ENHANCE ENTOM-OPATHOGENIC NEMATODE STORAGE STABILITY AND KEEP INFECTIVITY POST- APPLICATION SURVIVAL

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Abstract

The storage affect on entomopathogenic nematode (IJs) efficacy so we enhance the nematode storage and have gain new progenies have the ability to store and its infectivity were more than the origin nematode stored under the same conditions. This study carried out on four entomopathogenic nematodes species *Steinernema riobrave, Steinernema rarum, Heterorhabditis indica* and the isolate *Heterorhabditis* sp. (Hp2). At freshly origin ones (0 time storage) for this four nematodes, the infectivity, as measured by *Galleria mellonella* mortality (96.66, 80, 100 and 93.33%, respectively) and invasion rate (39, 37.33, 58.33, 31.66%, respectively).The two *Steinernema* species (*Steinernema riobrave, Steinernema rarum*) were stored 6 months recorded insect mortality (6.66 and 3.33%) and invasion rate (8.33 and 7.33%). The two *Heterorhabditis (Heterorhabditis indica* and the isolate *Heterorhabditis* sp. (Hp2)) stored 3 months only recorded insect mortality (43.33 and 30%) and invasion rate (17.66 and 15%) while the enhanced 6th progenies of the four species *Steinernema riobrave, Steinernema rarum, Heterorhabditis indica* and the isolate *Heterorhabditis* sp. (Hp2) at 0 time storage recorded insect mortality (100%) and invasion rate (100, 98, 99 and 98.66%, respectively) and when the 6th progenies of the two *Steinernema* (*Steinernema riobrave, Steinernema rarum*) were stored 6 months it recorded insect mortality (43.33%) and invasion rate (27and 23%) while the 6th progenies of the two *Heterorhabditis* (*Heterorhabditis indica* and the isolate *Heterorhabditis* sp. (Hp2)) after stored 3 months recorded (52.66 and 50%) so we have new nematodes progenies have infective juveniles can be stored up to several months and enhance its storage stability and post- application survival.

Keywords: Entomopathogenic nematode, *Steinernema*, *Heterorhabditis*, Storage, Enhance, Infectivity, Mortality, Invasion rate.

INTRODUCTION

Entomopathogenic nematodes (EPNs) from the Heterorhabditidae and Steinernematidae families are well-known biocontrol agents against numerous insect pests. The infective juveniles (IJs) are naturally occurring in the soil and their success in locating and penetrating the host will be affected by extrinsic/intrinsic factors that modulate their foraging behavior (Campos-Herrera and Gutiérrez2014). The infective juvenile of EPNs are specifically adapted to search for insect hosts, and are able to survive in the absence of a host for weeks, or even months through a reliance on energy reserves (Hatab and Gaugler 1999; Qiu and Bedding 2000 and Hass *et al.*, 2002).Age is one factor contributing to variability in EPN efficacy(Yoder *et al.;* 2004). Storage difficulties constitute one of the major obstacles to expand the use of EPNs as bioinsecticides. High demands for oxygen, sensitivity of some species to temperature variations, susceptibility to microbial contaminants, and toxicity from antimicrobial agents are factors that influence the quality of nematode storage in water (Grewal, 2000).

The survival of entomopathogenic nematodes under laboratory conditions is low. With the aim of evaluating substrates to extend the survival of entomopathogenic nematodes in addition to the ability to store nematodes in product form is critical to commercial success. EPNs can be stored in several substrates such as activated charcoal, alginate, vermiculite, clay, and porous foam, which provide a high surface area to volume ratio, as well as adequate interstitial space (Kaya and Stock, 1997); however, survival in these substrates varies according to species. Dutky *et al.*, (1964) stored *S. carpocapsae* in insulated jugs held at 7.1°C. With oxygenation of the jug's atmosphere every few weeks, some of the nematodes were reported to survive up to 5 years. Burman and Pye (1980) reported that aerated environments were able to maintain 100% survival of *S. carpocapsae* at 20°C, for a period exceeding 43 days by spraying with nitrogen down to oxygen tension of 0.75 mmHg. Klingler (1988) stored *Heterorhabditis* sp. in its culture substrate in Erlenmeyer flasks at a temperature of 6.0±0.4°C. He counted then the surviving infective juveniles after 1, 22, 42 and 63 days of storage, and found no significant decrease in their number. Wright *et al.*, (1997) mentioned IJs exhibited a rapid decline in infectivity after 90 days of storage. Fitters and Griffin (2004) stated that *H. megidis* IJs after the

two weeks of storage in water its infectivity declined. Consequently, investigating the parameters that influence EPN survival under storage and keeping its infectivity, an important aspect to be considered for their release in the field in biological control programs. So the objective of this work to study the efficacy of infective juveniles of progenies passing with enhancing method then stored up to several months at distilled water .

