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PHYSIOLOGICAL AND ULTRASTRUCTURE RESPONSES OF *Schistocerca gregaria* (FORSKAL) TO *Metarhizium acridum* AND FOUR BIO ACTIVE COMPOUNDS

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ABSTRACT

The effect of *Metarhizium acridum* and four bioactive compounds (Neem, L-Glutamic acid, *Schinus molle* and abamectin) in sole treatments and in combination with *M. acridum* was studied on the mortality of 5th nymphal instar of desert locust *Schistocerca gregaria* as well as the time mortality responses and the ultra-structure of females ovary. The obtained results showed that mortality in case of *Metarhizium acridum* treatment reached to 100% after 14 days, while in case of neem treatment the mortality reached to 60 % after 12 days, at the same time the mortality reached up to 20 % after 10 days post treatment with L- Glutamic acid, but such mortality reached to 50 % after 14 days post treatment with *Schinus molle* extract, finally the mortality reached to 60 % after 11 days of abamectin treatment. All the mixtures with *M. acridum* caused 100 % mortality by the 5th day post treatment. However the sole treatments caused long time to kill 50 % of treated insects (LT_{50}), where the LT_{50} of *M. acridum* was 7.261 day as the fastest treatment, while all mixtures caused accelerate in the mortality,but the best result was obtained in case of *M. acridum* + neem treatment which LT_{50} was only1.625 day.

Any way there were different effects on ultra-structure of the ovary as result of treatments used. Such effects varied between changes in nucleus shape to changes and destruction of nuclus it self.

Keywords: *Schistocerca gregaria, Metarhizium acridum*, plant extract, amino acid, L- Glutamic acid, *Schinus molle,* abamectin, bioassay, time mortality responses and ovary ultrastructure.

INTRODUCTION

The desert locust *S. gregaria* has been the most serious crop pest in many countries of Africa and Asia, it is major pest of many tropical and subtropical countries causing extensive damage to the foliar part of many plants particularly during years with locust outbreaks. (Abd El-Fattah, 2005 and Ceccato, *et al*., 2007).The current method for locust control based on applying synthetic chemical insecticides (Gamal *et al*., 2012). Entemopathogenic fungi *Metarhizium acridum*, has been commercialized and used successfully for biocontrol of grasshopper pests in Africa and Australia(Stuart *et al*., 2012) now produced commercially with trade names such as Green Muscle and Green Guard), characterized as safe pathogen to non-target organisms**,** and they can penetrate directly through the cuticle and not necessarily need to be ingested in order to initiate disease (Goettel *et al*., 1990) The application of Green Muscle consederd as a new technique to locusts and grasshoppers control (Mahgoub *et al*., 2011). Neem tree *Azadirachta indica* has been used for many years against several insect pests (Hamadah *et al*., 2013). Neem tree has strong insecticidal activity against *S. gregaria* (Nicol and Schmutterer, 1991). *S. molle* has active substances, such as terpens tanins, alcaloids, flavonoids, saponins, gums, linoleic acid, oleorresins, mostly in leaves and fruits (Hayouni *et al*., 2008), Abamectin is a natural product of the soil microorganism *Streptomyces avermitilis* and shows spectrum insecticide and acaricide with high pesticidal activity, (Qiao et al. 2012).Abamectine and enhanced the effect of *M. acridum* against desert locust nymphs in the field (Mohamed *et al.* 2014). L-Glutamic acid is known as excitatory transmitter at the neuromuscular junction of invertebrates (Wafford and Sattelle, 1989), while (Clements and May, 1974) showed that when *Schistocerca gregari* nerve-muscle exposed to glutamate caused a variety of responses, some of which were shown to be abnormal and were much more severely affected. *S. gregaria* had panoistic ovary, which is composed of number of ovarioles (Martoja, 1964). Each ovariole was surrounded by basal lamina and consists of follicle cells and oocytes. The aim of present work is study the effect of four bioactive compounds (at rate of 1 tenth of recommended dose of each compound) on acceleration mortality caused by *M. acridum* to achieve safe desert locust control procedures, as well as the effect of such treatments on the ultra- structure of female ovarian tissues.

