THESIS

BIOCHEMICAL AND MOLECULAR DETECTION OF CYPERMETHRIN AND ROTENONE RESISTANCE IN THE TROPICAL ARMYWORM, *SPODOPTERA LITURA* (FABRICIUS)

AJIN RATTANAPAN

GRADUATE SCHOOL, KASETSART UNIVERSITY
2007

THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Bioscience)
DEGREE

Bioscience
FIELD

Interdisciplinary Graduate Program
PROGRAM

TITLE: Biochemical and Molecular Detection of Cypermethrin and Rotenone Resistance in the Tropical Armyworm, *Spodoptera litura* (Fabricius)

NAME: Miss Ajin Rattanapan

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR
(Associate Professor Suraphon Visetson, Ph.D.)

COMMITTEE MEMBER
(Assistant Professor Lertluk Ngernsiri, Ph.D.)

COMMITTEE MEMBER
(Assistant Professor John Milne, Ph.D.)

GRADUATE COMMITTEE CHAIRMAN
(Assistant Professor Pattana Srifah Huehne, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN
(Associate Professor Vinai Artkongharn, M.A.)

THESIS

BIOCHEMICAL AND MOLECULAR DETECTION OF CYPERMETHRIN AND ROTENONE RESISTANCE IN THE TROPICAL ARMYWORM, *SPODOPTERA LITURA* (FABRICIUS)

AJIN RATTANAPAN

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Bioscience)
Graduate School, Kasetsart University
2007

Ajin Rattanapan 2007: Biochemical and Molecular Detection of Cypermethrin and Rotenone Resistance in the Tropical Armyworm, *Spodoptera litura* (Fabricius). Doctor of Philosophy (Bioscience), Major Field Bioscience, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Suraphon Visetson, Ph.D. 127 pages.

This dissertation was carried out to investigate resistance of the tropical armyworm, *Spodoptera litura* (Fabricius), against 3 insecticides, i.e., cypermethrin, standard rotenone and derris crude extract, and their effects on this insect's detoxification enzyme systems over 10 generations. Ethanolic derris extract gave high yields both as crude extract and in rotenone content, *ca*. 48.60 and 17.91%w/w, respectively. The toxicity of the 3 insecticides estimated by the dipping method showed that cypermethrin gave the best control of 3rd instar larvae at LC_{50}=9.93 (r^2=0.98) after 72 hours exposure. *In vitro* study of carboxylesterase, acetylcholinesterase (AChE) and glutathione-s-transferase (GSTs) enzymes revealed that the pH most suitable for homogenization and incubation was pH 10. After 24 hours exposure to the three insecticides, carboxylesterase was found to be the most important enzyme for insecticide detoxification. When larval instars were examined, cypermethrin and derris extracts seemed to express enhanced enzyme activity in all instars while rotenone seemed to inhibit these three enzymes' activities in some larval instars.

The toxicological response after sequential treatment with these insecticides through 10 generations was examined and it was found that cypermethrin gave the highest levels of toxicity and resistance development. The cypermethrin treatment gave a resistance ratio of 104.58 fold for the F_{10} generation, which was much higher than those for the other 2 treatments (standard rotenone, RR = 5.79; derris crude extract, RR = 3.14). Through 10 generations, carboxylesterase showed the greatest increase in activity and all enzymes were characterized as enhancing activity. Although standard rotenone and derris crude extract resulted in enhanced enzyme activities, the increased activities were slight when compared with those resulting from cypermethrin treatment. SDS-PAGE showed bands for carboxylesterase, two subunits of AChE, and GSTs at 60, 24 and 48, and 24 kDa, respectively, in every generation. RT-PCR characterized a part of the gene associated with expression of the carboxylesterase enzyme in the F_{10} generation of each insecticide-treated population as a single band of 553 bp with 99% identity of nucleic acid and amino acid sequences to a *S. litura* partial sequence in GenBank (accession number DQ445461). Five positions, T^{30}, C^{76}, A^{102}, G^{111} and C^{381}, of partial sequences were found different from those in the GenBank database. Four of these differences (C^{76}, A^{102}, G^{111} and C^{381}) belonged to the cypermethrin population while another one (T^{30}) belonged to every population of this study. In addition, the deduced amino acid of cypermethrin also gave a single substitution from E^{37} to D^{37} which meant that Aspartate (Asp) substituted Glutamate (Glu). The results indicated that cypermethrin gave much faster resistance than did rotenone, which was caused by enhancement of detoxification mechanisms. Therefore, rotenone is an alternative safe choice to control *S. litura*. These results provide evidence for novel gene mutations that may lead to changes in the carboxylesterase enzyme mechanism in *S. litura*.

_____ _____ ___ / ___ / ___

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and deepest gratitude to my super-advisor Associate Professor Dr. Suraphon Visetson for provide my valuable advice and encouragement during my study. I would like to sincerely appreciated and greatly indebted my others advisory committee, Assistant Professor Dr. Lertluk Ngernsiri and Assistant Professor Dr. John Milne for their guidance and encourage throughout my research throughout the study.

I especially thank my graduate school representative, Assistant Professor Dr. Vipa Hongtrakul for her greatly guidance.

I am grateful to Dr. Manthana Milne and Dr. Chaiwud Sudthongkong for their valuable guidance in my research. I am also grateful to Ms. Vasakorn Bullangpoti for her excellently helped in my experiments. Thanks are extended to academic staff and my nice friends in Bioscience program and Department of Zoology and Biology, Faculty of Science, Kesetsart University.

I appreciate the financial support provided by the Cooperative Research Network (CRN) Scholarship of Mahasarakham University by Commission on Higher Education, Ministry of Education, and the thesis and dissertation support fund, graduate school, Kasetsart University for their funded and promotion supported my study, research and puplications that made me possible to successful accomblish of this investigation.

Finally, I am grateful to my family for their moral support and to relatives for their love and encouragement.

Ajin Rattanapan
May 2007

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

BIOCHEMICAL AND MOLECULAR DETECTION OF CYPERMETHRIN AND ROTENONE RESISTANCE IN THE TROPICAL ARMYWORM, *SPODOPTERA LITURA* (FABRICIUS)

INTRODUCTION

Spodoptera litura (Fabricius) is distributed worldwide especially in Asia and Oceania. It is a member of the economically important polyphagous pest that infests crops more than 120 host plants and causes serious crop losses (Singh and Jalali, 1997). Information on distribution, host plant, bio-ecology and control strategies of *S. litura* has been reviewed in entire south and south-east Asia countries, Australia and Pacific Islands (European and Mediterranean Plant Protection Organization (EPPO), n.d.). In Thailand, this insect is one of the key pests in agricultural area and causes damage to many cultivated plants (Department of agriculture (DOA), 2003, 2004; Plant pest forecasting and early warning group, 2005) such as vegetables, rice paddy field, fruit plantation, forest tree, etc.

The chemical control of *S. litura* has been extensively reported in relation to various crops in India. Until 1968, this pest was held in check by methyl-parathion, but then resistance to this compound developed. Since then, numerous other organophosphorus, synthetic pyrethroid, cypermethrin and other types insecticides have been used, with appearance of resistance and cross resistance in many cases (Issa *et al.*, 1984; Abo-El-Ghar *et al*., 1986). Until today they are going strong to insecticide resistance. In Egypt Sawicki (1986) showed the synthetic pyrethroid can stop the new resistance appearance on cotton by applied one time per year. The successfully of insect pest management like this, Solangi *et al*. (2001) recommended that the selective insecticides should be applied when insect pest population reaches to economic threshold level and the less toxic chemical should be encouraged for suppressing their population. However, although the synthetic chemicals control have the advantage of being very rapid in its action and giving generally predictable results, but it has many disadvantages, especially it has often failed as this pest is reported to have developed resistance to many commercially available insecticides. And the ever increasing resistance in this species (Surendra and Reddy, 1994) to many synthetic insecticides has compounded the problem (Nandagopal *et al*, 1997). Moreover, the inappropriate and indiscriminate used cause to problematic not only increased the production cost but also health hazard to the growers and consumers as well as disruption of the natural balance. Furthermore, mismanagement like applying broad spectrum insecticides at high dosages or repeatedly using the same active ingredients can lead to the development of resistance in insect including *S. litura* and also contaminate in the environments and agricultural products.

Thus, the development of other alternative insecticides such biological control as a means of overcoming this problem should be considers. Numerous studies have been carried out on possible biological control such as parasites, predators, microorganism and nematode. Nevertheless, directly use of these bio-control agents has not apparently passed into successfully practice and due to improper use of

synthetic pesticides, many insects have acquired resistance against many insecticides. By the other hand, interestingly the efficacy in various anti-feedant, anti-growth, substances that decrease reproductive percentages, etc, in compounds or extract of natural chemical products that can control the pests population. It is therefore, recommended that the alternative insecticides such as botanical insecticides should be introduced to combat the problem of infestation caused by *S. litura*. Because of this insecticide was showed the controlling efficacy to many insect pests replacement the synthetic insecticides (DOA, 2005) and also showed friendly effects to human and environmental in previously reported (Ruamthum, 2002; Bullangpoti, 2004).

The information concerning about insecticide resistances in *S. litura* is limited in Thailand. While the effectively control this insect requires knowledge about insecticide resistance mechanism involving biochemical mechanism and molecular study can help perfect control. Because of in recent year, the biochemical assays have a useful method for carried out the resistance mechanism and the molecular techniques have influenced all biological disciplines. These techniques have the advantage over the traditional bioassay which the mechanism clarity. Furthermore, this knowledge also provides understanding and circumventing to insecticide resistance in this insect. However, although the bioassay is a traditional technique, but I still use it for the toxicological data in this study.

Therefore, in this research I studied on insecticide resistance of cypermethrin which is one of the worldwide use insecticides comparing with rotenone which is a popular botanical pesticide aganist *S. litura*. I also focused on comparative toxicity, efficiency, effects to detoxification enzyme system such as carboxylesterase, acetylcholinesterase and glutathione-S-transferase and emphasised on effects on gene associated with detoxification enzyme expression. Thus, the main objectives of this research as shown below;

1. To determine the toxicity of cypermethrin, standard rotenone and derris crude extracts with known amount of rotenone against larvae of *S. litura*.

2. To characterize the detoxification enzyme mechanisms, carboxylesterase, acetylcholinesterase and glutathion-S-transferase enzyme, in *S. litura* larvae against cypermethrin, standard rotenone and derris crude extracts.

3. To determine if *S. litura* can develop resistance to cypermethrin, standard rotenone and derris crude extracts

4. To study genes associated with detoxification enzyme expression against cypermethrin, standard rotenone and derris crude extracts in *S. litura*.

LITERATURE REVIEW

1. *Spodoptera litura* biology and economic importance

Spodoptera litura (Fabricius) is the cosmopolitan insect species. It has a number of common names including the tropical armyworm, armyworm, cluster caterpillar, rice cutworm, cotton leafworm or tobacco cutworm. It is belongs to Phylum Arthropoda, Subphylum Uniramia, Order Lepidoptera, Family Noctuidae and Subfamily Amphipyrinae.

The female moth of *S. litura* lays its eggs underneath the leaves (Figure 1A), in clusters of 200-300 eggs. The egg masses are usually covered with hair scales from the anal tuft of the female. After 3-4 days hatching will take place. The newly hatched caterpillars (Figure 1B) are tiny, blackish-green, and with a distinct black band on the first abdominal segment. There are six larval instars. The head capsule is black (Figure 1C). Pupation takes place in the soil in an earth cell. Pupa (Figure 1D) is 1.4-1.8 cm long with red brown in color. The adult emerges after 6-7 days depending on the temperature. The adult (Figure 1E) is a nocturnal moth with a 30 mm wingspan, grayish brown moths with wavy markings on upper wings. The male moth is distinguished from female moth by having shiny bluish forewings, slender abdomen and a prominent large continuous yellow oblique patch in the centre of the forewing. In the female moth the central oblique patch is discontinuous and marked with brown scales. The whole life-cycle takes about 30 days, and in wet tropics they may be as eight generations (Areekul *et al.*, 1963; Hill, 1983).

Artificial rearing techniques have been developed and widely used for maintaining cultures of many insect pests and their natural enemies. While many diets have been formulated for use in rearing different pests, the most elaborate diets include those for lepidopterous pests, such as European corn borer, the corn earworm, the southwestern corn borer, the cotton bollworm and the common armyworm. Many diets can be used to rear several related species. It is therefore not always necessary to develop a complete new diet for each pest. In certain cases, only slight modifications to widely adaptable diets, are necessary to obtain a suitable rearing medium for a given insects (Abdullah, 2001).

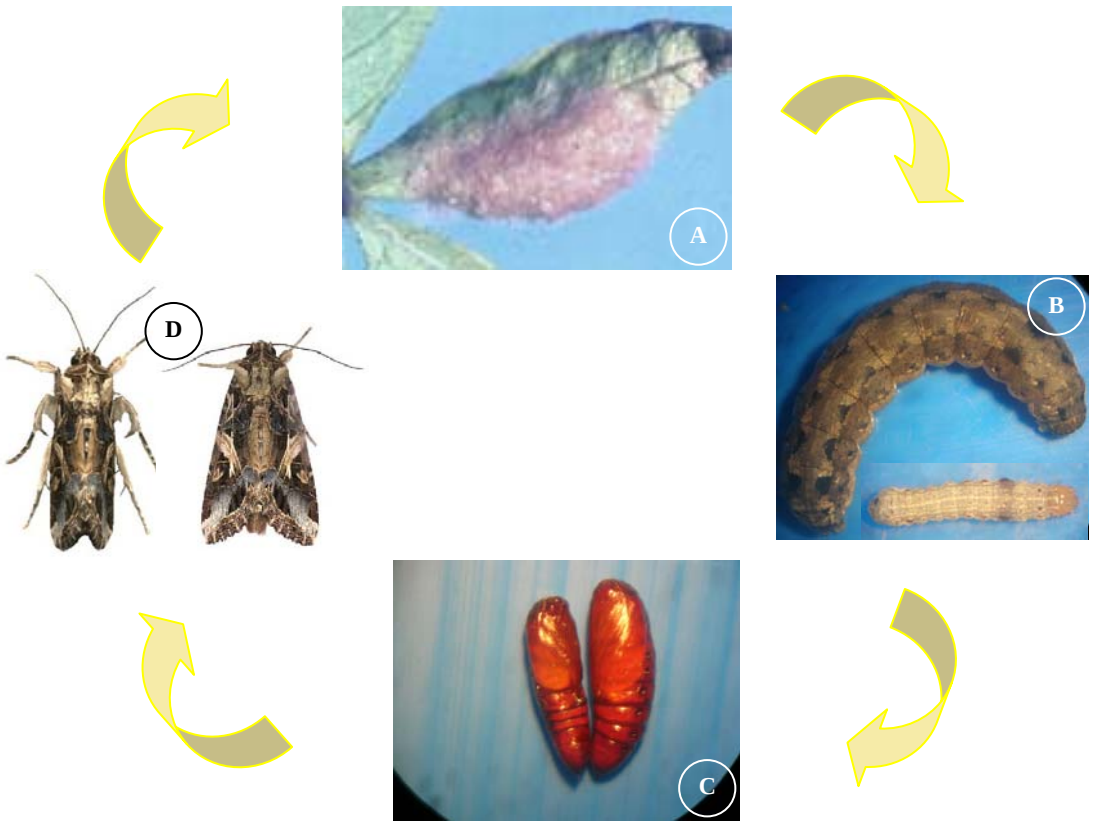


Figure 1 *Spodoptera litura* (F.); A = eggs, B = larvae, C = pupae, D = adults

S. litura was first recorded from New Zealand as a pest of tobacco and it has appeared in significant numbers in home garden and on crops (Cottier and Gourlay, 1955). It is a member of the economically important polyphagous pest that a serious attack many crop plants. The host range of this species covers over 40 families of economic importance crop species (Salama *et al*., 1970). Among the main crop species attacked in the tropics are *Colocasia esculenta*, cotton, flax, groundnuts, jute, lucerne, maize, rice soybeans, tea, tobacco, vegetables (aubergines, *Brassica*, *Capsicum*, cucurbit vegetables, *Phaseolus*, potatoes, sweet potatoes, *Vigna* etc.). Other hosts include ornamentals, wild plants, weeds and shade trees (e.g. *Leucaena leucocephala*, the shade tree of cocoa plantations in Indonesia). The damage is caused by the larvae feeding by chewing mainly on the foliage, besides young twigs, flower buds of many crops. In the early instars the larvae are gregarious in habit and hundreds of them are seen mining on soft tissues of a single leaf. The young larvae hang on silken threads produced from saliva and migrate to other leaves. In late instars, they become solitary and get distributed on leaves cutting holes on them by feeding. The grown up caterpillars also act as cutworms cutting the tender seedling of young plants in field. Under favorable conditions, if left unchecked the entire plantation are completely wiped out by this pest. The extent of damage varies from 80-100% in tobacco nurseries and 10-15% in planted crop (Prasad, 1997). In heavy infestation it can seriously defoliate a crop. The outbreak of this insect generally occurs with a good rainfall after a long dry spell (Boardman, 1977; Hill, 1983).

Larvae of *S. litura* is the most harmful insect pest of crops, which remains in the crop up to maturity and exhibits maximum damage during the vegetative phase of the plant where enough chlorophyll is present (Abdul Fatah *et al*., 1977). The pest emergence is found to be associated with food availability (Dass and Parshad, 1983) and suitable climatic condition where it attacks not only leaves but tunnels in the stem of the plant (Khoso, 1988). Mature larvae present a serious threat because of their extensive eating habits and the large numbers during an outbreak. The larvae consume about 90% of their total food intake in the last third of the larval stage (length 10 to 35 mm). Prior to this the small larvae and their limited damage is easily missed without careful inspection. Damage may not be noticed until the larvae are almost full-grown. Large larvae may invade from adjacent cereal crops or grasslands and eat out the entire area. Infestations are indicated by the eaten-out margins of leaves due to feeding of the older larvae and also by the faecal pellets around the base of the plant. The larvae often feed on the leaf blades leaving the midrib and so leaving the plant with a tattered appearance. Leaves up to 45 cm from ground level are stripped (Figure 2).

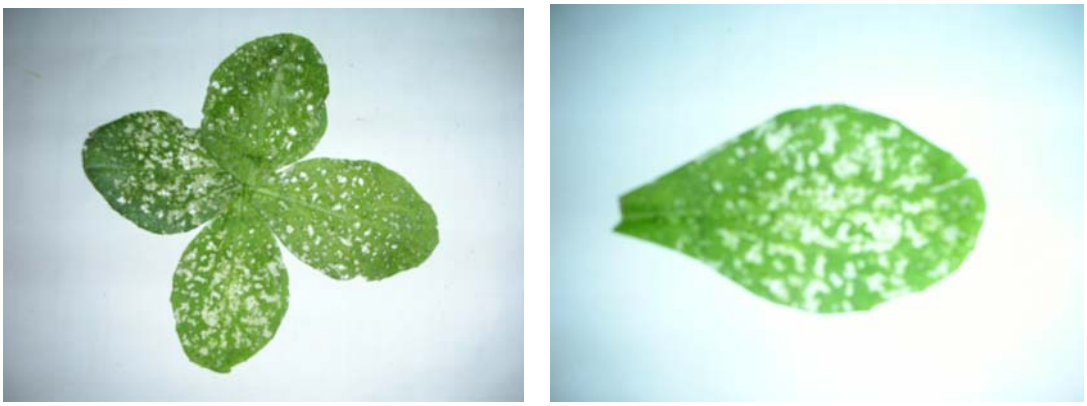


Figure 2 The characteristics of crops damage from *S. litura*.

The caterpillars cause severe defoliation resulting in huge loss. For example, the groundnut yield was reported to be 25.8 percent when the infestation level is at one larvae/plant seedling and 19 percent loss at flowering stages respectively, some season, a severe outbreak of this pest occurred on tobacco. On investigation it was observed crops such as groundnut, cotton, chillies and coriander before, after and along with tobacco were lead to greater incidence of *S. litura*. Resistance to insecticides, favorable weather conditions, cyclonic weather, and heavy rainfall following a prolonged dry spell also contributed to its outbreak. In heavy rainfall this insect had massive emergence of adults in a very short time and lead to widespread damage to plants. Samples of the pest were studied for insecticide resistance and the results indicated development of resistance to insecticides in this pest. The possible reasons for this outbreak were observed to be non-adoption of pest control measures/defective pest control measures undertaken, indiscriminate use of insecticides. These outbreaks suggest the necessity of adopting an environmental friendly control program for this pest. The insecticides strategy is a compatible manner to suppress the pest populations and maintain them below the economic injury level rather than total annihilation. With the advent of synthetic insecticides, chemical

control has become dominant in the pest management on crops. Several workers have test the efficacy of various types of insecticide against this pest and recommended insecticides like cypermethrin, endosulfan, etc for control its. But the use of insecticides is associated with problems like development of insecticide resistance in pest, toxicity to beneficial insects like parasitoids and predators and toxic residues in crop produce affecting the human health. Resistance in *Spodoptera* has been reported to all types of insecticides viz., organochlorines, organophosphates and synthetic pyrethroids. Its larvae reared on cotton showed tolerance to insecticides. Hence to mitigate these problems efforts have to be made towards judicious use of insecticides based on surveillance and economic thresholds for evolving a sound and ecological safe natural insecticides for this pest (Prasad, 1997).

In recent years *S. litura* have been causing increasing damage to cultivated crops worldwide. They are especially serious in the tropics and subtropics, including Thailand. They are polyphagous with feed on foliage, flowers and fruits from seedling stage up to harvest. When they are abundant, large population causes seriousness in plant injury, which results in decrease product. There infestations can result in total crops failed. High population of these insect can cause economic loss in many plants by reducing product. If control is necessary, they can be spraying synthetic insecticides. However frequent application of insecticides against them should be not practiced as this insect has tendency to develop resistance rapidly.

Geographical distribution

S. litura is distributed widespread in Asia, Oceania, North America (USA: Hawaii only) (EPPO, n.d.), Russia and UK (Aitkenhead, *et al.,* 1974) (Figure 3).

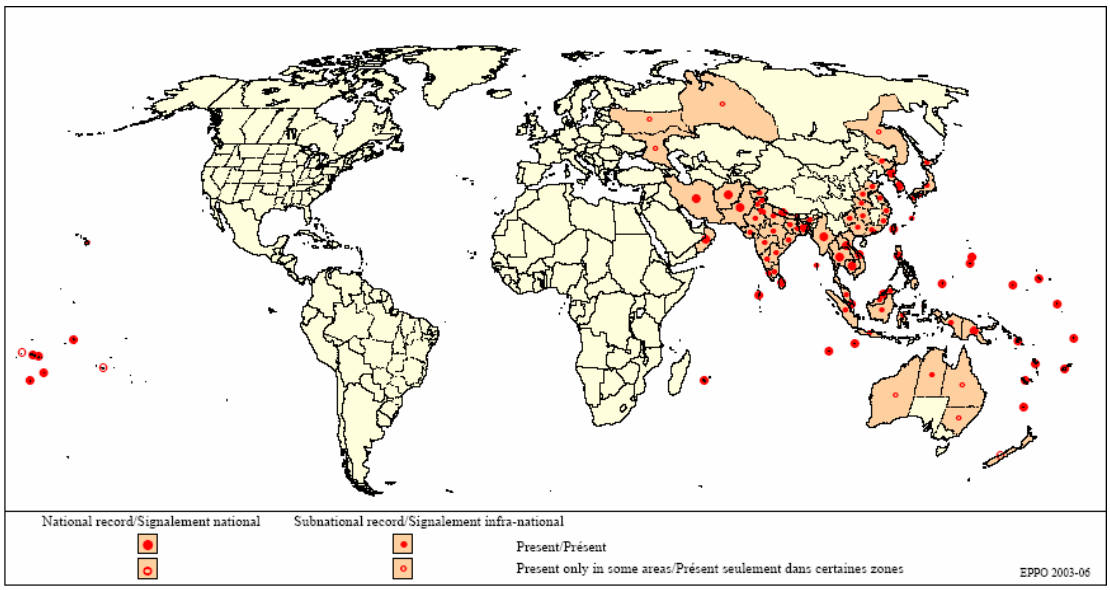


Figure 3 The geographical distribution map of *Spodoptera litura* (F.)
Source: EPPO (n.d.); Aitkenhead *et al.* (1974)

2. The *S. litura* control and insecticides effects

Currently, there are a lot of methods developed to control *S. litura* such as synthetic and natural insecticides. However, the major control is still various insecticides. Realizing this, the growers in Thailand make regular, usually 3-7 days/time, pesticide applications to their crops even when no pests are seen on them. And they were also used their tactics which many insecticides mixed application or altered kind of insecticides in a few short time in same area (data from personal contact). This is certainly not a proper control measure in pest management tactic from an environment standpoint. And they are short generation time and the excessive pesticide treatments to which they are subjected contribute to rapid development of resistance to insecticide. The knowledge of bionomics, materials application and biological traits is fundamental to control with several techniques to harmful this insect.

With the advent of synthetic insecticides, chemical control has become dominant in the pest management on crops. Several workers have tested the efficacy of various types of insecticides against this pest and recommended insecticides like synthetic pyrethroid (cypermethrin) for control them (Prasad, 1997). The primary action of pyrethroid is on the central nervous system which causes extensive disruption of nervous tissue. It effective both the peripheral and central nervous system of treated insects causing repetitive discharges followed by convulsion of insects. Application of higher concentrations of pyrethroid resulted in an acute blockage of nerve condition. The signs of toxic action of this insecticide in insects and mammals are very similar. The early signs of hyper excitation, tremoring and convulsion are followed by paralysis and death at lethal level of the insecticides (Perry *et al.*, 1998).

Although synthetic insecticides cause adverse effects on non-target organism and human being, they are still considers the only effective means to control insect pest among the farmers. The use of chemicals remains the most practical way of effectively reducing lepidopterous pest populations. Siddappaji *et al.* (1985) tested the efficacy of three synthetic pyrethroids, 0.004% deltamethrin, 0.009% cypermethrin and 0.06% fenvalerate against American bollworm larvae on pegion pea. It was found that the effective insecticide which gave 69.20, 50.40 and 46.30% decrease in damage, respectively. Singh and Nath (1998) evaluated 17 insecticides to control infestations of *S. litura* in groundnut fields in India where the most effective five insecticides in order of their efficacy against this pest were cypermethrin (0.016%), deltamethrin (0.003%), endosulfan (0.05%), fluvalinate (0.05%) and phosphamidon (0.05%). Mahadevan and Balasubramanian (1983) conducted the experiment of imichlovos and methomyl to control this insect, all applied at 0.5 kg a.i./ha, which gave over 75% control. Agnihotri *et al.* (1987) reported that cypermethrin at 60 g a.i./ha, permethrin at 90 g a.i./ha and fenvalerate at 120 g a.i./ha were more effective insecticides in controlling American bollworm than the traditional insecticides, carbaryl, acephat and quinalphos applied at 300 g a.i./ha. Suphangkasen *et al.* (1980) carried out field experiment to determine the effectiveness of three insecticides (fenvalerate, cypermethrin and decamethrin at 0.05, 0.10 and

0.015 kg a.i./ha respectively) against American bollworm on cotton at Takfa and Srisamrong field crop experiment station in Thailand. They observed that the lower rate of these insecticides still controlled this insect expect in case of heavy infestation where higher rates were needed. But the use of insecticides is associated with problems like development of insecticide resistance in pest, toxicity to beneficial insects like parasitoids and predators and toxic residues in crop produce affecting the human health.

In vegetable, Lee *et al.* (1986) decided the experiment to observe the effect of several insecticides which were registered for control aphid. The result showed that cypermethrin EC, methomyl LG, deltamethrin EC methidathion EC were highly effective, over than 90%, on the *Aphid citricola*. In pod borer control, Gohokar *et al.* (1994) reported that the treatment of cypermethrin 0.006% was found to be effective in controlling least than fenvalerate 0.01%, but gave the maximum yield higher than fenvalerate 0.01% and other treatments in this experiment. In pigeonpea pods in India, Baruah *et al.* (1997) reported that the bioeffective of cypermethrin 10%EC, 0.006% (45 g a.i./ha), fenvalerate 20%EC, 0.008% (60 g a.i./ha), deltamethrin 2.8%EC, 0.002% (25 g a.i./ha) and endosulfan 35%EC, 0.07% (525 g a.i./ha) against pod borer *Helicoverpa armigera*.

2.1 The synthetic insecticide used in this research: Cypermethrin

Cypermethrin has become one of the most important insecticides in wide-scale use. It has been said that "no pesticide is perfect, but the pyrethroids come close". This insecticide is a stomach and contact insecticide effective against a wide range of insect pests, particular leaf and fruit-eating Lepidoptera (Wothing, 1987). Its structure is based on pyrethrum, a natural insecticide which is contained in chrysanthemum flowers, but it has a higher biological activity and is more stable than its natural model. It was synthesised in 1974 and first marketed in 1977, by Shell (which has since sold their pesticide business to American Cyanamid). In 1988, pyrethroids amounted to 40% of the sales for insecticides for cotton treatment in the world (cypermethrin 8%) and cypermethrin is one of the most important insecticides for cereals and vegetables in the UK. There has been a dramatic increase in the use of cypermethrin for arable crops in the UK: from approximately 216,000 ha in 1988 to 1,582,000 ha sprayed in 1992, falling back to 863,000 ha in 1994. It is also used for impregnation of mosquito bed nets to prevent malaria, and extensively for indoor pests. As many patents for pyrethroids expire between 1993 and 1996, the market looks set to open up dramatically (Anonymous, 1995).

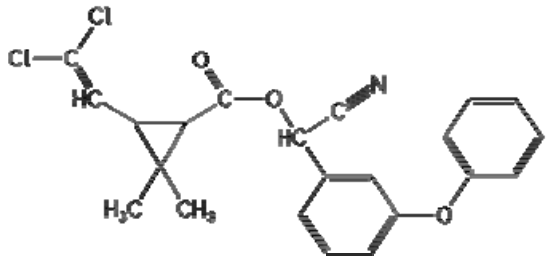
2.1.1 Common name: Cypermethrin

2.1.2 International Union of Pure and Applied Chemistry Nomenclature (IUPAC) name : (*RS*)-α-cyano-3-phenoxybenzyl (1*RS*,3*RS*;1*RS*,3*SR*)-3-(2,2-dichlorovinyl)-2,2 dimethylcyclopropanecarboxylate or (*RS*)-α-cyano-3-phenoxybenzyl (1*RS*)-*cis*-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate

2.1.3 Chemical Abstracts Service name (CAS) : cyano(3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate

2.1.4 Formulation: 35%EC

2.1.5 Chemical structure



2.1.6 Toxicity

2.1.6.1 Acute toxicity. Cypermethrin is classified by the World Health Organization (WHO) as moderately hazardous in class II based on acute oral LD_{50} toxicity. The class II poison is the LD_{50} for rats (the amount of the chemical lethal to one-half of experimental animals) is between 132 and 1,500 mg per kilogram (WHO, 2000, 2001). It interacts with the sodium channels in nerve cells through which sodium enters the cell in order to transmit a nerve signal. These channels can remain open for up to seconds, compared to the normal period of a few milliseconds, after a signal has been transmitted. Cypermethrin also interferes with other receptors in the nervous system. The effect is that of long-lasting trains of repetitive impulses in sense organs. Symptoms of poisoning include abnormal facial sensations, dizziness, headache, nausea, anorexia and fatigue, vomiting and increased stomach secretion. It is also a skin and eye irritant. Normally, symptoms should disappear after some days but severely exposed patients additionally may suffer from muscular twitching, comata and convulsive attacks. In such cases, symptoms may persist for some weeks. Most cases of pyrethroid poisoning have been reported in China (nearly 600 between 1983 and 1988, of which 45 involved cypermethrin). They occur among farmers, mostly after misuse. Recently, poisonings have as well been reported after indoor use of pyrethroids in Germany among pest controllers and private users (He, 1994).

2.1.6.2 Chronic toxicity. Chronic symptoms after exposure to pyrethroids have now been reported. Symptoms include brain and locomotory disorders, polyneuropathy and immuno-suppression and resemble the multiple chemical sensitivity syndrome (MCS).

2.1.6.3 Carcinogenic and mutagenic effects. Cypermethrin is classified by the US EPA as a weak category C oncogen - a possible human carcinogen with limited evidence of carcinogenicity in animals but no evidence of carcinogenicity in humans: it produced benign lung adenomas (tumours) at the highest dose level in female mice and has potential for liver carcinogenicity in

rodents. However, the view of WHO is that as there was no evidence of carcinogenicity in male mice and as the results of mutagenic studies have been mainly negative, it is concluded that there is no evidence for the carcinogenic potential of this insecticide. Cypermethrin was found to be genotoxic in mouse spleen and bone marrow but other tests have been negative (Amer *et al.*, 1993).

2.1.6.4 Resistance effects. As the pyrethroids are chemically relatively similar, a pest species resistant to one member of the pyrethroid family is often resistant to another or even to all types (Beugnet and Chardonnet, 1995). Replacing one pyrethroid by another may not therefore be appropriate when resistance occurs. Resistance against cypermethrin is reported widely for the tobacco budworm (one of the most important pests on many crops in the US and Mexico). Some *Heliothis* species, the most serious pests on cotton, also developed resistance against pyrethroids, which led to severe yield losses worldwide (Rosier, 1990). In *S. litura* Kodandaram and Dhingra (2006) showed 12.9 fold of resistance to cypermethrin in the third instar larvae of *S. litura* Punjab strain in India when applied by direct spray and leaf dip method, respectively. Armes *et al.,* (1997) reported the resistance of *S. litura* to cypermethrin was 2 to 197 fold in groundnut. The onset of resistance is frequently accompanied by increased doses of toxic pesticides, gradual loss of pest control and consequent loss of the farmer income and increase in pesticide hazard.

2.2 Botanical insecticides used in this research: Standard rotenone from Sigma and crude extracts of derris roots with known amount of rotenone.

Rotenone is classified by the WHO as a moderately hazardous in class II. It is a naturally occurring chemical with insecticidal obtained from the roots of several tropical and subtropical plant species belonging to the genera *Lonchocarpus* and *Derris*. It is a selective, non-specific insecticide, used for insect control. It is highly toxic to fish. Rotenone is an insect stomach and contact poison, kills slowly which takes about 2-3 days by being deprived of oxygen to the tissue cell, it exerts its toxic action by acting as a general inhibitor of cellular respiration, but causes insect to stop their feeding almost immediately (Ware, 1978, 1983). This substance is not a stable compound and breaks down when exposed to light, heat, oxygen and alkaline water into two common substances; carbon dioxide and water**.** It has a longer residual life than most botanical insecticides, its half-life is between 1-3 days. Nearly all its toxicity is lost in 5-6 days of spring sunlight, or 2-3 days of summer sunlight. It does not readily leach from soil and it is not expected to be a groundwater pollutant.

The common acute oral toxicity of rotenone is moderate for mammals, but there is a wide variation between species. It is less toxic for the mouse and hamster than for the rat; the pig seems to be especially sensitive. Recent studies have shown that in rats, rotenone is more toxic for females than males. It is highly irritating to the skin in rabbits and to the eyes. In rats and dogs exposed to rotenone in dust form, the inhalation fatal dose was uniformly smaller than the oral fatal dose. Rotenone is believed to be moderately toxic to humans with an oral lethal dose estimated from 300 to 500 mg/kg. A lowest lethal dose of 143 mg/kg has been cited in a child. Clinical

experience seems to indicate that children, in particular, are rather sensitive to the acute effects of rotenone. Human fatalities are rare, perhaps because rotenone is usually sold in low concentrations (1-2% formulation), and because its irritating action causes prompt vomiting. If the dust particle size is very small, and can enter deep regions of the lungs, rotenone's toxicity when inhaled may be increased. Acute local effects include conjunctivitis, dermatitis, sore throat, congestion, and vomiting. Inhalation of high doses can cause increased respiration followed by depression and convulsions. On the basis of rabbit studies, absorption through the intact skin is low (International Programme on Chemical Safety; IPCS, n.d.).

2.2.1 Standard rotenone

2.2.1.1 Plant name: *Derris elliptica; Lonchocarpus utilis; L. urucu; L. nicou;* (barbasco; cube; haiari; nekoe; timbo)

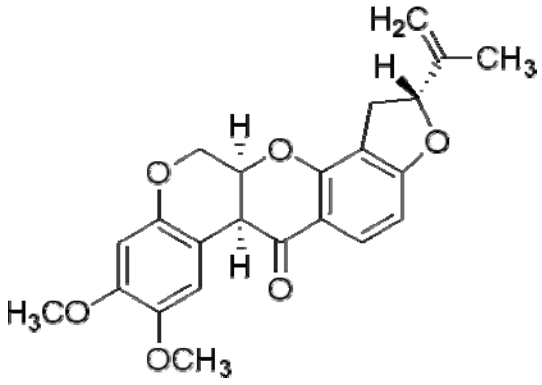
2.2.1.2 Common name: tubatoxin, tuba-root, derris-root, aker-tuba , cube-root

2.2.1.3 Synonyms of the chemical name: Derrin; Nicouline; Rotenonum; Tubatoxin

2.2.1.4 Purified: 95-98%

2.2.1.5 Active ingredient substance: (1,2, 12a-tetrahydro-8,9-dimethoxy-2(1-menthylethenyl (1) benzopyrono (2,4-b)furo (2,3-h) (1) benzophyran-6 (6H)-one

2.2.1.6 Chemical structure



2.2.1.7 Toxicity (Rahde, 1990)

- Acute toxicity. Ingestion, after ingestion, signs and symptoms are: numbness of oral mucous membranes, pharyngitis, nausea, vomiting and gastric pain. Muscle tremors, lethargy, respiratory stimulation followed by depressed respiration. In one case reported that cardiopulmonary arrest was the cause of death.

Animal experiments indicate that hypoglycaemia may occur. Inhalation, Rhinitis, coughing, and sneezing occur. In experimental acute poisoning, onset of symptoms is fast when dusts or fine powders are inhaled. Severe pulmonary irritation and asphyxia were present. Skin exposure, Skin irritation is observed after local application. Eye contact, rotenone was effected to the conjunctivitis and photophobia disease. Parenteral exposure, experimentally: vomiting, incoordination, muscle tremors, clonic convulsions and respiratory failure.

- Chronic toxicity. Ingestion, in animals, chronic ingestion causes growth depression. Inhalation, ulcerative rhinitis and complete but transient loss of smell. Irritation of the throat with partial destruction of the soft palate is as well as of the anterior pillars. Skin exposure, Occupational chronic exposure produces important immediate dermatitis characterized by a red-violet colour, slight oedema, and some itching. If contact is stopped, desquamation occurs and dermatitis became worse,with large papules. Eye contact; conjunctivitis.

- Carcinogenic and mutagenic effects. The carcinogenicity of rotenone is a controversial issue. It has been suggested that rotenone may cause tumours only in vitamin-deficient animals. About the mutagenicity is no mutagenic effects were reported in mouse bone marrow. And rotenone is also non-mutagenic in bacterial reversion tests.

- Interaction. When applied in low concentrations to plant foliage, rotenone catalyses the photoisomerization of dieldrin and other cyclodiene insecticide residues. However, photodecomposition was a predominant effect when residues of rotenone were combined with those of the methylcarbamate and phosphothionate insecticides.

2.2.2 *Derris elliptica* (Benth)

D. elliptica (Figure 4) is a large climber that is mainly cultivated in the tropics for its roots. It is known to be invasive in Fiji and western Polynesia (Pacific Island Ecosystems at Risk (PIER) 2000). In Hawai'i, it forms a dense canopy and smothers vegetation, fences, forest, pastures, and farm land. There are few locations of this aggressive invader on Maui (Starr *et al.*, 2003). *Derris* family has been proven as potent as many conventional synthetic pesticide. It has not appeared on the insecticide market for two decades because highly effective, synthetic pesticides have been introduced. However, with the development of resistant insects, the threat of contaminated food and high production cost problem, derris came back again in 1995 (Visetson and Milne, 2001). It is widely available in tropical, local plant in the eastern region of Thailand, locally it is known as “Lotin”. *Derris* is provides the rotenone compound that is a principal secondary plant substance. Rotenone is extracted from many tropical leguminosae such as *D. elliptica*, *Lonchocarpus nicou* and *Tephrosia vogelii*. It is contains 2-4 % w/w of active ingredient rotenone. It is believed to be effective against aphids, thrips, scale insects, mealybugs, spider mite and caterpillars (Zubairi *et al.*, 2007). Its mechanism of action in higher organisms is on interference with the electron transport chain at the inner mitochoria membrane. It has low toxicity to mammals, but is extremely toxic to fish (Matsumura, 1985).

