IDENTIFICATION AND CHARACTERIZATION OF PYRIPROXYFEN-RESPONSIVE PROTEINS FROM THE LARVAL HAEMOLYMPH OF *SPODOPTERA MAURITIA* BOISD. (LEPIDOPTERA:NOCTUIDAE)

Thesis submitted to
the University of Calicut in partial fulfilment of the
requirements for the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

by

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CERTIFICATE

This is to certify that this thesis entitled **"Identification and characterization of pyriproxyfen-responsive proteins from the larval haemolymph of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae)"** is an authentic work carried out by **Smt. Resmitha C.** in the Department of Zoology, University of Calicut under my supervision and guidance and no part thereof has been presented earlier for any other degree.

Calicut University
Date

Dr. Kannan V.M.

DECLARATION

I do hereby declare that this thesis entitled "**Identification and characterization of pyriproxyfen-responsive proteins from the larval haemolymph of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae)**" submitted to the University of Calicut in partial fulfillment for the Doctoral Degree in Zoology is a bonafide research work done by me under the supervision and guidance of Dr. Kannan V.M., Professor, Department of Zoology, University of Calicut and no part of the thesis has been presented by me for the award of any other degree or similar title.

Calicut University **Resmitha C.**
Date

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INTRODUCTON

Insects are the largest group of animals in the phylum arthropoda. They are the dominant multicellular life form on the planet, ranging in size from minute parasitic wasps at around 0.2mm to stick insects measuring 35cm in length. Insects are generally small in size and have a complex nervous system surrounded by an effective blood-brain barrier. Insects have a major role on earth. They pollinate the vast majority of world's 250,000 or so species of flowering plants. Insects are also important in nutrient recycling by disposing of carcasses and dung. They are the principal food source for many other animals such as birds and other vertebrates.

Insects can also have a huge negative impact on humans. One-sixth of all crops grown worldwide are lost to herbivorous insects and the plant diseases they transmit. Any insect that is harmful to human health, or does economic damage to domestic animals or crops constitutes a pest (Clark, 1970; Dempster, 1975). It is estimated that about 26 % of the potential food production was losing as food for herbivorous insects (Singh and Sharma, 2004). Almost all the crops face the loss due to insect pests. *Spodoptera mauritia* or rice swarming caterpillar is a major pest of paddy coming in the order lepidoptera. It is a sporadic pest which has six larval stages. They attack the paddy field in large swarms, feed on the paddy leaves, after finishing one field they march to the next field like an army operation. Hence they are known as army worm. In 2017 Kerala faced an attack by these

army worm in which they destroyed 200 ha of paddy field in just 4 days.

Hormones are chemical signals that are secreted directly into the blood and circulate in the body to regulate physiological, developmental and behavioural activities. These signals complement those from the nervous system, which provide short-term co-ordination. The activities of the two systems are closely linked and sometimes not clearly distinguishable. In insects, growth, development, metamorphosis and reproduction are regulated mainly by hormones. The important hormones are juvenile hormone, secreted by corpora allata, ecdysteroid or moulting hormone, secreted by the prothoracic gland and the neurohormones or neuropeptides secreted by the neurosecretory cells of segmental ganglia and brain. Corpora allata are small organs located adjacent to the brain and the prothoracic gland is located inside the first thoracic spiracle. In Lepidoptera the role of the prothoracic gland in ecdysis was discovered in silkworms in 1944, and the molting hormone, ecdysone and its structure determined in 1954 (Takeda, 2009). Ecdysone was the first hormone to be isolated from an insect species. In addition, the function of the corpora allata in Lepidoptera was also first discovered in silkworms in 1942. Larval ecdysis is induced by a molting hormone and the juvenile hormone controls larval development together with molting hormone. In insects, neuropeptides regulate many physiological and behavioral processes during development, reproduction, and senescence, and they maintain growth, homeostasis, osmoregulation, water balance, metabolism, and visceral activities.

Pest attack is a major problem faced by farmers. According to Dhaliwal and Arora loss in different crops due to pest was 25% in rice, 5 to 10% in wheat, 35% in oil seeds, 30% in pulses, 50% in cotton and 20% in sugarcane (Dhaliwal and Arora, 1996). In order to control the crop loss, pesticide use was promoted. In India during 1955-56 chemical pesticide use was 15 g/ha of gross cropped which was increased to 90 g/ha in 1965-66 (Birthal, 2004). In mid 1960s green revolution is introduced and it promoted the pesticide use, as a result the use of pesticides increased to 266 g/ha in 1975-76 and reached 404 g/ha in 1990-91 (Birthal, 2003). Despite the increased use of pesticides crop loss still is a major issue. The reason for this is explained as technological failure of chemical pesticides, rising pest problem and changes in production systems. (Atwal, 1986; Dhaliwal and Arora, 1996; Pradhan 1983)

Use of insect growth regulators (IGRs) is a new approach to control insect pests. IGRs are compounds that interfere with growth and development of insects. Generally they either disrupt the hormonal process or exoskeleton development. They affect certain physiological processes essential to the normal development of insects. Their mode of action is selective and potentially acts only on target species (Tunaz and Uygun, 2004). Based on the mode of action IGRs can be grouped in to chitin synthesis inhibitors and hormonal analogues. Chitin synthesis inhibitors disrupt the formation of new cuticle in the larvae by inhibiting the chitin biosynthesis. Hence the larvae fail to ecdyse. Hormonal analogues interfere with the action of insect hormones especially with the moulting hormone, ecdysone, and the juvenile

hormone. As these two hormones have major role in the metamorphosis and development of an insect, interfering their action should lead to the impaired development or death of the larvae. Advantages of hormonal analogues are that they are species-specific, less or zero toxicity to other animals, fast penetrance through the insect cuticle and they get degraded to non-toxic compounds in a short time period. Tebufenozide, methoxyfenozide, chromofenozide, halofenozide etc are some examples of IGRs which are ecdysone analogues. Many juvenile hormone (JH) analogues or mimics were discovered and among these Epofenonane, Methoprene, Hydroprene, Kinoprene, Pyriproxyfen etc. are the well known examples. Though there are articles on protein profile analysis accompanying the treatment with IGRs on some insect species, there are no studies on the effect of pyriproxyfen on *S. mauritia*. In this study we examined the effect of JH analogue, pyriproxyfen, on the haemolymph protein profile of the army worm, *Spodoptera mauritia*, to identify proteins altered on treatment with pyriproxyfen.

OBJECTIVES OF THE STUDY

1. To identify and characterize Juvenile Hormone (JH) analogue, pyriproxyfen,-responsive protein/s from the larval haemolymph of *Spodoptera mauritia.*

2. To identify the site of synthesis of the pyriproxyfen-responsive protein/s.

3. To understand the regulation of pyriproxyfen-responsive protein/s by JH analogues.

4. To identify JH analogue, pyriproxyfen,-responsive protein/s from other larval tissues.

REVIEW OF LITERATURE

2.1. AGRICULTURE & RICE CULTIVATION

Agriculture plays a major role in human welfare and also has a vital role in the economy. According to census 2011, in India, 54.6% of the total workforce is engaged in agriculture and allied sector activities. As per the land use statistics 2014-15 the net area sowed works out 43% of the total geographical area of the country (Annual report 2019-20, Department of agriculture, cooperation & farmers' welfare, Govt. of India). Farming practices have vital role in food security of a country. India occupied the largest area under rice cultivation and is one of the major centers of rice farming (Diwakar, 2014). Rice is a very important and essential part of the daily meal in the southern and eastern parts of India. For more than 50 % of the world population, rice is an important staple food crop. At the global level, rice occupies an area of about 161.8 million hectares, of which about 143.2 million hectares is in Asia. The largest area under rice crop in the world (43 m ha) is in India. (Mahajan et al., 2017) In Kerala, the most important food produced and consumed is rice. Kuttanad (Alappuzha), Thrissur and Palakkad are the large scale rice cultivating places in Kerala (Mukesh, 2015). According to the World rice statistics database, IRRI, in Kerala, the total rice harvested area in 2015 was 162100 hectare.

2.2. INSECT PLANT INTERACTON

In an agricultural field, a range of essential ecosystem services are provided by the inhabiting arthropods. Majority of arthropods species are beneficial however a small number damage the crop. The beneficial ones pollinate wildflowers and crops, recycle organic materials through decomposition, act as natural enemies that reduce herbivore damage, or reduce weed populations (Schmidt et al., 2015; Settle et al., 1996; Way and Heong, 1994; Westphal et al., 2015). Interactions between plants and insects can be beneficial to both or can be detrimental to the plant but beneficial to the insect. Insects utilize plants for food, shelter or for egg-laying sites. Plant-feeding insect species are numerous, constituting more than one-quarter of all macroscopic organisms. A very different view of insect-plant interaction focuses on the use of insects as biological control agents for weeds ("Interactions, Plant-Insect", 2020). In rice fields along with the growth of rice plants invasion of herbivores also occurs. If it is not effectively regulated, a small number of these herbivores can become pest. Many of these herbivore colonies will be consumed by generalist predators (Settle et al., 1996).

2.3. INSECTS AS PEST

It is estimated that, insect pests on an average cause 15-20% loss in yield in both principal food crops and cash crops (Rathee and Dalal, 2018). Considering monetary value, currently there is about $36 billion annual loss suffered by Indian agriculture (Dhaliwal et al., 2015). In the rice growing areas of the country the moderate to serious

attack of stem borer, gall midge, plant hoppers and other sporadic pests causes yield losses ranging from 21 to 51 percent (Pasalu et al., 2004). The major constraints limiting the agricultural productivity are diseases, insect pests and weeds. It is estimated that about 26% of the food production was eaten by herbivorous insects (Singh et al., 2004). In tropical Asia about 120–200 million tons of rice is lost annually due to insects pests, diseases, and weeds (Gianessi, 2014). Despite any physical, biological, or chemical crop protection the estimated global loss in average potential yield due to animal pest is 18% (Oerke, 2006).

2.4. PEST CONTROL METHODS

As there has been the need to maintain crops pest free, the pest control techniques are as old as agriculture. There are different traditional methods such as cultural and physical methods of pest control.

2.4.1. CULTURAL CONTROL

2.4.1.1. FARM LEVEL PRACTICES: The farm level practices helps to check the pests such as red hairy caterpillar, rice mealy bug, potato tuber moth, rice grass hopper, cotton whitefly, rice armyworm, rice stem borer, sorghum stem borer, sweet potato weevil etc. The methods include ploughing, puddling, pest free seed material, trimming and plastering, destruction of alternate host, flooding, pruning or topping, intercropping, water management, timely harvesting etc.

2.4.1.2. COMMUNITY LEVEL PRACTICES: This method includes crop rotation to break the life cycle of the pest, synchronized sowing for the dilution of pest infestation and crop sanitation which involves the destruction of insect infested parts and potential sources of infestation followed by disinfection of surfaces.

2.4.2. PHYSICAL CONTROL

Modification of physical factors can help to minimize or prevent problems related to pest. Some of them are the following.

2.4.2.1. MANIPULATION OF TEMPERATURE: Various methods are there in which the temperature plays a key role to prevent pests. The eggs of the pests of stored products can be killed by sun drying the seeds, burning torch against hairy caterpillars and flame throwers against locusts are effective methods. To kill fruit flies, the fruits and vegetables can be stored under cold condition (1 - 2^{o}C for 12 - 20 days).

2.4.2.2. MANIPULATION OF LIGHT: Light trapping, Infra-red seed treatment to kill all stages of insects especially in grains for storage. Lighting reduces the fertility of Indian meal moth, so providing light in storage go downs are helpful to reduce this pest.

2.4.2.3. MANIPULATION OF MOISTURE: Alternate drying and wetting rice fields helps to reduce brown plant hopper. Drying

seeds below 10% moisture level affects insect development. Flooding the field controls the cutworms.

2.4.3. MECHANICAL CONTROL

Mechanical control includes the use of mechanical devices or manual forces to reduce the pests. There are two types of mechanical control – mechanical destruction and mechanical exclusion.

2.4.3.1. MECHANICAL DESTRUCTION: Life stages of pest are killed by manual or mechanical force. Hand picking of caterpillars, sieving and winnowing, shaking the plants by passing rope across rice field to dislodge caterpillars etc. are some example for mechanical destruction.

2.4.3.2. MECHANICAL EXCLUSION: In mechanical exclusion, mechanical barriers prevent access of pests to hosts. Wrapping the fruits with polythene bag, netting, trenching, sand barrier to protect stored grains, water barrier such as ant pans etc. are some of them.

2.4.4. CHEMICAL CONTROL

Chemical control meant the use of chemical pesticides to control the pests. The chemicals used to control insect pests are termed as insecticides. Chemical control is the fastest way to destroy or prevent insect pests. But understanding of the potential hazards of pesticides to other organisms including humans and to the environment is essential. The continuous use of insecticide causes the development of insecticide resistance in pests. Use of pesticide is easy and fastest

way to reduce pest, but it has many disadvantages also. The use of chemical pesticides may adversely affect domestic animals, natural enemies of the pest and beneficial insects such as honey bees. Pesticides contaminate ground water and water bodies. Continuous use of pesticides destroys microorganisms in the soil, thereby reduces the fertility of the soil. Human beings also have adverse effects such as hypersensitivity, inflammation and immunosuppression (Aktar et al., 2009; Arias-Estévez et al., 2008). The insecticides can be grouped based on their chemical composition in to organochlorines (DDT, BHC etc.), organophosphates (Malathion, Temephos, Dichlorves, Fenthion etc.), carbmates (Propoxur, Carbaryl, Bendiocarb etc.) and pyrethroids (Cyfluthrin, Bifenthrin, Permethrin etc.) (Kaur et al., 2019). Organochlorines are polychlorinated derivatives of cyclohexane or polychlorinated biphenyls and cyclodiene. Organophosphates are the esters of phosphates, thiophosphates and dithiophosphate. Carbamates are esters of carbamic acids or thiocarbamic acids. Pyrethroids are synthetic mimics of naturally occurring pyrethrins found in the flowers of chrysanthemum species. Pyrethrins are the esters of chrysanthemic acid and pyrethric acid with alcohols.

2.4.5. APPLIANCES THAT HELPS TO CONTROL PESTS

2.4.5.1. PHEROMONE TRAP: Synthetic sex pheromones are used to attract and trap adult males. The traps are specially designed for this purpose. Water pan trap, sticky trap and funnel type models are available for pheromone based insect control.

2.4.5.2. YELLOW STICKY TRAP: Many insects prefer yellow colour. Tin boxes were painted with yellow colour and sticky

material like castor oil is smeared on the surface. The insects will attract to yellow colour and trapped on the sticky material.

2.4.5.3. LIGHT TRAPS: In the night, most adult insects are attracted towards light. This is the principle behind light traps. Different types of light traps are there.

Mercury vapour lamp light trap: Mercury vapour lamps are a specific type of lamp commonly used in moth traps. Light is emitted from mercury that is held within a bulb in excited state. Mercury vapour lamp light trap is used against a wide range of nocturnal insects. A 125W mercury lamp is better for this. Robinson trap is an example for Mercury vapour lamp light trap.

Incandescent light trap: This type of trap produces radiation by heating a tungsten filament. Small amount of ultraviolet and considerable visible lights are included in the spectrum of the lamp. A pan of kerosenated water is placed below the light source.

Black light trap: Black light is popular name for ultraviolet (UV) radiant energy. There are many varieties of black traps, but they all have black light or UV light in common. Usually flying insects are attracted and when they come in contact with electric grids, they become electrocuted and killed.

2.4.5.4. BAIT TRAP: To attract the insects some attractants placed in traps the attracted insect are killed in the trap. Fishmeal trap which is used against sorghum shoot fly is an example for bait

trap. Moistened fish meal is kept in polythene bag or plastic container inside the tin. A cotton piece soaked with insecticide (DDVP) is also kept along with the bait to kill the attracted flies.

2.4.5.5. PITFALL TRAP: The insects such as ground beetles and collembola, which are moving on the soil surface, can be trapped using pitfall trap. It consists of a plastic funnel, opening into a plastic beaker containing kerosene supported inside a plastic jar.

2.4.5.6. EMERGENCE TRAP: Most of the insects pupate in the soil hence we can trap the adults during the emergence by using suitable covers over the ground. A wooden frame shaped like a house roof covered with wire mesh is placed on soil surface and a plastic beaker fixed at the top of the frame to collect the emerging insects.

2.5. PESTS OF PADDY

There are around 1000 insect species recorded as pest on paddy, in which only about 24 insects and mites species act as key pest in different rice fields in India (Sain and Prakash, 2008). The humid and warm conditions of rice fields in South and South East Asia is adequate to the proliferation and survival of key insect pests such as brown plant hoppers, green leafhoppers, stem borer and leaf folders. In the rice fields in Asia stem borers appear every year and cause some

damage. The loss in yield due to borer attack can be up to 95% and those of leaf folders was 63 to 80% (Gianessi, 2014) Rice swarming caterpillar (*Spodoptera mauritia*) is a sporadic pest of paddy, but whenever it appears it will be in large numbers causing severe destruction (Pradhan and Jotwani, 1992). In eastern India, during the last few years, it has emerged as a major pest and caused high yield loss in the wet season rice production (Tanwar et al., 2010).

2.5.1. SPODOPTERA MAURITIA

In recent years the change in climatic condition made *Spodoptera mauritia* Boisd. (Rice swarming caterpillar) to become a major pest in states like Orrisa, Assam, and Telangana causing severe loss to paddy at nursery stage (Sain et al., 2008, Tanwar et al., 2010). In an outbreak of rice swarming caterpillar they become high in number and they swarm in large group from one field to other to attack the crop. The larvae can totally damage the rice plant hence it is very destructive (Catindig, n.d.). *Spodoptera mauritia* is a holometabolous insect. The first instar larvae hatches out on the third day after egg laying. It has six larval stages in which the late 4^{th}, 5^{th} and early 6^{th} instars are voracious feeders. The 6^{th} instar larvae moult in to prepupae and become pupae. After the development of about 7 days, the adult emerges from the pupae.