MATRIALS AND METHODS

Desert locust nymphs

Fifth nymphal instar of *S. gregaria* two days after final molting, were kindly obtained from desert locust colony maintained in Locust and Grasshoppers Research Dep., Plant Protection Research Institute, ARC, Cairo Egypt. Desert locust individuals were reared in the laboratory according to (Robert *et al.,* 2002). The colony was fortified with wild insect collect from the field each year.

Metarhizium acridum

The entomopathogenic fungus used during the study is *M. acridum* (formerly *Metarhizium anisopliae* var. acridum) Bischoff *et al.,* 2009, isolate (IMI330189) was kindly obtained from BASF, South Africa ,under the commercial name Green Muscle®. The spores were suspended in sterilized water; trace of Tween (80) was added. The concentration was adjusted to $5×10^8$ spores/ ml, each nymph received 5µl of the final solution.

Neem (Azaderachtin)

Azaderachtin, under the commercial name Safe-oil 0.03 % EC, at concentration of 1ml/liter distilled water.

Schinus molle extract

50 g of fresh aerial part of *S. molle*, were air-dried at lab temperature, dried in oven at 40°C till constant weight then ground to fine powder, add to

800 ml liter of distilled water in volumetric flask for 3 days and repeated 4 times, then filtrated. Combined filtrates were evaporated under reduced pressure using rotary evaporator apparatus until a minimum amount of solvent remained which gives (3 g) at last. The extract (brownish sticky) was stored in a refrigerator at 5 ºC and kept for using in different analysis. The concentration used was 1 g from extract added to 100 ml distilled water (Woo *et al.,* 1977).

Amino acid (L-Glutamic acid)

Molare solution from L-glutamic acid is prepared by addition of 147.13 gm \ litter distilled water. concentration are used as 0.1 from molar solution (Krasilnikov and Bakhramov, 1983) each nymph received 5µl of the final solution.

Abamectin

5-*O*-demethylavermectin A_{1a} (i) mixture with 5-*O*-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl) avermectin A_{1a} (ii).Under the commercial name, Agromic 1.8 % EC, was used at concentration of 1ml/liter distilled water, each nymph received 5µl of the final solution.

Mixtures

Metarhizium acridum was used in combination with Neem, *S. molle*, L-Glutamic acidand and Abamectin, the mixture solution contain the same concentration for each compound as in sole case.

Treatments

Thirty individuals of 5th instar nymphs were used in each treatment, divided into 3 replicates each. *M. acridum* , L-glutamic acid and Abamectin treatments were used as topical application, while Neem and *S. molle* were used as follow: 40 g of fresh clover were dipped in 100 ml of each used concentration, dried for 1 hr. in room temperature, then introduced to the nymphs. The mixtures were used as topical application, but in case of Neem and *S. molle* the nymphs were treated with *M. acridum* first then were feed on treated clover as described previously. Treated and untreated nymphs were kept in cages under laboratory conditions at 31 ± 0.5°C, also were feed and cleaned daily, mortality was observed and recorded daily. Extra five female 5^{th} nymphal instar per each treatment were treated as described previously, then at the 4^{th} day post treatment, these nymphs were subjected to dissection for Electron Microscope examination.

Transmission Electron Microscopy (TEM) examination

The insects were killed by twisting the head to break the "neck" membrane. The posterior tip of the abdomen was cut off and the head, with the gut attached, was removed. Ovary cleaned from the surrounding fat body and then it was dissected in ice-cold (0-5 °C) karnovsky fixative, pH 7.3 (Karnovsky, 1965). The tissue pieces were washed twice in a buffer for 30 min. The specimens were then dehydrated in grades of ethanol; 50, 70, 80, 90 and 100%. The specimens were cleared in toluene for 10 min and then embedded in the resin of choic Epon. Semithen sections are cut from these blocks (stained with toluidine blue) and examined by the light microscope (Spnrr, 1969). Ultrathin sections obtained from selected blocks were mounted on copper grids stained with uranyl acetate and lead citrate and then examined with Jol 1010 transmission electron microscope (Reynolds, 1963). This technique was carried out at Regional Center for Mycology and Biotechnology, Al-Azhar University, Madenit Nasr, Cairo.

