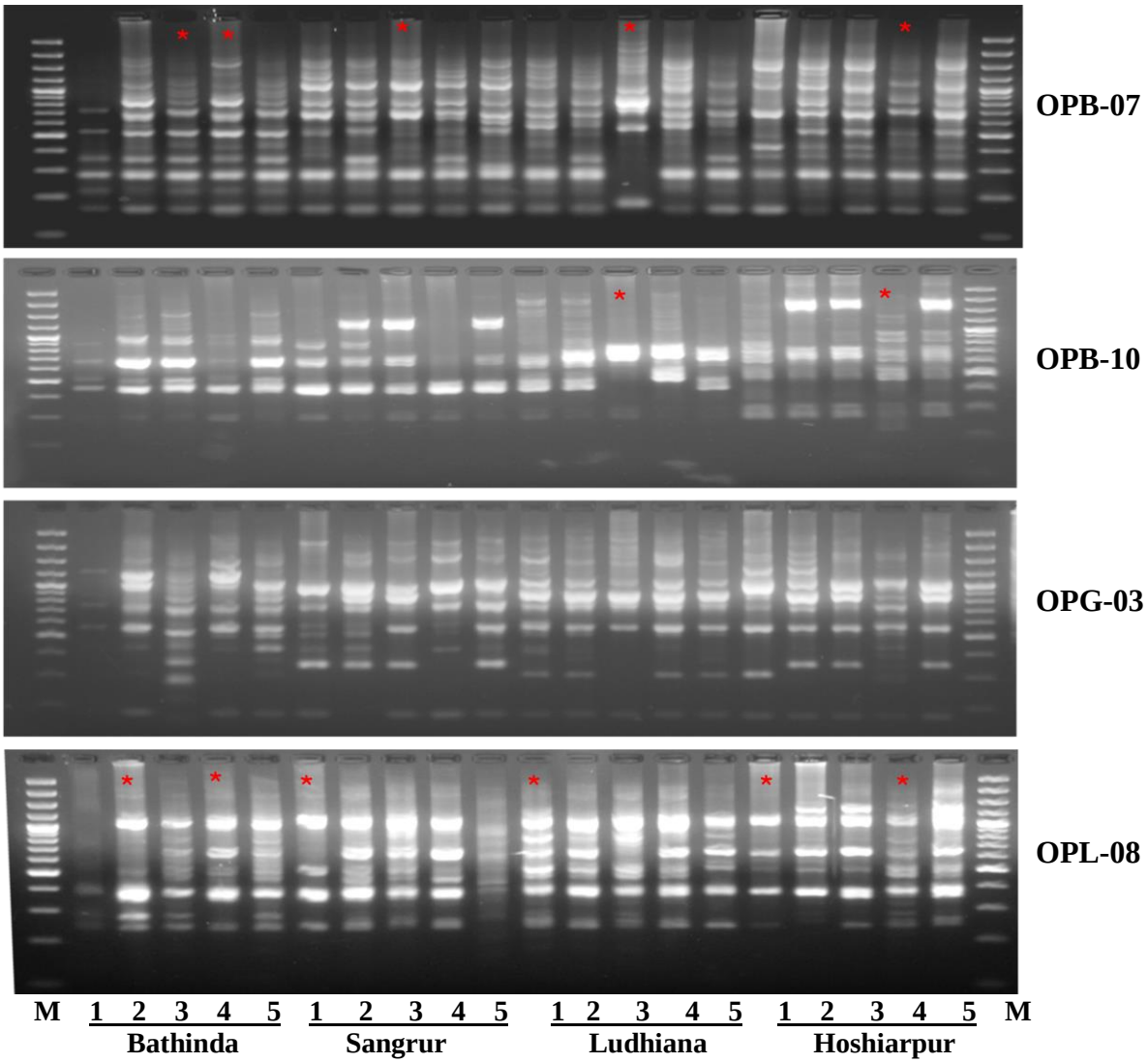


Fig. 9 Comparative RAPD-PCR amplification of isolated DNA from different *S. litura* populations using different RAPD primers.

1, 2, 3, 4, 5 represent larvae of each population

* Lanes showing DNA polymorphism among larvae of different populations

Table 21. Banding profile of RAPD-PCR Amplified DNA of different *S. litura* populations using different RAPD primers.

RAPD Primer	Size of amplified DNA bands (bp)			
	Bhatinda	**Sangrur**	**Ludhiana**	**Hoshiarpur**
OPB-07	**1800, 1500**, **1100**, **1000, 900**, **800**, **750, 700, 650, 550, 500, 450, 400, 350**, 275, 225, 160	**1800,** 1500, **1200**, 1100, 800, **750, 700,** 650**, 600, 550, 500, 450, 400,** 350, **300**, 275, 225, 160	**3000, 2400, 1800, 1500, 1200, 1100, 1000, 900,** 800, **750, 700, 650,** 600**, 550, 500, 450, 400, 350, 275, 225, 200, 160**	1500, **1200, 1150, 1100, 1000, 800, 750, 700, 650, 600, 500, 450, 400,** 350, 275, 225, 160
OPB-10	**2500, 1500, 1200, 1000, 900, 800, 700, 620, 500, 450,** 400**, 275,** 250	**2500, 1200, 800, 650**, 450, 300	**2500, 2000, 1500, 1200, 1000, 900**, 800, **700**, 650, **600, 500, 450**, 300	**3000, 2000, 1500, 1200, 1000**, 900, 800, 700, 650, 500, 350, 300, **200**
OPG-03	**2500**, **1500**, 1200, 1000, **900**, **800**, 750, 550, **500**, **400, 350, 300, 200, 180**	**2500**, **1500**, 1000, 900, **800**, 750, 550, **500**, **400, 350, 200**	**3000, 2500**, **2000**, 1500, **1200**, 1000, **900**, 800, 750, 550, **500**, **400, 350, 300, 200**	**3000, 2500**, **2000**, **1500**, **1200**, 1000, **900**, 800, 750, 550, 500, **400, 350, 300,** 200
OPL-08	**3000, 2500, 1200, 1100, 1000, 800, 700, 620, 500, 450,** 400**, 275,** 250	**3000, 2500, 1200, 1100,** 1000, **900, 800, 700, 620, 500, 450,** 400**, 275, 250**	3000, 2500, **1400**, 1200, 1100, 1000, 900, 800, 700, 620, 500, 400, **275**, 250	**2000,** 1400, 1200, 1100, 1000, **900, 800, 700,** 620, 500, **450,** 400, **275, 250**

Figures in bold represents the polymorphic marker bands.

Clustering analysis on the basis of the PCR-RAPD generated band sharing suggested that most of the individuals of population collected from Sangrur (the least susceptible population) clustered together. Population from Ludhiana was found to be the most homogenous of all the populations, except for one individual. There was no variation between two individuals of Hoshiarpur population, while two individuals from Bathinda region had 97 per cent genetic

similarity. In the populations collected from Hoshiarpur, one individual had more genetic variation than other individuals. Most of the individuals inspite of having intra-population genetic variations belonged to respective populations, a single individual each from Bathinda (B1), Hoshiarpur (H4) and Ludhiana (L3) find closer genetic similarity with larvae from other populations. This observation established existence of both inter- and intra- population variations amongst individuals forming different populations of *S. litura* that might be related to possibly similar differences in resistance to different insecticides. In a similar study, Janarthanan *et al* (2002) found that 8 out of 40 random primers exhibited distinguishable banding patterns showing polymorphism between 6 different ecotypes of *S. litura.*

4.4 Development of resistance specific molecular diagnostic markers for monitoring of insecticide resistance in *S. litura*, their evaluation and validation

4.4.1 RAPD-PCR amplification of *S. litura* field collected population and insecticide-selected population

In order to check genetic homogeneity amongst individuals from fenvalerate selected (R) and field collected Sangrur (S) *S. litura* populations, DNA isolated from five individuals each, was PCR amplified using a RAPD primer OPL-08. All the individuals in a specific population supported matching RAPD- banding profile establishing genetic homogeneity (Fig 11). Therefore, total DNA from all the individuals for each of the resistant and susceptible populations were separately pooled and designated as R and S for further study.