MATERIALS AND METHODS

Three entomopathogenic nematode species and local isolate were used in the present study the four nematodes were obtained from the laboratory of Insect Parasitic Nematodes, Plant protection Research Institute, Agriculture Research Centre, Egypt. *Steinernema riobrave, Steinernema rarum* and *Heterorhabditis indica* imported from USA, Florida, *Heterorhabditis* sp. (Hp2) originally isolated from a soil sample El-kasasin- Ismailia-Egypt. Continuous culturing of nematode juveniles for several cycles under optimum conditions of temperature and nematode density inside the full grown larvae of the greater wax moth, *Galleria mellonella*(El-lakwah, *et al.*, 2008). Full grown larvae of *G. mellonella* kept in 1.5 ml Eppendorf tubes, lined with double layer filter paper (Whatman No. 1), were subjected to nematode infection, at a dose level of 20 IJs/larva in 300 µl of distilled water and kept at 25°C, in the dark. All dead larvae were placed in extraction dishes after 48 hours (White, 1927*).* After 10 days, the first progeny of emerged juveniles was received in distilled water. Continuous culturing of nematode juveniles, for several cycles under the same conditions, was carried out to collect the new progenies until six progeny for *Steinernema* and *Heterorhabditis*. Each progeny was washed three times and concentrated to approximately 5000 IJs/ml of distilled water and stored at15°C. Invasion rate and mortality of the stored new progenies of the four species were determined according to (Koppenhöfer and kaya, 1999).

1. Invasion rate and mortality:

In each of the obtained nematode progenies, *G. mellonella* larvae were exposed to nematode infection, using 24-well tissue culture plates each 20 IJs/larva in 300 µl of distilled water were placed on the bottom of each well which lined with double layer filter paper (Whatman No. 1), A wax moth larva was placed in each well and the plate was covered with its lid and kept in the darkness incubator at 25°C.

Mortality were taken after 48hours and corrected according to Abbott's formula(Abbott,1925).For 4-5days, according to the species of nematode, all dead larvae were washed twice with distilled water to remove any nematode juveniles that attached to them, dried and dissected under a stereomicroscope. The number of nematodes inside each larva was counted in at least 10 larvae for each origin and stored progenies for at (one, two, three, four and six months) for the Steinernema species and (one, two, three months) for the *Heterorhabditis* species in addition to a control (0 time storage) at(20IJ/larva in 300 µl)of distilled water in 1.5 ml Eppendorf tubes, lined with double layer filter paper) and the invasion rate was calculated as percentage.

Statistical Analysis

The Percentage values in the present study were normalized using arcsine transformation. The significance of the main effects was determined by analysis of variance (ANOVA) using SAS program (SAS Institute,2002) The significance of various treatments was evaluated by Duncan's multiple range test ($P<0.05$) .

RESULTS

In Fig. 1 A, the mortality of the original progenies of the *S. riobrave*, *S. rarum*, *Heterorhabditis* sp. (Hp2) and *H. indica* to *G. mellonella* in the control condition(0 time storage) were (96.66, 80, 100 and 93.33%, respectively) and in Fig. 2 A, its invasion rate at0 time storage were (39,37.33,58.33 and 31.66%, respectively). Afterwards, the mortality increase through the progenies where the 1st progenies of the *S. riobrave*, *Heterorhabditis* sp. (Hp2) and *H. indica* recorded (100%) mortality to *G. mellonella* except species *S. rarum* recorded (100%) mortality at the 3rd progeny. The invasion rate increased gradually through the 1st, 2nd, 3rd,4th, 5th and 6th progenies of the four tested nematode, Where it recorded the highest invasion rate at the 6th progenies of the four nematode in the control condition (0 time storage) (100, 98, 99and 98.66%, respectively).

The origin and the 6 progenies for the *S. riobrave*, and *S. rarum* after stored (1, 2, 3, 4 and 6 months) and the two *Heterorhabditis (H. indica* and the isolate *Heterorhabditis* sp. (Hp2)) after stored (1, 2 and 3 months only) its mortality to the *G. mellonella* presented in Fig 1B, C , D , E and F where the invasion rate presented in Fig 2B, C , D , E and F. The effect of storage appears on the decreased of the

insect mortality and the invasion rate for all the progenies. After increasing the time of storage to 3 month the origin of the *S. riobrave*, *S. rarum*, *Heterorhabditis* sp. (Hp2) and *H. indica* in efficacy decrease so the insect mortality were (40, 16.66, 43.33 and 30%, respectively) and invasion rate were (18.33,17.33,17.66 and 15%, respectively) but the enhanced progenies try to keep its efficacy where the 6th progenies of the *S. riobrave*, *S. rarum*, *Heterorhabditis* sp. (Hp2) and *H. indica* after stored to 3monthes its mortality to *G. mellonella* were (93.33, 86.66, 93.33 and 93.33%, respectively) and invasion rate were (56, 52, 52.66 and 50%, respectively).When the 6th progenies of the two *Steinernema* (*S. riobrave, S. rarum*) were stored 6 months it recorded insect mortality (43.33%) and invasion rate (27and 23%, respectively) .Despite these, it were more than the invasion rate of the original of the *S. riobrave* and *S. rarum*(8.33 and7.33%, respectively) and its insect mortality(6.66 and 3.33%, respectively) at the same condition of storage .