Statistical analyses

Mortality data were subjected to probit analyses according to Finney (1971) to calculate time mortality responses and its regression lines.

RESULTS

Fig. (1) shows the accumulative mortality caused by *Metarhizium acridum* Neem, *Schinus molle*, L- Glutamic acid and abamectin used separately or in combination against *Schistocerca gregaria* 5^{th} nymphal instar. It's clear that *M. anisopliae* treatment caused slow mortality progress began by day 5, ended with 100 % of mortality by day 14 post treatment, while other treatments (used at 1 tenth of the recommended dose) showed same trend, where mortality in Neem treatment began by day 4 ended with 60% mortality by day 12, while *S. molle* mortality initiated by day 6 post treatment ended with 50 % by day 14, also in case of L- Glutamic acid the mortality began by day 6 ended with 20 % by day 10. Finally in abamectin treatment the mortality began little bet early by day 3 post treatment ended with 60 % by day 11.

While all mixtures seriously increased the efficacy of *M. anisopliae*, the mortality began by the first day post treatment, the mortality % were (10, 22, 28 and 24%) respectively for the mixture of *M. anisopliae* with *Schinus molle*, L- Glutamic acid, abamectin and Neem, respectively. Such mortality ended by day 5 with 100 %.

In the same line with these data fig. (2) and table (1) demonstrate mortality time responses of the previous treatments, it could be concluded that sole treatments: *M. anisopliae*, Neem, *Schinus molle*, L- Glutamic acid and abamectin needed long period to kill half of the population (LT_{50}). These LT_{50}s were 7.261, 10.489, 14.608, 46.568 and 9.055 days respectively, while LT_{90}s of the same treatments were 12.613, 27.815, 39.972, 462.937 and 29.907 days, respectively.

Although, mixtures greatly accelerate the mortality where LT_{50}s were 1.625, 2.414, 1.963, and 1.888 days for the mixtures of *M. anisopliae* with Neem, *Schinus molle*, L- Glutamic acid and abamectin, respectively, LT_{90}s of same treatments were 5.818, 5.496, 4.749, and 5.715 days, respectively.

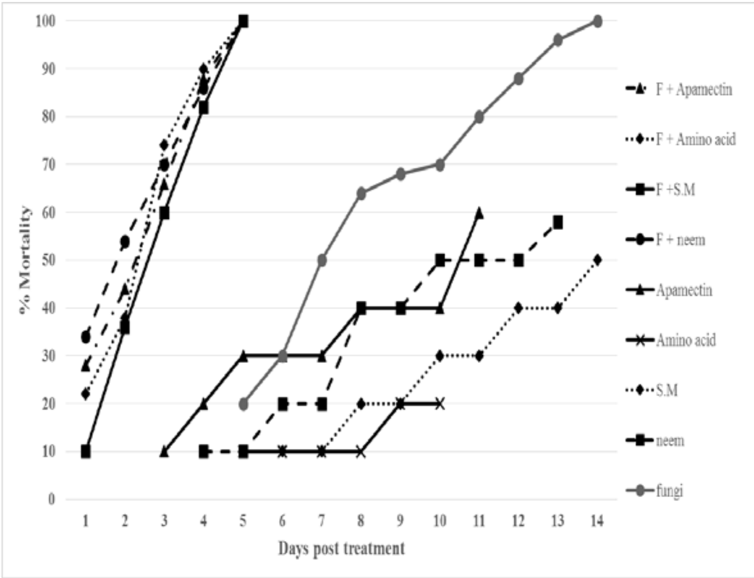

Fig. (1) Accumulative mortality due to *Metarhizium acridum* Neem, L-Glutamic acid, *Schinusmolle* and abamectin treatment that are used separately or in combination against *Schistocerca gregaria* 5th nymphal instar.

