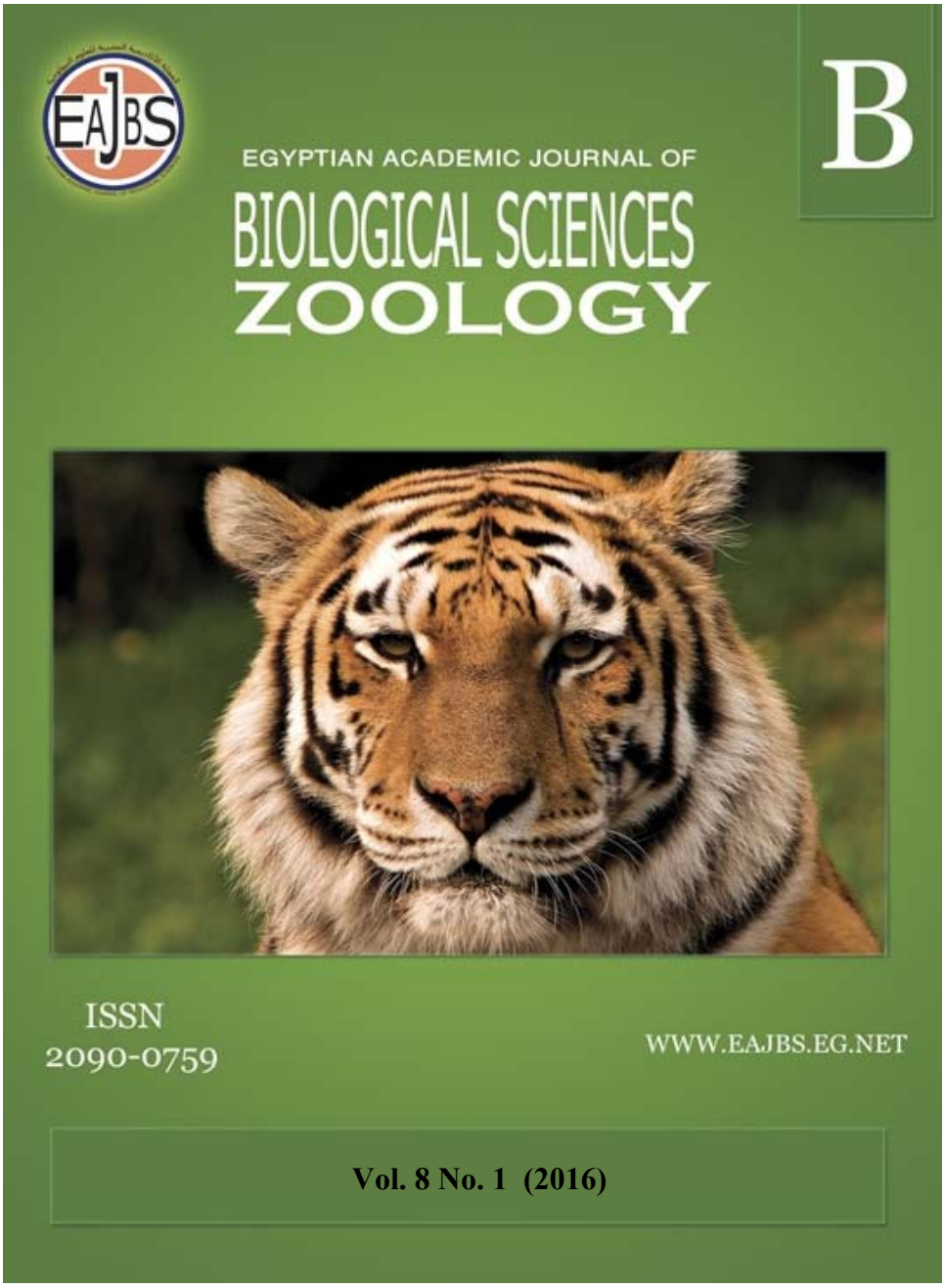

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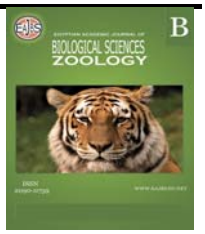

New Approaches For Extracting And Mass Rearing of Entomopathogenic Nematodes *In Vivo*