Statistical analysis in Table (1) showed no significant differences between nematode progeny of all tested nematodes in the control condition. On the other hand, found that significant differences between progeny nematode of all tested nematode species after storage for 1, 2,3,4 and 6 months.

Data in (Table,2) proved that found significant differences between all nematode progenies individual in the control condition and after storage for 1,2,3,4 and 6 months

DISCUSSION

We obtained new progenies after passing in the enhancing method, continuous culturing of nematode juveniles (El-lakwah, *et al.*, *2008*); under optimum condition of temperature 25 °C and nematode density 20 IJs/larva. Dunphy and Webster (1986) found that the differences in virulence were influenced by temperature. Density should be taken into account; Selvan *et al.,* (1993a) reported that the percentage penetration declined with increasing dose, so that several tests were carried out to choose the optimal temperature and nematode density. The new progenies, its invasion rate and mortality to *G. mellonella* were higher than the freshly original species and after storage, the new progenies infectivity were more than the original nematodes stored under the same conditions. Songbi and Glazer (2005) in their results showed that after storage of infective juveniles (IJs) of the entomopathogenic

nematode (EPN) *Steinernema feltiae* IS-6 at 23 ± 0.3 °C. for 6 months in calcium alginate granules insect mortality (100%).The present study thus corroborates this previous report since the 1st progenies of the *S. riobrave*, *Heterorhabditis* sp. (Hp2) and *H. indica*, freshly emerged, recorded (100%) mortality to *G. mellonella*. And from the3rd to the 6thprogenies for the four nematodes species mortality to *G. mellonella* were (100%) and when it stored at 15°C in distilled water at three months these mortality decreased but it ranged between (93.33and 86.66%) for the 6thprogenies of the four nematodes species. Woodring and Kaya, (1988) stated that *Heterorhabditis* can be stored only 2-4 months of storage at 4-10°C is considered good. That is similar to the two *Heterorhabditis (H. indica* and the isolate (Hp2) stored 3 months only in this presenting. Many reports mentioned that nematodes can be stored and survived well for several months. But its infectivity is affected by storage. IJs exhibited a rapid decline in infectivity after 90 days of storage (Wright *et al.*, 1997). The IJs of some EPN species become inactive when stored in water. A significant increase in infectivity towards *G. mellonella* larvae was reported in Fitters and Griffin (2004) for *H. megidis* IJs in the first two weeks of storage in water. And its infectivity declined after the two weeks of storage. The nematode species and the new progenies stored at temperatures of 15°C in our laboratory, Andaló*et al.,* 2011 mentioned that infectivity was best preserved at temperatures of 16 and 20°C gave rise to the least reduction in infectivities after 180 days of storage. In this way, lipids and infectivity are influenced by different storage temperatures for the species tested.

Yoder *et al.,* (2004), in their study, found that age and species of nematodes have effective influence on *G. mellonella* mortality and nematode penetration. Storage not effect on the latest new progenies as did on the original. This was related to the efficiency of the enhancement which occurred to this progeny and to its parents, Also at least in some situations, the nematodes become established recycle, and their offspring continue to control the target insect (Parkman *et al.*, 1993), especially if this offspring descending from parents passing in the enhancing method that make them having high mortality to their host and high invasion rate. Selvan *et al.,*(1993a) the longest infective juveniles were produced at the lowest nematode densities, indicating a tradeoff between size and number of progeny. Longer infective

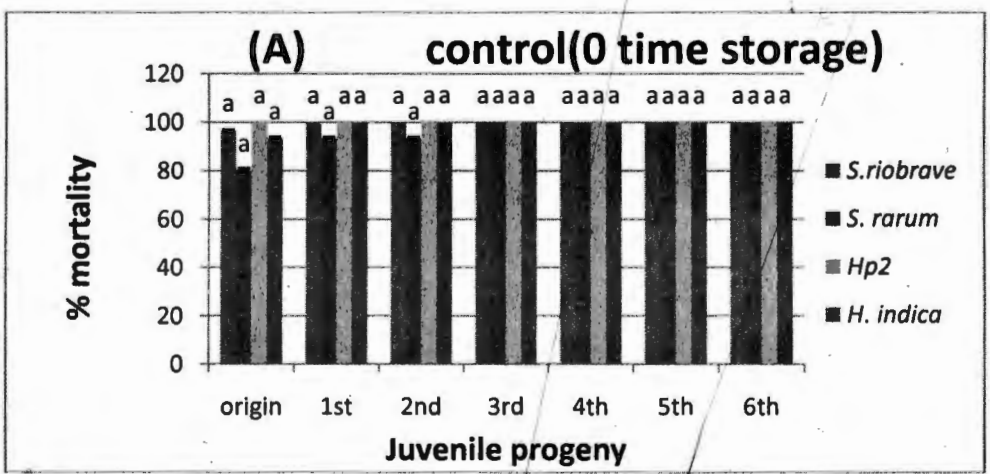
juveniles store more nutrients;(Selvan *et al.*, 1993b) they can be expected to survive for a long period than shorter nematodes. The low number of IJs were penetrated the *G. mellonella* larvae, thus having more amount of food, because of the suitable nutrient conditions. In that case, infective juveniles of nematode with high quality and efficacy are produced. This means that this progeny descended from the stronger nematode. So, their generation became stronger and acquired the genetic characteristics of its parents, in this manner, the strong descendant came from the strong parent. These results have important implications for storage and application of EPNs. The ability to store nematodes in product form is critical to commercial success. the storage affected on the infectivity of the four tested nematode but the new progenies obtained from the enhancing method can resisted the storage and recorded high infectivity so these data are useful for greater success in using stored entomopathogenic nematodes as biocontrol agents and by using this information it is possible to handle properly the situations for greater success in using EPNs.