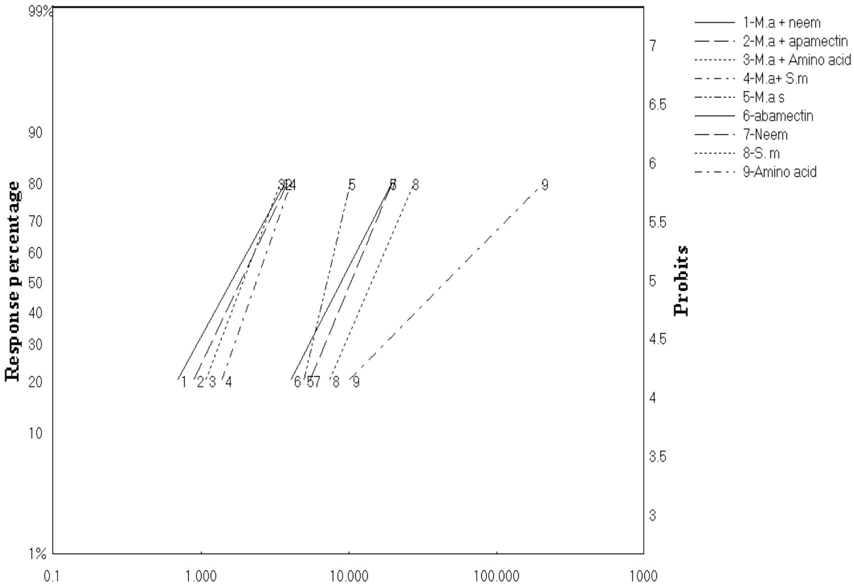

Fig. (2) Time mortality responses of *Metarhizium acridum* Neem, L-Glutamic acid, *Schinusmolle* and abamectin Treatment that are used separately or in combination against *Schistocerca gregaria* 5th nymphal instar.

Table (1) Time mortality responses of *Metarhizium acridum* Neem, L- Glutamic acid, *Schinus molle* and abamectin treatment that are used separately or in combination against *Schistocerca gregaria* 5th nymphal instar.

Line name	LT_{50} (Days)	Index	Slope	LT_{25} (Days)	LT_{90} (Days)
M. acridum	7.261	22.38	5.344	5.43	12.613
Neem	10.489	15.492	3.026	6.278	27.815
S.mlle	14.608	11.124	2.932	8.6	39.972
Amino acid	46.568	3.49	1.285	13.904	462.937
abamectin	9.055	17.946	2.47	4.828	29.907
M. acridum + neem	1.625	100	2.314	0.831	5.818
M. acridum + S.mlle	2.414	67.316	3.587	1.566	5.496
M. acridum + Amino acid	1.963	82.781	3.34	1.233	4.749
M. acridum + apamectin	1.888	86.07	2.664	1.054	5.715

Transmission Electron Microscopy (TEM) examination

Electron microscope of ovarioles for untreated insects illustrated in Fig. $3_{(1)}$, showed nucleus (N) which coated with nucleus membrane (Nm) and contain normal chromatin (Ch), cytoplasm have normal organelles as rough endoplasmic reticulum (RER), mitochondria (M), and Golgi bodies (Gb). Yolk bodies (Y) are appearing black.

While in case of treated insects with *M. anisopliae* illustrated in Fig. $3_{(2)}$, it's clear that treatment caused rupture in nucleus (RN), malformation in mitochondria, cytoplasm was damaged and vasculization (V) occured. The fungus hypha were clearly visible and wiedly spread in the cytoplasm which cause subversion of ovarioles.

Fig. $3_{(3)}$, shows ovary of treated insects with neem it's obvious that the shape of nucleus was irregular, the majority of chromatins were disappeared except gathering of few number in two spot, yolk was destroyed and decreased in size into small particles, while cytoplasm was destroyed and become incomprehensible, mitochondria were absent, and vasculization was occured.

Ovarioles of insects treated with L- Glutamic acid illustrated in Fig. $3_{(4)}$, showed nucleus destruction, with dark abnormal chromatin materials, the yolk was disappeared, while the mitochondria were enlarged, the cytoplasm was damaged and vasculization ocured near basement membrane (Bm).