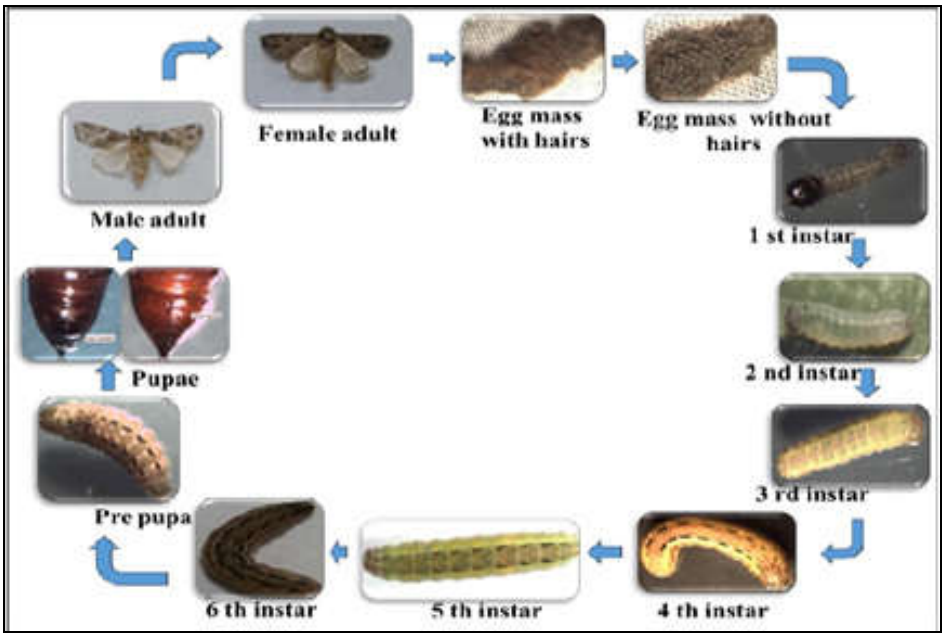


Figure 1: Life cycle of *Spodoptera mauritia* (Ramaiah and Maheswari, 2018)

2.5.2. SPODOPTERA MAURITIA PEST STATUS

Spodoptera mauritia has emerged as a regular pest in Odisha, Jharkhand, Bihar and Chhatisgarh. In Cuttack and Sonepur districts of Odisha, a severe outbreak occurred in 2008. During 2009 in Western Odisha 13 districts were suffered from severe outbreak of this pest recording about 80-90% damage in about 1.25 lakh ha of kharif paddy (Tanwar et al., 2010). It has been considered as a serious pest of paddy in Bangladesh. In India, earlier it was considered as a sporadic and minor pest of rice but it has emerged as serious pest of rice seedlings for the last one decade. The army worm attacks different plants in the family poaceae, but, rice is the main host of the caterpillar (Tanwar et

al., 2010). According to Mogili Ramaiah, in a study conducted at Regional Agricultural Research Station, Warangal, Telangana, rice swarming caterpillar observed maximally during the third week of August (Ramaiah et al., 2018). Mass army worm (*Spodoptera mauritia)* attack was reported form Thiruvalla and Upper Kuttanad of Kerala in 2017. Within 4 days the caterpillar destroyed 200 hectare paddy fields (Manorama News, 2017). Pillai reported in 2017 that 4,500 hectares of paddy out of 26,000 hectors sown in Kuttanad, Alappuzha, got destroyed by army worm infestation (Pillai, 2017).

2.5.3. PEST MANAGEMENT OF *SPODOPTERA MAURITIA*

The beginnings of agriculture is believed to be about 10,000 years ago and since then farmers have to compete with harmful organisms, collectively called pests for protection of crop products. These pests can be controlled by different methods including physical, biological or chemical measures (Oerke, 2006). Implementation of integrated pest management (IPM) will be effective for controlling the pest. Regular monitoring, mechanical and cultural methods, augmentation of natural enemies and controlled use of insecticides are the major components of IPM. (Tanwar et al., 2010) Opting resistant varieties, pheromone traps and biological control agents will helps the farmers to decrease damage by herbivore insects (Lv et al., 2015).

2.5.3.1. MONITORING

Regular monitoring in the field provides knowledge about the current pests and crop situation and it will be helpful in selecting the best combinations of the control measures for pest management. As the

moths are nocturnal they can be attracted by light, hence light trap can be used for monitoring the moth population. The severity of the pest can be understood by checking the number of moths attracted towards the light (Tanwar et al., 2010) According to Singh et al., 2004 pheromone traps are better than light and sticky traps. Sex pheromone baited traps allow visualizing population trends and can be used to time the application of pesticides or release of bioagents (Sood, 2010). As in the early stage the pest appear on the alternate host plants and they were completely defoliated by feeding of the caterpillar, observing the feeding symptoms can give an idea about the presence of larvae. In *S.mauritia* the pupation takes place in the soil, so digging the soil up to 6-9 inches helps to confirm the population (Tanwar et al., 2010).

2.5.3.2. PHYSICAL OR MECHANICAL CONTROLS

Knowledge of the pest biology is the base of this method. The simplest method to control insect pest is hand picking (Singh et al., 2004). Traps, nets, radiation etc. can be used as tools for pest control. Some pests such as insects can be controlled by changing the amount or water (Extension Pesticide Program of University of Hawaii's Manoa campus). *S. mauritia* larvae cannot swim hence in a flooded field they have to be stay on the defoliated plants. In this stage pouring kerosene oil (2 L/ hectare) to the water and dropping the larvae from the plant by shaken rigorously with the help of a rope stretched across the field. The fallen larvae will ultimately die due to the effect of kerosene oil. The grasses around the fields can be mechanically

destroyed before the paddy season as these grasses act as an alternate host for the pest (Tanwar et al., 2010).

2.5.3.3. CULTURAL CONTROL OF PEST

Cultural methods mainly aim to make crop environment less susceptible to pests. For that different crop production practices are used. Crop rotation, fallowing, managing of planting time and harvesting time are some of the cultural methods to control the pests. The suitable methods are selected based on knowledge of pest biology (Singh et al., 2004). In summer, ploughing the field helps to expose the larvae and pupae to birds. Flooding the infested field brings out the larvae to the surface, which also helps for predation by birds. Introducing bamboo perches in the field facilitate the predatory birds. Allowing ducks in the field is another option as they destroy the caterpillars. Severely infested fields can be isolated by digging a trench around the plot wherever possible, to prevent the spreading of caterpillar (Tanwar et al., 2010).

2.5.3.4. CHEMICAL CONTROL

When we cannot control the pest by other means we have to opt the chemical means such as use of pesticides. Pesticides may be synthetic or plant derived. Synthetic pesticides are man-made chemicals which are relatively inexpensive, fast acting and easy to use. Generally pesticides cause potential negative effect on the environment. Chlorpyriphos 20 EC, Quinalphos 25 EC, 0, Triazophos 40 EC, Dichlorvos 76 SL etc. are some synthetic pesticides against *S. mauritia* (Tanwar et al., 2010). Plant derived pesticides may be

purified chemicals from the plants or extracts of different parts of plants or raw crushed plant parts, mainly leaves. Neem, pongamia, tobacco and garlic formulations are some examples. As the botanicals have quick degrading property they are less harmful and it can be prepared by farmers themselves (Singh et al., 2004). One of the major problems is the pesticide resistance in insects.

2.5.3.5. BIOLOGICAL CONTROLS

Biological method includes control of pest using their natural enemies like parasitoids, predators, parasitic nematodes, bacteria and fungi (Singh et al., 2004).

2.5.3.5.1. Parasites/parasitoids

There are many parasites and parasitoids that act as natural enemies of the lawn army worm, *Spodoptera mauritia.* According to Beardsley the scelionid egg parasite *Telenomus nawai* Ashmead has been reported from the egg masses of *S. mauritia* in the Waialae-Kahala and Aina Haina regions of Honolulu (Beardsley, 1955). Many larval parasites of *S. mauritia* have been recorded. *Meterorus* sp., *Apanteles* sp., *Charops* sp. *Pseudoperichaeta orientalis* Wied., *Cuphocera varia* Fabr., *Pseudogonia cinerascens* Round, *Drino unisetosa* Bar, *Euplectrus euplexiae* Roh. & *Uplectrus* sp. *Sturmiopsis semiberbis* Bezzi., *Sturmia bimaculata, Isomera cinerascena* Rond., etc. are some examples (Alam, 1967; David and Ananthakrishnan, 2003). A solitary larval internal parasite *Apanteles marginiventris* (Cresson), a braconid wasp also recorded from Hawaii and it mainly oviposit on first instar larvae (Tanwar et al., 2010). Two another

braconid wasp species also reared from the larvae of *S. mauritia* in Hawaii. Parasitation by theses wasps kills the caterpillar before it attaining later instars which are the more destructive stages (Tanada and Beardsley, 1958). Murad in 1969 reported the isolation of *Hexamermis* sp., an entomopathogenic nematode from the dead larvae of the pest.

2.5.3.5.2. Predators

In India, *Andrallus spinidens*, the spiny soldier bug (Hemiptera: Pentatomidae) was reported as a potential predator to lepidopteran larvae (Rao, 1965). Ebadi and Ghaninia reported the applicability of mass rearing of *A. spinidens* on *Galleria melonella* Linnaeus under laboratory condition (Ebadi and Ghaninia, 2003). Chitra shanker et al. studied the biology and functional response of *A. spinidens* on *S. mauritia* in the laboratory. They reported that *A. spinidens* as a potential biocontrol agent against *S.mauritia* because they can be easily reared under laboratory conditions on *Corcyra cephalonica, Ephestia kuhnella* and *Spodoptera litura* (Shanker et al., 2017). Tanada and Beardsley reported that in Aina Haina-Wailupe Circle section of Honolulu two ant species *Monomorium floricola* Jerdon and *Pheidole megacephala* Fabricius found attacking *S. mauritia* eggs (Tanada et al., 1958). In South Africa, the pupae and pre pupae of *S. mauritia* were destroyed by Argentine ants (Dick, 1943). Hutson reported that in Ceylon, the caterpillars of *S. mauritia* were fed by the beetle *Cicindela sexpunctata* Fabricius and predatory bugs (Hutson, 1920). The vertebrate predators such as the house crow, jungle crow, cattle egret,

common mynah have also been found to predate on the larvae of this pest (Tanwar et al., 2010).

2.5.3.5.3. Nuclear polyhedrosis virus (NPV)

A nuclear polyhedrosis virus of *S.mauritia* was discovered In Hawaii and it might have entered together with its host (Bianchi, 1944; Tanada et al., 1958). The larvae died due to the virus showed typical nuclear polyhedrosis symptoms. Larvae infected in early instars die before fourth instar; they have a whitish appearance at death and rapidly darken after death. In the southern parts of India a disease of *S.mauritia* was believed to be bacterial disease but the symptoms was that of the nuclear polyhedrosis (Ananthanarayanan and Ramakrishna Ayyar, 1937).

2.6. INTEGRATED PEST MANAGEMENT

Integrated Pest Management (IPM) is an ecologically based strategy for pest control that aims at long-term solution which combines different types of pest control strategies such as cultural method, use of resistant varieties, modification of agronomic practices, habitat manipulation, biological and chemical control. It was in 1960 that IPM promoted as a strategy for pest control. The effective implementation of IPM needs skills in various areas such as pest monitoring and knowledge of pest dynamics. Pest control strategies are applied with minimum risks to beneficial and non-target organisms, human health and environment (Singh et al., 2004). Charles and Youngberg stated that, the IPM system sustains agricultural

productivity, minimizes environmental degradation, maintains quality of the life and promotes economic viability (Charles and Garth, 1990).

2.7. INSECT GROWTH REGULATORS

An insect growth regulator (IGR) is a chemical which interfere the growth and development of an insect and in turn inhibits its life cycle. An IGR did not have to be always toxic to its target organism, instead, it may cause various abnormalities to them (Siddall, 1976). Generally IGRs regulate the metamorphosis or interfere with the reproduction of the insect and this in turn controls them (Riddiford and Truman, 1978). IGRs may be chitin synthesis inhibitors or hormonal analogues such as juvenile hormone analog and ecdysone analogues. As humans do not use moulting hormones of insects and do not make chitin, IGRs believed to have little toxic to human (Schmutterer, 1985). Pyriproxyfen, methoxyfenozide, tebufenozide, diflubenzuron, hydroprene, methoprene etc. are some examples of IGRs.

2.7.1. CHITIN SYNTHESIS INHIBITORS

Cuticle is an exoskeletal structure in insects which is formed of epidermal cells. Procuticle is one of the different layers of cuticle and 30 to 60% of procuticle is chitin. The disruption of cuticle in insects during formation leads to lethality (Retnakaran et al., 1985). Chitin synthesis inhibitors (CSI) are generally used as larvicides. The biosynthesis of chitin is interfered by the treatment with CSIs (Gijswijt et al., 1979). The treated larvae fail to form new cuticle and prevented from moulting (Hammock and Quistad, 1981). The Benzoylphenylurea, diflubenzuron, was the first CSI in the market as

an insecticide and it was a potent agent against *Cydia pomonella* L. and *Spodoptera litura* Fabr. (Miyamoto et al., 1993). Chitin synthesis inhibitors effectively suppress the entire life cycle and development of insects (Verloop and Ferrell, 1977). These compounds can cause physiological disturbances by creating hormonal imbalance (DeLoach et al., 1981).

2.7.2. HORMONAL ANALOGUES

In insects the growth and development is controlled by different hormones such as the neuropeptide prothoracicotrophic hormones (PTTH) secreted by the brain, the steroidal moulting hormone 20-hydroxyecdysone (20E) secreted by prothoracic gland and the sesquiterpenoid juvenile hormones (JH) secreted by corpora allata. In the development of an insect the larvae undergoes larval moults. The moulting is regulated by the moulting hormone ecdysone and the development from egg to adult through larva and pupa is regulated by the titers of JH (Riddiford, 1996). Both these hormones change their roles in adults and regulate reproductive processes (Wyatt and Davey, 1996). Substances interfering with the action of insect hormones can be ecdysone analogue or juvenile hormone analogues.

2.7.2.1. NEUROHORMONES AS INSECT GROWTH REGULATORS

In insects neuropeptides act as master regulators of growth and development. The secretion of JH and ecdysteroids are influenced by neuropeptides. The first neuropeptide to be isolated and identified was the proctolin, a pentapeptide (Brown, 1975, Brown and Starratt, 1975).

Masler et al. reported that the insect pests can be controlled by disrupting the synthesis, release and degradation of neuropeptides (Masleret al., 1993). Crickets and Stick insects have a neuropeptide family that can inhibit JH biosynthesis (Lorenz et al., 1995).

2.7.2.2. ECDYSONE

Ecdysone or moulting hormone is synthesized by the prothoracic gland and it act as the precursor of 20-hydroxyecdysone (20HE), which is the most common steroid in insects. Ecdysone is released into the blood and gets converted in to20HE in target tissues. In each developmental stage, after feeding and growth, rapid increase in the level of ecdysteroids occurs. If the next is also a larval stage, ecdysteroid along with high level of juvenile hormone, circulate in the body and the expression of new larval characters occurs. If it is the last larval instar, metamorphosis is signaled by short pulses of ecdysteroid and the larva became adult (Dai & Adams, 2009).

2.7.2.3. ECDYSONE ANALOGUES

Ecdysone analogues are the substances that resemble the moulting hormone ecdysone and which can be used as insect growth regulator to control insect pests. Ecdysone analogues exert their toxicity by binding to the ecdysone receptor as does the natural insect molting hormone (Smagghe and Degheele, 1994; Wing, 1988). The most common effect of ecdysone analogue treatment is precocious lethal moult (Dhadialla et al., 1998). In 1970s itself the attempts to discover insecticides with ecdysone activity was made (Watkinson and Clarke, 1973). After several years, RH-5849, a potent ecdysone analog

was discovered (Aller and Ramsay, 1988). RH-5849 found to be effective against dipteran, coleopteran and lepidopteran pests (Wing, 1988; Wing and Aller, 1990). Further studies lead to the commercialization of other analogues of RH-5849 such as RH-5992, tebufenozide, RH-2485, methoxyfenozide and RH-0345, halofenozide. Out of these, both methoxyfenozide and tebufenozide showed selective toxicity to lepidopteran larvae (Hsu, 1991). Halofenozide showed efficacy against cutworms, scarabid beetle larvae and webworms (RohMid LLC, 1996). Another ecdysone analogue chromafenozide, ANS-118, was developed and registered under the trade names, MATRIC® and KILLAT®. It is used to control lepidopteran larval pests of fruits, rice, vegetables etc. in Japan (Reiji et al., 2000; Yanagi et al, 2000).

2.7.2.4. JUVENILE HORMONE

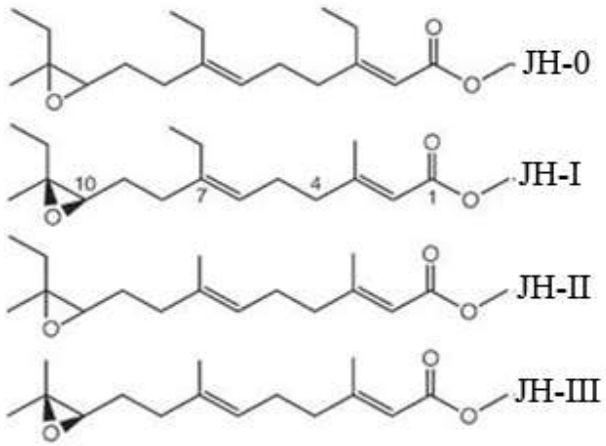
Juvenile hormone (JH) is a major hormone in insects that acts along with the moulting hormone ecdysone, to control the expression of larval specific genes to bring about morphogenetic effects. Determination of internal organs, colour and hardness of cuticle and related physiological processes are the morphogenetic effects occurring with the action of the hormone. At the end of larval development the level of JH decreases and the level of ecdysteroids rises and they program the expression of pupal characteristics and development of adults. In adults JH has gonadotropic functions hence the hormone reappears in adults. In addition to these functions JH involved in some polyphenisms such as caste determination in social insects and also in dormancy. A combination of synthesis and

degradation regulate the JH level in the haemolymph. Corpora allata synthesize JH which is promoted by allatotropins and suppressed by allatostatins.

Juvenile hormone is an acyclic lipophilic sesquiterpenoid derivative of farnesoic acid. The first natural JH was identified in 1967, JH isolated from the abdomen of silk moth by Roller and colleagues. The identified structure is a carbon skeleton with 15carbons substituted at 7^{th} and 11^{th} positions with ethyl groups. Other key structural features were at carbon 10 the presence of a methyl ester and an epoxide at carbon 11. This great achievement was followed by the discovery of a second JH, which was also from moths. These two molecules were called JH-I and JH-II respectively. They differ only at 7^{th} carbon, ethylated in JH-I and methylated in JH-II. JH-I and II are largely restricted to Lepidoptera. Within a few years JH-III was identified with the help of ^{14}C-labelled methionine. JH-III occurs in most of the insect groups. Its 7^{th} and 11^{th} carbons have methyl groups. Later, JH-0 and 4-methyl JH were identified from eggs of moth. Eight forms of JHs were identified (Goodman and Cusson, 2012; Schooley and Baker, 1985). From Lepidoptera five forms of juvenile hormone; JH 0, JH I, JH II, JH III, and 4- methyl JH I were reported (Bergot et al., 1981; Judy et al., 1973; Meyer et al., 1971; Roller et al., 1967). In higher Dipterans the bis-epoxide of JH and JH B3, is reported along with JH III (Cusson and Palli, 2000).

Juvenile hormone is a pleiotropic hormone and it regulates metamorphosis, reproduction, and behavior (Cusson, et al., 2000; Hiruma, 2003; Palli and Retnakaran, 2000; Riddiford, 1994, 1996;

Riddiford et al., 2003; Wyatt et al., 1996). Maintaining the larval status is the major function of JH. In Lepidoptera JH is absent during the last instar and 20E will increase, this in turn results in the regulation of metamorphosis towards pupation (Retnakaran et al., 1985). In adults JH has function in vitellogenin synthesis and adult diapauses. Juvenile hormone also shown role in pheromone production, migration, caste determination, antifreeze protein production, male accessory gland secretion, female sexual behavior, male accessory gland secretion etc. (Wyatt et al., 1996). As juvenile hormone has many functions in the development of an insect, for the production of target-specific insecticides JH biosynthesis interruption is a good strategy (Cusson et al., 2013).