4.4.2 Molecular analysis *S. litura* field population and insecticide- selected population

The molecular differences between field population of *S. litura* (S) and one selected with fenvalerate (R) were assessed through comparative RAPD- PCR (Random amplified polymorphic DNA- polymerase chain reaction) using six RAPD primers, OPB-07, OPB-10, OPG-03, OPL-08, OPN-10 and OPP-04. The comparative RAPD banding patterns of these amplified PCR products from fenvalerate- selected (R) and field collected (S) populations obtained with these primers is presented in Fig. 12.

All the primers amplified a total of 37 products (Table 22).

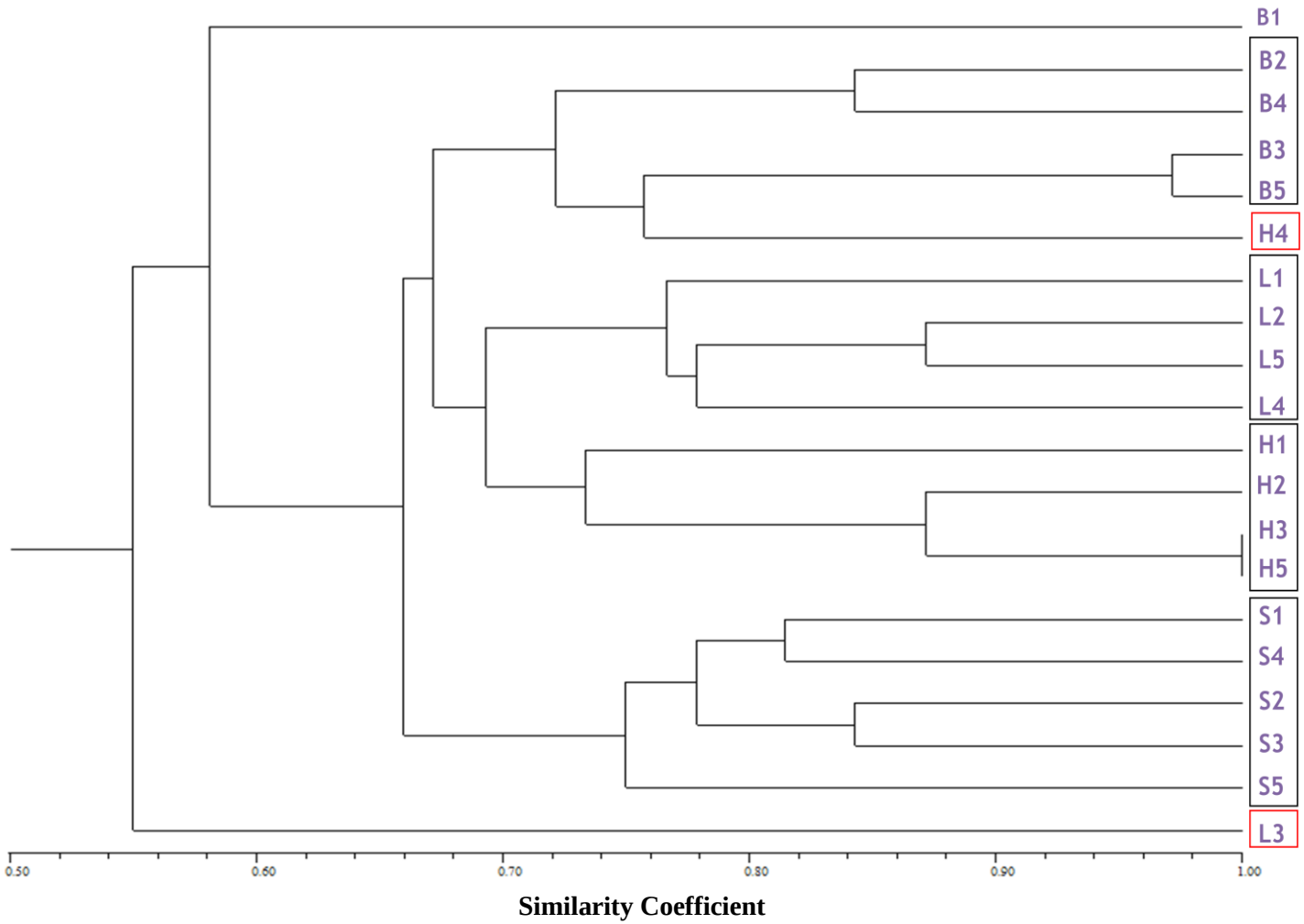
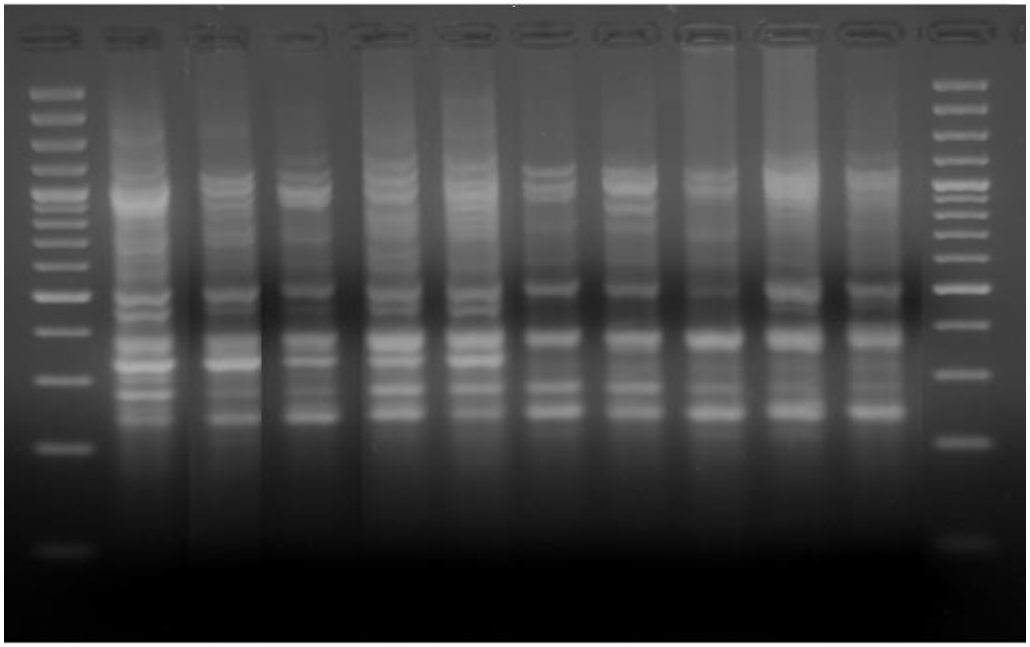


Fig. 10 RAPD-PCR based genetic relatedness dendrogram amongst individuals from different populations of *S. litura*



M R1 R2 R3 R4 R5 S1 S2 S3 S4 S5 M

Fig. 11 RAPD-PCR amplification of isolated DNA from individuals of fenvalerate selected (R) and field-collected (S) *S. litura* populations

