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COMBINED EFFECT OF ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* (METSCHINKOFF) AND NOVEL INSECTICIDES ON THE CELLULAR AND HUMORAL IMMUNE MECHANISMS OF *SPODOPTERA LITURA* (FABRICIUS)

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ABSTRACT

Combination of insect entomopathogenic fungi and microbial metabolites is a prospective method for highly polyphagous pest control. The effect of the entomopathogenic fungus, *Metarhizium anisopliae* (Metschinkoff) and Emamectin benzoate, Spinosad and Spinetoram on the cellular and humoral responses of *Spodoptera litura* (Fabricius) larvae {Cellular immune responses [Total haemocyte count (THC), Differential haemocyte count (DHC), phagocytosis, nodulation and encapsulation] and Humoral immune responses [lysozyme-like, phenoloxidase (PO) activities]} was studied. It is shown that the combination of these agents leads to a synergistic effect on *S. litura* mortality. Colonization of *S. litura* larvae by entomopathogenic fungus following inoculation with novel insecticides is shown. The larvae affected by combined treatments are characterized by a decrease in PO and lysozyme levels. In the initial stages of fungal infection and toxicosis (12 to 24 h posttreatment), increases in the activity of insect cellular and humoral responses, and these increases are suppressed later with time. A strong suppression of immune response of the larvae under combined treatment of *M. anisoplae* with Emamectin benzoate and mild response when combined with *M. anisoplae* and Spinosad, Spinotoram was observed, however the response was decreased after 24 hours of treatment. The changes in the response of immune system under combined treatment suggest the effect of synergism.

Keywords **:** *Spodoptera litura*, *Metarhizium anisopliae*, Emamectin benzoate, Spinosad, Spinetoram, Cellular & Humoral immune responses.

Introduction

The tobacco caterpillar, *Spodoptera litura* Fab (Noctuidae: Lepidoptera), is one of the polyphagous pests of many agricultural crops, with a wider geographic range throughout Asia,

Africa, Australia and New Zealand (Weinberger and Srinivasan, 2009). The crops such as cauliflower, chilli, groundnut, tobacco, cotton, sunflower, pulses, and castor are some of the important cultivated plants that serve as hosts of this notorious pest. Among the vegetables, cauliflower is one of the important hosts for *S. litura* and is considered as a serious pest next to Diamondback moth (DBM). Use of synthetic insecticides is indispensible for *S. litura* management in cauliflower. Synthetic pyrethroids and organophophates are being used widely for its management. Indiscriminate use of synthetic chemicals and development of resistance to synthetic chemicals in *S. litura* has been reported earlier (Kodandaram and Dhingra, 2007). In the past, this droves the farmers to move towards the newer chemistry insecticides to suppress this pest in various crops as they have proven to have an added advantage over the conventional insecticide due to its novel mode of action and low eco-toxicity. In spite of various advantages in using newer molecules, the occurrence insecticide resistance to some molecules including emamectin benzoate,

spinosad and spinetoram has also been documented worldwide (Ahmad *et al*., 2008; Chen *et al*., 2008; Mohamoudvand *et al*., 2011). Therefore, there is a need for alternative nonchemical vector control approaches. Classical biological control based on using various microorganisms, such as entomopathogenic fungi and bacteria, is a frequent tool for addressing this issue.

Among the biological agents employed for *S. litura* larvae control, bacteria from the genus *Bacillus* are the most widely used. In addition, products of the entomopathogenic fungi *Metarhizium anisopliae s.l.,* and *Beauveria bassiana s.l.* are actively being developed for use against *S. litura* adults and larvae (OrtizUrquiza, Luo & Keyhani, 2015). It should be noted that the insects can develop resistance to the *Bacillus thuringiensis* Berliner biological larvicide (Boyer *et al*., 2012). However, the resistance of insects to entomopathogenic fungi develops very slowly (Dubovskiy *et al*., 2013).

To reduce toxic effects on the environment and increase efficacy against *S. litura*, entomopathogenic fungi may be combined with other biocontrol agents or low doses of natural insecticides. For example, combined treatment with *Metarhizium* and mosquito predator species (Toxorhynchites) has shown additive or synergistic effects on the mortality of *Ae. aegypti* (Alkhaibari *et al*., 2018).

Synergistic effects between entomopathogenic fungi and some chemical insecticides (temephos, spinosad) (Vivekanandhan *et al*., 2018) or biological agents (*Azadirachta indica*) A. Juss (Badiane *et al*., 2017) on the mortality of mosquito larvae have been found. However, the physiological and biochemical aspects of this synergism were not considered.

The promising insecticides that can be effectively used for *S. litura* control is the avermectins, Spinosad and spinetoram. Avermectins are a class of macrocyclic lactones isolated from the soil actinomycete *Streptomices avermitilis* Kim and Goodfellow (Drinyaev *et al*., 1999) and include several commercial derivatives (ivermectin, abamectin, doramectin and eprinomectin) with the same mode of action-activation of glutamate-gated chloride channels, followed by uncontrolled influx of chloride ions into the cells, which leads to paralysis and death of the organism (Campbell *et al*., 1983). At the same time, avermectins are relatively safe for humans (Crump & Omura, 2011).