2.7.2.4.1. BIOLOGICAL ACTIONS OF JUVENILE HORMONE

MORPHOGENETIC EFFECTS

The morphogenetic effects of JH during juvenile stages have been linked with the actions of ecdysteroids. The expression of juvenile characters of the insect is promoted by the JH in the blood. In insects with incomplete metamorphosis, the effect is not so visible whereas in holometabolous insects the effects are extreme. In them the immature forms are worm like and lack most of the adult structures such as wings, antennae, compound eyes. The studies by Truman and colleagues found that JH and nutrient-dependent signals regulate the growth and differentiation of imaginal disc primordia, and JH helps to program proper scaling of tissues leading to normal-sized adults (Truman et al., 2006). In many insects during the larval stages itself considerable development of the gonads takes place. In such insects the mating of the adults occurs within hours of emergence, which means the well development of gonads during the larval and pupal stages. During the larval stage the development of gonads and gametes were promoted by JH, but the level of JH should be decreased for the complete development of them. Hence prior to the pupal stage a drop in the level of JH occurs and this drop serves both morphogenetic functions and gonadotropic functions.

EFFECTS OF JUVENILE HORMONE IN ADULT

The corpora allata retain in adult and the JH reappear to regulate reproductive functions in the adult. They promote the development of gametes. In females JH promotes vitellogenesis. They

promote the synthesis of lipo proteins and glycoproteins in the fat body and their uptake into the oocyte. In males JH is required for the growth of sperm. In both the cases of oocyte development and sperm development JH exerts both positive and negative influences

POLYPHENISM AND CASTE DETERMINATION

Polyphenism is the phenomenon in which two or more distinct phenotypes are produced by the same genotype. Most polyphenisms are controlled by JHs. Juvenile hormone acts at certain sensitive periods during development. Caste polyphenisms in social insects such as ants, bees, and termites are the most common one. Different types of phase polyphenisms seen in nonsocial insects. Locusts are an example which shows phase polyphenisms. Depending on population density they occur either in solitary or in migratory phases. Differences in both behavior and physiology are seen in these phases. Both JH and peptide neurosecretory hormones determine these two phases. In response to seasonal conditions, food quality and crowding, aphids shows at least two different types of phase polyphenism and JH is involved in specification of these forms. In short JH and other neurosecretory hormones are important determinants of polyphenisms and this determination results in distinct body forms, behaviors and reproductive physiologies in the adult stage.

2.7.2.5. JUVENILE HORMONE ANALOGUES

The secretion of a hormone from corpora allata that prevent metamorphosis was described by Wigglesworth and he called it juvenile hormone (JH) (Wigglesworth, 1936). Williams worked out the

physiology of JH and few years later he postulated the statement 'third generation pesticides'. He used this statement to describe the juvenile hormone as insect pest control agent which is environmentally safe and the less chance to develop pesticide resistance (Williams, 1961). William's claim got reliability by the development of synthetic JH analogues that were highly potent and more active than the native JH (Henrick et al., 1973).

2.7.2.6. JUVENILE HORMONE AND JUVENILE HORMONE MIMICS IN PEST MANAGEMET

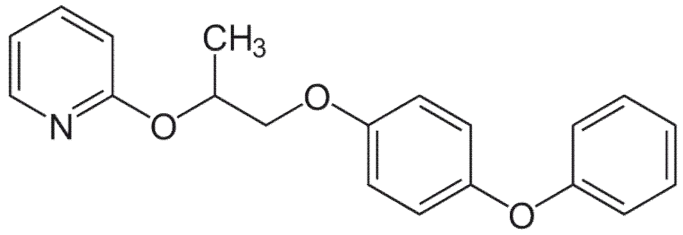
Williams in 1961 suggested that JH can be used as a control agent against insect pest as it is believed that insect will not develop JH resistance. The synthesis of JH was difficult and costly hence the realization concept of using JH as pesticide was delayed. However the production and use of many synthetic JH analogs soon became real. Many of the synthetic analogs showed several fold more activity than the native JH. This was the pioneer for the production of different analogues with various chemical structures (Bowers, 1968). Synthesis of many JHAs has been done in later years and their structure- activity relationships, relative potencies and effects on different species also studied (Romanuk, 1981; Slama et al., 1974). Juvenoids are natural JH analogues isolated from plants. ''paper factor'' from *Abies balsamea*, the balsam fir tree and juvocimenes from *Ocimum basilicum*, the sweet basil plant, are examples for naturally occurring JHAs (Bowers and Nishida, 1980). This may be a defense mechanism of plants to protect themselves from insects. There are many pest species such as fleas, fire ants, tsetse flies, mosquitos and cockroaches are vulnerable to JHAs.

The JHAs can be classified in to two groups: the terpenoid JHAs and the phenoxy JHAs. Methoprene and kinoprene are terpenoid JHAs whereas fenoxycarb and pyriproxyfen are phenoxy JHAs (Retnakaran et al., 1985). Time of application is important for the effectiveness of JHAs. In a study in tobacco hornworm larva, it was found that at day 2 of last instar larvae the JH began to decrease and on day 3 prothoracicotrophic hormone was released which in turn stimulate the secretion of ecdysone. The high level of ecdysone in the absence of JH induces pupal development. This indicates that after pupal commitment there is no morphological effect for the application of JHAs. In last instar larvae the sensitive period to JHAs is between the disappearance of JH and the appearance of ecdysteroid (Miyamoto et al., 1993; Riddiford, 1976). Normal adults will not develop, if pupae were treated with JHAs. Generally the adults are insensitive to the treatment of JHAs. In some cases JHA treatment make them sterile (Retnakaran et al., 1985).

PYRIPROXIFEN

Pyriproxyfen is a phenoxy JH analogue. KNACK®, SUMILARV®, ADMIRAL® are its trade names. Pyriproxyfen causes both morphogenetic effects and sterility (Retnakaran et al., 1985). In insects pyriproxyfen competes with native JH for the receptors in the binding site, acts like JH and thus keep the insect in juvenile stage (Sullivan and Goh, 2008). It was first registered in1991 to control public health pest in Japan and it is less toxic to mammals (Miyamoto et al., 1993). In *Drosophila melanogaster* Meigen, pyriproxyfen is

more effective than the first JH analogue, methoprene (Riddiford and Ashburner, 1991).



PYRIPROXYFEN

Hatakoshi et al. reported that treatment of pyriproxyfen to the last instar larvae of tobacco cut worm and tobacco horn worm resulted in the formation of supernumerary larvae (Hatakoshi et al.,1988). The same effect was reported in the German cockroach also (Reid et al., 1994). In pear psylla, *Casopsylla pyricola* the egg hatching was suppressed by the phenoxy JHAs, pyriproxyfen and fenoxycarb (Higbee et al., 1995). In *Bemisia tabaci* along with egg hatch the JHAs suppress adult formation also (Ishaaya and Horowitz, 1992). Same effect was reported in *Haematobia irritans* L. (Bull and Meola, 1993). In *Ziposcelis entomophila* (Enderlein) pyriproxyfen and methoprene showed a lethal effect on egg hatching (Ding et al., 2002). In honey bee vitellogenin synthesis was impaired by the JH analogue pyriproxyfen (Pinto et al., 2000). Liu and Chen reported that in *Lipaphis erysimi* (mustard aphid) pyriproxyfen showed good activity and it causes direct mortality, reduces longevity and inhibits progeny formation (Liu and Chen, 2000).

2.7.2.7. ANTI JUVENILE HORMONE AGENTS

The substances which prevent production of JH, degrading the JH or destroying the JH secreting gland are collectively known as anti juvenile hormone agents (Anti JHAs). They are a group of various elements that interrupt JH activity. These compounds are very effective in pest management. Bowers et al. reported two compounds from the plant *Ageratum houstonianum* with anti- JH activity and they were called 'precocenes' (Bowers et al., 1976; Bowers, 1976). In sensitive insect species physiological responses caused by these compounds were similar that in JH deficient insects. The effects of anti JHAs include precocious metamorphosis, sterility in adults, diapause induction, inhibition of sex attractant production, rhythms, embryogenesis, cuticular sclerotization etc. The precocenes interfere with the biosynthesis or secretion or transport of JH and did not interfere at receptor level. Hence the actions of precocenes are reversible by the treatment with JH III or JH analogues (Bowers, 1981). Santha P. C. and V.S.K. Nair reported that the treatment of last instar larvae of *Spodoptera mauritia* with precocene II resulted in high mortality and also a significant increase in the duration of the stadium (Santha and Nair, 1986).

2.8. EFFECT OF HORMONE ANALOGUES ON *SPODOPTERA MAURITIA*

The effects of treatment with ecdysterone or the combination of ecdysterone and a juvenile hormone analogue, hydroprene on larval-pupal transformation in the last instar larvae of *Spodoptera mauritia* were studied by E. Balamani and V.S.K. Nair. They treated neck-ligated and thorax ligated last instar day 4 larvae. They observed that 63% of neck-ligated and 84% of thorax ligated larvae moulted in to larval pupal intermediates on ecdysone treatment. Treatment with the combination of ecdysterone and hydroprene resulted in 80 % of headless pupae in the case of neck-ligated larvae and 60% of thorax ligated larvae were moulted in to larval pupal intermediates (Balamani and Nair, 1991). Diflubenzurone (DFB) a benzoylphenyl urea induced ecdysial failures, development of larval-pupal intermediates and deformed pupae and adults in fifth and sixth instar larvae of *Spodoptera mauritia*. In pupae, treatment with lower concentrations has no specific effects, they emerged normally. As the concentration of DFB increased, the pupae with normal eclosion decreased (Jagannadh and Nair, 1997). Sam Mathai and V.S.K. Nair studied the histomorphological changes in the ovary of *Spodoptera mauritia* induced by the JH analogue hydroprene and reported that treatment of last instar larvae with hydroprene had no adverse effect on the development of ovary but the treatment of pupae resulted in various histological and morphological abnormalities in the ovary. In adults hydroprene caused precocious growth and differentiation of ovarian follicles. Thus they indicated that the response of ovaries that are differentiating and that are differentiated to the JHA were different (Mathai and Nair, 1990). Effect of the hydroprene on food

consumption and activities of digestive enzyme in the last instar larvae of *S. mauritia* was studied by A. Sindhu and V. S. K. Nair. They reported that in last instar larvae the treatment of hydroprene resulted in supernumerary larvae and it showed increase in the activities of the digestive enzymes amylase, protease, trehalase and invertase. There was an increase in most of the nutritional parameters also (Sindhu and Nair, 2004). In the last instar larvae of *S. mauritia* hydroprene influenced on the wing disc differentiation also. In hydroprene treated larvae the wing disc showed only partial differentiation (Safarulla et al., 2003).

2.9. HAEMOLYMPH PROTEINS AND ITS ALTERATION ON TREATMENT WITH INSECT GROWTH REGULATORS

Insects have an open circulatory system through which the circulating fluid or haemolymph moves. Larval stages have relatively larger volume of haemolymph than the adult. Haemolymph contain haemocytes and 20-50% of the total water content in the body is seen in haemolymph. Haemolymph functions as a water storage pool, as a depot for other chemicals, plays an important role in immune system and it transports nutrients, hormones and metabolites. A major component in the plasma is proteins, the concentration of which range from 10 to100 mg/ml. Majority of the plasma proteins are synthesized by the fat body (Kanost et al., 1990). Storage proteins or hexamerins are the most abundant proteins in the larval haemolymph of holometabolous insects (Chandrasekar et al., 2008; Hahn and Wheeler, 2003; Levenbook, 1985; Pan and Telfer, 2001; Roberts, 1987). In the last instar, the concentration of storage protein is very high and they are synthesized by the fat body. In insects generally amino acids are reserving in the form of storage proteins (Wheeler et al., 2000). At the

end of last instar these proteins are taken back into the fat body and stored in protein granules. Break down of the storage proteins in to free amino acids are occur during metamorphosis and these amino acids are used to synthesize adult proteins. Hexamerins are high molecular weight proteins with homologous or heterologous subunits of an average 80 kDa molecular weight (Burmester et al., 1998; Burmester, 1999; Kanost et al., 1990; Telfer and Kunkel, 1991; Tysell and Butterworth, 1978). Based on the biochemical nature, the hexamerins can be grouped mainly in to three- arylphorins (rich in aromatic amino acids), Methionine-rich storage proteins and small organic molecule (JH, riboflavin etc.) binding hexamerins (Fujii et al., 1989; Haunerland, 1996; Jones et al., 1990; Ryan et al., 1985; Sakurai et al., 1988; Telfer and Massy, 1987; Willott et al., 1989). Arylphorins contain 16-21% of aromatic amino acids (Chandrasekar et al., 2008).

Hexamerins functions as a nutrient storage. It acts as an amino acid pool for the protein synthesis and energy source at the time of metamorphosis. In addition storage proteins functions in the transport of hormones and other small organic compounds. There are tyrosine-rich storage proteins which involve in the cuticle formation (Chandrasekar et al., 2008). During cuticle formation arylphorin has a role in sclerotizing system (Scheller et al., 1990). The lepidopteran arylphorins are involved in immune response and act as cytotoxic effectors in infections by bacteria (Beresford et al., 1997).

As storage proteins have important role in the development of an insect, it is important to examine the effect of IGR- insecticides like pyriproxyfen on the protein profile changes in insects. Saleh TA and Abdel-Gawad RM studied the effect of diflubenzuron and chromafenozide, two insect growth regulators on the total protein,

carbohydrate and lipid contents of haemolymph and body homogenate of 6th instar larvae of *Spodoptera littoralis*. In this study, they reported that there were differences between control and treated larvae in native protein, lipoprotein, glycoprotein, and protein bands in SDS PAGE (Saleh and Abdel-Gawad, 2018). The sub lethal (LC_{20} and LC_{30}) concentrations of the insect growth regulator flufenoxuron showed a decrease in total soluble protein with the increase in larval instar of *Tribolium castaneum* (Herbst). In general, no significant difference was observed in the protein profiles of treated when compared to control, but some protein bands (MW 50–97 kDa) decreased in treated ones (Salokhe et al., 2006). Changes in haemolymph proteins of *Spodoptera mauritia,* when exposed to nuclear polyhedrosis virus (NPV) was reported by G.H. Takei and M. Tamashiro. Lethal doses of NPV induced a general reduction of haemolymph protein whereas sub lethal doses caused an increase in certain haemolymph proteins (Takei and Tamashiro, 1975).

Though the effects of IGRs on many insects are studied, their effect on haemolymph protein profile changes is not explored to a great extent. There are no studies on the effect of the IGR, pyriproxyfen, on the protein profile changes in *S. mauritia*. Thus in this study we examined the effect of pyriproxyfen on the haemolymph protein changes in the larvae of *Spodoptera mauritia*.

MATERIALS AND METHODS

3.1. CHEMICALS

Knack IGR, the active ingredient of which is pyriproxyfen (11.23%) obtained from Valent Corporation, USA, JH III and Pyriproxyfen from Sigma Aldrich. Cycloheximide, Acrylamide, N,N,N',N'-Tetramethyl Ethylenediamine (TEMED), coomassie brilliant blue R-250 and G-250 and Schiff's reagent from Sisco Research Laboratories Pvt. Ldt.(SRL), Bis-Acrylamide, Tris base and sodium dodecyl sulphate(SDS) from Himedia Laboratories, Ammonium per sulfate from Sigma Aldrich. All other chemicals used were of analytical grade and mentioned in the methodology.

3.2. TEST ORGANISM - *Spodoptera mauritia* Biosd.

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Lepidoptera
Family	:	Noctuidae
Genus	:	Spodoptera
Species	:	*Spodoptera mauritia*

Spodoptera mauritia is a sporadic pest of paddy. It is known as rice swarming caterpillar, coming under the order noctuidae of

lepidoptera. It is a holometabolous insect with four life stages – egg, larva, pupa and adult.

EGG

The adult female lay egg as egg masses each of which contain 100-200 eggs covered with grey hairy structures. Egg is dull white in colour and spherical in shape. They became dark in colour near to hatching. In most cases the egg hatches on the third day. Rarely the incubation period extends up to 7 days. Major portion of the eggs hatches in the morning hours.

LARVAE

FIRST INSTAR LARVAE

The first instar larvae are about 2mm in length and light green in colour with a large black head capsule. Immediately after hatching the first instar larvae descents by means of silken threads. They feed on the tender green part of the grass and leave the veins. The larval period is 2-3 days.

SECOND INSTAR LARVAE

Second instar larvae are more greenish than the first instar larvae. They were characterized by the three longitudinal lines on the dorsal side of the body. The larvae were 3-3.5 mm in length and the larval period is 2-3 days.

THIRD INSTAR LARVAE

Third instar larvae were pale green in colour with a size of about 7mm and they have 3 longitudinal lines one on the dorsal side and other two on lateral sides. The third instar larvae were characterized by the dark reddish superspiracular lines. The larval period is 3-4 days.

FOURTH INSTAR LARVAE

In fourth instar larvae the three longitudinal lines became dull in colour and the larvae attain a gray colour. The dark reddish lines were still visible. They have an average length of 1.5cm. The larval period lasts for 3-4 days.

FIFTH INSTAR LARVAE

Fifth instar larvae feeds voraciously and they became 2.5-3.0 cm in length. They were greyish-black in colour. On the dorso-lateral side double rows of prominent black triangular markings are seen which is bordered with narrow white stripes. It is a characteristic feature of 5^{th} instar. The supraspiracular stripes became pale in colour. After about 3-4 days of larval period they moult in to 6^{th} instar.

SIXTH INSTAR LARVAE

Sixth instar larvae have a length of about 3.5cm and they are grayish black in colour. The triangular markings become wider and were darker than those of the fifth instar. Sixth instar larvae were voracious feeders during the first three days of the instar and later stops

feeding and enter in to wandering stage. The size of the wandering larvae was reduced and had an average length of 2.5cm. On the next day the wandering larvae became prepupae, which are characterized by highly wrinkled body. Within 24 hours prepupae became pupa. The sixth instar larval stage lasts for 5-6 days.

PUPAL STAGE

The newly formed pupae were light brown in colour and later became dark brown. The pupal period was about 7 and 8 days for male and female moth respectively.

ADULT

In females, the forewings are grayish brown in colour with wavy lines. In the middle of the wing there is a dark spot. In male moths, forewings are bright grayish in colour, the hind wings are brownish white and have a black margin. The male moth has immense tuft of hairs on forelegs whereas the tuft of hairs were absent in females. Within 1-2 days of emergence they underwent mating and started ovipositing shortly after mating.

Egg

3rd instar larva

4th instar larva

5th instar larva

6th instar larva

Prepupa

Pupa

Adult female

Adult male

Figure 2: Different stages in the development of *Spodoptera mauritia*

3.3. METHODS

3.3.1. Collection, rearing and maintenance of laboratory culture of larvae of *Spodoptera mauritia* Boisd.

During the night the adult moths were attracted by using fluorescent lamps. They were collected with the help of an insect sweeping net. The adults were kept in glass beakers closed with muslin cloth for mating and egg laying. They were provided with cotton balls dipped in 10% solution of honey as food. The adults lay eggs on the muslin cloth and on the side walls of the container. After egg laying the adults were removed from the container. After two days the eggs become dark in colour, which indicate that it is near to hatching. At that time, tender leaves of the grass *Ischaemum aristatum* was provided in the container which is the alternative host of the army worm. Immediately after hatching the first instar larvae descends on silken thread to the grass provided as food. The food is provided as and when needed and feacal matter and the food waste were removed occasionally. Larvae were kept at a relative humidity of 70-80% and at room temperature (28°C). As the larvae grow they were sorted based on the markings and kept in separate containers.

