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Cellular Immune Response of *Spodoptera litura* (Fabricius) to the Potential Entomopathogenic Fungus *Metarhizium anisopliae* (Metschinkoff) Sorokin

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ABSTRACT: This experiment was conducted at Insect Pathology laboratory, Department of Entomology and Central Instrumentation, PJTSAU, Rajendranagar, Hyderabad in order to study the cellular immune response of *Spodoptera litura* (Fabricius) to the entomopathogenic fungus *Metarhizium anisopliae.* It would be a cumbersome work to collect the data on hourly basis. Moreover working on the haemolymph and its characteristics of an insect is a very exquisite work. One of the most prevalent and pervasive entomopathogens, *M. anisopliae* is recognised to be a highly effective bioagent for the control of insects of several orders. A fungal suspension containing 2.03×10^8 spores ml^{-1} was applied to larvae in their third instar. Following inoculation, hemolymph samples from treated and untreated larvae were collected at 1, 3, 6, 12, 24, 48, and 72 hours. Plamatocytes (PLs) and Granulocytes (GRs) were found to be the major haemocytes involved in the immune responses of the lepidopteran insects. Total Haemocyte Count (THC), PLs, GRs, Phagocytosis and Nodulation were observed as a part of cellular immune response. The THC, PLs, GRs, Phagocytosis and Nodulation were increased up to 24 HAI and later decreased. Despite these insect defence mechanisms, fungal hyphae development was seen after 72 HAI, which finally caused the host to perish.

Keywords: *Spodoptera litura*, *Metarhizium anisopliae*, Cellular Immune Response, Total Haemocyte Count (THC), Plamatocytes (PLs), Granulocytes (GRs), Phagocytosis and Nodulation.

INTRODUCTION

Insects are able to successfully fight themselves against microorganism infection despite lacking the specific defence systems present in vertebrate animals, such as antibodies (Dunn, 1986). Insect cellular immunity is associated with the free mobility of haemocytes inside the hemocoel. They can assault foreign objects and form overlapping sheaths that lead to the formation of nodules, encapsulation, disintegration, melanization, and phagocytosis (Choi *et al*., 1997). In response to the invasion of pathogens like bacteria, insect humoral immunity depends on the creation or amplification of a variety of antibacterial proteins (Boman *et al*., 1991). Entomopathogenic fungi, like *Metarhizium anisopliae* (Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes), have the ability to produce toxins that act as inhibitors of the host defence reactions, destroying the haemolymph and cell nuclei and also causing a decrease in energy because the fungus uses the nutrients from the haemolymph. *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), commonly known as tobacco caterpillar, is one of the most damaging pests of tobacco, tomato, cauliflower, castor, groundnut, cabbage and other cruciferous crops and it has been documented to result in significant crop losses in some regions of India (Venkateswarlu *et al*., 2006). Microbial management is being prioritised because to this pest developing pesticide resistance. Numerous lepidopteran pests have been reported to be successfully controlled by *M. anisopliae*. The pathophysiology of *S. litura* cellular immunological response, however, has not been well investigated. The goal of the current study was to learn more about how *M. anisopliae* affected *S. litura* haemocytes.

REVIEW OF LITERATURE

Corrêa *et al*. (2021) evaluated the impact of Dopamine (DA) on the survival and cellular immune response of *Metarhizium anisopliae* blastospore inoculated *Rhipicephalus microplus*. Exogenous DA improved both tick survival (72 h post *M. anisopliae* inoculation) and the quantity of circulating haemocytes (24 h post treatment) in comparison to the control group. When exogenous DA was injected, the phagocytic index of tick haemocytes exposed to *M. anisopliae* did not change. In comparison to untreated ticks or ticks inoculated with the fungus alone, phenoloxidase

activity in the haemolymph of ticks injected with DA and the fungus or exclusively with DA was higher 72 hours after treatment. Both ticks treated with and without fungus had DA found in their haemocytes.

