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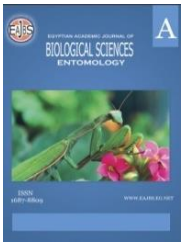

Corruption of the Hemocyte Profile and Immunosuppression in *Agrotis ipsilon* (Lepidoptera: Noctuidae) Larvae by Two Entomopathogenic Nematodes and their Symbiotic Bacteria

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ABSTRACT

The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), is widely distributed in the world. It is a polyphagous insect attacking nearly all vegetables and many economic field crops causing a great economic loss. The current research was conducted to evaluate the corruptive effects of entomopathogenic nematodes (EPNs), *Steinernema carpocapsae* (Weiser) and *Heterorhabditis bacteriophora* (Poinar) on the hemocyte profile of the *A. ipsilon* larvae and investigate the interaction between their immune defences and EPN-bacterium complex immunosuppression. For achieving this purpose, the newly moulted 5th instar larvae were infected with LC_{50} concentrations of *S. carpocapsae* and *H. bacteriophora* (21 & 62 IJs/ml, respectively) and the immune reactions were described at certain time intervals post-infection and recorded by photomicrographs. The most important results could be summarized as follows. Five main types of normal circulating hemocytes had been identified, *viz*., prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherulocytes (SPs) and oenocytoids (OEs). The immune responses of *A. ipsilon* larvae to EPNs were described as encapsulation, nodulation and phagocytosis. On the other hand, the EPN-bacterium complex exhibited different features of suppression on the immune system of *A. ipsilon* larvae. The major features were considerable deformations of the host hemocytes. At 24 to 48 hrs after infection with *S. carpocapsae* and *H. bacteriophora*, toxins produced by their symbiotic bacteria, *Xenorhabdus nematophilus* and *Photorahbdus bacteriophora*, respectively, attack *A. ipsilon* hemocytes leading to death. Because the insects have only innate immune responses against invading pathogenic microbes by immunocytes, the destruction of these hemocytes constitutes a promising approach to the biocontrol of insect pests. Therefore, the tested EPNs *S. carpocapsae* and *H. bacteriophora*, can be used in the integrated pest management of the dangerous insect, *A. ipsilon*.

INTRODUCTION

The black cutworm, *Agrotisipsilon* (Hufnagel) (Lepidoptera: Noctuidae), is widely distributed in the world, particularly in moderate and subtropical countries (Binning *et al*., 2015; Mishra, 2020; Rodingpuia and Lalthanzara, 2021; Bakr *et al*., 2021a). It is considered

one of the most dangerous species of underground pests and destroys more than 100 species of host plants (Binning *et al*., 2015; Liu *et al*., 2015; Bakr *et al*., 2021b; Hayat *et al*., 2021). The larvae attack many field crops, especially in the seedling stage causing damage of about 100% in some cases (Wang *et al*., 2021). *A. ipsilon* has a migratory behavior and flies thousands of kilometres under suitable conditions expanding its distribution range (Liu *et al*., 2015; Liu, 2015; Guo *et al*., 2016). Also, it has a high reproductive capacity (Mustu *et al*., 2021). In addition, it is a nocturnal insect and larvae remain buried in the soil during the day, and consequently its control is difficult (Andersch and Schwarz, 2003; Bento *et al*., 2007; Zhang *et al*., 2022). Therefore, *A. ipsilon* is a serious pest on various strategic economic crops worldwide (Yağcı *et al*., 2022).

In Egypt, the control measure of this insect pest still depends mainly on the application of synthetic insecticides (Vattikonda and Sangam, 2017). However, the intensive use of these insecticides usually causes many toxicological problems in the ecosystem (Haq *et al*., 2004; Holoubek *et al*., 2009; Tiryaki and Temur, 2010; Adrees *et al*., 2015). In addition, the widespread use of synthetic insecticides exhibits negative effects on the non-target beneficial insects and human health (Blacquière *et al*., 2012; Vattikonda and Sangam, 2017; Shahzad *et al*., 2020).

Therefore, many research institutions in the world have to search for new control alternatives to insecticides (Laznik and Trdan, 2012; Glare *et al*., 2016). These alternatives should be eco-environmentally safe (Liao *et al*., 2017; Kunbhar *et al*., 2018) and effective at low concentrations (Walkowiak *et al*., 2015). One of these alternatives is the biological control of insect pests. It is a highly promising approach because the entomopathogenic agents are safe for humans and the ecosystem (Amutha *et al*., 2021; Devi *et al*., 2021), as well as they have little or no effect on the non-targeted organisms (Jagodič *et al*., 2019).

Among the biological control agents, entomopathogenic nematodes (EPNs) have a potential role in killing the cutworms in soil (Kumar *et al*., 2022). They have been recorded as biologically potential control agents by many studies in the world (Lacey *et al*., 2015; Peçen and Kepenekci, 2022). The effectiveness and success of EPNs as biocontrol agents depend on their adaptability to the environment (Campos-Herrera *et al*., 2012). From the functional point of view, the infective juveniles (IJs) of EPNs have suppressed the immune responses of the insect host leading to death (Arthurs *et al*., 2004; Lewis and Clarke, 2012; Shapiro-Ilan and Brown, 2013; Kaliaskar *et al*., 2022). They may achieve this role alone or/and their symbiotic bacteria (Lewis and Clarke, 2012; Shapiro-Ilan and Brown, 2013; Kumar *et al*., 2015; Leonar *et al*., 2022; Kaliaskar *et al*., 2022). Also, EPNs can be used individually or in combination with other biocontrol agents, such as entomopathogenic bacteria and fungi in order to improve their efficacy in controlling insect pests (Laznik *et al*., 2012). For reviews, see Vashisth *et al*., 2013; Sujatha and Jeyasankar, 2018; Jagodič *et al*., 2019; Trdan *et al*., 2020; Askary and Abd-Elgawad, 2021; Kumar *et al*., 2022; Tomar *et al*., 2022 a; Shaurub, 2023).

The most virulent EPN families for use as biocontrol agents are Steinernematidae and Heterorhabditidae (Nematoda, Rhabditida). Symbiotic bacteria *Xenorhabdus*, associated with Steinernematidae (Kaya and Gaugler, 1993) and *Photorhabdus*, associated with Heterorhabditidae (Forst and Clarke, 2001; Silva, *et al*., 2002). After entering the haemocoel of the insect host, the nematode acts as a vector for the symbiotic bacteria, and, by interacting early with the host immune system, it prepares a favourable environment for its symbionts which produce natural products with insecticidal potential (Eleftherianos *et al*., 2018; Vicente-Díez *et al*., 2021). The EPNs, also, contribute with their own toxins and immune suppressors to the insect host (Chang *et al*., 2019).