3.3.2. Treatment to test toxicity of pyriproxyfen and LD_{50} calculation

Third, fourth, fifth and sixth instar larvae of *Spodoptera mauritia* were sorted out from the laboratory culture on the basis of moulting marks. Different concentrations of pyriproxyfen in acetone were applied topically on the dorsal side of the larvae on day 0 of each instar

using a Hamilton Micro-Syringe in a total volume of 2μL. To the control larvae an equal volume of acetone was applied in the same manner. For each experiment, at least 3 replicates were done and the number of larvae per experiment varied from 15 to 20. After 24 hours the mortality was recorded and from the average percentage mortality for different concentrations of pyriproxyfen, LD_{50} value for 3rd, 4th, 5th and 6th instar was calculated with the help of a plot of concentration of pyriproxyfen versus percentage mortality.

3.3.3. Exposure of larva to the JH-analogue, pyriproxyfen, for identification of pyriproxyfen responsive haemolymph proteins

Sub lethal concentration (LD_{10}) of pyriproxyfen for the 5th instar larvae of *S. mauritia* was taken for the treatment. Pyriproxyfen (Knack IGR), diluted in acetone was applied topically along the dorsal midline of meso and meta thorax and to the abdomen of 5th instar day 0 larvae using a Hamilton Micro-Syringe. To the control group an equal volume of acetone was applied in the same manner. After 24 hours, the haemolymph of both the control and test group were collected individually.

3.3.4. Collection of haemolymph

The treated larvae were anesthetized in a specimen tube using diethyl ether. One of the prolegs of larvae excised with the help of a sterilized scissors and the exuded haemolymph (with haemocytes) from each larva was drawn into separate micro centrifuge tubes and stored at -20°C.

3.3.5. Determination of the effect of pyriproxyfen on protein concentration in the haemolymph

Modified Lowry's method (Sandermann & Strominger, 1972) was used to determine the concentration of haemolymph protein. The haemolymph (with haemocytes) collected was treated with SDS (1% final) and centrifuged for 5 minutes at 9272xg. The supernatant was collected which contain SDS-soluble protein and it was used for protein estimation using bovine serum albumin (BSA) as standard.

3.3.5.1. Modified Lowry's Method for protein estimation

Reagents

1. 2x Lowry concentrate

a) Alkaline copper reagent

- Dissolved 20g sodium carbonate in 260ml distilled water
- Dissolved 0.4g cupric sulphate in 20ml distilled water
- Dissolved 0.2g sodium potassium tartarate in 20 ml distilled water

Mixed all the above solutions to make alkaline copper reagent

b) 1 % SDS solution : Dissolve 1g SDS in 100ml distilled water

c) 1M NaOH solution : Dissolve 4g NaOH in 100ml distilled water

Mixed 3 parts alkaline copper reagent with 1 part SDS solution and 1 part NaOH solution to make 2x Lowry concentrate.

2. 0.4N Folin- Ciocalteu reagent

3. Standard BSA solution

Dissolve 200mg BSA in 100 ml of 0.1N NaOH solution. Used 10x diluted solution of this as standard solution

Procedure

Added 1ml of 2x Lowry concentrate to 1ml of the sample, mix thoroughly and incubated at room temperature for 10 minutes. After the incubation, added 0.5ml of 0.4N Folin- Ciocalteu reagent very quickly and vortexed immediately and incubated for 30 minutes at room temperature. Read the absorbance at 680nm.

3.3.5.2. Bradford's dye binding method for protein estimation

Bradford's reagent: 0.06% coomassie brilliant blue G-250 in 6N HCl

Mixed equal volumes of sample and the reagent, mixed well and read the absorbance at 620nm (Bradford, 1976)

3.3.6. Electrophoretic analysis of haemolymph proteins

The haemolymph collected from treated and control group of larvae was treated with SDS (1% final) and centrifuged at 9272xg for 5 minutes. The supernatant containing SDS-soluble protein was collected. Theses samples were subjected to SDS-PAGE in a mini slab

gel under reducing conditions using 10% acrylamide according Laemmli's method (Laemalli, 1970). Comparison of the protein profile of the treated larvae with untreated was done to identify changes in the intensity of protein band and appearance of new polypeptides or disappearance of the existing ones.

3.3.6.1. SDS PAGE (Laemmli's method)

Reagents

1. Acrylamide solution

Dissolved 30g acrylamide and 0.8g bis acrylamide in about 50ml distilled water. Make up to 100ml. filtered and stored at 4ºC in amber coloured bottle.

2. Buffer 1 (pH 8.8)

Buffer for separating gel, 0.614M Tris buffer, adjusted the pH to 8.8 with HCl, made up to 100ml with distilled water and dissolved 164mg SDS

3. Buffer 2 (pH 6.8)

Buffer for stalking gel, 0.147M Tris buffer, adjusted the pH to 6.8 with HCl, made up to 100ml with distilled water and dissolved 108mg SDS

4. Buffer 3 (Chamber buffer/ running buffer, pH 8.3)

The buffer containing 0.025M Tris buffer, 0.192M glycine and 1% (w/v) SDS

5. Ammonium per sulphate (APS)

It is used as a catalyst for initiation of polymerization. 15mg/ml APS was used.

6. TEMED: Concentration - 0.733 to 0.777g/ml

7. Tracking/Loading dye (6x)

The 6x tracking dye contain 6% (w/v) SDS, 375mM tris HCl pH 6.8, 9% (v/v) β-mercaptoethanol, 48% (v/v) glycerol, and 0.03% (w/v) bromophenol blue.

6. Fixative

Mixed 75µL formaldehyde with 100ml of 50% methanol

7. Staining solution

To make 100ml staining solution mixed 12ml glacial acetic acid, 44ml methanol, 44ml distilled water and 60mg coomassie brilliant blue R-250

8. Destaining solution

Mixed 7.5ml glacial acetic acid with 5ml methanol and made up to 100ml

Gel preparation

Separating gel: To make 10% gel, mixed 6ml Acrylamide, 11ml buffer 1 (pH 8.8), 0.02ml TEMED and 0.9ml APS

Stalking gel: To make 3% gel, mixed 1ml Acrylamide, 8.5ml buffer 2 (pH 6.8), 0.01ml TEMED and 0.5ml APS

3.3.7. Regulation of expression of pyriproxyfen-responsive protein

3.3.7.1. Determination of the effect of increase in concentration of pyriproxyfen on pyriproxyfen-responsive protein

The effect of increase in pyriproxyfen on the level of pyriproxyfen-responsive protein was determined. To determine the effect, the fifth instar day 0 larvae of *S.mauritia* was treated with different concentrations of pyriproxyfen. The concentrations taken were 2µg, 4µg, 10µg and 20µg/ larva. After 24 hours, the haemolymph of the treated larvae were collected individually and subjected to SDS-PAGE to asses change in intensity of protein band with change in concentration of pyriproxyfen.

3.3.7.2. Effect of cycloheximide on the expression of pyriproxyfen-responsive protein

To understand the regulation of the expression of the pyriproxyfen-responsive protein, one set of larvae was treated with 25µg cycloheximide, a protein synthesis inhibitor, in acetone, and another set was treated with 25µg cycloheximide, along with the sub lethal dose of pyriproxyfen. It was topically applied as done in earlier experiments. The haemolymph was collected after 24 hours, loaded on 10% SDS-PAGE and compared with the haemolymph from control and pyriproxyfen treated larvae.

3.3.8. Identification of the JH analogue-responsive protein in fat body

The fat body (5mg) of 5th instar larvae of *Spodoptera mauritia* was dissected out and homogenized in insect ringer and centrifuged at 9272xg for 5 minutes at 4^{o}C. The supernatant was collected and used for TCA precipitation. TCA solution was added to the supernatant in such a way that the final concentration of the TCA was 10%. After the addition, the sample kept in the freezer for 1 hour. Then centrifuged at 9272xg for 10 minutes at 4^{o}C and collected the pellet. The pellet was washed thrice with acetone. After evaporating the excess acetone the pellet was re-suspended in 2% SDS and heated in boiling water bath for 5 minutes. This sample was centrifuged at 9272xg for 5 minutes at room temperature and the supernatant was subjected to 10% SDS-PAGE along with the haemolymph collected from the control larvae. Gel was stained to visualize the protein bands. The same procedure was used for the fat body collected from the test and control larvae and the protein bands obtained in the SDS-PAGE gel were analyzed.

3.3.9. Determination of glycosylation status of pyriproxyfen-responsive protein

The haemolymph from the test and control larvae were collected and the haemolymph proteins were separated by SDS-PAGE. To determine the glycosylation status of the identified pyriproxyfen-responsive protein, the gel was subjected to Periodic Acid- Schiff's (PAS) staining (Dubray and Bezard, 1982) Ovalbumin was used as positive control in the gel.

3.3.9.1. PAS staining

After running the gel to separate the proteins, it was soaked in 7.5% (v/v) acetic acid for 30 minutes and then in 0.2% (w/v) periodic acid for 2 hours. The periodic acid solution was removed and the Schiff's reagent was added to the gel, incubated for 1-1½ hours. Reddish-pink bands of stained glycoprotein will be visible. The Schiff's reagent was removed and the gel was soaked in 7.5% (v/v) acetic acid for 1 hour and subsequently stored in water. For detection of non-specific staining periodic acid solution was omitted from the procedure and replaced with water.

3.3.10. Determination of the sub unit composition of the identified pyriproxyfen-responsive protein

To determine whether the protein is a single subunit or multi-subunit protein, a polyacrylamide gel in native condition (alkaline PAGE) was done, and was followed by SDS PAGE. The heamolymph sample was loaded on to standard alkaline PAGE and run at voltage 25 mA in mini slab gel. After the run the gel was stained with colloidal coomassie stain without fixation. From the stained gel JH analog responsive protein band was excised. The gel pieces minced and mixed with 1x sample loading buffer, boiled for 5 min and loaded on to 10% SDS PAGE under reducing conditions.

3.3.10.1. Native or alkaline PAGE

Reagents

1. Solution A: pH 8.8-9.0

   1N HCl- 24ml

   Tris buffer- 18.1g

   TEMED- 0.12ml

   Distilled water to make up to 100ml

4. Solution B: pH 6.6-6.8

   1N HCl- 48ml

   Tris buffer- 5.98g

   TEMED- 0.46ml

   Distilled water to make up to 100ml

5. Solution C:

   Acrylamide- 28g

   Bis-acrylamide- 0.735g

   Distilled water to make up to 100ml

6. Solution D:

   Acrylamide- 20g

Bis-acrylamide-5.0g

Distilled water to make up to 100ml

7. Solution G:

Ammonium persulphate- 14mg/10ml

8. Solution E:

0.005% Bromophenol blue solution

7. Running buffer: pH 8.3

Dissolved 6g tris buffer and 28.8g glycine in distilled water. Made up to 1000ml

Gel preparation

Separating gel

Mixed equal volumes of solution A and solution C. Added solution G to this mixture in 1:1 ratio.

Stalking gel:

Mixed 1 part solution B, 1 part solution D, 4 part solution G and 2 part distilled water

Colloidal coomassie stain

- Ammonium sulphate-5g
- Alcohol (100% or 96%)-10ml

- Coomassie brilliant blue G-250- 20mg
- Phosphoric acid (85%)- 2.35ml
- Double distilled water- to make up to 100ml

(Added the chemicals in the above order)

This stain was used for colloidal coomassie staining of the gel

3.3.11. Identification of the protein by mass spectrometry

To identify the protein, the haemolymph of *Spodoptera mauritia* was separated on SDS-PAGE (10% acrylamide) and the pyriproxyfen responsive protein band was cut out from the gel after staining (Coomassie brilliant blue G-250 in water or colloidal coomassie stain). The protein from the gel is electro-eluted and loaded on to SDS-PAGE (12% acrylamide) to further resolve from contaminating protein, if any. The gel was fixed and stained normally and the single band was cut out and processed for LC-MS/MS analysis to identify the protein.

3.3.11.1. Mass spectrometry-procedure

The mass spectrometry was done in the proteomics facility at Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum using the method described by Shevchenko et al. (2007). The procedure is briefly described below.

3.3.11.1.1. Excision of protein bands

The excised bands were cut into cubes (ca. 1 x1 mm). The gel pieces were transferred into a micro centrifuge tube and spin them down on a bench-top micro centrifuge.

3.3.11.1.2. Destaining of gel pieces excised from Coomassie-stained gels

To the gel pieces ca. 100 µl of 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) was added and incubated with occasional vortexing for 30 minutes, depending on the staining intensity. To this 500 µl of neat acetonitrile was added and incubated at room temperature with occasional vortexing, until gel pieces become white and shrink and then the acetonitrile was removed.

Samples were now ready for in-gel digestion. Alternatively, they can be stored at -20ºC for a few weeks.

3.3.11.1.3. Saturating the gel pieces with trypsin

Added enough trypsin buffer to cover the dry gel pieces (typically, 50 µl or more, depending on the volume of a gel matrix) and left it in an ice bucket or a fridge. After ca. 30 min, checked if all solution was absorbed and if necessary, added more trypsin buffer. Gel pieces should be completely covered with trypsin buffer.

3.3.11.1.4. Extraction of peptide digestion products

To each tube, 100 µl of extraction buffer (1:2 (v/v) 5% formic acid/acetonitrile) was added and incubated for 15 minutes at 37ºC in a

shaker. To withdraw the supernatant, a pipette with fine gel loader tip was used to prevent clogging the needle of auto sampler injector or nano LC MS/MS column. The supernatant was collected into a PCR tube, dried down in a vacuum centrifuge. Dried extracts can be safely stored at -20ºC for a few months.

3.3.11.1.5. Redissolving tryptic peptides for further analysis

For further LC MS/MS analysis, 10–20 µl of 0.1% (v/v) trifluoroacetic acid was added into the tube, vortexed and/or incubated the tube for 2–5 minutes in the sonication bath and centrifuged for 15 minutes at 10,000 rpm in bench-top centrifuge and with drawed the appropriate aliquot for further analysis. The rest was dried down in a vacuum centrifuge and store at -20ºC as contingency.

4.1. Toxicity of pyriproxyfen to larvae of *Spodoptera mauritia*

The average percentage mortality for 3rd, 4th, 5th & 6th instar larvae of *Spodoptera mauritia* treated with different concentrations of pyriproxyfen (Knack IGR) was calculated. With increase in concentration of pyriproxyfen, the mortality increased in all the instars of larvae tested. (Table 1)

Table 1: Percentage mortality of 3rd 4th 5th & 6th instar larvae of *Spodoptera mauritia* treated with different amount of pyriproxyfen

Amount of pyriproxyfen applied/ larva	Average percentage mortality ± SE			
	3rd instar	4th instar	5th instar	6th instar
Control (0 µg)	0	0	0	0
5µg	14.4±2.1	12.5±2.5	…….	…….
10µg	25±5.0	27.5±6.4	……..	…….
25µg	85±2.9	70.8±5.1	15.8±2.0	…….
50µg	96.7±3.3	91.7±4.4	65.8±2.2	…….
100µg	100±0.0	100±0.0	75±2.9	8.13±2.8
125µg	…….	…….	96.7±3.3	13.8±1.3
200µg	…….	…….	……	32.5±2.5
300µg	…….	…….	…….	45±5.0
400µg	…….	…….	…….	73.3±1.7

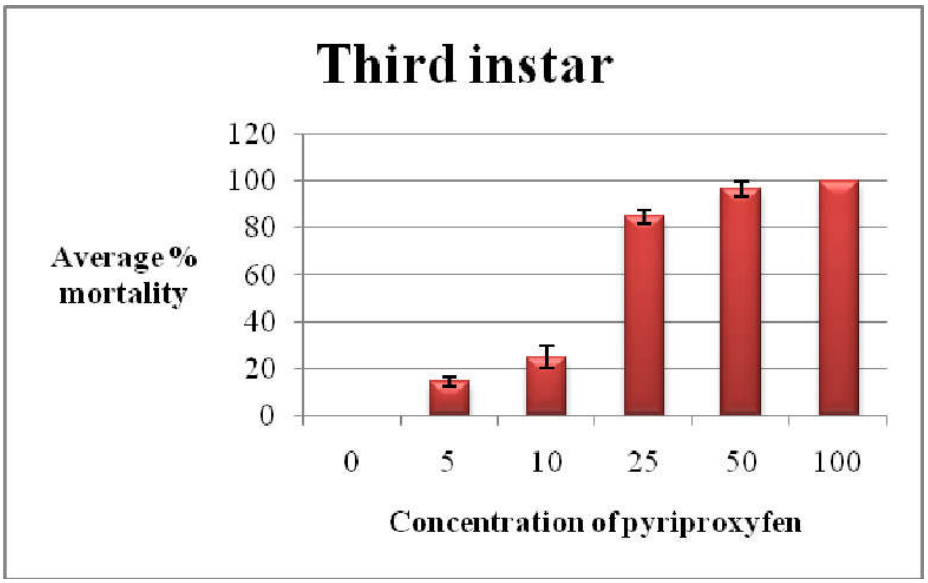


Figure 3: Graphical representation of the percentage mortality of *Spodoptera mauritia* 3rd instar larvae at different concentration of pyriproxyfen (µg/larva).

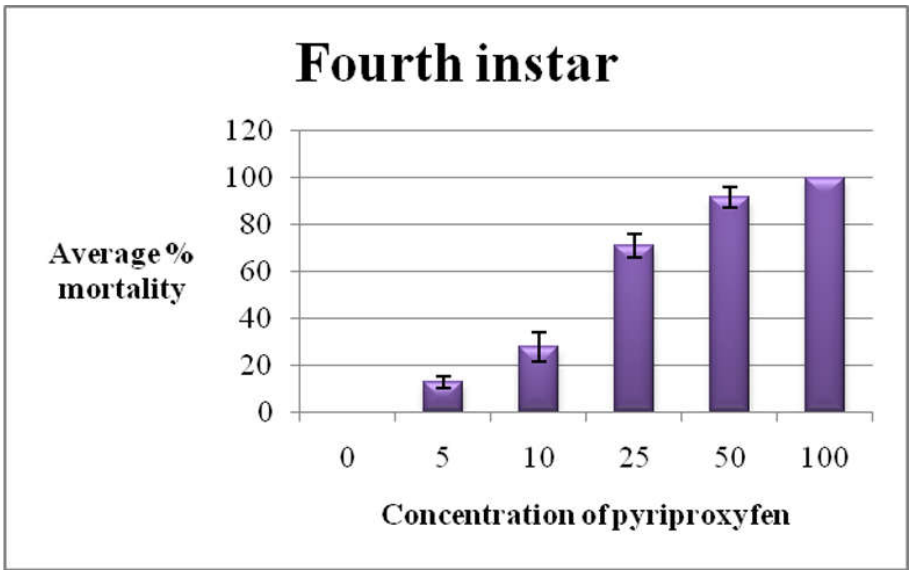


Figure 4: Graphical representation of the percentage mortality of *Spodoptera mauritia* 4th instar larvae at different concentration of pyriproxyfen (µg/larva).