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ABSTRACT

The dual-purpose of spongy traps for extracting and storage of EPNs was validated in this investigation. The spongy traps proved to be the most efficient method for recovering IJs from the insect cadavers (*Galleria mellonella*) comparing with the traditional white traps. Also, the spongy trap kept the viability of IJs for prolonged time (12 weeks). Based on these results, duplications of insect cadavers in spongy traps will be rather encouraging for nematode mass production. A linked study was continued for detection an artificial diet for *G. mellonella* larvae. This step is necessary towards the mass production of EPNs *in vivo*. The recommended artificial media divided into two parts: the first part is supplemented with old beewax used for feeding 1st and 2nd instars of insect larvae. The second part is supplemented with paraffin oil used for feeding the other instars of insect larvae. The present results demonstrated that, the insect larvae could live and develop on this artificial diet. Moreover, both nematodes of *Heterorhabditis bacteriophora* (Hb) and *Steinernema carocapcae* (Sc) have proven to propagate perfectly in insects fed on the artificial media. Full death of all treated insects by Sc or Hb occurred (mortality=100%). The pathogenicity and reproduction of Hb nematodes produced from insect larvae fed on artificial diet gave initial population (Pi), final population (Pf), Rate of reproduction (Rr) and Efficiency of conversion (Ec) values superior than those fed on beewax checks at 100 IJs/insect larva. Similary, the pathogenicity and reproduction of Sc nematodes produced from insect larval fed on artificial diet were equivalent to or superior than those produced from insects fed on beewax in all inoculum levels. These new methods require minimal expertise and capital investments for extracting, storage and mass production of the beneficial Entomopathogenic nematodes (EPNs).

INTRODUCTION

Entomopathongic nematodes (EPNs) including stienernematids and heterorhabditis are used to control a variety of economically insect pests (Shapiro-Ilan *et al*., 2002b; Grewal *et al.,* 2005). These nematodes gave control equivalent to or superior than the chemical standard insecticides. The infective juveniles (IJs) enter their host through natural openings or through the cuticle. Subsequently, nematodes release their bacterial symbioses, which are responsible for killing the host within 24-48 hours (Dowds and Peters, 2002). The nematodes feed on the bacterial cells and host tissues that has been metabolized by the bacterium and has 1-3 generations, depending

on host type. As the food resources in the host cadavers are depleted, a new generation of IJs is produced that emerges from the host cadaver in search of new host (Kaya and Gaugler, 1993).

All EPNs are cultured *in vivo* or *in vitro* for large-scale commercial production as well as laboratory experimentation or field testing (Friedman, 1990; Shapero-Ilan and Gaugler, 2002; Ehlers and Shapiro-Ilan, 2005). The *in vivo* is more costly than the *in vitro* methods. However, the *in vivo* method may be an important future sector in nematode commercialization in less-developed countries. Moreover, the *in vivo*, nematode qualities tends to be equal to or greater than the *invetro* technology (Gaugler and Georgis, 1991; Yang *et al*., 1997).

Extracting and storage difficulties constitute the major challenge to expand the use of EPNs insecticidally (Brown and Gaugler, 1997). For example, classical White traps do not provide adequate number of nematodes for wider applications. In addition, storage of IJs in aqueous suspension requires continuous refrigeration to maintain nematode for extended periods. Therefore, the present investigation has focused on improving techniques that are comparatively inexpensive, more efficacious, and easier to apply than the traditional methods.

Galleria mellonella larvae is the most common insect host used *in vivo* for nematode mass rearing (Woodring and Kaya, 1988). Propagation of this insect depends mainly on the available old beewax at any time and in adequate quantity. Thus, shortage in beewax supplies would in turn determine the nematode propagation inversely. Therefor, the present study was conducted to explore the possibility of developing an economically artificial diet for *G. mellonella*, which is being used for mass rearing of EPNs.

MATERIALS AND METHODS

Multiplication and maintenance of the entomopathogenic nematodes:

Nematodes in this investigation was represented by *Steinernema carpocapsae* (Sc) and *Heterorha bditisbacteriophora* (Hb).These nematodeswere obtained from Plant Protection Department, Faculty of Agriculture, Ain Shams, Shoubra El-Kheima. The nematodes were propagated on the fifth instar larvae of the greater wax moth (*Galleria mellonella* L.); a lepidopteran host that is highly susceptible to parasitic infection. Insect larvae were placed in a 9-cm-diam Petri dish lined with a moistened filter paper and exposed to about 100 IJ3s at 25 ºC. After 2 days, dead larvae (cadavers) were transferred to white trap dishes. After 10 days, all number of IJ3s was collected.