WHO (1992) reported 96 h – LC_{50} of 0.02 – 0.2 mg/L for different fish species and for daphnids (Water Fleas) exposed to rotenone. The LC_{50} value of *D. elliptica* observed by Akinbulumo *et al.* (n.d.) was found to be higher than other reported, this may be as a result of fish species, environmental factors, food or water parameters. Guerrero and Guerrero (1986) reported at 96 h the LC_{50} of 10 – 20 ppm for *Oreochromis niloticus* fingerlings exposed to *Derris* root powder. Crystalline rotenone has an acute oral LD_{50} of 60, 132 and 3,000 mg/kg for guinea pigs, rats and rabbits (Matsumura, 1985). Because the toxicity of derris powders exceeds that of the equivalent content of rotenone, it is obvious that the other esters in crude preparations have significant biologic activity. Acute poisoning in animals is characterized by an initial respiratory stimulation followed by respiratory depression, ataxia, convulsions, and death by respiratory arrest. The anesthetic-like action on nerves appears to be related to the ability of rotenone to block electron transport in mitochondria by inhibiting oxidation linked to $NADH_2$, this resulting in nerve conduction blockade (O'Brien, 1967; Corbett, 1974). The estimated fatal oral dose for a 70 kg man is of the order of 10 to 100 g. In Thailand, derris extracts showed high efficacy to *Callosobruchus maculatus* (F.), *Spodoptera litura* (F.), *Sitophilus zeamais* Motschulsky, *Aedes aegypti* L., *Oligonychus mangiferus* Rahman & Sapra (Nicholas *et al*., 1985; Chaikam, 2002; Sangsrichan, 2002). Worawong (2003) reported that the effectiveness of *Derris elliptica* Benth exhibited both contact and stomach poisons extract on broad mite *Polyphagotarsonemua latus* Banks, with an LC_{50} of 0.0037% (direct spray) and 0.035% (leaf dip bioassay) at one hour after treatment.

The detoxification enzyme mechanisms against rotenone in *S. litura* have not been investigated. This study is to be describes the root of derris extraction, laboratory efficacy test and mechanism of detoxification enzymes of rotenone for an environmental friendly and non toxic biopesticide.

Figure 4 *Derris elliptica* (Benth) in Thailand.
Source: Department of Agriculture (2005)

3. Resistance problem and resistant mechanism of *S. litura*

The resistance of an insect against insecticides comes from the detoxification enzyme. These enzymes are produce after exposure to the insecticides for a long period time. Their functions are metabolism of xenobiotics in the body (Dauterman and Hodgson, 1978; Yu, 1983, 1984; Small and Hemingway, 2000; Vontas *et al.*, 2001).

Resistance, as defined by the WHO, is the development of an ability in a given number of insects to tolerate doses of an insecticide which would prove lethal to the majority of individuals in a normal population of the same species (Brown, 1958). It is widely accepted that the development of insecticide resistance by insects is due to the selection of variants in the population carrying pre-adaptive genes for resistance. Insecticide resistance strains seem to have arisen as a result of natural selection as the insects that have genetic characteristic for resistance to insecticides. They can survive after selection pressure by using insecticide and can transfer this genetic characteristic to descendants from one generation to another generation. Growers prefer using insecticides for controlling insect pest because they are convenient, quick and satisfyingly effective. The growers later found that the effectively used insecticide become less effective, they then try to apply more frequently with higher dosage of insecticides. New expensive insecticide was used instead of the old one and a few insecticides were mixed and sprayed at the same time, so the survival insects from selection pressure of insecticide could develop resistance to insecticide with increasing rate (Ounchaichon, 1989). The rate at which resistance develops in a population depends on the frequency of resistance genes present in population, the nature of this gene (either single or multiple, dominant or recessive), the intensity of selection pressure and the rate at which the species breeds (Perry *et al.*, 1998).

Insecticide resistance is now an immense problem associated with the chemical control of insect pests. More than 500 species of arthropods have evolved resistance to pesticides (Georghiou and Lagunes-Tejeda, 1991). *S. litura* from the several areas are resistance or highly resistance to all classes of insecticide. In many case this pest is becoming more and more dangerous, occupying new habitats and substituting other pest species. Undoubtedly they have spread throughout worldwide because of the market distribution of plants or plant parts, but resistance developed to most pesticides applied frequency in protected and open field crops has been of great importance. Furthermore, mismanagement like applying broad spectrum insecticides at high dosages and using repeatedly the same active ingredients, etc., can lead to the development of resistance in this insect in very few years. Pesticide selective pressure therefore must be reduced and it is necessary to find alternative ways to control *S. litura* in general. The insecticide resistance is an important man-made example of natural selection. The factors governing the origin and spread of resistance-associated mutations are both of academic and of applied importance (Richard *et al.* 2004). An insect population may be termed resistant when its larval LC_{50} increases by a 10-fold (Chansang, 2003).

Resistance in *Spodoptera* has been report to all types of insecticide viz., organochlorines, organophosphate and synthetic pyrethroid. *Spodoptera* larvae reared on cotton showed tolerance to insecticides. Hence to mitigate these problems efforts have to be made toward judicious use of insecticides based on surveillance and economic thresholds for evolving a sound and ecologically safe control technology for this pest (Prasad, 1997). In case of synthetic insecticides resistance, twenty-two strains of the *S. litura* were collected from 8 locations in India between 1991 and 1996. Assayed in F_1 generation was showed various levels of resistance to commonly used insecticides. They observed that cypermethrin and fevalerate resistance levels in the field strains ranged 0.2 to 197-fold and fenvalerate 8 to 121-fold, respectively. Indicating that this insect resistance to cypermethrin higher than fenvalerate. Pretreatment with the synergist DEF, an inhibitor of esterases and the glutathione S-transferase system resulted in a 2-3 fold synergism with monocrotophos indicating that esterases and possibly glutathione S-transferase were at least to some extent contributing to organophosphate resistance (Armes *et al.*, 1997). Chau (1995) reported that an epidemic of soybean caterpillar, *S. exigua* and *H. armigera* occurred, destroying several thousands of soybean production in Mekong Delta, Vietnam where the cultivated were fell from 100,000 to 30,000 hectare per year because of the resistance of these caterpillars to insecticides. Thomas and Boethel (1995) observed the resistance of soybean looper to permethrin that increased rapidly in the 3rd and 4th generations when population density became higher and insecticides were applied repeatedly, then decreased suddenly during over-winter and slowly in the 1st and 2nd generations when insecticide spraying was suspended.

Insecticide resistance mechanism, Miller (1988) grouped the most common types of insecticide resistance into four categories namely behavioral resistance, penetration resistance, site-insensitivity (target-site resistance) and metabolic resistance (detoxification enzyme-based resistance). But Ishaaya (2001) categorized the insecticide resistance mechanism in 3 ways namely behavioral mechanism, physiological mechanism and biochemical mechanism which in general of an insect can develops. The two major forms of biochemical resistance are target-site resistance, which occurs when insecticide no longer binds to its target, and detoxification enzyme-based resistance, which occurs when enhanced levels or modified activities of esterases, oxidases or glutathione S-transferase enzyme to prevent the insecticide from reaching its site of action (Brogdon *et al.*, 1998).

Physiological resistance is the interplay of site-insensitivity and metabolic factors. Major physiological resistance to insecticide involves specific gene and is achieved via increased metabolism and decreased sensitivity of the target sites that are comprised of acetylcholinesterase, gamma-aminobutyric acid and para sodium ion channels. Three distinct kinds of target site resistance have been defined. There are cyclodiene-type, acetylcholoinesterase-type and knock down resistance-type. The enzyme detoxification of the metabolic resistance mechanism increasing in many types of insecticides can be occur from a variety of enzymes, these are monooxygenase, esterase and glutathione S-transferase (Miller, 1988). And the behavior resistance developed by insect is occurs when an insect strain develop the ability to avoid a lethal dose, rather than developing a physiological indifference to

the dose (O'Brien, 1967). Behavior adaptation is another way that insects use to avoid insecticides. Among behavior adaptations, selective feeding is the most ubiquitous mode (Abrahamson, 1989). Insect simply avoid plant parts or plant individuals rich in toxin. Sometime we found that insect adapt their behavior as they select of feeding site. The selection often depends on the difference between the chemical compositions of the different layer. For instance, some aphids are flight over feeding site on a bamboo lea(F.) The feeding site is sometimes limited to a specific part of the leaf by leaf minor, but aphid is also display area selection when sucking plant juice. If the egg is laid on the wrong site, the larvae often to refuses to feed and perishes (Pierre, 1998). Another example flies are avoid lay they eggs from contaminated insecticide areas. In mosquitoes they are change the fly habit and no lay eggs in contaminated insecticide areas, this behavior change is similar to flies (Pimsaman, 1988).

Many insects also display flexible behaviors when faced with inducible plant defense. Moreover, polyphagous insect almost have survival rate or resistant rate more than oligophagous insect and monophagous insect, respectively. Because they eat variety food source so they have opportunity if that food source is not suitable for them such as that food source is control by insecticide or amount of food source is not enough to eat in contrast with oligophagous and monophagous insect pest, both types will have problem if they cannot find suitable food source and may caused their population is decrased (Abrahamson, 1989).

4. Insecticide susceptibility or resistance test methods

There are currently two major approaches to test the insect susceptibility or resistance; the general test method to determine the overall toxicity through standard WHO susceptibility or resistance test methods including diagnostic test method and the specific test method using biochemical, immunologic, nucleic acid resistance probe to determine whether individual insects possess a mutant resistance allele. In addition, point mutation detection directly by PCR technique has been improved and reported (Williamson *et al.*, 1993; Williamson *et al.*, 1996). Aside from these two major approaches, there are some other methods for testing insect resistance. The demand for sensitive and specific method to detect and study resistance has stimulated the pursuit of molecular methods of analyzing insecticide resistance mechanism at the level of individual genes. Recent advances in molecular neurobiology have provided insight into the structure and function of target sites for neurotoxin pesticides, e.g. ligand-gated ion channel, voltage-gated ion channel and acetylcholinesterase resulting in reduced neuronal sensitivity which is particularly important in predicting the future of insecticide use in insect control programs (Soderlund and Bloomquist, 1989).

5. Metabolism of xenobiotic compounds and detoxification enzymes

Xenobiotics as well as active metabolites in the body called metabolic precursors or protoxicants. They entered by ingestion, skin and inhalation and follow by absortion, metabolism, temporary storage and distribution or excretion. The most important process in toxicology is metabolism. Because of in ecological basis, animal have no co-evolution to some plants or the new pesticides, that animal may not

produce enzymes that suitable for their needs and allergic to the compounds when they eat accidentally. The detoxification mechanism is one of important way for organisms to adapt themselves for survival such as insect use this way to resist many chemical insecticides that decrease toxicity result of chemical insecticides. Thus, in the toxicity metabolism, we can found the changing of detoxification mechanism in organisms (Visetson, 2001; Ishaaya, 2001). The metabolism is a process by which xenobiotics are metabolized to be the intermediate or final products. Xenobiotics are changed by enzymatic metabolism which catalyzed by microsomal enzymes in the organisms (Hodgson and Levi, 1997). They enter to the body tissues are non-polar lipophillic, a property that enable them to penetrate lipid membranes well. These xenobiotics are metabolized well by a number of relatively non-specific enzymes by the various tissues and accumulated in the body. Metabolism is taken place first at the portal of entry, the skin or in such organs as the lung epithelium intestine including gastrointestine tract and especially the liver with the highest metabolism. The xenobiotics can pass into gastrointestinal tract and they are transported to liver to metabolize.

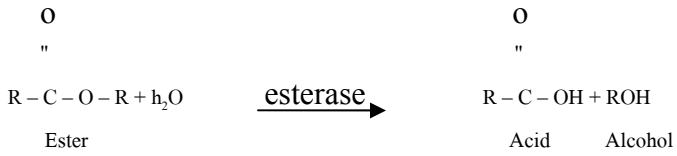
Two type reactions of metabolic transformation are known as metabolic transformation (phase I) and conjugation (phase II). The phase I enzymes role is the addition of chemically reactive groups to molecules. While the phase II process increases the overall polarity of the molecule to some degree it is usually not sufficient to cause a large increase in the rate of excretion of the primary metabolite. For this to be achieved large polar groups also need to be added, and phase II enzymes achieve this through interaction with the chemically reactive groups added during phase I metabolism (Plant, 2003). The systems of detoxification enzymes work like common enzymes. After xenobiotics enter into our body, they will be metabolized by microsomal enzymes into highly water soluble substances and then secreted out from body via bile, urine and sweat (Visetson, 1991). These enzymes are accumulated in the intestine and/or adipose tissue cells of insect. The detoxification enzymes work on various substrates to reduce poisons. Moreover, the detoxification enzymes can regulate and induce when xenobiotics are exposed. Usually animal have enzymes the functions of which are metabolism of xenobiotics in the body (Manson *et al.,* 1965). In insect, they have various behavior avoidance and various detoxification systems. There are three major detoxification enzymes in metabolism of xenobiotics, namely esterase, glutathione S-transferase and monooxygenase in order to detoxify xenobiotics or decrease poisons for survive. They provide fast mechanisms to detoxify the toxin (Dauterman and Hodgson, 1978; Yu, 1984 and Visetson, 2001).

5.1 Esterase, the esterases are specific enzyme (Kao *et al,* 1985). It is one of detoxification enzyme which catalyze the hydrolysis of ester, amide or phosphate linkage of insecticide. It metabolism of many insecticides are in phase I which have two types. A-type esterase is not inhibited by organophosphate. This esterase type are includes arylesterases, phosphatase, phosphotriesterase, amidase, paraoxonase and malaoxonase. These enzymes can be inhibited by tetraethyl pyrophosphate (TEPP), ethyl p-nitrophenyl phenylphosphorothioate (EPN), etc. and these inhibitors can synergize toxicity of insecticidal esters. B-type esterase is inhibited by several organophosphate insecticides due to irreversible phosphorylation of the active serine

site. This esterase type includes aliesterase, carboxylesterase and cholinesterase (Dauterman, 1985). The esterase enzymes play a significant role in the metabolism of several insecticidal in many reported. In this study, I focus on carboxylesterase and acetylcholinesterase which many reported show it play important role in resistance cause from this enzyme is a general detoxification mechanism in an insects (Small and Hemingway, 2000; Vontas *et al.,* 2001).

5.1.1 Carboxylesterase

5.1.1.1 Types of reaction. The carboxylesterase enzyme activities are involved in the metabolism of xenobiotics that induced insect resistance to organophosphate, pyrethroid (Dauterman, 1985) and chitin inhibitor insecticides (Ishaava and Degheele, 1988). In insect, esterases are found in cytosol, microsomes, mitochondria and nuclei. The highest specific activity of this enzyme was found in mitochondria fraction followed by in microsomal fraction; the activities in nuclei, cell debris and in the soluble fraction were relatively low (Zhu and Brindley, 1990). It mechanism is transforming the ester bond of xenobiotics to acid and alcohol as diagram below;



5.1.1.2 Role in resistance. There are many researches on the role of carboxylesterase enzyme to detoxify toxic in insect pests such as Haubruge *et al.,* (2002) showed specific resistance to malathion in a strain of *Tribolium castaneum* is due to a 44-fold increase in malathion carboxylesterase (MCE) activity relative to a susceptible strain, whereas non-specific esterase levels are slightly lower. In the sheep blowfly, *Lucilia cuprina*, the resistance is due to a change in a specific esterase isozyme, carboxylesterases (E3), in the resistant flies to organophosphate (OP) which an OP-resistant allele of the gene that encodes E3 differs at five amino acid replacement sites from a previously described OP-susceptible allele. (Newcomb *et al.,* 1997). Beranek (1974) reported the nine resistant clones of the peachpotato aphid (*Myzus persicae* (Sulz.)) and the black bean aphid (*Aphis fabae* Scop.) resitance to parathion and dimethoate, from different sources, all showed much the greate activity of carboxylesterase.

5.1.1.3 Method to determine activity. The most common method to study the multiple forms of insect esterases are by electrophoretic separation and determination of enzyme activity with color forming product. Kao, *et al.* (1985) found multiple forms of four types of esterases by using ion exchange chromatography. They presented the molecular weights of being 220 kDa. The endogenous inhibitors could denature enzymes and reduce their activities. Therefore, the appropriate buffered conditions should be used to solve this problem. Some researcher used distilled water as a homogenizing medium (Ishaaya and Degheele, 1988). Willaden *et al.* (1987) used phosphate buffer with DDT and EDTA. Price (1984) used phosphate

buffer without adding any other chemical. Yu (1990) washed the empty midgut of insect first with KCL then homogenized with Tris/HCL. After enzyme homogenized, it was centrifyged to sediment mitochondrial fragment. The hydrolysis product of carboxylesterase degradation of an artificial substrate can be reacted with a dye to give a color.

The substrates (*in vitro*) for detecting levels of general esterase activity were appropriate ways to study the involvement of carboxylesterase mechanisms in resistant insects. There are many substrates such as PNPA (paranitrophenyl acetate), ANA (alpha naphthylacetate), BNA, phenyl acetate and MTB (methyl thiobutylate) to detect the levels of general esterase activity (Soderlund and Bloomquist, 1990). Each assay was using different substrate to investigate enzyme activity by spectrophotometer with different absorbance. For instance, the pNPA assay for detect the carboxylesterase activity level is use absorbance at 400 nm.

5.1.2 Acetylcholinesterase

5.1.2.1 Type of reaction. The insecticides resistance mechanism can also detected by the insensitivity of AChE. For example, the irreversible of organophosphates bind to cholinesterase substance is causing the phosphorylation and deactivation of acetylcholinesterase. The accumulation subsequently of acetylcholine at the neuron synapse causes an initial over-stimulation followed by eventual exhaustion and disruption of neuron transmission in the central and peripheral nervous system.

5.1.2.2 Role in resistance. The insensitivity of AChE have been reported in several resistant strain of insect such as *Musca domestica* (De Jersey *et al.*, 1985), *Culex pipines* (Tand *et al.*, 1990) and *Latrodectus Hesperus* (Zhu and Brindly, 1990). It has accepted that reduce sensitivity of acetylcholinesterase is significant factor in resistance to organophosphate and carbamate compounds (Hama, 1983).

5.1.2.3 Method to determine activity. The artificial substrate, acetylthiocholine iodide (ATCH) is used to hydrolyze into acetate and thiocholine. The sulfhydryl group in thiocholine reacts with dithiobisnitrobenzoate (DTNB) to produce two compounds namely 2-nitrobenzoate-5-mercaptoathiocholoine and 5-thio-2-nitrobenzoate. The later compound has a yellow color and can be measured in a spectrophotometer. The second reaction is rapid and complete. Thus, the concentration of the final colored end product is a direct measure of the concentration of thiocholine produced.

5.2 Glutathione-S-transferases (GSTs), GSTs are a group of soluble detoxification enzyme which catalyze the conjugation of reduced glutathione with various compounds containing an electrophilic center, including insecticides (Chasseaud, 1979). GSTs are found in mammals, insects, protozoa, algae, fungal, bacterial and also in plants that found largely in the cytosol of insects (Jakoby, 1978). Structure of GST consists of three amino acid, glutamic acid, cysteine and glycine.

5.2.1 Types of reaction. GSTs are involved in the metabolism of many insecticides. Reactions are catalyzed by nonspecific enzymes include phase II conjugation of reactive metabolites formed by microsomal oxidations, as well as phase I with insecticides themselves. The conjugates are further metabolized to form mercapturic acids by several steps or excreted because of their increased water solubility. In this way compounds may be dealkalated, dehalogenated or cyanide may be released from organic thiocyanates (Visetson, 1991). The reaction as below;

$$RX + GSH \xrightarrow{GST} GSR + X^- + H^+$$

5.2.2 Role in resistance. GSTs are well established high levels of GSH S-transferases activity and are important in resistance to organophosphate and organochlorine insecticides (Motoyama and Dauterman, 1980). This enzyme has multiple forms and can be divided into three groups; GSH-S- aryl transferase, GSH-S-alkyl transferase and Epoxy S-transferase (Motoyama *et al.*, 1977). Different forms and amounts of GST present in different animal species and strains determine their tolerance of chemicals detoxified by GST.

5.2.3 Method to determine activity. GST activity can be measured spectrophotometrically. This is based on the catalysis by GST of the conjugation of DCNB or CDNB substrate with glutathione reduced form, to produce a yellow product at wavelength of 344 and 340 nm, respectively. This method can also be miniaturized for use in a microtitre plate reader (Kulpiyawat, 2001). And Visetson (1991) found that GST activity was very stable for 120 days when stored at -85°C.

5.3 Enzyme preparation conditions. Enzyme activity from insects is usually inhibited by a number of endogenous inhibitors such as xanthommatin in the head of the houseflies, guinones and tyrosinases from houseflies, proteolytic enzyme from southern armyworm.Several inhibitors are presented in whole homogenates of different insect species, sex, tissue and organ (Visetson, 1991). In addition, the accuracy of enzyme activities analysis depends on species, age, sex, exposure time, substrate, feeding behavior, solvent system and temperature (Visetson *et al.*, 2004). Tyler *et al.* (1993) also suggested that the most appropriate temperature for evaluated the insecticides toxicity in laboratory of most insect appears to be less than 25°C. A variety of chemicals protect activity of enzymes during homogenization. They are DTT, PVPP, EDTA, PVP, PMSF, reduced glutathione, mercaptoethanal, etc.

Recently, there are many assays for the determination of protein concentration in a solution as Biuret, Lowry and Bradford assay. The criteria for choice of a protein assay are usually based on convenience, availability of protein for assay, presence or absence of interfering agents, and need for accuracy. The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. The Bradford is

recommened for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis (Caprette, 2000).

6. The molecular study of gene responsible for detoxification enzyme expression

A diverse range of novel molecular (DNA, RNA) techniques are now available for ento-mological investigations. Both DNA and protein markers have revolutionized the biological sciences and have enhanced many fields of study, especially ecology. Relative to molecular techniques, allozymes are cheap, often much quicker to isolate and develop, even from minute insects such as aphids, thrips, parasitic wasps, early first instar caterpillar, etc. and subsequently easy to use. They display single or multi-locus banding patterns of a generally easily interpretable Mendelian nature, and the statistics for their analysis are well established. Molecular techniques are also suitable for use with small amounts of insect material and can be used with stored, dry or old samples. They have an expanding range of applications, many involving intra- and inter-specific discriminations (Loxdale and Lushai, 1998).

Insecticide resistant of insect is occur from mutation in gene. The enzyme synthetic is controlled by gene or DNA in an insect and it makes insecticides non toxic to insects. Detection of insecticide resistance in insects can be done by using molecular techniques with resistant gene, but not in the susceptible. This method can be done by PCR electrophoresis (Chansang, 2003). The biochemical/physiological mechanisms of resistance can be categorized as target site insensitivity (target site resistance), increased metabolic detoxification and sequestration or lowered availability of the toxicant. These are achieved at the molecular level by: point mutation in the ion channel portion of a GABA receptor subunit (cyclodiene insecticides), point mutation in the vicinity of the acetylcholinesterase (AChE) active site (organophosphorus and carbamate insecticide resistance), amplification of esterase genes (organophosphorus and carbamate insecticides), mutation linked genetically to a sodium channel gene (DDT and pyrethroid insecticides) and yet uncharacterized mutation leading to the up-regulation enzymes such as cytochrome P450 and glutathione S-transferase (many classes of insecticides). In several cases, the selection of a precisely homologous mutation has been observed in different insect species (Feyereisen, 1995; Enayati *et al.*, 2003; Soderlund and Knipple, 2003).

Since the past ten years have seen the elucidation of the molecular basis of insecticide resistance to many chemical insecticides. Target genes, mostly in the nervous system, have been identified and cloned from *Drosophila melanogaster* and resistance-associated mutations have been examined in a range of pest insects. More recently, with the advent of annotated insect genomes, resistance mediated by complex multi-gene enzyme systems such as esterase, cytochrome P450s and glutathione-S-transferases has also been elucidated. The cloning of these resistance genes has enabled to address fundamental questions relevant to the selection of these adaptive traits (Richard *et al.*, 2004). But in several insect including the *S. litura* are had not yet study.

6.1 SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis). This method is a common technique for evaluate the characteristic of the enzyme activity. It is the most commonly used electrophilic technique for protein analysis. This is due to the ability of the strong anionic detergent SDS, when used in the presence of disulfide bond cleaving reagents, to solubilize, denature and dissociate most protein to produce single polypeptide chains. The resulting SDS-protein complexes can then be separated according to molecular size by electrophoresis in gel containing SDS (Dunn, 1993).

6.2 Detection of gene responsible for expression of detoxification enzyme. Non-silent point mutates within structural genes are the most common cause of target site resistance. For selection of the mutation to occur, the resultant amino acid changes must reduce binding sites of the insecticide without causing a loss of primary functional of the target site. Therefore the number of possible amino acid substitutions is very limited. Hence, identical resistance-associated mutations are commonly found across highly diverged taxa. The degree to which function is impaired by the resistance mutation is reflected in the fitness of resistant individuals in the absence of insecticide selection. This fitness cost has important implications for the persistence of resistance in the field. The main sodium and GABA channel genes in insects have been cloned and their sequences compared in resistant and susceptible insects. Acetylcholinesterase-based resistance has been well characterized in *Drosophila* (Hemingway and Ranson, 2000), but the elucidation of this mechanism at the molecular level in *S. litura* not yet prove.

A number of techniques for the rapid detection of resistance-associated mutations have been reported. For example, a competitive polymerase chain reaction amplification of specific alleles (PASA) technique has been used to diagnose the azinphos-methyl-resistance allele of the AChE gene in the Colorado potato beetle (*Leptinotarsa* decemlineata) (Zhu and Clark, 1997). However, where DNA is of a lower quality, single-strand conformational polymorphism (SSCP) and mini-sequencing are more reliable technique has also been used to detect a cyclodiene insecticide resistance-associated mutation resulting from an alanine to serine or glysine substitution, which is common to several insect spices. This mutation can be detected by SSCP and or by PCR and restriction digestion, as the mutation results in the loss of *Hae*II restriction site (Ffrench-Constant *et al.*, 1993).

Detecting base changes in RNA. RNA sequencing is used to register the nucleotide arrangement of DNA. PCR with thermostable polymerases (Taq and T7) has revolutionized the ability to sequence specific templates of genomic DNA in target organisms. Usually, the PCR fragment is purified and ligated into plasmids. These are amplified by bacterial colonies and the insert is then removed from the bacterial cells, sequenced and run on an acrylamide gel prior to autoradiography (Fang *et al.,* 1993). Direct-sequencing or cycle-sequencing methods circumvent plasmid amplification. Here the PCR reaction is used to amplify templates for sequencing and radiolabelling occurs by the use of end-labelled primers or by the addition of labelled nucleotides (Hamby *et al.,* 1988). All these techniques use various radio-isotopes ([a 35 S] dATP, [a 33 P] dATP or [a 32 P] dATP) or can involve non-

isotopic labelling (silver staining, DIG labelling and colourfluors) methods. The fluoresence markers have enabled the procedure to become automated. There are other methods of quickly screening differences between RNA strands without sequencing. These include techniques that may not reveal the position of mutations, but indicate their presence in genomic DNA fragments. These are denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), constant denaturing gel electrophoresis (CDGE) and single stranded conformational polymorphism (SSCP) analysis (Loxdale and Lushi, 1998). In the first three techniques, DNA is denatured using formamide and urea (DGGE and CDGE) or temperature (TGGE) and the extent to which this occur correlates with the sequence, with even single base differences affecting the stability of the DNA double strand. Such methods have proved to have a success rate ^{3}95% in the detection of mutations in fragments up to 600 bp. In the last technique, band migration rates are altered as a result of secondary structure, and the sensitivity of the technique is highly dependent on the gel running conditions. Another group of techniques employs chemicals or proteins that act at sites where DNA mismatching has taken place (Mashal and Sklar, 1996).

MATERIALS AND METHODS

Materials

1. Selective (resistance) and non-selective (control) strains of the tropical armyworm, *S. litura*

2. Candidate insecticides
   2.1 Derris roots from Chonburi province for derris crude extrac
   2.2 Standard rotenone 98% from SIGMA
   2.3 Cypermethrin 35%EC, commercial available product

3. Equipment
   3.1 Shelves, box and equipments for *S. litura* mass rearing
   3.2 Blender and equipments for artificial diet preparation
   3.3 Stereomicroscope (Olympus)
   3.4 Soxhlet extractor
   3.5 Rotary evaporator (Buchi B-850)
   3.6 Hot air oven (Memmert-600)
   3.7 Autoclave
   3.8 Refrigerate high speed centrifuge
   3.9 Spectophotometer with Kinlab program
   3.10 Thermocycler (GeneAmp® model 9700)
   3.11 Electrophoresis (Bio-Rad)
   3.12 Mini protein equipment (Bio-Rad)
   3.13 HPLC (Hewlett Packard series 1100) with column (Synergi 4u hydro-RP 80A, 150 x x4.06 mm 4 micron)
   3.14 Cage and control room condition at 25 °C, 70%RH and 13:11 day/night for mouse experiment
   3.15 Mixer (BUCHI Mixer Model 5000)
   3.16 Plastic box
   3.17 Glass and plastic Petri-dish
   3.18 Glass bottle
   3.19 Beaker
   3.20 Flask
   3.21 Forcep
   3.22 Cotton pad
   3.23 Tissue
   3.24 Small fine brush
   3.25 Motar and pestle
   3.26 Ice box
   3.27 Balance
   3.28 Parafilm
   3.29 pH meter
   3.30 Micropipette
   3.31 The 1.5 ml polystylene cuvette tube

3.32 Microtube (1.5 ml appendorf tube, 1.5 ml Corling tube, 0.2 ml PCR tube)

3.33 Pipette tips (1.5 ml blue tip, 0.2 ml yellow tip, 0.0025 ml white tip)

4. Reagent

4.1 Ethanol 95%
4.2 Absolute ethanol
4.3 Methanol
4.4 Acetic acid
4.5 Polyvinyl polypyrrolidone (PVPP)
4.6 Potassium dihydrogen phosphate (KH_2PO_4)
4.7 Ethylene diamine tetra acetic acid (EDTA)
4.8 Glutathion reduce form (GSH)
4.9 1-chloro-2,4-dinitrobenzene (CDNB)
4.10 1,2-dichloro-4,nitrobenzene (DCNB)
4.11 Paranitrophenyl acetate (p-NPA)
4.12 Albumin from bovine serum
4.13 Coomassie Brilliant blue R
4.14 TAE (Tri-Acetate –EDTA buffer)
4.15 Tri-reagent (Sigma)
4.16 1-bromo-3-chloropropane (BCP)
4.17 Isopropanol
4.18 DNase-RNase-free water
4.19 Forward and reverse primer
4.20 Superscript III reverse transcriptase one step ® (Invitrogen)
4.21 Agarose (molecular biology grade)
4.22 Ethidium bromide
4.23 Distilled water
4.24 Lamda marker (lamda DNA, *Hin*d III, buffer of *Hin*d III)

Methods

The experimental analysis and the tropical armyworm, *S. litura* preparation

The experimental analysis

The Completely Randomized Design (CRD) with 5 replications and 30 sampling of *S. litura* larvae unit per replicate were used for all toxicity testing experiments. There are five concentrations of cypermethrin 35%EC, crude extracts of derris roots and standard rotenone (98%) with separate control treatments (distilled water). Mean differences of mortality and detoxification enzyme from each treatment were analyzed by analysis of variance. The LC_{50} statistic analysis and enzyme activity analysis were determined using SPSS (Statistical Package for the Social Sciences) for windows version 11.0 and Sigma plot program by computer analysis. About molecular analysis, the GENETYX analysis program version 7.0 was used for primer designed, sequence editing and sequence alignment. About sequence blasting was used the blast program from http://www.ncbi.nlm.nih.gov/.

The tropical armyworm, *S. litura* preparation

Collecting of *S. litura* sample

Eggs of the tropical armyworm, *S. litura* were collected from vegetable plantation in vegetable producing area at Damnern Sadoug district, Ratchaburi province, Thailand. The samples were kept in plastic boxes containing kale leaves and covering with a tissue paper. They were used to establish the laboratory populations.

Mass rearing of the *S. litura*

The mass rearing (Figure 5) and maintaining of the *S. litura* population were under laboratory at 25-30 ℃ with artificial diet. The day/night period was adjusted about 13:11 hours. Two cultures were established the mass production was commenced under laboratory conditions after the first generation established. After freshly emerge males and females were collect from their respective emergence cages and bring into a breeding box. Pairs of newly emerge moths were placed in well ventilated plastic containers. The inner wall of the containers was lined with paper to enable the adults to lay eggs. They were fed with 10 % honey solution on cotton wool placed in small glass cap. The eggs which were generally laid in batches on the paper are cut out. Freshly laid egg masses were kept for hatching in sterilized plastic boxes. After the newly hatch larvae were transfer to artificial diet, changed the diet twice a day (Rattanapan, *et al.,* 2005), fed until they are going to pupa stage and keep in a plastic container with stand for pupation. The pupae were collected in 3 days after all the larvae enter the pupation. The cleaned pupae with 0.1 % formalin, by soaked them into 40% formalin about 10 seconds and dry on tissue, were sex and kept on a lid over a tissue paper in adult emergence cage. When adult emerged and laid their

eggs, the eggs were collected for further rearing to get the larvae for toxicity test, detoxification enzymes activity bioassay and molecular analysis.

Artificial diet preparation

Distilled water was autoclave for prevented contamination in artificial diet. Agar was boiled in 350 ml of autoclaved distilled water and was mixed properly. The diet ingredients (Table 1) were blended together using blender in 400 ml autoclaved distilled water without agar. Then boiled agar was added into the blender for final blending. The diet was poured into plastic box. Diet was allowed to solidify at room temperature and was kept in the refrigerator until use.

Table 1 Compositions of artificial diet for mass rearing of *Spodoptera litura* (F.) larvae and insecticide bioassay studies.

Ingredient	Amount
Soaked mungbean	130.0 g
Baking yeast	10.0 g
Ascorbic acid	3.0 g
Sorbic acid	1.5 g
Methyl paraben	2.5 g
Casein	3.0 g
Choline chloride	0.5 g
Agar	15.0 g
Vitamin syrup	10.0 ml
Formalin (40%)	2.0 ml
Distilled water	750.0 ml

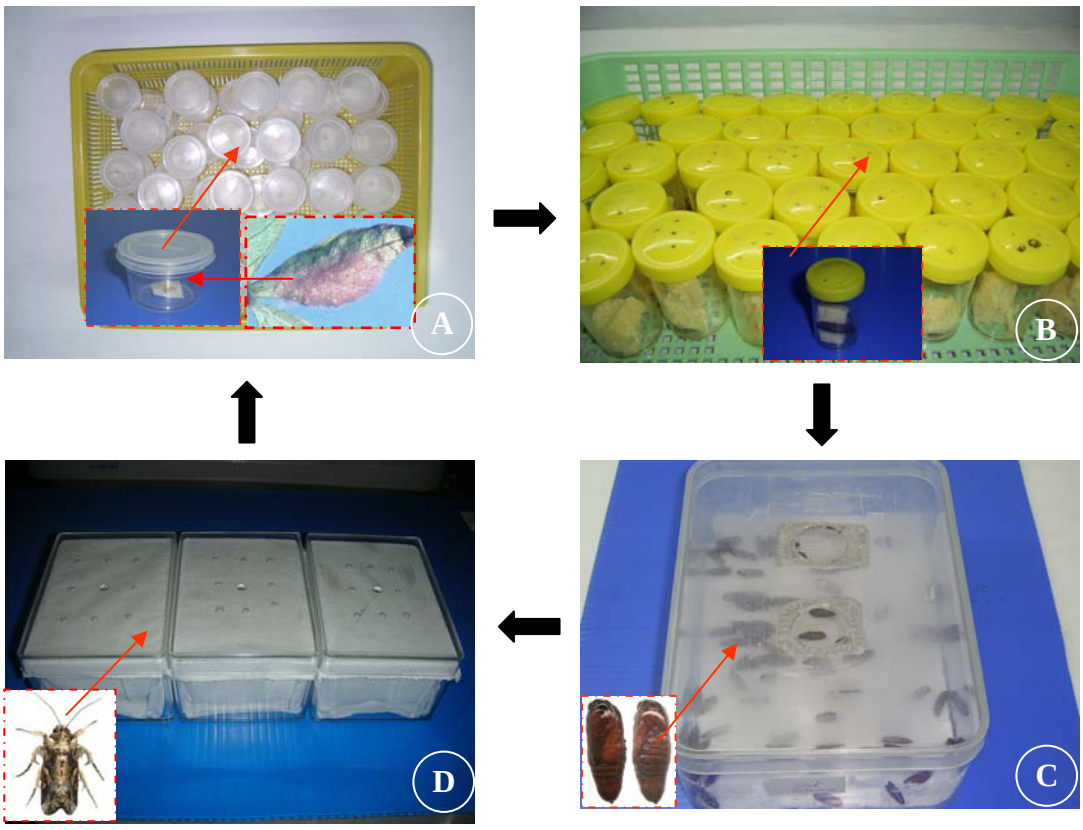


Figure 5 *Spodoptera litura* (F.) mass rearing with artificial diet for larvae feeding. A = eggs, B = larvae, C = pupae and D = adults

The processes of research

1. Toxicity determination of cypermethrin, standard rotenone and derris crude extracts against *S. litura* by bioassay method

1.1 Root of *Derris* extraction and quantitative

1.1.1 Derris extraction method

Roots of derris were collected on dry season from Chon Buri province. This sample was the same source of Visetson and Milne (2001). They were dried by Hot Air Oven (Memmert-600) and were grounded by mixer (BUCHI Mixer Model 5000) for powder. The extracted were by modified method from Visetson *et al.* (2004) with ethanol solvent using Soxhlet extractor at 75°C for 8 hours of extraction process. The derris extract were evaporated to remove solvent by rotary evaporator (BUCHI B-850) then the crude extracts were kept at 4°C until experiment commenced.

1.1.2 Quantitative analysis method

To analyze the amount of rotenone from dry powder of derris crude extracts. The rotenone content was separated using the high performance liquid chromatography (HPLC) by modified method from Hu *et al.* (2005). The ethanolic derris extract was diluted to 10 mg/ml concentration with acetone and tested by HPLC under the testing condition: equipment: US HP (Palo Alto, CA, USA) 1100 HPLC; column: Hewlett Packard (Palo Alto, CA, USA) (ODS2 Hypersil, 5 µm, 250 x 4.0 mm); detecting wavelength: λ 296 nm; scanning wavelength: 200–800 nm; mobile phase: 65 : 35 methanol : water; flow rate: 1 ml/min; testing sensitivity: AUFS = 0.001; column temperature: constant; amount of injection: 10 µl. The rotenone percentage determined by compared the HPLC value between derris crude extracrts and standard rotenone (98%).

1.2 To estimate the efficiency of cypermethrin, standard rotenone and derris crude extracts against *S. litura* method

In order to determine the efficiency of these three types of insecticides affects on *S. litura* should be elucidated before any attempt beyond would be made. Then the important effects such as toxin concentrations in term of LC_{50}, time exposure and age of organism were evaluated first. In this study, the lethal dose of these insecticides with different insecticide concentrations and time exposure durations to larva of *S. litura* was elucidated. Method of Jansiri (1994) was used. Three types of insecticide were used in this study. One type is the synthetic chemical insecticide which commercial formulation of cypermethrin (35%EC) was used. The other types are 98% standard rotenone from SIGMA and the crude extract of *Derris sp.* (rotenone substance), with known amount of rotenone substance were used as natural insecticide for the studies. They were treated to the third instar larvae by body dipping method. Five concentrations include the control (distilled water treated) of each insecticide and thirty larvae of the second day molted of the third instar per replicated were used.