Spinosyns are the newest class of insecticides, represented by spinosad and spinetoram. This are the fermentation metabolite of the actinomycetes, *Saccharopolyspora spinosa*, a soil inhabiting microorganism. These are novel insecticides with nicotinic acetylcholine receptor (nAChR) allosteric modulation as mode of action.

It is important that entomopathogenic fungi such as *Metarhizium* are adapted to terrestrial hosts and that in lepidopteran larvae, the fungi do not adhere to the cuticle surface and do not germinate through integuments into the hemocoel. Conidia ingested by larvae do not penetrate the gut wall (Butt *et al*., 2013). Thus, a ‘‘classic’’ host-pathogen interaction does not occur, and larval mortality is associated with stress induced by spore-bound proteases on the surface of ingested conidia (Butt *et al*., 2013). These authors suggest that fungal proteases cause an increase in the activity of caspases in the larva, which leads to apoptosis, autolysis of tissues and death of the larvae. The activation of immune mechanisms, cellular and humoral defence mechanisms occur in larvae infected with the fungus but is not sufficient to protect the larvae from death. During this process, particular superfamilies of enzymes such as glutathione-Stransferases (GST) and nonspecific esterases (EST) are usually involved in the biochemical transformation of xenobiotics (Li, Schuler & Berenbaum, 2007). Various hormones such as biogenic amines are involved in insect stress reactions. Among them, the role of the neurotransmitter dopamine (which serves as a neurohormone as well) in this process remain poorly understood. It is known that dopamine mediates phagocytosis and is involved in the activation of the pro-phenoloxidase (proPO) cascade, thus playing an important role in fungal and bacterial pathogenesis as well as in the development of toxicoses caused by insecticides (Wu *et al*., 2015). In addition, both pathogens and toxicants can lead to changes in the antimicrobial activity of insects which can affect the susceptibility of insects to pathogenic fungi (Polenogova *et al*., 2019).

Nodulation, encapsulation and phagocytosis are examples of cellular immune responses. Humoral immune responses include the production of various antimicrobial peptides, proteins, reactive oxygen and nitrogen species, lysozyme activating system and prophenoloxidase activating system, which results in the coagulation and melanization of haemolymph (Bogdan *et al*., 2000 and Nappi *et al*., 2000). Pattern Recognition Receptors (PRRs), found on the surface of insect cells, interact with Pathogen Associated Molecular Patterns (PAMPs), which are found on the surface of pathogens and include Peptidoglycans, Lipopolysaccharides (LPSs) and β-1,3 glucans, to cause the humoral response (Zhong *et al*., 2017).

We hypothesize that the interaction of entomopathogenic fungi with novel insecticides, Avermectins, spinosad and spinetoram can have a stable insecticidal effect at relatively low concentrations and is a promising combination for safe and effective *S. litura* control. It should be noted that the above-mentioned physiological reactions in *S. litura* larvae under the combined action of entomopathogenic fungi and insecticides have not yet been studied. The aims of this study were (1) to determine the susceptibility of *S. litura* larvae to combined treatment with emamectin benzoate, Spinosad, spinetoram and Metarhizium anisopliae and (2) estimate their immune mechanisms both cellular and humoral to M. anisopliae and emamectin benzoate, Spinosad, spinetoram in combination.

Materials and Methods

Insect Rearing

The *Spodoptera litura* larvae were collected from the college farm of College of Agriculture, Professor Jayashankar Telangana State Agricultural Univesrity (PJTSAU) and farmer fields in Rajendranagar, Hyderabad. The collected larvae were maintained on the artificial diet (Gupta *et al*., 2005) with Relative Humidity 70-75% and temperature 20±2˚c under laboratory conditions. The larvae which were in the stage to pupate were transferred into the tray containing soil. After pupation, the pupae were collected in a tray and covered with a muslin cloth. The emerged adults with a male and female ratio of 2:3, were transferred into the cages containing 15% sucrose solution (Santharam, 1985). The egg masses were collected and maintained in the Insect Pathology Laboratory, Department of Entomology, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad.

Inoculation procedure

Commercial formulation of entomopathogenic fungus, *Metarhizium anisopliae* used in the present study were sourced from a noted agri biotech company, AgriLife (India) Private Limited, Hyderabad. Topical application of entomopathogenic fungus has been done for inoculating the larvae. The third instar larvae were topically exposed by adding 10µl of the solution with a sampler device, following which they were placed on filter paper for 30 seconds and transferred two larvae per petri dish. Three to four days after development of symptom, insecticidal treatment (Table 1) was admistered and five such replications were maintained for the treatment. For control, castor leaves were dipped in 10µl of Tween 80 (0.05%) solution, then dried for 30 seconds and placed in the petri dish. Then the following immune responses were documented.