Rearing of the insect host (the Greater wax moth, *G. mellonella*):

*G. mellonella*were obtained from bee hives, Faculty of Agriculture, Ain Shams, Shoubra El-Kheima, and reared on old bee wax in transparent plastic jars at 28 ± 5 ^{o}C, in the laboratory. Eggs that laid in masses were gently removed and incubated in other rearing jars provided with old bee wax.

Preparing the new artificial diet media of *G. mellonella*:

Based on many previous laboratory trials, we concluded a new diet media which divided into two parts: the first part (hatching medium) used for feeding the newly hatched larvae (I &II insters). The second part (fatting medium) used for feeding the growing larvae (III, IV,V & VI instars). The recommended components of the diet media, after sterilization in autoclave, were:

Composition	Hatching medium	Fattening medium
Wheat flour	110 g	110 g
Wheat bran	220 g	220 g
Maize flour	220 g	220 g
Dried Yeast	55 g	55 g
Honey bee	100 cm^3	100 cm^3
Old bee wax	110 g	-----
Glycerine	110 cm^3	110 cm^3
Paraffin oil	-----	50 cm^3
Formaldehyde (10%)	1.5 cm^3	1.5 cm^3

Eggs of *G. mellonella* were divided into two groups, the first group was incubated in a rearing jar provided with old beeswax. The second group was incubated in a jar provided with the new sterilized artificial medium for feeding the hatched larvae. After few days (7-10 days), the jar was provided with the sterilized artificial medium supplemented with paraffin oil. *G. mellonella* larvae were reared on these media for several generations.

The new spongy trap technique:

In this study, the conventional white traps were included for comparison with the new spongy traps (one insect cadaver/trap). Insect hosts were inoculated by nematodes in a dish padded with absorbent paper. After 2 days, infected insect cadavers were transferred to the White traps; which consists of a dish covered with a filter paper on which the cadavers rest surrounded by water (Shapiro-Ilan, *et al*., 2012). As IJs emerge they migrate to the surrounding water trap by where they are harvested. On the same time, each cadaver was placed on a wetted spongy trap consists of 8 cm length x4 cm width x 5mm thickness which was placed in a petri dish and kept in a plastic bag. The amount of water in wetted sponge was calculated approximately as 10 ml of water /1gm of sponge. All traps maintained at at 25±3 ˚C. After 10 days, the cadavers were removed and the sponges were soaked in an amount of water then squeezed gently. The emerging nematodes were counted (Pf) and the rate of reproduction (Rr) was calculated comparing with those calculated by white trap.

Experimental methods:

1- Effect of the new artificial media on EPNs pathogenicity, reproductivity and efficiency of conversion:

Four increasing levels of *H. bacteriophora* or *S. carpocapcae,* nematode inocula included 100, 500, 1000, or 1500 IJs/ insect larva/petri dish were used to study the effect of the new media on their pathogenicity and reproductivity. One fullgrown larva of *G. mellonella* which reared on the new fatting media was weighed and placed in a 9-cm-diam Petri dish lined with a moistened filter paper and exposed to the previous nematode concentrations. After 2 days, Dead and alive larvae of all replicates were removed and percentage of mortality (M%) was determined per for each treatment. Cadavers of *G. mellonella* were divided into two groups. The first group: the infected larvae were dissected to estimate the percentage number of penetrating IJs (Pi%) per insect larva. The second group: the infected larvae were placed singly on the spongy trap for 10 days and the emerged IJ3s were collected to calculate the final population (Pf) and the rate of reproduction (Rr). The same experiment was repeated at the same time on *G. mellonella* which reared on old beewax for comparison and number of obtained nematodes per mg of insect host were estimated as indicator of the efficiency of conversion (Ec) . All treatments were replicated 5 times and maintained at 25±3 ˚C.

2- The efficiency of extracting nematodes from insect cadaver by the white trap or by the new spongy trap:

Two inoculum levels of EPNs (500 IJs and 1000 IJs of Hb or Sc) were tested in this experiment. Each inoculum level was added to one full grown larva of *G. mellonella* placed in an 9-cm-diam Petri dish lined with a moistened filter paper. After 2 days, all cadavers of *G. mellonella* in each inoculum level were divided into three groups.Those of the first group were dissected to estimate the number of penetrating IJs (Pi%) per insect larva. Cadavers of the second group were placed singly on the white trap. The third group were placed singly on the spongy trap. After 14 days, emerged IJ3s were collected and (Pf) per insect larva was calculated. All treatments were replicated 15 times and maintained at 25±3 ºC.