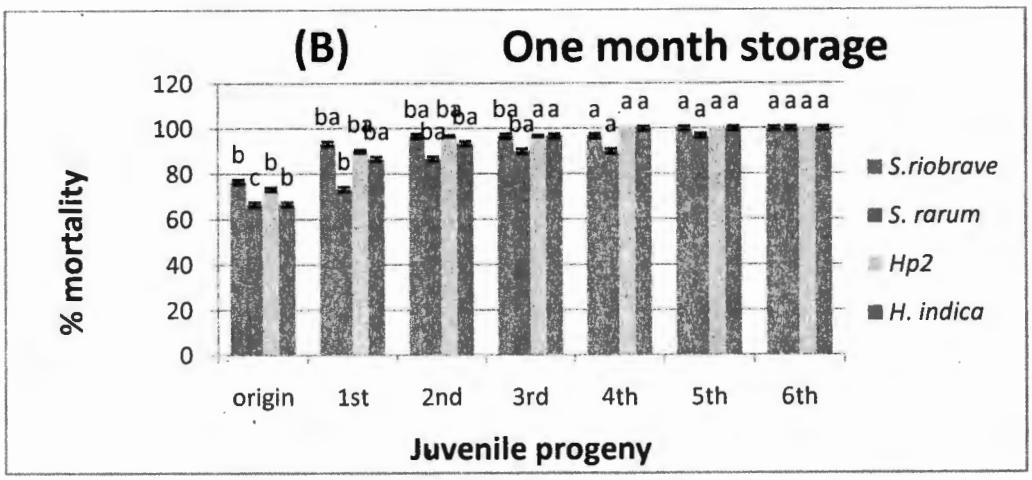
Table 1. One way ANOVA randomized of mortality in Galleria mellonella larvae subjected to Juvenile progeny of Steinernema riobrave, Steinernema rarum, Heterorhabditids sp. (Hp2) and Heterorhabditis indica.

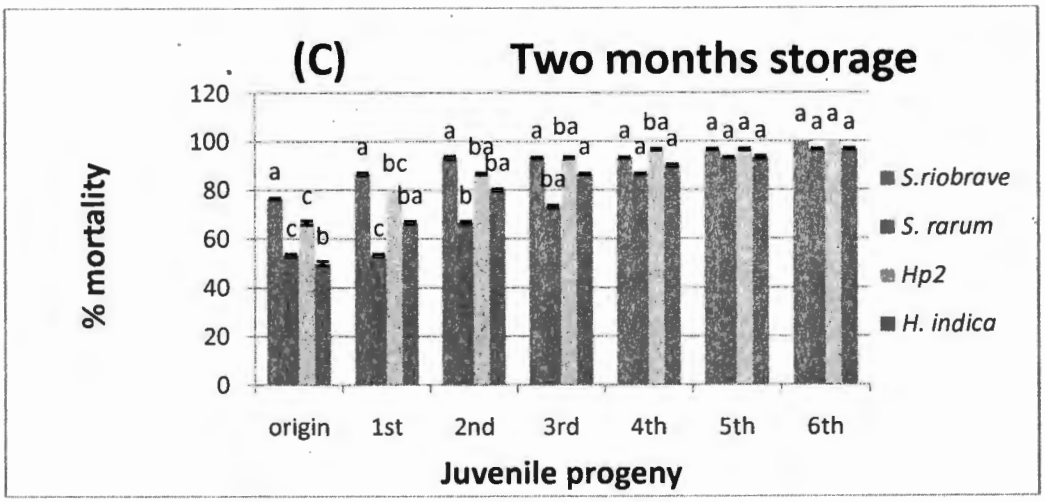
treatment		*S. riborave*	*S. rarum*	*Heterorhabditis sp.*	*H. indica*
Control	df	6	6	6	6
	F	0	0.05	0	0.01
	P	1	0.9993	1	1
One month	df	6	6	6	6
	F	2.41	12.29	2.44	2.96
	P	0.0853	<.0001	0.0824	0.0472
Two month	df	6	6	6	6
	F	1.84	15.19	7.86	4.17
	P	0.1645	<.0001	0.0008	0.0141
Three month	df	6	6	6	6
	F	11.82	15.77	10.59	19.67
	P	<.0001	0.0001	0.0002	<.0001
Four month	df	6	6		
	F	8.47	5.68		
	P	0.0006	0.0042		
Six month	df	6	6		
	F	3.09	5.83		
	P	0.0409	0.0035		