Fig. $3_{(5)}$, Illustrates ovarioles of treated insects with *Schinus molle* extract it's clear that the yolk was destroyed while spreation of numerous numbers of lipid droplets (L) were obvious, and vasculization was occurred and the mitochondria were absent.

Fig. $3_{(6)}$,Shows the ovarioles of treated insects with abamectin, it reveal that there were varying in shape and size of nucleus, while in some nucleus the chromatin became abnormal, the yolk destroyed and decreased in size also the cytoplasm was destroyed, the mitochondria was varied in size and shape finally vasculization occurred with large size.

Electron micrograph of ovarioles of treated insects with mixture from *M. anisopliae* and neem are illustrated in Fig. $4_{(7)}$,showed destruction of nucleus membrane, while the yolk was decreased in size and became pastel, cytoplasm was destroyed, vasculization occurred finally the hypha were spread in ovarioles.

Electron micrographe of ovarioles for insects treated with mixture from *M. anisopliae* and L- Glutamic acid illustrated in Fig. $4_{(8)}$,showed destruction of cytoplasm (DC) in the same time cytoplasm organelles disappeared, as well as the yolk, vasculization occurred and differed in size, the spread of hypha in ovarioles were obvius.

Fig. $4_{(9)}$, displays electron micrographe of ovarioles of treated insects with *M. anisopliae* and *S. molle* extract mixture it's clear that the yolk differed in size, vasculization occurred and the hypha were spread inside the ovarioles.

Ovarioles of treated insects with *M. anisopliae* and abamectin mixture demonstrated in Fig. $4_{(10)}$, showed destruction of the nucleus, although the chromatin congregated in a spot, while the cytoplasm was destroyed, also mitochondria was absent, but small vasculization occurred and hypha were appeared near the nucleus.

DISCUSSION

Many studies were done to evaluate the ability of *M. anisopliae* var. *acridum* to integrate with its other control agents in order to increase efficacy of *M.anisopliae* var. *acridum* for desert locust control operations *i.e* .integration with some insect growth regulators (IGRs) and antifeedant (El-Gamal *et al*., 2004), and with abamectin and D-limonene (Mohamed *et al.,* 2014). Fungi usually cause insect mortality by one or more of the following: nutritional deficiency, invasion and destruction of tissues, and release of toxins. Fungal species have numerous strains that differ in their virulence and pathogenicity. The pathogenicity of fungus may be associated with the production of enzymes and mycotoxins during the course of infection in an insect Tanada & kaya (1993). abamectine showed slow act against desert locust, this may be du to it's mode of action where Wolstenholme and Rogers 2005 indicated that, it is likely that abamectin bind to multiple sites (including glutamate and GABA) in insect chloride channels. In general, the chloride ion flux produced by the opening of the channel into neuronal cells results in loss of cell function and disruption of nerve impluses. Consequently, invertebrates are paralyzed irreversibly and stop feeding. *Schinus molle* hve diverse properties, such as insecticidal and repellent effects in different insects (Ferrero *et al*., 2006, 2007; Abdel-Sattar *et al*., 2009). *M. anisopliae* strain is compatible with neem and reduces survival of mosquito *Anopheles gambiae* adults after spraying (Fawrou *et al*., 2012).