Collection of haemolymph from infected larvae

Haemolymph was collected by cutting the tip of a proleg of the infected larvae from each treatment, previously anesthetized on ice using fine scissors and needle (Rosenberger and Jones, 1960). Gentle pressure was applied on the insect abdomen for getting more quantity of haemolymph (Barkat *et al.,* 2002). The collected haemolymph from the infected larvae was used to prepare blood smears, by spreading a drop (20µl) of haemolymph on a glass slide and smeared by drawing a second slide across the first at an angle of 45° and then dried at room temperature. The air-dried haemolymph smear slides were then dipped into methanol two times and air dried. Slides were then stained with Giemsa stain (diluted five times with phosphate-buffer saline (PBS) and filtered before use) for 20 min and then rinsed in distilled water. The smear was washed in 0.02% acetic acid followed by rinsing in distilled water. After drying, permanent microscopy slides were prepared using Permount or Canada balsam.

Characterization of the haemocytes

In order to identify haemocyte types, the shape, size and the cytoplasm constituents of cells were observed. About 10 slides (5 replications) were prepared from 10 untreated individuals for identifying different haemocyte types based on the identification keys set by Gupta (1979). The haemolymph drawn from the untreated individual was diluted with 0.9% NaCl solution, about 20µl of diluted haemolymph was placed on a slide using micropipette, stained with Giemsa stain and observed under the phase contrast microscope to identify different types of haemocytes.

Identification of cellular immune responses of *S. litura* to the combined treatment of *Metarhizium anisopliae* and test insecticides

Haemocytes count

Total haemocyte counts were calculated for *S. litura* larvae after infection with combined treatment of *M. anisopliae* and test insecticides (Emamectin benzoate, Spinosad and Spinetoram) and also from the untreated larvae in the control.

Determination of Total Haemocyte Count (THC)

The total haemocyte count of *S. litura* larvae were calculated after the inoculation with *M. anisopliae* and test insecticides by using Neubauer haemocytometer. The sampling of the haemolymph was done by using Thoma white cell pipette. Haemolymph was collected on a glass slide and quickly sucked up into Thoma white cell pipette up to the mark of 0.5. The end of the pipette was wiped and diluting fluid (glacial acetic

acid, 1 ml; distilled water, 100 ml; gentian violet, 0.3%) was drawn up to the mark of 11, for producing a dilution of 1/20, (1 part of blood: 20 parts of WBC fluid). The contents of the pipette were properly mixed and first three drops were discarded (Jones, 1962) and Neubauer's chamber was charged with the 200µl of the dilutant. Leave the haemocytometer for 2 minutes to settle the cells and reduce their movement. The haemocytes were counted from the four corner squares (white cell squares) in each of the two chambers according to the following formula (Gupta and Sutherland, 1968).

$$\frac{\text{Haemocytes in five 1 mm squares x dilution x Depth of chamber}}{\text{Number of 1 mm squares counted}} \times 100$$

Determination of Differential Haemocyte Count (DHC)

Differential Haemocyte Count (DHC) from the stained slides was carried out with the help of a cell counter by following the battlement method (Perveen and Ahmad, 2017). The film was examined systematically by observing traversed three fields along the edge and two fields down starting at the thin end of the smear. This sequence was continued until minimum of 200 cells were enumerated and various classes of haemocytes was also observed (Mahmood and Yousaf, 1985).

Phagocytosis

Phagocytic activity was determined by counting the cells containing spores in a Neubauer haemocytometer. Observations were made on phase contrast microscope (Zibaee *et al*., 2011).

Nodulation

Injected larvae were chilled on ice, haemolymph was collected in a capillary tube and 200µl was placed onto a haemocytometer for nodule counting (Zibaee and Malagoli, 2014).

Identification of humoral immune responses of *S. litura* to the combined treatment of *Metarhizium anisopliae* and test insecticides

Collection of haemolymph

Approximately 40 µl of haemolymph was obtained from each individual larva. Two larvae for each replication and five replications for each treatment were used for the study. The collected haemolymph was mixed with anticoagulant solution in the ratio of 4:5, immediately (400 µl haemolymph: 500 µl of anticoagulant solution) (0.01 M EDTA, 0.1 M glucose, 0.062 M NaCl, and 0.026 M citric acid, pH 4.6) (Azambuja *et al*., 1991).

PO preparation

The diluted haemolymph was centrifuged at 12,000 rpm for 5 min, and then the supernatant was removed and the pellet washed using phosphate buffer (K_2HPO_4 65 mM, KH_2PO_4 2.6 mM, NaCl 400 mM and NaN_3 3 mM; pH = 6.5) (two times) (Leonard *et al*., 1985). The pellet was homogenized after adding 500 µl of cold phosphate buffer, and then centrifuged at 12,000 rpm for 15 min. The Haemocyte Lysate Supernatant (HLS) was transferred into new microcentrifuge tubes. Samples (10 µl) were preincubated with phosphate buffer at 30°C for 30 min, then 50 µl of 10 mM L-dihydroxyphenylalanine (L-DOPA) as substrate was added and incubated for 5 min at 30°C. PO activity was measured using a spectrophotometer (S 2100 Diode Array spectrophotometer) at 490 nm wavelength. One unit was described as 0.01 absorbance increase at 490 nm/min (Zibaee *et al*., 2011). Activity in treated assays were compared with that of control.