Shahriari *et al*. (2021) showed that the cellular and antibacterial defences of the *Chilo suppressalis* walker larvae were improved after the injection of several natural entomopathogenic fungi. The total and differential haemocyte counts increased sharply in the first 3 and 6 hours after injections, but they gradually decreased 12 and 24 hours later. After a fungus infection, the production of nodules and phenoloxidase activity both increased over time. Antimicrobial peptides such as attacin1 and 2, cecropin1 and 2, gallerimycin, defensin, lysozyme and prophenoloxidase activating proteinase-3 all showed a similar pattern of transcription during fungal infection. In every instance, fungi injected larvae had more target gene transcription than control larvae.

Stączek *et al*. (2020) evaluated the cellular immunological response of the greater wax moth *Galleria mellonella* to *Aspergillus niger*-1,3-glucan. The creation of haemocyte aggregates, alterations in apolipophorin III localisation and changes in the Total Haemocyte Count (THC) and Differential Haemocyte Count (DHC) all pointed to the activation of *G. mellonella* cellular processes in response to the administration of *A. niger* α-1,3-glucan.

Seyedtalebi *et al*. (2017) conducted comparative studies on *Eurygaster integriceps*, *Ephestia kuehniella* and *Zophobas morio* when exposed to five different *B. bassiana* isolates to examine several defensive responses, such as cuticular lipids, phagocytic activity, nodulation and haemolymph phenoloxidasec activity. Despite TV being the most virulent strain, DE, a less virulent fungal isolate, triggered immune responses at high levels in the majority of tests. The most delicate host, *E. integriceps* had a decreased immunological response. *Z. morio* displayed the greatest nodulation and resistance. *E. kuehniella* had maximum phagocytic and phenoloxidase activities with a moderate sensitivity. The peak of the phagocytic activity occurred 30 minutes after the fungal injection. After injection, nodulation and phenoloxidase activity were seen at 6, 12 and 24 hours. At 24 hours, the highest nodulation rate was seen. The majority of isolates phenoloxidase activity peaked after 12 hours for *Z. morio* and *E. integriceps* and after 24 hours for *E. kuehniella*.

Zhong *et al.* (2017) studied the effect of the entomopathogenic fungus *Nomuraea rileyi* on the cellular immunological responses of *Helicoverpa armigera* and found that the fungal infection did not affect Total Haemoglobin Concentration (THC), but it did decrease responses mediated by haemoglobin mediated phagocytosis, nodulation and encapsulation. They found that *N. rileyi* infection significantly decreased the distribution of haemoglobin and came to the conclusion that *N. rileyi* reduced its host cellular immunological response, presumably by secreting exogenous, cytotoxic substances into the host haemolymph.

Shen *et al.* (2016) identified that within 0.5 hours of infection with *Beauveria bassiana* conidia, there was a significant reduction in the total number of free haemoglobin containing cells in *Ostrinia furnacalis* (Guenée) larvae (granulocytes, oenocytoids, plasmatocytes, prohaemocytes and spherulocytes). The collected haemolymph of *B. bassiana* infected larvae showed signs of phagocytosis and nodulation in addition to a decrease in free haemoglobin after 24 and 36 hours of infection. The research proved that *O. furnacalis* larvae protected themselves from *B. bassiana* by using haemocytes as well as phagocytosis and nodulation.

Hwang *et al*. (2015) conducted cellular studies on the last larva of the Japanese rhinoceros beetle *Allomyrina dichotomas* haemolymph. LysoTracker was used to stain whole haemocytes in order to determine whether these granules may be produced as phagosomes immunologically. At 4 hours following bacterial infection, more than 90% of the granulocytes still had the LysoTracker dye on them. Fluorescent microscopy supported the flow cytometry findings at 4 hours after bacterial infection, the red fluorescent signal was much higher (60.36%) compared to controls (5.08%).