Mortality rate was recorded for each concentration of the insecticide in 5 replicates at 24, 48 and 72 hours after treatment. Control mortality in the absence of insecticide was taken into account in deriving dose response. If mortality data in the control treatment appeared, the data would be corrected by Abbott's formula (Abbott, 1925). If the control batch mortality exceeds 20 %, the test should be repeated to reduce or eliminate the causes of such mortality (Anonymous, 1969). Raw data of bioassay was analyzed using Sigma Plot program analysis to obtain LC_{50}.

Abbott's formula

$$\% \text{ corrected mortality} = \frac{\% \text{ treatment mortality} - \% \text{ control mortality} \times 100}{100 - \% \text{ control mortality}}$$

1.2.1 Efficiency of cypermethrin method

Cypermehtrin 35%EC was diluted into 5 concentrations (0, 5, 10, 15, 20 ppm; ml/l = ppm) with distilled water which contain 100 ml of the each concentration. Thirty larvae of the second day molted of each instar were dipped in each concentration of insecticide for 20 seconds and dried on filter paper about a few minutes. They were released in plastic box with the artificial diet (30 larvae per box / replication). After that the larvae boxes were kept in the controlling condition rearing room. In control treatment (0% cypermethrin), larvae were dipped with distilled water. The efficiency of cypermethrin to all instars larva at 24, 48 and 72 hours were observed with larvae mortality. The mortality rate were recorded and processed as mention above for obtain the LC_{50} value.

1.2.2 Efficiency of standard rotenone

Standard rotenone 98% was diluted into 5 concentrations (0, 15, 25, 35, 45 ppm; mg/l = ppm) with distilled water which contain 100 ml of the each concentration. Thirty larvae of the second day molted of each instar were used per replication. The method and process were same 1.2.1.

1.2.3 Efficiency of derris crude extracts

Derris crude extracts was diluted into 5 concentrations (0, 20, 40, 60, 80 ppm; mg/l = ppm) with distilled water which contain 100 ml of the each concentration. Thirty larvae of the second day molted of each instar were used per replication. The method and process were same 1.2.1.

1.3 To elucidation the toxicity of cypermethrin, standard rotenone and derris crude extracts against all instars larvae of *S. litura*

In order to estimate the lethal dose of these three insecticides with was different larvae instars of *S. litura*. Method of Jansiri (1994) was used to obtain the LC_{50} value. Each insecticide was diluted into 5 concentrations with distilled water which contain 100 ml of the each concentration. Cypermehtrin 35%EC was diluted to 0, 5, 10, 15, 20 ppm, standard rotenone was 0, 15, 25, 35, 45 ppm and derris crude extracts was 0, 20, 40, 60, 80 ppm. They were treated to all instars larvae by body dipping method. Five concentrations include the control (distilled water treated) of each insecticide and thirty larvae of the second day molted of all instars per replicated were used. They were dipped into insecticide solution of each concentration about 20 minutes, dried on filter paper and then place to plastic box with artificial diet. The mortality rate was observed at 72 hours. Control mortality in the absence of insecticide was taken into account in deriving dose response. If mortality data in the control treatment appeared, the data would be corrected by Abbott's formula. If the control batch mortality exceeds 20 %, the test should be repeated to reduce or eliminate the causes of such mortality. Raw data of bioassay was analyzed using Sigma Plot program analysis to obtain LC_{50} value.

2. Characterization of the detoxification mechanism in *S. litura* larva against with cypermethrin, standard rotenone and derris crude extracts by biochemical method

2.1 To elucidate the pH optimal for detoxification enzyme mechanisms detection in *S. litura*

The optimum pH buffer for homogenization and incubation of enzyme activity detection were elucidated. Potassium phosphate buffer were used in this study. pH buffer varying in 4, 6, 7, 7.5, 8, 10, 12 and 14 series were observed by UV-visible spectrophotometer. This buffer pH optimal was tested the highest activity of three detoxification enzymes namely carboxylesterase, acetylcholinesterase and glutathione-S-transferase. The activity levels were test in 3rd instar larvae with pNPA substrate for carboxylesterase enzyme, Acetylthiocholine iodide for acetylcholinesterase (AChE), and 1,2-dichloro-4,nitrobenzene (DCNB) and 1-chloro-2,4-dinitrobenzene (CDNB) for glutathione-S-transferase (GSTs). The pH which highest activity level of substrate detected of each enzyme was selected to apply in this study. After screened the pH optimum and suitable substrate for detection of these enzymes were used this condition to elucidate the enzyme activity level in all instars larvae for detecting the highest activity level of each detoxification enzyme in ones of instar larvae.

2.2 To determine the changing of detoxification enzyme mechanisms in all instars larvae of *S. litura* against cypermethrin, derris crude extracts and standard rotenone by biochemical assay test

The activities of three detoxification enzymes in the 1st to 5th instars larvae were performed by biochemical assay. The specific substrate of each enzyme was used for enzyme activities detection with potassium phosphate buffer at pH 10 using UV-visible spectrophotometer. Thirty larvae per replication of each instar were used for each insecticide. The survival larvae were took to detect their detoxification enzyme activities after 24 hours exposure.

Insect preparation for enzyme extraction

Both of the treatment and control (distilled water treated) were used 0.5 g per replication of the second day after molted of the third instar larvae of *S. litura.* They were treated with cypermethrin, standard rotenone and derris crude extracts at LC_{50} concentration value which was obtained from the toxicity analysis of each insecticide. Survival larvae were separated from dead ones at the time of sampling. Sample of control and treated larvae were collected at 24 hours after treatment and were used for enzyme extraction.

Detoxification enzymes extraction method

Three detoxification enzymes of the third instar larva namely carboxylesterase, AChE and GSTs were detected from the enzyme extraction in this

study. Both treatment and control (distilled water treated) of the survival larvae after 24 hours exposure which Visetson and Milne (2001) and Yang *et al.* (2004) modified method were used. The whole insects were placed on ice and homogenized on cool mortar in 1 ml homogenization buffer (0.1 M potassium phosphate, pH 10, containing 1 mM EDTA and 10 mM glutathione reduced form) with plus of 0.05 g polyvinyl polypyrrolidone (PVPP). The homogenate was rinsed into 1.5 ml microtube and spin by centrifuge at 4 °C, 18,000 rpm for 5 minutes. The supernatant was decanted into a clean 1.5 ml microtube, place on ice and use immediately for carboxylesterase, AChE and GSTs assays with spectrophotometer.

Enzyme activity detection assays

2.2.1 Carboxylesterase activity detection method

Detection of carboxylesterase activity was measured by the pNPA assay from Visetson (2001) and Visetson *et al.* (2003, 2004) modified method. The mixture sample 3 ml/cuvette contained 0.1 M potassium phosphate buffer pH 10, enzyme sample and pNPA substrate. The reference standard contained 0.1 M potassium phosphate buffer pH 10, 0.001 M of EDTA in 0.1 M potassium phosphate buffer pH7 and pNPA substrate. Each cuvette was sealed by parafilm, made the solution mix together quickly and took to detect carboxylesterase activity. Absorbance examining method of pNPA was read immediately for 3 minutes at room temperature by spectrophotometer with Kinlab program at wavelength 400 nm. The yellow color of paranitrophenol was formed from hydrolysis reaction between pNPA and catalytic of enzyme (Mackness *et al.*, 1983).

2.2.2 Acetylcholinesterase activity detection method

Detection of Acetylcholinesterase (AChE) activity was measured by the Acetylthiocholine iodide assay from Visetson (2001) modified method. The mixture sample 3 ml/cuvette contained 0.1 M potassium phosphate buffer pH 10, enzyme sample and ATCL substrate. The reference standard contained 0.1 M potassium phosphate buffer pH 10, 0.001 M of EDTA in 0.1 M potassium phosphate buffer pH7 and ATCL substrate. Each cuvette was sealed by parafilm, made the solution mix together quickly and took to detect AChE activity. The reaction was generated by 0.01 M DTNB which shows the yellow color. Absorbance examining method of Acetylthiocholine iodide was read immediately for 3 minutes at room temperature by spectrophotometer at wavelength 412 nm with Kinlab program.

2.2.3 Glutathione-S-transferase activity detection method

Detection of Glutathione-S-transferase (GSTs) quantitative activity was measured by the DCNB assay from Visetson *et al.* (2002, 2003) modified method. The mixture reaction 3 ml/cuvette consists of 130 l of 10 mM GSH reduced form in 0.1 M potassium phosphate buffer pH 10 to give a final GSH concentration of 1 nM, and 0.1 M potassium phosphate buffer pH 10 (no GSH) to give a final volume of 1.31 ml, enzyme solution and DCNB substrate. The reference standard contained

0.01 M GSH in 0.1 M potassium phosphate buffer pH7, 0.001 M of EDTA in 0.1 M potassium phosphate buffer pH7 and DCNB substrate. Each cuvette was sealed by parafilm, made the solution mix together quickly and took to detect GST activity. Absorbance examining method of the product of DCNB was read immediately for 3 minutes at room temperature by spectrophotometer at wavelength 344 nm with Kinlab program. The product was formed by conjugation reaction of DCNB with glutathione and catalytic of GSTs (Chotimanothum, 2000).

Moreover, the CDNB assay from Visetson *et al.* (2002, 2003) modified method was used for analyze GSTs activity compare with DCNB assay. The reaction mixture 3 ml contained 0.1 M potassium phosphate buffer pH7, 0.01 M GSH in 0.1 M potassium phosphate buffer pH7, enzyme solution and CDNB substrate. The reference standard contained 0.01 M GSH in 0.1 M potassium phosphate buffer pH 10, 0.001 M of EDTA in 0.1 M potassium phosphate buffer pH7 and CDNB substrate. Each cuvette was sealed by parafilm, made the solution mix together quickly and took to detect GSTs activity. Absorbance examining method of the product of CDNB was read by spectrophotometer at wavelength 340 nm with Kinlab program. The product was formed by conjugation reaction of CDNB with glutathione and catalytic of GSTs.

Determine of the total protein content method

Bradford assay was used to analyze the quantity of total protein with bovine serum albumin as a standard. This method was modified from BioRad, Hercules, CA, USA. All of supernatant of most experiments were determined the protein concentration together when examined the enzyme activity. In each enzyme was used 0.5 ml of supernatant and add 5 ml of Bradford solution, mixed well (100 mg Coomasie Brilliant blue R250 in 50 ml 0f 95% ethanol then add 100 ml 85% hydrogen phosphoric acid (H_3PO_4) and make final volume to 1L with distilled water). Then incubated the solution at room temperature for 5 min and determined the protein content by spectrophotometer at 595 nm absorbance.

The chemical and protocol of carboxylesterase, AChE and GSTs activities and protein concentration analysis were described in appendix A and B.

3. Determination of resistance to cypermethrin, standard rotenone and derris crude extracts after sequential treated in *S. litura*

In order to observe the resistance if *S. litura* can develop to cypermethrin, standard rotenone and derris crude extracts after sequential treated in 10 generations. In this research I performed two main methods are the biochemical and molecular techniques to determine the insecticide resistance in *S. litura*. And I also used the bioassay technique in the first before that two techniques, this method is necessary because of it can give the toxicity value that related to the biochemical and molecular mechanisms. The monitoring of insecticide resistance in *S. litura* in this study were carried out by conducted the pressure in selection strain as a treatment population and no pressure in non-selection strain as a control population. From the result in topic 3, I preferred the third instar larvae of each generation were used in this procedure. The

insecticide effects were observed at 72 hours after exposure in 10 generation by different techniques.

Insects preparation

S. litura mass rearing used the same method as above. The monitoring of insecticide resistance in *S. litura* were carried out by conducted the pressure in selection strain as a treatment population and non pressure in non-selection strain as a control population. Two cultures were established the mass production from one pair of parent. The F_1 generation after two cultures was used to conduct the selection and non-selection populations. Non-selection strain was sequential treated with distilled water continuous 10 generations. The selection strains were separated into three populations, there are the cypermethrin, standard rotenone and derris crude extracts population. In each generation of each population, the samples of larvae were taken for toxicity test to obtain the LC_{50} value which was then used to treat that generation. The next generation used for obtain the toxicity data and the detoxification enzyme effects throughout 10 generations, except in 10^{th} generation the samples also used for analysis the gene associated with detoxification enzyme expressions.

3.1 Bioassay detection method

To estimate the effects of these three insecticides by the toxicological response after sequential treated 10 generations of *S. litura*. Method of Jansiri (1994) was used to obtain the LC_{50} value. Each insecticide was treated to 3^{rd} instars larvae by body dipping method. Five concentrations include the control (distilled water treated) of each insecticide and thirty larvae of the second day molted of 3^{rd} instars per replicated were used. They were dipped into insecticide solution of each concentration about 20 minutes, dried on filter paper and then place to plastic box with artificial diet. The mortality rate was observed at 72 hours. Control mortality in the absence of insecticide was taken into account in deriving dose response. If mortality data in the control treatment appeared, the data would be corrected by Abbott's formula. If the control batch mortality exceeds 20 %, the test should be repeated to reduce or eliminate the causes of such mortality. Raw data of bioassay was analyzed using Sigma Plot program analysis to obtain LC_{50} value.

3.2 Biochemical detection method

To characterize the effects of these three insecticides by biochemical response to detoxification enzyme system after sequential treated 10 generations of *S. litura*. Three detoxification enzymes mechanisms such as carboxylesterase, AChE and GSTs were revealed by *In vitro* studied. The sample preparation for enzyme extraction, in each generation, both treatment and control were treated with cypermethrin, standard rotenone and derris crude extracts at LC_{50} value concentration of each insecticide. Survived larvae were collected at 24 hours after exposure and used for enzyme extraction. Three detoxification enzymes were studies on their activity level compared between non-selection (control) and selection strains. The detoxification enzymes extraction method, the enzyme activity detection assays and

the determination of the total protein content method as same issue 2.2 The results revealed by UV-visible spectrophotometer with specific substrate.

3.3 Molecular detection method

3.3.1 Effects on protein characterization of detoxification enzymes

To characterize the protein molecular weight of these enzyme systems by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). This procedure was used according to the method of Reidy *et a*l. (1990). The supernatant from 3.2 of both treatment and control were determined the molecular weights by the Precision Plus Protein^{TM} Dual Color Standards (Bio-Rad) as a protein marker. Standard of carboxylesterase, AChE and GST enzymes at 99.99% purified from Sigma® were used as references.

The chemical and protocol of qualitative enzyme (SDS) analysis was described in appendix C.

3.3.2 Effect on gene associated with detoxification enzyme expressions

To evaluate the effects of these three insecticides on gene encoding for detoxification enzyme expressions after sequential treated in 10 generations of *S. litura*. In this research, I preferred study on gene associated with carboxylesterase enzyme that showed highly level of activities after exposed with three insecticides. The 1st generation of non-selection strain (control) and the 10th generation of selection strain (cypermethrin, standard rotenone and derris crude extracts) provided the sample for this procedure. In each population, three replications and one larva of 3rd instar per replicated were used. Reverse transcriptase – polymerase chain reaction was used to detect and analyze the gene associated with detoxification enzyme expressions. One pair of forward and reverse primers was used to amplify DNA fragment from carboxylesterase gene of *S. litura*. The primer designed from the advantages region of open reading frame of *S. litura* carboxylesterase gene from GenBank by GENETYX analysis program.

3.3.2.1 Carboxylesterase gene detection method

1) Total RNA isolation**.** The total RNA was extracted from the third instar larvae of *S. ltitura* using Tri-reagent as described by the manufacturer follow by Sudthongkong method (2005) (personal contact). The third instar larvae were homogenized with Tri-reagent in clean tube. Let the sample solution incubated for 5 minutes at room temperature and centrifuged at 12,000 g, 4°C for 10 min. Transferred supernatant into new clean tube then added the BCP (1-bromo-3-chloropropane), mixed gently for 15 sec and let sample solution stand for 15 min at room temperature, after that centrifuged at 12,000 g, 4°C for 15 min. Transferred supernatant into new clean, added isopropanol and mixed gently then incubated the sample solution for 10 min at room temperature followed by centrifuged at 12,000 g, 4°C for 15 min. Kept pellets and washed with 75% ethanol, vortex and centrifuged at

7,500 g, 4°C for 5 min. Air dried around 30 min at room temperature. RNA Eluted with DNase-RNase Free Distilled water into final volume at 50 µl. Incubated RNA solution in water bath at 60 °C for 15 min and RNA solution in -80 °C until use.

2) Reverse transciptase - polymerase chain reaction (RT-PCR)

2.1) Primer design. The primer for carboxylesterase gene fragment of *S. litura* amplification, were designed by Genetyx program. The 1,613 bp of accessed number DQ 445461 from Genbank database (http://www.ncbi.nlm.nih.gov/) were used. The specific primers were screened from the 55th to 76th and 587th to 607th position for forward and reverse primer, respectively (Figure 6). The primer sequences showed as below.

Primer name	Sequences 5' to 3'
Forward: SLcaF	GGC CTA AGT ATG GTG CAA GTG A
Reverse: SLcaR	TGG TTA CGT TGT TGG GGT CAC

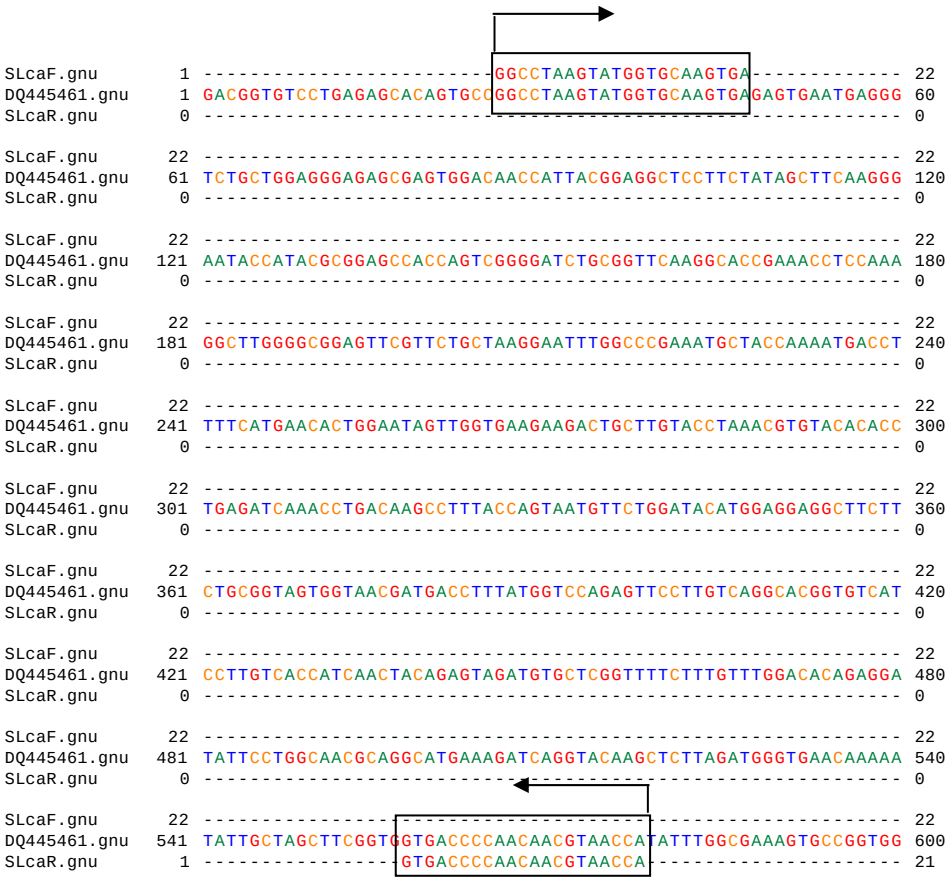


Figure 6 Position of primer SLcaF and SLcaR for caboxylesterase gene detection in *Spodoptera litura* (F.).

2.2) RT-PCR amplification. The total RNA is use for RT-PCR, construction of the cDNA library and primer extension analysis. The first strand cDNA is synthesized with Superscript III reverse transcriptase (Invitrogen). All reagents obtain from Invitrogen except primers, which were obtained from Quigen. PCR reaction contained of 20 ng RNA, 10 μM of each primer, 0.5 mM dNTPs, 1.5 mM MgCl, 2 U of superscript III reverse transcriptase and 2X PCR buffer. PCR was carried out by GeneAmp thermal cycler. The thermocycler programmed (Table 2) start at 1 cycle for cDNA synthesized which 60°C for 30 minutes. Next to 1 cycle for denaturation which 94°C for 2 minutes. Then 35 cycle for PCR amplification which composed of the DNA denaturing at 94°C for 30 seconds, DNA annealing at 59°C for 1 min and DNA extension at 72°C for 30 seconds. Finally of program, 1 cycle for final extension at 72°C for 10 minutes. PCR product was elucidated of DNA fragment by 0.8% agarose gel in TAE buffer. Ethidium-bromide was used for staining. PCR analysis was visualized and photograph by Gel Documentation. The RT-PCR products were determined by lamda DNA/*Hind*III marker. For sequencing and analysis, RT-PCR products were purified directly from the PCR reaction and then directly sequencing for gene analysis.

Table 2 The thermocycler programme of RT-PCR for carboxylesterase gene detection in *Spodopter litura* (F.).

Stage	Temperature (°C)	Time (min)
Stage1: cDNA Synthesis	60	30.0
	94	2.00
Stage2: PCR Cycle (35 cycle)		
Denature:	94	0.30
Annealing:	59	0.30
Extension	72	1.00
Sage3:		
Long Extension	4	Hold

3.3.2.2 Gene analysis. PCR products were sequenced by DNA sequencer at Bioservice Unit (BSU) of National Science and Technology Development Agency (NSTDA). The partial sequences were determined by compared with the GenBank database using BLAST program (http://www.ncbi.nlm.nih.gov/) to test identity. And were used the GENETYX analysis program for sequence editing, sequence alignments, translated nucleotide sequences to amino acid sequences and alignment for nucleotide sequences and amino acid sequence analysis.

8. Place and duration

Place: Department of Zoology, Faculty of Science, Kasetsart University; Bang Ken campus.
Department of Genetics, Faculty of Science, Kasetsart University; Bang Ken campus.

Duration: October 2005 to March 2007.

RESULTS AND DISCUSSIONS

Results

1. Toxicity determination of cypermethrin, standard rotenone and derris crude extracts against *S. litura*

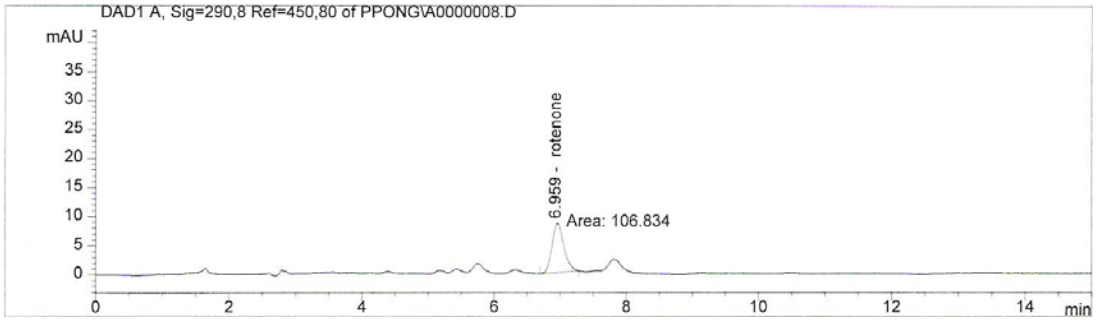
1.1 Root of derris extraction and quantitative

1.1 Root of derris extraction

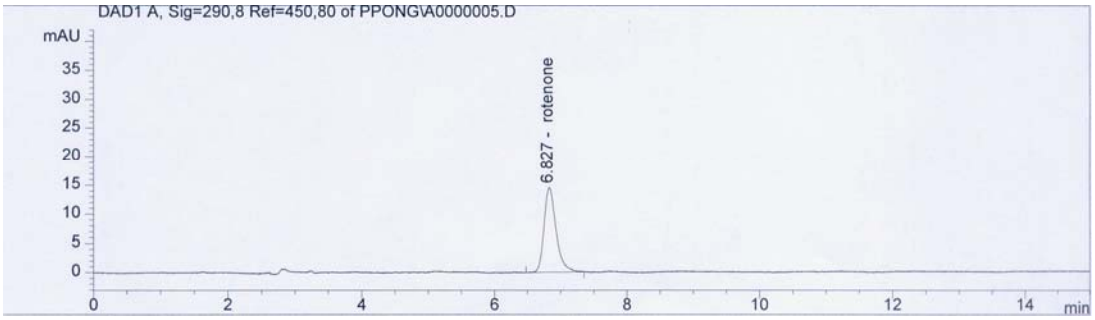
Roots of derris were extracted by Soxhlet's apparatus with ethanol solvent. The amount of crude extracts is shown high yield *ca.* 48.60 %w/w. The characteristic of dry crude extracts is rough solid substance and dark brown color.

1.2 Quantitative of crude extracts

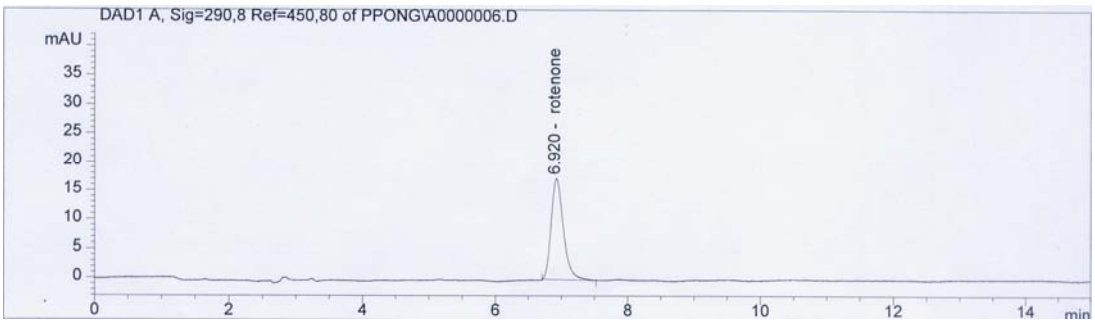
The crude extracts from roots of derris as analyzed the total content of rotenone. The yield of rotenone was separated from dried powder of crude extracts by HPLC with 3 replicated. Total rotenone percentage is shown high amount at 17.91 %w/w. The HPLC peak of rotenone amount is show in Figure 7.



A



B



C

Figure 7 HPLC chromatography A = dry ethanolic crude extracts from root of *Derris elliptica* (Benth) 5 ppm, B = standard rotenone 8 ppm and C = standard rotenone 10 ppm

1.2 Efficiency of cypermethrin, standard rotenone and derris crude extracts against *S. litura*

1.2.1 Efficiency of cypermethrin

The cypermethrin 35%EC was used to test the toxicity in larva of *S. litura*. Five concentrations of cypermethrin, included distilled water used as control 0, 5, 10, 15 and 20 ppm (ml/l = ppm) were treated with five replications. The mortality percentage at 24 hours were shown 0 ± 0, 8.89 ± 3.85, 32.22 ± 5.09, 66.67 ± 6.67 and

75.56 ± 5.09 respectively (Figure 8, Table 3). The mortality percentage values differed and significant increased when used high cypermethrin concentration. The simple linear regression was showed Y = (-5.11) + 4.17X (X means the concentration of cypermethrin and Y means the mortality percentage of treated larvae). Therefore, LC_{50} value of cypermethrin against *S. litura* larvar at 24 hours after exposure was 13.22 ppm at 0.95 of correlation coefficient (r^2) value (Table 3).

The mortality percentage at 48 hours after exposure was dramatically increased resulting 0 ± 0, 16.67 ± 3.33, 51.11 ± 1.92, 72.22 ± 5.09 and 88.89 ± 5.09 respectively (Figure 8, Table 3). The mortality percentage values differed among groups and significant increased when used high cypermethrin concentration. The simple linear regression was showed Y = (-0.88) + 4.66X. Therefore, LC_{50} value of cypermethrin against *S. litura* larvar at 48 hours after exposure was 10.92 ppm at 0.98 of correlation coefficient value (Table 3).

After 72 hours of exposure, the mortality percentage was stilled dramatically to increasing with showed the result that 0 ± 0, 18.89 ± 1.92, 54.44 ± 1.92, 80.00 ± 3.33 and 98.88 ± 1.92 respectively (Figure 8, Table 3). The mortality percentage values differed among groups and significant increased when used high cypermethrin concentration. The simple linear regression was showed Y = (-1.33) + 5.17X. Therefore, LC_{50} value of cypermethrin against *S. litura* larvar at 72 hours after exposure was 09.93 ppm at 0.98 of correlation coefficient value (Table 4). The mortality percentage values were increased when observed long time.

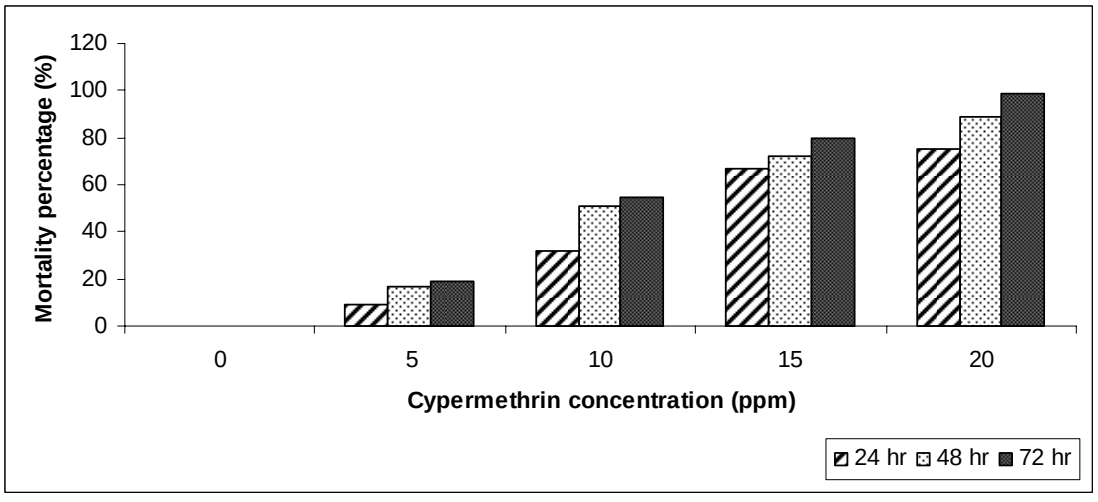


Figure 8 Mortality percentage of *Spodoptera litura* (F.) against cypermethrin at 24, 48 and 72 hours after exposure.

1.2.2 Efficiency of standard rotenone

The standard rotenone 98% formulation from Sigma was used to estimate the toxicity of *S. litura* larva. Five concentrations of standard rotenone, included distilled water used as control 0, 15, 25, 35 and 45 ppm (mg/l = ppm) were treated with five replications. The mortality percentage at 24 hours were shown 0 ± 0, 18.88 ±10.18, 37.78 ±3.85, 54.44 ±8.39 and 75.56 ± 5.09 respectively (Figure 9, Table 3). The mortality percentage values differed and significant increased when used high standard rotenone concentration. The simple linear regression was showed Y = (-3.06) + 1.68X (X means the concentration of standard rotenone and Y means the mortality percentage of treated larvae). Therefore, LC_{50} value of standard rotenone against *S. litura* larva at 24 hours after exposure was 31.58 ppm at 0.99 of correlation coefficient value (Table 4).

At 48 hours after exposure, the mortality percentage showed dramatically increasing with the result that 0 ± 0, 34.44 ± 8.39, 46.67 ± 3.33, 70.00 ± 3.33 and 87.78 ± 5.09 respectively (Figure 9, Table 3). The simple linear regression is showed Y = (-2.22) + 2.00X. The LC_{50} value of standard rotenone against *S. litura* larvar at 48 hours after exposure was 26.11 ppm at 0.99 of correlation coefficient value (Table 3). And after 72 hours of exposure, the mortality percentage was stilled dramatically increased with resulting that 0 ± 0, 31.11 ± 5.09, 51.11 ± 1.92, 80.00 ± 3.33 and 96.67 ± 3.33 respectively (Figure 9, Table 3). The mortality percentage values were increased when used high concentration. The simple linear regression was showed Y = (-0.96) + 2.19X. Thus, LC_{50} value of standard rotenone against *S. litura* larva at 72 hours after exposure was 23.27 ppm at 0.99 of correlation coefficient value (Table 4). The mortality percentage values were increased when observed long time.

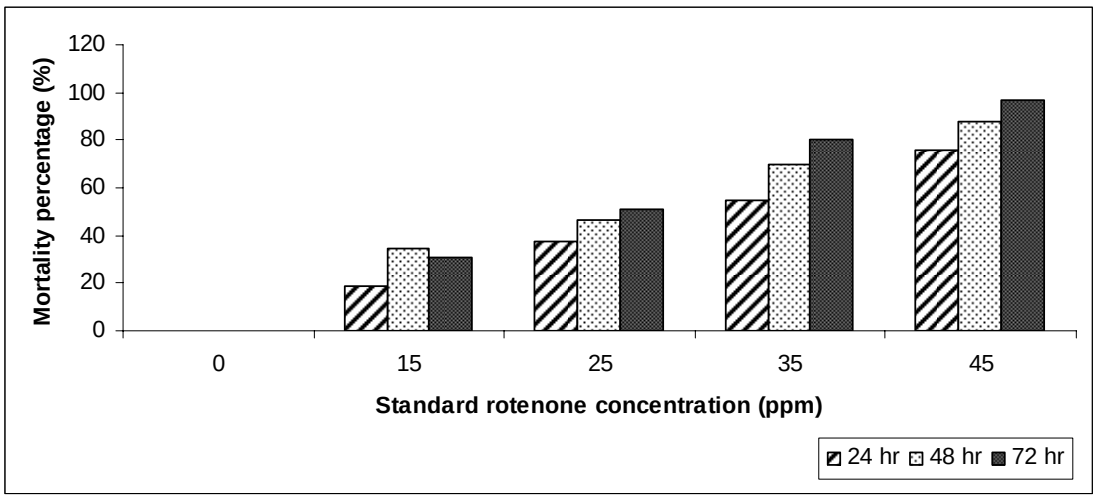


Figure 9 Mortality percentage of *Spodoptera litura* (F.) against standard rotenone at 24, 48 and 72 hours after exposure.

1.2.3 Efficiency of derris crude extracts

Crude extracts from roots of derris with ethanolic solvent was used to elucidate the toxicity in larva of *S. litura*. Five concentrations of derris crude extracts, included distilled water used as control 0, 20, 40, 60 and 80 ppm (mg/l = ppm) were treated with five replications. The mortality percentage at 24 hours were shown 0 ± 0, 24.44 ± 6.94, 37.78 ± 3.85, 57.78 ±13.87 and 68.89 ± 8.39 respectively (Figure 10, Table 3). The mortality percentage values differed and significant increased when used high derris crude extracts concentration. The simple linear regression was showed Y = 3.55 + 0.85X (X means the concentration of derris crude extracts and Y means the mortality percentage of treated larvae). Therefore, LC_{50} value of derris crude extracts against *S. litura* larva at 24 hours after exposure was 54.65 ppm at 0.98 of correlation coefficient value (Table 4).

The mortality percentage at 48 hours after exposure was increased with resulting that 0 ± 0, 30.00 ± 6.67, 51.11 ± 3.89, 77.78 ± 7.70 and 90.00 ± 8.82 respectively (Figure 10, Table 2). The simple linear regression was showed Y = 4.22 + 1.13X. The LC_{50} value of derris crude extracts against *S. litura* larvar at 48 hours after exposure was 40.51 ppm at 0.98 of correlation coefficient value (Table 3). And after 72 hours of exposure, the mortality percentage was stilled dramatically to increasing with showed the result that 0 ± 0, 34.44 ± 1.92, 53.33 ± 0.00, 82.22 ± 5.09 and 94.44 ± 5.09 respectively (Figure 10, Table 3). The mortality percentage values were increased when used high derris crude extracts concentration. The simple linear regression is showed Y = 1.18 + 5.55X. Therefore, LC_{50} value of derris crude extracts against *S. litura* larva at 72 hours after exposure was 37.67 ppm at 0.97 of correlation coefficient value (Table 4). The mortality percentage values were differed and increased when observed long time exposure.

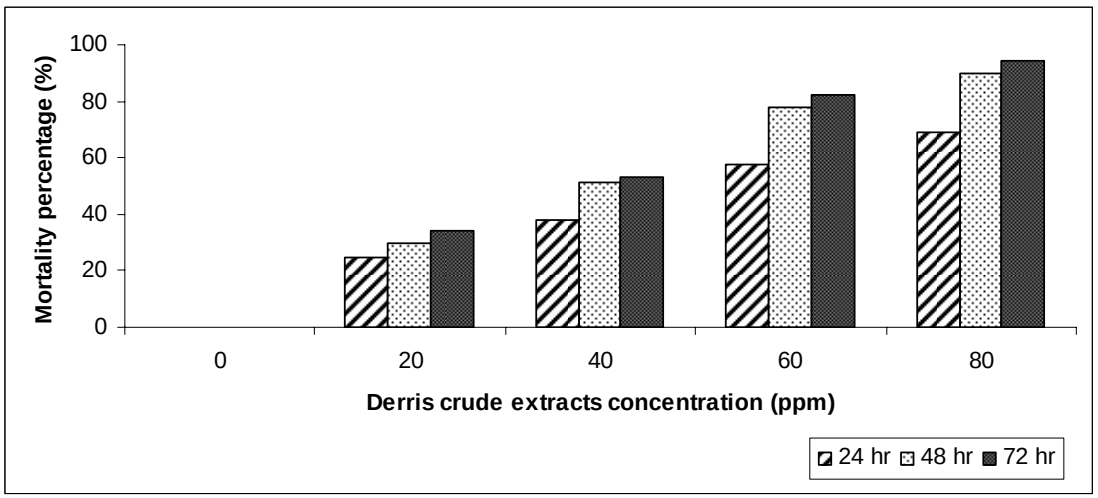


Figure 10 Mortality percentage of *Spodoptera litura* (F.) against derris crude extracts at 24, 48 and 72 hours after exposure.

As mentioned above, the mortality percentage of *S. litura* against different concentration and observed in different time exposure durations, every insecticide types showed high mortality rate at 72 hours. The highly concentration gave high mortality more than less concentration (Table 3).

In adiition to the toxicity, cypermethrin showed higher than others followed by standard rotenone and derris crude extracts. Most experiment showed highly correlated of mortality with r^2 value as 0.95-0.99, the r^2 is the correlation between concentration and mortality indicated the level of mortality correlated (Figure 11, Table 4).

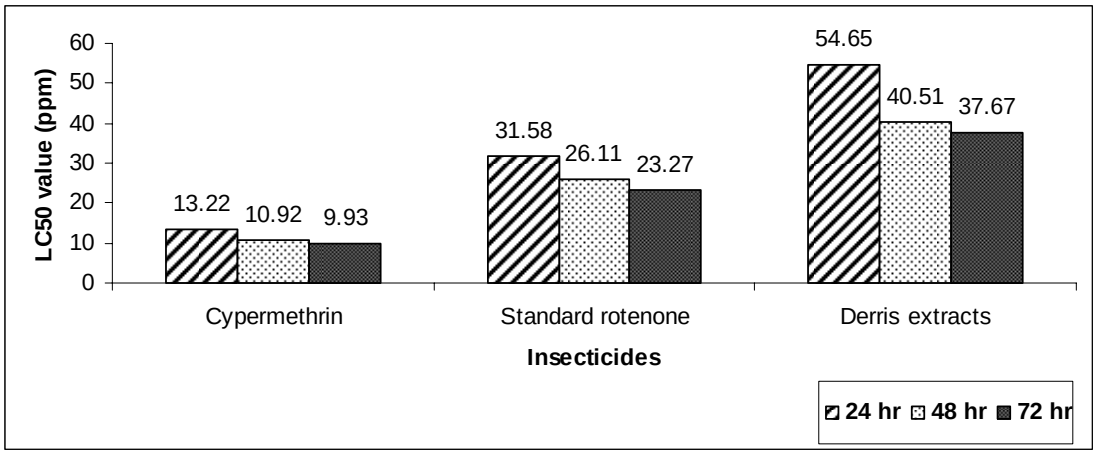


Figure 11 Comparison of LC_{50} value of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts at 24, 48 and 72 hours after exposure.