Lysozyme Activity

After collecting the haemolymph, the activity of lysozyme was evaluated according to Garriga *et al.* (2020) with slight modifications. Briefly, N-Phenylthiourea crystals were added to the samples to prevent activation of the PO system. Samples were centrifuged twice at 250 x g for 5 minutes at 4°C, and then at 1600 x g for 10 minutes at 4°C, collecting the supernatant. Cell-free hemolymph was diluted 1:10 with sterile PBS. Thereafter, 100 µl of the samples were added to 150 µl of *Micrococcus lysodeikticus* (0.45 mg/ml in 30mM phosphate buffer, pH 7.2; Sigma-Aldrich), with an optical density of 0.6-0.7, which was used as substrate to measure lysozyme relative activity. *M. lysodeikticus* alone and cell-free haemolymph without addition of the bacterium was used as control. Absorbance at 450 nm was measured every 30 s for 10 min. in 96-well plates using a Bio-Rad iMark™ Microplate Absorbance reader (Bruno *et al.,* 2021).

Data Analysis

Each treatment was replicated five times and the data was analyzed by using Completely Randomized Design (CRD). The data was subjected to the standard statistical analysis using techniques of analysis of variance. Differences between samplings were considered statistically significant at a probability more than 5% ($p \leq 0.05$).

Results

Cellular immune responses of *S. litura* when used in combination with *M. anisopliae* (Ma) and selected insecticides.

The cellular immune responses of *S. litura* in terms of important parameters were studied and outcome of the studies was discussed.

Determination of Total Haemocyte Count (THC) through light microscopy

The combined effect of *M. anisopliae* (Ma) and selected insecticides such as Emamectin benzoate, Spinosad and Spinetoram on larval immunity shows that there was an increase in the THC up to 12 HAI. However, the count decreased later (Fig. 1). The highest number of total haemocytes were recorded after treatment with Ma+ Spinetoram (159 ± 2.08^{a}) but there was no significant difference between Ma+ Spinetoram and Ma+ Spinosad treated larval immunity. On the other hand, Ma+ Emamectin benzoate treated larvae showed lowest number of haemocytes (72 ± 2.08^{c}) (Table 2).

Determination of Differential Haemocyte Count (DHC) through light microscopy

After the infection of *S. litura* larvae with combination of Ma and selected insecticides the PLs count was increased up to 12 HAI (Fig. 2) and GRs count was increased up to 24 HAI (Fig. 3). The highest number of PLs were observed at 12 HAI with Ma+ spinetoram (96 ± 1.53^{a}) but there is no significant difference with Ma+ Spinosad (91 ± 1.73^{a}). On the other hand, lowest was recorded in case of Ma+ Emamectin benzoate treated larvae (32 ± 2.52^{c}) (Table 3). The highest number of GRs were observed at 24 HAI with Ma+ spinetoram (78 ± 2.08^{a}) but there is no significant difference with Ma+ Spinosad (72 ± 2.52^{a}). On the other hand, Ma+ Emamectin benzoate treated larvae recorded the lowest DHC (53 ± 2.52^{b}) (Table 4).

Effect on phagocytosis

Injection of *S. litura* larvae with Ma and selected insecticides showed that the number of phagocytised cells increased up to 12 hours (Fig. 4). The maximum phagocytised cells were observed in Ma+ Spinetoram at 12 HAI but there is no significant difference between Ma+ spinetoram (26 ± 1.53^{a}) and Ma+ Spinosad (22 ± 1.53^{a}). On the other hand, Ma+ Emamectin benzoate treated larvae showed least phagocytised cells (3 ± 1^{b}) (Table 5).

Effect on Nodulation

Injection of *S.litura* larvae with Ma and selected insecticides showed that maximum nodule formation in Ma+ Spinetoram at 24 HAI but there is no significant difference between Ma+ Spinetoram (19 ± 1.53^{a}) and Ma+ Spinosad (16 ± 2.08^{a}). On the other hand, Ma+ Emamectin benzoate showed least nodulation (9 ± 1.53^{b}) (Fig. 5) (Table 6).

Effect on Encapsulation

Infection of *S.litura* larvae with Ma and selected insecticides showed that encapsulation was increased up to 12 HAI (Fig. 6). The maximum was observed in Ma+ Spinetoram at 12 HAI (18 ± 0.58^{a}) but there is no significant difference with Ma+ Spinosad (15 ± 2.08^{a}) On the other hand, Ma+ Emamectin benzoate treated larvae showed minimum encapsulation (5 ± 2.31^{b}) (Table 7).

Humoral immune responses of *S. litura* when used in combination with *M. anisopliae* (Ma) and selected insecticides

The humoral immune responses of *S. litura* in terms of important parameters were studied and outcome of the studies was discussed.

Effect on Phenol oxidase activity

Infection of *S.litura* larvae with Ma and selected insecticides showed that there is an increased PO activity (Fig. 7). The maximum PO activity was observed in Ma+ Spinetoram (2.05 ± 0.08^{a}) at 24 HAI but there is no significant difference with Ma+ Spinosad (1.92 ± 0.07^{a}). On the other hand, Ma+ Emamectin benzoate treated larvae showed least PO activity. (0.52 ± 0.15^{b}) (Table 8).

