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Review Article
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Suppressive strategies of entomopathogenic nematodes and their symbiotic bacteria against the hemocyte-mediated immune defenses of insects: A brief review

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Abstract

In the field of pest control, alternative approaches have been recently encouraged to avoid or minimize the hazards of synthetic insecticides and introduce new effective and safer biocontrol agents. The use of entomopathogenic nematodes (EPNs) is a safe and eco-friendly alternative to the synthetic pesticides for the control of various insect pests in different habitats worldwide. The major objective of this review was to gain a better understanding of the interactions between the cellular immune defences of insects and the EPN/bacteria complex immunosuppressive activity. It summarized recent literature on the major mechanisms of insect cellular immune defenses, such as encapsulation, nodulation, phagocytosis and increasing mitotic division of certain hemocytes. This review, also, described various qualitative and quantitative characters of the cellular immune defences of insects against suppressive strategies of the EPN/symbiotic bacteria complex. In conclusion, although few of the invading EPNs can be encapsulated, nodulated, or/and phagocytosized by the insect host, the majority of invading EPN/symbiotic bacteria usually overcome these defences by different mechanisms ending in the insect death. Therefore, many insect pests may be successfully controlled by using the appropriate concentration of effective EPNs under suitable biotic and abiotic conditions.

Key words: *Encapsulation, Haemocoel, Immunocytes, Larvae, Mitotic Division, Mortality, Nodulation, Phagocytosis, Pathogen, Pupa*

1. Introduction

In addition to the development of resistance in insect pests to almost all marketed insecticides, intensive and improper uses of these chemicals usually cause dangerous problems to all ecosystems including water, air and soil pollution (Haq *et al.*, 2004; Ibarra *et al.*, 2006; Tiryaki and Temur, 2010; Gunstone *et al.*, 2021). Also, synthetic insecticides detrimentally affect the economically beneficial natural enemies and insect pollinations (Calvo-Agudo *et al.*, 2019; Demok *et al.*, 2019) and harmfully affect the domestic animals and human health (Vattikonda and Sangam, 2017; Shahzad *et al.*, 2020) beside the accumulation of pesticide residues in food (Moustafa *et al.*, 2021). Also, synthetic insecticides have drastic impacts on non-target organisms including birds, fish, amphibians (Gill and Garg, 2014).

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To avoid these insecticidal hazards, several research institutions have intensively searching for safe and effective alternatives in the world (Derbalah *et al*., 2014; Glare *et al*., 2016). Biological control by natural enemies (parasitoids, predators and pathogens) is one of the eco-friendly control strategies of the insect pests. Biocontrol agents are highly promising because they are safe for humans and the environment (Amutha *et al*., 2021; Devi, 2021). Among the biocontrol agents, entomopathogenic nematodes (EPNs) have broad potential to kill the soil-dwelling insect pests (Lacey and Georgis, 2012; James *et al*., 2018) and other above-ground insects (Du Preez *et al*., 2021a, b; Kumar *et al*., 2022).

Recently, EPNs have received increasing attention worldwide to study their distribution, virulence, and usage in programs of pest control (Çağlayan *et al*., 2021; Ali *et al*., 2022) because they are harmless to non-target organisms and almost safe to human health and the environment (Kumar *et al*., 2022; Peçen and Kepenekci, 2022). Also, EPNs have high reproductive capacity, the ability to kill hosts quickly (within 24-72 h), high virulence, broad host range, and they can be mass-produced (Yaðci *et al*., 2021a, b). Unlike chemical insecticides, EPNs are target-specific with a wide range of insect pests (Devi and Nath, 2017; Husain *et al*., 2024).

In addition, there are symbiotic bacteria in the intestines of the nematode infective juveniles (IJs) (Forst and Clarke, 2001; Silva, *et al*., 2002). These symbiotic bacteria usually assist the EPNs to kill the insect host *via* providing the right conditions for nematode reproduction and nutrition inside the insect body (Lu *et al*., 2017; Eleftherianos *et al*., 2018). It is a mutualistic association or a type of symbiosis, where both organisms benefit from each other (Lacey *et al*., 2001). In other words, after entering the insect body through the natural openings, the IJ penetrates through the intestine wall and regurgitates its symbiotic bacteria into the insect haemocoel (Mastore *et al*., 2015). On the other side, the bacteria break down the host tissues, and provide food sources for the nematode, which feeds and multiplies on bacterial cells and degrading host tissues (Chang *et al*., 2019; Parks *et al*., 2022). For more detail, see reviews of Ghoneim and Bakr (2024) and Ghoneim and Hassan (2024). In this context, the major objective of the present review was to gain a better understanding of the interactions between the cellular immune defences of the insect host and the EPN/bacteria complex immunosuppressive activity.

2. The immune defences in insects: a synopsis

Insects have several defence reactions against the invading pathogens, including the morphological and immunological defences (Kunc *et al*., 2017). According to the current knowledge, a well-developed immune system has been characterized in vertebrates for the protection against various pathogens. This immune system generally includes two subsystems, innate immunity and acquired immunity (Janeway and Medzhitov, 2002). Innate immunity involves a generic recognition and response to foreign invaders that is only temporary. On the other hand, the acquired immunity consists of specialized cells that identify specific agents and ultimately produce an immunological memory (Hoffmann, 2003**)**. Insects appear to lack the acquired immune response that is characteristic of the vertebrates (Strand, 2008). Therefore, insects rely on their highly efficient innate immunity for defence against pathogens and other invaders (Berger and Jurcova, 2012).

In general, insect hosts defend themselves against entomopathogenic and parasitic infections by cellular and humoral immune reactions (Irving *et al*., 2005). The invading infective juveniles (IJs) of entomopathogenic nematodes (EPNs) must evade or suppress the host immune responses to ensure the release of their bacterial symbionts from guts (Brivio *et al*., 2002). When IJs succeed to pass through the host barriers (such as behavioral avoidance or physicochemical mechanisms) (Schmid-Hempel and Ebert, 2003) and physical barriers are breeched (Parsons and Foley, 2016), the immune responses of this host are activated to defend against these invading IJs (Jing *et al*., 2010).