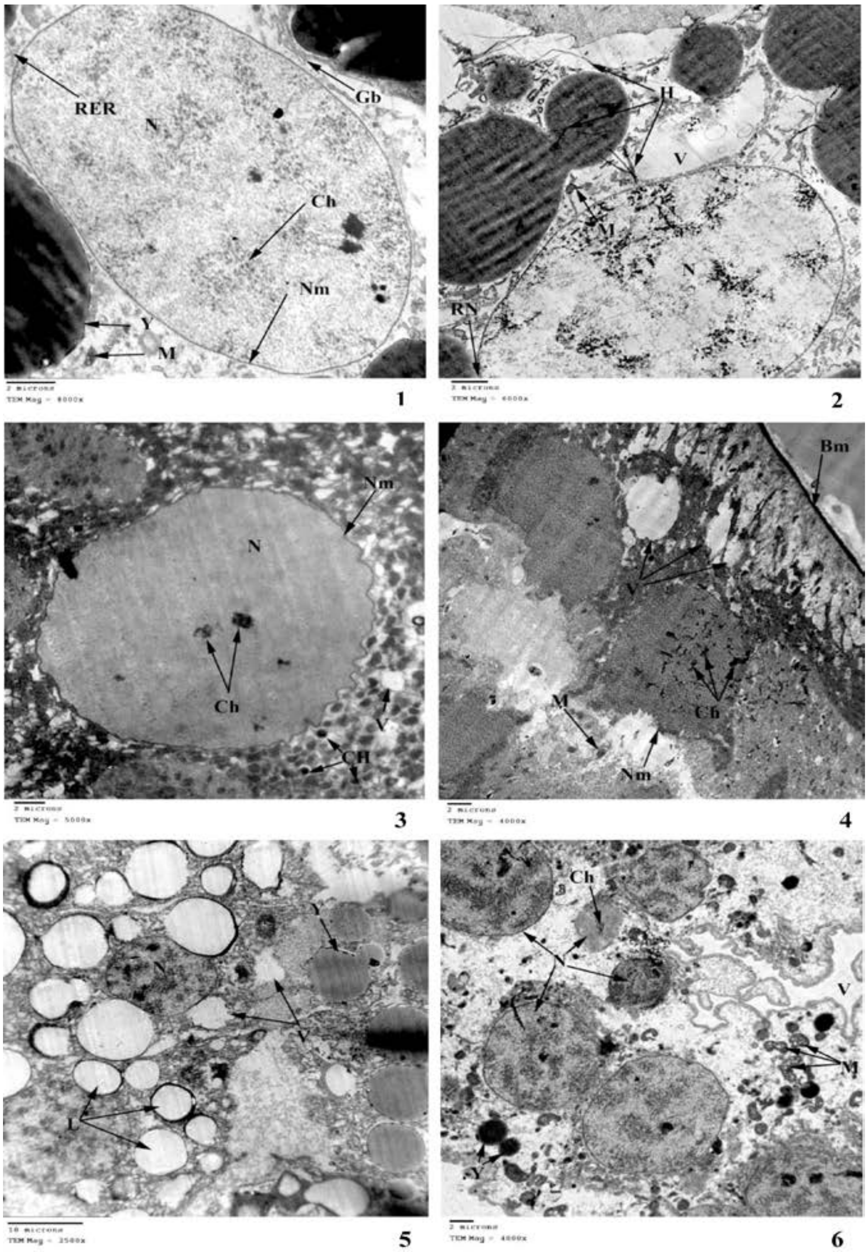

Fig.(3):1-:6 Electron micrograph of ovariols**1-**normal **2-** treated with *M. anisopliae* **3-** treated with neem **4-**treated with L- Glutamic acid **5-** treated with *S. molle.***6-** treated with abamectin**.**

basement membrane **(Bm)** chromatin **(Ch)** endoplasmic reticulum **(RER)** golgi bodies **(Gb)** hypha **(H)** lipid droplets **(L)** mitochondria **(M)** nucleus **(N)** nucleus membrane **(Nm)** rupture in nucleus **(RN)** vasculization **(V)**