In Egypt, several surveys carried out in the Egyptian soils revealed that the families Heterorhabditidae and Steinernematidae are more pathogenic than other nematode families

for biological control of some insect pests (Yuksel and Canhilal, 2018; Aashaq *et al*., 2020 Koppenhöfer *et al*., 2020; Yüksel *et al*., 2022) including *A. ipsilon* (Mathasoliya *et al*., 2004; Fetoh *et al*., 2009; Seal *et al*., 2010; Ebssa and Koppenhöfer, 2011; Khattab and Azazy, 2013; Hassan *et al*., 2016: Sobhy *et al*., 2020; Devi *et al*., 2021; Nouh, 2021, 2022; Ghoneim *et al*., 2022, 2023). However, more than 100 *Steinernema* and 16 *Heterorhabditis* have been described in the world until now (Shapiro-Ilan *et al*., 2017; Koppenhöfer *et al*., 2020; Bhat *et al*., 2020). Since the innate immune system of insects includes cellular and humoral responses and their hemocytes play a major role in the cellular immune response of insects to the entomopathogens, the current research was conducted to investigate the corruptive effects of EPNs, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, on the hemocyte profile of *A. ipsilon* larvae and the interaction between their immune defences and EPN-bacterium complex immunosuppression.

MATERIALS AND METHODS

I. The Experimental Insect:

A sample of eggs of the black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) was kindly obtained from the susceptible strain culture maintained for several generations in Plant Protection Research Institute, Doqqi, Giza, Egypt. Using this egg sample, a culture of the insect was established under constant conditions (27±2°C and 65±5% R.H.) at Department of Zoology and Entomology, Faculty of Science, Al-Azhar University, Cairo, Egypt. Raising technique was carried out according to Abdin (1979) with the improvement of El-Shershaby (2010). The newly hatched larvae were kept in suitable jars andprovided every day with clean castor bean leaves *Ricinus communis*for feeding. After moulting into the 4th instar, larvae were reared in a few numbers, in separate jars, to avoid crowding and cannibalism. Sawdust and fresh castor bean leaves were renewed daily until pupation. The pupae were then placed in plastic jars covered with muslin and fitted with filter paper, as an oviposition site for future moths. After the adult emergence, each jar was provided with a piece of cotton wool soaked in a 10% sugar solution and renewed every two days for feeding moths.

2. The Tested Entomopathogenic Nematodes (EPNs):

Imported two species of EPNs, *Heterorhabditis bacteriophora* (Poinar) (Heterorhabditidae) and *Steinernema carpocapsae* (Weiser) (Steinernematidae), were supplied by Dr. El-Sadawy, National Research Centre, Doqqi, Giza, Egypt, for the mass culturing of each EPN. The last instar larvae of the greater wax moth *Galleria mellonella* (Pyralidae: Lepidoptera) were used as hosts according to (Shamseldean *et al*., 2008). Five live *G. mellonella* larvae were placed in a Petri dish with approximately 100 live EPNs, 20 EPNs/ml, with a few drops of deionized water for each tested EPN. The infective juveniles (IJs) of each EPN species will enter and infect the larvae through their natural openings. Symbiotic bacteria carried within the guts of the EPNs are released after they penetrate their hosts. The EPNs complete one to three generations before they emerge from the dead larvae (cadavers). Petri dishes were stored for a week in a dark place at 20℃±2 ℃. After six days, larvae were checked for infection.

3. Larval Infection of *A. ipsilon* with EPNs:

For infection of the *A. ipsilon* 5th instar larvae, a series of concentrations of eachEPN was prepared as follows: *H. bacteriophora*: 200.0, 100.0, 50.0, 25.0 and 12.0 IJs/ml, and *S. carpocapsae*: 100.0, 50.0, 25.0, 12.0 and 6.0 IJs/ml. After recording the mortality rates at different time intervals, LC_{50} of *S. carpocapsae* was calculated as 21 IJs/ml, while LC_{50} of *H. bacteriophora* was 62 IJs/ml. For investigating the *A. ipsilon* larvae responses to these EPNs and the symptoms of EPN-symbiotic bacteria immunosuppression, the previously

described experiment was repeated using only the LC_{50} concentrations.

4. Larval Haemolymph for The Investigation of *A. ipsilon*-EPN Interactions:

The haemolymph samples were collected from the treated and control 5th instar larvae at different time intervals. Each haemolymph sample was obtained by amputation of one or two prothoracic legs, from the coxa of the larva using fine scissors. Gentle pressure was done on the thorax for obtaining haemolymph drops by a non-heparinized capillary tube. Haemolymph was drawn into Eppendorff Pipetman containing a few milligrams of phenoloxidase inhibitor (Phenylthiourea) to prevent tanning or darkening and then diluted 5× with saline solution 0.7%. Collected haemolymph samples were then centrifuged at 2000 r.p.m. for 5 min, and only the supernatant fractions were used for assay directly or frozen until use. Three replicates were used and the haemolymph of two individuals was never mixed. In these haemolymph samples, the main types of hemocytes were identified and the different symptoms of larval immune responses and EPN-symbiotic bacteria suppression of the insect immune system were carefully inspected. The available hemocyte deformities were recorded. Photomicrographs of these deformities were prepared using a light microscope provided with a camera at a magnification of 10 X 40 = 400

5. Data Analysis:

Data obtained were analyzed by the Student's *t*-distribution, and refined by Bessel correction (Moroney,1956) for the test significance of the difference between means using GraphPad InStat© v. 3.01 (1998).

RESULTS

1. Identification of the Normal Hemocyte Types in *Agrotis ipsilon* Larvae:

The present study investigated the deformities of hemocytes of 5th instar larvae of *A. ipsilon* by the infection with entomopathogenic nematodes (EPNs): *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* (Poinar) (Rhabditida: Heterorhabditidae). At first, the freely circulating hemocytes in 5th instar larvae of *A. ipsilon* were identified in five main types, *viz*., prohemocytes (PRs), granulocytes (GRs), plasmatocytes (PLs), spherulocytes (SPs) and Oenocytoids (OEs). These hemocyte types were distinguished based on the morphological characteristics and staining technique.

2. Immune Responses of *A. ipsilon* Larvae to EPNs:

The innate immune system of insects includes cellular and humoral responses. Hemocytes play a major role in the cellular immune response to entomopathogens in insects. The success of parasitism depends mainly on the immunesuppression induced by the tested EPNs, *S. carpocapsae* and *H. bacteriophora*, and their symbiotic bacteria when released from the nematode guts into the insect haemocoel.