Kwon *et al*. (2014) studied six types of circulating haemocytes. They discovered granulocytes, specialized phagocytes that mediate the encapsulation and phagocytosis of pathogens. The granulocytes phagocytosed potentially dangerous chemicals in vivo after being immunologically or morphologically stimulated. Additionally, they demonstrated that autophagy is linked to phagocytosis by granulocytes and that activating autophagy could be a successful strategy to get rid of pathogens in this system and active granulocytes accumulated a lot of autophagic vacuoles.

MATERIAL AND METHODS

Insect rearing

Infested plants from the student farm, college farm and farmer fields in Rajendranagar, Hyderabad, were used to gather *S. litura* larvae. The collected larvae were reared in lab environment with a relative humidity (RH) of 70–75% and a temperature of 22± 2 °C. To avoid contamination, the larvae were raised on castor leaves in separate trays. The collected larvae were also reared on artificial diet.

Preparation of Artificial Diet:

The following ingredients or substances are used to prepare 80g of diet:

The ingredients needed to prepare an artificial diet.

Ingredients	Quantity
Chickpea flour	80 g
Sorbic acid	0.85 g
Wheat germ	30g
Methyl paraben	1.65 g
Dried Yeast	26.5 g
L- ascorbic acid	2.65 g
Agar-agar	10 g
10 % Formaldehyde	7 ml
Antimould	1 ml
Distilled water	600 ml

With the exception of agar-agar, formaldehyde, and antimould, all of the aforementioned diet components were completely combined in 400 cc of water before being heated for 5 to 10 minutes. By heating 200 ml of water at 100°C in a separate container, agar-agar was dissolved. The previously prepared mixture and agar-agar solution were then mixed and mashed together. Formaldehyde and antimould were added as the last two components, and the mixture was stirred for a further two to three minutes. The mixed liquid was immediately poured onto enamel trays, with a thickness of 1.1 to 1.5 cm. Before storing these diet trays in the refrigerator to be utilised, they were allowed to cool. With a 15% sucrose solution for moth feeding, adults that had emerged were transferred to oviposition cages for egg laying (Santharam, 1985). In order to preserve the *S. litura* culture in a lab for limitless generations, the female eggs were extracted.

Inoculation procedure

The fungus solution was injected into *S. litura* larvae after they had reached their third instar of development. Castor leaves are dipped in either a treatment pathogen dose or a control to accomplish this. Since entomopathogenic fungi were not primarily transmitted by oral infection, the larvae were topically exposed by adding 10 l of the solution with a sampler device. The control group of larvae received 10µl of a Tween 80 (0.05%) solution.

Haemolymph collection

The larvae were anaesthetized on ice before collecting the haemolymph by cutting the tip of a proleg. To obtain extra haemolymph, light pressure is applied on the insect abdomen (Barakat *et al*., 2002). Once the haemolymph collected, blood smear is prepared by spreading a drop of the haemolymph onto a glass slide, smeared it conventionally by slicing a second slide at a 45° angle across the first slide and let the smear is allowed to dry at room temperature. The haemolymph smear slides were air dried after being twice submerged in methanol. Giemsa stain was then used to stain the slides for 20 minutes, followed by a washing in distilled water. Giemsa stain was diluted five times with phosphate-buffer saline (PBS) and filtered before use. The smear is rinsed in distilled water after being washed with 0.02% acetic acid. After drying, Permount or Canada balsam was used to create permanent microscope slides.

Haemocyte characterization. The shape, size and cytoplasmic components of cells were scrutinised in order to distinguish between different types of haemocytes. From 10 untreated individuals, about 10 slides (10 replications) were made in order to identify the various haemocyte types using the identification keys set by Gupta (1979).

Identifying the cellular immune responses of *S. litura* against *M. anisopliae*.

Haemocytes count. THC and DHC were calculated for *S. litura* larvae prior to infection and after infection with *M. anisopliae*.