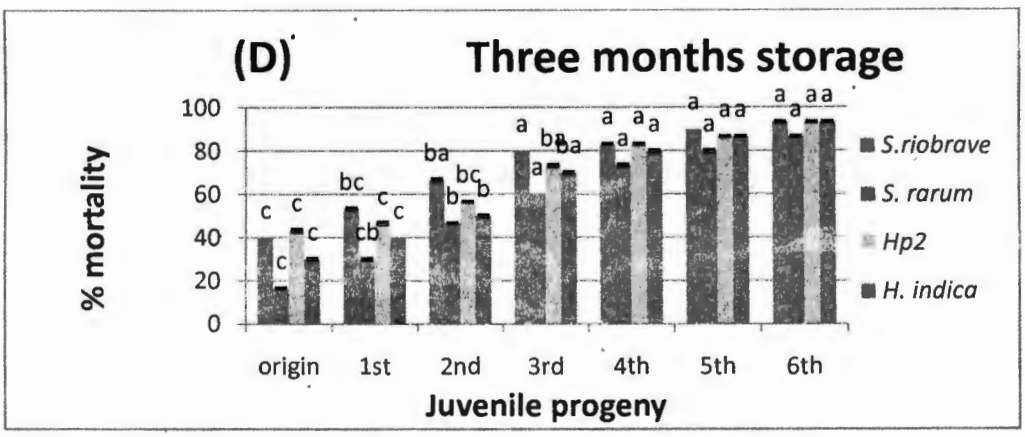
Table 2. One way ANOVA randomized of Penetration rate in *Galleria mellonella* larvae subjected to Juvenile progeny of *Steinernema riobrave*, *Steinernema rarum*, *Heterorhabditids* sp. (Hp2) and *Heterorhabditis indica*.

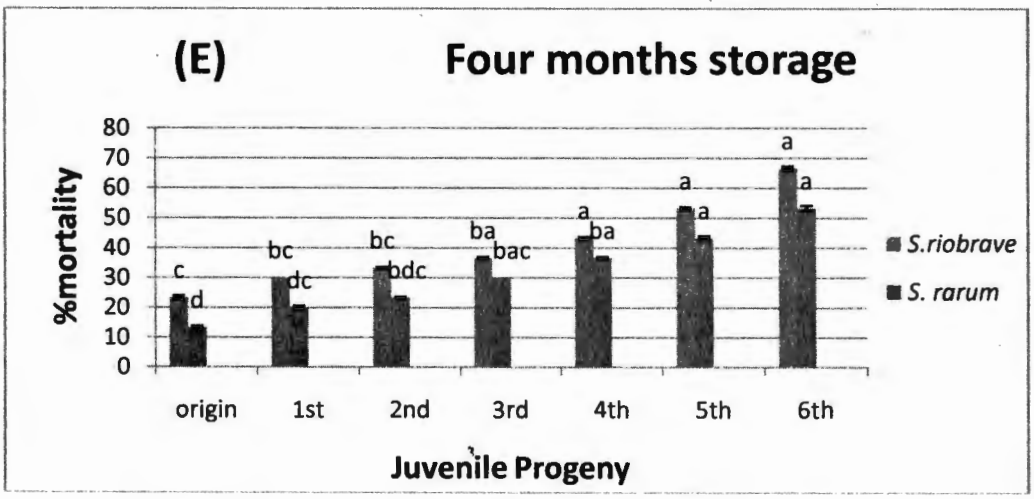
treatment		*S. riborave*	*S. rarum*	*Heterorhabditis sp.*	*H. indica*
Control	df	6	6	6	6
	F	52.82	121.61	74.79	177.27
	P	<.0001	<.0001	<.0001	<.0001
One month	df	6	6	6	6
	F	47.76	51.18	20.48	58.31
	P	<.0001	<.0001	<.0001	<.0001
Two month	df	6	6	6	6
	F	40.35	50.78	18.68	65.09
	P	<.0001	<.0001	<.0001	<.0001
Three month	df	6	6	6	6
	F	73.19	63.19	42.75	41.85
	P	<.0001	<.0001	<.0001	<.0001
Four month	df	6	6		
	F	27.52	35.56		
	P	<.0001	<.0001		
Six month	df	6	6		
	F	31.95	34.72		
	P	<.0001	<.0001		




(B) One month storage
% mortality
120
100
80
60
40
20
0
origin
1st
2nd
3rd
4th
5th
6th
Juvenile progeny
S.riobrave
S. rarum
Hp2
H. indica


(C) Two months storage
% mortality
120
100
80
60
40
20
0
origin
1st
2nd
3rd
4th
5th
6th
Juvenile progeny
S.riobrave
S. rarum
Hp2
H. indica