Table 3 Mortality percentage of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts at 24, 48 and 72 hours after exposure.

Dose [1] (ppm)	No.of larvae/ replicated	No. of repeated	% average mortality [3]		
			24 hr	48 hr	72 hr
Cypermethrin					
0 [2]	30	5	0.00 ± 0.00[a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
5	30	5	8.89 ± 3.85 [b]	16.67 ± 3.33 [b]	18.89 ± 1.92 [b]
10	30	5	32.22 ± 5.09 [c]	51.11 ± 1.92 [c]	54.44 ± 1.92 [c]
15	30	5	66.67 ± 6.67 [d]	72.22 ± 5.09 [d]	80.00 ± 3.33 [d]
20	30	5	75.56 ± 5.09 [e]	88.89 ± 5.09 [e]	98.88 ± 1.92 [e]
Rotenone	30				
0	30	5	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
15	30	5	18.88 ±10.18[b]	34.44 ± 8.39 [b]	31.11 ± 5.09 [b]
25	30	5	37.78 ±3.85[c]	46.67 ± 3.33 [c]	51.11 ± 1.92 [c]
35	30	5	54.44 ±8.39 [d]	70.00 ± 3.33 [d]	80.00 ± 3.33 [d]
45	30	5	75.56 ± 5.09 [e]	87.78 ± 5.09 [e]	96.67 ± 3.33 [e]
Derris extracts					
0	30	5	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]
20	30	5	24.44 ± 6.94 [b]	30.00 ± 6.67 [b]	34.44 ± 1.92 [b]
40	30	5	37.78 ± 3.85 [c]	51.11 ± 3.89 [c]	53.33 ± 0.00 [c]
60	30	5	57.78 ±13.87[d]	77.78 ± 7.70 [d]	82.22 ± 5.09 [d]
80	30	5	68.89 ± 8.39 [e]	90.00 ± 8.82 [e]	94.44 ± 5.09 [e]

[1] 1 ppm = insecticide 1 ml in distilled water 1 L (cypermethrin)
1 ppm = insecticide 1 mg in distilled water 1 L (standard rotenone and derris crude extracts)
[2] Control = distilled water
[3] Means ± SD followed by a common latter within the same column are significant at $p<0.05$ of DMRT

Table 4 LC_{50} value, regression equation and correlation coefficient of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts at 24, 48 and 72 hours after exposure.

Insecticides	Time (hr)	Regression equation [2]	LC_{50} [1] (ppm)	r^2 [3]
Cypermethrin	24	Y = (-5.11) + 4.17X	13.22	0.95
	48	Y = (-0.88) + 4.66X	10.92	0.98
	72	Y = (-1.33) + 5.17X	9.93	0.98
Rotenone	24	Y = (-3.06) + 1.68X	31.58	0.99
	48	Y = (-2.22) + 2.00X	26.11	0.99
	72	Y = (-0.96) + 2.19X	23.27	0.99
Derris extracts	24	Y = 3.55 + 0.85X	54.65	0.98
	48	Y = 4.22 + 1.13X	40.51	0.98
	72	Y = 1.18 + 5.55X	37.67	0.97

[1] 1 ppm = insecticide 1 ml in distilled water 1 L (cypermethrin)
1 ppm = insecticide 1 mg in distilled water 1 L (standard rotenone and derris crude extracts)
[2] Regression equation; Y=mortality percentage and X=insecticides concentration
[3] r^2 is a correlation determination between the concentration and mortality value

1.3 Toxicity of cypermethrin, standard rotenone and derris crude extracts against all instars larvae of *S. litura*

The results exhibited higher concentration dramatically increasing toxicity than less concentration (Figure 12, Table 5). The long time duration at 72 hours after exposure were also gave high toxicity than 24 and 48 hours after exposure in all instars larvae in most experiment. On the other hand, the toxicity value of each treatment was different in each instars larvae, although the toxicity similar increased from 1st to 5th instars larvae of most insecticides.

Cypermethrin showed highly toxicity than other insecticides. The lethal dose in term of LC_{50} showed different value that significant increasing from 1 to 5 instars larvae at 2.08, 2.92, 9.93, 11.64 and 18.38 ppm, respectively. The correlation between concentration and mortality showed r^2 as 0.95-0.97 in 2 to 5 instar indicated that the effect of cypermethrin were highly correlated of mortality in these instars, except the 1st instar that showed r^2 less than other as 0.87 (Figure 12, Table 5).

The standard rotenone resulted high toxicity following cypermethrin. The lethal dose was significantly ($p<0.01$) increasing from 1 to 5 instars larvae at 5.07, 8.12, 23.27, 39.44 and 124.79 ppm, respectively. In 3rd to 5th instar were showed high correlated of mortality with r^2 value as 0.95-0.98 than 1st to 2nd instar that gave r^2 as 0.78 and 0.84, respectively (Figure 12, Table 5).

And the crude extracts of derris roots revealed the least toxicity compare with cypermethrin and rotenone. The lethal dose significantly increased from 1 to 5

instars larvae did showed LC_{50} value at 3.79, 9.51, 37.67, 94.23 and 160.01 ppm, respectively. Most instars larvae showed high correlated of mortality with r^2 value as 0.93-0.97, accept 2nd instar is shown r^2 as 0.84 (Figure 12, Table 5).

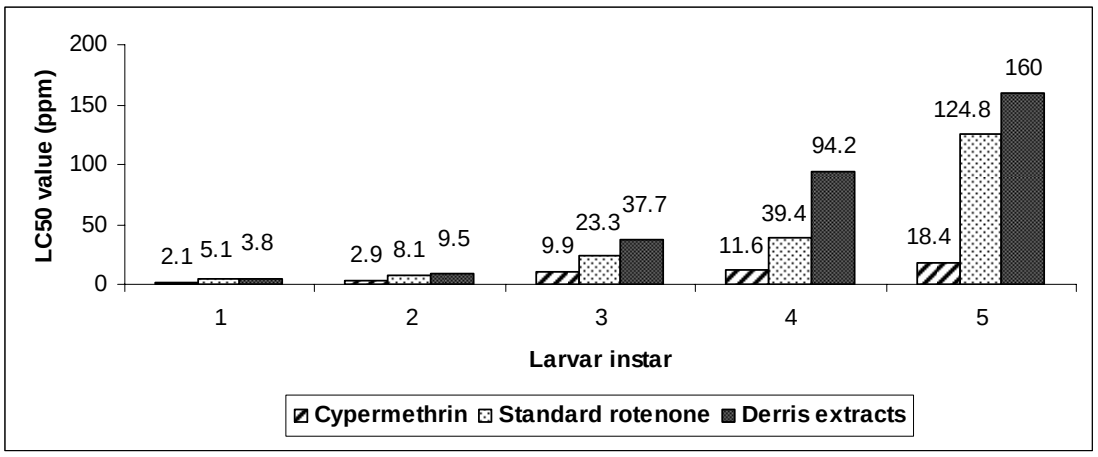


Figure 12 LC_{50} value comparison of all instars larvae of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts at 72 hours after exposure.

Table 5 LC_{50} value and correlation coefficient (r^2) of all instars larvae of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts at 72 hours after exposure.

Stage of larvae	Cypermethrin		Rotenone		Derris	
	LC_{50} (ppm)	$r^{2\,(1)}$	LC_{50} (ppm)	r^2	LC_{50} (ppm)	r^2
1st instar	2.08	0.87	5.07	0.78	3.79	0.93
2nd instar	2.92	0.95	8.12	0.84	9.51	0.84
3rd instar	9.93	0.99	23.27	0.98	37.67	0.97
4th instar	11.64	0.98	39.44	0.95	94.23	0.97
5th instar	18.38	0.97	124.79	0.95	160.01	0.93

(1) r^2 is a correlation determination between the concentration and mortality value

2. Characterization of detoxification mechanism of *S. litura* against cypermethrin, standard rotenone and derris crude extracts

2.1 pH optimal for detoxification enzyme mechanisms detection in *S. litura*

2.1.1 Suitable pH buffer for carboxylesterase

The detection of carboxylesterase activity was determined by pNPA method with paranotrophenyl acetate as substrate using UV-visible spectrophotometer at 400 nm absorbance. 0.5 g of the 3rd instar larvae were used to elucidate the enzyme

activity. The result showed the highest activity of carboxylesterase enzyme at pH 10 that means the pH 10 is the best pH range for carboxylesterase enzyme activity detection (Figure 13).

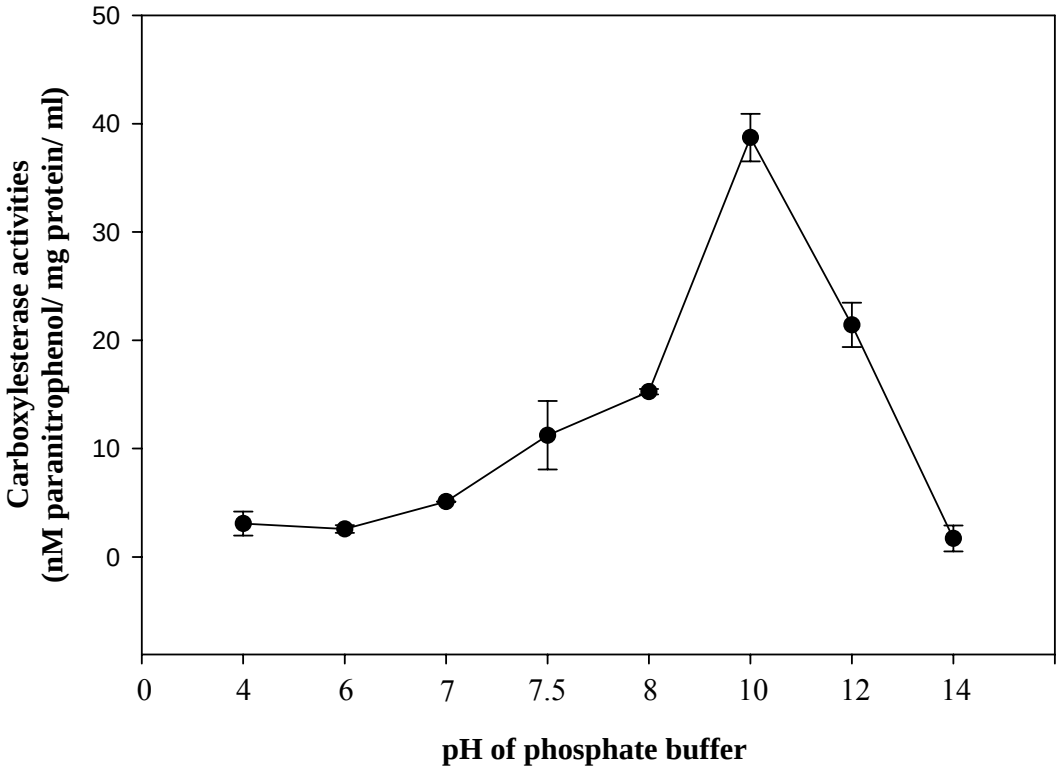


Figure 13 Carboxylesterase enzyme activity of *Spodoptera litura* (F.) with various pH range of potassium phosphate buffer.

2.1.2 Suitable pH buffer for acetylcholinesterase

The detection of acetylcholinesterase activity was determined by acetylthiocholine iodide as substrate using UV-visible spectrophotometer at 412 nm absorbance. 0.5 g of the 3rd instar larvae were used to elucidate the enzyme activity. The result showed the highest activity of acetylcholinesterase enzyme at pH 10 that means the pH 10 is the best pH range for acetylcholinesterase enzyme activity detection (Figure 14).

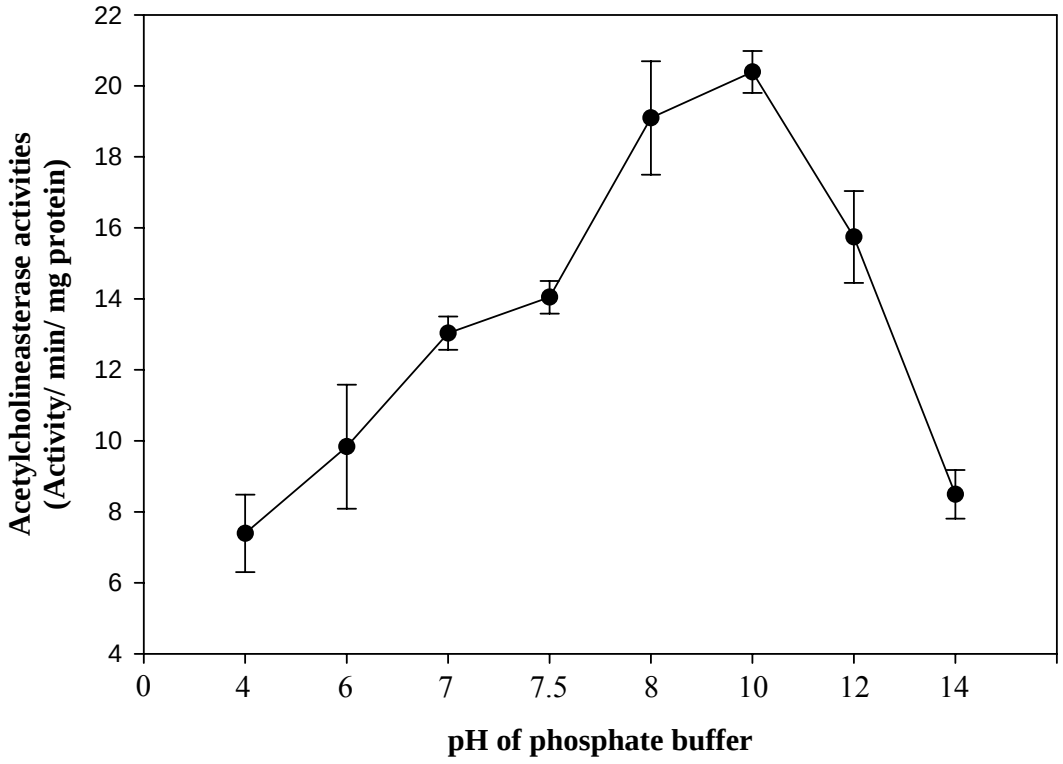


Figure 14 Acetylcholinesterase enzyme activity of *Spodoptera litura* (F.) with various pH range of potassium phosphate buffer.

2.1.3 Suitable pH buffer for glutathione-S-transfersase

The detection of glutathione-S-transferase (GSTs) activity was done by using UV-visible spectrophotometer. 0.5 g of the 3rd instar larvae were used to elucidate the enzyme activity. However, in this procedure, I conducted two methods that high efficacy for GSTs activity detection in animal. Two substrates were used to compare the GSTs activity level. The first substrate is dichloronitrobenzene (DCNB) was analyzed with absorbance at 344 nm. It showed the highest activity of GSTs enzyme at pH 10 that means the pH 10 is the best pH range for GSTs enzyme activity detection with DCNB substrate (Figure 15). Another substrate is chlorodinitrobenzene (CDNB) that can analyzed with absorbance at 340 nm. The result showed the fluctuation activity level (Figure 16). Therefore, I selected the DCNB substrate for GSTs activity detection in this study.

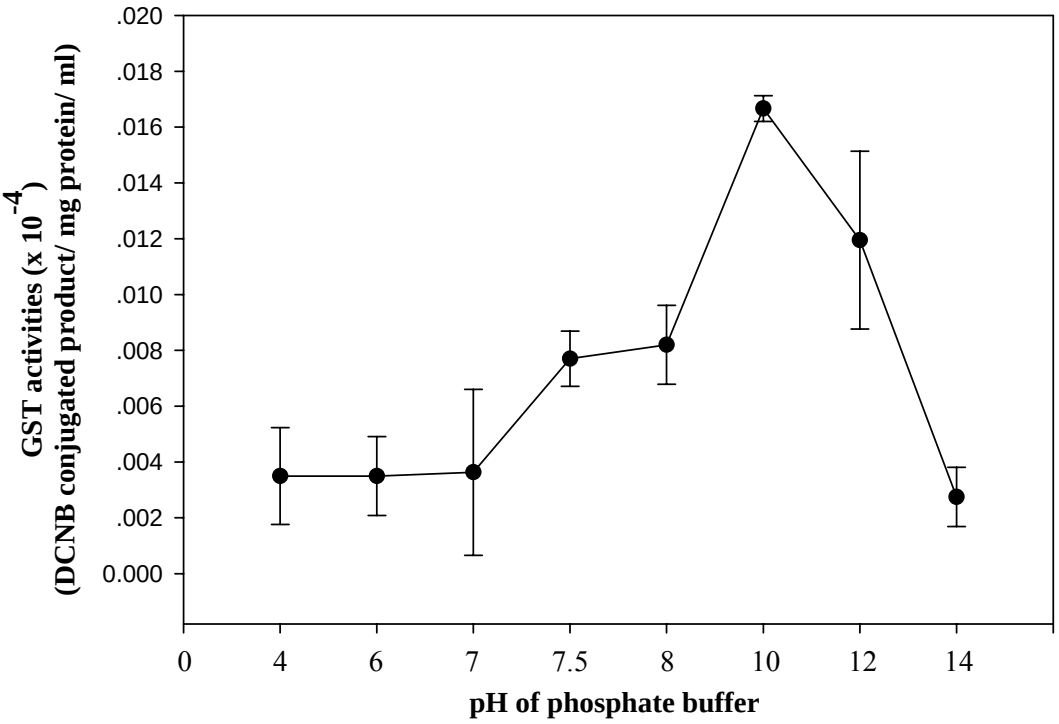


Figure 15 glutathione-S-transferase enzyme activity of *Spodoptera litura* (F.) with various pH range of potassium phosphate buffer using DCNB substrate.

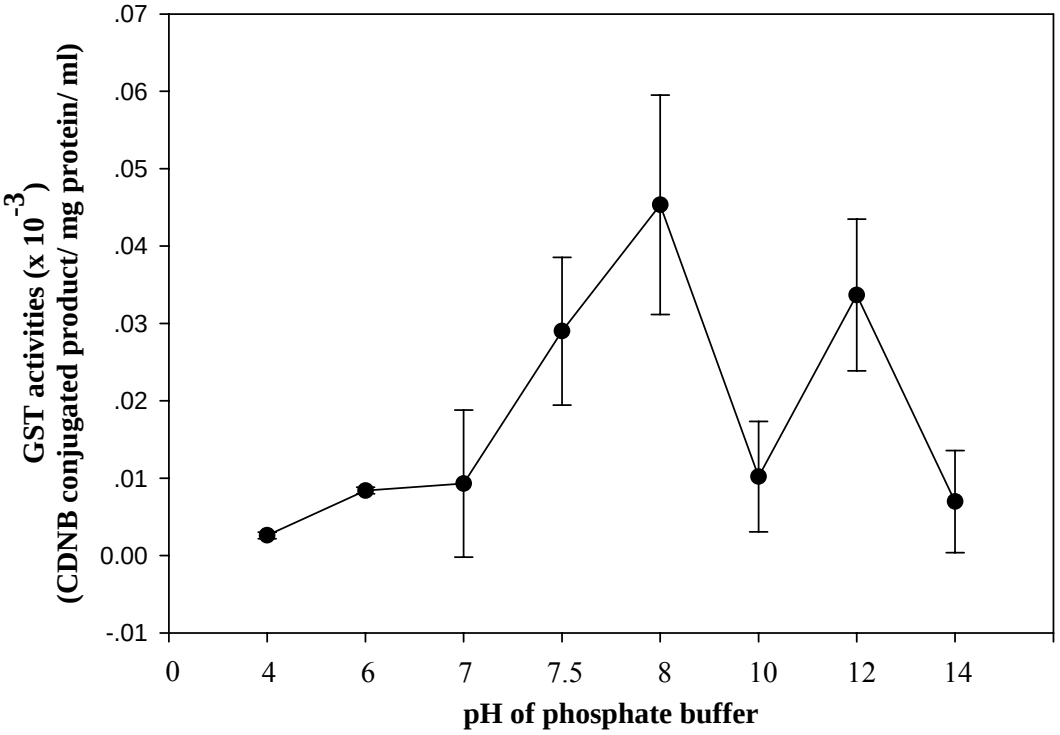


Figure 16 glutathione-S-transferase enzyme activity of *Spodoptera litura* (F.) with various pH range of potassium phosphate buffer using CDNB substrate.

2.2 Detoxification enzyme mechanisms in all instars larvae of *S. litura* against cypermethrin, standard rotenone and derris crude extracts

The results founded that the characteristic of each enzyme were different in each instars larvae as below.

2.2.1 Carboxylesterase activity

The activities of carboxylesterase in all instars larvae were done by PNPA assay with pNPA substrate. After 24 hours the absorbance at 400 nm showed high activity in every treatment of all instars larvae. The correction factor when compare between control and treatment showed cypermethrin and derris crude extracts were increased the carboxylesterase activity of all instars larvae while standard rotenone was decreased this enzyme activity (Table 6). The synthetic chemical insecticide, cypermethrin showed increased carboxylesterase activity with 0.36 to 0.68 fold. Rotenone substance showed decreased activity with 1.00 to 1.04 fold and crude extracts of derris roots showed increased activity with 0.32 to 0.37 fold. The third instars larvae of all treatments revealed the highest correction factor that means the third instars larvae is the best acts in carboxylesterase activity. However, most of experiments were resulting no different of protein concentration in all instars larvae of *S. litura* (Table 6).

Table 6 Carboxylesterase activity and correction factor of various instars larvar of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts at 24 hours exposure.

Insecticides	Stage of larva development				
	Instar 1 [CF] (3)	**Instar 2 [CF]**	**Instar 3 [CF]**	**Instar 4 [CF]**	**Instar 5 [CF]**
Cypermethrin					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity (1,2)	10.56 ± 4.12	12.64 ± 3.10	15.57 ± 1.83	14.86 ± 1.74	16.38 ± 1.44
Treatment					
- protein conc.	30.71 ± 0.23	50.45 ± 0.16	89.45 ± 0.28	138.42 ± 0.26	198.57 ± 0.16
- activity	20.59 ± 7.84	24.52 ± 2.60	25.95 ± 2.73	29.71 ± 5.88	45.50 ± 1.62
	[0.51]	[0.52]	[0.60]	[0.50]	[0.36]
Rotenone					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity	29.01 ± 1.50	29.98 ± 1.70	31.58 ± 1.29	39.43 ± 3.09	43.96 ± 2.81
Treatment					
- protein conc.	30.45 ± 0.10	50.38 ± 0.15	89.56 ± 0.26	139.18 ± 0.12	198.23 ± 0.20
- activity	28.33 ± 1.98	29.13 ± 2.20	30.33 ± 1.29	39.10 ± 3.86	43.72 ± 3.23
	[1.02]	[1.02]	[1.04]	[1.00]	[1.00]
Derris extract					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity	10.03 ± 3.70	11.00 ± 2.66	16.85 ± 1.23	14.11 ± 1.97	15.62 ± 0.49
Treatment					
- protein conc.	30.49 ± 0.11	50.49 ± 0.20	89.53 ± 0.10	138.33 ± 0.11	198.37 ± 0.40
- activity	31.47 ± 6.19	32.61 ± 6.20	45.84 ± 3.66	38.18 ± 0.12	43.59 ± 11.65
	[0.32]	[0.33]	[0.37]	[0.36]	[0.36]

(1) Means ± SD, 5 replicates
(2) The unit of carboxylesterase activity is activity product/mg protein/min
(3) CF is a correction factor = (average enzyme activity of control)/(average enzyme activity of treatment)

2.2.2 Acetylcholinesterase activity

The activities of AChE in all instars larvae were done by acetylthiocholine iodide substrate. After 24 hours the absorbance at 412 nm showed high activity next to carboxylesterase activity in every treatment of all instars larvae. The correction factor when compare between control and treatment showed cypermethrin and derris crude extracts were increased the AChE activity of all instars larvae (Table 7). Cypermethrin showed increased the activity with 0.14 to 0.99 fold. While the rotenone substance had efficiency different from the synthetic chemical insecticide, cypermethrin that showed decreased AChE activity in 1st to 3rd instar

between 1.00 to 1.03 fold but 4th to 5th instars larvae showed increased the activity with 0.98 to 0.99 fold, respectively. For the crude extracts of derris roots is act same cypermethrin with showed increased this enzyme activity in all instars larvae with 0.14 to 0.94 fold. The third instars larvae of all treatments revealed the highest correction factor that means the third instars larvae is the best acts in AChE activity. And most of experiments resulted no different of protein concentration in all instars larvae (Table 7).

Table 7 Acetylcholinesterase activity and correction factor of various instars larvar of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts at 24 hours exposure.

Insecticides	Stage of larva development				
	Instar 1 [CF] (3)	**Instar 2 [CF]**	**Instar 3 [CF]**	**Instar 4 [CF]**	**Instar 5 [CF]**
Cypermethrin					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity(1,2)	3.30 ± 0.21	3.70 ± 0.97	11.59 ± 0.47	5.56 ± 0.52	2.90 ± 0.78
Treatment					
- protein conc.	30.71 ± 0.23	50.45 ± 0.16	89.45 ± 0.28	138.42 ± 0.26	198.57 ± 0.16
- activity	4.65 ± 2.62	8.82 ± 2.02	11.71 ± 2.67	9.27 ± 7.97	20.74 ± 19.95
	[0.71]	[0.42]	[0.99]	[0.60]	[0.14]
Rotenone					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity	4.55 ± 0.84	7.48 ± 0.04	12.04 ± 0.10	9.50 ± 0.84	18.89 ± 0.64
Treatment					
- protein conc.	30.45 ± 0.10	50.38 ± 0.15	89.56 ± 0.26	139.18 ± 0.12	198.23 ± 0.20
- activity	4.48 ± 1.10	7.42 ± 2.05	11.65 ± 0.40	9.52 ± 1.25	19.21 ± 3.00
	[1.01]	[1.00]	[1.03]	[0.99]	[0.98]
Derris extract					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity	2.22 ± 0.28	3.70 ± 1.04	11.69 ± 0.53	5.52 ± 0.37	2.87 ± 0.51
Treatment					
- protein conc.	30.49 ± 0.11	50.49 ± 0.20	89.53 ± 0.10	138.33 ± 0.11	198.37 ± 0.40
- activity	2.40 ± 2.31	4.45 ± 1.06	12.5 ± 6.95	11.49 ± 10.35	20.82 ± 9.50
	[0.93]	[0.83]	[0.94]	[0.48]	[0.14]

(1) Means ± SD, 5 replicates
(2) The unit of acetylcholinesterase activity is activity product/mg protein/min
(3) CF is a correction factor = (average enzyme activity of control)/(average enzyme activity of treatment)

2.2.3 Glutathione-S-transferase activity

GSTs activity level of all instars larvae was done by DCNB substrate. After 24 hours the absorbance at 344 nm showed the least activity compare with carboxylesterase and AChE activity in every treatment of all instars larvae. But the correction factor when compare between control and treatment showed cypermethrin and derris crude extracts were increased this enzyme activity of all instars larvae. While standard rotenone showed decreased enzyme activity in 1^{st} to 3^{rd} instars larvae but the 4^{th} to 5^{th} instars larvar were showed increased the activity (Table 8). Cypermethrin showed increased the activity with 0.01 to 0.04 fold. The rotenone substance has high efficiency than cypermethrin that showed decreased this enzyme activity in 1^{st} to 3^{rd} instar with 1.02 to 1.05 fold and showed decreased the activity in 4^{th} to 5^{th} instar with 0.82 to 0.86 fold, respectively. The crude extracts of derris roots is act same cypermethrin with showed increased this enzyme activity with 0.02 to 0.08 fold. However, third instars larvae of all treatments still revealed the highest correction factor that means the third instars larvae is the best acts for this enzyme activity. And most of experiments resulted no different of protein concentration in all instars larvae (Table 8).

Table 8 Glutathione-S-transferase activity and correction factor of various instars larvar of *Spodopter litura* (F.) against cypermethrin, standard rotenone and derris crude extracts with at 24 hours exposure.

Insecticides	Stage of larva development				
	Instar 1 [CF] (3)	**Instar 2 [CF]**	**Instar 3 [CF]**	**Instar 4 [CF]**	**Instar 5 [CF]**
Cypermethrin					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity(1,2)	0.11 ± 0.01	0.11 ± 0.01	0.47 ± 0.06	0.34 ± 0.08	0.35 ± 0.05
Treatment					
- protein conc.	30.71 ± 0.23	50.45 ± 0.16	89.45 ± 0.28	138.42 ± 0.26	198.57 ± 0.16
- activity	11.23 ± 0.40	11.28 ± 0.22	11.78 ± 0.63	11.18 ± 0.11	11.61 ± 0.86
	[0.01]	[0.01]	[0.04]	[0.03]	[0.03]
Rotenone					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity	5.97 ± 0.02	6.41 ± 0.02	8.10 ± 0.08	7.44 ± 0.13	9.22 ± 0.12
Treatment					
- protein conc.	30.45 ± 0.10	50.38 ± 0.15	89.56 ± 0.26	139.18 ± 0.12	198.23 ± 0.20
- activity	5.76 ± 0.65	6.27 ± 1.18	7.66 ± 0.42	9.02 ± 1.03	10.69 ± 0.69
	[1.03]	[1.02]	[1.05]	[0.82]	[0.86]
Derris extract					
Control					
- protein conc.	30.53 ± 0.10	50.37 ± 0.10	89.35 ± 0.21	138.13 ± 0.11	198.33 ± 0.11
- activity	0.12 ± 0.02	0.15 ± 0.03	0.05 ± 0.05	0.38 ± 0.03	0.39 ± 0.02
Treatment					
- protein conc.	30.49 ± 0.11	50.49 ± 0.20	89.53 ± 0.10	138.33 ± 0.11	198.37 ± 0.40
- activity	5.52 ± 0.66	6.43 ± 0.12	6.03 ± 0.15	5.12 ± 0.02	5.07 ± 0.57
	[0.02]	[0.02]	[0.08]	[0.07]	[0.08]

(1) Means ± SD, 5 replicates

(2) The unit of GST activity is DCNB conjugated product/mg protein/min, each enzyme activity value multiple with 10^{-4}

(3) CF is a correction factor = (average enzyme activity of control)/(average enzyme activity of treatment).

3. Determination of resistance to cypermethrin, standard rotenone and derris crude extracts in *S. litura*

3.1 Detection by bioassay technique

3.1.1 Toxicity effect of cypermethrin in 10 generations of *S. litura*

The toxicity of cypermethrin in 10 generations was shown in Table 9. Non-selection strain had no different toxicity throughout 10 generations. This toxicity value is similarity of F_1 selection strain was showed LC_{50} at 11.23 ppm, non-selection strain showed LC_{50} at 10.13-12.51 ppm. The correlation efficiency showed high correlated of mortality with r^2 value as 0.95 to 0.99. In the selection strain, the toxicity values were high increased from F_1 to F_{10} with LC_{50} value from 11.23 to 1,059.40 ppm. After 3rd generation, the toxicity value showed high level and proceeded increasingly to onward generations. The correlation efficiency showed high correlated of mortality with r^2 value as 0.91 to 0.99. The resistance was developed high level from 3rd generation and proceeded increasingly to onward generations. At 10th generation of selection strain, it was showed high level *ca.* 94.33 times when compared with the toxicity value of 1st generation and showed *ca.* 98.27 times when compared with 1st generation of non-selection strain (Figure 17). Trend of resistance ratio was highly increased to 104.58 fold when compared between the toxicity values of non-selection strain and selection strain in the 10th generation (Figure 18).

Table 9 LC_{50} value, regression equation, correlation coefficient (r^2) and resistance ratio (RR) of 3rd instars larvae of *Spodoptera litura* (F.) against cypermethrin at 72 hours exposure.

G	Non-selection strain			selection strain			RR(3)
	Regression equation(1)	LC_{50} (ppm)	r^2 (2)	Regression equation	LC_{50} (ppm)	r^2	
F_1	Y =(-2.00) + 4.82X	10.78	0.99	Y =(-1.33) + 4.57X	11.23	0.99	0.09
F_2	Y = (-1.11) + 4.88X	10.47	0.99	Y = (-1.77) + 1.00X	51.77	0.98	4.94
F_3	Y = 0.88 + 4.42X	11.11	0.97	Y = (-3.33) + 0.80X	66.66	0.99	6.00
F_4	Y = (-3.11) + 4.37X	12.15	0.98	Y = 0.22 + 3.33X	212.13	0.98	17.47
F_5	Y = (-5.11) + 4.62X	11.92	0.95	Y = (-2.34) + 0.17X	307.88	0.96	25.83
F_6	Y = (-4.44) + 4.35X	12.51	0.96	Y = (-6.47) + 0.11X	513.36	0.92	41.04
F_7	Y = (-4.66) + 4.57X	11.96	0.95	Y = (-4.55) + 0.08X	681.88	0.93	57.01
F_8	Y = (-3.11) + 4.48X	11.85	0.96	Y = (-3.81) + 0.06X	896.83	0.91	75.68
F_9	Y = (-3.55) + 5.11X	10.48	0.99	Y = (-4.15) + 0.06 X	902.50	0.91	86.12
F_{10}	Y = (-2.22) + 5.15X	10.13	0.99	Y = (-2.97) + 0.05X	1059.40	0.93	104.58

(1) Regression equation: Y=mortality percentage and X=insecticide concentration
(2) r^2 is a correlation determination between t he concentration and mortality value
(3) RR is a resistance ratio: toxicity value of selection strain/ toxicity value of non-selection strain
G = Generation

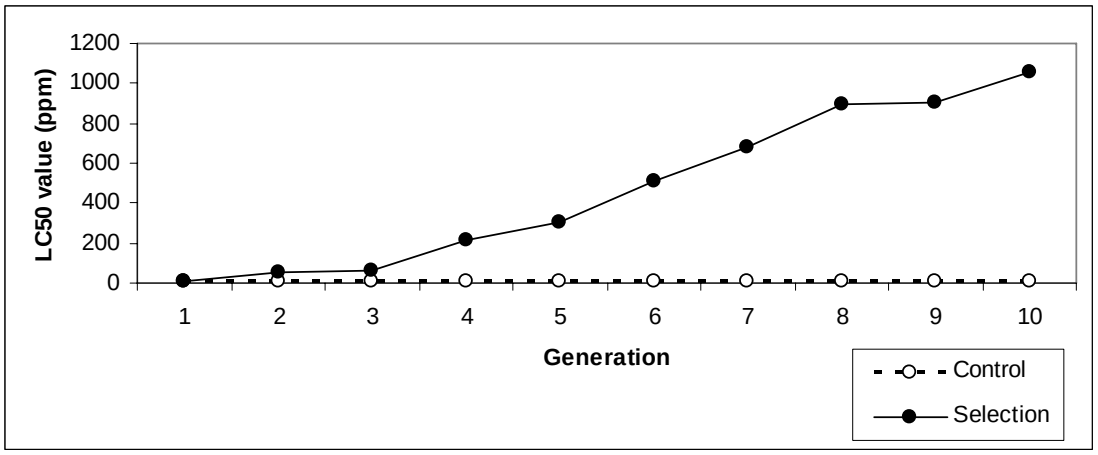


Figure 17 Resistance development of *Spodoptera litura* (F.) against cypermethtrin in 10 generations.

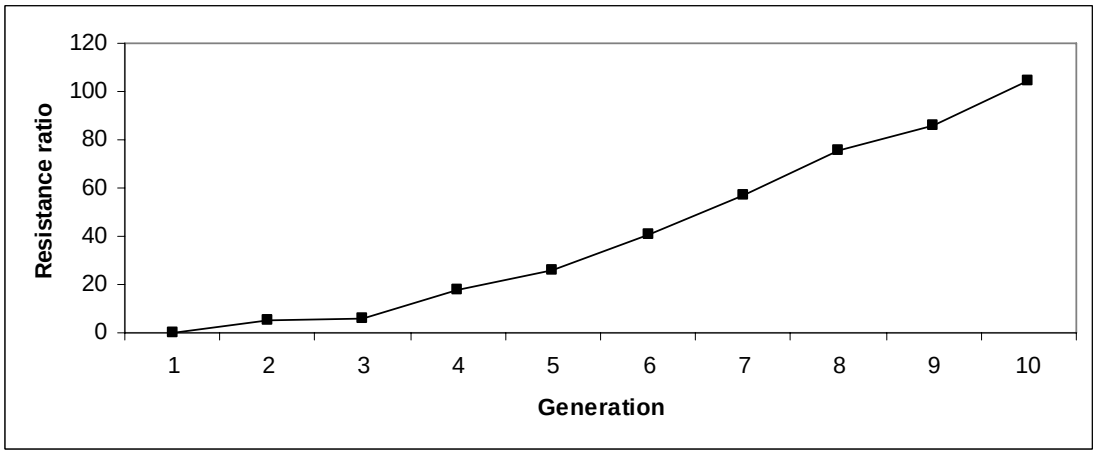


Figure 18 Trend of resistance ratio of *Spodoptera litura* (F.) against cypermethtrin in 10 generations.

3.1.2 Toxicity of standard rotenone against *S. litura* for 10 generations

The results of rotenone substance toxicity from standard rotenone for 10 generations were shown in Table 10. The toxicity values were shown LC_{50} at 23.39-27.56 ppm. The correlation efficiency showed high correlated of mortality with r^2 value as 0.96 to 0.99. In selection strain, F_1 to F_4 generation were showed no different in toxicity similarity with non-selection strain that gave LC_{50} at 23.39-31.63 ppm. The toxicity values were highly increased at F_5 and slightly increasing from F_6 to F_{10} with LC_{50} value from 96.15 to 143.16 ppm. The correlation efficiency showed high correlated of mortality with r^2 value as 0.95 to 0.99. The resistance development showed high level after 4^{th} generation and proceeded slightly increased to onward generations. At 10^{th} generation of selection strain, it was showed its level *ca.* 5.69 times when compared with the toxicity value of 1^{st} generation and showed *ca.* 5.91 times when compared with 1^{st} generation of non-selection strain (Figure 19). Trend of

resistance ratio was increased to 5.79 fold when compared between the toxicity values of non-selection strain and selection strain in the 10th generation (Figure 20).

Table 10 LC_{50} value, regression equation, correlation coefficient (r^2) and resistance ratio (RR) of 3rd instars larvae of *Spodoptera litura* (F.) against standard rotenone at 72 hours exposure.