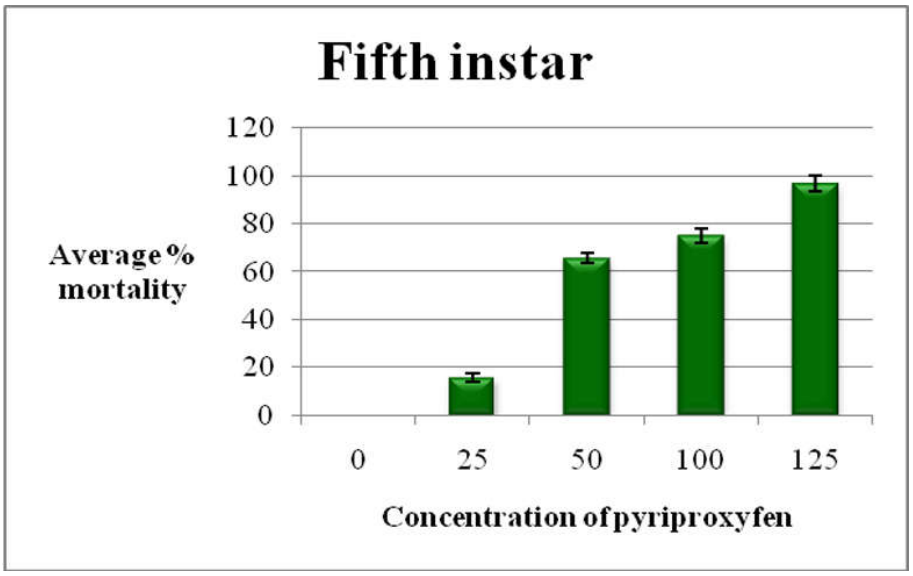


Figure 5: Graphical representation of the percentage mortality of *Spodoptera mauritia* 5th instar larvae at different concentration of pyriproxyfen (µg/larva).

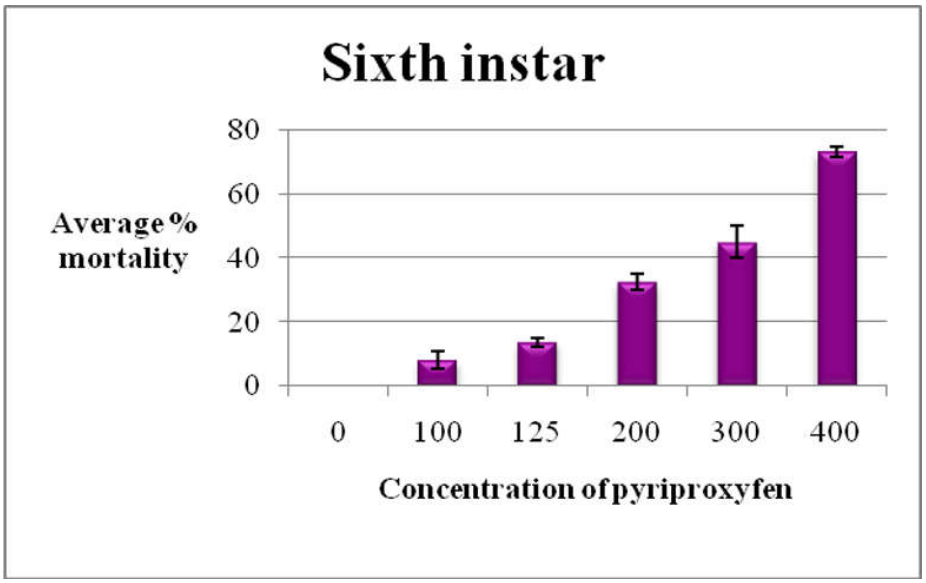


Figure 6: Graphical representation of the percentage mortality of *Spodoptera mauritia* 6th instar larvae at different concentration of pyriproxyfen (µg/larva).

4.2. Calculation of LD_{50} value

The LD_{50} value (24 hours) of pyriproxyfen for the 3rd, 4th, 5th & 6th instar larvae of *Spodoptera mauritia* was calculated from a plot of concentration of pyriproxyfen versus percentage mortality (Fig. 3,4, 5, 6), using the data obtained from toxicity study (Table 1). The LD_{50} value increases with the increase in larval instar (Table 2).

Table 2: LD_{50} value (24 hours) of pyriproxyfen for 3rd, 4th, 5th & 6th instar larvae of *Spodoptera mauritia*

SL. NO.	LARVAL INSTAR	LD_{50} VALUE (µg) (MEAN ±SE)
1	THIRD	14.13±2.67
2	FOURTH	15.85±3.67
3	FIFTH	39.81±2.61
4	SIXTH	316.20±2.64

4.3. Effect of pyriproxyfen on larval size and haemolymph protein concentration

Exposure of 5th instar day 0 larvae of *S.mauritia* to sub lethal concentration of pyriproxyfen (LD_{10}) led to an increase in size of the larvae within a week (Fig.7) and a statistically significant ($p<0.05$) increase (7%) in SDS-soluble haemolymph protein concentration after 24 hours compared to control (Table 3).

Figure 7: Test and control 5th instar larvae of *S. mauritia* showing difference in size on exposure to pyriproxyfen after 7 days.

Table 3: The increase in haemolymph protein concentration of *Spodoptera mauritia* 5th instar larva on treatment with pyriproxyfen

Sl. No.	Sample	Concentration of haemolymph protein (µg/µl) ± SE	p value
1.	Control	3.02±0.02	0.0283
2.	LD_{10} (4µg of pyriproxyfen /larvae)	3.23±0.03	

4.4. Effect of Pyriproxyfen on haemolymph protein profile

Exposure of 5th instar larvae of *S.mauritia* to sub lethal concentration (LD_{10}) of pyriproxyfen (Knack IGR) led to an increase in size of the larvae (Fig.7). Equal volume of haemolymph SDS-soluble protein (processed identically) from treated and untreated larvae were loaded onto 10% SDS -PAGE to assess changes in protein profile. Treatments with pyriproxyfen lead to an increase in intensity of a protein band with molecular weight of 83kDa (Fig.8). Analysis of the bands in gel doc showed that the 83kDa band in the test is 6.22±0.24 times intense than the band in the control.

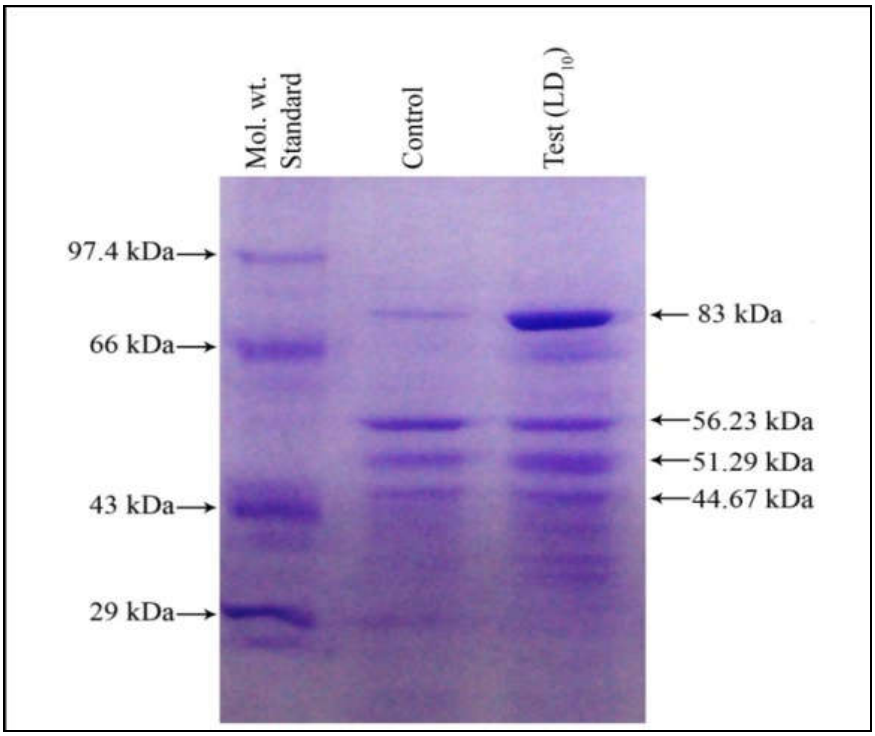


Figure 8: SDS-PAGE (10%) of haemolymph (3µl) of *Spodoptera mauritia* 5th instar larva

In addition to Knack IGR (4 µg/larvae), the effect of JH III (5µg/larvae) and pure pyriproxyfen (40 µg/larvae) were also tested on 5th instar day 0 larvae, and similar increase on 83kDa protein was observed. (Fig.9). Analysis of the bands in gel doc showed that the 83kDa band in the JH treated haemolymph is 6.56 times, in the Knack IGR treated haemolymph it is 6.43 times and in the pure pyriproxyfen treated haemolymph it is 2.66 times intense than the same band in the haemolymph from control larvae.

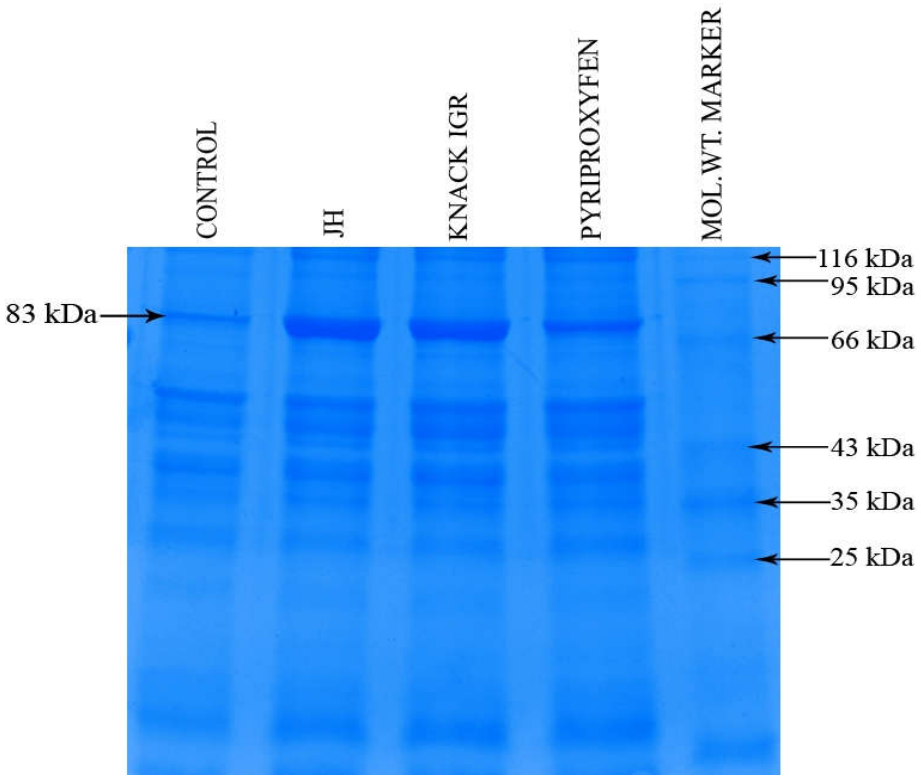


Figure 9: SDS-PAGE (10%) of haemolymph (3µl) of *Spodoptera mauritia* 5th Instar larvae showing the effect of JH, Knack IGR and pure pyriproxyfen on protein profile.

The protein profile of untreated larvae, many individual of same instar were analyzed to check whether there is any difference in the intensity of the identified pyriproxyfen responsive protein band in individual larvae of the same instar. The comparison revealed that there is no remarkable difference in the intensity of the 83 kDa band (Fig. 10) across different larva of the same instar (5^{th} instar). The difference in the intensity based on the lowest intense 83kDa band ranges from 0.08 to 1.95 times. The average difference is 0.65 ± 0.26 times. This difference in intensity is statistically not significant (p value = 0.1833). If there was a slight difference in the same, that difference in intensity was not only visible in the 83 kDa band but in other bands also, indicating that the difference is due to the overall variation in protein concentration in the larvae.

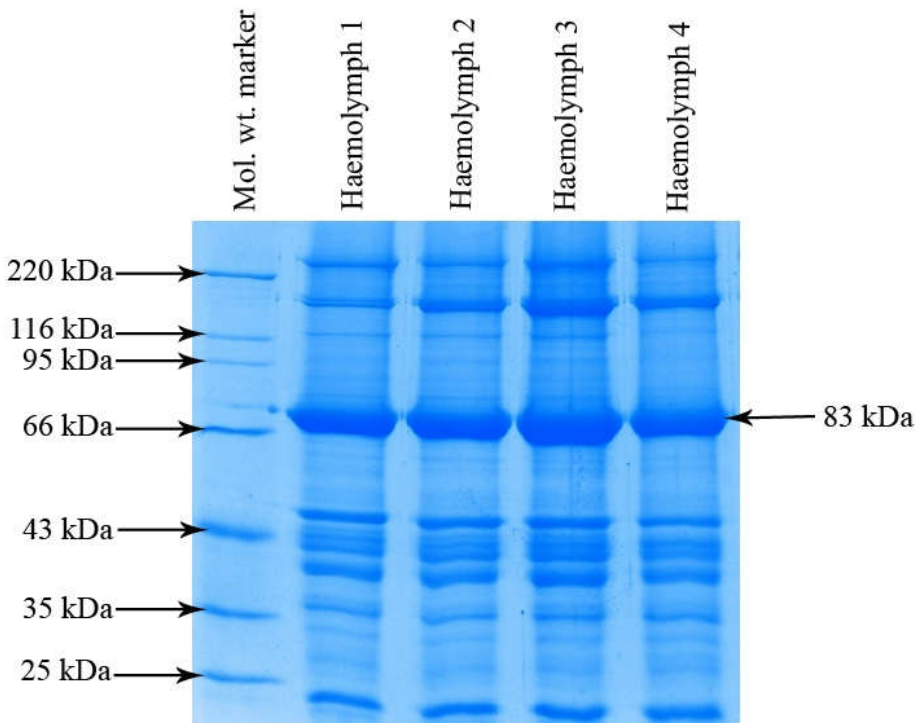


Figure 10: SDS-PAGE (10%) Gel electrophoresis- haemolymph of different untreated 5th instar day 1 *S. mauritia* larvae loaded in each well.

4.5. Regulation of expression of pyriproxyfen-responsive protein

4.5.1. Effect of increase in concentration of pyriproxyfen on pyriproxyfen-responsive protein

Fifth instar larvae of *S.mauritia* was treated with different concentrations (2µg, 4µg, 10µg, and 20µg) of pyriproxyfen to determine the effect of increase in concentration of pyriproxyfen on the level of pyriproxyfen-responsive protein. The haemolymph of the treated larvae were collected after 24 hours and subjected to SDS-PAGE. The intensity of the pyriproxyfen- responsive protein band was increased with increasing concentration of pyriproxyfen (Fig.11). Compared with haemolymph from 2µg pyriproxyfen treated larvae, the 4µg treated larval haemolymph showed 1.24 times increase, when compared with haemolymph from 4µg pyriproxyfen treated larvae the 10µg treated larval haemolymph showed 1.2 times increase and the 20µg pyriproxyfen treated larval haemolymph showed 1.13 times increase in the 83 kDa band intensity in comparison with 10 µg treated haemolymph.

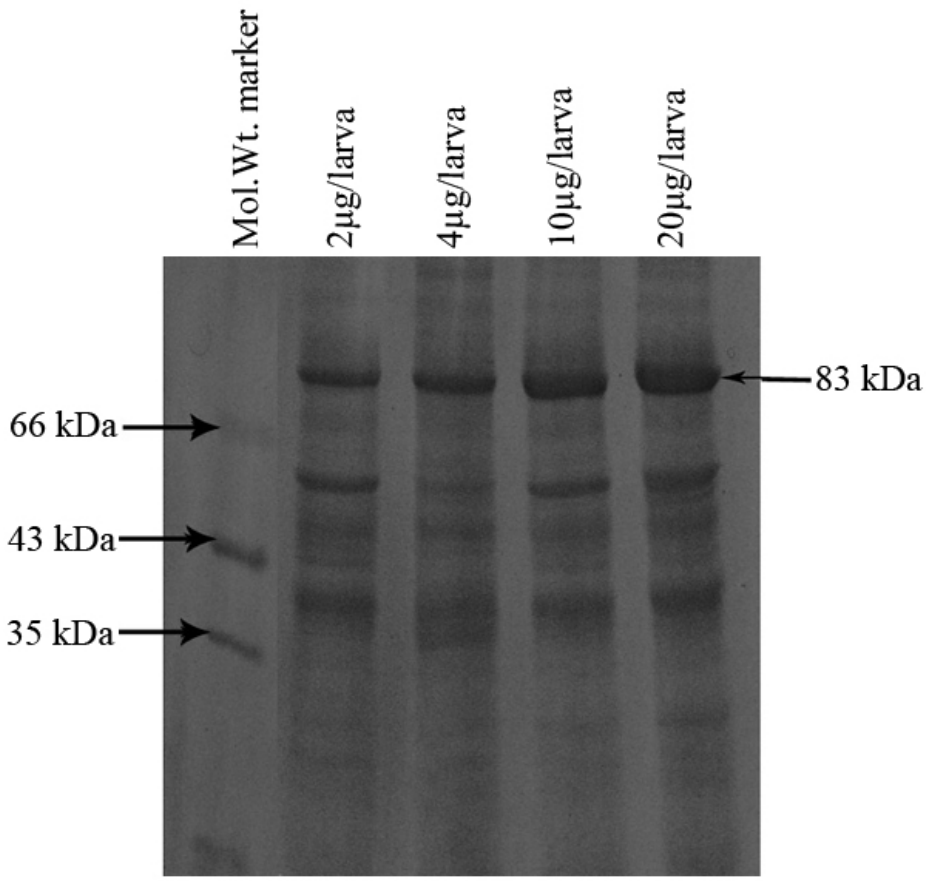


Figure 11: SDS-PAGE (10%) Gel electrophoresis haemolymph of *S. mauritia* 5th instar larvae treated with different concentrations of pyriproxyfen.

4.5.2. Effect of cycloheximide on level of expression of pyriproxyfen-responsive protein.

To understand the regulation of the pyriproxyfen-responsive protein, the larvae were treated with cycloheximide, a protein synthesis inhibitor, along with pyriproxyfen and also a control treated with cycloheximide alone. The level of a protein is the result of rate of synthesis and degradation. When compared to control the pyriproxyfen treatment led to an increase of 2.89 fold in the intensity of the pyriproxyfen-responsive protein. On concomitant treatment with cycloheximide, the level compared to control, increased only 1.35 fold. Thus there is an approximately 50% reduction in increase in protein concentration induced by pyriproxyfen.. (Fig. 12). This indicates that the protein level is increased on exposure to pyriproxyfen by induction of synthesis of this protein. Cycloheximide alone treatment led to a decrease of 74% protein band intensity compared to control.