Determination of Total Haemocyte Count (THC). Using a Neubauer haemocytometer, the total haemocyte count of *S. litura* larvae were determined both before and after infection with *M. anisopliae*. Using a Thoma white cell pipette, the haemolymph was sampled. On a glass slide, haemolymph was collected and swiftly sucked into a Thoma white cell pipette up to the 0.5 mark. The pipette tip was cleaned and then dilution fluid (1 ml of glacial acetic acid, 100 ml of distilled water, and 0.3% gentian violet) was drawn up to the number 11 mark to create a dilution of 1/20, or 1 part blood to 20 parts WBC fluid. After thoroughly mixing the contents of the pipette and discarding the first three drops, the dilutant was charged into Neubauer chamber. The four corner squares (white cell squares) in each of the two chambers was counted for haemocytes using the formula below (Gupta and Sutherland 1968).

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

Determination of Differential Haemocyte Count (DHC)

Differential haemocyte count (DHC) from the stained slides was carried out using a cell counter and the battlement technique (Perveen and Ahmad 2017). The film was carefully examined by travelling three fields along the edge and two fields down, beginning at the thin end of the smear. This process was repeated until at least 200 cells were counted, at which point the proportion of each type of haemoglobin was calculated. (Mahmood and Yousaf 1985).

Cellular immune responses. In the larva of *S. litura*, cellular immunological responses (phagocytosis and nodulation) against *M. anisopliae* were recognised and measured.

Phagocytosis. By counting the cells harbouring spores on a Neubauer haemocytometer, phagocytic activity was quantified. On a phase contrast microscope, observations were taken (Zibaee *et al*., 2011).

Nodulation. Injected larvae were cooled on ice and haemolymph was collected in a capillary tube, 200 µl of haemolymoh was transferred onto a haemocytometer for nodule counting (Zibaee and Malagoli 2014).

RESULTS AND DISCUSSION

Total Haemocyte Count (THC). The total haemocytes were increased up to 12 HAI with *M. anisopliae* and later decreased. The THC was more in *M. anisopliae* treated larvae when compared to Tween 80 treated larvae. (Table 1 and Graph 1)

Plasmatocytes (PLs) count. The no. of PLs were increased up to 6 HAI with *M. anisopliae* and later decreased. The PLs count was more in *M. anisopliae* treated larvae when compared to Tween 80 treated larvae. (Table 2 and Graph 2)

Granulocytes count (GRs). The no. of GRs were increased up to 12 HAI with *M. anisopliae* and later decreased. The GRs count was more in *M. anisopliae* treated larvae when compared to Tween 80 treated larvae. (Table 3 and Graph 3)

PHAGOCYTOSIS. The no. of Phagocytised cells were increased up to 24 HAI with *M. anisopliae* and later decreased. The Phagocytised cells was more in *M. anisopliae* treated larvae when compared to Tween 80 treated larvae (Table 4 and Graph 4).

NODULATION. The no. of nodules formed were increased up to 24 HAI with *M. anisopliae* and later decreased. The Phagocytised cells was more in *M. anisopliae* treated larvae when compared to Tween 80 treated larvae. (Table 5 and Graph 5)

The above results were in accordance with the Sanehdeep *et al*. (2011) who observed cellular immune responses of *S. litura* larvae at different time intervals when inoculated with the entormopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin.

Corrêa *et al*. (2021) evaluated the impact of Dopamine (DA) on the survival and cellular immune response of *Metarhizium anisopliae* blastospore inoculated *Rhipicephalus microplus*. Exogenous DA improved both tick survival (72 h post *M. anisopliae* inoculation) and the quantity of circulating haemocytes (24 h post treatment) in comparison to the control group.

Black *et al.* (2022) studied the immunological reactions of third instar *Helicoverpa zea* that had been exposed to four different pathogenic groups-viruses, bacteria, fungi and entomopathogenic nematodes by taking samples at three different points after infection. At 4, 24 and 48 hours after infection. The THC , DHC, Phagocytosis and Nodulation increased up to 12-24 HAI and then gradually decreased with time.