(D) Three months storage
% mortality
100
80
60
40
20
0
origin
1st
2nd
3rd
4th
5th
6th
Juvenile progeny
S.riobrave
S. rarum
Hp2
H. indica



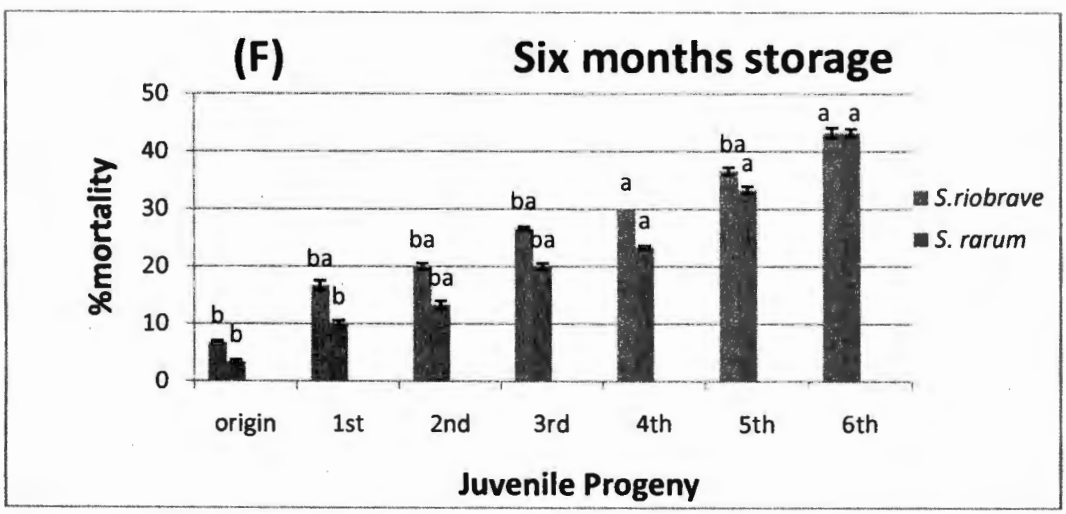
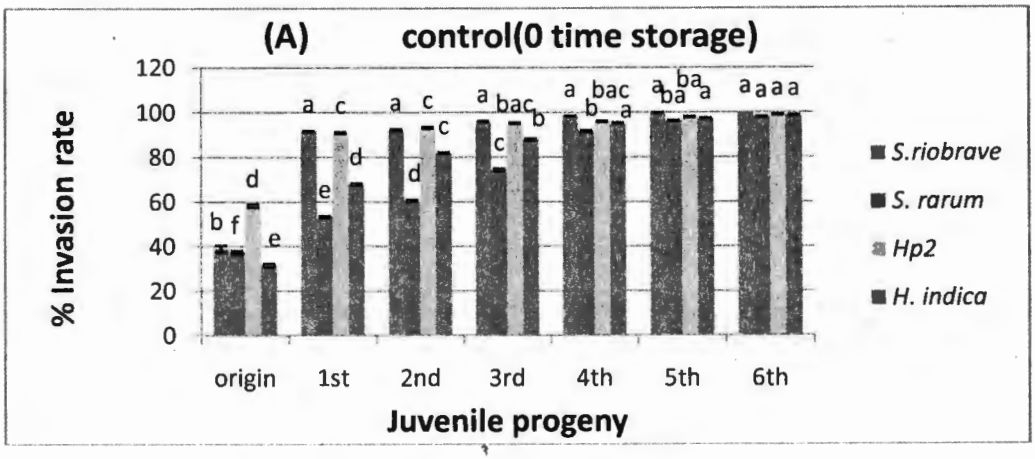
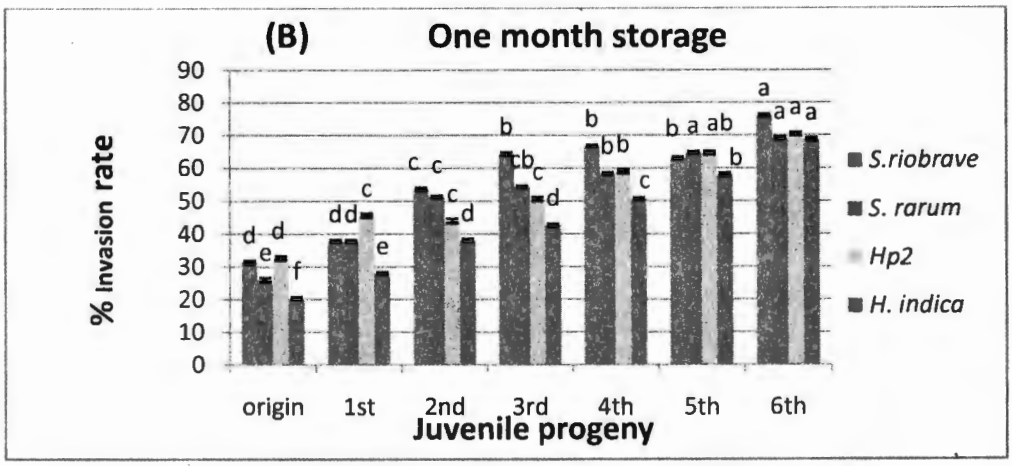
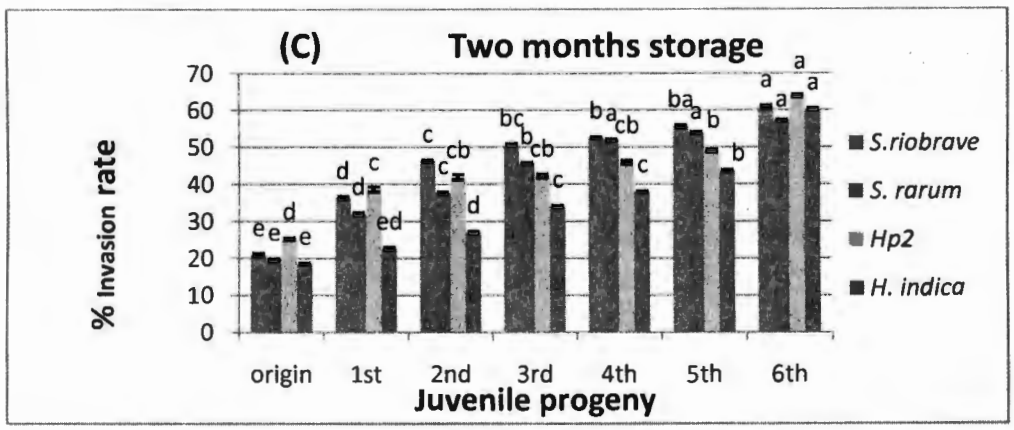