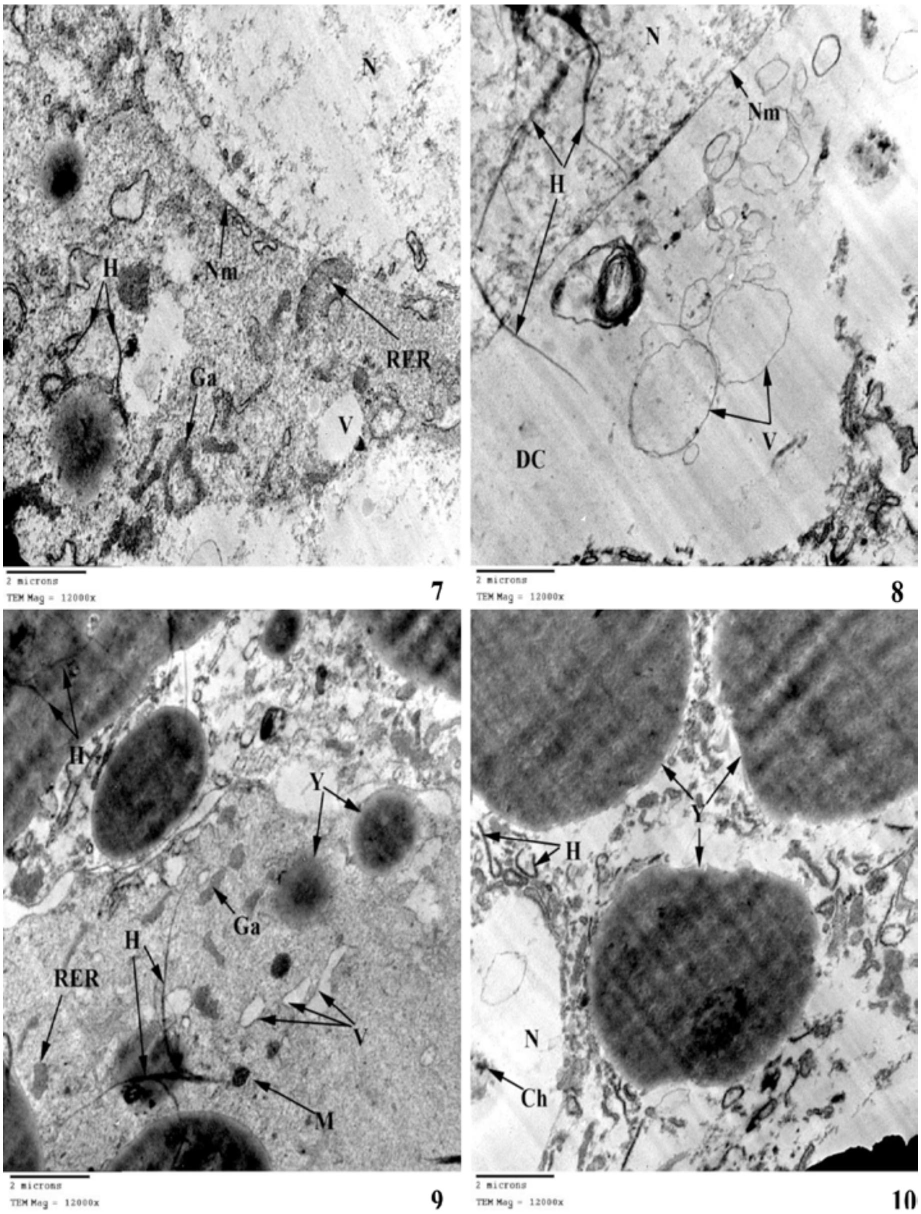

Fig.(4): 7-10 Electron micrograph of ovariols**7-**treated with mixture of *M. anisopliae* and neem **8-** treated with mixture of *M. anisopliae* and L-Glutamic acid. **9-** treated with mixture of *M. anisopliae* and *S. molle*.**10-**treated with with mixture of *M. anisopliae* and abamectin.

basement membrane **(Bm)** destroyed cytoplasm **(DC)** chromatin **(Ch)** endoplasmic reticulum **(RER)** golgi apparatus **(Ga)** hypha **(H)** lipid droplets **(L)** mitochondria **(M)** nucleus **(N)** nucleus membrane **(Nm)** rupture in nucleus **(RN)** vasculization **(V)**

Mohamed, *et al.,* (*2008*) examined the ovarian follicle of adult *S. gregaria* by transmission electron microscopy and showed that, the follicular epithelium of vitellogenic follicles is composed a single layer of more or less columnar to cuboidal cells without nuclei. While Tobe and Pratt (1975) observed four distinct periods, an early growth period, previtellogenic period, vitellogenic period and finally chorionation, which preceeds ovulation in *S. gregaria*. During vitellogenesis reserve materials were deposited in ooplasm and consequently oocytes become voluminous and filled with numerous yolk spheres (Simiczyjew and Margas 2001). John and James (1989) observed that the apical ooplasm of *S. gregaria* was rich in ribosomes and mitochondria, while rough endoplasmic reticulum (rER) and golgi complexes occupied the basal and basolateral regions of the cells. These ultra structures point to an increase in the activity of follicular cells associated with the synthesis and secretion of egg precursors. Similarly, newly emerged 5th instar nymphs of *S. gregaria* treated with cascade, rice bran extract and karate, synthetic pyrethroids each at LC_{50} produced disturbance in protein synthesis of the ovary, which reflected an inhibition of ovarian maturation and showed a degeneration of ovarioles and oocytes, disintegrated mitochondria, enlarged vacuoles and cracked yolk bodies mostly in two halves (John and James., 1989), (Ferenz., 1993) and (Hussein *et al*., 2008).