In the initial stage after infection with EPNs, the response of the host hemocytes appeared as immune-component cells. This response was achieved by the encapsulation, as shown in Plate 1 (I., A and B). Hemocytes of *A. ipsilon* larvae were able to recognize the EPN *H. bacteriophora*, as a foreign target, and adhered to their cuticle surface in multiple layers. These layers consist mostly of GRs and PLs. On the other hand, the hemocytes appeared to be unable to recognize *S. carpocapsae*, as clearly shown in Plate 1 (I, C). Once the EPN releases its symbiotic bacteria in the host haemocoel, multicellular hemocytes aggregate to entrap large numbers of bacteria forming a nodulation structure [Plate 1 (II- A, B and C)]. Other phenomena appeared in this stage, after 3-6 hr of infection, like phagocytic activity of hemocytes which was evaluated by the ingestion of symbiotic bacteria of the tested EPNs. Bacteria were ingested by PLs [Plate 1 (III, A and B)] and GRs [Plate 1 (III, C and D)]. PLs had a phagocytic activity lower than GRs toward both tested EPNs. During this

stage, new hemocytes were produced to replace the damaged hemocytes by increasing the mitotic division [Plate 1 (IV, A, B and C)] .

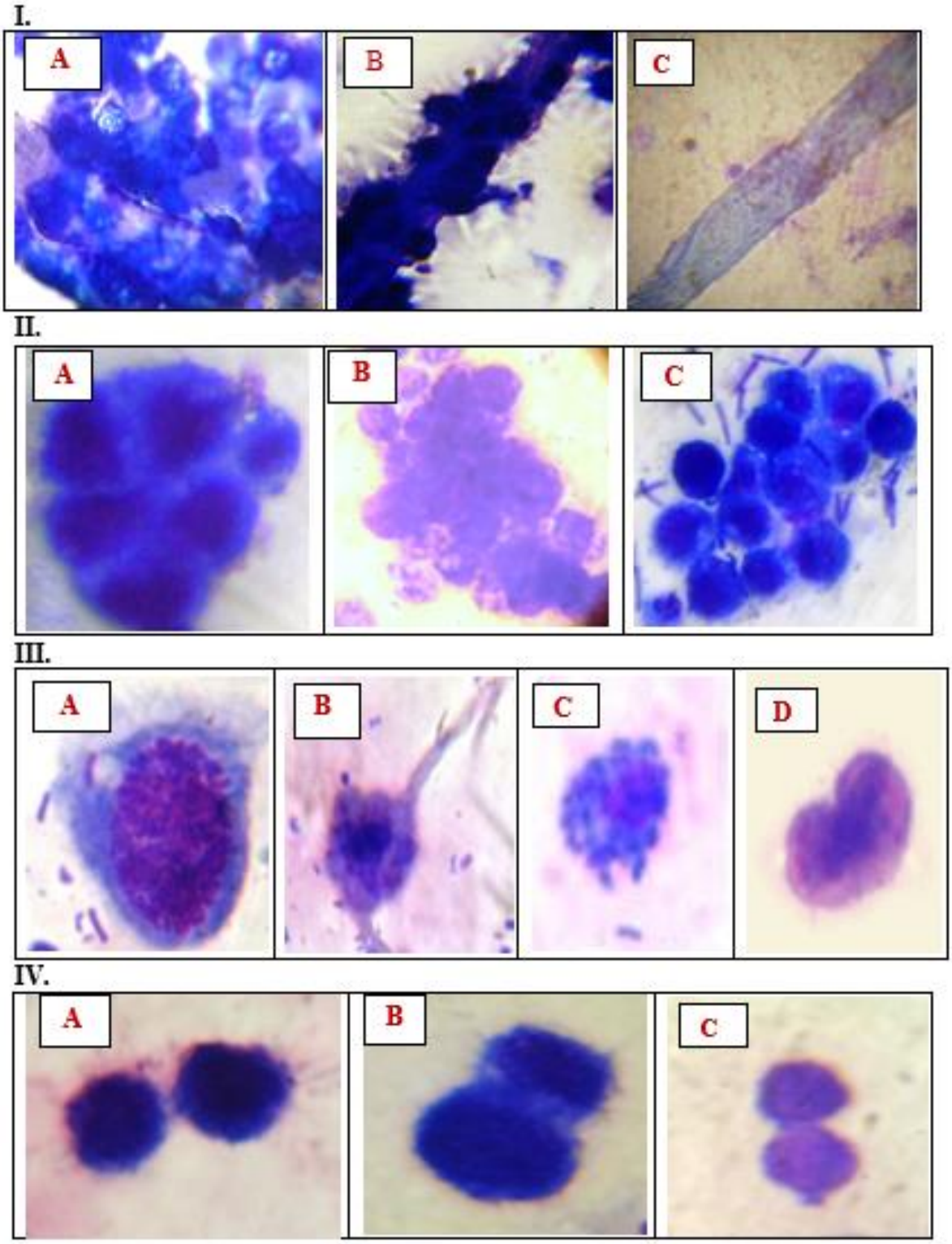

Plate 1: Photomicrographs of the *A. ipsilon* hemocyte responses toward *S. carpocapsae* and *H. Bacteriophora* infection. I. Larval response by encapsulation. II. Larval response by nodulation. III. Larval response by phagocytosis. IV. Larval response by increasing the mitotic division.

3. EPN-Bacterium Complex Immune Suppression:

Plate 2 (i. A) obviously demonstrated that the infective juveniles (IJs) of *H. bacteriophora* seemed to be able to escape from encapsulation by shedding off its cuticle and subsequently, the nematode escaped from the attached hemocytes. Three to six hr post-infection, partial encapsulation was observed in Plate 2 (i. B). The encapsulation processes were lacking at 12 hr post-infection. On the other hand, *S. carpocapsae* could not be recognized by the hemocytes of *A. ipsilon* larvae [Plate 2, i. (C and D)] .