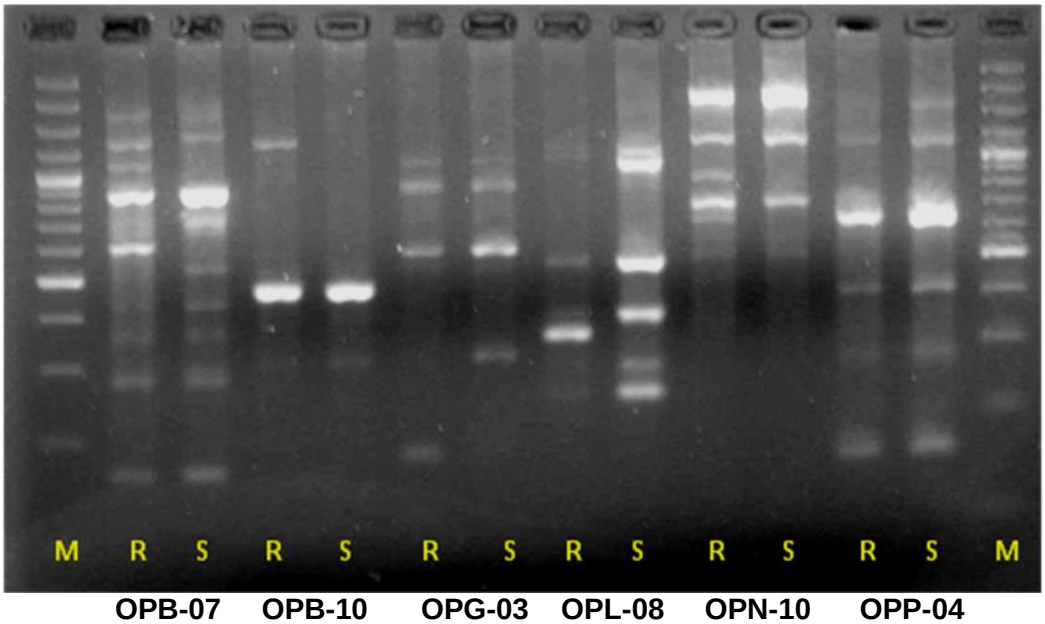


Fig. 12 Comparative RAPD-PCR amplification of isolated DNA from fenvalerate selected (R) and field-collected (S) *S. litura* populations using different RAPD primers.

Table 22. Banding profile of RAPD-PCR Amplified DNA of fenvalerate selected (R) and field-collected (S) *S. litura* populations using different RAPD primers

RAPD Primer	Size of amplified DNA bands (bp)
OPB-07	1800, **1500**, **1350, 1100**, 850, 700, **600, 550**, 450, 375, 280, 180
OPB-10	**1350**, 480, **300**
OPG-03	1100, 900, 600, **310**, **195**
OPL-08	1200, **1000**, 510, **400**, **350**, **290**, **250**
OPN-10	2000, 1200, **900**, 700, 680
OPP-04	**1800**, 1180, 625, 400, 180

Figures in bold represent the polymorphic marker bands

RAPD markers can produce a sufficient number of DNA markers for distinguishing between resistant and susceptible insects. The bands present in selected population and missing in the unselected population and vice-versa can be used to differentiate between such populations. Heckel *et al* (1995) had compared DNA from susceptible and resistant strains using RAPD methods. Of 117 primers tested, 75 showed polymorphism. Of the 223 such bands, 105 were found only in the susceptible strain and 118 only in the resistant strain. Shah *et al* (2002) had used RAPD markers for investigating its role in pesticide resistance monitoring in susceptible and propargite- resistant strains of *Tetranychus urticae* Koch. The DNA products obtained with the primer B8 were reproducible but inconsistent causing heterogeneity of PCR banding patterns. Ferguson and Pineda (2010) also used RAPD- PCR to identify polymorphic genomic DNA that would discriminate among cyromazine-resistant, abamectin-resistant, and susceptible *Liriomyza trifolii* leaf miners. The amplicons that were either amplified or those that disappeared in the individuals surviving the effect of insecticides selection pressure can serve as the potential RAPD markers for the identification of resistance at an early stage and could help in the pest management programmes. Sharma *et al* (2006) reported that RAPD-PCR analysis produced amplicons distinctively present in imidacloprid selected whiteflies.

4.4.2 Molecular analysis of mt COI fragment of variants of *S. litura*

The polymorphic products obtained from each primer ranged from 1 (Primers OPN-10 and OPP-04) to 5 (Primers OPB-07 and OPL-08) establishing availability of unique RAPD markers which could be related to insecticide resistance/ susceptibility or differences due to some other phenotype between insecticide resistant and susceptible individuals/ populations.

Although RAPD markers show a high level of polymorphism that might be able to differentiate between putatively resistant and susceptible individuals, designing primers based on them appeared to be very cumbersome. They are not feasible unless previously tested and validated. In the meantime, the mitochondrial COI sequences gained importance and universal acceptance as taxonomically important gene regions. Previous studies also corroborated on the changes in insecticide resistance in different insects with changes in nucleotide sequences in this region (Cariño *et al* 1994, Liu and Scott 1996, Maitra *et al* 2000, Catania *et al* 2004, Schlenke and Begun 2004, Feyereisen 2005, Amichot *et al* 2004, Zhu and Snodgrass 2003). Therefore, specific molecular makers based on mtCOI region were developed.

4.4.2.1. PCR amplification of COI fragment

A molecular correlation between pooled DNA of five individuals each of fenvalerate-selected (R) and field-collected (S) individuals with differing banding pattern representing different genotypes was derived based upon nucleotide sequences of phylogenetically important DNA region- mitochondrial cytochrome oxidase I (COI). The PCR amplifications of COI regions using region specific primers are shown in Fig. 13.

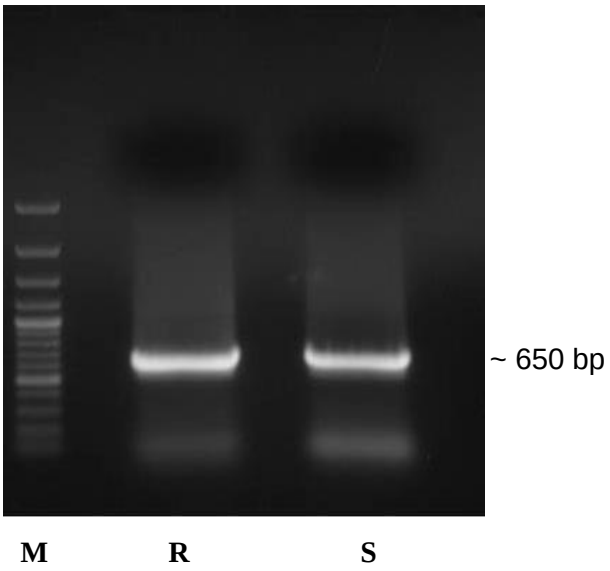


Fig. 13 PCR amplification of mt COI region (650 bp) with mt CO I specific primer set

COI primers helped consistent amplification of ~ 650 bp DNA fragments both from the pooled DNA from susceptible and resistant larvae. Size of these fragments was similar to the expected size of respective gene region reported for related lepidopteran species (GenBank Accession No. JQ064566.1) data for which was available in the GenBank database

(www.ncbi.nlm.nih.gov/pubmed/). This supported the specificity of the respective region specific primers and validity of the amplified COI gene fragments from the total DNA of all the different *S. litura* genotypes under study.

4.4.2.2 Cloning of PCR-amplified COI genes from variants of *S. litura*

i) Cloning of amplified COI gene fragments

After the purification of amplified COI gene fragments from the corresponding cut agarose bands, agarose gel electrophoresis of 10µL of purified DNA sample confirmed the presence of adequate amount of DNA (~100ng/ 5 µL) of expected size (~650 bp) in both the R and S samples. After the cloning of PCR amplified COI fragments, analysis of the isolated plasmids DNA showed that all the selected clones (five from R and three from S samples) were of equal expected size (~3.65 kbp) establishing correct cloning of amplified COI gene fragments of ~650 bp (Fig.14). This was indicative of existence of amplified DNA fragments of similar size as cloned insert DNA in the cloned plasmids.