G	Non-selection strain			selection strain			RR(3)
	Regression equation (1)	LC_{50} (ppm)	r^2 (2)	Regression equation	LC_{50} (ppm)	r^2	
F_1	Y = (-2.53) +2.17X	24.21	0.99	Y = (-4.55) +2.17X	25.14	0.98	1.04
F_2	Y = (-3.75) +1.95X	27.56	0.96	Y = (-5.35) +1.83X	30.25	0.95	1.09
F_3	Y = (-2.04) +3.11X	26.03	0.97	Y = (-5.44) +1.88X	29.49	0.97	1.13
F_4	Y = (-5.71) +2.25X	24.76	0.97	Y = (-1.56) +1.63X	31.63	0.96	1.27
F_5	Y = (-5.81) +2.19X	25.48	0.97	Y = 5.77 + 0.46X	96.15	0.98	3.77
F_6	Y = (-5.44) +2.21X	25.09	0.98	Y = 4.22 + 0.44X	104.04	0.99	4.15
F_7	Y = (-5.57) +2.17X	25.61	0.97	Y = 4.88 + 0.36X	125.33	0.97	4.89
F_8	Y = (-3.57) +2.29X	23.39	0.98	Y = (-1.56) +0.38X	135.68	0.99	5.80
F_9	Y = (-3.91) +2.26X	23.85	0.98	Y = (-2.04) +0.37X	140.64	0.99	5.89
F_{10}	Y = (-4.62) +2.21X	24.71	0.98	Y = (-2.97) +0.37X	143.16	0.99	5.79

(1) Regression equation: Y=mortality percentage and X=insecticide concentration
(2) r^2 is a correlation determination between the concentration and mortality value
(3) RR is a resistance ratio: toxicity value of selection strain/ toxicity value of non-selection strain
G = Generation

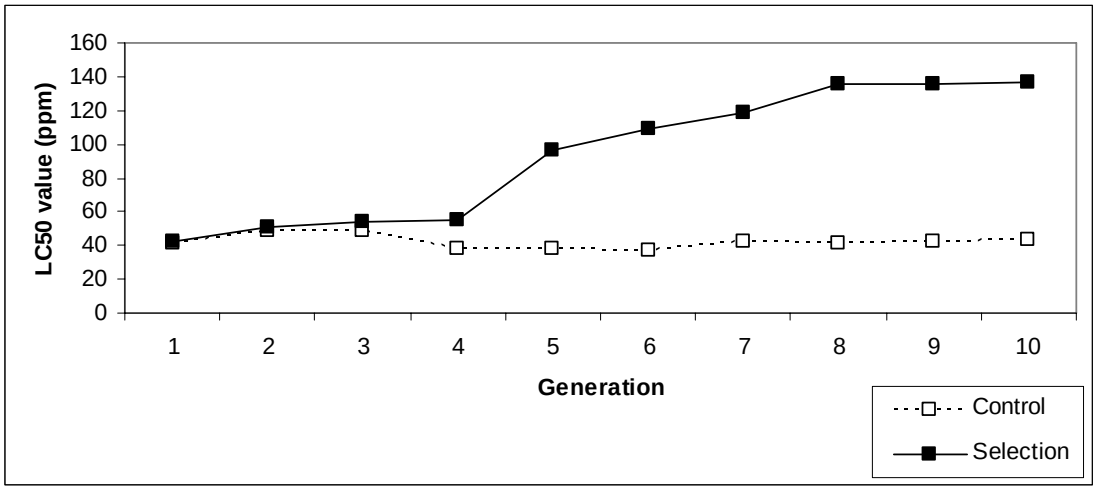


Figure 19 Resistance development of *Spodoptera litura* (F.) against standard rotenone in 10 generations.

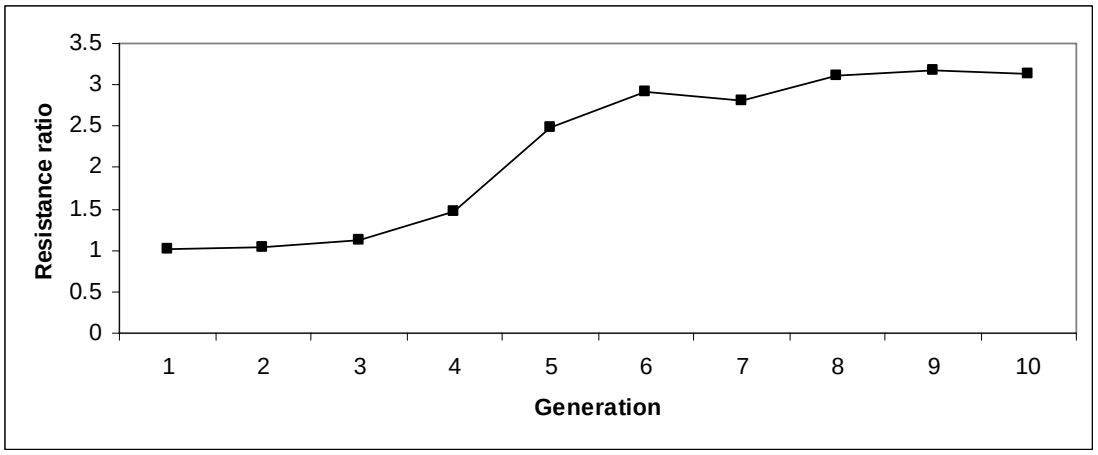


Figure 20 Trend of resistance ratio of *Spodoptera litura* (F.) against standard rotenone in 10 generations.

3.1.3 Toxicity of derris crude extracts for 10 generations of *S. litura*

The toxicity values were shown LC_{50} at 37.67-49.07 ppm (Table 11). The correlation efficiency showed high correlated of mortality with r^2 value as 0.97 to 0.99. In selection strain, F_1 to F_4 generation were showed no different in toxicity similarity with non-selection strain that gave LC_{50} at 42.19-55.13 ppm. The toxicity values were highly increased at F_5 and slightly increase from F_6 to F_{10} with LC_{50} value from 96.16 to 136.35 ppm. The correlation coefficien of selection strain showed high correlated of mortality with r^2 value of 0.93 to 0.99. The resistance development showed high level after 4^{th} generation and proceeded slightly increased toward 10 generations. At 10^{th} generation of selection strain, it was showed *ca.* 3.23 times when compared with the toxicity value of 1^{st} generation and showed *ca.* 3.27 times when compared with 1^{st} generation of non-selection strain (Figure 21). Trend of resistance ratio was increased to 3.14 fold when compared between the toxicity values of non-selection strain and selection strain in the 10^{th} generation (Figure 22).

Table 11 LC_{50} value, regression equation, correlation coefficient (r^2) and resistance ratio (RR) of 3rd instars larvae of *Spodoptera litura* (F.) against derris crude extracts at 72 hours exposure.

G	Non-selection strain			selection strain			RR(3)
	Regression equation(1)	LC_{50} (ppm)	r^2 (2)	Regression equation	LC_{50} (ppm)	r^2	
F_1	Y = 4.22 + 1.10X	41.62	0.98	Y = 4.44 + 1.08X	42.19	0.98	1.01
F_2	Y = 0.44 + 1.01X	49.07	0.98	Y = (-0.66) +0.99X	51.17	0.98	1.04
F_3	Y = (-1.77) +1.07X	48.38	0.97	Y = (-3.55) +0.99X	54.09	0.98	1.12
F_4	Y = 1.77 + 1.28X	37.68	0.99	Y = (-7.33) +1.04X	55.13	0.96	1.46
F_5	Y = 4.00 + 1.19X	38.66	0.98	Y = 0.96 + 0.51X	96.16	0.99	2.49
F_6	Y = 4.66 + 1.21X	37.47	0.98	Y = (-3.41) +0.49X	109.0	0.96	2.91
F_7	Y = (-1.33) +1.22X	42.07	0.99	Y = 0.82 +0.43X	118.18	0.93	2.80
F_8	Y = 3.55 + 1.13X	41.12	0.99	Y = (-1.35) +0.38X	135.13	0.99	3.11
F_9	Y = (-2.66) +1.23X	42.81	0.98	Y = (-1.63) +0.38X	135.86	0.99	3.17
F_{10}	Y = (-2.00) +1.20X	43.33	0.98	Y = (-0.45) +0.37X	136.35	0.99	3.14

(1) Regression equation: Y=mortality percentage and X=insecticide concentration
(2) r^2 is a correlation determination between the concentration and mortality value
(3) RR is a resistance ratio: toxicity value of selection strain/ toxicity value of non-selection strain
G = Generation

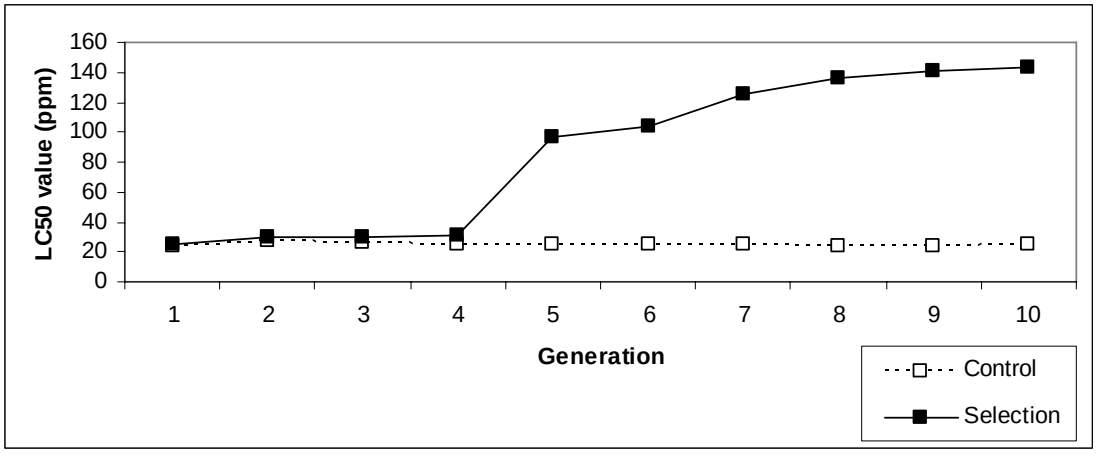


Figure 21 Resistance development of *Spodoptera litura* (F.) against derris crude extracts in 10 generations.

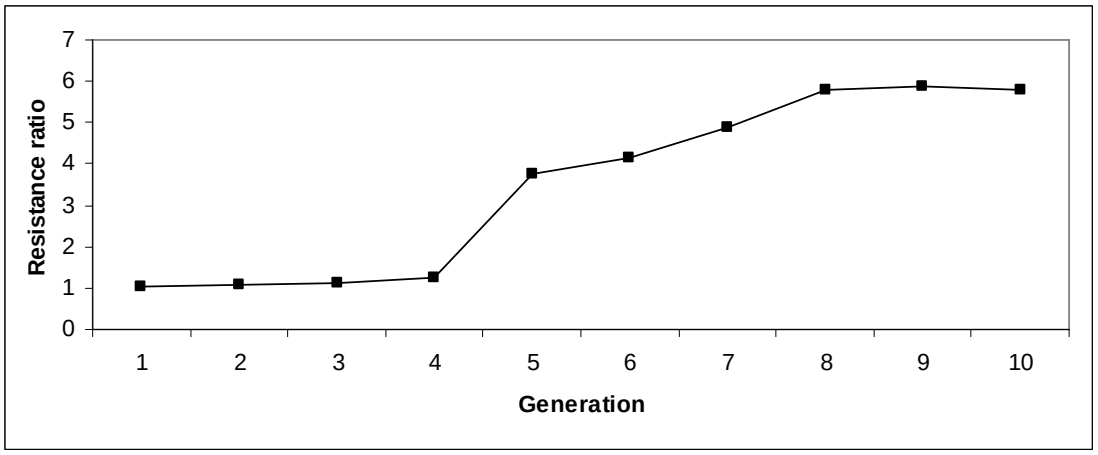


Figure 22 Trend of resistance ratio of *Spodoptera litura* (F.) against derris crude extracts in 10 generations.

3.2 Detection by biochemical technique

3.2.1 Effect on carboxylesterase activity

The carboxylesterase enzyme activities were examined using PNPA assay with pNPA substrate at 400 nm absorbance. The results showed that these three insecticides acts which enhanced characteristic of the carboxylesterase enzyme activities in *S. litura* larvar (Figure 23, Table 12). This activity showed which no different significant on protein concentration in most experiments. The synthetic chemical insecticide, cypermethrin showed decreased this enzyme activity level in F_1, but increased in F_2 and came back decreased in F_3 and F_4 with similar level. Then they were increased high activity level toward 10 generations in the 10^{th} generation, the enzyme activity values were shown in Table 12. The enzyme activity efficiency which shows as the correlation factor showed *ca.* 0.67 to 1.03 fold in 10 generations. As a correlation factor of 10 generations showed that cypermethrin is acts induction of carboxylesterase enzyme activities with activated high level of activity.

The standard rotenone showed decreased of carboxylesterase enzyme activity levels in F_1 to F_4 generations. Then it showed highly increased at F_4 to F_5 and after that it was slightly increase toward generations. The correlation factor showed *ca.* 0.69 to 1.00 fold (Table 12). As a correlation factor of 10 generations showed that standard rotenone acts as induction of carboxylesterase enzyme activities with activated the level of enzyme activity but slightly activated when compared with cypermethrin.

Derris crude extracts showed the fluctuation activity of carboxylesterase enzyme throughout 10 generations. With showed increased enzyme activity in F_1 to F_4 generations. But in F_5 to F_6 generation were decreased activity and came back to increased in F_7 to F_8. Then it decreased activity in F_9 and F_{10} generation. All of activities changing, they were changed with similar level of activity value throughout 10 generations. The correlation factor showed *ca.* 0.94 to 1.00 fold (Table

12). As a correlation factor of 10 generations showed that derris crude extracts was induction of carboxylesterase enzyme activities with activated level of enzyme activity similarity to standard rotenone resulted the slightly activated than cypermethrin.

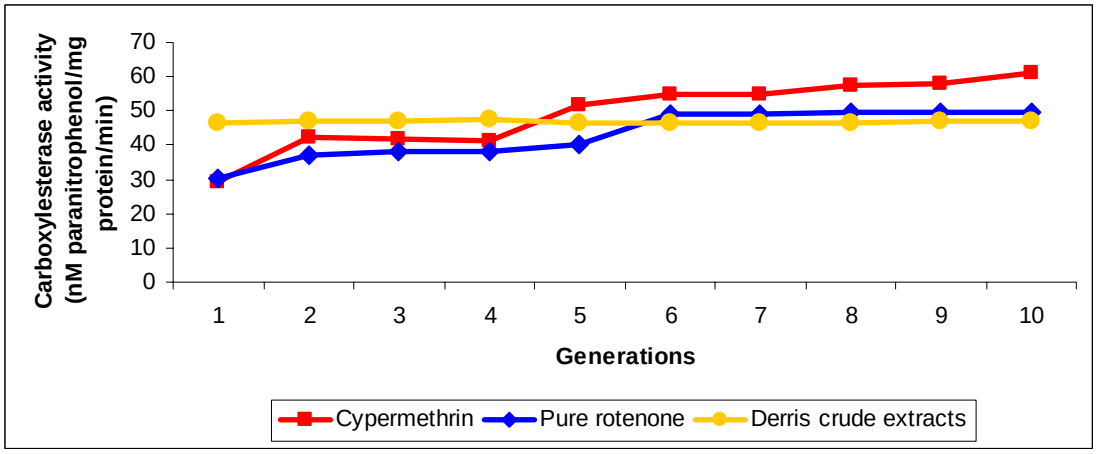


Figure 23 Carboxylesterase activity in *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts after 24 hours exposure in each of 10 generations.

Table 12 Carboxylesterase activity, correlation factor and protein content of *Spodoptera litura* (F.) after sequential treated with LC_{50} value each of cypermethrin, standard rotenone and derris crude extracts at 24 hours exposure in 10 generations.

G	Cypermethrin		Rotenone		Derris extracts	
	Protein[(1)] conc.	**Enzyme[(1,2)] activity [CF] [(3)]**	**Protein conc.**	**Enzyme activity [CF]**	**Protein conc.**	**Enzyme activity [CF]**
F_1C	89.35 ± 0.21	29.95 ± 2.73	89.35 ± 0.21	30.33 ± 1.29	89.35 ± 0.21	45.84 ± 3.66
T	89.37 ± 0.12	29.46 ± 0.44 1.01	89.42 ± 0.21	30.32 ± 2.06 1.00	89.46 ± 0.20	46.75 ± 0.14 0.98
F_2 C	89.36 ± 0.23	44.00 ±1.11	89.37 ± 0.17	37.10 ± 2.13	89.46 ± 0.23	44.75 ± 0.91
T	89.84 ± 0.38	42.41 ±2.16 1.03	89.68 ± 0.21	37.05 ± 1.11 1.00	89.92 ± 0.42	47.00 ± 0.03 0.95
F_3 C	89.61 ± 0.15	42.94 ±2.11	89.39 ± 0.14	37.90 ± 0.60	89.65 ± 0.19	44.19 ± 1.52
T	89.65 ± 0.34	41.56 ±0.08 1.03	89.40 ± 0.17	37.89 ± 0.68 1.00	89.45 ± 0.20	47.06 ± 0.04 0.94
F_4 C	89.65 ± 0.47	42.52 ±2.11	89.43 ± 0.11	37.99 ± 0.62	89.32 ± 0.10	45.75 ± 0.08
T	89.65 ± 0.25	41.52 ±2.11 1.02	89.98 ± 0.17	37.98 ± 0.95 1.00	89.36 ± 0.19	47.61 ± 0.03 0.96
F_5 C	89.65 ± 0.19	42.99 ±1.33	89.32 ± 0.07	37.56 ± 1.55	89.40 ± 0.09	46.82 ± 0.41
T	89.99 ± 0.22	51.62 ± 1.77 0.83	89.61 ± 0.16	40.12 ± 0.41 0.93	89.54 ± 0.17	46.55 ± 1.30 1.00
F_6 C	89.42 ± 0.04	42.49 ±2.04	89.57 ± 0.10	37.51 ± 1.46	89.39 ± 0.14	46.86 ± 0.94
T	90.00 ± 0.38	54.78 ± 1.06 0.77	89.65 ± 0.19	49.21 ± 1.41 0.76	89.41 ± 0.17	46.65 ± 0.84 1.00
F_7 C	89.44 ± 0.09	42.53 ±1.82	89.39 ± 0.14	36.47 ± 0.86	89.57 ± 0.58	45.41 ± 1.02
T	90.05 ± 0.42	54.97 ± 1.57 0.77	89.65 ± 0.19	49.35 ± 1.40 0.73	89.77 ± 0.93	46.68 ± 1.29 0.97
F_8 C	89.57 ± 0.06	42.38 ±1.27	89.75 ± 0.70	34.33 ± 0.13	89.50 ± 0.18	46.65 ± 0.31
T	89.62 ± 0.19	57.21 ± 0.74 0.74	89.83 ± 0.62	49.37 ± 0.67 0.69	90.25 ± 0.32	46.68 ± 0.84 0.99
F_9 C	89.38 ± 0.12	41.94 ±0.87	89.37 ± 0.22	34.33 ± 0.69	89.50 ± 0.50	46.79 ± 3.00
T	89.42 ± 0.16	58.10 ± 1.47 0.72	89.67 ± 0.49	49.39 ± 1.13 0.69	89.65 ± 0.19	46.76 ± 1.31 1.00
F_{10}C	89.96 ± 0.49	40.98 ±1.95	89.79 ± 0.52	34.32 ± 1.04	89.38 ± 0.15	46.78 ± 3.65
T	90.11 ± 0.34	60.92 ± 1.14 0.67	89.65 ± 0.19	49.42 ± 0.87 0.69	90.02 ± 0.45	46.76 ± 0.67 1.00

(1) Means ± SD, 5 replicates

(2) The unit of carboxylesterase activity is nM paranitrophenol/mg protein/min

(3) CF is a correction factor = (average enzyme activity of control)/(average enzyme activity of treatment)

G = Generation

C = Control

T = Treatment

3.2.2 Effect of acetylcholinesterase activity

The activity of AChE enzyme was examined by ATCL assay with acetylthiocholine iodide substrate at 412 nm absorbance. The results after treated with these three types of insecticides in each population were showed enhanced the AChE enzyme activities of *S. litura* larvar (Figure 24). All of experiments showed no different in protein concentration of enzyme activity expression. The activity against cypermethrin showed no different in decreasing of enzyme activity level from F_1 to F_4. Then they were increased the activity level toward generations. The correlation factor showed *ca.* 0.56 to 1.12 fold (Table 13). As a correlation factor of 10 generations showed that cypermethrin was an inducer of AChE enzyme activities with activated high level activity.

Standard rotenone showed some effects on AChE enzyme activity with decreased the enzyme activity levels in F_1 to F_5 generations. Then it showed slightly increased the activities after F_5 toward 10 generations with similar levels. The correlation factor showed *ca.* 0.73 to 1.10 fold (Table 13). As a correlation factor of 10 generations showed that standard rotenone is the inducer substance of AChE enzyme activities with can activate high level of enzyme activity but slightly activated when compared with cypermethrin.

Effects of derris crude extracts against AChE enzyme showed fluctuation activity between increased and decreased of enzyme activity levels from F_1 to F_5. From F_6 to F_7 generations showed increased the activity and decreased in F_8. Then it resulted in increasing activity from F_9 to F_{10} with similar level. The correlation factor showed *ca.* 0.93 to 1.02 fold (Table 13). As a correlation factor of 10 generations they showed that derris crude extracts acts as induction of AChE enzyme activities with can activate the level of enzyme activity similarity to standard rotenone but slightly activated than cypermethrin.

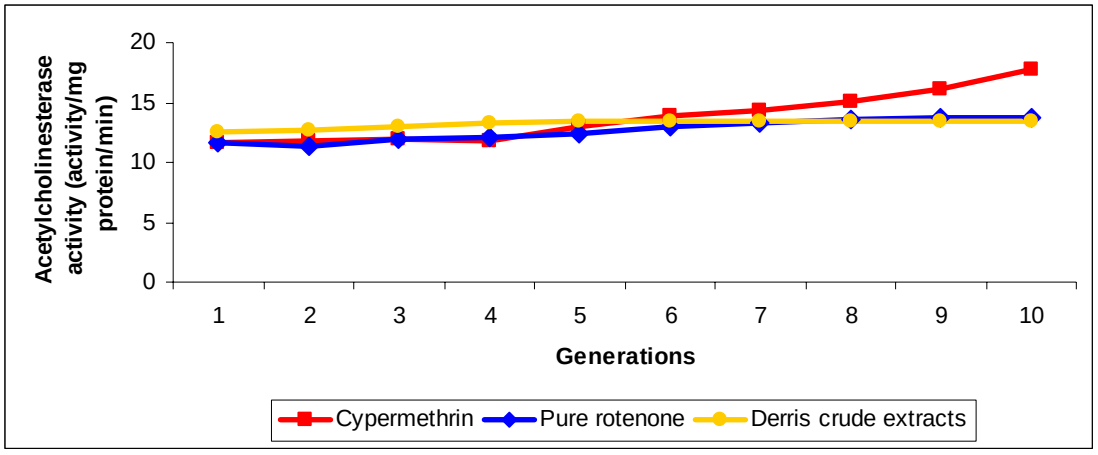


Figure 24 Acetylcholinesterase activity in *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts after 24 hours exposure in each of 10 generation.

Table 13 Acetylcholinesterase activity, correlation factor and protein content of *Spodopter litura* (F.) after sequential treated with LC_{50} value each of cypermethrin, standard rotenone and derris crude extracts after 24 hours exposure in 10 generations.

G	Cypermethrin		Rotenone		Derris extracts	
	Protein[(1)] conc.	**Enzyme[(1,2)] activity [CF][(3)]**	**Protein conc.**	**Enzyme activity [CF]**	**Protein conc.**	**Enzyme activity [CF]**
F_1 C	89.35 ±0.21	11.71 ±2.67	89.35 ±0.21	11.65 ±0.40	89.35 ±0.21	12.50 ±6.95
T	89.37 ±0.12	11.70 ±0.61 1.00	89.42 ±0.21	11.63 ±0.48 1.00	89.46 ±0.20	12.49 ±2.80 1.00
F_2 C	89.36 ±0.23	12.18 ±1.15	89.37 ±0.17	12.06 ±1.43	89.46 ±0.23	12.94 ±0.41
T	89.84 ±0.38	11.75 ±0.69 1.03	89.68 ±0.21	11.33 ±0.62 1.06	89.92 ± 0.42	12.70 ±1.70 1.01
F_3 C	89.61 ±0.15	12.18 ±0.48	89.39 ±0.14	12.92 ±0.38	89.65 ±0.19	13.26 ±0.64
T	89.65 ±0.34	11.96 ±0.10 1.01	89.40 ±0.17	11.90 ±0.51 1.08	89.45 ±0.20	13.01 ± 0.82 1.01
F_4 C	89.65 ±0.47	13.24 ±0.68	89.43 ±0.11	13.39 ±0.48	89.32 ±0.10	13.58 ±0.38
T	89.65 ±0.25	11.73 ±0.51 1.12	89.98 ±0.17	12.15 ±0.25 1.10	89.36 ±0.19	13.25 ±0.23 1.02
F_5 C	89.65 ±0.19	13.01 ±0.71	89.32 ±0.07	12.86 ±0.42	89.40 ±0.09	13.59 ±0.32
T	89.99 ±0.22	13.03 ±0.65 0.99	89.61 ±0.16	12.39 ±0.90 1.03	89.54 ±0.17	12.37 ±0.54 1.09
F_6 C	89.42 ±0.04	13.02 ±0.68	89.57 ±0.10	12.68 ±0.32	89.39 ±0.14	13.24 ±0.46
T	90.00 ±0.38	13.94 ±0.44 0.93	89.65 ±0.19	12.94 ±0.81 0.97	89.41 ±0.17	13.39 ± 0.28 0.98
F_7 C	89.44 ±0.09	12.01 ±0.32	89.39 ±0.14	12.27 ±0.45	89.57 ±0.58	13.20 ±0.46
T	90.05 ±0.42	14.40 ±0.35 0.83	89.65 ±0.19	13.28 ±0.90 0.92	89.77 ±0.93	13.41 ±0.91 0.98
F_8 C	89.57 ±0.06	11.70 ±1.52	89.75 ±0.70	11.80 ±0.85	89.50 ±0.18	13.47 ±1.33
T	89.62 ±0.19	15.01 ±0.21 0.77	89.83 ±0.62	13.65 ±0.93 0.86	90.25 ±0.32	13.42 ±0.95 1.00
F_9 C	89.38 ±0.12	10.46 ±2.87	89.37 ±0.22	10.92 ±0.85	89.50 ±0.50	12.63 ±1.39
T	89.42 ±0.16	16.19±1.03 0.64	89.67 ±0.49	13.74 ±0.29 0.79	89.65 ±0.19	13.45 ±0.36 0.93
F_{10}C	89.96 ±0.49	10.04 ±1.22	89.79 ±0.52	10.06 ±0.20	89.38 ±0.15	12.72 ±1.32
T	90.11 ±0.34	17.76±0.73 0.56	89.65 ±0.19	13.75 ±0.43 0.73	90.02 ±0.45	13.47 ±0.57 0.94

(1) Means ± SD, 5 replicates

(2) The unit of acetylcholinesterase activity is activity/mg protein/min

(3) CF is a correction factor = (average enzyme activity of control)/(average enzyme activity of treatment)

G = Generation

C = Control

T = Treatment

3.2.3 Effect of glutathione-S-transferase activity

GSTs enzyme activity was done by DCNB assay with DCNB substrate at 344 nm absorbance. After treated with three insecticides in each population they were showed acts as enhanced the GSTs enzyme activities of *S. litura* larvar (Figure 25). Most of experiments showed no different on protein concentration of enzyme activity expression. Effects of cypermethrin revealed increase the GSTs activity level to onward generations with similar level. The correlation factor showed *ca.* 0.66 to 0.99 fold (Table 14). As a correlation factor of 10 generations showed that cypermethrin is acts induction of GSTs enzyme activities with activated high level activity.

Standard rotenone was showed effects to GSTs which increase the activity level to onward generations with similar level. The correlation factor showed *ca.* 0.76 to 0.99 fold (Table 14). As a correlation factor of 10 generations showed that standard rotenone is acts induction of GST enzyme activities with can activate continuous more level of enzyme activity but slightly activated than cypermethrin.

Effects of derris crude extracts to GSTs enzyme activity showed no increased the levels in F_1 to F_5 generations. The activity level was dropped in F_6 and then showed slightly increased to onward generations. The correlation factor showed *ca.* 0.85 to 0.99 fold (Table 14). As a correlation factor of 10 generations showed that derris crude extracts is acts induction of GSTs enzyme activities with can activate the level of enzyme activity similarity of standard rotenone resulted but slightly activated than standard rotenone and cypermethrin.

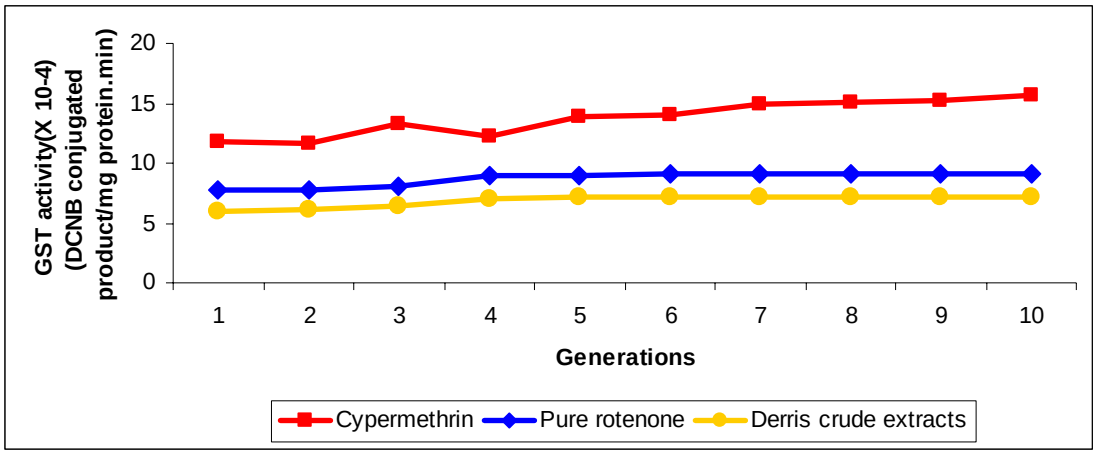


Figure 25 Glutathione-S-transferase activity of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts 24 hours exposure in each of 10 generations.

Table 14 Glutathione-S-transferase activity, correlation factor and protein content of *Spodopter litura* (F.) after sequential treated with LC_{50} value each of cypermethrin, standard rotenone and derris crude extracts at 24 hours exposure in 10 generations.

G	Cypermethrin		Rotenone		Derris extracts	
	Protein[(1)] conc.	**Enzyme[(1,2)] activity [CF][(3)]**	**Protein conc.**	**Enzyme activity [CF]**	**Protein conc.**	**Enzyme activity [CF]**
F_1 C	89.35 ±0.21	11.78 ±0.63	89.35 ±0.21	7.66 ±0.42	89.35 ±0.21	6.03 ±0.15
T	89.37 ±0.12	11.79 ±0.54 0.99	89.42 ±0.21	7.76 ±0.24 0.98	89.46 ±0.20	6.04 ±0.03 0.99
F_2 C	89.36 ±0.23	11.62 ±0.24	89.37 ±0.17	7.73 ±0.17	89.46 ±0.23	6.06 ±0.09
T	89.84 ±0.38	11.67 ±0.41 0.99	89.68 ±0.21	7.79 ±0.80 0.99	89.92 ±0.42	6.13 ±0.13 0.98
F_3 C	89.61 ±0.15	11.61 ±0.19	89.39 ±0.14	7.78 ±0.04	89.65 ±0.19	6.05 ±0.03
T	89.65 ±0.34	13.35 ±0.23 0.86	89.40 ±0.17	8.02 ±0.03 0.97	89.45 ±0.20	6.42 ±0.06 0.94
F_4 C	89.65 ±0.47	11.72 ±0.25	89.43 ±0.11	7.79 ±0.03	89.32 ±0.10	6.06 ±0.02
T	89.65 ±0.25	12.27 ±0.42 0.95	89.98 ±0.17	8.95 ±0.05 0.86	89.36 ±0.19	7.08 ±0.08 0.85
F_5 C	89.65 ±0.19	11.77 ±0.25	89.32 ±0.07	7.87 ±0.01	89.40 ±0.09	7.07 ±0.08
T	89.99 ±0.22	13.81 ±0.07 0.85	89.61 ±0.16	8.99 ±0.05 0.87	89.54 ±0.17	7.09 ±0.13 0.99
F_6 C	89.42 ±0.04	11.77 ±0.22	89.57 ±0.10	7.92 ±0.03	89.39 ±0.14	7.05 ±0.15
T	90.00 ±0.38	13.98 ±0.30 0.84	89.65 ±0.19	9.05 ±0.04 0.87	89.41 ±0.17	7.09 ±0.80 0.99
F_7 C	89.44 ±0.09	11.61 ±0.10	89.39 ±0.14	7.95 ±0.03	89.57 ±0.58	7.04 ±0.16
T	90.05 ±0.42	14.98 ±0.15 0.77	89.65 ±0.19	9.08 ±0.03 0.87	89.77 ±0.93	7.09 ±0.24 0.99
F_8 C	89.57 ±0.06	11.11 ±0.17	89.75 ±0.70	7.59 ±0.04	89.50 ±0.18	7.02 ±0.09
T	89.62 ±0.19	15.03 ±0.01 0.73	89.83 ±0.62	9.09 ±0.04 0.83	90.25 ±0.32	7.10 ±0.07 0.98
F_9 C	89.38 ±0.12	10.71 ±0.06	89.37 ±0.22	7.16 ±0.06	89.50 ±0.50	7.00 ±0.09
T	89.42 ±0.16	15.24 ±0.03 0.70	89.67 ±0.49	9.11 ±0.05 0.78	89.65 ±0.19	7.10 ±0.07 0.98
F_{10}C	89.96 ±0.49	10.41 ±0.01	89.79 ±0.52	6.92 ±0.04	89.38 ±0.15	6.99 ±0.80
T	90.11 ±0.34	15.66 ±0.02 0.66	89.65 ±0.19	9.11 ±0.03 0.76	90.02 ±0.45	7.10 ±0.01 0.98

(1) Means ± SD, 5 replicates

(2) The unit of GST activity is DCNB conjugated product/mg protein/min, each enzyme activity value multiple with 10^{-4}

(3) CF is a correction factor = (enzyme activity of control)/(enzyme activity of treatment)

G = Generation

C = Control

T = Treatment

3.3 Detection by molecular technique

3.3.1 Effect on protein characteristic of detoxification enzyme

The characteristics of enzyme with protein qualities determination were evaluated. The presence of enzyme system in most of experiments was estimated by Sodium Dodecyl Sulphate Polyacrelamide Gel Electrophoresis (SDS-PAGE) assay. Three detoxification enzymes; carboxylesterase, AChE and GSTs were studied. Protein samples from F_1 to F_{10} were used for compared the molecular weight between non-selection and selection strains. Enzyme supernatant was carried out on 7.5 to 15 % gradient gels. The results showed that every treatment presented the same molecular weight of protein throughout generations. This characteristic indicated that the carboxylesterase enzyme possessed the molecular weight of 60 kDa. AChE enzyme possessed the molecular weight of 24 kDa. While the GSTs enzyme possessed two unit of molecular weight at 24 and 48 kDa (Figure 26 to 30).

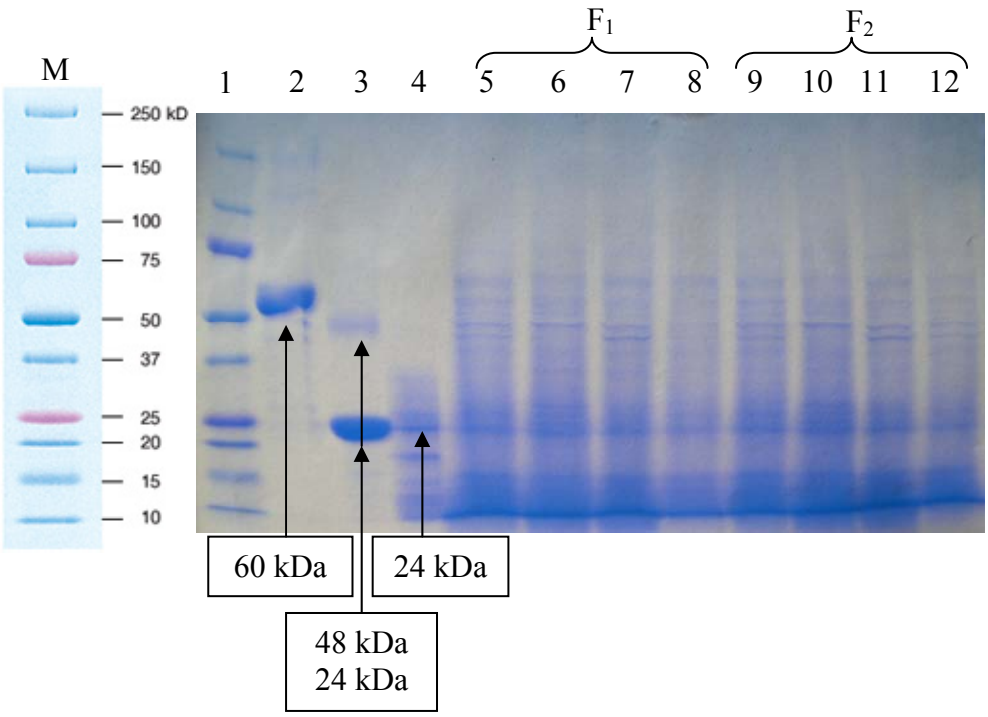


Figure 26 Molecular weight of carboxylesterase, acetylcholinesterase and glutathione-S-transferase in 1st and 2nd generations of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts.

Lane M : Precision Plus Protein™ Dual Color Standards of Bio-Rad
Lane 1 : protein marker
Lane 2 : carboxylesterase marker
Lane 3 : GSTs marker
Lane 4 : AChE marker
Lane 5,9 : non-selection strain of F_1 and F_2 as control
Lane 6,10 : cypermethrin of F_1 and F_2
Lane 7,11 : standard rotenone of F_1 and F_2
Lane 8,12 : derris crude extracts of F_1 and F_2

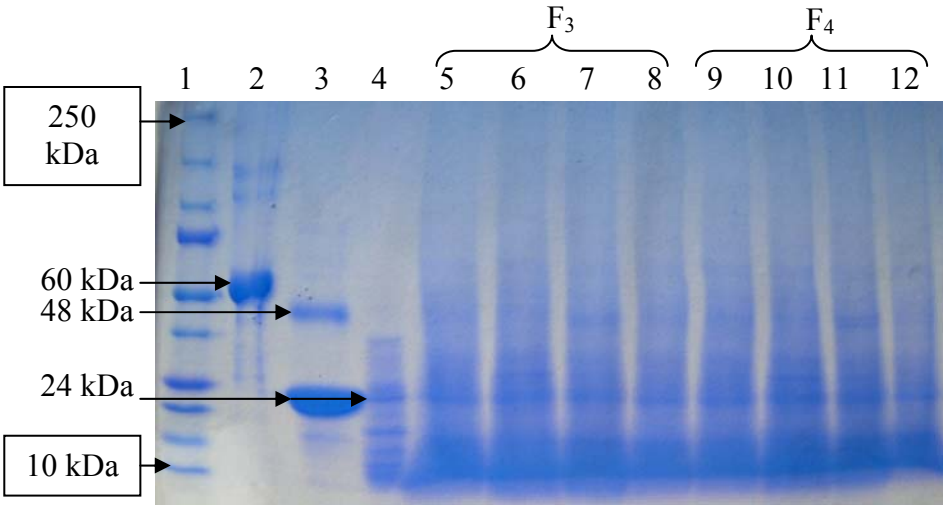


Figure 27 Molecular weight of carboxylesterase, acetylcholinesterase and glutathione-S-transferase in 3rd and 4th generations of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts.

Lane 1 : protein marker
Lane 2 : carboxylesterase marker
Lane 3 : GSTs marker
Lane 4 : AChE marker
Lane 5,9 : non-selection strain of F_3 and F_4 as control
Lane 6,10 : cypermethrin of F_3 and F_4
Lane 7,11 : standard rotenone of F_3 and F_4
Lane 8,12 : derris crude extracts of F_3 and F_4

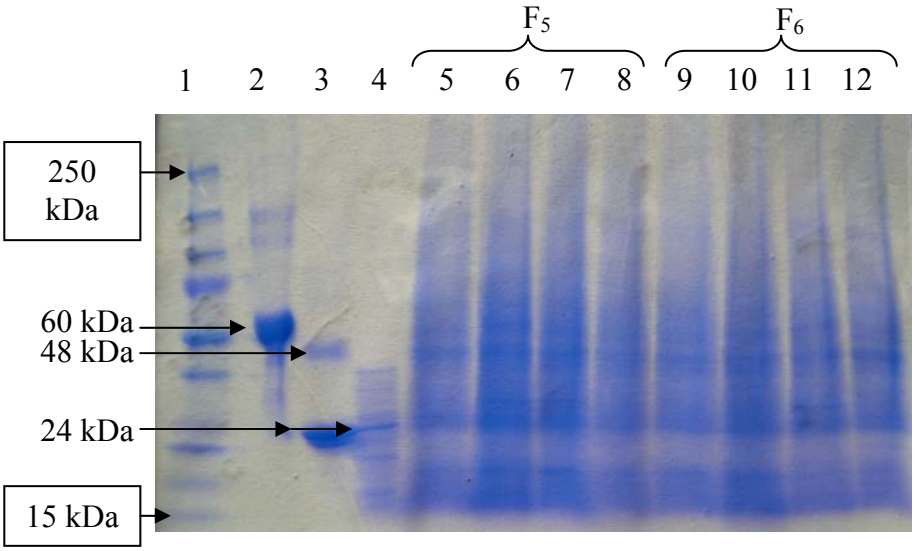


Figure 28 Molecular weight of carboxylesterase, acetylcholinesterase and glutathione-S-transferase in 5th and 6th generations of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts.