With regard to the cellular immune defense in insects, there are various immunocyte-mediated immune responses, like phagocytosis, nodulation, encapsulation and clotting (Lavine and Strand, 2002; Ribeiro and Brehelin, 2006; Strand, 2008). These cellular defense reactions are predominantly achieved by some of the hemocyte types, like plasmatocytes (PLs) and granulocytes (GRs), particularly in the lepidopterous insects (Browne *et al*., 2013; Vlisidou and Wood, 2015). These events eventually lead to the rapid and transient synthesis of antimicrobial polypeptides by the fat body (a functional equivalent to the mammalian liver and adipose tissues), hemocytes and the midgut epithelial cells (Walter, 2008).

In this regard, it should be give a brief perspective on the insect hemocytes. Hemocytes make up about 10% of the haemolymph volume. They are nucleated cells that do not contain respiratory pigments (Mullett *et*

al., 1993). As previously reported in the review of Ghoneim *et al*. (2021), there are several types of the insect hemocytes. The most common types are prohaemocytes (PRs), PLs, GRs, spherulocytes (SPs), adiphohaemocytes (ADs), coagulocytes (CGs) and oenocytoids (OEs). Not all of these hemocyte types exist in all insect species. Also, characteristic features of the circulating hemocytes are slightly differing in various insect species (for some detail, also, see Siddiqui and Al-Khalifa, 2014; Er *et al*., 2017). For example, Ghoneim *et al*. (2023) identified five main types of normal circulating hemocytes, *viz*., PRs, PLs, GRs, SPs and OEs in the 5th instar larvae of the black cutworm *Agrotis ipsilon* (Lepidoptera: Noctuidae). This result was in agreement with some previously reported results of the same number of basic hemocyte types in larvae of the same lepidopterous insect (Awad, 2012; El-Khayat *et al*., 2020; Shaurub *et al*., 2022).

Over last few decades, the insect circulating hemocytes have received much attention worldwide because these cells are responsible for different physiological functions, such as cell development and differentiation; metabolic processes; reproductive potential; endocrine regulation; distribution of nutritive materials and hormones to various sites throughout the insect body; coagulation to prevent loss of blood and wound healing; preservation of an insect homeostasis; defence immune reactions against pathogens invading the insect haemocoel; as well as the detoxification of xenobiotics and other foreign materials (for some detail, see: Siddiqui and Al-Khalifa, 2014; Chavan *et al*., 2018).

In addition to the previously mentioned contribution of different hemocyte types in the immune functions, PLs and GRs are considered as key players in the cellular immunity, since their morphology is dramatically disrupted when they encounter the pathogens, as well as they have been observed engulfing and killing pathogens (Kwon *et al*., 2014; Hwang *et al*., 2015). Moreover, PLs and GRs, play immune functions associated with phagocytosis and encapsulation in most Lepidoptera and some Coleoptera (Lavine and Strand, 2002; Manachini *et al*., 2011). Among Lepidoptera, the immune response of caterpillars, including the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) and tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae), depends on the activities of two main hemocyte populations, PLs and GRs that recognize the pathogens and parasites (Nardi *et al*., 2003).

3. Qualitative characterization of the cellular immune defenses of insects against EPN immune-suppressive activity

3.1. Major cellular immune mechanisms in insects against EPNs

Encapsulation of EPNs by the insect larvae: As previously mentioned in the present review, EPNs have free-living IJs in their life cycle, within their intestines some symbiotic bacteria exist. The IJs enter the haemocoel of the insect through some openings (like mouth, anus and spiracles). Upon entry into the body of host insect, IJs penetrate through the gut wall and regurgitate their symbiotic bacteria into the insect haemocoel (Mastore *et al*., 2015). Both EPNs and their symbiotic bacteria contribute to host mortality (Dowds and Peters, 2003), which usually occurs within 24-72 hr resulting from toxemia or septicemia in the late phase of infection (Shapiro-Ilan *et al*., 2018).

The immediate response against the invading EPNs is encapsulation and the response to their symbiotic bacteria is phagocytosis, or nodulation in the case of a large load (Marmaras and Lampropoulou, 2009). The cellular encapsulation results in a multilayer cellular capsule (overlapping layers of cells) and or a melanin coat that encloses and kills the intruder or invader (Ling *et al*., 2005). For instance, Ono *et al*. (2020) conducted a study to compare between the cellular encapsulation in two lepidopterous insects (*G. mellonella* and the oriental armyworm *Mythimna separata*) after infection with EPNs *Caenorhabditis elegans* and *Steinernema carpocapsae*). Their results indicated a difference in immunity against EPNs between the two insects largely depending on the capabilities of their hemocytes. In respect of the contribution of certain hemocyte types in the encapsulation process, GRs were reported to contact a foreign targeted body, disintegrate or degranulate liberating material that endorses attachment of PLs and subsequently multiple layers of PLs form the capsule. For more detail, see review of Ghoneim *et al*. (2021).

In the same area of research, results of Wang *et al*. (1995) revealed that *H. bacteriophora* was initially encapsulated in larvae of the Japanese beetle *Popillia japonica* (Coleoptera: Scarabaeidae) and only 10% of EPN 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 *M. sexta*. Later on, Sheykhnejad *et al*. ("https://ejbpc.springeropen.com/articles/10.1186/

s41938-019-0173-1#ref-CR22"2014) found the cellular response of the rose sawfly *Arge ochropus* (Hymenoptera: Argidae) weaker to *S. carpocapsae* than to *H. bacteriophora*. In the lined click beetle *Agriotes lineatus* (Coleoptera: Elateridae), only 6% encapsulation of the EPN *Steinernema feltiae* was achieved, but 24% encapsulation of *H. bacteriophora* (Rahatkhah *et al*., 2015). Also, Istkhar and Chaubey (2019) showed that *Steinernema abbasi* had strong capability to escape encapsulation responses in larvae of the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) leading to the death within a very short time. In addition, the encapsulation of *S. feltiae* and *H. bacteriophora* by the prepupae of Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) showed a more frequent encapsulation of *S. feltiae* than of *H. bacteriophora* (Ebrahimi *et al*., 2011). In agreement with these reported results, recent results of Ghoneim *et al*. (2023) revealed that once *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 ENP's cuticle surface in multiple layers. These layers were mostly formed by GRs and PLs whereas *S. carpocapsae* seems to be unrecognized by the infected larvae. Depending on all reported results, it is well-known that the encapsulation responses of the insects varied according to the insect species and EPN species.