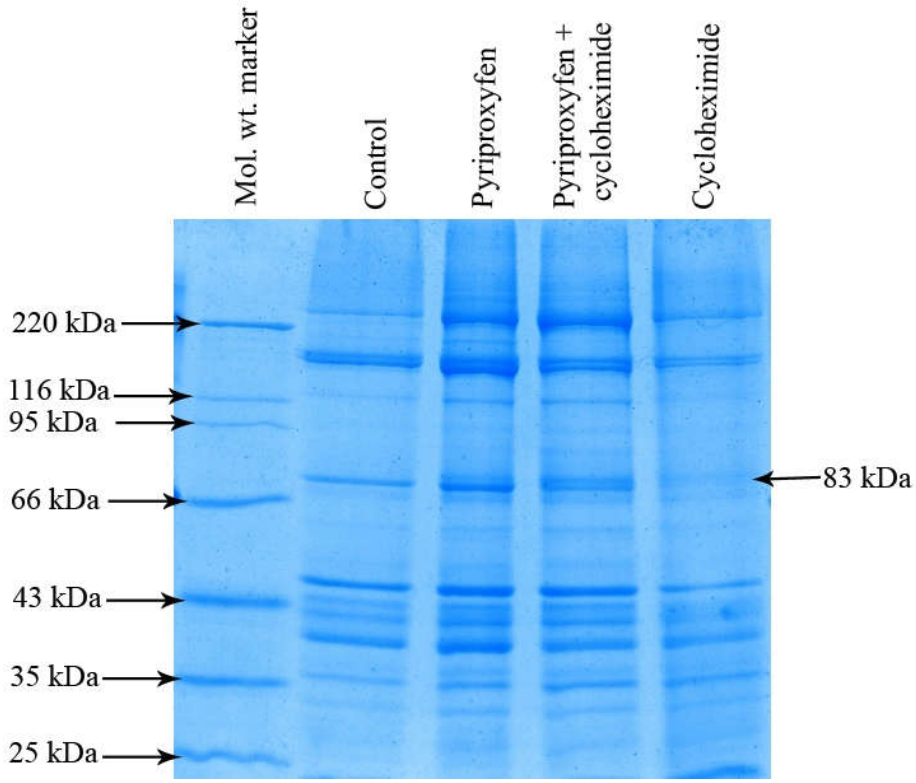


Figure 12: SDS-PAGE (10%) of the haemolymph showing the effect of cycloheximide on protein expression in 5th instar larvae of *Spodoptera mauritia*

4.6. Identification of the JH analogue-responsive protein in fat body

The fat body extract of *Spodoptera mauritia* was subjected to 10% SDS-PAGE and it is found that there is a protein band in the fat body extract which corresponds to the identified pyriproxyfen-responsive protein in molecular weight (Fig 13). When the same experiment was done with the fat body extract from control and test larvae, the band corresponds to the 83 kDa protein band was more intense in the sample from test than from control larva. Thus it is likely that the protein identified in fat body is the pyriproxyfen- responsive protein found in haemolymph indicating that this protein is expressed /localized in fat body. Hence, one of the sites of synthesis/storage of the identified pyriproxyfen- responsive protein is the fat body.

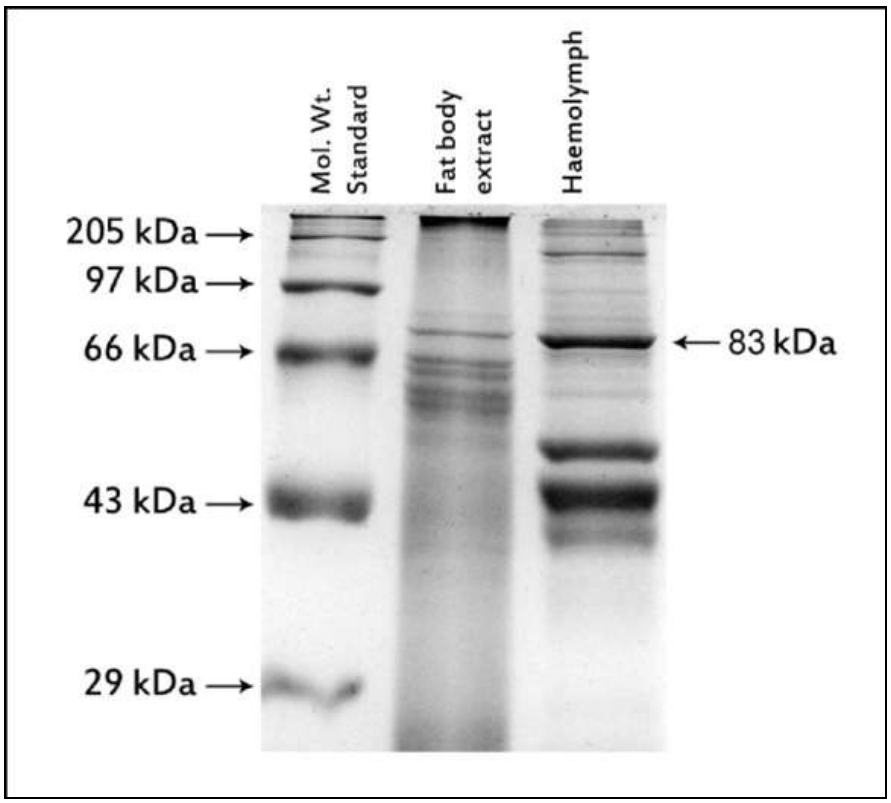


Figure 13: SDS-PAGE (10%) of fat body extract and haemolymph of 5th instar larvae of *Spodoptera mauritia*

4.7. Glycosylation status of pyriproxyfen-responsive protein

On Periodic Acid- Schiff's (PAS) staining of the SDS-PAGE separated haemolymph proteins, the band corresponding to the JH analogue responsive protein (83 kDa) was seen in reddish pink colour (Fig 14) which indicates that the identified pyriproxyfen- responsive protein is a glycoprotein.

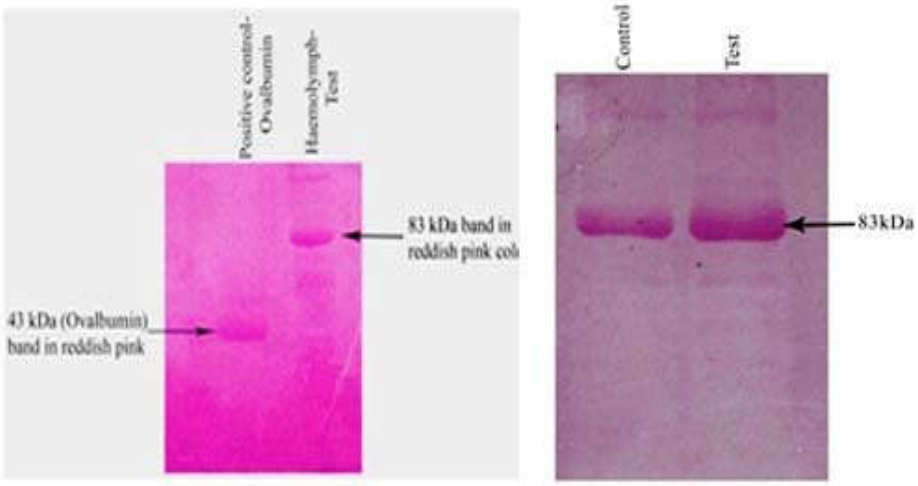


Figure 14: PAS stained SDS-PAGE (10%) of haemolymph proteins of 5th instar *Spodoptera mauritia* larvae. (A) with positive control ovalbumin and haemolymph from pyriproxyfen treated larvae. (B) Test-haemolymph from larva treated with pyriproxyfen and control- acetone treated larval haemolymph.

4.8. Determination of the subunit composition of the identified protein

To know the subunit composition of the native protein, alkaline PAGE was done. It was found that the pyriproxyfen- responsive protein on alkaline PAGE is a high molecular weight protein running above ferritin which is of molecular weight 440kDa. The molecular weight of the native protein is ≈500kDa. When the pyriproxyfen-responsive protein band was cut out from the alkaline PAGE gel and loaded on to SDS-PAGE under reducing conditions, this protein is converted in to a single polypeptide of around 83kDa on SDS-PAGE (Fig. 15) indicating that the protein is a multimer, a hexamer of 83kDa subunits.

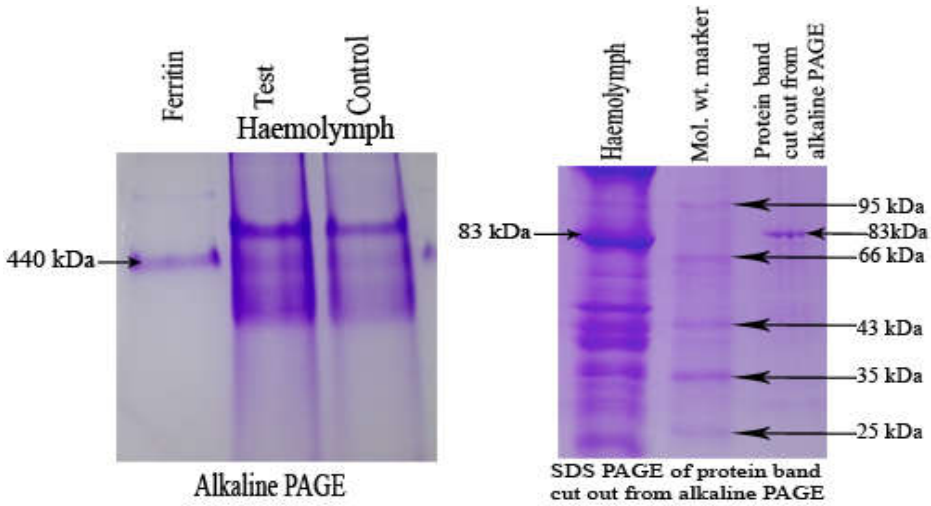


Figure 15: (A) Alkaline PAGE of haemolymph of 5th instar larvae of *S. mauritia* and (B) SDS PAGE of protein band cut out from alkaline PAGE

4.9. Identification of the JH analog responsive protein by LC-MS/MS analysis

Based on the confidence score and mass obtained in LC-MS/MS sequencing, it is identified that the JH analog responsive protein is similar to Arylphorin subunit (OS=*Spodoptera litura* OX=69820 GN=SL-3 PE=2 SV=1) of *Spodoptera litura*. The chromatogram and mass spectrum are given in Fig. 16 and 17 respectively. The highest number of unique peptides (84) matched with that of arylphorin subunit of *S.litura* (Table 4). The sequence of peptides matched with that of *S.litura* is given in peptide list (Table 5)

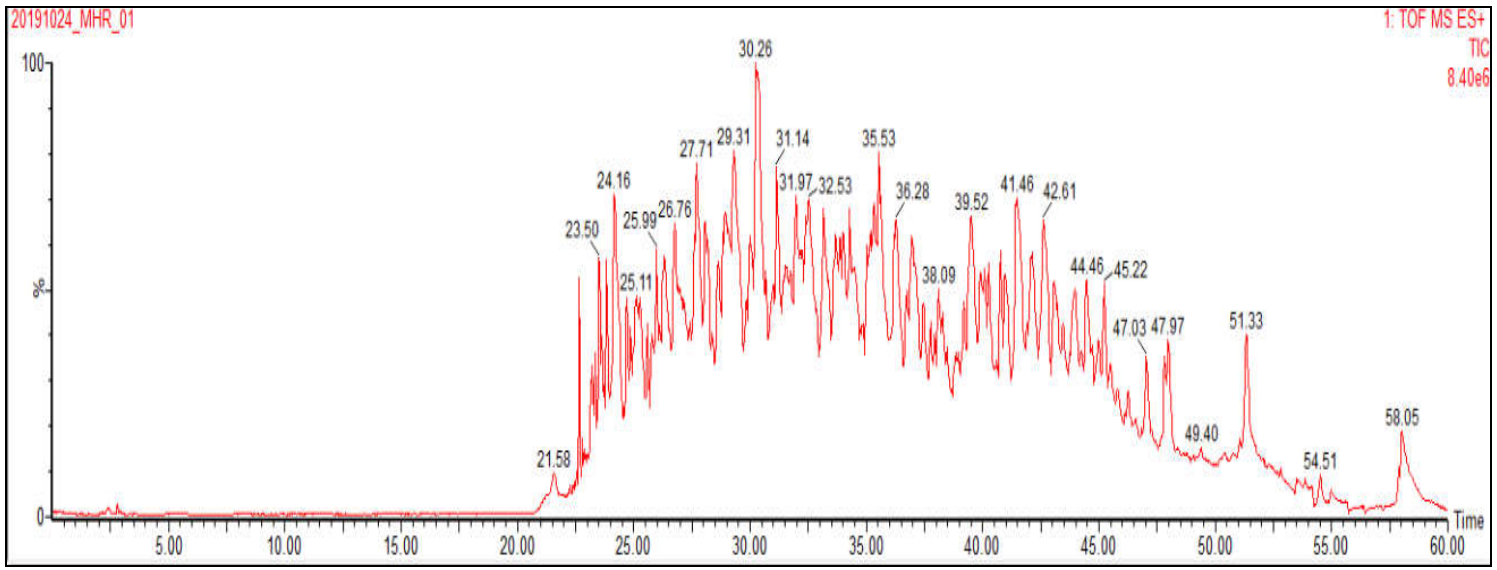


Figure 16: LC- MS/MS Chromatogram of pyriproxyfen-responsive protein

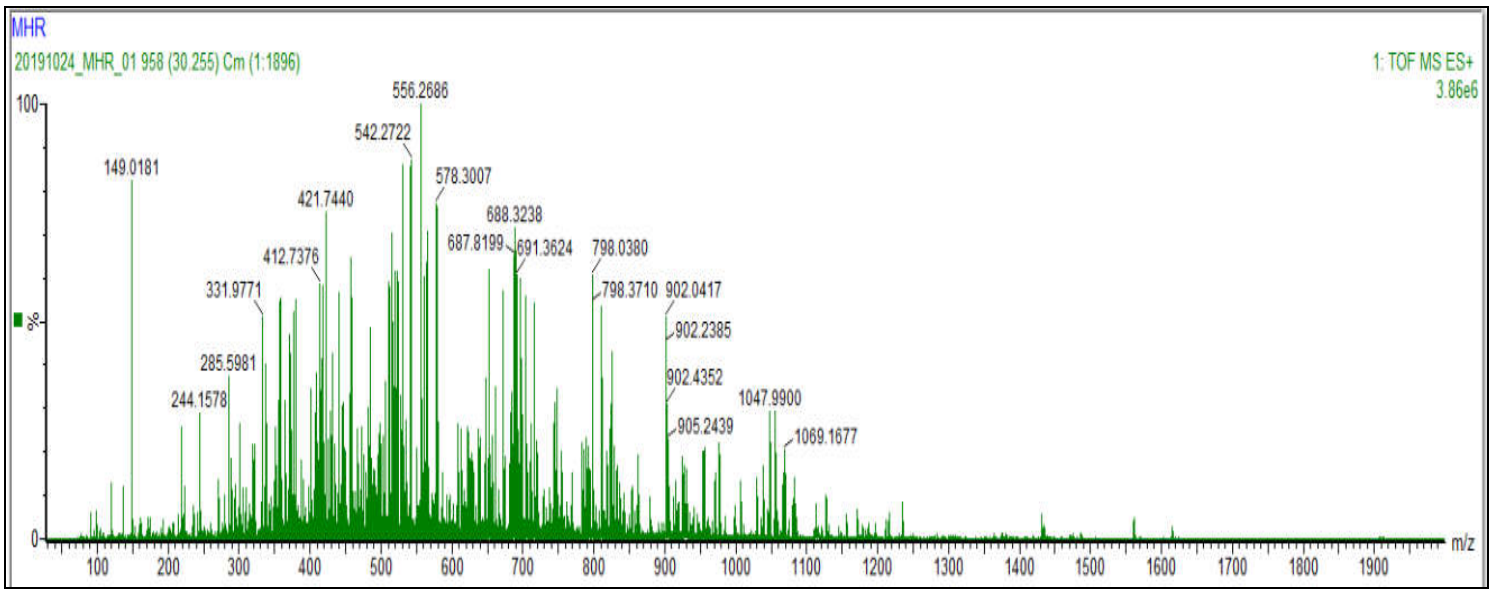


Figure 17 : Mass spectrum of the peptides from pyriproxyfen-responsive protein

Table 4: Protein list

Accession	Peptide count	Unique peptides	Confidence score	Mass	Description	Normalized abundance	
						MHR	
						20191024_ MHR_01	20191024_ MHR_02
A0A0G2YN85; A0A1S7D5J6;Q1HE32	17	17	86.4231	42221.1442	Actin OS=*Spodoptera litura* OX=69820 PE=2 SV=1	4729.2182	4305.9683
A0A0N7I630	3	2	13.3434	17364.5206	Putative odorant binding protein OBP11 OS=*Spodoptera litura* OX=69820 PE=2 SV=1	161.90596	172.53452
A0A0P0ELD1	1	1	5.3789	9773.2715	Putative acyl-CoA binding protein ACBP2 OS=Spodoptera litura OX=69820 PE=2 SV=1	3609.1242	4014.4396
A0A1J0M185	13	13	75.5469	73721.954	Putative carboxylesterase CXE30 OS= *Spodoptera litura* OX=69820 PE=2 SV=1	5351.8154	5386.7927
A7IT76	19	19	108.0711	77817.9815	Transferrin OS=*Spodoptera litura* OX=69820 PE=2 SV=1	27548.331	28638.586
E2F395;E0XN32	53	48	471.8113	71807.0591	Heat shock protein 70 cognate OS=*Spodoptera litura* OX=69820 GN=Hsc70 PE=2 SV=1	19638.936	19651.015
E7D2J5	12	7	101.9184	71049.0157	Heat shock protein 70 OS=*Spodoptera litura* OX=69820 GN=hsp70 PE=2 SV=1	1765.2532	1927.1885
I3QQD6	7	6	34.6676	27259.7975	Small heat shock protein 27.2	9324.4181	7580.1096

					OS=*Spodoptera litura* OX=69820 GN=HSP27.2 PE=2 SV=1		
J7EMH2;J7EJI5	12	12	54.6834	44663.1036	Elongation factor 1-alpha (Fragment) OS=*Spodoptera mauritia* OX=134409 GN=EF1-A PE=3 SV=1	18500.448	17166.007
Q3ZPT5	17	17	92.9462	80278.1902	Prophenol oxidase OS=*Spodoptera litura* OX=69820 PE=2 SV=1	1499.6692	1522.0759
Q9U5K4	**87**	**84**	**461.4975**	**84112.6912**	**Arylphorin subunit OS=*Spodoptera litura* OX=69820 GN=SL-3 PE=2 SV=1**	**150629.07**	**153978.99**
Q9U5K5	82	80	533.779	89479.1795	Methionine-rich storage protein OS= *Spodoptera litura* OX=69820 GN=SL-1 PE=2 SV=1	65234.159	61547.742
Q9U5K6	12	7	66.7764	90801.8893	Moderately methionine rich storage protein OS= *Spodoptera litura* OX=69820 GN=SL-2 beta PE=2 SV=1	3991.0303	4024.9581
Q9U5K7	16	11	73.6422	90290.2251	Moderately methionine rich storage protein OS= *Spodoptera litura* OX=69820 GN=SL-2 alpha PE=2 SV=1	4599.237	4467.0559
T2FEW4	1	1	7.2485	24392.9566	Beta-N-acetylglucosaminidase 3 (Fragment) OS=*Spodoptera litura* OX=69820 PE=2 SV=1	6928.1116	7202.8445

Table 5: List of peptides from the identified protein from *Spodoptera litura*

							Best identificati on						Normalized abundance		Spectral counts	
													MHR		MHR	
Peptide identifier	Ions used in quantitation	Ions	Deconvoluted peptide ions	Deconvoluted charges	Retention time (min)	Neutral mass	Score	Sequence	Modifications	Accession	Description	Maximum CV	20191024_MHR_01	20191024_MHR_02	20191024_MHR_01	20191024_MHR_02
32.65_1647.7407n	1	1	#13	2	32.6515333	1647.740721	8.1058	VPYDMSVQPDNMPR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.346430695	135402.1074	136067.108	1	0
32.73_1647.7413n	0	1	#253	3	32.7310833	1647.741264	8.1058	VPYDMSVQPDNMPR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.943087256	42637.56912	43210.0555	1	1
47.85_1908.9761n	0	2	#16,#187	2,3	47.8498333	1908.976085	8.6764	LGEVFFYYYQQLLAR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	10.65195199	102157.051	87845.9005	2	2
39.49_1057.5564n	1	1	#38	2	39.4868333	1057.556378	7.7606	TFFQFLQK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.683061873	186488.3257	190980.6	1	1
39.52_1057.5572n	0	1	#279	1	39.51765	1057.557178	7.7606	TFFQFLQK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	3.484754353	16365.47295	17192.3695	1	1
32.07_1938.9316n	2	2	#89,#49	2,3	32.0660833	1938.931642	5.7549	YTFMPSALDFYQTSLR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.612063875	129996.7839	134889.25	2	2
33.80_1506.7487n	0	2	#90,#114	2,3	33.7966667	1506.748685	8.2427	DLHQYSYEIIAR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.100002801	77388.10174	77278.7327	2	2
26.86_742.3648n	0	2	#123,#110	1,2	26.8560417	742.3648014	7.6868	YYLER		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.643269506	63364.11464	65777.8737	2	2
35.76_1950.8565n	0	1	#266	2	35.7610667	1950.856504	8.4353	YHANGYPVNIEDDWMK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	17.15100872	14362.81628	18327.3467	1	1
35.79_1950.8590n	0	1	#111	3	35.7918667	1950.858995	8.4353	YHANGYPVNIEDDWMK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	8.380931304	49879.71099	56164.0931	1	1