Fig (1): The % mortality of *G. mellonella* larvae subjected to Juvenile progeny of *Steinernema riobrave*, *Steinernema rarum*, *Heterorhabditis indica* and *Heterorhabditids* sp. (Hp2) at (A) control (0time storage), (B) one month, (C) three months (D) four months and (E) six months storage The statistics analysis for each species is made separately. Bars indicate Standard error of mean. Columns within each species annotated with the same letter are not significantly different (Duncan's multiple range; $P<0.05$).


(A) control(0 time storage)
% Invasion rate
120
100
80
60
40
20
0
origin
1st
2nd
3rd
4th
5th
6th
Juvenile progeny
S.riobrave
S. rarum
Hp2
H. indica


(B) One month storage
% Invasion rate
90
80
70
60
50
40
30
20
10
0
origin
1st
2nd
3rd
4th
5th
6th
Juvenile progeny
S.riobrave
S. rarum
Hp2
H. indica


(C) Two months storage
% Invasion rate
70
60
50
40
30
20
10
0
origin
1st
2nd
3rd
4th
5th
6th
Juvenile progeny
S.riobrave
S. rarum
Hp2
H. indica

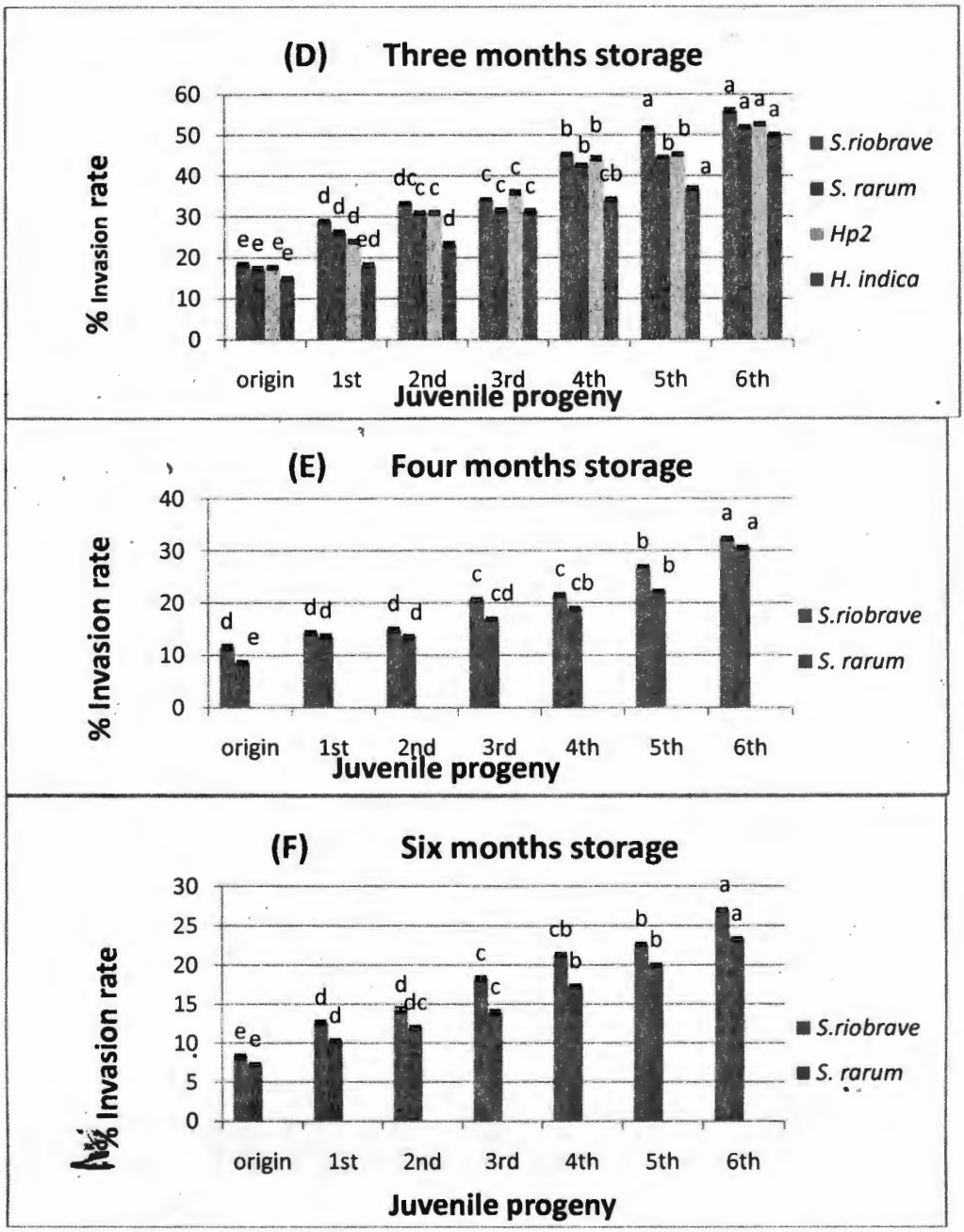


Fig (2): The % Invasion rate of Juvenile progeny of *Steinernema riobrave*, *Steinernema rarum*, *Heterorhabditids indica* and *Heterorhabditids* sp. (Hp2) to *G. mellonella* larvae at (A) control (0time storage), (B) one month, (C) three months (D) four months and (E) six months storage. The statistics analysis for each species is made separately. Bars indicate Standard error of mean. Columns within each species annotated with the same letter are not significantly different (Duncan's multiple range; $P<0.05$).

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زيادة استقرار التخزين والعدوى للنيماتودا الممرضة للحشرات لمرحلة التطبيق الحيوى