The infection of *A. ipsilon* with *S. carpocapsae* and *H. bacteriophora* caused several pathological deformations in the host hemocytes. Through infection, the hemocytes were undergone to considerable structural changes. The PRs showed vacuoles in the cytoplasm and the nuclei appeared as darkly stained [Plate 12, ii, (A)]. Also, the PRs showed vacuolization in the cytoplasm, granulosis of the nucleus and distortion of the cell membrane [Plate 2, ii, (B and C)]. Both nematode species exhibited remarkably drastic effects on GRs. These hemocytes were characterized by highly granulated and deeply stained nucleoplasm and cytoplasm [Plate 2, ii, (D and E)]. In addition, distortion in the cell membrane, lysed cells and high vacuolization in the cytoplasm were observed in Plate 2 (ii. F). As obviously shown in Plate 2 (ii. G), spindle-shaped PLs appeared darkly stained. Also, data of the present study revealed great variations in PLs volume, vacuolization in the cytoplasm, distortion of the cell membrane and granulosis of nucleus [Plate 2, ii. (H)] . Moreover, PLs were observed lysing in addition to small pink and dark staining bodies which represent pieces of denatured nuclear materials [Plate 2 (ii. I)] . With regard to SPs, infection of both EPNs *S. carpocapsae* and *H. bacteriophora* resulted in great variations in the cell shape and volume [Plate 12, ii. (J)] , vacuolization in the cytoplasm [Plate 2, ii. (K)] , and distortion of cell membrane [Plate 2, ii. (L)] . OEs were observed with distortion in the cell membrane, vacuolization in the cytoplasm, granulosis of the nucleus and very darkly stained nucleoplasm [Plate 2, ii. (M, N and O)] .

Once EPNs were released to the symbiotic bacteria, the cellular immune response of *A. ipsilon* larvae was activated, however, these responses were ultimately ineffective, as the bacteria replicated and secreted a number of toxins within the haemocoel. These bacteria toxins disrupted the cytoskeletons of hemocytes, resulting in malformed hemocytes [Plate 3 (A, B, C, D, E, F and G)], and pseudopods could not be formed. Since the ability of the hemocytes to extend pseudopods was essential for phagocytosis with their disruption, phagocytosis processes were suppressed. At 24 to 48 hrs after infection with both *S. carpocapsae* and *H. bacteriophora*, the toxin produced by the symbiotic bacteria, *Xenorhabdus nematophilus* and *Photorahbdus bacteriophora*, respectively, attack *A. ipsilon* hemocytes. As clearly demonstrated in Plate 3 (H, I, J, K and L), symbiotic bacteria filled the haemocoel cavity and suppressed the *A. ipsilon* immune system.

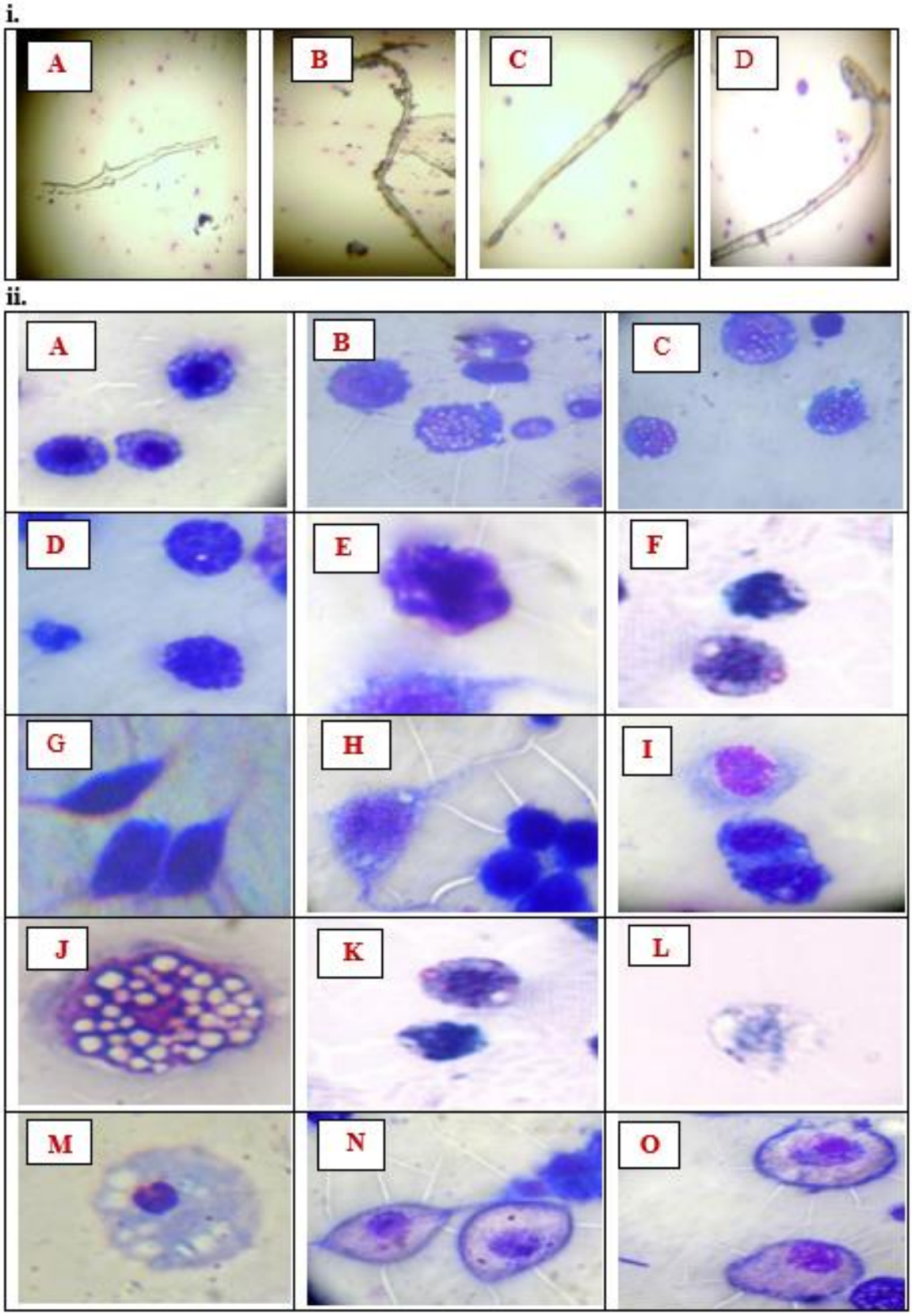

Plate 2: Responses of the infective juveniles of *S. carpocapsae* and *H. bacteriophora* toward the *A. ipsilon* hemocytes. i. EPN reactions by changing their body surface and/or secrete molecules participate in immune evasion. ii. EPN reactions bysecretion of molecules participate in the suppression of host defences.