Nodulation (Nodule formation) of EPNs by the insect larvae: Functionally, 'nodulation' in insects is a cellular immune process whereby hemocytes recognize a foreign body, like EPN, and insulate it within the haemocoel as well as aggregate large numbers of invading symbiotic bacteria (Lavine and Strand, 2002; Marmaras and Lampropoulou, 2009). The enzyme phenoloxidase (PO) in haemolymph can hydroxylate tyrosine and oxidize *o*-diphenols to quinones (Gorman *et al*., 2007). These quinones undergo a series of additional enzymatic and non-enzymatic reactions leading to melanin synthesis in the final stages of nodulation against invading body (Zibaee *et al*., 2011). As found in a recent study, once IJs of EPNs (*S. carpocapsae* and *H. bacteriophora*) release their symbiotic bacteria into the haemocoel of *A. ipsilon* 5th instar larvae, highly spreading of aggregation hemocytes appeared (nodule formation) (Ghoneim *et al*., 2023**)**. This finding was in corroboration with the reported results of some authors, such as Dean *et al*. (2004 a, b). Also, Hassan *et al*. (2016) recorded the formation of multicellular hemocytes aggregates that entrap large number of symbiotic bacteria of EPN that infect 5th instar larvae of *A. ipsilon*.

Phagocytosis as a cellular immune activity of the insect larvae against EPNs: As clearly shown in the current literature, specific insect hemocytes migrate towards and engulf several targets, including yeast, bacteria, apoptotic bodies, cell debris from damaged tissues and pathogens, in a process called 'phagocytosis' (Marmaras and Lampropoulou, 2009). It is important to shed some light on the hemocytes responsible for the phagocytosis process in insects. Both GRs and PLs have been shown to be capable to phagocytosis (Kwon *et al*., 2014; Melcarne *et al*., 2019). These two hemocyte types are recognized as the immunocompetent cells in most lepidopterans (Brillard *et al*., 2001). Depending on the insect species and antigen type and concentration, they participate in phagocytosis with the PLs playing a dominant role (Tojo *et al*., 2000). In addition, PLs of *M. sexta* larvae are the major hemocytes involved in phagocytosis of non-self-microsphere beads, whereas GRs are apparently the only hemocytes phagocytizing the self-dead cells (Ling and Yu, 2006). Ling *et al*. (2005) observed PLs and GRs of *M. sexta* performing different phagocytotic roles, with the PLs being the major hemocyte type involved in phagocytosis of non-self microsphere beads, while GRs were the only hemocyte type that participate in the phagocytosis of self dead cells. A decline of PLs population in *M. sexta*, known as hyperphagocytic cells, recognize and attach to large numbers of entomopathogens and act as nuclei for subsequent nodule formation (Dean *et al*., 2004 a, b). In the scarabaeid beetle *Cetonischema aerugnninosa* (Coleoptera), GRs and OEs are only the hemocyte types involved in phagocytosis (Giulianini *et al*., 2003). Ayyad *et al*. (2001) injected the 3rd instar larvae of *P. surcoufi* with 20 and 40 IJs of *H. bacteriophora*. The released symbiotic bacterium *Ph. luminescens* was poorly phagocytosized by the GRs. In *D. melanogaster*, only PLs are involved in the phagocytosis (Meister and Lagueux, 2003). Also, OEs were reported to play a role in this process (Giulianini *et al*., 2003).

As shown in the available literature, the following release of the symbiotic bacteria *Photorhabdus* spp. from the IJs into the insect haemolymph, the first response of the host immune system is to phagocytose or encapsulate the invading bacteria (Eleftherianos *et al*., 2010 a, b). Hassan *et al*. (2016) investigated the interaction of EPNs *S. glaseri* and *H. bacteriophora* with their symbiotic bacteria on the hemocytes in larvae *A. ipsilon*. They found *S. glaseri* escaped the encapsulation and overcome host immunity faster than *H. bacteriophora* which recognized by the host hemocytes. The hemocytes PRs, PLs or GRs are phagocytic cells in *A. ipsilon* instar larvae. Phagocytosis was expressed in *A. ipsilon* hemocytes after infection with *H. bacteriophora* at a higher rate, compared to *S. glaseri* infection. In a recent study on larvae of the same insect, phagocytosis process was observed toward symbiotic bacteria of *S. carpocapsae* and *H. bacteriophora*, as well as the PLs and GRs had been

considered the main phagocytic cells (Ghoneim *et al*., 2023). Also, this finding was in consistent with results of Hassan and Ibrahim (2010).

Increasing mitosis in the insect larvae as a defense reaction against EPNs: As reported by some authors (Gardiner and Strand, 2000; Saito and Iwabuchi, 2003), the maintenance of hemocyte populations in insects is thought to be regulated by mitotic division of the circulating hemocytes and by production and release of hemocytes in the hematopoietic organs (for some detail, see review of Ghoneim *et al*., 2021). As shown in the available literature, Hassan and Ibrahim (2010) detected an increasing mitotic division, as response to EPN infection into 6th larval instar of *S. littoralis*. Some years later, Salem *et al*. (2020) observed a hemocyte response as immunocompetent cells after the EPN application on *G. 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 (King and Hillyer, 2013; Kwon *et al*., 2014) recorded this stimulation after the bacterial infection on other insect species. To a great extent, these reported results can be substantiated by results of Ghoneim *et al*. (2023) who 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 reviewed before. Therefore, *A. ipsilon* larvae increased the mitotic division to maintain homeostasis.