3- Effect of storage period on nematode viability in spongy trap:

The previous two inoculum levels (500, and 1000 IJs) of*H. Bacteriophora*or *S. carpocapcae* were used to study the effect of reservation of emerged IJs using the spongy trap. One full grown larva of *G. mellonella* was placed in an 9-cm-diam Petri dish lined with a moistened filter paper and exposed to the previous concentrations. After 2 days, dead larvae were removed and cadavers of *G. mellonella* were placed singly on the spongy trap. After 14 days, emerged IJ3s were collected to calculate (Pf).

The viability percentage of IJs were estimated after 2, 4, 10, and 12 weeks from emerging. All treatments were replicated 5 times and maintained at 25±3 ºC.

Statistical analysis:

The data of all experiments were statistically analyzed using analysis of variance to check the significance of the differences between treatments using SAS program (2005) and separation between means was applied by Tukey test.

RESULTS

Effect of the new artificial media on EPNs pathogenicity, reproductivity and efficiency of conversion:

In case of Hb, Table (1) and Fig (1) indicated that, *G. mellonella* larvae fed on the artificial diet or beeswax at low inoculum level (100 IJs) induced a high nematode pathogenicity (represented by Pi, Pf, Rr and Ec) in comparison with other high inoculum levels.

Table 1: Effect of insect host nutrition on initial invasive populations (Pi) and Final populations (Pf) of *H. bacteriophora* (Hb) at different nematode concentration levels.

Nematode inoculum/insect larva	Insect host nutration	Pi	Pi %	Pf x100	Rr
100 IJs	Beewax medium	20^{f}	20.0^{cd}	935.7^{c}	4708.0^{b}
		±2.160	±2.160	±49.37	±303.4
	artificial medium	16^{f}	16.0^{d}	2217.0^{a}	14085.5^{a}
		±2.160	±2.160	±65.21	±1719.1
500 IJs	Beewax medium	172^{e}	34.4^{ab}	1130.0^{bc}	658.5^{c}
		±10.708	±2.142	±35.59	±28.6
	artificial medium	224^{de}	44.8^{a}	1617.0^{b}	720.5^{c}
		±26.280	±5.256	±213.57	±11.6
1000 IJs	Beewax medium	307^{cd}	30.7^{bc}	1206.0^{bc}	391.2^{c}
		±26.192	±2.619	±173.27	±30.9
	artificial medium	397^{bc}	39.7^{ab}	1431.3^{bc}	357.7^{c}
		±53.721	±5.372	±293.43	±39.5
1500 IJs	Beewax medium	484^{b}	32.3^{b}	929.0^{c}	192.5^{c}
		±39.632	±2.642	±42.59	±6.8
	artificial medium	601^{a}	40.1^{ab}	1338.7^{bc}	223.3^{c}
		±24.097	±1.606	±76.18	±17.7
F. Value		108.77**	18.81**	16.08**	123.6**
L.S.D.		98.975	11.178	515.43	2138

Numbers followed by the same letters did not differ significantly in their effects while, different letters had a statistically significant differences.

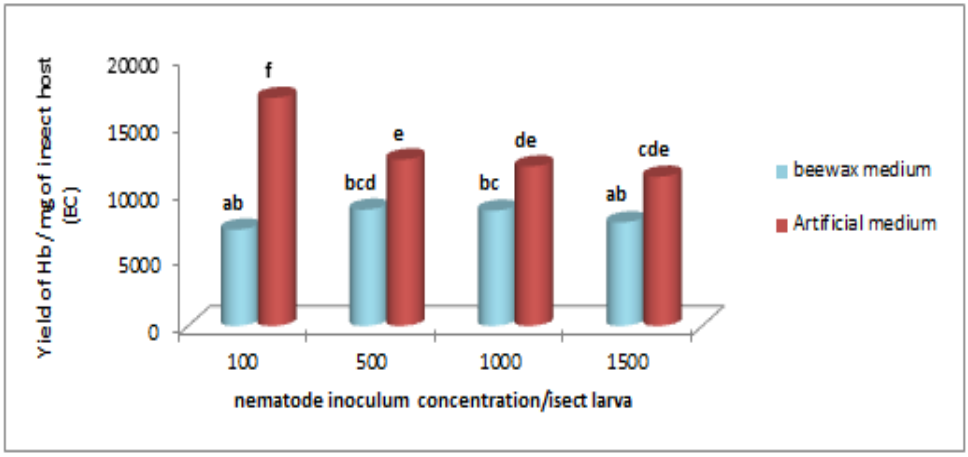

Fig. 1: Number of *H. bacteriophora* yield per mg of insect host(Efficiency of conversion=Ec) which fed on beewaxor artificial diet.The same letters above bars have no significant differences, while different letters above bars indicatestatistically significant differences in their effects.