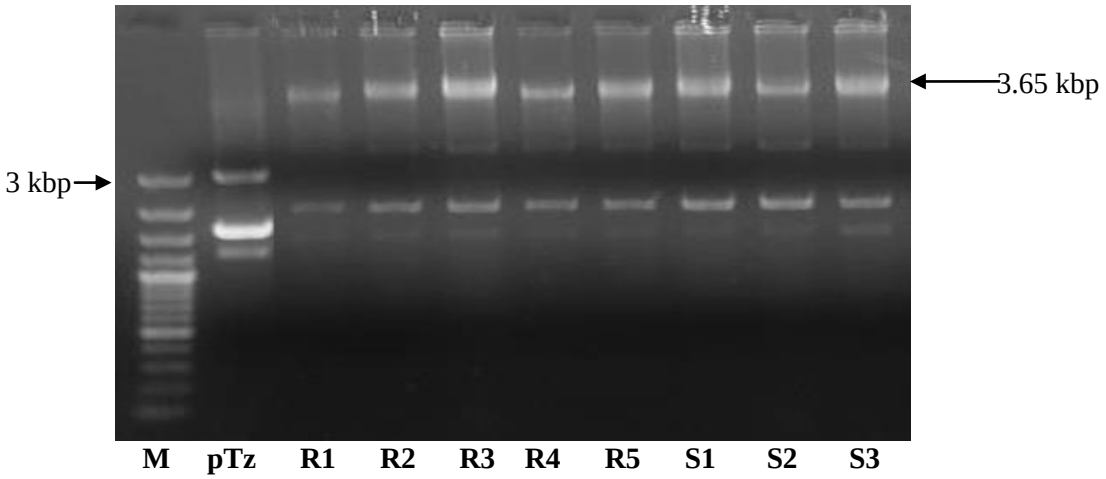


Fig. 14 Recombinant plasmids from different COI clones (vector + insert)
R1 R2 R3 are individuals from population of *S. litura* selected with fenvalerate and S1, S2, S3 are individuals from susceptible population of *S. litura*

ii) Analysis of recombinant clones for cloned COI gene fragment inserts by PCR amplification

The true identity of the insert sequences in the recombinant plasmids was confirmed by PCR amplification of cloned DNA from recombinant plasmid DNA by M_{13} universal primers (sites preknown to be present in the plasmid vector and around the cloning site) and

region specific COI primers (end sequences in the cloned COI gene fragments).

The amplification of insert fragments of COI genes was performed using universal M_{13} primers, which was expected to add 150 bp (from vector) to the cloned fragment and COI primer. Such PCR amplification of the all the different recombinant plasmid DNAs with M_{13} primers resulted in amplification of ~ 800 bp DNA fragments (Fig.15). This was equivalent to ~ 650 bp size of cloned amplicons for COI gene fragments.

Similar PCR amplification of the recombinant plasmid DNA with COI primers resulted in amplification of ~ 650 bp DNA fragments that represented the whole of the cloned COI fragment that was originally amplified from the total DNA of *S. litura* larva using same primers. Thus all the recombinant clones for all the different COI from both the *S. litura* populations proved true to the identity of correspondingly cloned amplicon DNA and were suitable for custom sequencing.

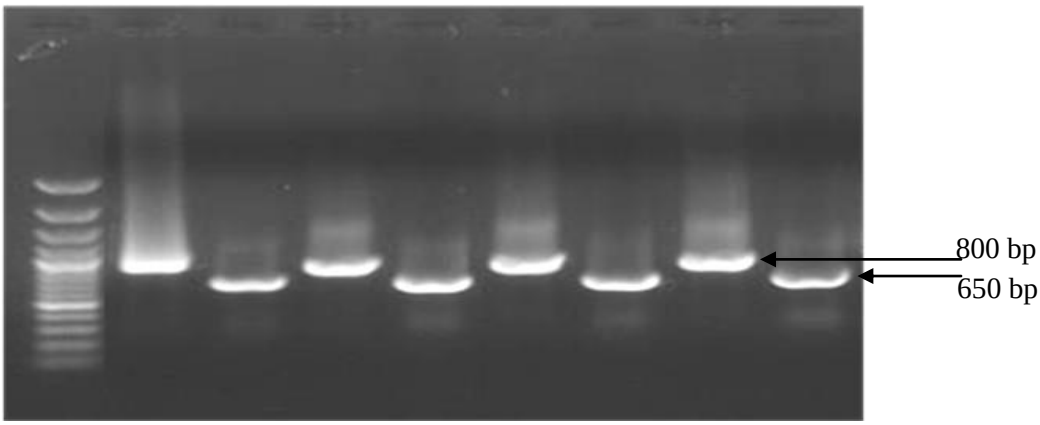


Fig. 15 PCR amplification of COI plasmid DNA with M_{13} and COI primer sets

iii) Restriction of plasmids: The restriction of the resulting recombinant plasmids with EcoR I and Pst I (sites bordering the cloning site) was expected to increase the size of the cloned fragment by 72 bp from vector (as per Fig 2). This restriction of recombinant plasmids released fragments of ~725 bp from the vector in case of all the individual recombinant plasmids corresponding to the cloned COI amplicon (Fig 16). This showed the presence of ~650 bp (722-72) cloned fragments in all the clones thus establishing true identity of the cloned fragments based upon their sizes.

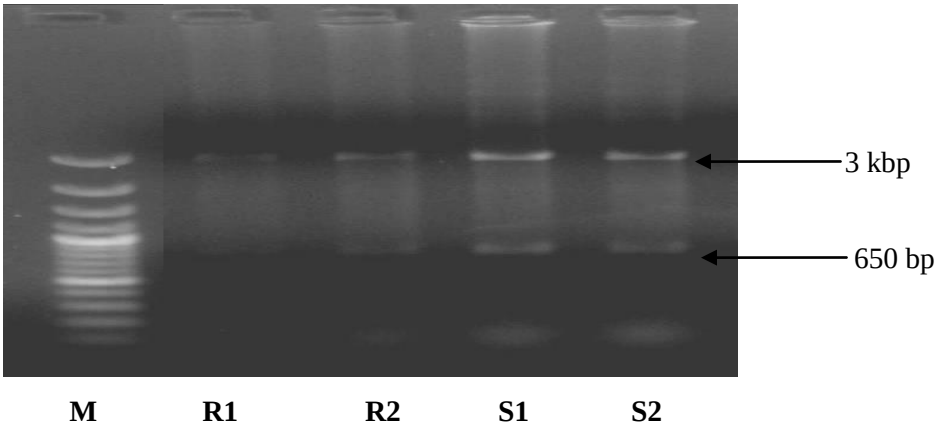


Fig. 16 Restriction of recombinant plasmids using Pst1 and EcoR1 releasing insert mt COI DNA (650 bp) from Vector plasmid DNA (3.0 kbp)

4.4.2.3. Custom sequencing of cloned COI DNA fragments

i) Custom sequencing of cloned COI DNA fragments

The sequencing grade recombinant plasmid DNA from single selected clone as representative of COI fragments from the two *S. litura* variants was purified and custom sequenced through services of 'M/S Xcelris Labs, Ahmedabad' for both the complementary strands using $M_{13}F$ in one set and $M_{13}R$ primers in another set of sequencing reactions. The raw data on the individual clones and for the both the complimentary DNA strands was proof-read for any misread bases by comparison with chromatograms of original sequence, completing the full length sequence for each fragment and by correcting for any misread base in each sequence. The whole sequence of the each individual strand was completed by manual aligning of the sequence of one strand with that of the reverse complimentary sequence for the corresponding sequence obtained with alternate M_{13} primer. This helped in both locating the vector sequences and cloned COI sequences in corrected sequences.

In the corrected sequences for each clone, the corresponding sequences for COI primers were located by search function of 'DNA Club'. These primer sequences were bordered by the vector specific sequences CTAG on the 5' end and AATCG on the 3' end of cloned insert sequence. The final sequences of the COI DNA fragments corresponding to both the variants of *S. litura* were submitted to 'GenBank Database', which assigned specific GenBank accession number to COI sequences from both the submitted sequences (Table 23).