3.2. Suppressive activity of EPN/symbiotic bacteria complex against the insect immune defences

3.2.1. How EPNs overcome the immune defences of larvae?

It is important to mention that the IJs of EPNs have suppressed the immune responses of the insect hosts leading to death (Shapiro-Ilan and Brown, 2013). They may achieve this role alone or with assistance of their symbiotic bacteria (Leonar *et al*., 2022; Kaliaskar *et al*., 2022). Two main strategies employed by EPNs to evade the host immune defences including molecular mimicry and modulation of the host immune response (Rougon-Cardoso *et al*., 2016). In the early stages of infection, EPNs primarily rely on mimicry by displaying surface molecules that interfere with hemocyte surveillance to prevent recognition (Brivio and Mastore, 2018). Later on, both EPNs and their symbiotic bacteria use strategies for inhibiting the humoral and cellular responses of the insect host, based on the release of toxins, inhibitors and proteases (Balasubramanian *et al*., 2009), *i.e*., direct manipulation or modulation of the host defences involves the excretion/secretion of effector molecules. In this regard, *S. carpocapsae* excretion/secretion products (ESPs) have been linked to the host immunosuppression during the initial stages of invasion to promote a suitable growing environment for their mutualistic bacteria (Chang *et al*., 2019; Parks *et al*., 2022).

In addition, it has evident that the EPNs are able to effectively kill their hosts independently through their excretion/secretion products, linked to host immunosuppression during the initial stages of infection (Lu *et al*., 2017; Jones *et al*., 2022). EPNs themselves should be considered, because shortly after infection, and before the bacteria are released from the guts of invading IJs of EPNs, there is a significant reduction in the total hemocyte count of an insect host suggesting that the nematode itself is capable of suppressing the host immune system. This may be beneficial to their endosymbiotic bacteria (for some detail, see Shapiro-Ilan *et al*., 2018; Bobardt *et al*., 2020). On the other side, several authors (Stock and Blair, 2008; Bode, 2009; Laznik *et al*., 2010) reported that the symbiotic association of IJs of EPNs with the bacterial endosymbionts is one among factors influencing the EPN virulence, *i.e*., symbiotic bacteria of EPNs play a crucial role in the EPN pathogenicity by releasing a wide range of secondary metabolites into host haemolymph.

However, more attention should be paid to this regard. It was reported in the current literature that *S. carpocapsae* is able to destroy the antibacterial peptides by its secreted compounds that have proteolytic activity suppressing the insect defences (Götz *et al*., 1981). Also, some authors (Lavine and Strand, 2002; Toubarro *et al*., 2010) demonstrated that Sc-KU-4, serine protease inhibitor, can inhibit the hemocyte aggregation which is the primary step in cellular encapsulation and requires the activation of GRs and PLs resulting in the entrapment of the invading EPN. Thus, EPN expresses a serine protease inhibitor during the invasive stage that is capable of targeting recognition proteins, thus impairing host defenses (Toubarro *et al*., 2013).

In agreement with the previously reported works, recent results of Ghoneim *et al*. (2023) revealed the capability of IJs of *H. bacteriophora* to escape from the encapsulation process in *A. ipsilon* larvae by shedding their cuticle. This result can be understood, since loss of the external layer of body surface of *H. bacteriophora* can be considered as a strategic mechanism to escape from host immunological surveillance (Hassan *et al*., 2016). Subsequently, the EPN escaped from the attached hemocytes and the encapsulation processes were

lacking at 12 h post-infection. In the same study of Ghoneim *et al*. (2023), *S. carpocapsae* could not be recognized by hemocytes of *A. ipsilon* larvae. This result was explained by the interaction of surface molecules of *S. carpocapsae* with its host and might be due to other active compounds which have been suggested to be actively secreted/excreted by EPNs into host haemocoel.

Furthermore, Balasubramanian *et al*. (2010) observed severe morphological disorders in the hemocytes of *G. mellonella* larvae after infection with *S. carpocapsae*. In Egypt, 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 6th larval instar of *S littoralis* after infection with *S. carpocapsae* and *H. bacteriophora*. In addition, infection of *G. mellonella* larvae 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 (Salem *et al*., 2014). In corroboration with those reported results, Ghoneim *et al*. (2023) observed many cytopathological disorders of the hemocytes of *A. ipsilon* larvae after infection with *S. carpocapsae*, which could be described as: distortion of the cytoskeletons of hemocytes, highly vacuolization of the cytoplasm, granulation of nucleus, and rupture of the cell membrane (Figures 1 and 2).