Effect on Lysozyme activity

Infection of *S. litura* larvae with Ma and selected insecticides showed that lysozyme activity was increased up to 12 HAI in Ma+ spinetoram (3.92 ± 0.25^{a}) and Ma+ Spinosad (3.82 ± 0.41^{a}) treated larvae and in case of Ma+ Emamectin benzoate it was increased up to 24 HAI (Fig. 8). The maximum Lysozyme activity was observed in Ma+ Spinetoram but there is no significant difference between Ma+ spinetoram and Ma+ Spinosad. On the other hand, Ma+ Emamectin benzoate treated larvae showed least lysozyme activity (0.59 ± 0.21^{b}) (Table 9).

Discussion

We showed a synergistic effect between avermectins, spinosyns and *Metarhizium* (Ma) fungi on *Spodoptera litura* larva. A similar effect was shown previously in insects (Colorado potato beetle, cotton moth) (Anderson *et al*., 1989; Asi *et al*., 2010; Tomilova *et al*., 2016). The cellular and humoral responses were initially increased and then later decreased in all the treatments. Interestingly, the larvae

treated with avermectins accumulated a lower amount of conidia, but this dose was sufficient for a synergistic effect on mortality. It is likely that reduced accumulation of conidia was due to disturbance of feeding. For example, decrease in quantity of consumed food under the influence of avermectins was shown for insects (Akhanaev *et al*., 2017). We observed a decrease in PO and Lysozyme activity under the influence of the fungus (Ling & Yu, 2005; Yassine, Kamareddine & Osta, 2012; Yaroslavtseva *et al*., 2017; Chertkova, Grizanova & Dubovskiy, 2018).

We suggest that some fungal metabolites inhibit the PO cascade of *S. litura* larvae. It was shown on insects that *Metarhizium* secondary metabolites (e.g., destruxins) may reduce the number of PO-positive haemocytes (Huxham, Lackie & McCorkindale, 1989) and these metabolites may upregulate serine protease inhibitors, which inhibit proPO cascade (Pal, Leger & Wu, 2007).

A similar effect was observed at 12 HAI for lysozyme activities. Combined treatment leads to either inhibition or containment of the activation of these enzymes. Thus, we assume that the physiological causes of the observed synergism lie in the initial stages of the development of infection and toxicosis. We have previously shown the cytotoxic effect of avermectins on haemocytes, leading to their death (Tomilova et al., 2016). Additionally, the cytostatic and cytotoxic effects of the avermectins complex on various cells of warm-blooded animals are well known (Sivkov, Yakovlev & Chashov, 1998; Korystov *et al*., 1999; Maioli *et al*., 2013).

Conclusion

In conclusion, the survival and immunological reactions of *Spodoptera litura* larvae under the combined action of avermectins and entomopathogenic fungi, spinosyns and entomopathogenic fungi were studied. The synergism observed under the combined action of these agents appears to be associated with immunological changes in the early stages of toxicosis and infection. In particular, inhibition of the activity of a number of enzymes (PO and Lysozyme) is observed under the combined treatment associated with the detoxifying and immune systems. Further investigations may be focused on studying the development of toxicoses and mycoses as well as the development of preparative forms based on fungi and spinosyns, avermectins for *S. litura* control in natural conditions.

Table 1: Insecticides used in experimentation

Insecticides	**g a.i./ ha**	**Quantity required (ml or g/ lit.) of water**
Emamectin benzoate 5 SG	10	0.4
Spinosad 45 % SC	75	0.3
Spinetoram 11.7 SC	50	0.5

Table 2: Changes in Total Haemocyte Count (THC) in *S. litura* larval haemolymph infected in combination with of *M. anisopliae* and selected insecticides

Treatment	**1HAI**	**3HAI**	**6HAI**	**12HAI**	**24HAI**	**48HAI**	**72HAI**
Ma+ Emamectin benzoate	98±2.31^{b}	102±2.52^{c}	106±2.52^{c}	85±2.08^{c}	72±2.08^{c}	69±1.53^{c}	54±2.08^{c}
Ma+ Spinosad	141±1^{a}	159±2^{a}	161±1.15^{a}	182±2.65^{a}	154±1.53^{a}	98±3^{b}	96±2.08^{b}
Ma+ Spinetoram	158±1.73^{a}	164±1.53^{a}	166±1.15^{a}	189±2.31^{a}	159±2.08^{a}	104±2.52^{ab}	93±2.52^{b}
Control	132±1.53^{a}	138±1^{b}	125±2.08^{b}	131±2.52^{b}	112±1.53^{b}	111±2.52^{a}	119±2.65^{a}

*Different alphabets denote statistical differences among treatments (P≤0.05).

Table 3: Changes in the total Plasmatocytes (PLs) number in *S. litura* larval haemolymph infected in combination with *M. anisopliae* and selected insecticides

Treatment	**1 HAI**	**3 HAI**	**6 HAI**	**12 HAI**	**24 HAI**	**48 HAI**	**72 HAI**
Ma+ Emamectin benzoate	42±2.65^{c}	49±1.53^{c}	51±1.73^{b}	54±2^{b}	32±2.52^{c}	29±2.52^{c}	23±2.52^{c}
Ma+ Spinosad	72±2.65^{a}	78±1.53^{a}	82±2.65^{a}	88±3^{a}	91±1.73^{a}	42±1.53^{b}	31±2.08^{b}
Ma+ Spinetoram	78±2.52^{a}	83±1.53^{a}	88±1.73^{a}	92±2.31^{a}	96±1.53^{a}	53±2.08^{a}	37±2.52^{ab}
Control	61±1^{b}	62±2.08^{b}	54±2.52^{b}	52±2.65^{b}	58±1.73^{b}	47±2.08^{b}	42±1.53^{a}

*Different alphabets denote statistical differences among treatments (P≤0.05).