27.63_856.4067n	0	2	#146,#127	1,2	27.6288917	856.4067119	7.788	NYEYIR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.233527341	29026.61714	29958.1892	2	2
29.16_673.4199n	0	1	#131	2	29.1592	673.4199434	7.2761	IFIGPK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.321119188	45620.62569	46481.0137	0	1
36.32_960.4676n	0	1	#265	1	36.3157	960.4676047	7.8159	FSIFYER		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	8.954468933	9111.659313	10343.5154	1	1
36.35_960.4713n	0	1	#134	2	36.3465	960.4713437	7.8159	FSIFYER		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.654823688	54573.39522	53310.9999	1	1
23.50_1232.5412n	0	3	#529,#141,#135	1,2,3	23.5000167	1232.541208	8.1907	SNDYNLHNEK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.0758182	53458.24201	52651.0492	2	2
33.78_948.4236n	0	2	#283,#137	1,2	33.7812667	948.4235557	6.9125	DFETFYK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.983010419	32717.7566	33648.3421	2	2
40.12_1954.9954n	0	3	#1996,#163,#227	2,3,4	40.1210333	1954.9954	7.8517	MRDEAIALFHVLYYAK	[1] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	16.01860567	91104.77172	72566.0044	2	2
35.57_1299.6232n	0	3	#1352,#175,#3667	1,2,3	35.5685167	1299.623247	7.6615	DPAFYQLYQR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.309953515	35785.00785	36973.4324	2	2
29.98_1663.7329n	0	2	#195,#446	2,3	29.9784083	1663.732945	7.9761	VPYDMSVQPDNMPR	[5] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	5.88490188	62786.78286	68239.1028	2	2
23.82_940.5026n	0	3	#1789,#237,#273	1,2,3	23.8158111	940.5025812	7.5704	FVEYQKK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	11.48505582	17405.75764	14791.0066	2	2
27.64_839.3812n	0	1	#262	2	27.6443	839.381186	0	NYEYIR	[N-term] Ammonia-loss	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	3.223015271	4633.367624	4849.48331	1	0
25.05_1069.5946n	0	1	#287	3	25.0457333	1069.594586	6.0489	TGTLPKYYK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.112577832	23410.94894	23782.223	1	1
25.23_1069.5898n	0	1	#695	2	25.2306	1069.589843	6.0489	TGTLPKYYK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.950689112	6693.607737	6784.21085	1	1
33.64_1966.8526n	0	2	#804,#292	2,3	33.6426	1966.852563	8.2189	YHANGYPVNIEDDWMK	[15] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.5975365	19011.94141	19723.4074	2	2
44.06_3236.5228n	0	2	#374,#750	3,4	44.06235	3236.522783	7.4931	YTFMPSALDFYQTSLRDPAFYQLYQR	[4] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	9.52331238	20616.29931	18014.8764	2	2

41.53_1954.9115n	0	2	#397,#737	2,3	41.52555	1954.911458	7.6708	YTFMPSALDFYQTSLR	[4] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	8.801615711	10903.15059	12350.3762	2	2
33.80_686.3260n	0	1	#459	1	33.7966667	686.3259538	0	ETFYK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	4.995398478	3506.738497	3763.54529	1	1
28.43_1037.5004n	0	1	#479	2	28.4325667	1037.500428	7.7161	SDVASDAVFK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.770393555	3872.378319	3914.79901	1	1
37.46_2771.3353n	0	1	#535	3	37.4608333	2771.335339	5.639	DNNNYVFYANYSNSLSYPNKEQK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.056672122	20089.17033	20073.076	0	1
37.68_2771.3323n	0	1	#4390	4	37.6765333	2771.332348	5.639	DNNNYVFYANYSNSLSYPNKEQK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	11.47386844	1713.126895	1456.00712	0	1
23.80_934.4739n	0	2	#6218,#547	1,2	23.7952667	934.4739051	7.118	AEFKSPEK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	4.002474489	3541.676447	3346.72257	1	1
33.81_1035.4291n	0	2	#1964,#582	1,2	33.812075	1035.429104	0	DLHQYSYE		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.758554934	5245.037784	5189.07138	2	0
26.89_1679.7270n	0	2	#690,#2320	2,3	26.88685	1679.727016	7.5191	VPYDMSVQPDNMPR	[5] Oxidation M[12] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	6.112833492	6091.16069	6641.52146	2	2
32.70_728.3260n	0	2	#693,#713	1,2	32.7002833	728.3260266	0	PDNMPR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	3.137184282	9778.817319	10222.5117	2	2
29.92_728.3277n	0	1	#2111	2	29.9167833	728.3277141	0	PDNMPR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	12.62358536	538.0165192	643.479485	1	1
29.92_728.3269n	0	1	#2399	1	29.9167833	728.3269107	0	PDNMPR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	13.87435275	513.3898445	625.081246	1	1
23.50_1128.4826n	0	1	#697	2	23.5000167	1128.482593	0	NDYNLHNEK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.534072624	2874.568775	2812.87406	1	1
29.01_572.3126n	0	1	#712	1	29.0051333	572.3125509	5.2322	TVEPK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	12.58785719	698.7776013	584.549206	1	0
36.32_613.2838n	0	1	#774	1	36.3157	613.2838236	0	FYER		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.636797122	2511.224203	2570.0342	1	1
33.80_930.4115n	0	1	#908	2	33.7966667	930.4115454	0	DFETFYK	[C-term] Dehydrated	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.175244616	1358.597004	1381.36674	1	0

33.77_1148.5127n	0	1	#1005	2	33.7658667	1148.512748	0	DLHQYSYEI		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.806183366	2829.338177	2861.78084	1	1
36.13_1491.7923n	0	2	#4843,#1092	2,3	36.1308167	1491.792326	5.5778	INVKSDVASDAVFK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	5.960993398	2113.103257	2299.07935	0	2
33.78_600.3577n	0	2	#1118,#3364	1,2	33.7812667	600.3577106	0	EIIAR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.444853324	1906.334362	1945.68916	1	0
23.81_565.3202n	0	2	#1173,#4268	1,2	23.810675	565.3202324	0	YQKK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	11.79861169	977.1145281	826.630292	0	2
36.61_1154.6180n	0	1	#1222	2	36.61095	1154.617972	7.7528	IIDYLIDYK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	3.222906688	2585.687389	2706.2883	1	1
36.32_813.4010n	0	2	#1301,#6176	1,2	36.3157	813.4009542	0	SIFYER		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.112800007	1789.045371	1791.90159	2	2
26.56_759.3923n	0	2	#6087,#1338	1,2	26.5607833	759.3923175	6.8024	WLEQGK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	21.88424634	451.4635268	616.766821	2	2
44.46_1953.9798n	0	1	#1410	3	44.4630667	1953.979846	6.3768	FLDTYEKTFFQFLQK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	15.67464678	1171.082801	937.387367	1	1
29.92_935.4059n	0	1	#1427	2	29.9167833	935.4059438	0	VPYDMSVQ	[5] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	13.10517431	1070.452449	1289.10711	1	0
32.13_1921.9027n	0	1	#1541	3	32.1277	1921.902683	0	YTFMPSALDFYQTSLR	[N-term] Ammonia-loss	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	4.526269703	1681.075391	1792.24082	1	1
33.80_743.3230n	0	2	#1593,#2564	1,2	33.7966667	743.3229801	0	DLHQYS		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.250549879	1702.446236	1732.82337	2	2
41.00_1373.6636n	0	1	#1778	2	41.0017333	1373.663574	7.5164	FYELDWFVQK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	12.16481662	1051.696886	1249.65514	1	1
30.07_744.3218n	0	2	#1888,#1953	1,2	30.07085	744.3218076	0	PDNMPR	[4] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	6.089509155	1291.814705	1408.06993	0	2
32.73_1630.7102n	0	1	#1903	3	32.7310833	1630.710154	0	VPYDMSVQPDNMPR	[N-term] Ammonia-loss	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.251181803	2994.653	3005.30967	1	0
43.27_3014.4773n	0	1	#1925	3	43.2740833	3014.47727	5.6763	KGENVFENYILDDKPFGYPFDRPVR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.845728907	2677.402539	2571.77676	1	0

26.44_870.5266n	0	1	#1955	2	26.4375333	870.5265808	6.0656	LNILKDR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.372671041	577.3510032	580.401893	1	0
39.49_662.3733n	0	1	#2120	1	39.4868333	662.3733206	0	QFLQK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.368182186	1120.973844	1159.15593	1	0
32.73_856.3862n	0	1	#2524	2	32.7310833	856.3861857	0	QPDNMPR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.854469735	1072.066585	1044.31431	1	0
27.61_561.2901n	0	1	#2708	1	27.6134833	561.2901066	0	EYIR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	3.216901653	316.2603933	330.983198	1	1
47.88_1891.9483n	0	1	#2716	3	47.88065	1891.94832	0	LGEVFFYYYQQLLAR	[N-term] Ammonia-loss	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	7.552567739	595.2949626	534.935332	1	0
33.81_906.3858n	0	2	#2730,#3244	1,2	33.812075	906.3857587	0	DLHQYSY		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	3.898296653	1140.85594	1205.53447	0	2
33.77_888.3775n	0	1	#3772	2	33.7658667	888.3774658	0	DLHQYSY		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.37283726	470.2410544	479.460209	1	1
38.13_472.3032n	0	1	#2831	1	38.12585	472.3032471	6.9283	INVK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	18.84278363	331.1672822	253.294422	1	0
27.55_1414.7142n	0	1	#2873	3	27.5518667	1414.714238	5.2646	YYKFSIFYER		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	30.51538247	359.7249443	557.679011	1	0
38.03_1095.6300n	0	1	#3066	2	38.0334167	1095.629968	5.4076	INHKPFNVK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	8.272278956	396.1611609	445.386521	0	1
30.86_1426.7735n	0	1	#3091	3	30.8591167	1426.773531	5.2187	EPFFLYELTIR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.214991728	479.1929836	464.413876	0	1
32.70_692.2824n	0	1	#3104	1	32.7002833	692.2824391	0	VPYDMS		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	3.982757233	657.2759102	695.369581	1	0
33.80_1261.5958n	0	1	#3214	2	33.7966667	1261.595774	0	DLHQYSYEII		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	3.013668402	818.5849935	854.232489	0	1
30.38_2022.9907n	0	1	#3288	5	30.3789833	2022.990709	6.0328	DDSVSINEIYKWLEQGK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	1.925578626	1333.356834	1370.16775	1	0
39.49_534.3148n	0	1	#3305	1	39.4868333	534.3148074	0	FLQK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	0.61863073	467.1241749	463.055217	1	0

35.79_802.3392n	0	1	#3829	2	35.7918667	802.3392048	0	YHANGYP		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	10.81537942	201.6879816	235.091232	1	0
39.13_979.5290n	0	1	#3850	2	39.12995	979.5289784	5.6066	LAAQYGMVK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	26.39207919	170.0049364	248.016098	0	1
40.15_1152.6280n	0	1	#4028	2	40.15185	1152.628037	0	LFHVLYYAK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	26.54581542	761.7653775	520.984005	0	1
40.15_1039.5446n	0	1	#4102	2	40.15185	1039.544577	0	FHVLYYAK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	7.671520772	584.9092268	524.716636	1	1
42.16_2886.3759n	0	1	#4122	4	42.15975	2886.375901	6.0464	GENVFENYILDDKPFGYPFDRPVR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	15.68704545	591.1062343	473.064038	1	1
47.85_1216.6171n	0	1	#4265	2	47.8498333	1216.617136	0	YYYQQLLAR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	10.91404808	463.3544795	396.96055	1	1
35.56_465.2319n	0	1	#4421	1	35.55825	465.2319214	0	YQR		Q9U5K4	Arylphorin subunit OS= *Spodoptera litura* OX= 69820 GN= SL-3 PE=2 SV=1	13.80794616	256.9663956	211.251082	0	1
47.85_1550.7431n	0	1	#4598	2	47.8498333	1550.743137	0	LGEVFFYYYQQL		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	4.920016157	491.8481541	458.776155	0	1
30.89_946.5395n	0	1	#4634	2	30.8899167	946.5394701	5.1819	NKKPLNSF		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.873690846	185.111795	192.790799	1	0
29.92_807.3462n	0	2	#4748,#6844	1,2	29.9167833	807.3461697	0	VPYDMSV	[5] Oxidation M	Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	18.71877301	275.6351135	359.733606	1	1
47.88_1663.8248n	0	1	#4754	2	47.88065	1663.824758	0	LGEVFFYYYQQLL		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	11.81857183	492.6815429	416.685785	0	1
47.88_1309.5991n	0	1	#4787	2	47.88065	1309.599121	0	LGEVFFYYYQ		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	8.768463111	409.2733094	361.484479	1	1
30.04_791.3523n	0	1	#4795	1	30.0400333	791.3523468	0	VPYDMSV		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	22.08995025	158.7640465	217.543039	0	1
39.49_523.2427n	0	1	#5142	1	39.4868333	523.242703	0	TFFQ		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	8.651870669	174.3946303	197.123344	1	0
30.35_1532.8025n	0	1	#5244	2	30.3481667	1532.802456	5.9984	TFFQFLQKAEFK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	25.3782093	136.0127226	195.503687	1	0

39.52_911.4517n	0	1	#5288	1	39.51765	911.4517436	0	TFFQFLQ		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	10.4959191	298.8871251	346.808983	1	0
35.82_1245.5648n	0	1	#5644	2	35.8226833	1245.564779	0	PVNIEDDWMK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	31.53389512	369.3440437	234.663469	0	1
35.76_705.2859n	0	1	#5728	1	35.7610667	705.2858829	0	YHANGY		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	8.488253894	125.3102862	141.313306	0	1
39.13_2343.1711n	0	1	#6216	3	39.12995	2343.171124	6.2833	FKSGYYPQLPAHYINYVQR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	9.145943088	251.1921869	285.928642	0	1
23.50_1014.4511n	0	1	#6328	2	23.5000167	1014.451125	0	DYNLHNEK		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	14.11745953	115.9975435	141.72481	0	1
33.77_656.2904n	0	1	#6329	1	33.7658667	656.29039	0	DLHQY		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	6.597724801	181.6907004	199.473121	1	1
47.85_599.3718n	0	1	#6843	1	47.8498333	599.3718419	0	QLLAR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	12.34031791	126.7253636	106.384387	1	1
35.53_578.3148n	0	1	#7163	1	35.5274333	578.3148294	0	LYQR		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	2.146440468	121.8068843	118.164686	0	1
33.64_1128.5420n	0	1	#7262	2	33.6426	1128.541958	0	YHANGYPVNI		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	12.52270002	131.1483798	109.811644	1	1
35.59_721.3082n	0	1	#7297	1	35.58905	721.3081764	0	DPAFYQ		Q9U5K4	Arylphorin subunit OS=*Spodoptera litura* OX= 69820 GN=SL-3 PE=2 SV=1	41.73629891	83.57668739	45.4872982	1	1

Table 4 shows the LC-MS/MS protein list. The list shows all the proteins identified with confidence. From the table we can understand that the protein matching with the mass of identified pyriproxyfen-responsive protein has a high confidence score (461.4975), and the protein is arylphorin subunit of *Spodoptera litura*. Table 5 shows the peptide list from the arylphorin subunit of *Spodoptera litur*a. There are 84 unique peptides matching with the arylphorin subunit, and it is the highest number considering the number of peptides matching with other proteins in the list. So, considering the confidence score, the mass obtained and the number of matching peptides, we can confirm that the identified pyriproxyfen-responsive protein is arylphorin of *Spodoptera mauritia*. The figure 16 and figure 17 represent the LC-MS/MS chromatogram and LC-MS/MS spectrum of the identified protein respectively. The chromatogram shows the relative abundance of the ions with time and the LC-MS/MS mass spectrum shows the relative abundance of peptides with the mass by charge ratio. Thus the pyriproxyfen-responsive protein identified from *Spodoptera mauritia* larvae is the storage protein, arylohorin, of *Spodoptera mauritia.*

DISCUSSION

5.1. Toxicity of pyriproxyfen to larvae of *Spodoptera mauritia* and calculation of LD_{50} value

When the 3rd instar day 0 *Spodoptera mauritia* larvae treated with 5µg, 10µg, 25µg, 50µg and 100µg pyriproxyfen per larva the average percentage mortality after 24 hours was found to be 14.38±2.13, 25±5.0, 85±2.88, 96.67± 3.33 and 100±0.0 percentage respectively. From this mortality data we calculated the LD_{50} 24 hours, and it is found to be 14.13±2.67µg. Mahmoudvand et al. studied the effect of Pyriproxyfen on Life Table Indices of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and reported LC_{50} value based on a leaf dip bioassay is 1.223 g L^{-1} in 3rd instar larvae (Mahmoudvand et al., 2015). In ten-day-old larvae of Indian meal moth *Plodia interpunctella* (Lepidoptera: Pyralidae), different concentrations (0.02, 0.04, 0.08, 0.16, and 0.3 ppm) of pyriproxyfen was incorporated in the artificial diet. The result was increased larval period, decreased longevity of adults and reduction in number of eggs compared to control (Ghasemi et al., 2010).

In 4th instar day 0 *Spodoptera mauritia* larvae different concentrations of pyriproxyfen, 5µg, 10µg, 25µg, 50µg and 100µg per larva showed an average percentage mortality of 12.5±2.50, 27.5±6.37, 70.83±5.07, 91.67±4.41 and 100±0.0 percentages respectively. In this case the LD_{50}, 24 hour for 4th instar larvae is 15.85±3.67µg. Freshly

moulted 4th instar larvae of citrus swallowtail *Papilio demoleus* (Lepidoptera: Papilionidae) was topically administrated 7.5, 15, 30 and 60µg/1µl/larva of pyriproxyfen. This treatment induced a delay in both larval– larval ecdysis and larval–pupal ecdysis. There is also reduced frequency of pupation, increased ecdysial failure and mortality and inhibition of adult emergence (Singh and Kumar, 2011). Shaurub et al. reported that in the 4th instar larvae of *Spodoptera littoralis*, treatment with different concentrations of pyriproxyfen between LC_{50} and LC_{90} resulted in the formation of shrunken larvae and larval pupal intermediates. The emerged adults were malformed ones (Shaurub et al., 2020).