Moreover, number of nematodes obtained from insect larvae fed on the artificial diet medium was comparatively higher than those fed on beeswax.

In case of Sc, the results in Table (2) and Fig. (2) showed a variety of comparative nematode pathogenicity (Pi, Pf, and Rr) according to the insect host nutrition and or nematode inoculum levels. In this respect, insects fed on artificial diet recorded the highest reproduction rate (Rr) at the lowest inoculum level (100 IJs). As with the efficiency of conversion (Ec), it appeared that, numbers of nematode per mg of insect larvae which fed on artificial diet were significantly higher than those fed on beewax at inoculum levels of 100 IJs and 1500 IJs. It is stressed in this contention that, full death occurred in all treated insect larvae by Hb or Sc (i.e, insect mortality =100%).

Table 2: Effect of insect host nutrition on initial invasive populations (Pi) and Final populations (Pf) of *S. carpocapcae* (Sc) at different nematode concentration levels.

Nematode inoculum/insect larva	Insect host nutration	Pi	Pi %	Pf x100	Rr
100 IJ	Beewax medium	24^{d}	24.0^{abc}	714.0^{d}	3004.8^{b}
		±3.265	±3.265	±46.676	±221.0
	artificial medium	29^{d}	29.0^{ab}	1269.0^{b}	4397.2^{a}
		±2.160	±7.788	±63.890	±371.2
500 IJs	Beewax medium	160^{b}	32.0^{a}	864.0^{cd}	542.3^{d}
		±14.719	±2.943	±50.622	±30.2
	artificial medium	95^{c}	19.0^{bc}	1136.7^{bc}	1195.5^{c}
		±6.799	±1.3597	±74.087	±77.7
1000 IJs	Beewax medium	229^{a}	22.9^{abc}	1232.3^{b}	536.9^{d}
		±13.441	±1.344	±128.779	±30.5
	artificial medium	258^{a}	25.8^{abc}	2001.0^{a}	775.5^{cd}
		±12.754	±1.275	±104.731	±4.6
1500 IJs	Beewax medium	252^{a}	16.8^{c}	994.0^{bcd}	391.6^{d}
		±17.204	±1.146	±173.120	±42.3
	artificial medium	250^{a}	16.7^{c}	1876.0^{a}	751.8^{cd}
		±16.753	±1.116	±74.422	±20.6
F. Value		134.92**	5.71**	43.43**	172.92**
L.S.D.		42.371	11.555	340.49	543.03

Numbers followed by the same letters did not differ significantly in their effects while, different letters had a statistically significant differences.

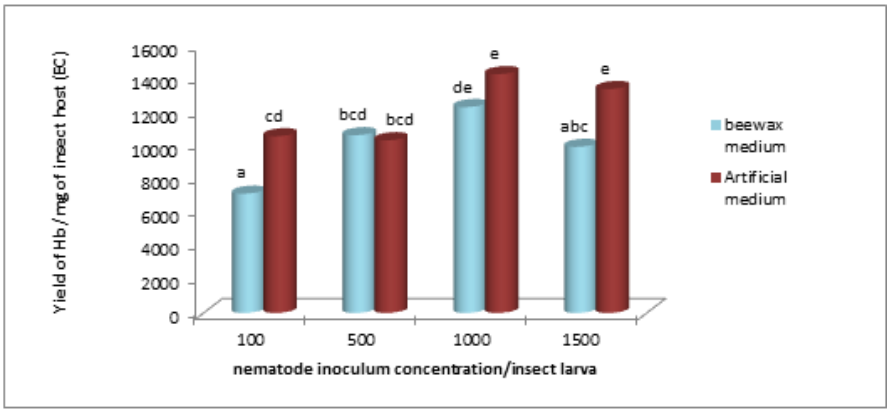

Fig. 2: Number. of *S. carpocapcae*yield per mg of insect host (Efficiency of conversion=Ec) fed on beewax or artificial diet. The same letters above bars have no significant differences, while different letters above bars indicate statistically significant differences in their effects.