Table 4: Changes in the total Granulocytes (GRs) number in *S. litura* larval haemolymph infected in combination with *M. anisopliae* and selected insecticides

Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Ma+ Emamectin benzoate	21±2.08^{c}	25±1.53^{c}	38±2^{b}	51±1.53^{b}	53±2.52^{b}	29±1.89^{a}	17±2.65^{c}
Ma+ Spinosad	42±2.08^{ab}	59±2.65^{a}	63±2.52^{a}	68±1^{a}	72±2.52^{a}	38±1.53^{a}	25±2.08^{b}
Ma+ Spinetoram	46±2.52^{a}	54±1.53^{ab}	67±1.15^{a}	72±2.52^{a}	78±2.08^{a}	32±2.08^{a}	29±1.53^{b}
Control	39±1.53^{b}	52±1.53^{b}	32±2.65^{b}	43±2.08^{c}	49±1.73^{b}	35±2.08^{a}	37±2^{a}

*Different alphabets denote statistical differences among treatments (P≤0.05).

Table 5: Number of phagocytised cells observed in *S. litura* larval haemolymph after infection in combination with |*M. anisopliae* and selected insecticides

Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Ma+ Emamectin benzoate	3±0.58^{a}	4±1.53^{b}	5±1.53^{b}	6±1.53^{b}	3±1^{b}	-	-
Ma+ Spinosad	4±1.53^{a}	9±2.08^{a}	17±1.73^{a}	22±1.53^{a}	20±2.08^{a}	12±2.65^{a}	1±0.50^{a}
Ma+ Spinetoram	6±1.53^{a}	12±1.15^{a}	19±1.53^{a}	26±1.53^{a}	23±1.73^{a}	16±1^{a}	1±0.50^{a}
Control	2±0.58^{a}	3±0.58^{b}	1±0.58^{b}	2±0.58^{b}	1±0.58^{b}	2±0.58^{b}	1±0.50^{a}

*Different alphabets denote statistical differences among treatments (P≤0.05).

Table 6: Number of nodules formed in *S. litura* larval haemolymph after infection in combination with *M. anisopliae* and selected insecticides

Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Ma+ Emamectin benzoate	-	1±0.58^{b}	5±0.58^{c}	8±1.53^{c}	9±1.53^{b}	4±1.73^{b}	-
Ma+ Spinosad	2±0.58^{a}	9±1.53^{a}	10±1.53^{b}	13±1.53^{b}	16±2.08^{a}	10±1.53^{a}	2±0.58^{a}
Ma+ Spinetoram	3±1.15^{a}	11±1.70^{a}	15±1.73^{a}	18±1.53^{a}	19±1.53^{a}	12±1.53^{a}	1±0.58^{a}
Control	1±0.58^{a}	3±2.08^{b}	2±0.58^{c}	3±1.53^{d}	4±1.15^{b}	2±0.58^{b}	1±0.58^{a}

*Different alphabets denote statistical differences among treatments (P≤0.05).

Table 7: Number encapsules observed in *S. litura* larval haemolymph after infection in combination with *M. anisopliae* and selected insecticides

Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Ma+ Emamectin benzoate	-	3±1.53^{b}	4±0.58^{b}	5±2.31^{b}	1±0.58^{b}	-	-
Ma+ Spinosad	4±2.52^{a}	9±1.20^{a}	9±0.58^{a}	15±2.08^{a}	9±2.31^{a}	2±0.58^{a}	1±0.50^{a}
Ma+ Spinetoram	8±2^{a}	10±1.53^{a}	11±2.08^{a}	18±0.58^{a}	12±1.53^{a}	3±0.58^{a}	3±0.58^{a}
Control	3±1.53^{a}	1±0.58^{b}	2±0.58^{b}	1±0.58^{b}	1±0.58^{b}	2±0.58^{a}	1±0.50^{a}

*Different alphabets denote statistical differences among treatments (P≤0.05).

Table 8: Phenoloxidase (PO) specific activity (µmol/min) measured in *S. litura* larval haemolymph after infection with *M. anisopliae* and selected insecticides

Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Ma+ Emamectin benzoate	0.61±0.03^{a}	0.68±0.10^{b}	0.69±0.02^{c}	0.71±0.17^{b}	0.52±0.15^{b}	0.48±0.15^{a}	0.32±0.10^{c}
Ma+ Spinosad	0.97±0.27^{a}	1.12±0.05^{a}	1.32±0.04^{a}	1.45±0.04^{a}	1.92±0.07^{a}	0.62±0.06^{a}	0.52±0.08^{ab}
Ma+ Spinetoram	1.17±0.12^{a}	1.19±0.05^{a}	1.49±0.05^{a}	1.57±0.14^{a}	2.05±0.08^{a}	0.73±0.12^{a}	0.59±0.10^{b}
Control	0.91±0.09^{a}	0.82±0.06^{b}	0.89±0.09^{b}	0.72±0.10^{b}	0.68±0.18^{b}	0.85±0.08^{a}	0.98±0.02^{a}

*Different alphabets denote statistical differences among treatments (P≤0.05).