In the case of 5th instar *S. mauritia* larvae there was no mortality when treated with lower concentrations of pyriproxyfen, hence higher concentrations was applied. When the 5th instar day 0 larvae treated with 25µg, 50µg, 100µg and 125µg per larva the average percentage mortality after 24 hours was found to be 15.83±2.01, 65.83±2.21, 75±2.89 and 96.67±3.33 percentage respectively. The LD_{50} was 39.81±2.61µg. Ahmad Khawar et al. reported that when 5th instar hoppers of desert locust, *Schistocerca gregaria* Forsk. (Acrididae), sprayed with 5ml/L of pyriproxyfen, after 24 hours, showed 65% mortality in field condition and 84% mortality under laboratory conditions (Ahmad et al., 2020). Study by Zhao et al. found that pyriproxyfen can significantly affect the growth and development of 5th instar larvae of *Bombyx mori*. They administrated pyriproxyfen through feed in 5th instar larvae of *B.mori*. At 48, 72 and 96 hours of exposure pyriproxyfen caused reduction in weight by 8.94%, 10.34%

and 6.41% respectively and the duration of 5th instar was more than that of control group. The cocooning rate was also decreased in the test group and it was 30.32% only. (Zhao et al., 2020)

The sixth instar *S. mauritia* larvae needed higher amount of pyriproxyfen for mortality. Day 0 larvae of sixth instar treated with 100µg, 200µg, 300µg and 400µg of pyriproxyfen per larvae. After 24 hours, the mortality was found to be 8.13±2.77, 13.75±1.25, 32.5±2.5, 45±5.0 and 73±1.67 percentage respectively. The LD_{50} value was 316.20±2.64µg. The 6th instar larvae of *Spodoptera litura* (Lepidoptera: Noctuidae) was treated with pyriproxyfen and diofenolan by Singh et al. The topical administration of sub-lethal doses (0.5, 1.0, 2.5 & 5µg/ µl/larvae) of these JHAs revealed that they severely hampered the metamorphosis and development with prolonged larval duration, ecdysial failure, mortality, formation of larval - pupal mosaics, reduced pupation and formation of abnormal pupae, complete suppression of adult emergence and production of adultoids (Singh and Kumar, 2015). In *Spodoptera litura* topical application of pyriproxyfen showed wing abnormalities and morphological ovarian abnormalities and significant differences in number and hatchability of eggs. Application of 0.125µg of pyriproxyfen to day 0, 6th stadium larvae and 0.1 ng to day 1 female pupae resulted in the reduction of the total number of eggs oviposited and their hatchability. Day 1 pupal stage treated with 0.3 ng of pyriproxyfen showed wing abnormalities and about 40% of female adults showed morphological ovarian abnormalities (Nomura and Miyata, 2000). In two different Spodoptera species, *S. littoralis* and *S.*

frugiperda, 6th instar larvae were inhibited from the transformation from larvae to pupae on exposure to 1µg pyriproxyfen or fenoxycarb (El-Sheikh et al., 2016). It is clear that the lower concentrations of pyriproxyfen itself disrupt the larval development, which leads to the failure of healthy adult emergence. Hence for most of the studies, sublethal concentrations were used. At relatively higher concentration pyriproxyfen causes death of the larvae in 24 hours after application. From our toxicity experiments it is clear that in 3rd, 4th, 5th and 6th instars the mortality was increased with increase in concentration of pyriproxyfen indicating the concentration dependence. In the case of LD_{50}, the value increased with increase in instars or size of the larvae as expected.

5.2. Effect of pyriproxyfen on haemolymph protein concentration and larval size

The fifth instar day 0 *S. mauritia* larvae when treated with 4µg/larva (LD_{10} concentration) of pyriproxyfen showed a 7% increase in haemolymph total protein. As the p value is <0.05, the increase in test compared to control is statistically significant. The effect of pyriproxyfen on synthesis or degradation of proteins/peptides in the haemolymph will be the reason for the increase in haemolymph protein concentration. In *Schistocerca gregaria* (desert locust) nymphs, after 1 day of treatment, pyriproxyfen and lufenuron elevated the protein level (Ghoneim et al., 2012). In the last instar larvae of *Spodoptera littoralis* the haemolymph protein concentration increased with the treatment of methoprene, hydroprene or kinoprene (Fouda and Amer, 1990). In the hemipteran insect *Eurygaster integriceps* Puton, treatment of the adults

with 0.74 and 1.49µg/mg insect of pyriproxyfen, lead to a decrease in the concentration of haemolymph protein. The protein concentration was lower than the control after 24 and 48 hours, but it reached to that of control after 120 hours (Zibaeeet al., 2011). In our study, the 5th instar day 0 larvae treated with sub lethal concentration of pyriproxyfen was considerably larger than the control, which became a supernumerary larva. Sindhu and Nair reported the treatment with JHA, hydroprene, to the 6th instar larva of *S.mauritia* lead to increased food consumption and formation of a supernumerary larva (Sindhu et al., 2004). Generally at the end of larval development the JH level decreases and the metamorphosis is triggered by ecdysone. Addition of JH at the end of larval development leads to formation of supernumerary larvae (Truman and Riddiford, 2002). Parthasarathy and Palli studied the action of JH analogues on metamorphosis of *Tribolium castaneum*, red flour beetle, and they found that JH analogue application on the penultimate and final instar larvae lead to prolonged larval lifespan and the formation of supernumerary larvae. The JH analogues blocked larval-pupal metamorphosis (Parthasarathy and Palli, 2009).

5.3. Effect of Pyriproxyfen on haemolymph protein profile

The protein profile of the haemolymph of fifth instar day 0 larvae of *Spodoptera mauritia* treated with LD_{10} concentration of pyriproxyfen (4µg/larvae) were analyzed by SDS-PAGE, after 24 hours of exposure, there was an increase in intensity of the major protein band (83 kDa) in the treated compared to control. The effect of JH III and pure pyriproxyfen were also tested and similar increase in

83kDa protein band was observed. As pyriproxyfen is acting through the JH receptor, JH is also supposed to increase the protein concentration. To check that we have treated the larvae with JH III which is the more active JH in lepidoptera to control moulting and an increase in the 83kDa protein band was observed. In our studies we used the insecticide Knack IGR, the active ingredient of which is pyriproxyfen, for all the experiments except where specified. Knack IGR is developed in USA to control silver leaf whitefly (*Bemisia argentifolii*) in cotton. Along with pyriproxyfen it contains aromatic petroleum distillates to promote the penetrance of the insecticide through the cuticle of the pest. To check whether the effect was due to the pyriproxyfen or due to the other substances in the Knack IGR formulation we treated the larvae with pure pyriproxyfen and the same effect as that of Knack IGR was observed indicating that the active ingredient, pyriproxyfen, in the Knack IGR is responsible for the observed effect.

When the difference in the protein profile of the haemolymph of untreated individual larvae was checked, the difference in the intensity of 83 kDa band ranges from 0.08 to 1.95 times. From a baseline value the average difference is 0.65 ± 0.26 times, which is statistically not significant (p value > 0.05). This indicates that the observed alteration is not due to variation in the protein band in individual larva.

During the experiments, sometimes, an aggregate upper band is formed especially on prolonged freezing of the samples. To remove the aggregation, additional treatment which includes addition of 2µl of β-mercaptoethanol before final heating was required.

5.4. Regulation of expression of pyriproxyfen-responsive protein

5.4.1. Effect of increase in concentration of pyriproxyfen on pyriproxyfen-responsive protein

The SDS-PAGE analysis of haemolymph collected from the *S. mauritia* larvae treated with different concentrations (2µg, 4µg, 10µg and 20µg/ larva) of pyriproxyfen showed an increase in the intensity of pyriproxyfen-responsive 83kDa protein band with the increasing concentrations of pyriproxyfen. It means that the effect of pyriproxyfen on pyriproxyfen-responsive 83kDa protein level is concentration dependent. Zhang et al. studied the role of JH binding protein in the Cotton Bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in response to hormone and starvation and they identified two *JHBP*(Juvenile Hormone Binding Protein) genes, *HaJHBP1* and *HaJHBP2*. In their study they reported that treatment with the JH analogue, methoprene (2.5µg) in 4th instar larvae of Cotton Bollworm increased the expression levels of *HaJHBP1* and *HaJHBP2*. The expression of *HaJHBP1* was increased to 204% after 24 hours of treatment (Zhang et al., 2019).

5.4.2. Effect of Cycloheximide on level of expression of pyriproxyfen-responsive protein.

The concentration of a protein is maintained by a balance between the synthesis and degradation process. On treatment with cycloheximide, a protein synthesis inhibitor, there is a considerable decrease in the intensity of the pyriproxyfen-responsive protein band when compared to pyriproxyfen alone treated larvae. This indicates

that, on exposure to pyriproxyfen, the protein level is increased by induction of synthesis of this protein. A moderate mortality rate was observed in leaf-cutting worker ants on high concentration of cycloheximide ingestion (Sousa et al., 2018). In larvae, pupae and adults of *Spodoptera littoralis*, cycloheximide showed a moderate toxic effect. It prevented the adult emergence by impairing development and cause sterilization of this pest (Ghoneim and Basiouny, 2018).

5.5. Identification of the JH analogue-responsive protein in fat body

A protein band corresponds to the identified pyriproxyfen-responsive protein in molecular weight was found in the SDS-PAGE analysis of fat body extract of *S. mauritia*. The same band in fat body extract showed increase in intensity on treatment with pyriproxyfen. Thus pyriproxyfen- responsive protein is expressed /localized in the fat body. Hence one of the sites of synthesis/storage of the identified pyriproxyfen- responsive protein is the fat body. Chauhan et al. reported the presence of high molecular weight hexamerin proteins (82-86 kDa) in the fat body of *Spodoptera litura* F. (Chauhan et al., 2017) During postembryonic development of *Corcyra cephalonica*, expression of arylphorin hexamerin (84kDa) in tissue specific manner by the larval fat body and regulation of its gene by 20E was also reported (Manohar et al., 2010; VenkatRao et al., 2015). Burmester reported that the hexamerins do not accumulate in the fat body, during the active feeding phase of the larvae the hexamerins are synthesized in the fat body and immediately released to the haemolymph

(Burmester, 2002). In the early instars, proteins are synthesized in the fat body (the main site of protein synthesis) and subsequently released into the surrounding haemolymph (Shigematsu and Takeshita, 1968) which, in later instars are sequestered from haemolymph into the fat body.

5.6. Glycosylation status of pyriproxyfen-responsive protein

Glycosylation status of the pyriproxyfen - responsive protein was analyzed by PAS staining and the identified pyriproxyfen - responsive protein band appeared in pink colour indicating that the pyriproxyfen - responsive protein is a glycoprotein. Studies on 5th instar *Antheraea mylitta*, Indian tropical non-mulberry tasar silkworms, revealed three potential N-linked glycosylation sites within the arylphorin protein-coding region (Dutta et al., 2020) Zhu, et al. noticed that in the 306 and NB strains of *Bombyx mori the* arylphorin protein was heavily O-glycosylated (Zhu et al., 2019). Tang et al., (2010) reported four potential N-glycosylation sites in storage hexamerins from *Spodoptera exigua*, *SeHex* (amino acids 75, 209, 479 and 647), and one potential site (amino acid 47) in *SeSP1*.

5.7. Determination of the subunit composition of the identified protein

The pyriproxyfen- responsive protein on alkaline PAGE is a high molecular weight (≈500kDa) protein. This band when cut out and loaded on to SDS-PAGE under reducing conditions, gave a single polypeptide of around 83kDa on SDS-PAGE indicating that the protein is a hexameric multimer. So, the pyriproxyfen-responsive protein

identified in SDS-PAGE is a subunit of hexamerin family of storage proteins. Telfer et al. (1991) reported that storage hexamerins are composed of six subunits of ~80 kDa with a native molecular weight around 500 kDa. They are the widely distributed and most abundant storage proteins that accumulate in the hemolymph or fat body of insects.

5.8. Identification of the JH analog responsive protein by LC-MS/MS analysis

The subunit molecular weight, glycosylated nature of the identified protein and the abundance in the haemolymph are indicates that the identified pyriproxyfen –responsive protein in SDS-PAGE is a subunit of hexamerin. To further confirm this we have identified the amino acid sequence of peptides from pyriproxyfen- responsive protein by LC-MS/MS analysis. From the data obtained, it is found that the pyriproxyfen-responsive protein is similar to arylphorin subunit of *Spodoptera litura*. Thus the identified pyriproxyfen-responsive protein is a storage protein, arylphorin, of *S.mauritia.*

Storage proteins or hexamerins are also known as larval serum proteins (LSP). In holometabolous insects, they are synthesized by larval fat body, secreted into the haemolymph and they accumulate at high levels during the late larval stage. In some cases the level of storage proteins reaches 60% of all soluble proteins of the organism (Munn and Greville, 1969; Chrysanthis et al., 1981).

Eleven storage protein-coding genes were identified from the genome of the mosquito *Culex quinquefasciatus*, eight of which

encode proteins similar to the *Drosophila melanogaster* LSP1 subunits and the other three showed similarity with the LSP2 polypeptide of *D. melanogaster*. The mature peptides had molecular weight ranges between 76 kDa and 83 kDa (Martins et al., 2013).

Storage hexamerins are composed of six subunits of ~80 kDa with a native molecular weight around 500 kDa. They are the widely distributed and most abundant storage proteins that accumulate in the hemolymph or fat body of insects (Telfer et al., 1991). Storage proteins include the hexamerins, juvenile hormone-related protein, riboflavin-binding hexamerin precursor, methionine-rich storage protein (storage protein 1, SP1), very-high-density lipoprotein, tyrosine-rich proteins and hemocyanin-related proteins (Wang, 2001). Thus the pyriproxyfen-responsive protein identified here is the sub unit of hexamerin family of storage proteins, arylphorin. Lepidoptera shows a wide diversity of hexamerins. Five different types of hexamerins such as arylphorins, arylphorin-like hexamerins, methionine-rich storage protein (MRSP), moderately methionine-rich storage protein (MMRSP) and riboflavin-binding hexamerins (RbH) have been identified from Lepidoptera. They differ in terms of evolutionary history and amino acid composition (Burmester, 2015). Phylogenetic analysis done by Wang and his colleagues revealed the expression of five different types of hexamerins in *Spodoptera exigua*, they are storage protein rich in methionine residue (MRSP), Storage protein moderately rich in methionine (MMRSP), Hexamerin with high composition of aromatic amino acids (Arylphorin), Arylphorin-like hexamerin and Riboflavin-binding hexamerin (RbH). They found that

the hexamerins act as the storage protein during metamorphosis, expressed in fat body and insecticide exposure can influence their expression (Wang et al., 2019). The crystal structure of *Bombyx mori* arylphorins reveals that it a hetero-hexamer, composed of a trimer of the tight SP2/SP3 hetero-dimer (Hou et. al, 2014). Two storage hexamerins have been cloned and characterized from *Spodoptera exigua* (Tang et al., 2010). SL-1, SL-2 and SL-3 are storage proteins from *Spodoptera litura* with molecular sizes between 400 and 450 kDa, and are composed of subunit(s) which range in size from 70 to 80 kDa. SL-3 is an arylphorin and the other two are methionine rich storage proteins (Zheng et al., 2000). The hexamerins are not only functions as storage proteins but also act as carriers of hormones, and participate in metamorphosis, moulting, and reproduction. Hence the storage proteins are crucial for insect development. They are also affected by treatment with insecticides. So the storage proteins are ideal targets for designing better insect control agents. It will be worth examining the role played by these proteins in the physiology of insects on exposure to the juvenile hormone analogue, pyriproxyfen. Also the identification of storage proteins altered on exposure to IGRs will help understand whether they will contribute to the insecticide resistance and will pave way for designing better insect control strategies.

SUMMARY

Insects are the largest group of animals in the phylum arthropoda. Insects have both positive and negative impact on humans. Negative impact is mainly in the form of pests. Almost all the crops

face the loss due to insect pests. *Spodoptera mauritia* or rice swarming caterpillar is a major pest of paddy coming in the order lepidoptera. . In India, earlier it was considered as a sporadic and minor pest of rice but it has emerged as serious pest of rice seedlings for the last one decade. In this study we examined the effect of juvenile hormone mimic, pyriproxyfen on the mortality and protein profile changes in the haemolymph of larvae of *Spodoptera mauritia*. It was found that after 24 hours of the treatment of 3rd, 4th, 5th and 6th instar day 0 larvae of *Spodoptera mauritia*, with increase in concentration of pyriproxyfen led to increase the mortality. From the mortality data, the LD_{50} value of pyriproxyfen for the 3rd, 4th, 5th and 6th instar larvae of *Spodoptera mauritia* was found to be 14.13±2.67, 15.85±3.67, 39.81±2.61and 316.20±2.64µg respectively. The LD_{50} value also increased with increase in the instar. The exposure of 5th instar day 0 larvae of *Spodoptera mauritia* to sub lethal concentration (LD_{10}) of pyriproxyfen led to the formation of supernumerary larvae and increase in haemolymph protein concentration compared to control. It also leads to an increase in intensity of an 83 kDa haemolymph protein band. The intensity of the pyriproxyfen- responsive protein band was increased with increasing concentration of pyriproxyfen. The experiment using cycloheximide, a protein synthesis inhibitor, revealed that the increase in protein concentration was the result of increased protein synthesis. The identified pyriproxyfen- responsive protein is a glycoprotein. From the native PAGE and followed by SDS-PAGE of the band cut out from the native PAGE, it is found that the identified protein is a hexamer with subunit molecular weight of 83 kDa and a native molecular weight around 500 kDa . From LC-MS/MS data, it is found

that the pyriproxyfen-responsive protein is similar to Arylphorin subunit of *Spodoptera litura*. Arylphorin is a storage hexamerin in Lepidoptera. The identified protein is present in fat body and is one of the sites of synthesis/storage of this protein. Thus the identified pyriproxyfen-responsive protein is a storage protein, arylphorin of *S.mauritia.* Identifying the storage protein altered in response to the IGRs will help in understanding the role played by this protein in the altered physiology of the insect on exposure to IGRs. Whether the increase in storage protein on exposure to pyriproxyfen, offers resistance towards the insecticide is worth investigating.

CONCLUSIONS

In this study we examined the effect of juvenile hormone mimic, pyriproxyfen on the mortality and protein profile changes in the haemolymph of larvae of *Spodoptera mauritia.*From the toxicity study, it was found that after 24 hours of the treatment of 3rd, 4th, 5th and 6th instar day 0 larvae of *Spodoptera mauritia*, with increase in concentration of pyriproxyfen the mortality increased. The LD_{50} value of pyriproxyfen for the 3rd, 4th, 5th and 6th instar larvae of *Spodoptera mauritia* was found to be 14.13±2.67, 15.85±3.67, 39.81±2.61and 316.20±2.64µg respectively. The exposure of 5th instar day 0 larvae of *Spodoptera mauritia* to sub lethal concentration (LD_{10}) of pyriproxyfen led to the formation of supernumerary larvae and increase in haemolymph protein concentration compared to control. It also leads to a concentration dependent increase in intensity of an 83 kDa haemolymph protein band. The increase in the intensity of this protein band was due to the increase in protein synthesis. The identified pyriproxyfen- responsive protein is a glycoprotein and it is a hexamerin with a subunit molecular weight of 83 kDa and a native molecular weight around 500 kDa. From LC-MS/MS data, it is found that the pyriproxyfen-responsive protein is similar to Arylphorin of *Spodoptera litura*. The identified protein is present in fat body and fat body is the site of synthesis/storage of this protein. Thus the identified pyriproxyfen-responsive protein is a storage protein, arylphorin of *S.mauritia.* Whether the increase in storage protein on exposure to

pyriproxyfen led to any advantage to the larvae to overcome the effect of pesticide is worth investigating.

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