Zibae *et al*. (2014) conducted studies on Immune response of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) larvae to different entomopathogenic fungi including isolates BB1, BB2 and BB3 of *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicilium lecanii* & *Isaria fumosoroseus* and revealed that the probability of resistance development in *C. suppressalis* might be decreased by using several fungal extracts in the field.

The increase in number of haemocytes was observed within the 12 HAI which was followed by a decrease up to 72 HAI. During pathogenic challenges, this kind of variation in hemocyte quantity has already been seen in *Schistocerca gregaria* L. (Orthoptera: Acrididae) (Gunnarsson & Lackie, 1985), *Melanoplus sanguinipes* Fabricius (Orthoptera: Acrididae) (Bidochka & Khachatourians, 1987), *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae) (Chouvenc *et al*., 2009), *Galleria mellonella* L. (Lepidoptera, Pyralidae) (Sewify & Hashem 2001), *Periplaneta americana* L. (Blattaria: Blattidae) *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae) (Hung & Boucias 1992), *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) (Zibaee *et al*., 2011) and *Oxya japonica* Thunberg (Orthoptera: Acrididae).

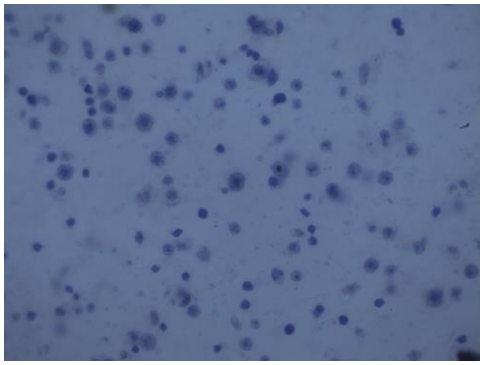


Fig. 1. Total Heamocyte Count (THC).

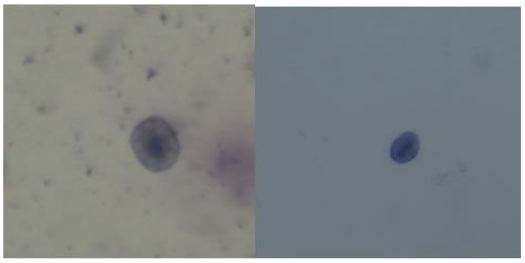


Fig. 2. Plasmatocytes (PLs).

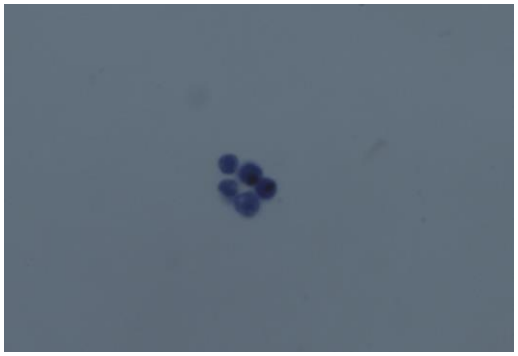


Fig. 3. Granulocytes (GRs).

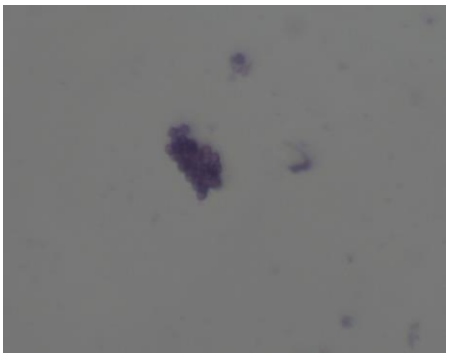


Fig. 4. Nodulation .