Table 23. GenBank accession numbers of COI region variants of *S. litura*

Population	Variant designation	GenBank Accession No.
Fenvalerate selected population of *S. litura* (SGR-Sel)	SL-1	KC864790
Field collected population of *S. litura* (SGR)	SL-2	KC864791

ii) Nucleotide sequences of COI region variants of *S. litura*

a) Edited sequences of COI region of *S. litura* **variants under study**

1) Fenvalerate selected population of *S. litura* (SGR-Sel), SL-1 (GenBank Accession No. KC864790)

ATTCAACCAATCATAAAGATATTGGAACATTATATTTTATTTTTGGAATTTGAGCA
GGAATAGTAGGAACTTCCTTAAGTTTACTAATTCGAGCTGAATTAGGAACTCCAG
GGTCATTAATTGGAGATGATCAAATTTATAATACTATTGTAACAGCTCATGCTTTT
ATTATAATTTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGACT
TGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCACGTTTAAATAATATAA
GTTTTTGACTTTTACCACCTTCTTTAACCTTACTAATTTCAAGTAGAATTGTAGAA
AATGGAGCAGGAACTGGATGAACAGTTTACCCCCCCCTCTCCTCTAATATTGCTC
ATGGTGGAAGATCAGTAGATTTAGCTATTTTTTCCCTTCACTTAGCTGGAATTTCA
TCTATTTTAGGAGCTATTAACTTTATTACTACTATTATTAATATACGATTAAATAA
TTTATCATTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACTGCATTTTT
ATTATTATTATCTTTACCTGTTTTAGCTGGAGCTATTACTATATTATTAACTGATCG
AAATTTAAATACATCATTTTTTGATCCAGCAGGAGGAGGTGACCCTATTCTTTATC
AACATTTATTT

2) Field collected population of *S. litura* (SGR), SL-2 (GenBank Accession No. KC864791)

ATTCAACCAATCATAAAGATATTGGAACATTATATTTTATTTTTGGAATTTGAGCA
GGAATAGTAGGAACTTCCTTAAGTTTACTAATTCGAGCTGAATTAGGAACTCCAG
GGTCATTAATTGGAGATGATCAAATTTATAATACTATTGTAACAGCTCATGCTTTT
ATTATAATTTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGACT
TGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCACGTTTAAATAATATAA
GTTTTTGACTTTTACCACCTTCTTTAACCTTACTAATTTCAAGTAGAATTGTAGAA
AATGGAGCAGGAACTGGATGAACAGTTTACCCCCCCCTCTCCTCTAATATTGCTC
ATGGTGGAAGATCAGTAGATTTAGCTATTTCTTCCCTTCACTTAGCTGGAATTTCA
TCTATTTTAGGAGCTATTAACTTTATTACTACTATTATTAATATACGATTAAATAA
TTTATCATTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACTGCATTTTT
ATTATTATTATCTTTACCTGTTTTAGCTGGAGCTATTACTATATTATTAACTGATCG
AAATTTAAATACATCATTTTTTGATCCAGCAGGAGGAGGTGACCCTATTCTTTATC
AACATTTATTT

3) TNAU reference sequence *S. litura* (GenBank Accession No. JQ064566.1)

ATTCAACCAATCATAAAGATATTGGAACATTATATTTTATTTTTGGAATTTGAGCA
GGAATAGTAGGAACTTCCTTAAGTTTACTAATTCGAGCTGAATTAGGAACTCCAG
GGTCATTAATTGGAGATGATCAAATTTATAATACTATTGTAACAGCTCATGCTTTT
ATTATAATTTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGACT
TGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCACGTTTAAATAATATAA
GTTTTTGACTTTTACCACCTTCTTTAACCTTACTAATTTCAAGTAGAATTGTAGAA
AATGGAGCAGGAACTGGATGAACAGTTTACCCCCCCCTCTCCTCTAATATTGCTC
ATGGTGGAAGATCAGTAGATTTAGCTATTTTTTCCCTTCACTTAGCTGGAATTTCA
TCTATTTTAGGAGCTATTAACTTTATTACTACTATTATTAATATACGATTAAATAA
TTTATCATTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACTGCATTTTT
ATTATTATTATCTTTACCTGTTTTAGCTGGAGCTATTACTATATTATTAACTGATCG
AAATTTAAATACATCATTTTTTGATCCAGCAGGAGGAGGTGACCCTATTCTTTATC
AACATTTATTT

iii) Derived protein sequences of COI region variants of *S. litura*

1) SGR-Sel (GenBank Accession No. KC864790)

TNHKDIGTLYFIFGIWAGMVGTSLSLLIRAELGTPGSLIGDDQIYNTIVTAHAFIMIF
FMVMPIMIGGFGNWLVPLMLGAPDMAFPRLNNMSFWLLPPSLTLLISSSIVENGA
GTGWTVYPPLSSNIAHGGSSVDLAIFSLHLAGISSILGAINFITTIINMRLNNLSFDQ
MPLFVWAVGITAFLLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGGDPILYQHLF

2) SGR (GenBank Accession No. KC864791)

TNHKDIGTLYFIFGIWAGMVGTSLSLLIRAELGTPGSLIGDDQIYNTIVTAHAFIMIF
FMVMPIMIGGFGNWLVPLMLGAPDMAFPRLNNMSFWLLPPSLTLLISSSIVENGA
GTGWTVYPPLSSNIAHGGSSVDLAISSLHLAGISSILGAINFITTIINMRLNNLSFDQ
MPLFVWAVGITAFLLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGGDPILYQHLF

3) TNAU (GenBank Accession No. JQ064566.1)

TNHKDIGTLYFIFGIWAGMVGTSLSLLIRAELGTPGSLIGDDQIYNTIVTAHAFIMIF
FMVMPIMIGGFGNWLVPLMLGAPDMAFPRLNNMSFWLLPPSLTLLISSSIVENGA
GTGWTVYPPLSSNIAHGGSSVDLAIFSLHLAGISSILGAINFITTIINMRLNNLSFDQ
MPLFVWAVGITAFLLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGGDPILYQHLF

4.2.3 Multiple alignments of COI nucleotide sequences and derived protein sequences

Multiple alignment of COI nucleotide sequences for both the fenvalerate susceptible (SGR) and resistant variants (SGR-Sel) of *S. litura* as well as Reference *S. litura* (TNAU) is given in Fig. 17. This alignment established existence of no difference in the COI sequence from SGR-Sel type and reference type (TNAU). However, this alignment identified existence of a single base/ nucleotide polymorphism at nucleotide position 421 represented by substitution of T in SGR-Sel type/ TNAU type with C in SGR type. This single base polymorphism corresponded to substitution of single amino acid phenylalanine in the SGR-Sel type/ TNAU type with serine in SGR type at amino acid position in the predicted protein sequence of COI gene region under study (Fig. 18).