All treatments effect on ovarioles of *S. gregaria,* this effect vary from treatment to other as damaged in cytoplasm, uneven shape of nucleus, rupture in nucleus, destroyed in nucleus membrane, disappeared of chromatin, malformation in mitochondria, destroyed of yolk, lipid droplets, vasculization, and hypha which spread in ovarioles in treatment of *M. anisopliae* in alone case or in mixture with other treatments. All this unnatural phenomena may be due to effect of all treatments on central nerve cord by plugging of sodium and potassium channels, which ultimately stimulate the endocrine system and later on it may affect development of reproductive system, this result are in parallel to (Gupta *et al*., 1992) and(Gupta, 1979).

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الاستجابات الفسيولوجيه وتحت تركيبيه للجراد الصحراوى عند معاملته بفطره الميتاريزيم اكريديوم واربع مركبات ذات نشاط حيوى

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تم دراسة تأثير فطر الميتاريزيم اكريديوم وكذلك 4 مركبات نشطه حيويا (النيم و الحمض الأمين جلوتاميك ومستخلص نبات الشينس مولى و الأبامكتين) بعشر الجرعه المقرره منفرده او مخلوطه مع الفطر على نسبه الموت والتغيرات الهستولوجيه الدقيقه لأنسجة المبيض لحوريات العمر الخامس للجراد الصحراوى واظهرت النتائج ان نسبه الموت فى حاله فطر الميتاريزيم انيسوبلى وصلت الى 100% بعد 14 يوم اما عند استخدام النيم فكانت نسبه الموت 60% بعد 12 يوم وعند استخدام الحمض الأمينى جلوتاميك فكانت نسبه الموت 20% بعد 10 ايام وعند استخدام مستخلص من نبات الشينس مولى اصبحت نسبة الموت 50% بعد 14 يوم اما عند استخدام الأبامكتين فكانت نسبه الموت 60% بعد 11 يوم بينما جميع المخاليط ادت الى 100% موت بعد 5 ايام. بينما عند دراسه تاثير المواد سالفه الذكر فى الحاله المنفرده على المده الزمنيه اللازمة لاحداث نسب موت 25%، 50% و 90% لحشره الجراد الصحرواى وجد انها تستغرق وقت طويل وكان فطر الميتاريزيم اشدها تاثيرا حيث وجد ان المده الزمنيه اللازمه لاحداث نسبه 50% موت هى 7،261 يوم ، بينما عند خلط فطر الميتاريزيم مع المواد الاخرى كلا على حده فانها تقوم بتسريع عمليه الموت بصوره ملحوظه وكان اسرع المخاليط موتا مخلوط فطر الميتاريزيم مع النيم حيث كانت المده الزمنيه اللازمه لاحداث 50% موت هى 1،625 يوم .

وعند دراسه تاثير المواد السابقه على التغيرات الواقعه على مبيض حشره الجراد ودراسه التاثير بواسطه استخدام الميكرسكوب الالكترونى النافذ فوجد ان هناك تغيرات حدثت وان تلك التغيرات تنوعت واختلفت من معامله الى اخرى وشملت انفجار النواه ، تغير فى شكل النواه ، تجمع الكروماتين ، وجود فجوات ، تغير فى شكل وحجم الميتوكوندريا، ظهور قطرات زيتيه وفى المعاملات التى تحتوى على فطر الميتاريزيم انيسوبلى فان الخيوط الفطريه كانت ظاهره جليا فى مبيض الحشره .