Table 9: Lysozyme specific activity (µmol/min) measured in *S. litura* larval haemolymph after infection with *M. anisopliae* and selected insecticides

Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Ma+ Emamectin benzoate	0.35±0.12^{a}	0.43±0.12^{c}	0.48±0.06^{c}	0.59±0.21^{b}	0.73±0.10^{c}	0.34±0.06^{c}	0.32±0.06^{b}
Ma+ Spinosad	0.98±0.34^{a}	1.92±0.20^{a}	2.86±0.09^{a}	3.82±0.41^{a}	3.01±0.13^{b}	0.92±0.10^{ab}	0.77±0.08^{a}
Ma+ Spinetoram	1.18±0.28^{a}	2.1±0.17^{a}	2.97±0.09^{a}	3.92±0.25^{a}	3.87±0.12^{a}	1.01±0.06^{a}	0.65±0.06^{a}
Control	0.67±0.08^{a}	1.17±0.29^{b}	2.01±0.17^{b}	1.29±0.17^{b}	0.87±0.16^{c}	0.74±0.08^{b}	0.62±0.06^{a}

*Different alphabets denote statistical differences among treatments (P≤0.05).

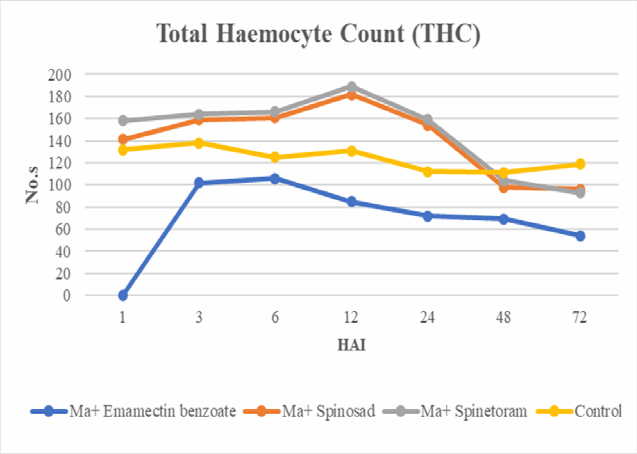

Fig. 1: Total Haemocyte Count (THC) in *S. litura* larval haemolymph at different Hours After Infection (HAI) with *M. anisopliae* and selected insecticides.

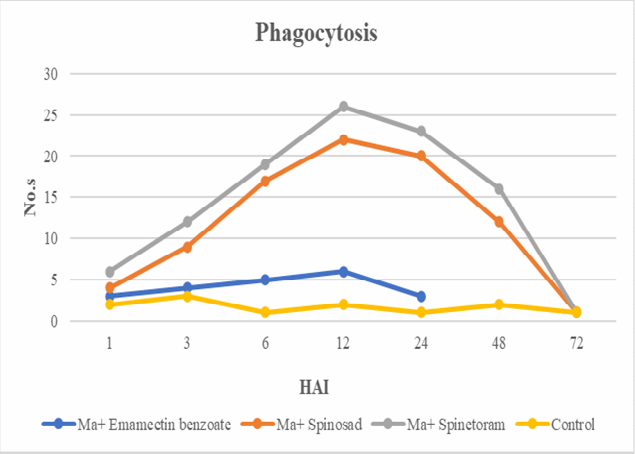

Fig. 4: Number of phagocytised cells observed in *S. litura* larval haemolymph at different Hours After Infection (HAI) with *M. anisopliae* and selected insecticides.

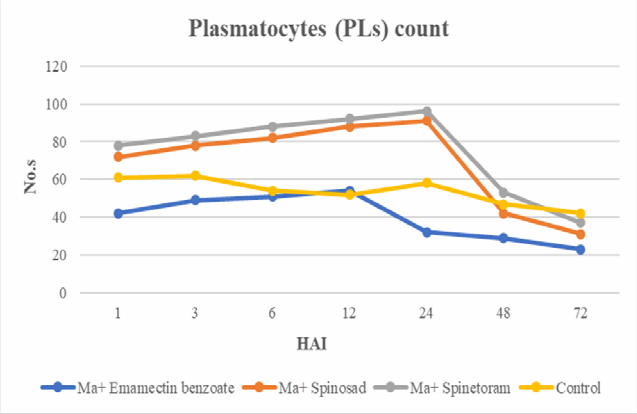

Fig. 2: Plasmatocytes (PLs) count in *S. litura* larval haemolymph at different Hours After Infection (HAI) with *M. anisopliae* and selected insecticides.