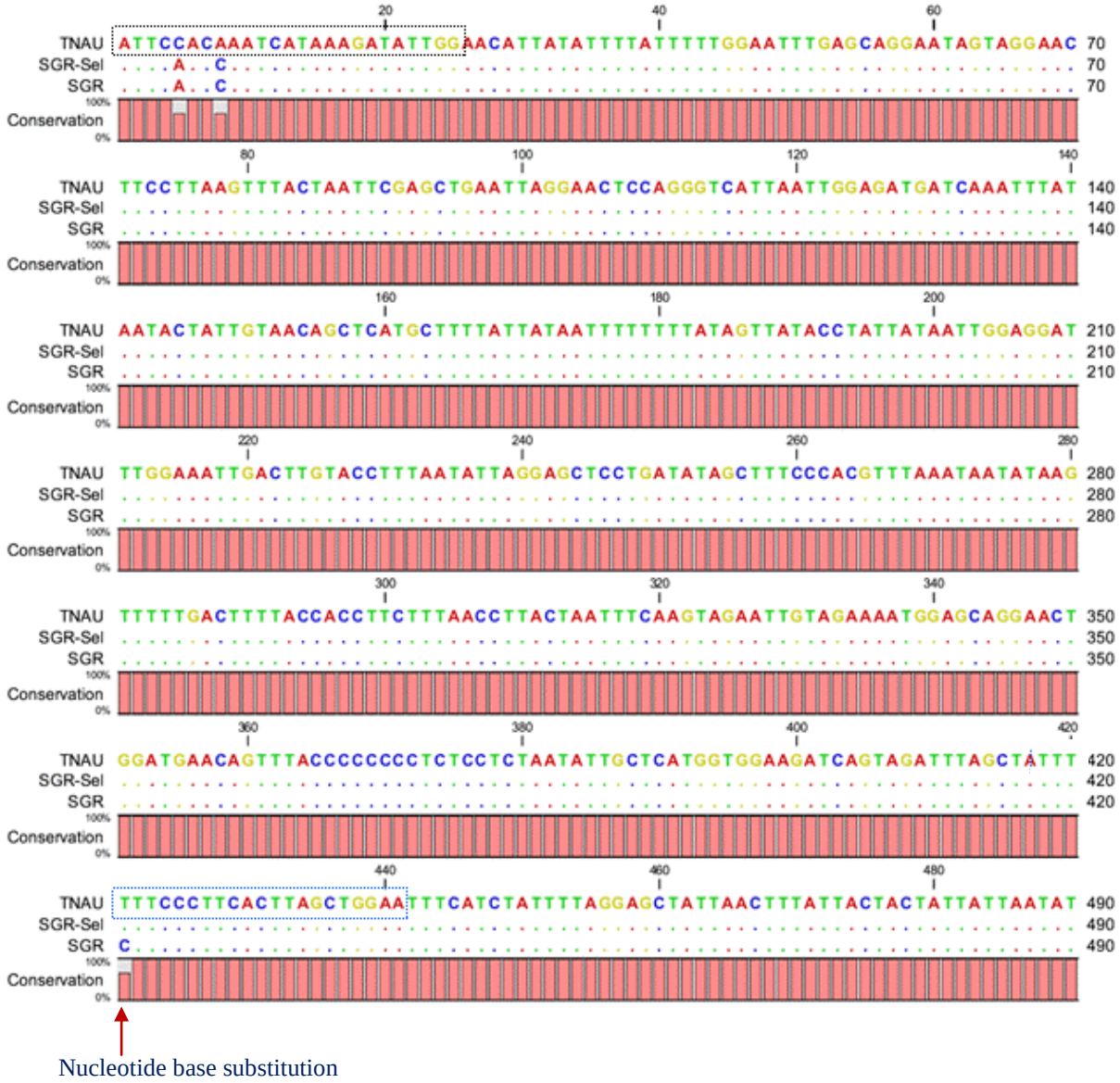


Fig. 17 Multiple alignment of mitochondrial COI gene sequences specific to *S. litura*

SGR-Sel fenvalerate selected population of *S. litura*

SGR field collected population of *S. litura*

Fig. 18 Multiple alignment of derived protein sequences of COI region specific to *S. litura*

4.4.3 Developing specific molecular markers based on COI gene sequences

Existence of single nucleotide polymorphism between the SGR and SGR-Sel/ TNAU type *S. litura* samples provided opportunity for development of COI based specific primers for possible exploitation in differentiating amongst these *S. litura* types. Therefore, based upon this polymorphism three specific primers that were designed and developed are given below:

Primer	Nucleotide sequence	Position in sequence	Remark
SL-F	ATTCAACCAATCATAAAGATATTGG	1- 25	Common Forward primer Lep-F
SL-R1	TTCCAGCTAAGTGAAGGGAAA	441- 421	Reverse primer Lep-R specific for SGR-Sel variant
SL-R2	TTCCAGCTAAGTGAAGGGAAG	441- 421	Reverse primer Lep-R Specific for SGR variant

Primers SL-R1 and SL-R2 differed w.r.t. terminal 3' nucleotide (A or G) only

4.4.4 Evaluation of specific molecular markers based on COI gene sequences

The specific primers that were designed based on COI gene sequences were custom synthesized and evaluated on the two populations under study, the field- collected SGR population and the fenvalerate –selected SGR-Sel population. Both the primers were able to yield a 441 bp amplicon as desired (Fig. 20). The primer set SL-F & S-R1 designed for SGR-Sel population could successfully amplify 441 bp product in the SGR-Sel variant and not in the SGR. Similarly, primer set SL-F & SL-R2 could amplify only in the SGR variant. Thus, both the primers were found to be specific.

4.4.5 Validation of specific molecular markers for differentiating between resistant and susceptible populations

The molecular markers developed specifically to differentiate between the resistant and susceptible populations of *S. litura* were validated by testing against field populations collected from different locations of Punjab. The genomic DNA from eight individuals each of four populations collected from Hoshiarpur, Sangrur, Ludhiana and Bathinda were PCR amplified with the specific primer set SLF R1. The amplified PCR products from different individuals of different populations obtained are presented in Fig 21. The specific primer amplified DNA in all the eight individuals from Sangrur population (found to be the most

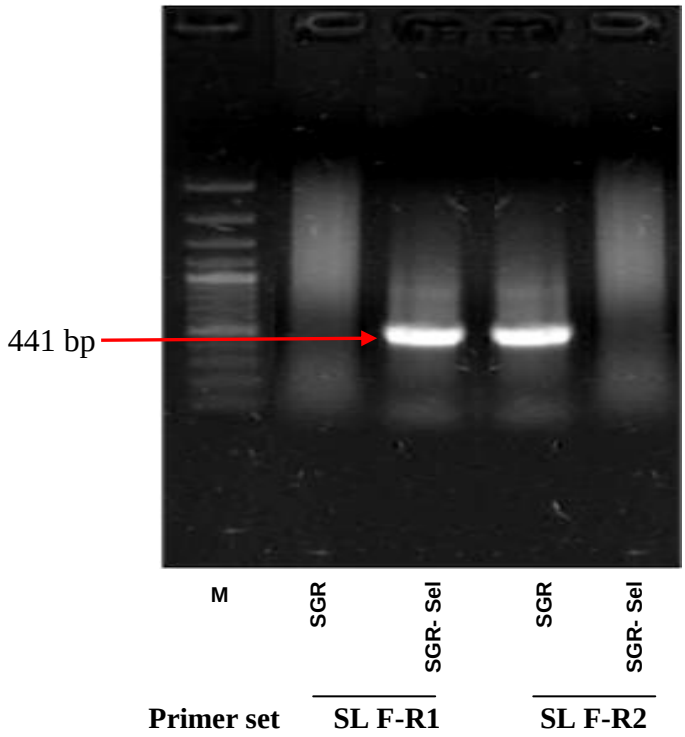


Fig. 20 PCR amplification of isolated DNA from resistant and field- collected *S. litura* variants with specific primer sets

resistant) while it amplified DNA in only 3 individuals of population from Bathinda. It was unable to amplify DNA in any of the individuals in populations collected from Ludhiana and Hoshiarpur. There is a possibility that the absence of amplification could result from the populations of Ludhiana and Hoshiarpur being susceptible to fenvalerate. Though, these populations were found to be less resistant to the insecticide, still the specific primer set designed may not be able to differentiate between resistant and susceptible individuals as effectively in populations from locations other than Sangrur.

From the bioassay studies, it can be concluded that population from Sangrur was found to be the least susceptible for most of the insecticides, except for chlorpyriphos, chlorantraniliprole and novaluron which were least toxic to population from Bathinda, and pyridalyl (least toxic to Ludhiana population). Population from Hoshiarpur was found to be the most susceptible for all insecticides except acephate and pyridalyl to which Bathinda population was most susceptible. Thiodicarb among the conventional group of insecticides and chlorantraniliprole among the newer insecticides were found to be the most toxic against all the test populations of *S. litura*. The synergistic effect of TPP (6.16) and PBO (3.72) in the least susceptible population suggested the involvement of esterases and mixed function oxidases in providing resistance to various insecticides. Synergistic studies in the fenvalerate-selected population indicated the possible role of mixed function oxidases, esterases and to

some extent glutathione S- transferases in imparting resistance to pyrethroids in *S. litura*. Biochemical studies provided evidence that multiple detoxification enzymes and AChE insensitivity were found to be involved in imparting resistance in *S. litura* towards various insecticides. Selection with fenvalerate increased the activities of MFOs, esterases and GST. The activities of MFO, esterases and insensitivity of AChE can be used as biochemical tools for monitoring insecticide resistance in *S. litura*. RAPD markers established the presence of inter and intra- population variations that might be related to insecticide resistance. Molecular makers based on COI region were developed, tested and validated for detection of resistant individuals in *S. litura* populations.