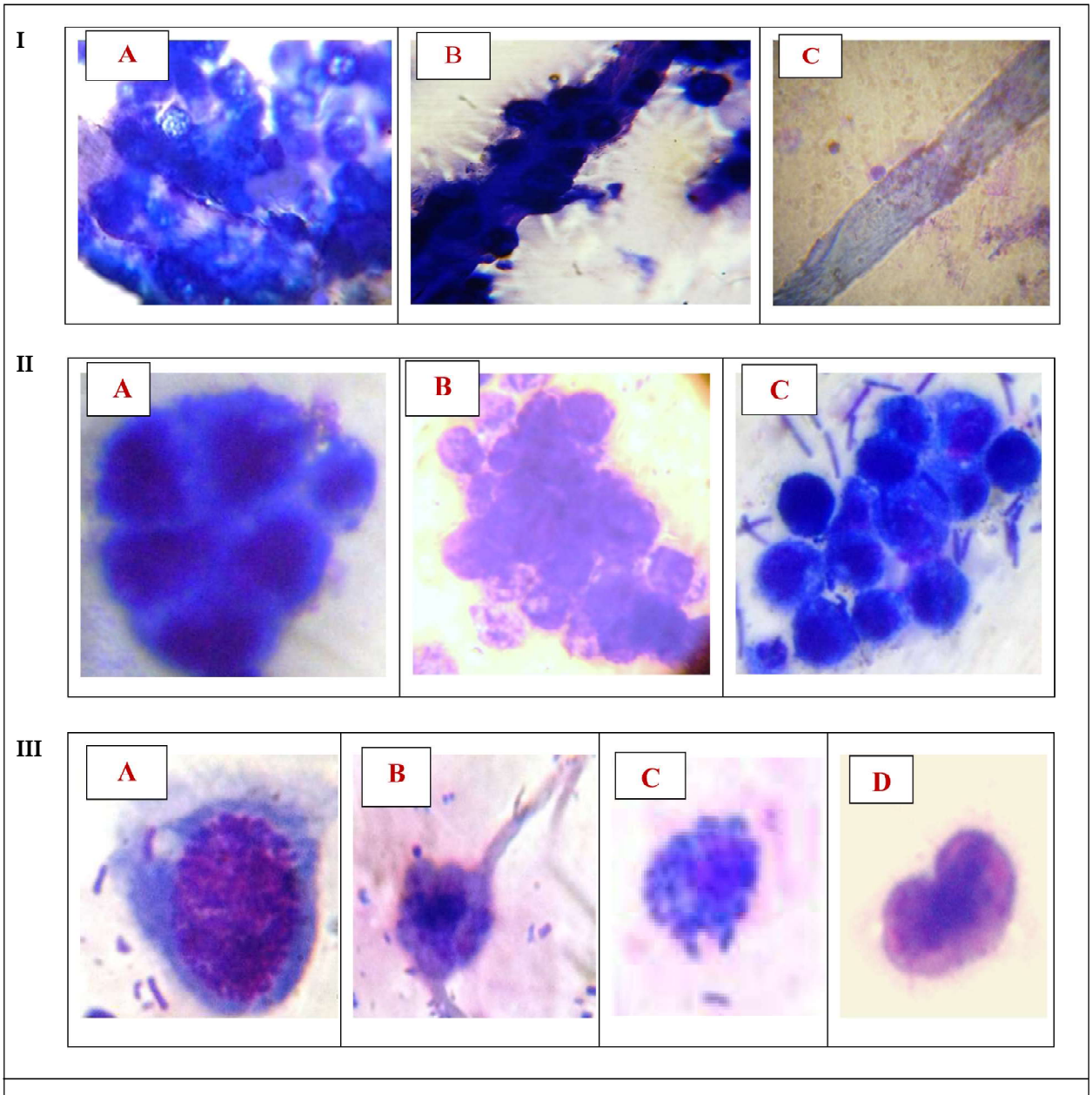
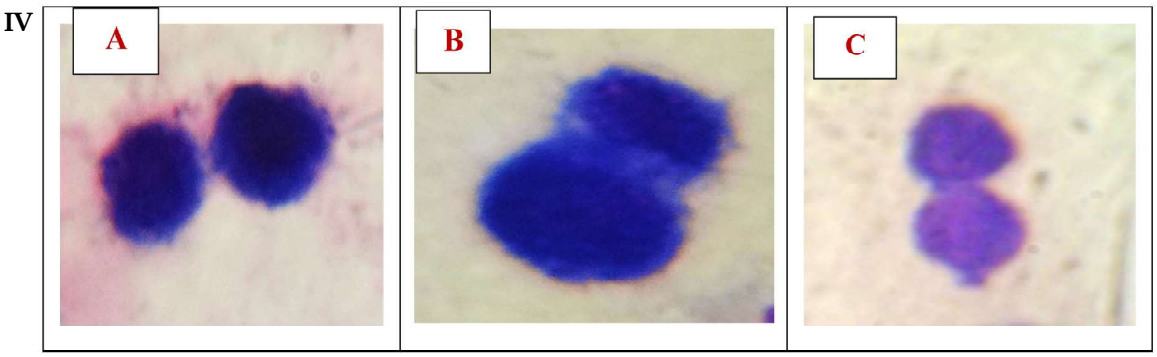
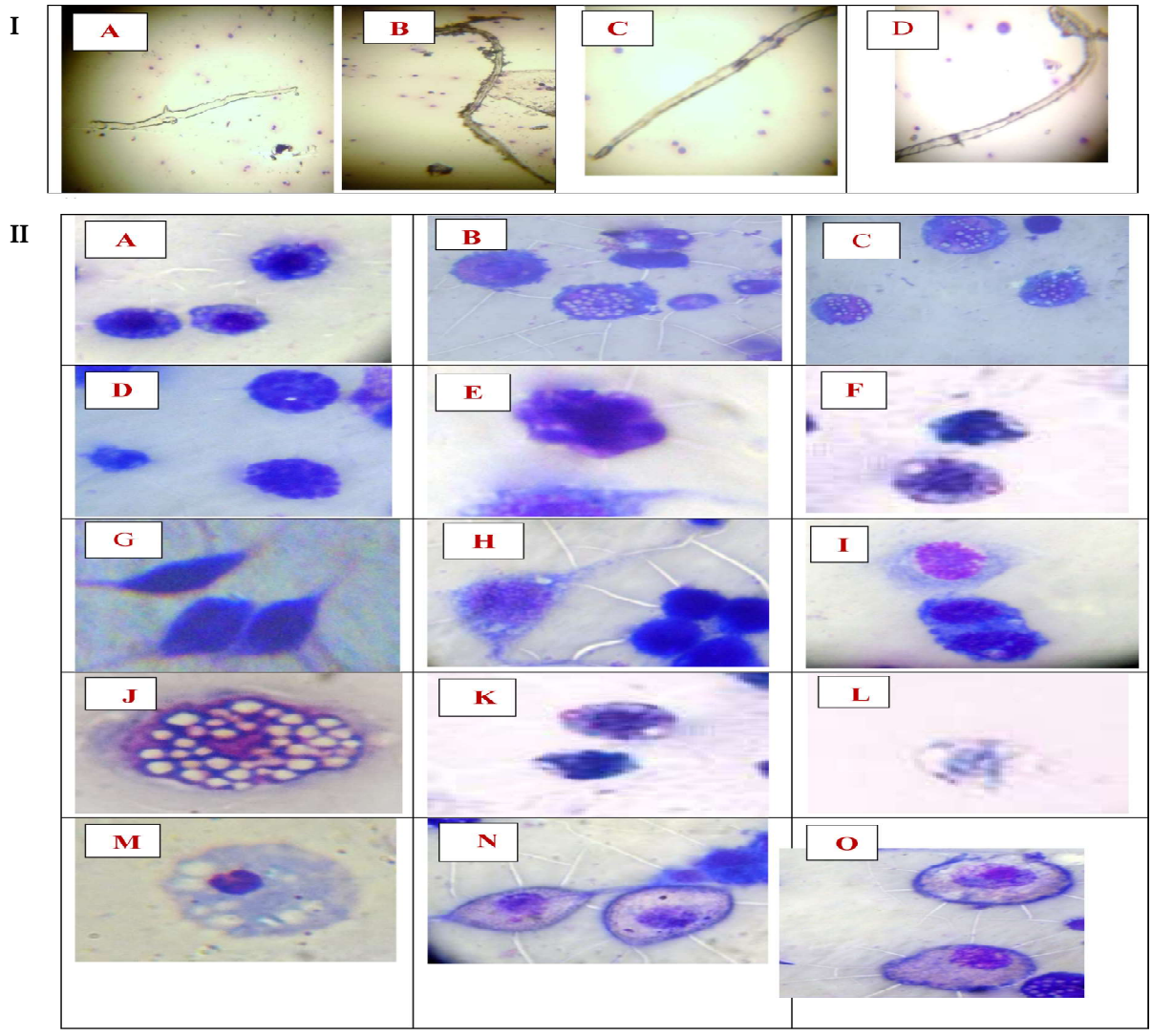