Lane 1 : protein marker
Lane 2 : carboxylesterase marker
Lane 3 : GSTs marker
Lane 4 : AChE marker
Lane 5,9 : non-selection strain of F_5 and F_6 as control
Lane 6,10 : cypermethrin of F_5 and F_6
Lane 7,11 : standard rotenone of F_5 and F_6
Lane 8,12 : derris crude extracts of F_5 and F_6

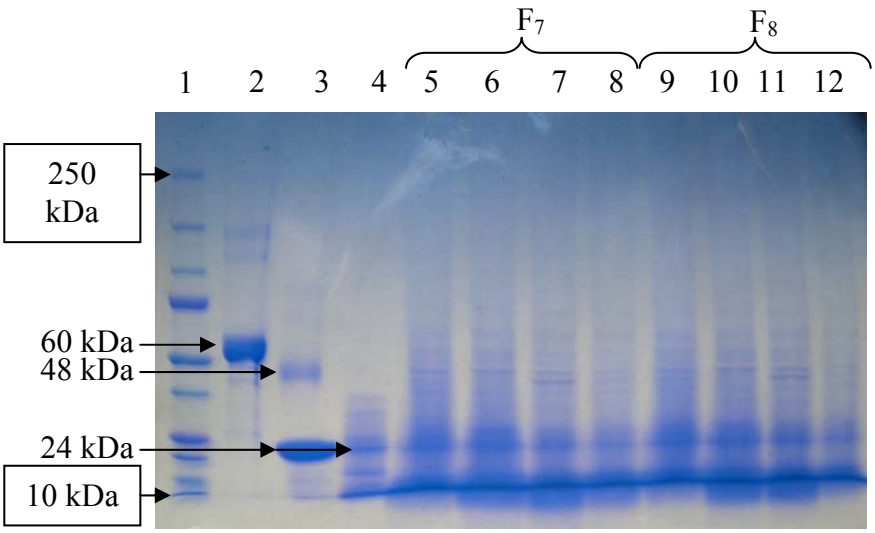


Figure 29 Molecular weight of carboxylesterase, acetylcholinesterase and glutathione-S-transferase in 7th and 8th generations of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts.

Lane 1 : protein marker
Lane 2 : carboxylesterase marker
Lane 3 : GSTs marker
Lane 4 : AChE marker
Lane 5,9 : non-selection strain of F_7 and F_8 as control
Lane 6,10 : cypermethrin of F_7 and F_8
Lane 7,11 : standard rotenone of F_7 and F_8
Lane 8,12 : derris crude extracts of F_7 and F_8

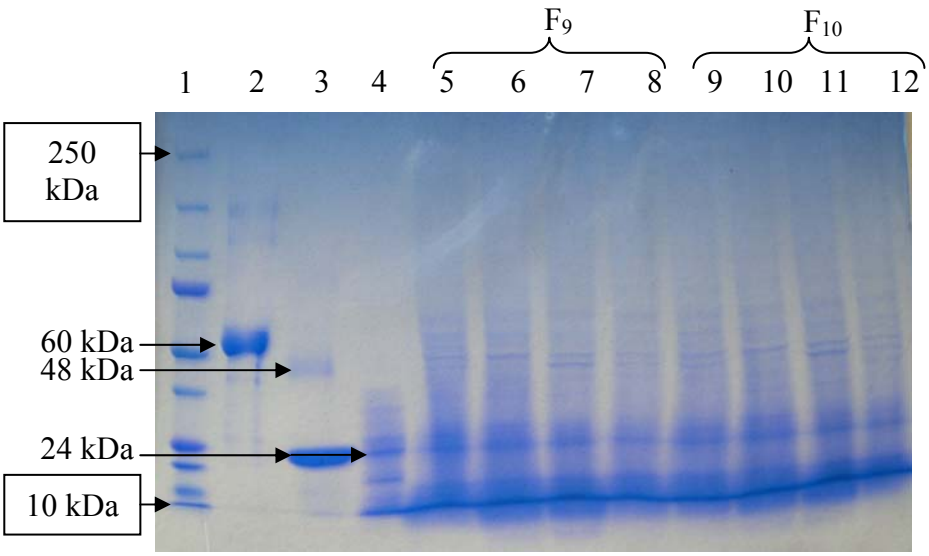


Figure 30 Molecular weight of carboxylesterase, acetylcholinesterase and glutathione-S-transferase in 9th and 10th generations of *Spodoptera litura* (F.) against cypermethrin, standard rotenone and derris crude extracts.

Lane 1 : protein marker
Lane 2 : carboxylesterase marker
Lane 3 : GSTs marker
Lane 4 : AChE marker
Lane 5,9 : non-selection strain of F_9 and F_{10} as control
Lane 6,10 : cypermethrin of F_9 and F_{10}
Lane 7,11 : standard rotenone of F_9 and F_{10}
Lane 8,12 : derris crude extracts of F_9 and F_{10}

3.3.2 Effect on gene associated with detoxification enzyme expressions

A single PCR product was obtained from non-selection population as control and the 10th generation of cypermethrin, standard rotenone and derris crude extracts population with 3 replications and one larva per replicated. The results showed as below.

3.3.2.1 Carboxylesterase gene detection

After derived the sequences from GenBank for primer designed and was synthesized on different annealing temperature to find out the suitable temperature condition for gene amplification. The result showed suitable annealing temperature at 59 °C that can detect DNA fragment approximately 553 bp of carboxylesterase gene of *S. litura* by RT-PCR (Figure 31 and 32).

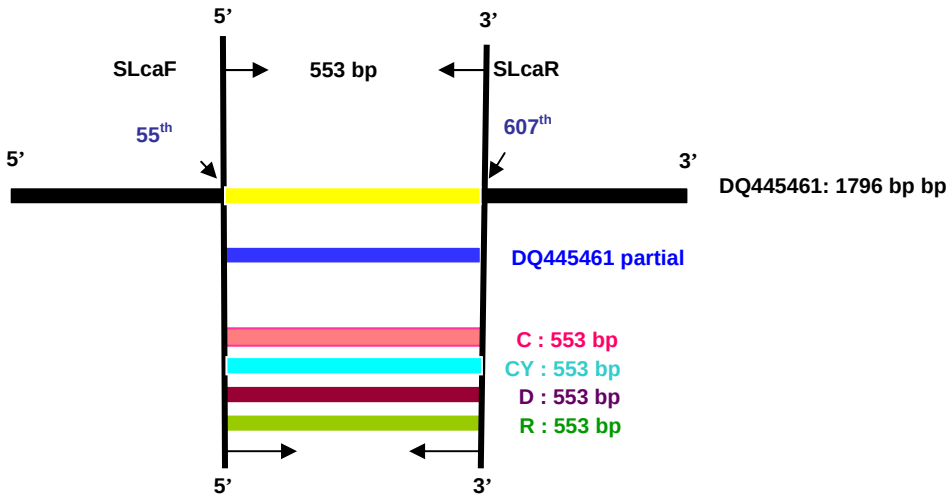


Figure 31 Schematics of carboxylesterase gene structure from Genbank (acc: DQ445461) and carboxylestrease PCR amplification. C= control, CY= cypermethrin, D= derris crude extracts and R= standard rotenone. The location and direction of PCR primers are indicated by arrow.

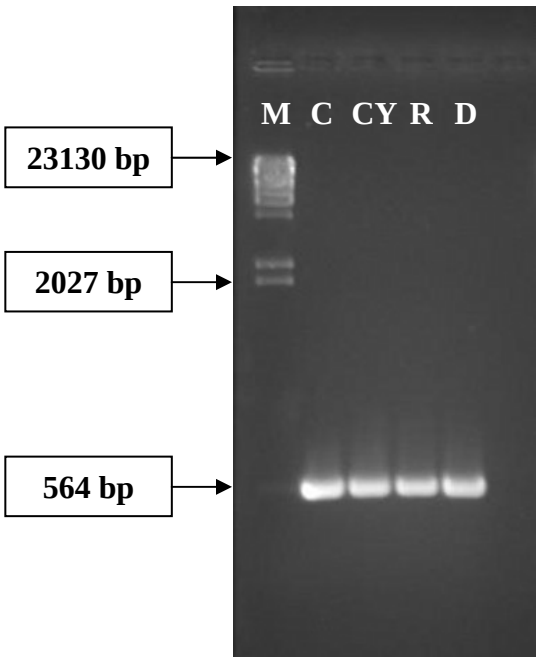


Figure 32 RT-PCR product of carboxylesterase gene in *Spodoptera litura* (F.) from non-selection strain as control (C), cypermethrin (CY), standard rotenone (R) and derris crude extracts (D) population; M = Lamda DNA/*Hind*III Marker.

3.3.2.2 Carboxylesterase gene Analysis

Gene amplification and alignment

Partial sequence of carboxylesterase gene of *S. litura* was analyzed. DNA was directed sequencing. The editing and analyzing of DNA sequences were done by GENETYX analysis program. The result showed the partial sequence of carboxylesterase gene of *S. litura* that 553 bp in most experiments. This result showed 99% identity with *S. litura* from GenBank (www.ncbi.nlm.nih.gov). When aligned each sequences compared with carboxylesterase gene from GenBank, they were showed different at five positions as Figure 33.

First, at 30th position of 553 bp founded the base of most experiment different from GenBank database that showed thymine base but all of experiment and non-selection population in this study showed the cytosine base.

Second, at the 76th position of 553 bp founded the thymine base in non-selection, standard rotenone and derris crude extracts populations different from GenBank data and cypermethrin population that showed the cytosine base.

On the third different position, the guanine base was resulted at the 102rd position of 553 bp in cypermethrin population different from other experiment and GenBank data that showed adenine. This result is similarity of the different at 111th position of 553 bp that showed cytosine base in cypermethrin population while

other experiment and GenBank data showed guanine, this is the fourth position of differentiation.

Finally, the fifth differentiation occurred at the 381st position of 553 bp similar the second that founded the adenine base in non-selection, standard rotenone and derris crude extracts populations different from GenBank data and cypermethrin population that showed the cytosine base.

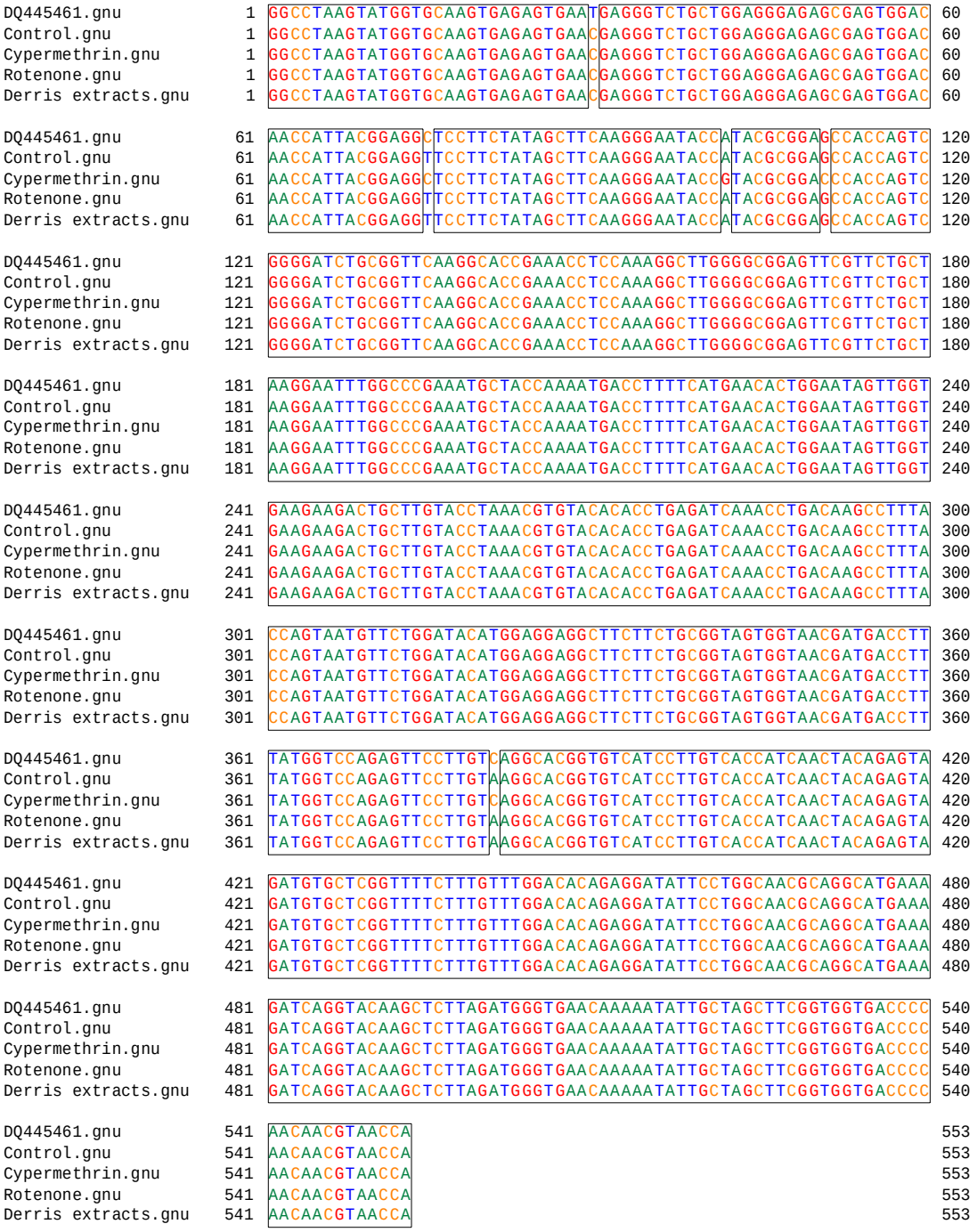


Figure 33 Sequence alignment of partial carboxylesterase gene in *Spodoptera litura* (F.) from GenBank sequence, control sequence and sequences from 10th generations after sequential treated with cypermethrin, standard rotenone and derris crude extracts.
In block = the same sequences
Outside block = the different sequences

Amino acid analysis

After founded the base differentiation in the experiment of *S. litura* against sequential insecticides continuous 10 generations, the amino acids were revealed by GENETYX analysis program. The result showed interestingly at the fourth position of differentiation, the 111th position of 533 bp that showed cytosine base in cypermethrin population while other experiment and GenBank data showed guanine. This position is consisted of GAG codon that coding the glutamate (Glu, E) amino acid in most of experiment and GenBank data. Whereas in the cypermethrin population nucleotide at this position changed from GAG to GAC which code for aspartate (Asp, D) substituted the glutamate as shown in Figure 34. Whilst the other positions of base differentiation coded for the same amino acid of GenBank database.

```
DQ: GGCCTAAGTATGGTGCAAGTGAGAGTGAATGAGGGTCTGCTGGAGGGAGAGCGAGTGGAC 60
    G  L  S  M  V  Q  V  R  V  N  E  G  L  L  E  G  E  R  V  D
 C: ............................*............................. 60
    G  L  S  M  V  Q  V  R  V  N  E  G  L  L  E  G  E  R  V  D
CY: ............................*............................. 60
    G  L  S  M  V  Q  V  R  V  N  E  G  L  L  E  G  E  R  V  D
 R: ............................*............................. 60
    G  L  S  M  V  Q  V  R  V  N  E  G  L  L  E  G  E  R  V  D
 D: ............................*............................. 60
    G  L  S  M  V  Q  V  R  V  N  E  G  L  L  E  G  E  R  V  D

DQ: AACCATTACGGAGGCTCCTTCTATAGCTTCAAGGGAATACCATACGCGGAGCCACCAGTC 120
    N  H  Y  G  G  S  F  Y  S  F  K  G  I  P  Y  A  E  P  P  V
 C: ..............*.............................................. 120
    N  H  Y  G  G  S  F  Y  S  F  K  G  I  P  Y  A  E  P  P  V
CY: .........................................*........*......... 120
    N  H  Y  G  G  S  F  Y  S  F  K  G  I  P  Y  A (D) P  P  V
 R: ..............*.............................................. 120
    N  H  Y  G  G  S  F  Y  S  F  K  G  I  P  Y  A  E  P  P  V
 D: ..............*.............................................. 120
    N  H  Y  G  G  S  F  Y  S  F  K  G  I  P  Y  A  E  P  P  V

DQ: GGGGATCTGCGGTTCAAGGCACCGAAACCTCCAAAGGCTTGGGGCGGAGTTCGTTCTGCT 180
    G  D  L  R  F  K  A  P  K  P  P  K  A  W  G  G  V  R  S  A
 C: ..................................................... 180
    G  D  L  R  F  K  A  P  K  P  P  K  A  W  G  G  V  R  S  A
CY: ..................................................... 180
    G  D  L  R  F  K  A  P  K  P  P  K  A  W  G  G  V  R  S  A
 R: ..................................................... 180
    G  D  L  R  F  K  A  P  K  P  P  K  A  W  G  G  V  R  S  A
 D: ..................................................... 180
    G  D  L  R  F  K  A  P  K  P  P  K  A  W  G  G  V  R  S  A
```

```
DQ: AAGGAATTTGGCCCGAAATGCTACCAAAATGACCTTTTCATGAACACTGGAATAGTTGGT 240
    K  E  F  G  P  K  C  Y  Q  N  D  L  F  M  N  T  G  I  V  G
 C: ............................................................ 240
    K  E  F  G  P  K  C  Y  Q  N  D  L  F  M  N  T  G  I  V  G
CY: ............................................................ 240
    K  E  F  G  P  K  C  Y  Q  N  D  L  F  M  N  T  G  I  V  G
 R: ............................................................ 240
    K  E  F  G  P  K  C  Y  Q  N  D  L  F  M  N  T  G  I  V  G
 D: ............................................................ 240
    K  E  F  G  P  K  C  Y  Q  N  D  L  F  M  N  T  G  I  V  G

DQ: GAAGAAGACTGCTTGTACCTAAACGTGTACACACCTGAGATCAAACCTGACAAGCCTTTA 300
    E  E  D  C  L  Y  L  N  V  Y  T  P  E  I  K  P  D  K  P  L
 C: ............................................................ 300
    E  E  D  C  L  Y  L  N  V  Y  T  P  E  I  K  P  D  K  P  L
CY: ............................................................ 300
    E  E  D  C  L  Y  L  N  V  Y  T  P  E  I  K  P  D  K  P  L
 R: ............................................................ 300
    E  E  D  C  L  Y  L  N  V  Y  T  P  E  I  K  P  D  K  P  L
 D: ............................................................ 300
    E  E  D  C  L  Y  L  N  V  Y  T  P  E  I  K  P  D  K  P  L

DQ: CCAGTAATGTTCTGGATACATGGAGGAGGCTTCTTCTGCGGTAGTGGTAACGATGACCTT 360
    P  V  M  F  W  I  H  G  G  G  F  F  C  G  S  G  N  D  D  L
 C: ............................................................ 360
    P  V  M  F  W  I  H  G  G  G  F  F  C  G  S  G  N  D  D  L
CY: ............................................................ 360
    P  V  M  F  W  I  H  G  G  G  F  F  C  G  S  G  N  D  D  L
 R: ............................................................ 360
    P  V  M  F  W  I  H  G  G  G  F  F  C  G  S  G  N  D  D  L
 D: ............................................................ 360
    P  V  M  F  W  I  H  G  G  G  F  F  C  G  S  G  N  D  D  L

DQ: TATGGTCCAGAGTTCCTTGTCAGGCACGGTGTCATCCTTGTCACCATCAACTACAGAGTA 420
    Y  G  P  E  F  L  V  R  H  G  V  I  L  V  T  I  N  Y  R  V
 C: ............................................................ 420
    Y  G  P  E  F  L  V  R  H  G  V  I  L  V  T  I  N  Y  R  V
CY: ..................*......................................... 420
    Y  G  P  E  F  L  V  R  H  G  V  I  L  V  T  I  N  Y  R  V
 R: ............................................................ 420
    Y  G  P  E  F  L  V  R  H  G  V  I  L  V  T  I  N  Y  R  V
 D: ............................................................ 420
    Y  G  P  E  F  L  V  R  H  G  V  I  L  V  T  I  N  Y  R  V

DQ: GATGTGCTCGGTTTTCTTTGTTTGGACACAGAGGATATTCCTGGCAACGCAGGCATGAAA 480
    D  V  L  G  F  L  C  L  D  T  E  D  I  P  G  N  A  G  M  K
 C: ............................................................ 480
    D  V  L  G  F  L  C  L  D  T  E  D  I  P  G  N  A  G  M  K
CY: ............................................................ 480
    D  V  L  G  F  L  C  L  D  T  E  D  I  P  G  N  A  G  M  K
 R: ............................................................ 480
    D  V  L  G  F  L  C  L  D  T  E  D  I  P  G  N  A  G  M  K
 D: ............................................................ 480
    D  V  L  G  F  L  C  L  D  T  E  D  I  P  G  N  A  G  M  K
```

```
DQ: GATCAGGTACAAGCTCTTAGATGGGTGAACAAAAATATTGCTAGCTTCGGTGGTGACCCC 540
    D  Q  V  Q  A  L  R  W  V  N  K  N  I  A  S  F  G  G  D  P
 C: ............................................................ 540
    D  Q  V  Q  A  L  R  W  V  N  K  N  I  A  S  F  G  G  D  P
CY: ............................................................ 540
    D  Q  V  Q  A  L  R  W  V  N  K  N  I  A  S  F  G  G  D  P
 R: ............................................................ 540
    D  Q  V  Q  A  L  R  W  V  N  K  N  I  A  S  F  G  G  D  P
 D: ............................................................ 540
    D  Q  V  Q  A  L  R  W  V  N  K  N  I  A  S  F  G  G  D  P

DQ: AACAACGTAACCA                                                553
    N  N  V  T
 C: ............                                                 553
    N  N  V  T
CY: ............                                                 553
    N  N  V  T
 R: ............                                                 553
    N  N  V  T
 D: ............                                                 553
    N  N  V  T
```

Figure 34 Partial nucleotide sequence and deduced amino acid sequence of carboxylesterase gene in *Spodoptera litura* (F.) at 10^{th} generations after sequential treated with cypermethrin, standard rotenone and derris crude extracts.

DQ = DQ 445461
C = non-selection strain as a control
CY = selection strain; cypermethrin
R = selection strain; standard rotenone
D = selection strain; derris crude extracts
Dot = the same bases
Star = the different bases
(D) = a single amino acid substitution from glutamate (Glu, E) to aspartate (Asp, D)

Discussion

The information concerning about insecticide resistances in *S. litura* is limited in Thailand. Although the synthetic insecticides still to advantage of being very rapid in its action and giving generally predictable results. But it has many disadvantages with often failed in this insect pests is reported to have developed resistance. And the ever increasing resistance in *S. litura* to many synthetic insecticides has compounded the problem. Thus, the botanical insecticide is one of alternative chance to control them. Because of this insecticide was showed the controlling efficacy to many insect pests without resistance problem and also showed friendly effects to human and environmental in previously reported. The successful researches on botanical extracts against some key agricultural pests have been revealed by several scientists. Such as Ruamthum (2002) used the nutsage (*Cyperus rotundus* L.) to control *Pomacea canaliculata* L. Bullangpoti (2004) used the fruit rind of magosteen (*Garcina mangostana* L.), rambutan seed (*Nephilium lappaceum* L.) and kaffir lime leave (*Citrus hystix* DC.) to control *S. oryzae*. Saisongkroh (2006) use the sugar apple (*Annona squamosa*) seed and chilli (*Capsicum frutescans* L.) to control *S. litura*.

Therefore, in this research I compared the resistance development and mechanisms of toxicity between synthetic chemical, cypermethrin and botanical insecticides, derris crude extracts and standard rotenone in lavae of *S. litura*. All of results as mentioned below.

1. Toxicity determination of cypermethrin, standard rotenone and derris crude extracts against *S. litura*

1.1 Derris crude extracts and their quantitative approaches

In this study, roots of *D. elliptica* from Eastern region of Thailand were extracted by Soxhlet's apparatus with ethanol as solvent. Yield of derris crude extracts showed high crude amount and rotenone content. The amount of crude extracts is shown high yield *ca.* 48.60 %w/w. The characteristic of dry crude extracts is rough solid substance and dark brown color. This amounts were higher than derris roots extract from Visetson *et al.* (2005) which showed *ca.* 33.54 %w/w, and higher than other plant materials extracted with the methods such as sesame seeds, siam weeds and galanga rhizomes which showed *ca.* 43.22, 36.71 and 32.25 %w/w, respectively (Visetson *et al.*, 2005). These may due to different plant or due in part to different active compounds showing different chemical properties. Some active compounds may show stronger or weaker solubility than the other so although using the same solvent method of extraction and common temperature, the yield of different compounds are eventually different (Bullungpoti, 2004). Visetson *et al.* (2003) also mentioned this situation that the yield of compounds extracted may vary dramatically from one batch to the other owing to other components other than the active principle covalently bound one another. If the different of yield production was may be due in kind of palnts to the latter reason. This means tannin, alkaloid and protein as well as other active compounds may interact one another in different species. For the different resulted from Visetson *et al.* (2005) although used the same kind and same

part of plant that the roots of *D. elliptica.* It is can be happened because the different yield may occurred from plant source or came from the extraction processed such as temperature extraction. Thus in my research the different yield of crude extracts may occurred from these reasons mention above.

Moreover, crude extracts of derris roots were analyzed the total content of rotenone. Yield of rotenone was separated from dried powder of crude extracts by HPLC was shown in Figure 7. The results also showed high rotenone content ca 17.91%w/w. This rotenone percentage is higher than the rotenone compound from Visetson and Milne (2001) who determined the rotenone content from crdue extracts of derris roots by HPLC at 8.6%w/w. Promsattha and Sangwanit (2004) reported the active ingredient of rotenone from roots of derris from eastern Thailand at 4.78%. The results from Arnason *et al.* (1989) who stated the rotenone percentage of the native eastern tropic plant *Derris* spp. that showed about 5% and the native western hemisphere plant *Lonchocarpus* spp. That showed 13%. The different of crude extracts yield and rotenone content that mention above, they may due to the plant fertile, plant source, plant age, extraction method, extraction process and separation technique.

Therefore before process the plant extraction, we should concern about the specific limit factors of each extract. For example, the extraction method that is the important factor of plant extract, we should select the appropriate method for each plant such as if we need rotenone from root of derris Visetson and Milne (2001) showed the Soxhlet method was suitable than the stirring soaking method. Because of the temperature during Soxhlet extraction had a greater influence on rotenone solubility in ethanol than did the stirring soaking extraction. Moreover, we should concern about plant fertility because of it can effect to yield of active ingredient substance.

1.2 Efficiency of cypermethrin, standard rotenone and derris crude extracts against *S. litura*

As resulted, the mortality rates were increased when the toxic substance concentration and the time exposure duration increasing. The toxicity values were shown in Figure 8-10 and Table 3-4. The highest concentration of all insecticides was showed highest mortality percentage in most experiment. The different time exposure also gave different mortality rate. High toxin concentration and long time exposure had affected to various insect similar the reported of Saisongkroh *et al.* (2005) who showed highest mortality of *S. litura* against ethanolic chilli extracts at highest concentration with more than 71.42% mortality in every treatment. Or the time of 7 days resulted 100% morality of rice moths against with ECO_2FUME, this result was reported by Chankeawmanee *et al.* (2005). When considered the toxicity of these three insecticides they were gave high toxicity when going on long time exposure. As the Figure 10 and Table 4, they were shown the highest toxicity value of all insecticides at 72 hours after exposure. From the data can conclude that the concentration and time exposure are affected toxicity to organisms including insects.

After 72 hours exposure to cypermethrin, It was gave the highest toxicity when compared with the other which LC_{50} value at 9.93 ppm. The standard rotenone showed LC_{50} at 23.27 ppm followed by derris crude extracts (LC_{50} at 37.67 ppm). Moreover, most of experiment showed highly correlated of mortality with r^2 value as 0.95-0.99, the r^2 is the correlation between concentration and mortality that indicated the level of mortality correlated.

The different efficiency of different toxin or the same toxin substance in the same species of organism normally occurred due to the tolerance ability of organism or individual to each substance. For instance the ability tolerance of organism to difference toxin, Saisongkroh (2006) who stated the toxicity of seed of sugar apple and chili crude extracts for controlling *S. litura* which LC_{50} value at 1.642 and 4.88%w/v, respectively. The resulted from Dwivedi *et al*. (1999) who reported the toxicity value of fenvalerate which LC_{50} value at 700 ppm to control *S. litura*. The example of individual ability tolerance to the same toxin that stated by Dwivedi *et al*. (1999) who reported the toxicity value of cypermethrin which LC_{50} value at 500 ppm to effective control of *S. litura*. The efficacy of cypermethrin to control *S. litura* with LC_{50} value at 0.016% was reported by Singh and Nath (1998). The effect of rotenone, Morimoto *et al.* (2006) showed the insect antifeedant of rotenoids from *Pterocarpus marcrocarpus* Kruz. in term of ED_{50} value at 1.05 $\mu M/cm^2$ in *S. litura*. These results can be occurred because of different organism or individual have different ability or efficiency to avoid or tolerance the toxic substance such as different in detoxification mechanism ability.

1.3 Toxicity of cypermethrin, standard rotenone and derris crude extracts against all instars larvae of *S. litura*

From the Figure 12 and Table 5 showed that the higher concentration dramatically increasing toxicity than less concentration. The long time duration at 72 hours after exposure were also gave high toxicity than 24 and 48 hours after exposure in all instars larvae of most experiment. On the other hand, the toxicity value of each treatment was different in each instars larvae, although the toxicity similar increased from 1^{st} to 5^{th} instars of most insecticides. The cypermethrin showed highly toxicity than the other with LC_{50} value at 2.08, 2.92, 9.93, 11.64 and 18.38 ppm, respectively with increased the toxicity level from 1^{st} to 5^{th} instars larvae. The standard rotenone resulted high toxicity following cypermethrin. It was showed LC_{50} value at 5.07, 8.12, 23.27, 39.44 and 124.79 ppm, respectively with increased the level. And the crude extracts of derris roots revealed the least toxicity compare with two substances above did showed increased level of LC_{50} value at 3.79, 9.51, 37.67, 94.23 and 160.01 ppm, respectively. The different toxic level occurred from the different of organism or individual have different ability or efficiency to avoid or tolerance the toxic substance as mentions and samples according to topic 1.2 on above.

The toxicity value of cypermethrin is higher than that of rotenone substance and derris crude extracts *ca.* 2.44 – 10.72 and 1.82 – 13.15 times, respectively. They results their effective efficacy when applied with high doses compared with the synthetic chemical substance. This result is similar to Visetson and

Milne (2001) who stated the toxicity value of cypermethrin that highly than derris crude extracts from Soxhlet and stirring soaking method at 1.195 and 1.194 times, respectively in diamondback moth larvae, *Plutella xylostella* Linn. The reported of Chansang (2003) who showed more toxicity value of permetrhin than *Bacillus thuringiensis* var. *israelensis* (Bti) and Bti standard at 2.17 and 7.7 times, respectively with mg/L of LC_{50} value in susceptible strain of *Aedes aegipti*. This result suggests that the synthetic chemical insecticide is high toxic and different toxicity in organism, we should concern about their toxic and consideration in appropriate use.

The mortality rate in term of LC_{50} value of all instars larvae considerably to the larvae age. Most of substance showed highly efficiency in 1^{st} to 3^{rd} more than 4^{th} to 5^{th} instar. Especially the last instar larvae showed their affected when highly dose exposed them as showed in Figure 15 and Table 4 indicated that the mortality rate is depend on the age of *S. litura*. This result is related to the report of Saisongkroh *et al.* (2005) who recommended the early instars larvae is the best suitable stage for controlling of *S. litura* by ethanolic chilli extracts. Thus, this result suggests that the appropriate stage for the effective control of *S. litura* is should be manage in the early instar better than the late instars larvae.

In addition, as a Table 5 the mortality rates were showed high correlated of concentration and mortality percentage. In the population against the synthetic chemical substance, cypermethrin showed r^2 as 0.87 to 0.99. The population against rotenone substance, standard rotenone showed r^2 as 0.84 to 0.98 and derris crude extracts showed r^2 as 0.84 to 0.97. This result suggests that the effect of all three substances to mortality rate of *S. litura* were highly correlated.

2. Characterization of detoxification mechanism of *S. litura* against cypermethrin, standard rotenone and derris crude extracts

2.1 The pH optimal for detoxification enzyme mechanisms detection in *S. litura*

The optimum pH buffer for homogenization and incubation of enzyme activity detection were investigated. This pH buffer was tested the highest activity of three important detoxification enzymes namely carboxylesterase, acetylcholinesterase and glutathione-S-transferase that use for mechanism elucidation in this study as Figure 13-16. Two esterase enzymes are play a role in phase I reaction and GSTS is in phase II reaction. Carboxylesterase activity was measured because this enzyme system is a generally important detoxification mechanism in insects, including *S. litura* while AChE play important affected to nervous system and GSTs activity was determined because it is likely to be the most important enzyme in *S. litura* resistance to synthetic insecticides, this recommend was reported from Domínguez-Gil and McPheron (2000). The suitable pH for obtain the accuracy activity of enzyme is necessary to investigate before study the enzyme mechanism. Because of different pH range can give different enzyme activity level or different organism effect to different pH optimum for enzyme acitivity detection. Such as the reported of Zhang *et al.* (2004) who stated that the caboxylesterase activity showed different level in different

pH range. In this study, the potassium phosphate buffer was applied. The pH buffer series varying in 4, 6, 7, 7.5, 8, 10, 12 and 14 ranged were used.

The optimum pH of these three enzymes was used specific substrate to reveal the activity by UV-visible spectrophotometer with different absorbance value in each enzyme. The results showed the highest activity of all enzymes at pH 10 as showed in figure 16 – 19 that means the pH 10 is the best pH range suitable for these three enzymes activity detection. The pH buffer value at 10 that I used in this study different from the other such as Saisongkroh (2005; 2006) who study the activity of general esterase and GSTs in *S. litura* was used pH 7 for this enzyme activity detection. Steven *et al.* (1994) used pH optimum ranging between 6.6 and 8.0 for malathion carboxylesterase activity detection in sheep blowfly *Lucilia curprina*. Or Domínguez-Gil and McPheron (2000) used pH 7.4 to study the effect of diet on detoxification enzyme activity of *Platynota idaeusalis*.

Moreover, the specific substrate is also important for enzyme activity detection. In this study I screened the suitable substrate for each enzyme simultaneously of pH suitable study. The result showed pNPA substrate response to carboxylesterase activity more than NA (data not show because can not detected, the chemicals and protocol of NA assay were described in appendix A). This result related to Visetson (1991), Thummasarangkoon (2000), Bullangpoti (2004) and Saisongkroh (2006) who used pNPA substrate to detect esterase enzyme activity. About AChE I performed the acetylthiocholine substrate following Visetson (1991). In addition, for GSTs activity detection I compared the efficiency of two substrates DCNB and CDNB. The result of DCNB showed unfluctuated activity level as Figure 19. Therefore, I selected the DCNB substrate for GSTs activity detection in this study that related to the study of Saisongkroh (2005; 2006) who used the DCNB substrate to detect the GSTs activity in *S. litura*.

This resulted suggests that the suitable pH buffer and specific substrate are important for enzyme activity accuracy detection as mention above. Therefore, we should elucidate the pH buffer suitability before study on enzyme mechanism.

2.2 Detoxification enzyme mechanisms of all instars larvae of *S. litura* against cypermethrin, standard rotenone and derris crude extracts

The activities of three detoxification enzymes of the second days 1^{st} to 5^{th} instars larvae were done by biochemical assay. I preferred the second day larvae for this procedure following Rajurkar *et al.* (2003) who stated the second day larvar is suitable for GSTs activity detection. Thus, I performed the second day larvar in my detoxification enzyme activity detection method. The specific substrate of each enzyme was used for enzyme activities detection with potassium phosphate buffer at pH 10 using UV-visible spectrophotometer. Each instar of insect samples was treated with LC_{50} value of each insecticide. After 24 hours the survival larvae were took to detect their detoxification enzyme activities. The results founded that the characteristic of each enzyme seem to be induce in each instars larvae of all treatment.

The induction is increase when the concentration of substance increasing. And the induction rate increase when the correction factor value decrease.

Carboxylesterase enzyme, as Table 6, showed the highest activity from other which increased the activity of all instars larvae after treated with cypermethrin and derris crude extracts while the rotenone substance trends to be decreased this enzyme activity. The activities were changed increasing from 1st to 5th instars in all treatments, especially in the 4th – 5th instar of cypermethrin treatment was high increasing as table 4. This characteristic showed that the cypermethrin is trend to be resistance in the late instars larvae. This is due to the late instars larvae are strong focus to develop themselves to the mature stage. Then they may have some behavioral, physiological and metabolic detoxification adaptation to protect themselves such as the penetration and excretion ability including metabolism changing that described by Liu *et al.* (2006). In evolution of animal-plant interaction basic pointed that some animal prefer to produce high population in next generation for protect their population from the other disturb. Thus, the age of organism can be related to toxins and their effect, the stage is mention by Abrahamson (1989). Moreover, the correction factor when compare between control and treatment showed cypermethrin and derris crude extracts seems to be induce enzyme activity with 0.32 to 0.32 fold. While rotenone substance seems to be inhibit the activity with 1.00 to 1.04 fold. Furthermore, the third instars larvae of all treatments showed the highest correction factor that means the third instars larvae is the best effects in carboxylesterase activity although the protein content showed no different changing in most of experiments of all instars larvae.

AChE, as Table 7, showed high activity next to carboxylesterase activity in every treatment of all instars larvae with similarity of carboxylesterase characteristics. Which cypermethrin and crude extracts of derris roots is act the same with showed induce this enzyme activity in all instars larvae. While the rotenone substance was has efficiency different from both substances. The enzyme activity was also high changing in the 5th instars larvae. The correction factor also showed cypermethrin and derris crude extracts trend to be induce which CF as 0.14 – 0.99 and 0.14 – 0.94, respectively, but standard rotenone trend to be inhibit this enzyme activities which CF as 0.98 – 1.03. Moreover, the third instars larvae of all treatments also showed the highest correction factor that means the third instars larvae is the best effects in carboxylesterase activity although the protein content showed no different changing in most of experiments of all instars larvae as a Table 5. These characteristics can occur due to reasons as mention above.

GSTs, as Table 8, showed the least activity compare with two types of esterase enzyme in every treatment. But the correction factor when compare between control and treatment were showed induced this enzyme activity of all instars larvae after treated with cypermethrin and derris crude extracts. Even though the rotenone substance showed inhibit enzyme activity in 1st – 3rd instars and induce in the 4th – 5th instars and the 5th instars was highly changing activity level than other as Table 6. However, neither the GSTs expressed induce nor inhibit the activities were increased from 1st to 5th instars, this result is similar the resulted from Rajurkar *et al.* (2003)

who showed that the GSTs activity levels were increasing from 1st to 5th instars lavae of *H. armigera*. The correction factor also indicated that cypermethrin and crude of derris extracts trend to be induce this enzyme activity in all instars, but rotenone substance trend to be inhibit in 1st – 3rd and induce in 4th – 5th instars. And the third instar also showed highly effective than the other similar with two types of enzyme characteristic as mention above.