The efficiency of extracting nematodes from insect cadaver by the white trap or by the new spongy trap:

Table (3) showed that, spongy trap is more effective than that of the white trap in all inoculum levels in both tested nematode species.

Table 3: Number of extracted nematodes by using two different methods (spongy and white trap).

Extracting method	Nematode inoculum	*H. bacteriophora* (x100)	*S. carpocapcae* (x100)
Spongy trap	100 IJs	1410 ^{a}±32.66	1287 ^{a}±24.50
	500 IJs	1244^{c} ±78.383	1092^{d} ±40.008
	1000 IJs	1382^{a} ±66.952	1255^{ab} ±40.824
White trap	100 IJs	1360 ^{a}±16.33	1193 ^{c}±81.65
	500 IJs	1073^{d} ±58.787	1014^{e} ±33.536
	1000 IJs	1307^{b} ±80.833	1222^{bc} ±1.632
F. Value		6.73*	23.06**
L.S.D.		229.9	106.15

Numbers followed by the same letters not differ significantly in their effects while different letters statistically significant differences.

Effect of storage period of nematodes on their viability in spongy trap:

Fig. (3) demonstrated that, reservation of EPNs in spong kept their viability for a long time (more than 10 weeks). Generally, the viability of *H. bacteriophora* IJs was 23% after 12 weeks of reservation while decreased in *S. carpocapcae* up to 16% after the same time.

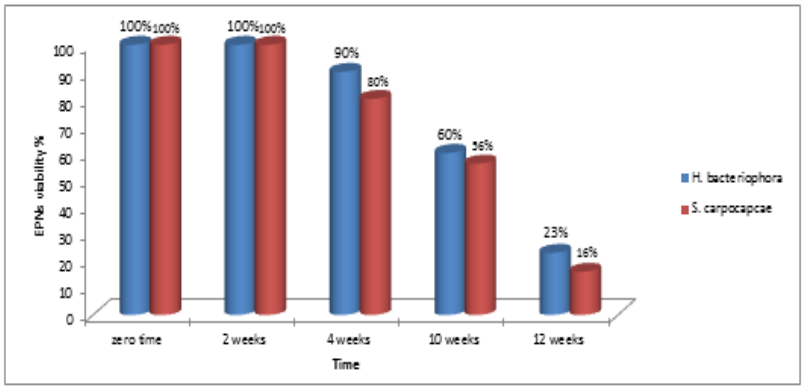

Fig. 3: Survival of *H. bacteriophora* and *S. carpocapcae* by the time of storage in spongy trap.

DISCUSSION

The present data proved that, the numbers of IJs extracted from insect cadaver in spongy traps were higher than those extracted by white traps. In this respect, Kung, et.al., 1991, pointed that, emergences of IJs can be induced by environmental factors. This was true in the present investigation where cadavers inside the petri dishes of the spongy traps exposed to low RH (below 70%) while cadavers in white traps exposed to high RH (above 90%).

The results indicated that, the average of viable nematodes decreased as storage period increased. Such conclusion was supported by data obtained by Qui and Bedding 2000, who reported that, the survival tax of IJs, under aerated and cooled conditions, diminished slightly in the six first weeks (91%), falling in the seventh week (78%) and in eighth week (55%) of storage. As general trend, data showed that, the survival of both Hb and Sc during storage periods were nearly the same. This finding was contradicted by Shang, P. K. *et al*., 1990, who indicated that, the locomotion and aggregation in heterorhabditids is greater than in steinernematids, which in turn, leading to the loss of energy reserves.This could be a main factor that maintains heterorhabditid nematodes alive for a short time whether in the aqueous suspension or in the soil. Presumably, however, this behavior is of little importance inside the confined tiny porous of the moistened sponge and likely, these nematodes could change their behavior temporarily (e.g., ambushing or crusing behavior).

To date, the available data in review does not provide adequate information about quite inexpensive artificial diet for mass rearing of *G. mellonella* under simple laboratory conditions. However, our trails have implications to suspect improving in mass production of EPNs through rearing *G. mellonella* on an artificial diet. This diet contains paraffin oil (a by- product of petroleum distillation) as a source of lipid instead of feeding the larvae of insects on the old beewax which is more expensive and is currently rare.