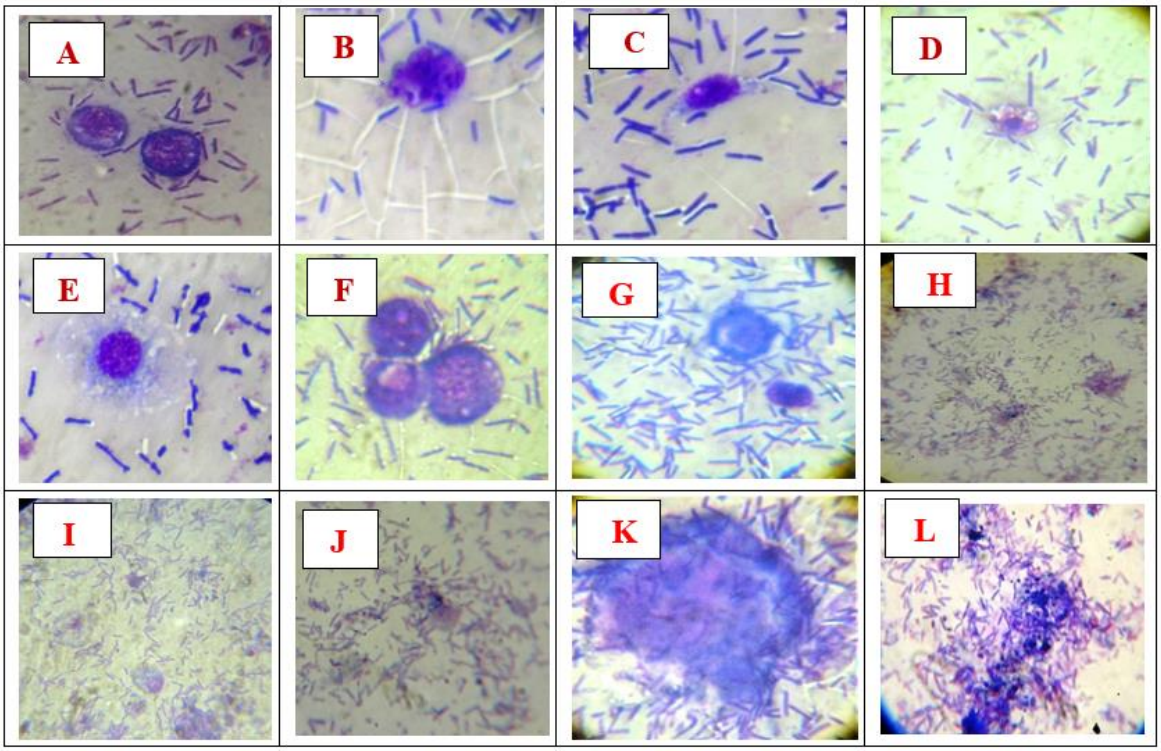

Plate 3: Responses of symbiotic bacteria of *S. carpocapsae* and *H. bacteriophora* toward the *A. ipsilon* hemocytes

DISCUSSION

1. The Main Hemocyte Types in Haemolymph of *Agrotis ipsilon* Larvae:

In insects, the most common types of hemocytes are prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherulocytes (SPs), adiphohaemocytes (ADs), coagulocytes (CGs) and oenocytoids (OEs). It is important to point out that not all of these hemocyte types exist in all insect species (Lavine and Strand, 2002; Meister and Lagueux, 2003; Lamprou *et al*., 2007; Wang *et al*., 2010; Manachini *et al*., 2011) because their diagnostic characters are slightly or greatly differing in various insects (Ribeiro and Brehelin, 2006; Browne *et al*., 2013). Moreover, there is confusion between various hemocyte types, such as PRs and PLs as well as GRs and ADs (for review, see Ghoneim, 2019).

In the present study, the freely circulating hemocytes in the haemolymph of 5th instar larvae of *A. ipsilon* had been identified into five main types, *viz*., PRs, GRs, PLs, SPs and OEs. The present finding was in agreement with some reported results of similar hemocyte types in the larvae of *A. ipsilon* (Awad, 2012; El-Khayat *et al*., 2020; Shaurub *et al*., 2022). On the other hand, Abd El-Aziz and Awad (2010) identified five types of hemocytes in haemolymph of *A. ipsilon* larvae but adipohemocytes (ADs) instead of OEs. In contrast, our result was in disagreement with the reported results of Xiang *et al*. (2022) who identified six types (PRs, PLs, GRs, SPs, OEs and cystocytes) in the haemolymph of 6th instar larvae of *A. ipsilon*. Moreover, eight hemocyte types (PRs, PLs, GRs, SPs, OEs, Spindle cells, ADs and Cystocytes) had been recognized in 5th instar larvae of *A. ipsilon* by Sayed *et al*. (2023). Also, three hemocyte types in haemolymph of *G. mellonella* larvae were observed under a fluorescence microscope (PLs, GRs, and PRs) (Izzetoglu, 2012). In addition, Wu *et al*. (2016) used cytological and morphological analyses for the differentiation of four types of hemocytes in *G. mellonella* (PLs, GRs, SPs and OEs). Er *et al*. (2017) distinguished four

types of circulating hemocytes in the last instar larvae of the same insect (GRs, PLs, PRs and OEs). Also, Ghoneim *et al*. (2017) recognized six main hemocyte types in the haemolymph of full-grown larvae of the pink bollworm, *Pectinophora gossypiella*, *viz*., PRs, PLs, SPs, OEs, GRs and ADs. Many authors (Gadelhak, 2005; Manachini *et al*., 2011; Al Dawsari and Alam, 2022) reported six types of hemocytes in the larval haemolymph of the red palm weevil *Rhynchophorus ferrugineus*, *viz*., PRs, GRs, OEs, PLs, SPs and CGs.

To understand this controversial identification, may be due to the used nomenclature of hemocytes and complicated comparisons of hemocyte categories in insects of various orders (Nardi, 2004; Huang *et al*., 2010). In addition, the differencein types of the recognized hemocytes may be attributed to several technical difficulties and the characteristics adopted by other researchers (George and Ambrose, 2004; Ribeiro and Brehelin, 2006). Various techniques often yield considerable differences in information about the types, number, distribution and functions of hemocytes (for detail, see Qamar and Jamal, 2009; Pandey and Tiwari, 2011; Pandey and Tiwari, 2012). Therefore, the hemocyte classification has been recommended to be revised several times in the same insect species (for review, see Ghoneim *et al*., 2021).

2.Interaction between Immune Defenses of *Agrotis ipsilon* Larvae and Entomopathogenic Nematode-Bacterium Complex Immune-Suppression:

It is important to point out that the hemocytes are involved in different physiological functions in insects, such as development and metamorphosis, detoxification of xenobiotics and immunity against pathogens. Therefore, the circulating hemocytes provide an excellent model system to study cell development, differentiation and their role in the immune system (Rosales, 2011; Pandey and Tiwari, 2012).