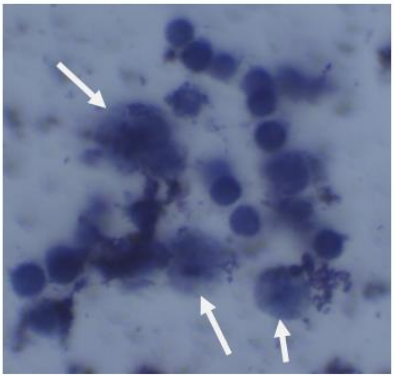


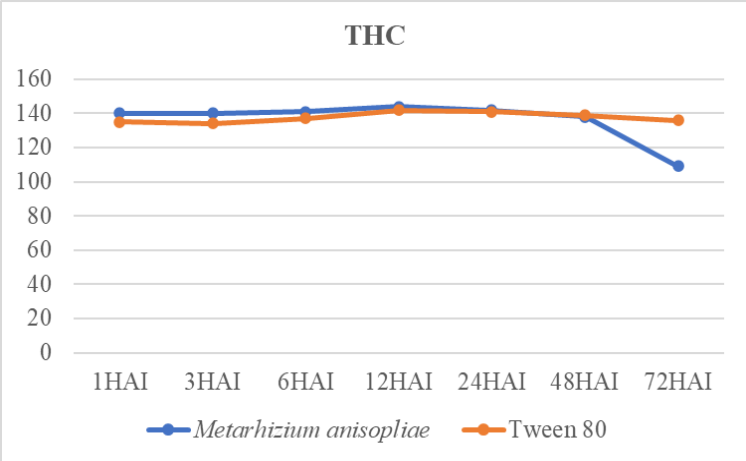
Fig. 5. Phagocytosis.

Fig. 6. 3 HAI of *S. litura* with *M. anisopliae.*

Fig. 7. 72 HAI of *S. litura* with *M. anisopliae.*

Table 1: Changes in total haemocyte numbers of *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

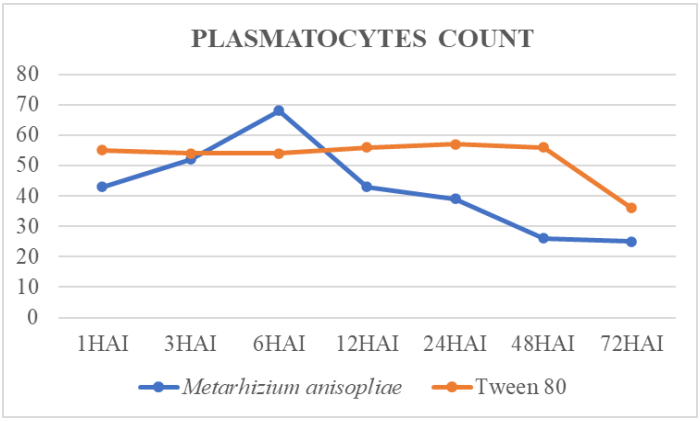
Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Metarhizium anisopliae	140±2.52	140±2.52	141±1.25	144±3	142±1.53	138±2.08	109±1.73
Tween 80	135±2.65	134±2	137±0.88	142±1.73	141±1.15	139±0.58	136±1.53



Graph 1. THC in *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

Table 2: Changes in Plasmatocytes (PLs) numbers of *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

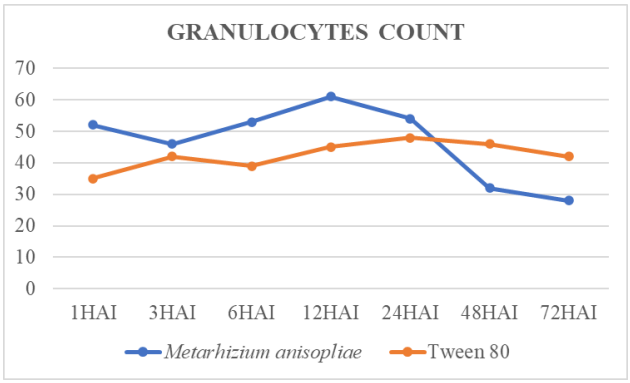
Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Metarhizium anisopliae	43±2.52	52±2.31	68±1.53	43±1.53	39±2.08	26±2.65	25±1.73
Tween 80	55±2	54±1.73	54±2.08	56±1.53	57±2.52	56±2.08	36±2.65