As the explanatory above, the carboxylesterase expressed highly activity than AChE and GSTs. This data is support by the resulted from Domínguez-Gil and McPheron (2000) and Small and Hemingway (2000) who stated the carboxylesterase enzyme system is a generally important detoxification mechanism in insects and that has play important role in several of insecticide resistance. However, although GSTs enzyme give the less activity, it is still important to insecticide resistance mechanism because an increase in GSTs activity is associated with resistance to insecticides due to GSTs play a central role in detoxified to xenobiotic and endogenous compounds that noted from Vaz *et al.* (2004) Moreover, all of substances seems to express with induce characteristic except standard rotenone that showed high efficiency with can inhibit three detoxification enzymes in some instars larvae. This is once primary cause to insecticide resistance. However, although the crude extracts of derris characterized induce the enzyme activity but slightly than the synthetic chemical substance, cypermethrin *ca.* 1.59 – 1.00 times in carboxylesterase, 0.76 – 1.00 times in AChE and 0.375 - 0.5 times in GSTs activity. Thus, this result indicated that the cypermethrin tendency to resistance faster than other.

In addition, the study for revealed the toxicity of each instars larvae of *S. litura* with investigated the detoxification enzyme mechanism have useful for observed trend to be resistance of this insect in the future were showed. Because of some animal including insect usually use this mechanism for protect themselves from toxic substances. The mechanism can occur in different characteristics due to many factors such as toxic substances factor as may depend on type and/or dose, or organism factor as may depend on type, sex and/or age. From this resulted, type of toxic substance and age of insect were affected to the mechanisms of enzymes.

3. Determination of resistance to cypermethrin, standard rotenone and derris crude extracts in *S. litura*

3.1 Detection by bioassay technique

The bioassay is a primary usual technique for evaluated the substance toxicity including insecticide resistance with toxicity value determination in term of LC_{50}. After treated with three insecticides the results showed cypermethrin is highly toxicity than standard rotenone and derris crude extracts. And it was also showed the highest resistance ratio than the other as Table 9-11. The toxicity values of selection strain were increased in all population continuous from 1st to 10th generations. The cypermethrin, standard rotenone and derris crude extracts populations increased the toxicity value reached to 1,059.40, 143.16 and 136.35 ppm of LC_{50}, respectively with the high correlated of mortality which r^2 value as 0.91 to 0.99. This result is support

by the report of Abdullah (2001) who showed the resistance strain under laboratory selection of *S. exigua* was developed high level of resistance to cypermethrin continuous throughout 12 generations.

While the non-selection strain of all treatments were gave similarity of toxicity value throughout 10 generations with high correlated of mortality as 0.95 to 0.99. In the cypermethrin and standard rotenone treatment the toxicity values seem to be decreased from 5^{th} and 6^{th} generations, respectively. In the derris crude extracts was showed similarity of toxicity from 1^{st} to 10^{th} generations. The decreased of toxicity in non-selection strain was reported by Patarasiriwong (1990) that after 5 generations of diamondback moth in laboratory without cypermethrin exposure, LC_{50} decreased from 690 to 99 ppm for BKS strain, 677 to 3 ppm for BBS strain and 536 to 10 ppm for KKS strain.

About the toxicological response in term of the toxicity value at LC_{50} in selection strains, the cypermethrin population, showed high level after 3^{rd} generation and proceeded increasingly to onward generations. This result is different from the toxicity values of standard rotenone and derris crude extracts population that were showed highly increased at F_5 and slightly increasing from F_6 to F_{10} generation. This phenomenal is result to the resistance development of each population that cypermethrin population was increased in high level after 3^{rd} generation and proceeds increasingly to onward generations together as Figure 17. While the development of resistance in standard rotenone and derris crude extracts population that highly increased at F_5 and slightly increasing from F_6 to F_{10} generation as Figure 19 and 21, respectively. This result is similarity to the result of Abdullah (2001) who described the resistance development of *S. exigua* was high level development in 12 generation of cypermethrin population than neem extract and Bt populations that developed equal fluctuated throughout 12 generation and develop slightly increased, respectively.

At 10^{th} generation of selection strains, the toxicity values were showed highly than the 1^{st} generation of both selection and non-selection strains. When comparison the toxicity value between the 1^{st} and 10^{th} generation, the synthetic chemical insecticide population, cypermethrin showed the highest level *ca.* 94.33 times. The rotenone substance population, standard rotenone showed next to high level *ca.* 5.69 times and derris crude extracts showed the least level *ca.* 3.23 times. And when compared with 1^{st} generation of non-selection strain, the toxicity value showed higher than compared with the 1^{st} generation of selection strain. Cypermethrin population that showed *ca.* 98.27 times, standard rotenone showed *ca.* 5.91 times and derris crude extracts showed *ca.* 3.27 times. This result is can occur because of the resistance insects often develop from susceptible after a few generations of being toxic treated in laboratory with reported of Chau (1995) that said the beet armyworm had become resistance to pyrethroids such as cypermetrhin, fenvalerate and deltamethrin in short time under laboratory.

Moreover, resistance levels in term of resistance ratio at LC_{50} value were revealed for resistance mechanisms. When compared between the toxicity values of

non-selection strain and selection strain in the 10^{th} generation the synthetic chemical insecticide population, cypermethrin revealed highly increased to 104.58 fold as Figure 18. The rotenone substance population, standard rotenone and derris crude extracts were showed 5.9 and 3.14 fold as Figure 20 and 22, respectively. This result indicated that the synthetic chemical substance is highly toxic than the botanical substance approximately 17.72 to 33.31 fold. Brown and Pal (1971) stated that at the beginning of selection process, a slight increase in LD_{50} may be independent of specific genes for resistance. Thomas and Boethel (1995) observed that permethrin resistance strain of *Pseudoplusia includens* exhibited 93 fold resistant after 9 generation under laboratory. Aldosari *et al.* (1996) founded that selection of the Marana strain of beet armyworm for resistance to cyflythrin in 9 generations resulted 70.7 fold increased at LD_{50}. Ahmad and McCaffery (1988) reported high level resistance of *Helicoverpa armigera* in Thailand with 102 fold and 82 fold increase at LD_{50} to *cis*-cypermethrin and *trans*-cypermethrin, respectively. A rapid increasing the resistance ratio was observed in *S. exigua* to cypermethrin by Abdullah (2001) he founded the resistance ration increased sharply to onward generation resulting 95.83 fold at LC_{50} level in 12^{th} generations.

Vollinger (1986) described the development resistance of *P. xylostella* was showed resistance ratio of 1 and 2 fold at 11^{th} and 21^{st} generations, respectively. Abdullah (2001) reported *S. exigua* that against neem extracts showed resistance development of 1 – 1.44 fold at 12^{th} generations. All of these results support the resistance development in *S. litura* continuous against cypermethrin, standard rotenone and derris crude extracts. As the results indicated that cypermethrin is more affective than two substances when we use continuous for long time. Therefore the botanical insecticides may be the best alternative way for controlling the *S. litura.*

3.2 Detection by biochemical technique

This technique is an advanced assay of insecticide resistance using the biochemical reaction to determine the detoxification mechanism. The results showed that two types of esterase enzyme revealed high activity than the activity of phase II enzyme as GSTs. Especially, the carboxylesterase enzyme was showed high activity level than the other. Though, the most of experiments were showed similarity in protein content. Moreover, every substance characterized all enzymes activity into inducible characteristics.

3.2.1 Effects on carboxylesterase enzyme activity

Carboxylesterase enzyme showed the highest activity from other which trend to be induces the activity of all generations after treated with three insecticides. The activities were changed increasing from 1^{st} to 10^{th} generations with similarity level in standard rotenone and derris crude extracts populations, except in the cypermethrin treatment was high increased level. In the 1^{st} – 4^{th} generation of cypermethrin and standard rotenone populations seem to be showed inhibited enzyme activity and after that showed induced the activity to the 10^{th} generation. While derris crude extracts population showed fluctuated expression of activity throughout

generation as Table 12 and Figure 23. Nevertheless, although the standard rotenone acts similar with cypermethrin but it acts in lower level than cypermethrin about 1.23 times of carboxylesterase activity. This characteristic showed that the cypermethrin is trend to be resistance in the late generations. This is may be due to the late generations are strongly to develop themselves from xenobiotics because of they were sequential treated with same toxin for a long time. Then they may adapted some ability to protect themselves such as ability of behavioral adaptation, ability of physiological adaptation and ability of biochemical adaptation including ability of enhanced detoxification that described by Oakeshott *et al*. (2003) and Liu *et al.* (2006) who said esterase enzyme increased activities for enhancing the metabolism of insecticides.

Moreover, when compared the sensitivity with induction rate value of carboxylesterase between 1st generation of non-selection and 10th generation of selection strain (enzyme activity of 10th generation of selection per enzyme activity of 1st generation of non-selection strain) founded that the cypermethrin population showed high sensitivity than standard rotenone and derris crude extracts with 2.03, 1.62 and 1.02 times, respectively.

3.2.2 Effects on acetylcholinesterase enzyme activity

AChE showed high activity next to carboxylesterase activity in every treatment of all generations with similarity to carboxylesterase characteristics. Which cypermethrin and standard rotenone are act the same with showed inhibit this enzyme activity in 1st – 4th and 1st- 5th generations, respectively, but this inhibit revealed in induce characterization. While the derris crude extracts is has efficiency different from both substances which showed fluctuated induce activity throughout generations as Table 13 and Figure 24. The characteristics are similar to carboxylesterase enzyme that mention above because of they are play roles in the same reaction group. About the AChE resistance mechanism Andrews *et al.* (2004) said in *A. gossypii*, it is shown to be the elevation presented the multiple forms of insecticide-insensitive AChE by their association with either resistance specifically to the carbamate pirimicarb or resistance more generally to carbamates and certain organophosphates.

And when compared the sensitivity with induction rate value of AChE between 1st generation of non-selection and 10th generation of selection strain founded that the cypermethrin population showed high sensitivity than standard rotenone and derris crude extracts with 1.52, 1.18 and 1.07 times, respectively. While Gao *et al.* (2001) showed sensitivity between susceptible and resistance strain of *H. armigera* at 186 and 85 times of difference in heads of adults and 1,010 and 105 times of difference in heads of larvae based on a comparison for 2.8s and 8.7s of AChE forms, respectively.

3.2.3 Effects on Glutathione-S-transferase enzyme activity

GSTs enzyme was showed the least activity compare with two types of esterase enzyme in every treatment of all generations as Table 14 and Figure 25. Interestingly, all of substances showed induced the expression of GSTs. Cypermethrin showed high activity than standard rotenone and derris crude extracts 1.72 and 2.21 fold, respectively in 10^{th} generation. About the increasing of GSTs activity level in resistance strain Rajurkar *et al.* (2003) was stated the GSTs enzyme is responsible for endosulfan resistance *H. armigera* as increased the GSTs activity levels. The reported from Liu *et al.* (2006) who stated the elevated GSTs activities have been implicated in resistance in many insect species. Because of GSTs are soluble dimeric proteins involved in the metabolism, detoxification, and excretion of a large number of endogenous and exogenous compounds. Moreover, when compared the sensitivity with induction rate value of GSTs between 1^{st} generation of non-selection and 10^{th} generation of selection strain founded that the cypermethrin population showed high sensitivity than standard rotenone and derris crude extracts with 1.33×10^{-4}, 1.19×10^{-4} and 1.17×10^{-4} times, respectively.

As mention above, the carboxylesterase expressed highly activity than AChE and GSTs. This data is support by the resulted from Domínguez-Gil and McPheron (2000), Small and Hemingway (2000), Vontas *et al.* (2001) and Visetson *et al.* (2005) who stated the carboxylesterase enzyme system is a generally important detoxification mechanism in insects and that has play important role in several of insecticide resistance with detoxification enzyme mechanism that produce by an insect when passed for long time of insecticide exposure. Moreover, all of substances effective to detoxification enzyme mechanism with express characteristic as induction. However, although the botanical substance, standard rotenone and crude extracts of derris characterized enhancement the detoxification enzyme activity but this activity is slightly than the synthetic chemical substance, cypermethrin *ca.* 1.72 – 2.21, 1.20 – 1.32 and 1.72 – 2.21 times in carboxylesterase, AChE and GSTs activity, respectively when compared between enzyme activities of these three treatments at 10^{th} generation (enzyme activity of cypermethrin per enzyme activity of standard rotenone or derris crude extracts).

Therefore, as the result indicated that the synthetic chemical insecticide, cypermethrin tendency to resistant faster than the botanical substance, standard rotenone and crude extracts of derris roots in *S. litura* cause from enhancement of detoxification mechanism. This is related to the statement of Brogdon and McAllister (1998) who said the biochemical resistance in term of detoxification enzyme-based resistance, which occurs when enhanced levels or modified activities of esterases, oxidases, or GSTs prevent the insecticide from reaching its site of action. And Li *et al.* (2007) who stated that the xenobiotic resistance in insects has evolved predominantly by increasing the metabolic capability of detoxificative systems.

3.3 Detection by molecular technique

Molecular technique is also the advance method for detect the insecticide resistance mechanism. Insecticide resistance of insects is fundamental occurred from mutation in gene. The detoxification enzyme synthetic including carboxylesterase enzyme is controlled by gene or genetic material in an insect, when changing in the high levels it can makes insecticides non toxic to insects. Molecular detection of insecticide resistance in insects can be done by DNA technology. Moreover, the quality of protein is useful for enzyme mechanism can be done by protein technology.

3.3.1 Effect on detoxification enzyme protein molecular weight

The characteristic of enzyme with protein qualities determination should be evaluates because of it is useful for detoxification mechanism. The presented of three detoxification enzyme systems in most of experiments that estimated by SDS-PAGE showed no different in protein quality. The results of every treatment were presented the same molecular weight of protein throughout generations as Figure 26 to 30. This characteristic indicated that the carboxylesterase enzyme possessed the molecular weight approximately 60 kDa in 10 generations of *S. litura*. The result is similarity to the molecular mass of carboxylesterases of susceptible and resistant aphids *A. gossypii* were 59.10 and 59.26 kDa, respectively by Moores *et al.* (1997). The predicted physical properties of *A. gossypii* carboxylesterases are well within the range of other resistance-associated esterases of carboxylesterases molecular mass usually range from 50 to 70 kDa Sun *et al.* (2005).

About AChE enzyme, the enzyme standard marker is possessed many bands of molecular weight, but the strongly bands of both enzyme marker and the samples showed that at 24 kDa as protein standard marker. This result is different from the purification and characterization of AChE from oriental fruit fly *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) by Hsiao *et al.* (2004) They showed the molecular mass of purified AChE was 116 kDa for its native protein (non-reduced form) and 61 kDa for its subunits (reduced form). While Le and Han (2002) study on purification and characterization of AChE from cotton aphid (*Aphis gossypii* Glover) founded the molecular mass of the purified AChE that about 63,500 Dalton. About these several characteristics of AChE Hsiao *et al.* (2004) said it can occur because of these biochemical properties may have structural differences from those of other insect species. That is related to the reported of Gao *et al.* (2002) who founded the AChE molecular weight of greenbug *Schizaphis graminum* was 65.4 kDa and they said this value is smaller than the previous reported of Gao and Zhu (2001) and they suggested that this protein is lightly because of some reaction in organism probably affected the accuracy of molecular mass as demonstrated in the butyrylcholinesterase of chick by Treskatis *et al.* (1992) and esterase of small brown planthoppers by Sakata and Miyata (1994), and Small and Hemingway (2000).

While the GSTs enzyme possessed two subunit of molecular weight at 24 and 48 kDa. One subunit of this result at 24 kDa is similarity to the report from Bullangpoti (2004) who estimated the GSTS protein quality in *S. oryzae* with showed

molecular weight at 26 kDa that response to enzyme activity against plant extracts. And similarity to the molecular weight of these enzymes that found in the *Tribolium castaneum* done by Reidy *et a*l. (1990). The reported of Rajurkar *et al.* (2003) who studies on GSTs levels of endosulphan resistance in *H. armigera* showed molecular weight of GSTs at 30 kDa. About many subunit of GSTs protein, it is can occur because of following the reason of Duck-Oung and Lee (2005) who study on protein in whole bodies of two-spotted spider mites, *Tetranchus urticae*, compared between non-diapause and diapause condition by one- and two- dimensional electrophoresis showed severall protein numbers. And twenty-four proteins that have higher quantity expressed in diapause were selected at the range of low molecular weight as 5-20 kDa. However, although GSTs presented many subunits in this study, but two subunits are can remarkable with protein marker and standard GSTs enzyme at 24 and 48 kDa which showed strong and clearity bands as Figure 30-34.

Moreover, the protein quality of these three detoxification enzymes can elute on 7.5 to 15 % of polyacrylamide gel, although did not use the gradient gels. Such as, the experiments of Bullangpoti (2004) who used 10 and 15% SDS gel for expressions of esterase and GSTs in *S. oryzae*, respectively. The reported of Vararattanavech (2002) who showed the expression of GSTs on 15% SDS gel. However, about the presented of many subunits of AChE and GSTs enzymes may occur because of this is a crude protein extracts. This problem can resolute by enzyme purification before separate that was not done in this study. Therefore, these characteristics indicated the carboxylesterase, AChE and GSTs enzyme in *S. litura* possesses the molecular weight of 60, 24 and 48, 24 kDa, respectively that responsible to their activities against the synthetic chemical and botanical substances. In addition, the carboxylesterase characteristic indicated this enzyme is highly associated with three toxin substance effects than other which possessed the single band of molecular weight approximately 60 kDa that was confirmed in the range size of insecticide resistance-associated with carboxylesterase enzyme.

3.3.2 Effect on gene associated with detoxification enzyme expressions

The effective of these three insecticidal to gene associated with detoxification enzyme expressions was elucidated using RT-PCR technique. The carboxylesterase gene that associated with carboxylesterase enzyme preferable to this study, because of it is showed highly level of activities after exposed with three insecticides. As Figure 31 to 32, a single PCR product approximately 553 bp was successful amplified and determined by lamda DNA/*Hind*III marker. About the gene amplification conferring metabolic resistance of carboxylesterase enzyme, some scientist was done such as the carboxylesterase gene amplification conferring metabolic resistance to organophosphates and carbamates in aphids was done by Foster *et al.* (2003). The amplification of carboxylesterase gene that strong resistance conferring to organophosphorus insecticide in the blowfly *Lucilia cuprina* was by Heidari *et al.* (2004).

Partial sequence of carboxylesterase gene of all treatments in *S. litura* was directed sequencing, editing and analyzing by GENETYX analysis

program. As the results showed 99% identity both the nucleic acid and amino acid levels with carboxylesterase gene of *S. litura* in accession number DQ 445461 from GenBank (www.ncbi.nlm.nih.gov). When aligned the sequences compared with carboxylesterase gene from GenBank, as Figure 33, most of populations in this study were showed interestingly base different from GenBank database. That is the occurring at the T^{30} position of 533 bp founded the base of most experiment including non-selection population as control in this study showed cytosine base (C) while in the GenBank database showed thymine base (T). It is can occur because of this position in the conserve region showed Y, that means this position is can present either T or C base. This is according to the principal of genetics. As a result presented base C in all population of experiments, thus, indicated that the 30^{th} position of 553 bp in this study is a C not T as showed in the database of GenBank.

Second, at the C^{76} position of 533 bp founded the thymine base in non-selection, standard rotenone and derris crude extracts populations different from GenBank data and cypermethrin population that showed cytosine base. This chance is can occur because of this position in the conserve region is showed Y, that means this position is can present either T or C base. As a result presented base T in all population of experiments accepted cypermethrin population, thus, according to the situation of T^{30} position indicated that the 76^{th} position of 553 bp in this study is a T not C as showed in the database of GenBank. Therefore, when the cypermethrin population presented the C indicated that it is mutated from this population.

On the third different position, the guanine base was resulted at the A^{102} position of 553 bp in cypermethrin population different from other experiment and GenBank data that showed adenine. This result is similarity to the different at G^{111} position of 533 bp that presented cytosine base in cypermethrin population while other experiment and GenBank data showed guanine, this is the fourth position of differentiation. This sutiation is can occur because of the A^{102} and G^{111} position in the conserve region is showed R and S, respectively. That means the A^{102} position is can present either A or G base and the G^{111} position is can present either G or C base. As a result base G presented at the A^{102} position and base C presented at the G^{111} position in cypermethrin population. While most of experiments included the database from GenBank are showed A and G at the 102^{nd} and 111^{th} position, respectively. Therefore, when the cypermethrin population presented different base from other indicated that it is mutated from this population.

Finally, the fifth differentiation occurred at the C^{381} position of 553 bp similar the second that founded the adenine base in non-selection, standard rotenone and derris crude extracts populations different from GenBank data and cypermethrin population that showed cytosine base. This chance is also can occur because of this position in the conserve region is showed M, that means this position is can present either C or A base. As a result presented base A in all population of experiments accepted cypermethrin population, thus, according to the situation of T^{30} position indicated that the 381^{st} position of 553 bp in this study is a A not C as showed in the database of GenBank. Therefore, when the cypermethrin population presented the C indicated that it is mutated from this population.

This characteristics of gene associated with insecticide resistance normally occurred such as the reported of Foster *et al.* (2003) who showed the single-point mutations conferring target-site resistance (kdr) to pyrethroids and DDT in aphids and houseflies. Heidari *et al.* (2004) were shown two mutations in LcalphaE7 of the gene encoding carboxylesterase E3 that enhance the enzyme ability to hydrolyse insecticides in the resistance of blowfly *Lucilia cuprina* to organophosphorus insecticides. About the changing of gene structural, Scott (1995) confirmed that the genetic mutation in insecticide resistance is associated with the activities of esterase enzyme group. Suzuki and Hama (1998) suggested structural gene substitution participate in the overproduction of esterase in the resistant cotton aphid.

After founded the base differentiation in the experiment of *S. litura* against sequential insecticides continuous 10 generations, the amino acids were revealed and showed that the interestingly result at the fourth position of differentiation. The G^{111} position of 533 bp showed cytosine base in cypermethrin population while other experiment and GenBank data that showed guanine. This position is consisted of GAG codon that coding the glutamate (Glu, E) amino acid in most of experiment and GenBank data. While in this position of cypermethrin population deduced nucleotide changing different from other with coded to aspartate (Asp, D) amino acid as Figure 34. Because of this position is consisted of GAC codon cause to amino acid coded changing to aspartate substituted the glutamate. However, this amino acid changing is presented in the nearby amino acid of acidic group.

About the changing of amino acid substitutions cause from the nucleic acid changing in the genome, many scientists were presented. For example, Newcomb *et al*. (1997) and Campbell *et al*. (1998) showed the single substitutions of Gly^{137} to Asp^{137} or Trp^{251} to Leu^{251} in the catalytic center of blow fly, *Lucilia cuprina* in two different cross-resistance pattern favoring either diethyl (parathion/diazinon) or dimethyl (malathion) types of organophosphates, respectively. Claudianos *et al*. (1999) showed amino acid substitution at Gly^{137} to Asp^{137} position in the orthologous *_E7* gene of housefly, *Musca domestica* (*Md_E7*) caused structural modification led to the ali-esterase-based resistance. Martinez-Torres *et al.* (1999) founded the knockdown resistance (*kdr*) gene conferred to DDT and pyrethroid insecticides in the housefly, *M. domestica* has revealed two amino acid mutations, Lue^{1014} to Phe^{1014} and Met^{918} to Thr^{918}, that are associated with *kdr* and *super-kdr* resistance phenotypes. Zhou *et al.* (2005) reported the full length of the cDNA opening reading frame of aphis, *A. gossypii* carboxylesterases gene is 1581 bp, encoding 526 amino acids showed four amino acid substitutions. There are Thr^{210} to Met^{210}, Asn^{294} to Lys^{294}, Gly^{408} to Asp^{408} and Ser^{441} to Phe^{441} in resistant strain to omethoate that is associated with carboxylesterases.

As mention above, this result indicated that the nucleic acid and amino acid changing in carboxylesterase gene are presented the up-regulated expression of carboxylesterase enzyme with enhanced it activities is correlated with insecticide resistance. Several lines of evidence suggest that carboxylesterase genes involved in insecticide resistance, and perhaps insecticide detoxification genes in general, may share an evolutionary association with genes involved in insecticide metabolisms.

This resulted related to the reported of Heidari *et al.* (2004) they were showed substituted the nearby amino acids for some expected to affect the efficiency of the enzyme by changing aspartate to glutamate or histidine was revealed the less effective than aspartate in improving organophosphate hydrolase activity. This is following the statement of Li *et al.* (2007) who stated that many molecular mechanisms lead to enhancements in xenobiotic metabolism. The genomic changes that lead to amplification, overexpression, and coding sequence variation in the three major groups of genes encoding metabolic enzymes, i.e., cytochrome P450 monooxygenases (P450s), GSTs and esterases, including the carboxylesterase. Differences in the selective regime imposed by insecticides may account for the relative importance of regulatory or structural mutations in conferring resistance.

CONCLUSION AND RECOMMENDATION

Conclusion

The monitoring of synthetic and botanical insecticides resistance in the tropical armyworm, *S. litura* was done in the toxicity test of each compound, the efficiency comparison between synthetic and botanical insecticides, the detoxification enzyme mechanisms elucidation against these insecticides, and the effective determination after sequential treated with these insecticides in 10 generations of *S. litura*. Moreover, the natural botanical insecticide, derris crude extracts was done in this research.

The ethanolic crude extracts of *D. elliptica* roots by Soxhlet method gave high yield was 48.60 %w/w. The characteristic of dry crude extracts is rough solid substance and dark brown color. Moreover, when analyzed the total content of rotenone from dried powder of crude extracts by HPLC. The results also showed high rotenone content was 17.91%w/w.

Efficacy of the synthetic insecticide, cypermethrin and botanical insecticides, standard rotenone and derris crude extracts was estimated by lava body dipping method with different concentration of insecticide, exposure time and various instars larvae of *S. litura* for obtain the toxicity value in term of LC_{50}. The results showed the toxicity value dramatically increasing when increased the insecticide concentration with significant different level ($p<0.05$) and increased the time exposure duration with not significant in statistical ($p<0.05$). The synthetic insecticide, cypermethrin showed the best control to 3rd instar larvae of *S. litura* with LC_{50} value was 9.93 ppm (r^2 = 0.98). Followed by the botanical insecticides, standard rotenone and derris crude extracts were showed LC_{50} value at 23.27 and 37.67 ppm with r^2 0.99 and 0.97, respectively at 72 hours after exposure. In various instars larvae, cypermethrin showed the highest efficiency to all instars larvae of *S.litura* than standard rotenone and derris crude extracts approximately 2.44 – 6.78 and 1.82 – 8.71 times at LC_{50} (ppm) with r^2 0.84 – 0.99, respectively of 1st to 5th instars larvae. Moreover, every insecticide showed the best effected to the 3rd instar lava with gave highly r^2 value than other as 0.97 – 0.99.

The characterization of carboxylesterase, AChE and GSTs mechanisms in all instars larvae were determined by *In vitro* study with specific substrate using UV-visible spectrophotometer. As the results, carboxylesterase showed the role to detoxify than the other after 24 hours exposure with three insecticides. Both of cypermethrin and derris crude extracts seems to express with induce characteristic while standard rotenone that showed high efficiency with can inhibit three detoxification enzymes in some instars larvae. However, although the crude extracts of derris characterized induce the enzyme activity but it is slightly than cypermethrin *ca.* 1.59 – 1.00, 0.76 – 1.00 and 0.37 - 0.5 times of carboxylesterase, AChE and GSTs activity, respectively. And these enzymes more affected the 3rd instar larvae than

other. Thus, type of toxic substance and age of insect were affected to the mechanisms of enzymes. Moreover, the pH buffer is suitable for homogenization and enzyme incubation was done at pH 10. In addition, the elucidation of detoxification enzyme mechanism not only revealed for toxin effected but also useful for observed trend to be resistance of this insect in the future. Therefore, from the result indicated that the cypermethrin tendency to resistance faster than other.

The determination effects of the synthetic and botanical insecticides after sequential treated continuous 10 generations in *S. litura* was done by bioassay, biochemical and molecular techniques.

The bioassay technique revealed the toxicological response in term of LC_{50}. Cypermethrin population, showed highly level of toxicity and resistance development after 3rd generation and proceeded increasingly to onward generations. Different from standard rotenone and derris crude extracts population that showed high increased of toxicity and resistance development at F_5 and slightly increasing from F_6 to F_{10} generation. The resistance levels in term of resistance ratio at LC_{50} value were revealed for resistance mechanisms. Cypermethrin population showed highly increased than rotenone substance and derris crude extracts with 104.58, 5.9 and 3.14 fold, respectively. This result indicated that the synthetic chemical substance is highly toxic than the botanical substance approximately 17.72 to 33.31 fold, respectively.

The biochemical technique revealed the detoxification enzyme mechanism characterizations. The results showed carboxylesterase and AChE enzyme that revealed high activity than GSTs. Especially, the carboxylesterase enzyme was showed high activity level than the other. Though, the most of experiments were showed similarity in protein content. Moreover, every substance characterized all enzymes activity into inducible characteristics. However, although the botanical substance, standard rotenone and crude extracts of derris characterized enhancement the detoxification enzyme activity but this activity is slightly than the synthetic chemical substance, cypermethrin approximately 1.72 – 2.21, 1.20 – 1.32 and 1.72 – 2.21 times in carboxylesterase, AChE and GSTs activity, respectively. Therefore, as the result indicated that the synthetic chemical insecticide, cypermethrin tendency to resistant faster than the botanical substance, standard rotenone and crude extracts of derris roots in 10 generations of *S. litura* cause from enhancement of detoxification mechanisms.

The molecular technique is advance method performed to characterized the detoxification system qualities and analyzed gene associated with detoxification enzyme expressions. SDS-PAGE of most experiments gave no different of each protein quality in 10 generation of *S. litura* that possessed the molecular weight of carboxylesterase as 60 kDa, two subunit of AChE as 24 and 48 kDa, and GSTs as 24 kDa. About the gene associated with detoxification enzyme expressions characterized by RT-PCR gave a single band approximately 553 bp with 99% identity nucleic acid and amino acid to *S. litura* (DQ445461 GenBank). Five positions were founded that different from GenBank database there are T^{30}, C^{76}, A^{102}, G^{111} and C^{381} position of

553 bp. Moreover, four positions (C^{76}, A^{102}, G^{111} and C^{381}) of all nucleic acid differences are only belonging to the synthetic insecticide, cypermethrin population while another one (T^{30}) belong to every population in this study. In addition, the deduced amino acid of cypermethrin gave single substitution at E^{37} to D^{37} that means Aspartate (Asp) substituted Glutamate (Glu).

In addition, according to the results, this is somewhat anew in characterized of gene mutations leading to the carboxylesterase enzyme mechanisms in *S. litura* especially in Thailand.

Recommendation

1. The efficiency of derris crude extracts, standard rotenone and common available synthetic insecticide to continuous used longer than this study should be estimate in order may be detecting more effects to detoxification mechanisms and gene associated with detoxification enzymes.

2. The isolation and purification of the active compound, rotenone substance from derris crude extracts by Soxhlet method with ethanol solvent system should be investigates the toxicity in *S. litura* and another insect to evaluate their efficiency and effects to detoxification mechanisms in the future research.

3. The toxicity investigate of derris crude extracts in the field should be done in the future research and should be concern about economic problem when compare to another insecticide before develop to commercial product.

4. The improvement of Soxhlet apparatus for the highest yield of crude extracts should be concern.

LITERATURE CITED

Abbott, W.S. 1925. Method for computing the effectiveness of an insecticide. **J. Econ. Entomol.** 18: 265-267.

Abdul Fatah, M. I., Y.S. Saleem and M.I. Abdul Majeed. 1997. Effect of larval diet on the development and fecundity of Cotton Leafworm, (*Spondoptera littorals* (Boised) **Zeitschrit fur Angewandate Entomol.** 84: 311-315.

Abdullah, M.D. 2001. **Resistance Development and Effects of Cypermethrin, Neem Extracts and *Bacillus thuringiensis* on *Spodoptera exigua* (HUBNER) on Vegetable Soybean.** M.S. thesis, Kasetsart University. Bangkok Thailand.

Abo-El-Ghar, M.R., M.E. Nassar, M.R. Riskalla and S.F. Avd-El-Ghafar. 1986. Rate of development of resistance and pattern of cross-resistance in fenvalerate and decamethrin-resistance strains of *Spodoptera littioralis*. **Agri. Reser. Rev.** 61: 141-145.

Abrahamson, W.G. 1989. Plant-animal interaction: an overview. *In* W.G. Abrahamson, eds., **Plant-Animal Interactions.** McGraw-Hill Publishing, NY. Pp. 1-22.

Agnihotri, A.P., K.P. Srivastava, V.T. Gaibhiye and N.K. JA. 1987. Relative efficacy of some synthetic pyrethroids and other commonly used insecticides against bollworm and their residues in cotton. **Rev. Appl. Entomol. Ser. A.** 75(1): 35.

Ahmad, M. and A.R. McCaffery. 1988. Resistance to insecticides in a Thailand strain of *Heliothis armigera* (Hubner) (Lepidoptera: Noctuidae). **J. Econ. Entomol.** 81(1): 45-48.

Aitkenhead, P., C.R.B. Baker and G.W.D. de Chickera. 1974. An outbreak of *Spodoptera litura*, a new pest under glass in Britain. **Plant Patho.** 23: 117-118.

Akinbulumo, M.O., O.A. Fagbenro and E.A. Fasakin. n.d. **Acute toxicity of ethanolic extracts of *Derris elliptica* roots to O*reochromis niloticus* fingerlings.** Department of Fisheries, Federal University of Technology, Nigeria.

Aldosari, S.A., T.F. Watson, S. Sivasupramaniam and A.A. Osman. 1996. Susceptibility of field population of beet armyworm (Lepidoptera: Noctuidae) to cyfluthrin, methomyl, and profenofos, and selection for resistance to cyfluthrin. **J. Econ. Entomol.** 89(6): 1359-1363.

Amer, S.M., A. Abd-EL, S. Ibrahim and K.M. El-Sherbeny. 1993. Induction of chromosomal aberrations and sister chromatid exchange in vivo and in vitro by the insecticide cypermethrin. **J. Appl. Toxicol.** 13(5): 341-345.

Andrews, M.C., A. Callaghan, L. M. Field, M. S. Williamson and G. D. Moores. 2004. Identification of mutations conferring insecticide-insensitive AChE in the cotton-melon aphid, *Aphis gossypii* Glover. **Insect Mol. Biol.** 13(5): 555–561.

Anonymous. 1969. World health organization expert committee on insecticides, p. 46. *Cited by* K. Ounchaichon. Insecticide Resistance. **Entomol. Zool. Gaz.** 11(1): 44-47. (in Thai)

_____. 1995. Cypermethrin - a synthetic pyrethroid. **Pestic. News** 30: 20-21.

Areekul, S., B. Wanleeluk, A. Vatanatungum, U. Skulpanich, O. Prachoubmoa, P. Teeravet and Y. Singhasenee. 1963. **Insect pest of corn in Thailand.** Department Entomology and Plant Pathogen. Kasetsart University, Thailand. 205p. (In Thai).

Armes, N.J., J.A. Wightman, D.R. Jadhav and G.V.R Rao. 1997. Status of insecticides resistance in *Spodopter litura* in Andhra Pradesh. India. **Pestic. Sci.** 50(3): 240-248.

Arnason, J.T., B.J. Philogene and P. Morand. 1989. **Insecticides of Plant Origin.** American chemical society. Washington, DC. USA. 213p

Baruah, A.A., L.H. Chauhan and R. Chauhan. 1997. Bioefficacy of synthetic pyrethroids on the pod damage by pod borer, *Heliothis armigera* (Hubner) in pigeonpea. **Legume Res.** 20(2): 87-90.

Beugnet, F. and L. Chardonnet. 1995. Tick resistance to pyrethroids in New Caledonia. **Vet. Parasitol.** 56(4): 325-338.

Boardman, L.A. 1977. Insectary culture of *Spodoptera litura* (Lepidoptera: Noctuidae). **N. Z. Entomol.** 6(3): 36-41.

Brogdon, W.G. and J.C. McAllister. 1998. Insecticide Resistance and Vector Control. **Emerging Infect. Dis.** 4(4): 605-613.

_____., _____ and J. Vulule. 1998. Heme peroxidase activity measured in single mosquito identifies individuals expressing the elevated oxidase mechanism for insecticide resistance. **J. Am. Mosq. Cont. Assoc.** 13: 233-237.

Brown, A.W.A. and R. Pal. 1971. Nature and characterization of resistance. *In* WHO **Insecticide Resistance in Arthropods**. Monograph Series 38, Geneva.

_____. 1958. Nature and characterization of resistance. *In* WHO **Insecticide Resistance in Arthropods.** Monograph Series, vol. 38. Geneva, 240 pp.

Bullangpoti, V. 2004. **Effects of Some Plant Extracts on Toxicity and Activities of Esterase and Glutathione-S-transferase in Rice Weevils (*Sitophilus oryzae* L.).** M.S. thesis, Kasetsart University, Bangkok Thailand.

Campbell, P. M., R. D. Newcomb, R. J. Russell, and J. G. Oakeshott. 1998. Two different amino acid substitutions in the ali-esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blow fly, *Lucilia cuprina.* **Insect Biochem. Mol. Biol.** 28: 139-150.

Chaikam, V. 2002. **Efficiency of *Stemona* sp. And *Derris scandens* Benth.** M.S. thesis, Khon Kean University. Khon Kean Thailand. (in Thai)

Chankeawmanee, B., N. Leuwthong and P. Teuwthong. 2005. Toxicity Test of ECO_2FUME for Controlling Some Stored Product Insect Pests. *In* **The 7th national plant protection.** November 2-4, 2005, Cheing Mai Thailand.

Chansang, U. 2003. **Evaluation of Methodologies for Control of the Dengue Vector, *Aedes aegypti*, in Thailand.** Ph.D. thesis, Mahidol University, Bangkok Thailand.

Chasseaud, L.F. 1979. The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic reagents. **Adv. Cancer. Res.** 29: 175-274.

Chau, L.M. 1995. Integrated pest management: A strategy to control resistance of *Spodoptera exigua* and *Heliothis armigera* caterpillars to insecticides on soybean in the Mekong Delta. **Pestic. Sci.** 43(3): 873-878.

Chotimanothum, B. 2000. **Studies on Phosphine Resistance and Detoxification Enzyme Activity of Lesser Grain Borer, *Rhizopertha dominica* (Coleoptera: Bostrichidae) in Central Thailand.** M.S. thesis. Kasetsart University, Bangkok Thailand.

Claudianos, C., R.J. Russell, and J.G. Oakeshott. 1999. The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blow fly. **Insect Biochem. Mol. Biol.** 29: 675-686.

Corbett, J.R. 1974. **The Biochemical Mode of Action of Pesticides.** Academic Press, New York.

Cottier, W. and E.S. Gourlay. 1955. New horticultural pest found on Nelson tobacco. **N.Z. J. Agri.** 91(4): 349-51.

Dass, R. and B. Parshad. 1983. Influence of the age of *Spodoptera Litura* (F.), eggs on parasitization by *Telenomus litura* Nixon (hymenoptera: Scelionide). **J. Entomol. Res.** 7: 18: 20.

Dauterman, W.C. 1985. Insect metabolism: extramicrosomal, pp 713-730. *In* Kerkut G.A. and L.I. Gilbert, eds. **Comprehensive insect physiology, biochemistry and pharmacology.** Vol. 12, Pergamon Press, Oxford.

_____. and E. Hodgson. 1978. Biochemistry of insect, pp.541-577. *In* M. Rocktion, eds. **Detoxification mechanism in insect.** Academic Press, New York.

Depatment of agriculture. 2003. **Plan of production control.** Available Source: http://www.doa.go.th/gap/food_safety/asparagus/asparagus-3.doc, Febuary 12, 2005.

_____. 2004. **Good Agricultural Practice (GAP) for vegetables.** Available Source: http://agritech.doae.go.th, Febuary 12, 2005.

_____. 2005. Natural insecticides for agriculture. Available Source: http://agriqua.doae.go.th/news/bio.doc, Febuary 12, 2005.

Domínguez-Gil, O.E. and B.A. McPheron. 2000. Effect of diet on detoxification enzyme activity of *Platynota idaeusalis* (Walker) (Lepidoptera: Tortricidae) larvae strains. **Rev. Fac. Agron.** (LUZ) 17: 119-138.