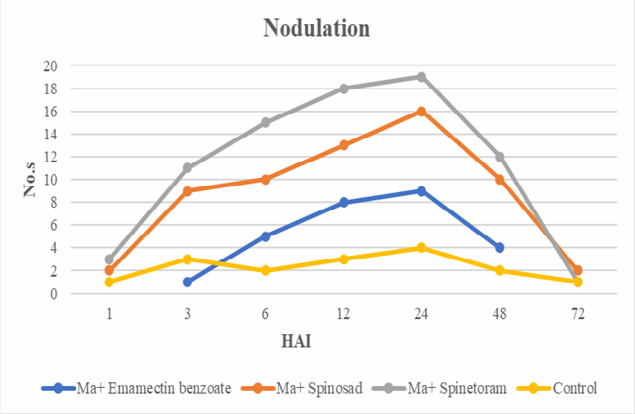

Fig. 5: Number of nodules formed in *S. litura* larval haemolymph at different Hours After Infection (HAI) with *M. anisopliae* and selected insecticides.

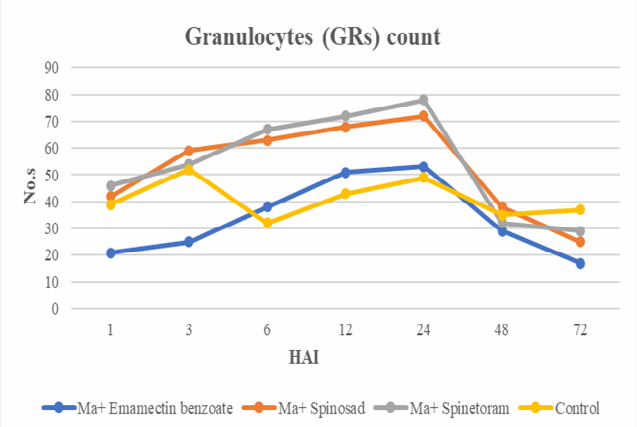

Fig. 3: Granulocytes (GRs) count in *S. litura* larval haemolymph at different Hours After Infection (HAI) with *M. anisopliae* and selected insecticides.

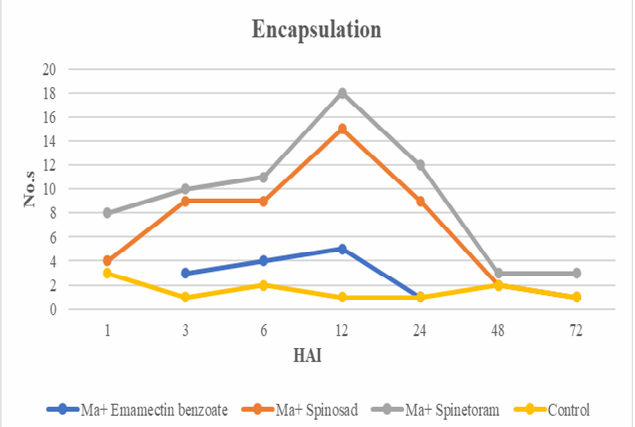

Fig. 6: Number of encapsules observed in *S. litura* larval haemolymph at different Hours After Infection (HAI) with *M. anisopliae* and selected insecticides.

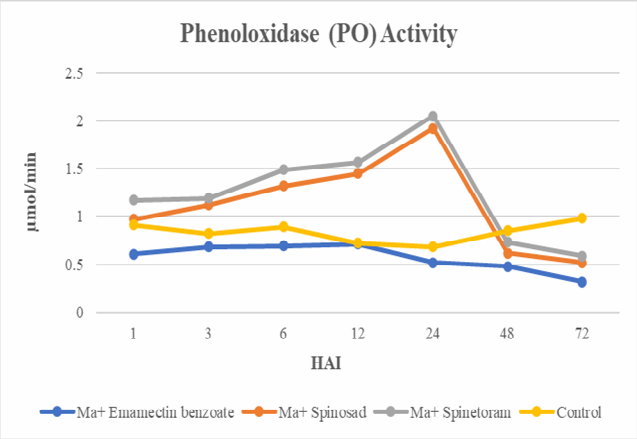

Fig. 7: Phenoloxidase (PO) activity in *S. litura* larval haemolymph at different Hours After Infection (HAI) with *M. anisopliae* and selected insecticides.

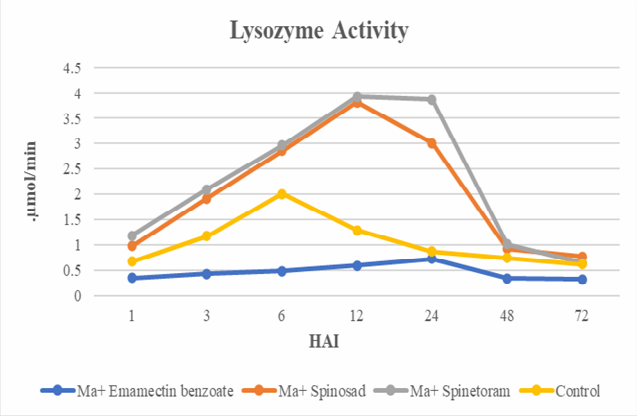

Fig. 8: Lysozyme activity in *S. litura* larval haemolymph at different Hours After Infection (HAI) with *M. anisopliae* and selected insecticide.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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