Graph 2. Plasmatocytes (PLs) count in *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

Table 3: Changes in Granulocytes (GRs) numbers of *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

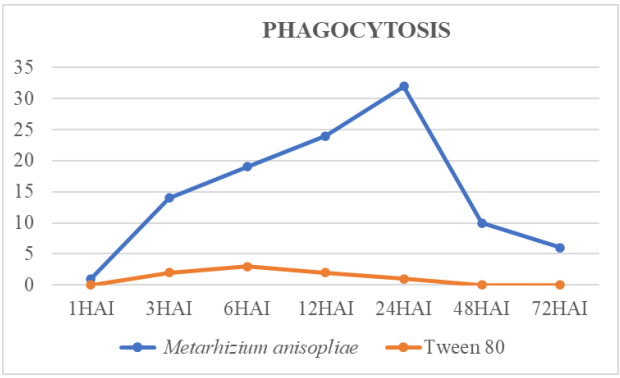
Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Metarhizium anisopliae	52±2	46±2.65	53±2.08	61±2.52	54±2.08	32±2.08	28±2.08
Tween 80	35±2.08	42±2.89	39±2.08	45±2.08	48±2.89	46±2.52	42±2.52



Graph 3. Granulocytes (GRs) count in *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

Table 4: Phagocytosis observed in *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

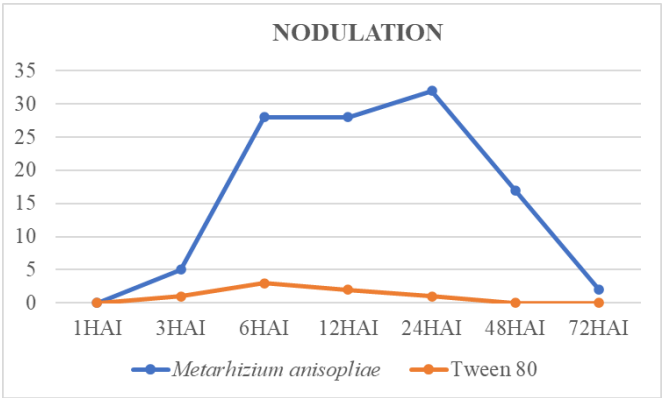
Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Metarhizium anisopliae	1±0.58	14±1.73	19±1.53	24±1.53	32±1.53	10±0.58	6±1.15
Tween 80	-	2±0.58	3±0.58	2±0.58	1±0.58	-	-



Graph 4. Phagocytosis observed in *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

Table 5: Number of nodules formed in the haemolymph of *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

Treatment	1 HAI	3 HAI	6 HAI	12 HAI	24 HAI	48 HAI	72 HAI
Metarhizium anisopliae	-	5±1.15	28±1.73	28±1.53	32±2.52	17±2.65	2±0.58
Tween 80	-	1±0.58	3±0.58	2±0.58	1±0.58	-	-



Graph 5. Nodules formation in the haemolymph of *S. litura* larvae at different HAI with *M. anisopliae* and Tween 80.

CONCLUSIONS

It was observed that when *S. litura* larvae were treated with the fungal suspension the immune response was increased up to 24 HAI and later decreased. The immune response was high in fungi treated larvae up to specific period of time when compared with the control (Tween 80). Despite these insect defence mechanisms, fungal hyphae development was seen after 72 HAI, which finally caused the host to death.

FUTURE SCOPE

To acquire a greater understanding of how the insect is reacting, more research should recognise the advantages of utilising both cellular and humoral tests, numerous entomopathogens and sample sites. Future

research on the ecology and evolution of disease resistance in *S. litura* can build on this basis.

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Conflict of Interest. None.

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