On the other hand, entomopathogenic nematodes (EPNs) are usually used as biological control agents for pest insects instead of hazardous synthetic insecticides (Ali *et al*., 2005; Dillman and Sternberg, 2012; Lacey *et al*., 2015). Most virulent EPNs belong to the families Steinernematidae and Heterorhabditidae. In addition to the EPNs themselves, there are symbiotic bacteria, *Xenorhabdus* associated with Steinernematidae (Kaya and Gaugler, 1993) and *Photorhabdus* associated with Heterorhabditidae (Forst*et al*., 1997; Forst and Clarke, 2001; Silva, *el al*., 2002). These symbiotic bacteria contribute to the ability of nematodes to kill the host providing the right conditions for nematode reproduction, providing nutrients and inhibiting the growth of other microorganisms in the insect host (Eleftherianos *et al*., 2018).

The present study was carried out to investigate the corruptive effects of (LC_{50}) concentrations of two EPNs, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, on the hemocytes of 5^{th} larval instar of the black cutworm,*Agrotisipsilon*. Among the host immune defences, encapsulation, nodulation, phagocytosis and increasing mitotic division were determined. In addition, this study tried to answer the question, of how both EPNs and their symbiotic bacteria overcome the host's immune defences. Also, the present study revealed the role of certain hemocytes in *A. ipsilon* 5^{th} instar larvae for overcoming the infection of *S. carpocapsae* and *H. bacteriophora*, as should be discussed in the following paragraphs.

2.1. The Immune Defences of *A. ipsilon* against EPNs:

A critical step for the successful infection of entomopathogen is overcoming the host's immune defences (Grewal *et al*., 2005; Beckage, 2008). As reported by some authors for insects (Medzhitov and Janeway, 2002), parasites can stimulate several immune reactions and sometimes lead to the differential expression of effector genes, but any immune reaction is preceded by the interaction of characterized pattern-recognition receptors (PRRs) that used to interact specifically with a broad range of foreign antigenic compounds, commonly named pathogen-associated molecular patterns (PAMPs) lead to defensive reactions. PAMPs from

invaders are recognized by free or cell-associated host receptors (PRRs). Hemocytes play a major role in several defensive functions against foreign targets, and in general, they need to be activated by the presence of PAMPs and/or by endogenous soluble factors. Following this interaction, hemocytes become stimulated and initiate defense mechanisms, such as encapsulation, nodulation, phagocytosis and proPO-AS release (Schmidt *et al*. 2001; Hultmark, 2003; Dubovskiy *et al*., 2016). Some authors (Schmidt *et al*., 2001; Sigle and Hillyer, 2016) reported that both encapsulation and nodule formation as the primary means of cellular immunity are accompanied with melanization. It has been proved that OEs produce the precursor of prophenoloxidase (pro-PO) which is a key enzyme for melanin and contributes to immune melanization. For more details, see reviews of Nappi and Vass (2001); Eleftherianos *et al*., (2007); Marmaras and Lampropoulou (2009); Rosales (2011) and Dubovskiy *et al*. (2016).

2.1.1. Encapsulation of EPNs by *A. ipsilon* larvae:

When insects are infected with large targets, like parasites, protozoa and EPNs, they can encapsulate them by multiple layers of certain hemocytes in a process called'cellular encapsulation' (Ling *et al.*, 2005). The formation of cellular capsules mainly requires two hemocyte types: GRs and PLs leading to the killing of the invader (Lavine and Strand, 2002). For some detail, the formation of a capsule begins within 30 min from parasite entry and the initial steps involve GRs and humoral pattern-recognition receptors (PRRs) as opsonic factors, i.e., GRs release chemotactic components, called PL-spreading peptides (PSP), that attract PLs and increase their adhesive properties (Clark *et al*., 2001; Srikanth *et al*., 2011). Many studies have reported that PRRs are needed to stimulate the aggregation of PLs on the surface of the target or to the earlier layers of the GR capsule (Bulet*et al*., 1999). These immunocompetent hemocytes result in a thick multicellular capsule that separates the parasite, avoiding nutritional exchanges with the host's environment (Schmidt *et al*., 2001**)**. In addition, the toxic effects of melanin, which exist within the inner layers of the capsule, may contribute to killing the trapped parasite (Vass and Nappi, 2000; Nappi and Vass, 2001). In this context, results of the current study revealed that once the EPN *H. bacteriophora* invaded *A. ipsilon* 5th instar larvae and reached the haemocoel, most of them were recognized as a foreign target of the hemocytes. Then, hemocytes adhered to ENPs cuticle surface in multiple layers. These layers are mostly formed from GRs and PLs whereas the EPN *S. carpocapsae* seems to be unrecognized by the infected larvae.

The present results were in agreement with the reported results of Wang *et al*. (1994, 1995), since *H. bacteriophora* was initially encapsulated in larvae of the Japanese beetle *Popillia japonica* and only 10% of the nematodes could escape the encapsulation after 24 h by an unknown mechanism. Also, Li *et al*. (2009) reported that *H. bacteriophora* was recognized by (>99 %) value, while the EPN *Steinernema glaseri* showed only (28%) recognition in the larvae of the tobacco hornworm *Manduca sexta*. Five years later, Sheykhnejad *et al*. (2014) found the cellular response of the rose sawfly *Arge ochropus* weaker to *S. carpocapsae* than to *H. bacteriophora*. In the beetle *Agriotes lineatus*, only 6% encapsulation of *Steinernema feltiae* was done, but 24% encapsulation of *H. bacteriophora* (Rahatkhah*et al*., 2015). Some years later, Istkhar and Chaubey (2019) showed that *Steinernema abbasi* had a strong capability to avoid encapsulation responses in the cotton bollworm *Helicoverpa armigera* larvae leading to the death of larvae within a very short time. With few exceptions, similar cellular encapsulation was reported for the armyworm moth *Pseudalaetia unipuncta* (Cruz *et al*., 2001) and humoral encapsulation was observed for the marsh crane fly *Tipula oleraceae* (Peters *et al*., 1997). In addition, the encapsulation of *S. feltiae* and *H. bacteriophora* by the prepupae of Colorado potato beetle *Leptinotarsa decemlineata* showed a more frequent encapsulation of *S. feltiae* than of *H. bacteriophora* (Ebrahimi *et al*., 2011). In view of our results and these reported results,

it is well-known that the encapsulation responses of the insects varied according to the insect species and EPN species.