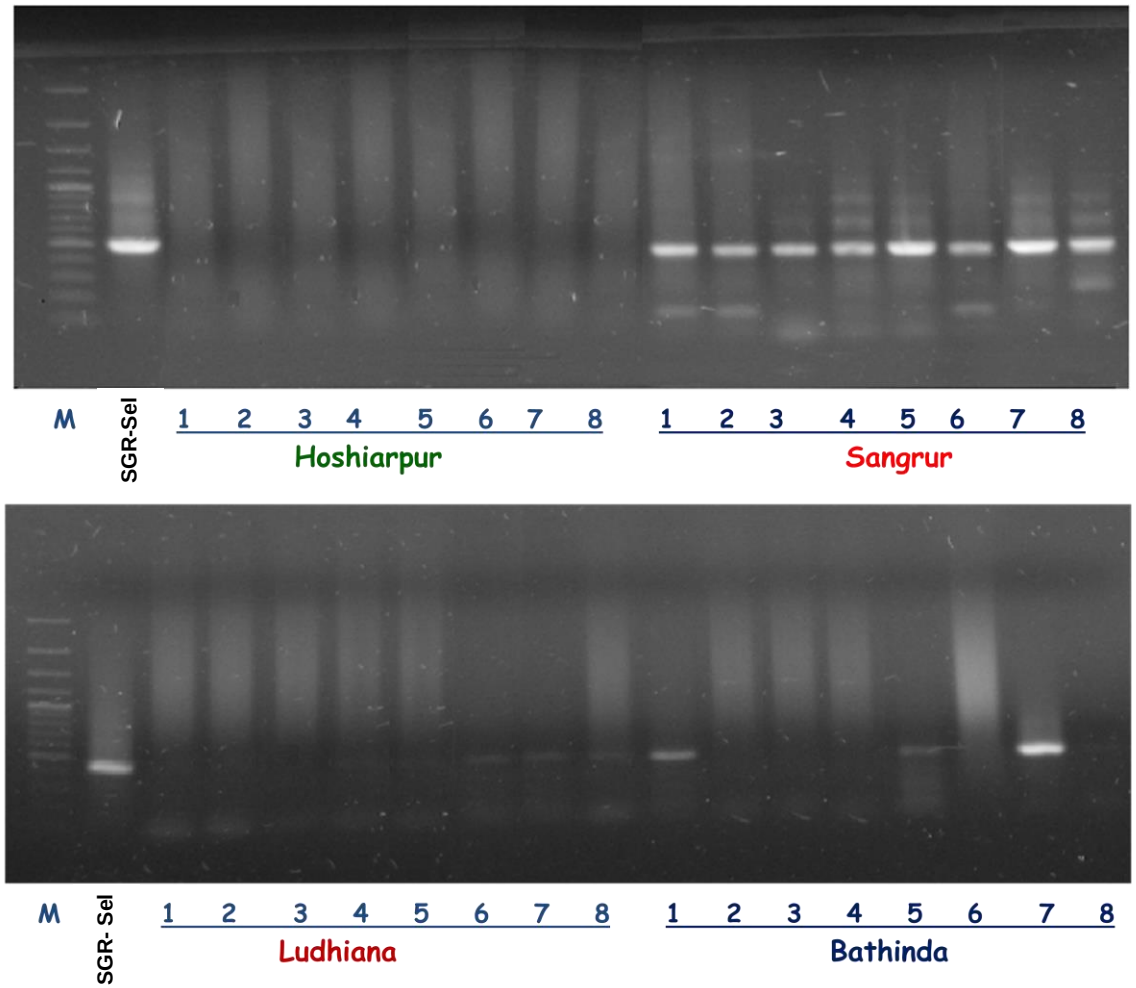


Fig. 21 PCR amplification of isolated insect DNA with SL F-R1 primer set for differentiating between resistant and susceptible populations of *S. litura*

Chapter V

SUMMARY

Tobacco caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is an important sporadic, polyphagous and potentially destructive insect pest causing heavy foliage damage to more than 115 species of plants. Several insecticides (including those with new chemistries) have been recommended for the control of this pest in many crops including cotton, pulses and vegetables. The presence of this pest on different crops throughout the year has widely exposed it to insecticides that resulted in rapid development of resistance. The insecticide resistance must be continuously monitored and must form an integral part of chemical control, to enable the detection of resistance as early as possible and to take necessary measures thereof. Insecticide resistance involves mainly three mechanisms viz., decreased penetration, enhanced detoxification and target site insensitivity. Synergists are the tools for elucidating resistance mechanisms and overcoming metabolic resistance, while biochemical analyses have been conducted to assess the mechanism of resistance. So far, no systematic, comprehensive studies had been done on the status of insecticide resistance in *S. litura* populations in Punjab. Moreover, the biochemical studies had not been conducted to assess the basis/ mechanisms of insecticide resistance involved in case of *S. litura* populations of the region. Therefore, populations of *S. litura* were collected from four regions of the state to assess the level of resistance developed.

The lowest LC_{50} value of fenvalerate was found against population from Hoshiarpur, while the maximum was observed against Sangrur population. Not much increase in fenvalerate resistance levels were observed over the past 6 years. They ranged from 2.8 to 14-fold; however, when compared to baseline values provided in 1992, it was found that resistance levels were about 117 to 578- fold. The lowest LC_{50} value of chlorpyriphos was found against populations from Hoshiarpur and Ludhiana, while the maximum was observed against Bathinda and population followed by Sangrur. The resistance levels ranged from 5 to 33- fold in *S. litura* populations when compared to a baseline value provided in 2007. There is not much change in resistance with respect to chlorpyriphos in Punjab. The lowest LC_{50} value of acephate was found against population from Bathinda, while the maximum was observed against Sangrur population, resistance ratio being < 7 fold and the lowest LC_{50} value was set as the baseline value. The susceptibility status of *S. litura* of Punjab seems to remain unchanged over the years, with no apparent indications of resistance development. Thiodicarb was the most toxic insecticide among all the conventional insecticides to all the tested *S. litura* populations. The lowest LC_{50} value of thiodicarb was found against population from Hoshiarpur, while the maximum was observed against Sangrur population. Not much increase

in thiodicarb resistance levels were observed over the past 6 years. They ranged from 2.5 to 15 fold. The lowest LC_{50} value of indoxacarb was found against population from Ludhiana, while the maximum was observed against population from Sangrur. There was no change in the susceptibility status of Ludhiana and Hoshiarpur populations to indoxacarb in the recent times. The population from Sangrur developed a 15- fold resistance. The difference between toxicity values of pyridalyl against all the populations was found to be unapparent. The resistance levels ranged from 18 to 24- fold in *S. litura* populations when compared to a baseline value provided in 2007. Chlorantraniliprole was the most toxic insecticide among all the newer insecticides to all the tested *S. litura* populations. The low LC_{50} value of chlorantraniliprole was found against population from Hoshiarpur and Ludhiana, while the maximum was observed against population from Bathinda. The resistance levels ranged from 2.5 to 50- fold in *S. litura* populations when compared to a baseline value provided in 2007. The lowest LC_{50} value of novaluron was found against population from Hoshiarpur, while the maximum was observed against Bathinda population, resistance ratio being 4.7 fold. There were no apparent indications of resistance development and the LC_{50} value of novaluron from Hoshiarpur was set as the baseline value.