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أجريت الدراسة لمعرفة أثر التخزين على كفاءة النيماتودا الممرضة للحشرات لذلك قمنا بتحسين النيماتودا للحصول على أجيال لا تتأثر كفاءتها بالحفظ ،وكانت كفاءتها فى العدوى اكثر من كفاءة النيماتودا الأصلية بعد ان مرت بنفس ظروف الحفظ.

تم تطبيق البحث باستخدام أربع أنواع من النيماتودا الحشرية *(Steinernema riobrave, Steinernema rarum, Heterorhabditids indica* and the isolate *Heterorhabditids* sp. (Hp2)) حديثة الإنتاج التي لم تحفظ وكانت كفائتها التى قيست بقدرتها على قتل يرقة الشمع الكبرى كالتالي(٩٦.٦٦و٨٠و١٠٠و٩٣.٣٣%) ومعدل اختراق(٣٩و٣٧.٣٣و٥٨.٣٣و٣١.٦٦%).

النوعين من جنسى (*Steinernema riobrave, Steinernema rarum*)حفظت لمدة ٦ أشهرر أعطت نسبة موت (٦.٦٦و٣.٣٣%) ومعدل اختراق (٨.٣٣و٧.٣٣%).

النوعين من جنسى *(Heterorhabditids indica* and the isolate *Heterorhabditids* sp. (Hp2) حفظت لمدة ٣ أشهر وأعطت نسبة موت (٤٣.٣٣و ٣٠%) ومعدل اختراق (١٧.٦٦و ١٥%).

بينما الجيل المحسن السادس من الاربع أنواع *(Steinernema riobrave, Steinernema rarum, Heterorhabditids indica* and the isolate *Heterorhabditids* sp. (Hp2)). التى لم تحفظ وحديثة الخروج اعطت نسبة موت ١٠٠% ومعدل اختراق (١٠٠و٩٨,٩٩و٩٨.٦٦%) وعندما حفظ الجيل السادس من(*Steinernema riobrave, Steinernema rarum*) لمدة ٦ اشهر أعطت نسبة موت(٤٣.٣٣%) ومعدل اختراق (٢٧و٢٣%) فى حين الجيل السادس من النوعين (*Heterorhabditids indica* and the isolate *Heterorhabditids* sp. (Hp2)عندما حفظت لمدة ٣أشهرأعطت نسبة موت (٨٣.٣٣و٨٠%) ومعدل أختراق (٥٢.٦٦و٥٠%). وبذلك الطور المعدى من الاجيال المحسنة يمكن ان تحفظ لعدة أشهر مع استقرار فى معدلات العدوى أثناء تطبيقاتها الحيوية بعد ذلك.