Figure 1: Photomicrographs of the *A. ipsilon* hemocyte responses toward *S. carpocapsae* and *H. bacteriophora* infection; I. Larval response by encapsulation; II. Larval response by nodulation; III. Larval response by phagocytosis; IV. Larval response by increasing the mitotic division



Source: Ghoneim et al. (2023)

Figure 1 (Cont.)



Source: Ghoneim et al. (2023)

Figure 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 by secretion of molecules participate in suppression of host defenses

3.2.2. How the symbiotic bacteria overcome the larval immune defenses?

As previously reviewed, some studies have shown that EPNs play an active role alone in the nematode/symbiotic bacteria complex for killing the insect host through suppression of its immune system. On the other hand, several studies concluded a synergistic action of EPN with their symbiotic bacteria to overcome the insect's immune system, causing its death within 24 to 48 h (Lu *et al*., 2017), as experimentally proved for the beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae) larvae (Darsouei *et al*., 2017). In this context, also, it is important to give an insight into the crucial role of symbiotic bacteria in the overcoming of host immune defences. After a variable time of entering host haemocoel, IJs begin to release their symbiotic bacteria from their guts into the host circulatory stream. The action of bacteria, supported by the immunosuppression processes, was found to be supported by EPN (Chang *et al*., 2019; Parks *et al*., 2022). Shortly, EPNs rearrange the environment (the host body) in a favorable fashion that promotes the survival and reproduction of symbiotic bacteria (Tomar *et al*., 2022).

On the other hand, the symbiotic bacteria *Xenorhabdus* synthesized and released antibiotic compounds within the insect haemolymph that suppressed the competing pathogens (Vallet-Gely *et al*., 2008). By 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 (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. In this area of research, Ghoneim *et al*. (2023) studied the virulence of EPNs *S. carpocapsae* and *H. bacteriophora* against *A. ipsilon* larvae in Egypt. Once IJs released their symbiotic bacteria, the cellular immune response of larvae is activated. However, these responses appeared ultimately ineffective, as the bacteria replicate and secrete a number of toxins within the host haemocoel. These toxins disrupted the cytoskeletons of hemocytes, resulting in malformed hemocyte. Then, the symbiotic bacteria, *X. nematophilus* and *Ph. bacteriophora* replicate, filled the host haemocoel and suppressed the *A. ipsilon* immune system.

4. Quantitative characterization of insect immune defenses against the EPN immune-suppressive activity

4.1. Basic knowledge

As previously mentioned in the present review, circulating hemocytes of insects are involved in different physiological functions, such as development and metamorphosis, detoxification of xenobiotics and immune defences against pathogens and other invaders (Li *et al*., 2007). Also, certain hemocytes can take part in humoral reactions, hormone transport, wound repair and antimicrobial activity (Pandey and Tiwari, 2012). However, functions ascribed to a given hemocyte type vary owing to variation in the observation techniques, insect species and other factors. For some detail, see review of Ghoneim (2019).

Increased cellular immunity in an insect after exposure to entomopathogens may be used as an indicator to the hemocyte involvement in the achievement of immunity mechanisms, like increasing phagocytic activity and haemolymph encapsulation (Dubovskiy *et al*., 2008). In insects of Lepidoptera, maintenance of hemocyte populations during the larval stage depends on both proliferation of cells already in circulation and the release of hemocytes from hematopoietic organs (Yamashita and Iwabuchi, 2001; Ghoneim, 2019). Also, some studies investigated the responses of certain hemocytes of insects against the invading EPNs, depending on the rapid increase of populations during an infection, which indicate their responsibility for several cellular immune defenses in insects (Rahatkhah *et al*., 2015).

In this context, the insect haemogram profile, including total hemocyte count (THC) and differential hemocyte counts (DHCs), represents a very good indicator of the insect physiology and the environmental adaptability in each stage of the insect in response to stress (Bardoloi *et al*., 2016; Ghoneim, 2019) and act as a valuable tool for the investigation of toxic effects of the insecticidal materials on biocontrol agents, like pathogens (Baishya *et al*., 2015). Therefore, disturbances or alterations in THC and DHCs, in the insect circulating haemolymph

against a pathogen, provide high indicative criteria for the determination of the cellular immune reactions (Altunta[o] *et al*., 2012). In view of these considerations, some special attention should be paid to the disturbance in THC and DHCs in the insect circulating haemolymph after infection with EPNs.

4.2. Disturbed THC in haemolymph of the insect larvae by immunosuppressive activity of EPN/bacteria complex

The pattern of THC response in an insect varies according to several factors, such as the developmental stages of the insect and their physiological processes, and species-specific host–pathogen interactions. Moreover, THC disturbance is expected to be a fast and sensitive response upon exposure to entomopathogens (Manachini *et al*., 2011; Hillyer and Strand, 2014).

To the best of our knowledge, limited information exists in the available literature concerning the assessment of EPNs on THC in haemolymph of insects. The 3rd instar larvae of *P. surcoufi* were injected with 20 and 40 IJs of *H. bacteriophora* by Ayyad *et al*. (2001) in Egypt. According to their results, injection with 20 IJs led to decreasing THC up to 40 h post-injection, except for an increase at 20 h. On the other hand, injection with 40 IJs led to increasing THC during few hours post-injection, except for a reduction at 40 h. In Iran, Abdolmaleki *et al*. (2017) investigated THC in the haemolymph of cabbage white butterfly *Pieris brassicae* (Lepidoptera: Pieridae) larvae when they were challenged with EPN *H. bacteriophora*. Their results recorded an increase of THC early after injecting IJs into haemolymph. However, at 7 h post-injection with IJs, THC was significantly decreased. With regard to *A. ipsilon*, THC in haemolymph of 5th instar larvae considerably increased, only at 6 h post-infection with *H. bacteriophora* or *S. carpocapsae*. On the other hand, THC was significantly decreased, at both 24 and 48 h post-infection. In addition, the infected larvae exhibited a higher cellular immune defense against *H. bacteriophora* than against *S. carpocapsae*, at early time interval, but remarkably inhibited defense was found at 24 and 48 h post-infection **(**personal unpublished data**)**.