The present results demonstrated that, the insects fed on artificial medium produced a high numbers of Hb progeny (based on Pf, Rr and Ec) at 100 IJs/ insect larva. This finding was supported by Shapiro-Ilan *et al*, (2008) who found that, *H. indica* production efficiency was improved by supplementing insect (*Tenebrio molitor*) diet with lipids for enhancing host susceptibility and infection rates. Nevertheless, in both food media the average numbers of nematode progeny (Pf and Rr) decreased as the infective juveniles / insect larva (Pi and Pi%) increased. In fact, the nematode feeds on the host tissues that has been metabolized by the symbiotant bacterium and has 1-3 generations, depending on host size and numbers of invading nematode (Pi). However, when the food resources in the host cadaver are depleted by the high numbers of invasive nematodes, the new generation are ceased and few numbers of nematode only emerges from the host cadaver. The present data fit this model.

As with the pathogenicity of Sc, the present results showed a variety of comparative Pi, Pf and Rr values irrespective to insect nutration or nematode inoculum levels. Similar results were obtained by Shapiro-Ilan *et al*, (2012) who detected effects of *T. molitor* host diet on *H. indica* but not on *S. riobravae*. In fact, steinernematids are amphimectic in the first and in the following generations. Therefore, steinernematids require at least two IJs, a male and a female, to invade the host insect to produce progeny, but heterorhabditids need only one IJ to penetrate into the host as the resulting heterorhabditid adult is self-fertilise. These findings could contribute to apparent conflicts among the present data sets of Sc pathogenicity.

In conclusion, detection of spongy traps for extracting and storage of EPNs as well as detection of new artificial diet for insect host (*G. mellonella*) may be a crucial step towards successful nematode production and application. Moreover, these spongy traps and artificial diet are feasible for countries with low labor costs and with limited expertise.

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ARABIC SUMMERY

طريقة جديدة لاستخلاص نيماتودا الحشرات واكثارها حيويا

عبد الله كساب وانتصار حلمى طه
قسم وقاية النبات كلية الزراعة جامعة عين شمس

تم استخدام الاسفنج لهدفين هما: استخلاص وتخزين النيماتودا الممرضة للحشرات.
اظهر استخدام الاسفنج فى استخلاص الاطوار المعدية من يرقات ديدان الشمع المصابة كفاءة اعلى من الاستخلاص بالطريقة التقليدية (white trap) كما ان الاسفنج حافظ على حيوية النيماتودا لفترة اطول (12 اسبوع).
تم عمل بيئة صناعية جديدة لتربية يرقات ديدان الشمع لاستخدامها في اكثار النيماتودا حيويا.
قسمت البيئة الصناعية الجديدة الى جزأين: **الجزء** الاول (مزود بشمع نحل قديم) استخدم لتغذية العمر اليرقى الاول والثانى، اما **الجزء** الثانى (مزود بزيت بارافين) لتغذية الاعمار اليرقية الاخرى.
وقد اوضحت الدراسة ما يلى :

1- امكانية نمو وتطور يرقات ديدان الشمع الكبيرة على البيئة الصناعية الجديدة .

2- عند دراسة تاثير النيماتودا الممرضة للحشرات من الجنسين :

Heterorhabditis bacteriophora (Hb), *Steinernema carpocapcae* (Sc)

بتركيزات (100و 500و 1000و 1500) اظهرت النيماتودا الممرضة للحشرات من كلا الجنسين قدرة على التكاثر على ديدان الشمع المرباه على البيئة الجديدة بكفاءة حيث كانت نسبة الموت 100% .

3- في حالة نيماتودا Hb كانت اعداد (Pi , Pf, Rr, Ec) اعلى من المتحصل عليها عند استخدام اليرقات المرباة على بيئة الشمع القديم وذلك عند تركيز 100.

في حين انه **عند** استخدام نيماتودا Sc فكانت **الاعداد** اعلى من او تساوى الناتجة من الديدان المرباة على الشمع القديم.

وعليه فانه يمكن استنتاج الاتى من البحث:

1- يمكن استخدام الاسفنج فى استخلاص النيماتودا الممرضة للحشرات وتخزينها عليه بكفاءة.

2- يمكن استخدام البيئة الصناعية الجديدة فى تربية ديدان الشمع الكبيرة (لاستخدامها فى تربية النيماتودا الممرضة للحشرات) بكفاءة عالية و بتكاليف منخفضة.