The synergists TPP, PBO and DEM had a synergistic effect of 6.16, 3.72 and 1.73 fold in the relatively resistant population collected from Sangrur. This suggests the involvement of mainly esterases and mixed function oxidases in providing resistance in S. litura populations to organophosphates, pyrethroids and other insecticides. PBO had highest synergistic effect on the fenvalerate- selected resistan population (6.21- fold) followed by TPP (5.74- fold) and DEM (1.89- fold) indicating the possible role of mixed function oxidases, esterases and to some extent glutathione S- transferases in imparting resistance to pyrethroids in *S. litura*. A strong positive correlation (r = 0.98) was observed between MFO activity and fenvalerate resistance for different populations of *S. litura*. The activity was 4.26, 2.32 and 1.41- fold in the resistant populations SGR-Sel, SGR and HSP, respectively as compared to SUS population. GST activity was 1.72 and 1.31 times higher in the SGR and SGR-Sel populations than the SUS population, indicating some role in resistance towards insecticides mainly pyrethroids. A strong positive correlation (r = 0.98) was observed between esterase activity and fenvalerate resistance for different populations of *S. litura*. It was 3.70-, 2.14- and 1.40- fold in the resistant populations SGR-Sel, SGR and HSP, respectively compared with SUS population. All the resistant populations had significantly higher activity of AChE than the susceptible population. The activity was also positively correlated with fenvalerate resistance (r= 0.96). MFOs activity increased 1.84 times in the population from Sangrur after selection with fenvalerate, followed by esterase activity that increased 1.73 fold. GST activity increased 1.3 times while there was no change in the

activity of AChE. This showed that MFOs followed by esterases and to some extent GSTs were responsible for pyrethroid resistance in the pyrethroid-selected population. A decrease in the sensitivity of AChE to inhibition by chlorpyriphos, as evident from higher I_{50} values, resulted in increased resistance in *S. litura*. Activities of MFOs, esterases and insensitive AChE can be used as biochemimcal tools for monitoring insecticide resistance in *S. litura*.

Total DNA from five larvae of four populations was PCR amplified using four RAPD primers, OPB-07, OPB-10, OPG-03and OPL-08. Comparative RAPD banding patterns of these amplified PCR products from different individuals of different populations were obtained. Existence of such a high level of polymorphism suggested that all the populations were heterogeneous mixed genotype populations. Clustering analysis on the basis of the PCR-RAPD-generating band sharing indicated that most of the individuals of population collected from Sangrur (the least susceptible population) clustered together. Population from Ludhiana was found to be the most homogenous of all the populations, except for one individual. Thus RAPD markers established the presence of inter and intra population variations that might be related to insecticide resistance. However, they were not very efficient in differentiating between putatively resistant and susceptible individuals. After the purification of amplified COI gene fragments from the corresponding cut agarose bands, they were cloned. The true identity of the insert sequences in the recombinant plasmids was confirmed by PCR amplification of cloned DNA from recombinant plasmid DNA by M_{13} universal primers (sites preknown to be present in the plasmid vector and around the cloning site) and region specific COI primers (end sequences in the cloned COI gene fragments). The sequencing grade recombinant plasmid DNA from single selected clone as representative of COI fragments from the two *S. litura* variants was purified and custom sequenced. The nucleotide sequences of the two COI region variants were submitted to GenBank and Accession Numbers were granted. Alignment of the sequences identified existence of a single base/ nucleotide polymorphism at nucleotide position 421 represented by substitution of T in SGR-Sel type/ TNAU type with C in SGR type. This indicated substitution of amino acid phenylalanine by serine in SGR type. Based on this polymorphism, specific primers were designed and developed. These resistance specific molecular diagnostic markers were tested and validated for detection of resistant individuals in *S. litura* populations.

The present study, thus, presents a comprehensive resistance profile of *S. litura* populations collected from diverse areas of Punjab. The information generated for eight insecticides can be used for monitoring insecticide resistance in future work. Biochemical tools in terms of activities of insect's detoxification enzymes such as MFOs, esterases as well as the decreased sensitivity of AChE can be used as relatively less- laborious means of

insecticide monitoring. Furthermore, the information obtained on resistance mechanisms involved can serve as the basis for developing rarional resistance management strategies. The specific molecular diagnostic markers developed in the study can be exploited for early detection of insecticide resistance developed in *S. litura* populations.

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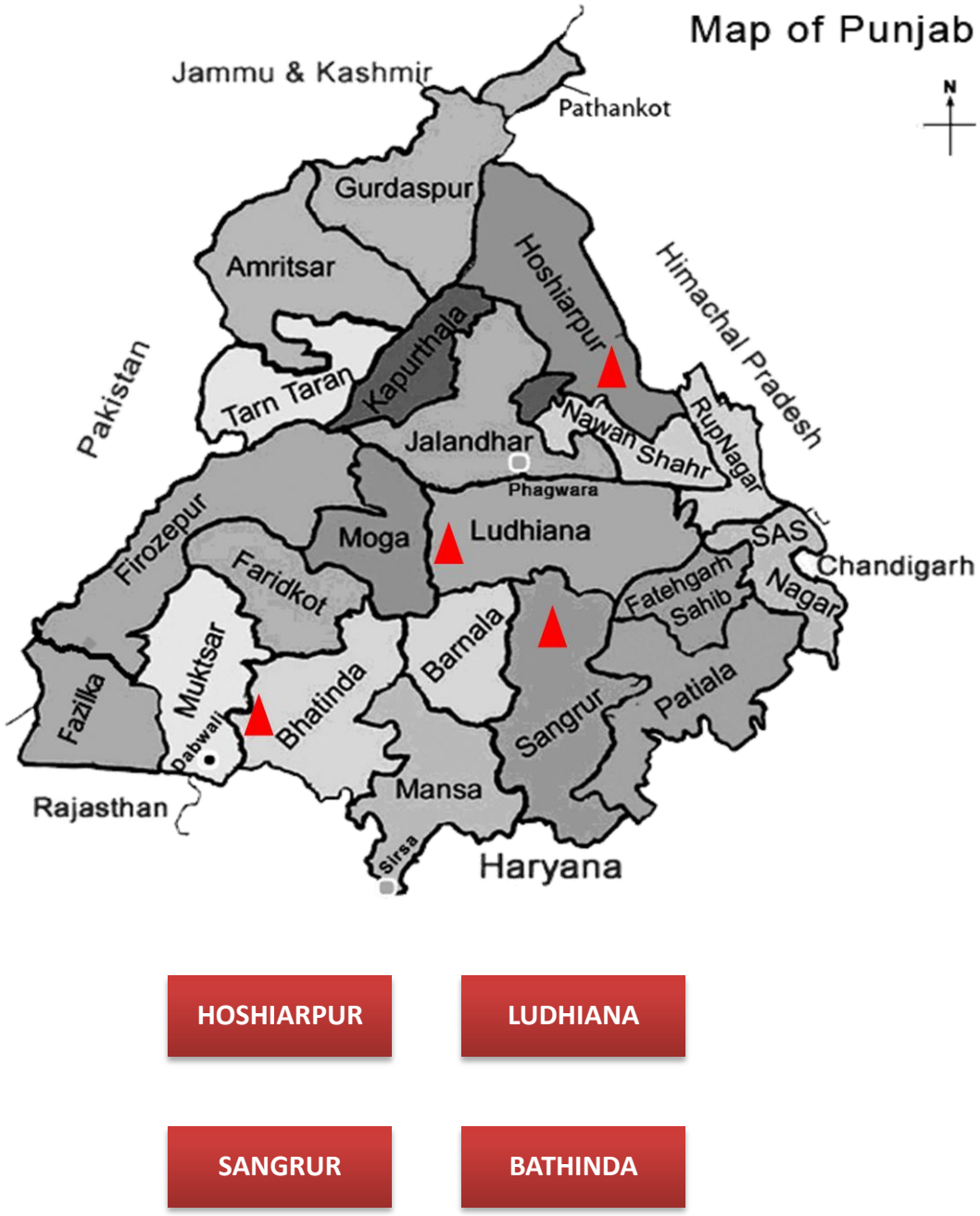

Plate 1 Collection of *Spodoptera litura* populations from various locations in Punjab

Plate 2 Rearing of *S. litura* and insecticide treatment using leaf disc bioassay technique

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

Plate 3 Development of *S. litura* on castor

(a) Eggs (b) 1st instar larvae (c) 2nd instar (d) 3rd instar (e) 4th instar (f) 5th instar (g) prepupa (h) pupae (i) adult male (j) adult female