For understanding the increasing THC in larval haemolymph, at the early stage of the EPN infection and then decreasing count later on, it is important to mention that THC disturbances can be used as a good indicator to the cellular immune defenses. In other words, EPN-infected larvae exhibited a highly immune defense by increasing THC in haemolymph early (at 6 h post-infection) indicating fast cellular immune response of larvae against the invading EPNs at this time. This early THC increase may be due to the promotion of mitotic division of hemocytes by increasing ecdysteroid biosynthesis in haemolymph or haematopoiesis which has been activated in response to EPN infection (George and Ambrose, 2004; Kurt and Kayis, 2015) or the release of hemocytes that adhered on surfaces (sessile haemocytes) within the haemocoel (Ghasemi *et al*., 2013a).

In contrast, the remarkable decrease of THC in haemolymph of the EPN-infected larvae, at later time intervals post-infection, may indicate an active immunosuppressive action of the invading EPNs on the target host larva. This reduction in THC can be ascribed to the metabolites of tested EPNs and/or their symbiotic bacteria (Vilcinskas *et al*., 1997) because symbiont bacteria of EPNs have been reported to a detrimentally affect the status of host hemocytes, causing a decrease in the THC (Brivio *et al*., 2005; Rahatkhah *et al*., 2015). In addition, the reduction of THC may be considered as a result of the inhibition of larval hematopoietic function and cell proliferation (Zhu *et al*., 2012). The reduced number of mitotic hemocytes can be considered to interpret the reduced THC (Er *et al*., 2017).

In this context, it may be informative to review some studies investigating the THC disturbance in haemolymph of different insects as indicated their cellular immune reactions against some entomopathogens other than EPNs. For instance, Hoch *et al*. (2004) reported a significant increase of THC in larval haemolymph of the gypsy moth *Lymantria dispar* (Lepidoptera: Erebidae) after infection with an entomopathogenic microsporidia, *Vairimorpha* sp. With regard to the immune response of the tropical aquatic dipteran (midge) *Chironomus Xanthus* (Diptera: Chironomidae), Ajamhassani *et al.* (2013) found an increase in THC after infection with increasing concentrations of the Entomopathogenic bacterium *Bacillus thuringiensis* subs. *kurstaki*. A similar trend of increased THC was recorded by Grizanova *et al*. (2014) for *G. mellonella* larvae after infection with sublethal doses of spore–crystal mixtures of *B. thuringiensis* var. *galleria*, since an increase of THC was recorded on the second day of exposure but decreased later on. On the contrary, Abd El-Aziz and Awad (2010) recorded a decrease of THC by 44.9% in *A. ipsilon* larvae, 48 h post-infection with *B. thuringiensis* var. *kurstaki*. In Egypt, also, Gabarty (2011) determined a significant reduction of THC in *A. ipsilon* larvae of 3rd, 4th and 6th instars after infection of 2nd instar larvae with LC_{50} of *B. bassiana* or *M. anisopliae*.

On the other hand, THC in haemolymph of the sunn pest *Eurygaster integriceps* (Hemiptera: Scutelleridae) increased 3 h after injection with *B. bassiana*. However, interaction between the pathogen-immune reactions might lead to a decline of THC later on (Zibaee *et al*., 2011). According to the study of Ajamhassani *et al*. (2013) on larvae of the fall webworm moth *Hyphantria cunea* (Lepidoptera: Erebidae), a significant increase of THC was observed after injection of certain isolates of *B. bassiana* and *Isaria farinosae*, but then THC was declined. In Egypt, Sayed *et al.* (2023) recently found a significant reduction in THC of *A. ipsilon* larvae after infection with the entomopathogenic fungus *Metarhizium anisopliae* and exposure to gamma radiation in.

4.3. Deteriorated DHCs in haemolymph of the insect larvae by immunosuppressive activity of EPN/bacteria complex

As previously mentioned in the present review, hemocytes of insects perform important role in various biological, physiological and immune functions, such as coagulation, phagocytosis, encapsulation of foreign bodies, detoxification of metabolites and the defense against xenobiotics or microbial infection. Each hemocyte type has engaged in specific function(s). Therefore, increasing or decreasing population of certain hemocyte type is a prerequisite event for performing this function (Gelbic *et al*., 2006). On the other hand, increasing populations of some hemocyte types and decreasing populations of others may be due to the transformation of some types into others for achieving the phagocytic function or other tasks against the biotic targets, like bacteria, fungi, nematodes, yeast and apoptic bodies (da Silva *et al*., 2000). According to the available literature, some studies recorded a considerable influence of pathogenesis on the DHCs in some insects, such as the red cotton stainer *Dysdercus koenigii* (Hemiptera: Pyrrhocoridae)(Tikku *et al*., 1992) and the maize stalk borer *Busseola fusca* (Lepidoptera: Noctuidae)(Mochiah *et al*., 2003). In the following sections, we reviewed the quantitatively disturbed populations of the hemocyte types that identified only in 5th instar larvae of *A. ipsilon, viz*., PRs, PLs, GRs, SPs and OEs (Ghoneim *et al*., 2023), as for example.