2.1.2. Nodulation of EPNs by *A. ipsilon* Larvae:

As reported by some authors (Marmaras and Charalambidis 1992; Dubovskiy, 2016), the host hemocytes release humoral factors that form multicellular hemocytes aggregates, called "nodules" against many symbiotic bacterial cells. This process leads to the trapping of bacterial cells. These nodular aggregates may adhere to host tissues and larger nodules may eventually be encapsulated by the hemocytes. Results of the present study revealed that, once EPNs release their symbiotic bacteria into the haemocoel of *A. ipsilon* larvae, highly spreading of aggregation hemocytes appeared (nodule formation). This finding was in corroboration with the reported results by many authors, such as Dean *et al*. (2004 a, b) who found the infection of *M. sexta* larvae with nematode-symbiotic bacterium *Photorhabdus luminescens* resulted in the appearance of hemocytes with an extreme spreading ability that may play a role in nodule formation. Also, Hassan et al. (2016) recorded the formation of multicellular hemocytes aggregates that entraps a large number of symbiotic bacteria of EPN that infected the 6th instar larvae of *A. ipsilon.*

2.1.3. Phagocytic Activity of *A. ipsilon* larvae against EPNs:

Phagocytosis is the first response of the insect hemocytes to small particles, such as bacteria, yeast, or protozoa. In insects, both GRs and PLs have been shown to be capable of phagocytosis (Tojo *et al*., 2000; Kwon *et al*., 2014). OEs were reported, also, to take a role in phagocytosis (Giulianini *et al*., 2003). However, the target particle is first recognized by phagocytic receptors of the insect host that activate different signalling pathways in the cell (Jones *et al.*, 1999). These signals lead to massive changes in the plasma membrane that extends pseudopods around the particle, forming a cup that moves into the cell. Within a few minutes, the membrane closes at the distal end, leaving a new plasma membrane-derived phagosome (Yeung *et al.*, 2006). The efficacy of phagocytosis depends on the structure of the surface of the invading pathogen and on the involved hemocytes. Also, the presence of microbial factors, such as glucans, PGNs, or LPS, can increase the phagocytic rate of hemocytes. Phagocytosis can be enhanced by the interaction between foreign sugars (free or conjugated oligosaccharides) and haemolymph sugar-binding proteins (Ni and Tizard, 1996). In addition, the process may be stimulated by the same components released after proPO-AS activation (Wang and Jiang, 2004). In the present study on *A. ipsilon* larvae, the phagocytosis process was observed toward symbiotic bacteria of the tested EPNs, as well as the PLs and GRs had been considered the main phagocytic cells. This result was consistent with the results of Hassan and Ibrahim (2010). In addition, Eleftherianos *et al*. (2010 a, b) reported that the following release of the symbiotic bacteria *Photorhabdus* into the insect haemolymph by the infective juveniles, the first response of the host immune system is to phagocytose or encapsulate the invading bacteria.

2.1.4. Increasing Mitotic Division in *A. ipsilon* Larvae as A Reaction against EPNs:

Results of the current investigation recorded an increase of mitotic division in the larval hemocytes of *A. ipsilon* as a response to the infection with EPNs, *S. carpocapsae* and *H. bacteriophora*, which stimulated several immune reactions, like encapsulation, nodulation and phagocytosis, as discussed before. These processes used up and damaged a lot of hemocytes, and subsequently, new hemocytes were produced to replace the damaged hemocytes for immune defence. Therefore, the *A. ipsilon* larvae increased mitotic division to maintain homeostasis. These results were consistent with the reported results of Hassan and Ibrahim (2010) since an increasing mitotic division, as a response to EPNs infection into the 6th larval instar of the cotton leafworm *Spodoptera littoralis*, was recorded. Recently, Salem *et al*. (2020) observed a hemocyte response as immunocompetent cells after the nematode application on the greater wax moth *Galleria mellonella* larvae. However, this

response was done by phagocytosis or encapsulation of the foreign body or increasing the mitotic division. On the other hand, some authors (Nakahara *et al*., 2003; King and Hillyer, 2013; Kwon *et al*., 2014) recorded this stimulation after the bacterial infection on other insect species.

2.2. EPN-Bacteria Complex Immune Suppression against *A. ipsilon*:

2.2.1. How EPNs Overcome the Immune Defences of Larvae?

The success of EPNs and their symbiotic bacteria for insect infection depends on the interaction of each other of the nematode-bacteria complex with the insect immune system (Eleftherianos *et al*., 2010 b; Castillo *et al*., 2011).There are two main strategies by which EPNs overcome the immune defences of the host: mimicry processes and the host's immunosuppression. Mimicry processes can be achieved through the synthesis of molecules that are antigenically related to the host (usually called "self-proteins") and are exposed on the surface of the EPN's body (Weston *et al*., 1994). In addition, mimicry could be a form of camouflage based on the acquisition of host molecular compounds or tissues which cover the parasite body surface (Kathirithamby *et al*., 2003). The relationship between the body surface of the parasite and the immune responses of the insect host has been extensively investigated (Brivio *et al*., 2002). For some detail, inhibition of the host defences is usually achieved by the excretion/secretion of various compounds that interfere with and neutralized many immune processes exerted by the host in response to infection. After the nematode enters the host body, there are two phases. In the first phase, EPNs use a mimic to become unrecognizable by proPO-AS and by immunocompetent hemocytes in the insect host. In the second phase, both EPNs and their symbiotic bacteria use strategies aimed at inhibiting the humoral and cellular responses, basing on the release of toxins, inhibitors and proteases (Simões and Rosa, 1996; Balasubramanian *et al*., 2009).

Results of the present investigation showed that the infective juveniles (IJs) of *H. bacteriophora* appeared to be able to escape from the encapsulation process in *A. ipsilon* larvae by shedding their cuticle which could be an escape mechanism from host immune defence. Our results were comparable with those reported results of Peters et al. (1997) and Hassan *et al*. (2016) since loss of the external layer of the body surface of *H. Bacteriophora* could be considered as a strategic mechanism to escape from host immunological surveillance. Subsequently, the EPN escaped from the attached hemocytes. The encapsulation processes were lacking at 12 hr post-infection. On the other hand, the EPN *S. carpocapsae* did not be recognized by hemocytes of *A. ipsilon* larvae. This could be explained by the interaction of surface molecules of *S. carpocapsae* with its host and may be due to other active compounds which have been suggested to be actively secreted/excreted by EPNs into host haemocoel. This interpretation was based on some of the reported results, where the EPNs secreted or excreted proteases (ESPs), apoptosis-inducing factors, protease inhibitors and other active compounds into the host tissues (Balasubramanian *et al*. 2009; Toubarro *et al*., 2009; Toubarro *et al*., 2013; Lu *et al*., 2017). In general, serine, cysteine and aspartic proteases, secreted by EPNs, are involved in some tasks, such as invasion, digestion of the host tissues and evasion of host immune responses (Hao *et al*., 2009; Hao *et al*., 2010; Jing *et al*., 2010).