Duck-Oung, J. And K.Y. Lee. 2005. Identification of low molecular weight diapause-associated protein of two-spotted spider mite. *Tetranychus urticae*. **Entomol. Res.** 35(4): 213-218.

Dunn, M.J. 1993. **Gel electrophoresis protein.** Bios Scientific Publishers Ltd., United Kingdom.

Dwivedi, S.C., B. Mathur and B. Mathur. 1999. Effect of synthetic pyrethroids and organophosphates on hatching of larvae of *Spodopter litura.* **J. Ecotoxic. Environ. Monito.** 9(1): 19-22.

Enayati, A.A., H. Vatandoost, H. Ladonni, H. Townson and J. Hemingway. 2003. Molecular evidence for a kde-like pyrethroid resistance mechanism in the malaria vector mosquito *Anopheles stephensi*. **Med. Vect. Entomol.** 17: 138-144.

European and Mediterranean Plant Protection Organization (EPPO). n.d. **The geographical distribution of *Spodoptera litura* (F.)** Available Source: http://www.eppo.org/QUARANTINE/quarantine.htm, March 20, 2005.

Fang, Q., W.C. Black, H.D. Blocker, and R.F. Whitcomb. 1993. A phylogeny of New World *Deltocephalu*s-like leafhopper genera based on mitochondrial 16S ribosomal DNA sequences. **Molec. Phylogen. Evolu.** 2**:** 119–131.

Feyereisen, R. 1995. Molecular biology of insecticide resistance. **Toxicol. Lett.** 82/83: 83-90.

Ffrench-Constant, R., J.C. Steichen, T. Rocheleau, K. Aronstein, and R.T. Roush. 1993. A single-amino acid substitution in a g-aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in *Drosophila* populations. **Proc. Natl. Acad. Sci. USA** 90: 1957-1961.

Foster, S.P., S. Young, M.S. Williamson, I. Duce, I. Denholm and G.J. Devine. 2003. Analogous pleiotropic effects of insecticide resistance genotypes in peach-potato aphids and houseflies. **J. Heredity** 91(2): 98-106.

Gao, J.R., S. Kambhampati and K.Y. Zhu. 2002. Molecular cloning and characterization of a greenbug (*Schizaphis graminum*) cDNA encoding acetylcholinesterase possibly evolved from a duplicate gene lineage. **Insect Biochem. Mol. Biol.** 32: 765-775.

Gao, X.-W., X.-G. Zhou and G.B.-Z. Zhen. 2001. A comparison of sensitivity to inhibitor among acetylcholinesterase (AChE) molecular forms of resistance and susceptible strains in *Helicoverpa armigera.* **Entomologia Sinica** 8(1): 49-54.

Georghiou, G.P. and A. Lagunes-Tejeda. 1991. **The occurrence of resistance to pesticides in arthropods.** Publication GAPP/Misc/91-1. FAO, Rome.

Gohokar, R.T., S.M. Thakre and M.N. Borle. 1994. Chemical control of gram pod borer *Heliothis armigera* (Hubner) by different synthetic pyrethroids and insecticides. **PKV Res. J.** 8(2): 41-43.

Guerrero, R.D. and L.A. Guerrero. 1986. **Uses of *Derris* root power for management of fresh water ponds.** Aquatic Biosystems, Bay, Laguna, Philippines. pp. 125-127.

Hamby, R.K., L. Sims, L. Issel and E.A. Zimmer. 1988. Direct ribosomal RNA sequencing: Optimization of extraction and sequencing methods for work with higher plants. **Plant Mol. Biol. Reporter** 6: 175–192.

Haubruge E, M. Amichot, A. Cuany, J.B. Berge and L. Arnaud. 2002. Purification and characterization of a carboxylesterase involved in malathion-specific resistance from Tribolium castaneum (Coleoptera: Tenebrionidae). **Insect Biochem. Mol. Biol.** 32(9):1181-90.

He, F. 1994. Synthetic pyrethroids. **Toxicol.** 91: 43.

Heidari, R., A.L. Devonshire. B.E. Campbell, K.L. Bell, S.J. Dorrian, J.G. Oakeshott and R.J. Russell. 2004. Hydrolysis of organophosphorus insecticides by in vitro modified carboxylesterase E3 from Lucilia cuprina. **Insect Biochem. Mol. Biol.** 34(4): 353-363.

Hemingway, J. and H. Ranson. 2000. Insecticide resistance in insect vectors of human disease. **Ann. Rrv. Entomol.** 45: 371-391.

Hill, D.S. 1983. **Agricultural insect pest of the tropics and their control.** 2nd. Cambridge University Press, New York. 746p.

Hodgson, E. and P.E. Levi. 1997. **Introduction to biochemical toxicology.** 2nd Appleton and Lange. Norwalk, Connecticut. 1245p.

Hsiao, Y.M., J.Y. Lai, H.Y. Liao and H.T. Feng. 2004. Purification and characterization of acetylcholinesterase from oriental fruit fly [*Bactrocera dorsalis* (Hendel)] (Diptera: Tephritidae). **J. Agric. Food Chem.** 52(17): 5340-5346.

Hu, M.Y., G.H. Zhong, Z.T. Sun, H.M. Liu and X.Q. Liu. 2005. Insecticidal activities of secondary metabolites of endophytic *Pencillium* sp. in *Derris elliptica* Benth. **J. Appl. Entomol.** 129(8): 413–417.

International Programme on Chemical Safety (IPCS). n.d. **Rotenone Health and Safety Guide**. Number 4, 73.

Ishaava, J. and D. Degheele. 1988. Properties and toxicological significance of diflubenzuron hydrolase activity in *Spodoptera littoralis* larvae. **Pestic. Biochem. Physiol.** 32: 180-187.

Ishaaya, I. 2001. Biochemical processes related to insecticide action. *In* I. Ishaaya, eds. **Biochemical Sites of Insecticide Action and Resistance**. Springer, Berlin-Heidelberg- New York, chapter 1, p. 1-16.

Issa, Y.H., M.E. Keddis, M.A. Ayad and M.A. El-Guindy. 1984. Survey of resistance to organophosphorus insecticides in field strains of the leafworm during 1980-1984 cotton-growing seasons. **Bull. Entomol. Socie. Egypt, Econ. Series.** 14: 399-404.

Jakoby, B. 1978. The glutathione-S-transferase: A group of multifunctional detoxification protein. **Adv. Enzymol. Relat. Areas Mol. Biol.** 46: 383-414.

Jansiri, T. 1994. **Insecticide Resistance Monitoring and Mapping for Brownplant hopper, *Nilaparvata lugens* (Stal.) in Thailand.** M.S. thesis, Kasetsart University, Bangkok Thailand.

Kao, L.R., N. Motoyama and W.C. Dauterman. 1985. The purification and characterization of esterase from insecticide-resistant and susceptible houseflies. **Pest. Biochem. Physiol.** 23: 228-239.

Khoso, A.W. 1988. **Growing Vegetable in Sindh**. 1st ed. Ahmed Brothers. Printers, Nazimabad, Karachi, p: 43-63.

Kodandaram, M.H. and S. Dhingra. 2006. Variation in the Susceptibility and Resistance of *Spodoptera litura* (Fab) (Delhi and Punjab Populations) to Various Synthetic Pyrethroids. **Resist. Pest Manag. News.** 16(1): 9-12.

Kulpiyawat, T. 2001. **Resistance and Resistant Mechanisms of Some Acaricides in African Red Mite, *Eutetranychus africanus* (TUCKER) (Acari: Tetranychidae).** Ph.D. Thesis, Kasetsart University, Bangkok Thailand.

Le, F. and Z. Han. 2002. Purification and characterization of acetylcholinesterase from cotton aphis (*Aphis gossypii* Glover). **Arch. Insect Biochem. Physiol.** 51(1): 37-45.

Lee, H.R., S.Y. Na, H.M. Park and Y.W. Kwon. 1986. Control efficacy of the several insecticides on the dominant aphis of apple tree and vegetables. Research reports of the rural development administration plant environment, mycology and farm productx utilization. **Korea Res.** 28(2): 60-64.

Li, X., M.A. Schuler, and M.R. Berenbaum. 2007. Molecular Mechanisms of Metabolic Resistance to Synthetic and Natural Xenobiotics. **Ann. Rev. Entomol.** 52: 231-253.

Liu, N.N., F. Zhu, Q. Xu, W.J. Pridgeon and X.W. Gao. 2006. Behavioral change, physiological modification, and metabolic detoxification: mechanisms of insecticide resistance. **Acta Entomol. Sinica** 49(4): 671-679.

Loxdale, H.D. and G. Lushai. 1998. Molecular markers in entomology. **Bull. Entomol. Res**. 88: 577–600**.**

Mackness, M.I., C.H. Walker, D.G. Rowlands and N.R. Price. 1983. Esterase activity in homogenates of three strains of the rust red flour beetle, *Tribolium castanemun* (Herbst). **Comp. Biochem. Physiol.** 74: 1172.

Mahadevan, N.R. and M. Balasubramanian. 1983. Efficiency of some newer insecticides against tobacco caterpillar, *Spodoptera litura* (F.) and green peach aphid, *Myzus persicae* S. on tobacco. **Pestology** 5(1): 20-21.

Manson, H.S., J.C. North and M. Vanneste. 1965. Microsomal mixed-function oxidation: the metabolism of xenobiotics. **Fed. Proc.** 24: 1172.

Martinez-Torres, D., A.L. Devonshire and M.S. Williamson . 1999. Molecular studies of knockdown resistance to pyrethroids: cloning of domain II sodium channel gene sequences from insects. **Pestic. Sci.** 15(3): 265-270.

Mashal, R.D. and J. Sklar. 1996. Practical methods of mutation detection. **Cur. Opinions Genet. Develop.** 6: 274–280.

Matsumura, F. 1985. **Toxicity of Insecticides.** Plenum Press, New York.

Miller, T.A. 1988. Mechanism of resistance to pyrethroid insecticides. **Parasitol. Today** 4: 8-12.

Mohn, D.L. 2001. **Oriental Leafworm Moth (Noctuidae Amphipyrinae *Spodoptera litura* (Fabricius).** Available source: www.ccs-hk.org/.../Spodoptera-litura.html, June 15, 2005.

Moores, G.D., X.W. Gao, I. Denholm and A.L. Devonshire. 1997. Characterization of insensitive acetylcholinesterase in insecticide resistant cotton aphids, *Aphis gossypii* Glover (Homoptera: Aphididae). **Pestic. Biochem. Physiol.** 56: 102-110.

Morimoto, M., H. Fukumoto, M. Hiratani, W. Chawasiri and K. Komai. 2006. Insect antifeedant, Pterocarpans and pterocarpol in heartwood of *Pterocarpus carnicarpus* Kruz. **Biosci. Biotec. Biochem.** 70(8): 1864-1868.

Motoyama, N. and W.C. Dauterman. 1980. Glutathione S-transferase: their roles in the metabolism of organophosphorus insecticides. **Rev. Biochem. Toxicol.** 2:49-69.

_____., A.P. Kulkani, E. Hodgson and W.C. Dauterman. 1978. Endogenous inhibitors of glutathione S-transferases in house flies. **Pestic. Biochem. Physiol.** 9: 255-262.

Nakasuji, F. and T. Matsuzaki. 1977. The control threshold density of the tobacco cutworm *Spodoptera litura* on eggplants and sweet peppers in vinylhouse. **App. Entomol. Zool.** 12: 184-189.

Nandagopal, V., S.K. Yadav, J.B. Misra, A. Bandyopadhyay, V. Singh, M.S. Basu and P. Narayan. 1997. Status of *Spodoptera litura* resistance in ground in India. *In* J.A. Wightman and G.V. Ranga Rao, eds. ***Spodoptera litura* in India: procesding of the national scientists forum on *Spodoptera litura* (F.).** ICRISAT Asia.

Newcomb, R.D., P.M. Campbell, D.L. Ollis, E. Cheah, R.J. Russell, and J.G. Oakeshott. 1997. A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. **Proc. Natl. Acad. Sci.** U.S.A. 94: 7464-7468.

Nicholas, B., L. Crombie, and W.M. Crombie. 1985. Rotenoids of *Lonchocarpus salvadorensis:* Their effectiveness in protecting seeds against bruchid predation. **Phytochem.** 24(12): 2881-2883.

Oakeshott, J.G., I. Horna, T.D. Sutherland and R.J. Russell. 2003. The genomics of insecticide resistance. **Genome Biol.** 4(1): 202.1-202.4.

O'Brien, R.D. 1967. **Insecticides, Action and Metabolism.** Academic Press, New York.

Ounchaichon, K. 1989. Insecticide resistance. **Entomol. Zool. Gaz.** 11(1): 44-47. (in Thai)

Pacific Island Ecosystems at Risk (PIER). 2000. **Invasive Plant Species: *Derris elliptica.*** Available Source: http://www.hear.org/pier/deell.htm, January 7, 2007.

Patarasiriwong, V. 1990. **Monitoring, Mapping and Resistance Assessment of Diamondback Moth (*Plutella xylostella* L.) Against Certain Insecticides in Thailand.** M.S. thesis. Kasetsart University, Bangkok Thailand.

Perry, A.S, I. Yamamoto, I. Ishaaya and R. Perry. 1998. Synthetic pyrethroids. pp. 92-107. In: **Insecticides in Agriculture and Environment.** Norosema publishing house. New Delhi.

Pimsaman, S. 1988. **Insecticides.** Faculty of Agriculture, Khon Kean University. 175pp.

Plant pest forecasting and early warning group. 2005. **Good Agricultural Practice (GAP) For Asparagus.** Department of agriculture. Available Source: http://plantpro.doae.go.th/Forecast/summarylastmonth/1245.html, March 20, 2005.

Plant, N. 2003. **Molecular Toxicilogy.** School of Biochemical and Life Science, University of Surrey, Guildford, UK. 150pp.

Prasad, G.R. 1997. Integrated management of *Spodoptera litura* (F.) *In* J.A. Wightman, and G.V. Ranga Rao, eds. ***Spodoptera litura* in India: Proceeding of the National Scientists Forum on *Spodoptera litura* (F.).** ICRISAT Asia.

Price, N.R. 1984. Carboxylesterase degradation of malathion *in vitro* by susceptible and resistance strains of *Tribolium castanemun* (Herbst) (Coleoptera: Tenebrionidae). **Comp. Biochem. Physiol.** 77(c): 95-98.

Promsattha, R. and A. Sangwanit. 2004. **Study on Botanical Pesticides from *Derris* spp.** Agriculture Toxic Substance Division. Department of Agricualture, Ministry of Agriculture and Coorperatives. (In Thai)

Rahde, A.F. 1990. **Rotenone.** Poison Centre of Porto Alegre. London Group.

Rajurkar, R.B., Z.H. Khan and G.T. Gujar. 2003. Studies on levels of glutathione-S-transferase, its isolation and purification from *Helicoverpa armigera*. **Cur. Sci.** 85(9): 1355-1360.

Rattanapan, A., S. Visetson, J. Milne, L. Ngernsiri, C. Sudthongkong and V. Bullangpoti. 2005. Molecular detection of gene responsible for expression of detoxification enzyme in *Spodoptera litura* (F.) *In* **The 7th national plant protection.** November 2-4, 2005, Cheing Mai Thailand.

Reidy, G.F., H.A. Rose, S. Visetson and M. Murry. 1990. Increased glutathione-S-transferase activity and glutathione content in an insecticide resistant strain of *Tribolium castaneum* Herbst. **Pestic. Biochem. Physiol.** 36: 269 - 276.

Richard, H.C, P.J. Daborn and G.L. Goff. 2004. The genetics and genomics of insecticide resistance. **Trens in Genet.** 20: 163-170.

Ruamthum, W. 2002. **Effect of Nutgrass (*Cyperus rotundus* L.) Corn Extracts on Esterase and Glutathione-S-transferase Level in Digestive System of the Golden Apple Snails (*Pomacea canaliculat* Lamarck)**. M.S. thesis, Kasetsart University. (in Thai)

Saisongkhroh, B., Visetson S., Milne M., Mekton M. and A. Rattanapan. 2005. Toxicity of chili extract to glutathione-S-transferase activity in *Spodoptera litura* (F.). *In* **The 11th National Plant Protection Conference.** November 2-4, 2005, Cheing Mai Thailand.

Saisongkroh, B. 2006. **Esterase and Glutathione-S-transferase Activities in *Spodoptera litura* (F.) After Exposure to Some Plant Extracts.** M.S. thesis, Kasetsart University, Bangkok Thailand. (In Thai)

Sakata, K. and T. Miyata. 1994. Biochemical characterization of carboxylesterase in the small brown planthopper *Laodelphax striatellus* (Fallen). **Pestic. Biochem. Physiol.** 50: 247-256.

Salama, H.S., N.Z. Dimetry and S.A. Salem. 1970. On the host preference and biology of the cotton leaf worm *Spodopteral;ittoralis*. **Zei. Ang. Entomol.** 67: 261-166.

Sangsrichan, C. 2002. **Ecological of *Oligonychus mangiferus* (Rahman & Sapra) and Botanical Control.** M.S. thesis, Kasetsart University. Bangkok Thailand. (in Thai)

Sawicki, R.M. 1986. Resistance to synthetic pyrethroids can be contered successfully. **Agribusiness worldwide** 8.

Scott, J.A. 1995. The molecular genetics of resistance: resistance as response to stress. **Florida Entomologist** 78(3): 399-414.

Siddappaji, C., A.R. Kumar and H.K. Sanhappa. 1985. Synthetic pyrethroids for control of pod borer on pegion pea. **Pestic.** 19(12): 29-30.

Singh, A.P. and P. Nath. 1998. Insecticidal management of tobacco caterpillar, *Spodoptera litura* (Fab.) infestation in groundnut. **Shashpa** 2(2): 203-206.

Singh, S.P. and S.K. Jalali. 1997. Management of *Spodoptera litura* (Fabricius) (Lepidoptera : Noctuidae). *In* J.A. Wightman and G.V. Ranga Rao, eds. ***Spodoptera litura* in India: Proceeding of the National Scientists Forum on *Spodoptera litura* (F.).** ICRISAT Asia.

Small, J.G. and J. Hemingway. 2000. Diferential glycosylation produces heterogeneity in elevates esterase associated with insecticide resistance in brown planthopper *Nilaparvata lugens* Stal. **Insect Biochem. Molec. Biol.** 30: 443-453.

Soderlund, D.M. and J.R. Bloomquist. 1989. Neurotoxic action of pyrethroid insecticides. **Ann. Rev. Entomol.** 34: 77-96.

_____. and D.C. Knipple. 2003. The molecular biology of knockdown resistance to pyrethroid insecticide. **Insect Biochem. Molec. Biol.** 33: 563-577.

Solangi, B.K., F.C. Oad, V.Suthar, N.M. Soomro, A.W.Gandahi, N.L.Oad, Z.A.Bhutto and D.Sindh. 2001. Population and Damage Fluctuation of Tobacco Cutworm (*Spodoptera litura* (F.)) In Relation to Age of Turnip Crop. **J. Biol. Sci.** 1(5): 382-383.

Starr, F., K. Starr and L. Loope. 2003. ***Derris elliptica.*** United States Geological Survey, Biological Resources Division Haleakala Field Station, Maui, Hawai'i.

Sun, L., X. Zhou, J. Zhang and X.W. Gao. 2005. Polymorphisms in a Carboxylesterase Gene Between Organophosphate-Resistant and -Susceptible *Aphis gossypii* (Homoptera: Aphididae). **J. Econ. Entomol.** 98(4): 1325-1332.

Suphangkasen, P., M. nachapong, P. Jansukho, P. Ramsiri, T. Wongsaree, P. Suwanpong, P. Jidee and S. Thongpanchang. 1980. Field trial on dosage rates of synthetic pyrethroids against bollworm on cotton. pp. 140-143. *In:* **Ann. Report on Cotton Insect Res.** Entomology and Zoology Division, Department of Agriculture, Bangkok, Thailand.

Surendra, C.N.U. and G.P.U. Reddy. 1994. Preliminary assessment of resistance in tobacco caterpillar, *Spodoptera litura* (F.) to insecticide. **Andhra Agric. J.** 31: 85-91.

Suzuki, K. and H. Hama. 1998. Carboxylesterase of the cotton aphid, *Aphis gossypii* Glover. Isoelectric point variants in an organophosphorus insecticide resistant clone. **Appl. Entomol. Zool.** 33: 11-20.

Taylor, M.J.F., D.G. Heckel, T.M. Brown, M.E. Kreitman and B. Black. 1993. Linked of pyrethroid insecticide resistance to a sodium channel locus in the tobacco budworm. **Insect Biochem. Mol. Biol.** 23:763-775.

Thomas, J.D. and D.J. Boethel. 1995. Inheritance of permethrin resistance in soybean looper (Lepidoptera: Noctuidae). **J. Econ. Entomol.** 88: 1536-1541.

Thummasarangkoon, W. 2000. **Efficiency of Lemon Grass (*Cymbopogon winterianus* Jewitti) Extracts on Mortality and the Level of Detoxification Enzymes in Larva of *Culex pipien quinquefasciatus*.** M.S. thesis, Kasetsart University, Bangkok Thailand.

Treskatis, S., C. Ebert and P.G. James. 1992. Butylrylcholinesterase from chicken brain is smaller than that from serum: its purification, glycosylation, and membrane association. **J. Neurochem.** 58: 2236-2247.

Vararattanavech, A. 2002. **Enzymatic Studies of Recombinant Yeast and Insect Glutathione–S – transferase**s. M.S. Thesis, Mahidol University, Bangkok Thailand. (In Thai)

Vaz Jnr, I.S., S. Imamura, K. Ohashi and M. Onuma. 2004. Cloning, expression and partial characterization of a Haemaphysalis longicornis and a Rhipicephalus appendiculatus glutathione S-transferase. **Insect. Mol. Biol.** 13(3): 329.

Visetson, S. 1991. **Insecticide Resistance Mechanism in the Red Rust Flour Beetle (*Tribolium castaneum* Burst).** Ph.D. Thesis. The University of Sydney, Australia.

_____. 2001. Effect of azadirachtin from various Thai neem extracts on some detoxification enzyme activity in *Callosobruchus maculates* (F.), pp. 38-46. *In* **The 20th ASEAN/2nd APRC Seminar on Posthavest Technology.** Septemper 11-14, 2001. Lotus hotel pang suan kaew. Chingmai Thailand.

_____. and M. Milne. 2001. Effect of root extract from derris (*Derris eliptica* Benth) on mortality and detoxification enzyme levels in the diamondback moth larvae (*Plutella xytostella* L.). **Kaestsart J. (Nat.Sci.)** 35:157-163.

_____., _____, J. Milne and P. Kanasutre. 2003. Synergistic effects of sesame oil with cypermethrin on the survival and detoxification enzyme activity of *Plutella xytostella* L. larvae. *In* **The 6th International Conference on Plant Protection in the Topic "Globolization and Plant Protection in Developing Economics"** 11-14 August 2003. Pan Pacific Hotel.

_____., _____, _____, V. Bullangpoti and A. Rattanapan. 2005. Similarities and diferences in toxicity and characteristic of monooxygease activity in the diamondback moth larvae (*Plutella xylostella* Linn.), subteranean termites (*Coptotermes* spp.) and mouse against some allelochemicals and conventional pesticides. *In* **The 7th National Plant Protection.** November 2-4, 2005, Cheing Mai Thailand.

_____., Khanananukhulchai and J. Milne. 2004. Detoxification mechanism of larvae of diamond backmoth (*Plutella xytostella* L.) against various allelochemicals in Thailand. *In* **The 2nd Asian International Conference on Ecotoxicology and Environmental Safety.** September 26-29, 2004, BP Samila beach hotel, Songkla Thailand.

_____., W. Purivirojkul, P. Kannasutra and H. Rose. 2002. Insecticide resistance mechanisms in various strains of rust-red flour beetle (*Tribolium castanemun* (Herbst)). **Agr. Sci. J.** 33(6 Supply.): 318-324.

Vollinger, M. 1986. The possible development of resistance against neem seed kernel extract and deltamehtrin in *Plutella xylostella.* Pp. 543-554. *In* H. Schmutterer and K.P.S. Ascher (eds.). **Natural Pesticides from the Neem Tree and Other Tropical Plants**: Proc. 3rd Int. Neem Conf, Nairobi, Kenya.

Vontas, G.J., J.S. Graham and H. Jenet. 2001. Gultathione-S-transferase as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. **Biochem. J.** 357: 65-72.

Ware, G. 1978. **Pesticides – Theory and Application**. W H Freeman & Co, New York.

_____. 1983. **Pesticides – Theory and Application**. W H Freeman & Co, New York.

Whyard, S., R.J. Russell and V.K. Walker. 1994. Insecticide resistance and malathion carboxylesterase in the sheep blowfly,*Lucilia cuprina.* **Biochem. Genet.** 3(1-2): 9-24.

Wilkins, R.M., S. Ahmed and D. Mantle. 1999. Comparative effect of fenitrothion treatment on intreacellular protease activities in insecticide-resistant and susceptible strains of *Musca domestica* L. **Comp. Biochem. Physiol.** Part C 124: 109-116.

Willaden, P., R.V. Mckenne and G.A. Riding. 1987. Purification and characterization an esterase from the tick *Boophilus microplus*, pp. 56-57. *Cited by* S. Visetson. **Insecticide Mechanism Rust Red Flour Beetle, *Tribolium castaneum* (Herbst).** Ph.D. Thesis. University of Sydney, Sydney.

Williamson, M.S., D. Marinez-Torres. C.A. Hick and A.L. Devonshire. 1996. Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to purethroid insecticides. **Mol. Gen. Genet.** 252: 51-60.

_____., I. Denholm, C.A. Bell and A.L. Devonshire. 1993. Knockdown resistance (kdr) to DDT and pyrethroid insecticides maps to a sodium channel gene locus in the house fly (*Musca domestica*). **Mol. Gen. Genet.** 240: 17-22.

Worawong, K. 2003. **Effectiveness of *Derris elliptica* Benth. Extract on Broad Mite *Polyphagotarsonemua latus* Banks.** Master thesis, Khon Kean University. Khon Kean Thailand. (in Thai)

World Health Organization (WHO). 2000. **Recommended Classification of Pesticide by Hazard**, WHO/PCS/01.4.

_____. 2001. **Recommended Classification of Pesticide by Hazard**. WHO/PCS/01.4.

_____. 1992. **United Nations International, Environment Programme Labour Organization on Chemical Safety**. Health and Safety Guide No 73.

Wothing, C.R. 1987. **Pesticide Manual**. A world compendium. British crop protection council. UK, 1081 p.

Yang, Y., Y. Wu, S. Chen, G.J. Devine, I. Denholm, P. Jewess and G.D. Moores. 2004. The involvement of microsomal oxidase in pyrethroid resistance in *Helicoverpa armigera* from Asia. **Insect Biochem. Mol. Biol.** 34: 763-773.

Yu, S.J. 1983. Induction of detoxifying enzymes by allelochemicals and host plant in the fall armyworm. **Pestic. Biochem. Physiol.** 12: 330-336.

_____. 1984. Interaction of allelochemicals with detoxification of insecticides susceptible and resistant fall armyworm. **Pestic. Biochem. Physiol.** 14: 275-281.

_____. 1990. Liquid chromatography determination of permethrin esterase activity in six phytophagous and entomophagous insects. **Pestic. Biochem. Physiol.** 36: 237-241.

Zhang, P., J.J. Wang, Z.M. Zhao, W. Dou and Y. Chen. 2004. Effects of simulated acid rain on the physiology of carmine spider mite, *Tetranychus cinnabarinus* (Boisduvals) (Acari: Tetranychidae). **J. App. Entomol.** 128(5): 342-247.

Zhu, K.Y. and W.A. Brindley. 1990. Acetylcholinesterase and its reduced sensitivity to inhibition by paraoxon in organophosphate-resistant *Lygus herperus* Knight (Hemiptera: Miridae). **Pestic. Biochem. Physiol.** 36:22-28.

Zubairi, S.I., M.R. Sarmidi, S. Ngadiran, H. Yaakob, K. Anuar and A. Kamal. 2007. **Enviromental Friendly Bio-Pesticide.** Chemical Engineering Pilot Plant. University of Teknologi Malaysia. Available Source: http://www.cepp.utm.my/rnd_bio.php, March 12, 2007.

APPENDICES

Appendix A

Enzyme activity analyzed method

Appendix A

Enzyme activity analyzed method

This research using modified method of Yang *et al.* (2004) and Visetson and Milne (2001) that's described below;

Appendix A1 The reagent for analyzed

1. Buffer : Potassium phosphate buffer (KH_2PO_4)
   Potassium dihydrogenthiophosphate (M.W = 136.09)

   0.1 M = M.W / L
   = 13.609 g / ddw 1 L
   Adjust pH

2. 1mM EDTA
   0.45224 g/ ddw 1 L

3. 1L 0.1 M Phosphate buffer + 1 ml 1mM EDTA

4. 10 mM GSH
   0.15g GSH reduced form in 50 ml 0.1 M Phosphate buffer

5. PVPP: 50%w/w material

Appendix A2 Enzyme Isolation Method

Homogenized material with 2000 µl 0.1 M PPB + EDTA
1000 µl 10 mM GSH
50% w/w PVPP
Centrifuged at 18,000 rpm, 4ºC 5 min in refrigerated centrifuge

Appendix A3 pNPA assay

This protocol is modified from Visetson (2001) and Visetson *et al.* (2003, 2004) for analyze the carboxylesterase activity using spectrophotometer at absorbance 400 nm (3 min)

Stock solution:

- 1 ml 1mM EDTA + 0.1 M Phosphate buffer
- 10 mM Glutathione reduced form (0.15 g GSH / 50 ml buffer)
- Substrate: 0.12 M paranitrophenylacetate(pNPA) (0.1 g / 5 ml ethanol)

Appendix Table A1 pNPA assay protocol

Solution reference	Blank	Sample
0.1 M Phosphate buffer	2900 µl	2900 µl
Substrate	50 µl	50 µl
0.1 M Phosphate buffer + EDTA (Homogenized buffer)	50 µl	-
Supernatant (enzyme)	-	50 µl

Enzyme activity (nM paranitrophenol product/ mg protein/ml)
= O.D x 58.8235 x (total volume)

Appendix A4 NA assay

This protocol is modified from Wen and Hin (1994); Gilham and Lehner (2005) for analyze the carboxylesterase activity using spectrophotometer at absorbance 600 nm (3 min)

Stock solution:

- 4 ml of 0.42 mM Naphthyl acetate in ethanol
- 96 ml 100 mM Tris –HCl (pH 7.0) containing 100 mg Gum Arabic and 200 mg sodium dioctyl sulfosuccinate
- 0.02 M Phosphate buffer pH 7.8
- Substrate: 0.10 M napthylacetate NA) (0.1 g/ 5ml ethanol)

Method

- 1.5 ml Stock solution (Substrate solution)
- 1 ml enzyme containing sample prepared in 100 mM Tris –HCl (pH 7.0)
- Reactions are performed at 28 °C and are terminated after 10 min.
- Terminated reaction by 0.5 ml 0.3% fast blue B salt solution (with 3.5 % SDS)

"*Enzyme is determinate from standard curves made from alpha – naphthol"*

Appendix A5 CDNB assay protocol

This protocol is modified from Visetson *et al.* (2002, 2003) for analyzed glutathione-s-transferase activity using spectrophotometer at absorbance 340 nm (3 min)

Stock solution:

- 1 ml 1mM EDTA + 0.1 M Phosphate buffer
- 10 mM Glutathione reduced form (0.15 g GSH / 50 ml buffer)
- Substrate: 150 mM CDNB (0.152 g / 5 ml ethanol)

Appendix Table A2 CDNB assay protocol

Solution reference	Blank	Sample
0.1 M Phosphate buffer	1150 µl	1150 µl
0.1 M Phosphate buffer + GSH	20 µl	-
Supernatant (enzyme)	-	20 µl
Equilibrate 35 °C for ca. 3 min…..		
Substrate	10 µl	10 µl

Enzyme activity (CDNB conjugated product/ mg protein/ ml)
=(O.D x 1.316) / (9.6 x 1000)

Appendix A6 DCNB assay
This protocol is modified from Visetson *et al.* (2002, 2003) for analyzed glutathione-s-transferase activity using spectrophotometer at absorbance 344 nm (3 min)

Stock solution:
- 1 ml 1mM EDTA + 0.1 M Phosphate buffer
- 10 mM Glutathione reduced form (0.15 g GSH / 50 ml buffer)
- Substrate: 150 mM DCNB (0.144 g / 5 ml ethanol)

Appendix Table A3 DCNB assay protocol

Solution reference	Blank	Sample
0.1 M Phosphate buffer +GSH	1100 µl	1100 µl
0.1 M Phosphate buffer	200 µl	-
Supernatant (enzyme)	-	200 µl
Equilibrate 35 °C for ca. 3 min…..		
Substrate	10 µl	10 µl

Enzyme activity (mM conjugated product/ mg protein/ ml)
= (O.D x 1.316) / (10 x 1000)

Appendix A7 Acetylthioiodide assay

This protocol is modified from Visetson et al. (2001) for analyzed cholinesterase activity using spectrophotometer at absorbance 412 nm (3 min)

Stock solution:

- 0.1mM Phosphate buffer pH7.5
- Substrate: 0.1 M acetylthiocholine iodide (ATCL) in 0.1 M phosphate buffer (0.1446 g/ 5 ml PPB) : ((F.)w. 289.2)
- 0.01 M DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) in phosphate buffer (0.0198 g/ 5ml PPB)

Appendix Table A4 AChE assay protocol

Solution reference	Blank	Sample
0.1 M Phosphate buffer	2765 µl	2765 µl
0.01 M DTNB	75 µl	75 µl
0.1 M Phosphate buffer + EDTA (Homogenized buffer)	100 µl	-
Supernatant (enzyme)	-	100 µl
Substrate (ATCL)	60 µl	60 µl

Enzyme activity (activity/ mg protein/ ml)
= O.D x 73.529 x total volume (ml)

Appendix B

Bradford method

Appendix B
Bradford method

This research use bovine serum albumin (BSA) as standard

Appendix B1 Stock solution:

- 100 mg Coomassie Brilliant Blue R250 in 50 ml 95% ethanol then add 100 ml 85% H_3PO_4 and make final volume to 1L by distilled water.

Appendix Table B1 Bradford assay protocol

Solution reference	Volume
Sample	0.5 ml
Bradford solution	5 ml
Incubate at room temp. for 5 minutes….	
Determine by spectrophotometer at 595 nm	

Appendix C

SDS-PAGE method

Appendix C
SDS-PAGE method

Appendix C1 SDS-PAGE (Laemmli) Buffer System

1. Acrylamidw/ Bis (30%T, 2.67 %C) [Bio-Rad cat no. 161-0125, 150 g]

87.6 g	acrylamide	(29.2 g/ 100 ml)
2.4 g	N'N'- bis –methylene acrylamide	(0.8 g/ 100ml)

Make to 300 ml with deionized water.
Filter and store at 4˚C in the dark (30 days maximum)

2. 10% (w/v) SDS [Bio-Rad cat no. 161-0416]

dissolve 10 SDS in 90 ml water with gentle stirring and bring to 100 ml with deionized water.

3. 1.5M Tris-HCl, pH 8.8: resolving gel buffer [Bio-Rad cat no. 161-0798, 1L]

27.23 g	Tris base	(18.15 g/ 100 ml)
80 ml	deionized water	

Adjust to pH 8.8 with 6 N HCl, Bring total volume to 150 ml with deionized water and store at 4˚C.

4. 0.5 M Tris –HCl, pH 6.8: Stacking gel buffer [Bio-Rad cat no. 161-0799]

6 g	Tris base
60 ml	deionized water

adjust to pH 6.8 with 6N HCl. Bring total volume to 100 ml with deionized water and store at 4 ˚C.

5. Sample buffer (SDS reducing buffer)

3.55 ml	deionized water
1.25 ml	0.5 M Tris-HCl, pH 6.8
2.5 ml	Glycerol
2.0 ml	10% (w/v) SDS
0.2 ml	0.5 %(w/v) bromophenol blue
9.5 ml	Total volume

Store at room temperature
USE: Add 50 µl β-Mercaptoethanol to 950 µl sample buffer prior to use. Dilute the sample at least 1:2 with sample buffer and heat at 95˚C for 4 minutes.
6. 10x Electrode (Running) buffer, pH 8.3 [Bio-Rad cat no. 161-0772, 5L]

30.3 g Tris base
144.0 g Glycine
10.0 g SDS

Dissolve and bring total volume up to 1000 ml with deionized water. Do not adjust pH with acid and base. Store at 4˚C.

If precipitation occurs, warm to room temperature before use.

USE: Dilute 50 ml of 10x stock with 450 ml deionized water for each electrophoresis run. Mix thoroughly before use.

7. 10% APS (fresh daily)

100 mg ammonium persulfate
Dissolved in 1 ml of deionized water

Appendix C2 Gel Formulation (10 ml)

1. Prepare the monomer solution by mixing all reagent except TEMED and 10% APS Degas the mixture for 14 minutes.

30% Degassed

Percent Gel	DDI H_2O (ml)	Acrylamide/bis (ml)	*Gel Buffer (ml)	10% w/v SDS (ml)
4%	6.1	1.3	2.5	0.1
5%	5.7	1.7	2.5	0.1
6%	5.4	2.0	2.5	0.1
7%	5.1	2.3	2.5	0.1
8%	4.7	2.7	2.5	0.1
9%	4.4	3.0	2.5	0.1
10%	4.1	3.3	2.5	0.1
11%	3.7	3.7	2.5	0.1
12%	3.4	4.0	2.5	0.1
13%	3.1	4.3	2.5	0.1
14%	2.7	4.7	2.5	0.1
15%	2.4	5.0	2.5	0.1
16%	2.1	5.3	2.5	0.1
17%	1.7	5.7	2.5	0.1

- Resolving Gel Buffer -15M Tris-HCl, pH 8.8 (Seperating/ Runnung Gel)
- Stacking Gel Buffer- 0.5 M Tris-HCl, pH 6.8

2. Immediately prior to pouring the gel, add:

For 10 ml monomer solution:

Resolving Gel: 50µl 10% APS and
5 µl TEMED

Stcking Gel:	50 µl 10% APS and 10 µl TEMED

Swirl gently to initiate polymerization.

NOTE: Prepare any desired volume of monomer solution by using multiples of the 10 ml receipe. The volumes of APS and TEMED must be adjusted accordingly.

WARNING: The catalyst concentration is very important! Webbing and incomplete well formation can result from inaccurate catalyst concentration.

An important principle of SDS-PAGE is to reduce each protein to the 'least common denominator,' that is, to a solution of individual polypeptide chains. Ideally, the only structure left in a protein sample for SDS-PAGE is the primary structure of individual polypeptides. The proteins are *denatured*, that is, they are no longer functional. Sample preparation usually falls short of that ideal, which you will discover as you analyze your results.

CIRRICULUM VITAE

NAME : Miss Ajin Rattanapan

BIRTH PLACE : Nakhon Si Thammarat, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE
1989	Rajamangala Univ. of Technology	B.Sc.(Agriculture)
2000	Khon Kaen Univ.	M.Sc. (Entomology)

POSITION : Lecturer

WORK PLACE : Faculty of Science, Mahasarakham University

ACADEMIC EXPERIENCE :
- 2005 Oral Presentation at 7th National Plant Protection Conference. November 2-4, Chiang Mai, Thailand
- 2006 Oral Presentation at 58th International Symposium on Crop Protection. May 23, Ghent, Belgium.
- 2006 Poster Presentation at 11th IUPAC International Congress of Pesticide Chemistry. August 6-11, Kobe, Japan.

SCHOLARSHIP :
- 1998 Research Scholarship for M.Sc. Thesis from Biodiversity Research and Training Program (BRT), Thailand.
- 2003 Cooperative Research Network (CRN) Scholarship of Mahasarakham University by Commission on higher Education, Ministry of Education, Thailand.
- 2006 Research Scholarship for Ph.D. Thesis from Graduate School, Kasetsart University, Thailand.
- 2006 Promotion Supporting Fund for Poster Presentation at 11th IUPAC International Congress of Pesticide Chemistry. August 6-11, Kobe, Japan.