Disturbed PRs population in insect larvae by the EPN/symbiotic bacteria complex: In a study, Ayyad *et al*. (2001) injected the 3rd instar larvae of *P. surcoufi* with 20 and 40 IJs of *H. bacteriophora*. Based on their results, PRs population increased at 40 h post-infection. In another study, a remarkable decrease of PRs population was recorded in haemolymph of 5th instar larvae of *A. ipsilon* at both 6 h and 24 h post-infection with *S. carpocapsae* and *H. bacteriophora*, but significantly increased at 48 h post-infection **(**personal unpublished data**)**. Some authors (Ling *et al*., 2005; Liu *et al*., 2013) reported PRs as stem cells transforming into other hemocytes, like PLs and GRs, and thus their population was decreased due to immune activity. Other authors (Lavine and Strand, 2002; Ribeiro and Brehelin, 2006) reported that the exact function of PRs is still disputable. At the early phase of infection (6 h and 24 h post-infection), PRs population is decreased because some of them transform into other immunocytes. In addition, the changes in PRs population may be due to some factors including inhibition of their mitotic division, or to the inhibited activity or destruction of hematopoeitic organs responsible for their production (Zibaee *et al*., 2012; Ghoneim, 2019). Therefore, the reduction of PRs population in larvae at the early phase of EPNs infection may be due to the effects of EPNs on these vital processes.

Disturbed PLs population in insect larvae by the EPN/ symbiotic bacteria complex: As previously mentioned in the current review, phagocytosis is an essential mechanism of the cellular immune responses of insects against entomopathogens. It is worthy to mention that the role of PLs in phagocytosis is still debatable, since some authors suggested their role as phagocytes, particularly in Lepidoptera like *A. ipsilon*, and thus they should be highly produced (Jiravanichpaisal *et al*., 2006; Ýzzatoglu, 2012). On the other hand, some authors reported no phagocytic function of PLs (Beaulaton, 1979). In general, Strand (2008) reported that PLs can act as the main mediators of cellular immunity in larvae of lepidopteran insects.

As shown in the available literature, injection of the 3rd instar larvae of *P surcoufi* with 40 IJs of *H. bacteriophora* resulted in a decreasing PLs population, at 40 h (Ayyad *et al*., 2001). In a study on *A. ipsilon*, PLs population pronouncedly increased in haemolymph of larvae at both 6 & 24 h post-infection with EPN *S. carpocapsae* or *H. bacteriophora* indicating a considerable immune defense against the invading EPNs. Later on, the same EPNs exerted a high immunosuppressive action on PLs, since the hemocyte count was considerably decreased **(**personal unpublished data**)**. Apart from EPNs, PLs population increased in *O. japonica* larvae 12 h post-infection with *M. anisopliae* (Anggraeni *et al*., 2011). To understand the increasing PLs population in haemolymph of larvae, at early phase of the EPN, it could be attributed to the differentiation of PLs by mitosis in the hematopoietic organs (Kurihara *et al*., 1992a, b) which may be triggered by increased haemolymph ecdysteroids (Nakahara *et al*., 2003). On the other hand, decreased PLs population at late time post-infection

can be attributed to the fact that these hemocytes are highly polymorphic and can be converted into other hemocyte types (George, 1996).

Disturbed GRs population in insect larvae by the EPN/ symbiotic bacteria complex: Although GRs represent the most abundant hemocytes in insect haemolymph, some authors (Karaçalý *et al*., 2000; Strand, 2008) reported little phagocytic activity of them. On the other hand, the main function of GRs in lepidopterous insects is phagocytosis, as found in the larvae of the European gypsy moth *Lymantria dispar* (Butt and Shields, 1996) and *S. littoralis* (Costa *et al*., 2005**)**. Ayyad *et al*. (2001) injected the 3rd instar larvae of *P. surcoufi* with 40 IJs of *H. bacteriophora* and recorded decreasing GRs population, at 40 h post-injection. In a certain study, the GRs population in 5th instar larvae of *A. ipsilon* significantly increased only at 6 h post-infection with *S. carpocapsae* or *H. bacteriophora* but were significantly decreased at both 24 and 48 hr post-infection **(**personal unpublished data). This increase of GRs population in the larval haemolymph may be explained by the transformation of some hemocytes into GRs (George and Ambrose, 2004). On the other hand, decreased GRs population may be interpreted by the death of a number of them owing to their challenge toxic molecules produced by EPNs (Costa *et al*., 2005). Also, decreased GRs population may be due to their differentiation into other hemocyte types (Liu *et al*., 2013).

Disturbed SPs population in insect larvae by the EPN/ symbiotic bacteria complex: In Lepidoptera, like *A. ipsilon*, SPs are quite different from GRs overloaded with phagocytosed material (for review, see Ghoneim, 2019). The exact function of these hemocytes is still unknown but Sass *et al*. (1994) suggested their responsibility for transporting the cuticular components and Akhurst (1982) believed its contents of heparin-like molecules which probably prevent the coagulation of the haemolymph. Unfortunately, the current literature lacks any information concerning the effects of EPNs or other entomopathogens on SPs population in insects.

Disturbed OEs population in insect larvae by the EPN/ symbiotic bacteria complex: OEs were reported to play a crucial role in the phenoloxidase cascade when an immune challenge occurs (Shaurub, 2012; Ghoneim *et al*, 2021). Ayyad *et al*. (2001) injected the 3rd instar larvae of *P. surcoufi* with 40 IJs of *H. bacteriophora*. Depending on their results, OEs population increased at 40 h post-infection. On the contrary, remarkably declined OEs population in haemolymph of *A. ipsilon* 5th instar larvae was observed at 6, 24 and 48 hr post-infection with *S. carpocapsae* or *H. bacteriophora*, denoting EPNs immunosuppressive effects on these immunocytes **(**personal unpublished data). For some detail, decreased OE population may be due to degeneration of some OEs for releasing precursors of proPo that likely play a role in melanization of haemolymph and an important immunity protein in insects (Ribeiro and Brehelin, 2006; Ghasemi *et al*., 2013b**)**, since OEs were suggested to play an important role in phenoloxidase (PO) cascade when an immune challenge occurs (Strand, 2008).

5. Conclusion

This described cellular immune defences in the insects, it should be completed by detailed humoral immune components to give a complete picture of immune reactions in insects against EPNs. Although the infected insect try to defend itself against the invading EPN/symbiotic bacteria, *via* morphological and innate immune defences, and few of them are encapsulated, nodulated, or/and phagocytosized, but the majority of invading EPN/symbiotic bacteria usually overcome these defences by different mechanisms ending in the insect death. Therefore, many insect pests may be successfully controlled by using the appropriate concentration of effective EPNs under suitable biotic and abiotic conditions. In our view, study of these conditions is a fundamental task for the future of pest control by EPNs.

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