The present results were, also, in agreement with those reported results of Götz *et al*. (1981) since the EPN *S. carpocapsae* was able to destroy the antibacterial peptides by its secreted compounds that have proteolytic activity suppressing the insect's defences. Also, Lavine and Strand (2002) reported that Sc-KU-4, a serine protease inhibitor inhibited the hemocyte aggregation which is the primary step in cellular encapsulation and requires the activation of GRs and PLs and results in the entrapment of the invading EPN (Toubarro *et al*., 2010). Also, *S. carpocapsae* exhibited a damaging strategy of host immune components by proteolytic secretions (Balasubramanian *et al*., 2010). According to Toubarro *et al*.

(2013), EPN expresses a serine protease inhibitor during the invasive stage that is capable of targeting recognition proteins, thus impairing host defences. Recently, Garriga *et al*. (2020) reported that both *S. carpocapsae* and its symbiotic bacterium *X. nematophila* avoided the cellular defences of the spotted wing fruit fly *Drosophila suzukii* larvae and depressed their humoral response. On the basis of other reported results, the toxic activity of *S. carpocapsae* secretions, when injected caused insect death after a few hours from the injection, indicating that nematodes secretions are independent of bacteria release (Laumond *et al*., 1989; Simões and Rosa, 1996; Snyder *et al*., 2007). Also, the role of *S. carpocapsae* secretion in insect mortality has been provided by work on the proPO-AS activity obtained from a study with live or dead nematodes, or isolated cuticles (Mastore *et al*., 2015) and by results on secreted compounds isolated from the activated EPNs (Lu *et al*., 2017).

In addition, results of the current study recorded that both tested EPNs, especially *S. carpocapsae*, caused many cytopathological disorders of the hemocytes of infected larvae which could be described as a distortion of the cytoskeletons of hemocytes, high vacuolization of the cytoplasm, granulation of nucleus, and rupture of the cell membrane. These results were in corroboration with those reported results by Balasubramanian *et al*. (2010) who purified trypsin-like serine protease from *S. carpocapsae* that possess potent activity against *G. Mellonella* hemocytes by blocking hemocyte spreading and causing severe morphological changes to the hemocytes. Also, Hassan and Ibrahim (2010) observed several cytopathological features, including variation in the cell volume, vacuolization in the cytoplasm, distortion in the cell membrane and pycnosis of nuclei in hemocytes of the 6th larval instar of *S littoralis* after infection with *S. carpocapsae* and *H. bacteriophora*. In addition, Salem *et al*. (2014) reported that the infection of *G. mellonella* with *S. carpocapsae* induced several cytopathological detritions. During infection, the hemocytes undergo considerable structural changes. The contents of GRs seemed to be swelled giving the cells an extremely vacuolated appearance.

2.2.2. How EPN Symbiotic Bacteria Overcome *A. ipsilon*'s Immune Defences?

After a variable time of EPN entering haemocoel of the host, the EPN begins to release its symbiotic bacteria from the gut into the circulatory stream. The action of bacteria, supported by the immunosuppression processes, was supported by EPN (Hassan *et al*., 2016). Several studies have described the physiological disturbance of the host due to the release, and proliferation of symbiotic bacteria and the production of their protein toxins (Chattopadhyay *et al*., 2004). Shortly, EPNs rearrange the environment (the host body) in a favourable manner that promotes the survival and reproduction of symbiotic bacteria (Tomar *et al*., 2022b). In the present work, once EPNs released their symbiotic bacteria, the cellular immune response of *A. ipsilon* larvae was activated. However, these responses appeared ultimately ineffective, as the bacteria replicate and secrete a number of toxins within the haemocoel. These toxins disrupted the cytoskeletons of hemocytes, resulting in malformed hemocyte. Then, the symbiotic bacteria, *Xenorhabdus nematophilus* and *Photorahbdus bacteriophora* replicate, filled the host haemocoel and suppressed the *A. ipsilon* immune system. Our results were in agreement with the findings of Dunphy and Webster (1988) and Herbert *et al*. (2007) who reported that when *Xenorhabdus* spp. were released into the *G. mellonella* haemolymph, adhered to the surface of hemocytes and damaged the cells, which become vacuolated, unable to adhere to surfaces and finally die. Also, Ribeiro *et al*. (2003) reported intracellularly hemocyte changes, such as selective vacuolization of the endoplasmic reticulum, cell swelling, and cell death by colloid-osmotic lysis in *S. littoralis* infected with *X. nematophilus.* In addition, Reynolds and French-Constant (2004) examined the effects of two strains of *Photorhabdus*, W14 and K122, on the Tobacco hornworm *Manduca sexta* larvae. *Photorhabdus* reduced the host hemocyte viability and caused considerable changes in the actin cytoskeleton morphology of different types of

hemocytes. At the same time, *Xenorhabdus* synthesized and released antibiotic compounds within the insect haemolymph that suppressed the competing pathogens (Vallet-Gely *et al*., 2008). In this way, they have suitable conditions that promote their reproduction and allow the parasites to complete their development (Richards and Goodrich-Blair, 2009). At the virulent phase, *Xenorhabdus* demonstrates a typical morphological phenotype recognizable by the presence of various surface structures such as pili/fimbriae, flagella and the outer membrane vesicles (OMVs) containing virulence factors (Khandelwal *et al*., 2003; Herbert Tran and Goodrich-Blair, 2009; Ellis and Kuehn, 2010). These structures interact with the host and affect its recognition by hemocytes; they also prevent phagocytosis and nodulation processes (pili/fimbriae), promote adhesion and invasion of host tissue (flagella), or release proteases, lytic factors and phospholipase C (OMVs), therefore contributing to the larvicidal activity (Brivio *et al*., 2018). Thus, the lethal effect of symbiotic bacteria is achieved by the immune evasive/depressive and toxic effect of both the external structures and of the secondary metabolites secreted by the bacteria, and the total effect of these toxic components caused a severe metabolic and functional disturbance that led to death by septicemia of the insect target. In the present study, it could be concluded that the EPN immune mechanisms showed a clear variation in the approaches used by the different species of EPN to suppress the host immune response.

Conclusion:

In the present study, the immune responses of *A. ipsilon* larvae to EPNs were described as encapsulation, nodulation and phagocytosis. On the other hand, the EPN-bacterium complex exhibited different features of suppression on the immune system of *A. ipsilon* larvae. The major features were considerable deformations of the host hemocytes. At 24 to 48 hrs after infection with both *S. carpocapsae* and *H. bacteriophora*, toxins produced by the symbiotic bacteria attacked the *A. ipsilon* hemocytes leading to death. Because the insects have only innate immune responses against invading pathogenic microbes by immunocytes, the destruction of these hemocytes constitutes a promising approach to the biocontrol of insect pests. Therefore, the tested EPNs *S. carpocapsae* and *H. bacteriophora*, can be used in the integrated pest management of the dangerous insect, *A. ipsilon*.

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