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Artificial solid media for *in-vitro* mass production of two Egyptian nematodes

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Entomopathogenic nematodes (EPN) have several important attributes that make them excellent candidates for biological control of soil insects. These nematodes can be produced by in vivo by baiting technique on insects and commercially by in vitro solid/liquid culturing.

This study is an attempt for in vitro mass production of two native isolates of EPNs, *Heterorhabditis bacteriophora* (BA1) and *Steinernema carpocapsae* (BA2), isolated in the Egyptian soil. Three different synthetic media were used and their yields was compared with that *in vivo* produced on the 6th instar larvae of the greater wax moth, *Galleria mellonella*. The results indicated that among the three media tested, only modified dog biscuit medium recorded the positive results with respect to successful mass production of the Egyptian strain *H. bacteriophora* BA1. The largest amount yield was obtained from dog biscuit medium, the infective stages number was ranged between 10–12.5 millions with an average 11.345 millions of IJs for *H. bacteriophora* BA1, and ranged between 6.8-8.95 millions with an average 7.78 millions of IJs for *S. carpocapsae* BA2.

Keywords: Entomopathogenic nematodes; *Steinernema*; *Hetrerhabditis*; production; media; Egypt

INTRODUCTION

In Egypt the entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* are proved to be efficient against an extraordinarily broad spectrum of pests both in laboratory and field especially in the last two decades after the breakthrough in formulation of EPNs for foliar application (Saleh et al. 2009 and 2001; Hussein and Abdel Raoaf, 2012; Hussein et al. 2015; Hussein and El-Mahdi, 2019). EPNs are safe for non-target vertebrates and to the environment, they live in mutualistic symbioses with bacteria from the genera *Xenorhabdus* and *Photorhabdus,* respectively (Boemare, 2002). In Egypt, EPNs were successfully produced in vivo using the great wax moth, *Galleria mellonella*. Yet a real trial to use in vitro techniques was introduced to the agricultural sector (Abdel Rahman and Hussein, 2007).

Mass production of EPNs under monoxenic techniques on artificial media with the goal of producing, cost effectively, large numbers of infective nematodes has been realized 30 years before Dutky et al.(1964). Entomogenous nematode can be produced *in vitro* produced axenically or monoxenically, in solid and liquid phase fermentation. *In vitro* production of steinernematids and heterorhabditids can be done on agar media with both nutrients and oil (Wouts, 1981), or dog food (Hara et al. 1981; Friedman, 1990). Currently, EPNs are produced on solid or liquid cultures (Bedding, 1984). Cultures on sponge and liquid media were used for industrial mass production of nematodes.

Successful progress in the mass culturing of EPNs on synthetic media was a main step in their

marketing as bio-pesticides. Liquid cultures are considered to be the most economical method for large scale commercial production, however, the quality of some produced infective juveniles (IJs) may be less than that produced *in vivo* (Gaugler and Georgis, 1992). Moreover, the cost of such technology for a developing country like Egypt consider unaffordable and *in vitro* production of EPNs on solid culture with some modifications can rise to large scale with less cost. Therefore, the present study examines the effects of nutritional components of three modified synthetic solid diets on the growth and reproduction of *H. bacteriophora* BA1 and *S. carpocapsae* BA2. The virulence of the produced IJs from each medium was estimated. The data is used to select media appropriate for the industrial mass production of these two nematode species.

MATERIALS AND METHODS

Two experiments were conducted; the first one was to study ability of native EPNs to be produced on 3 modified media. The second was to assess the virulence of the produced IJs against 6th instar larvae of the wax moth larvae, *Galleria mellonella* which were cultured according to Metwally, et al. (2012).

Nematode inoculum

The tested nematodes were sterilized according to Lunau et al. (1993). Infective juveniles were surface sterilized using hyamine (0.1%) solution and washed three times. The IJs were concentrated in distilled water by precipitation, and the final suspension was spread onto nutrient agar medium (in 9-cm Petri dishes) previously inoculated with a culture of *X. nematophila* and *P. luminescens*. The gravid females were collected after 3 days from the petri dishes and their eggs were isolated and surface sterilized by alkaline lysis buffer. The eggs were rinsed thoroughly and transferred to 24-well plates containing 0.3 ml of TSB liquid medium (4% tryptic- soy-broth + 0.5% yeast extract) in each well, for 2 d of incubation at 25^{o}C. After incubation, the 2-d-old juveniles were used as inocula for the first experiment

Isolation and production of the bacterial symbionts

Cadavers of greater wax moth larvae, *G. mellonella*, infected with two native nematodes; *H. bacteriophora* BA1 and *Steinernema carpocapsa* BA2 were surface sterilized in 70% alcohol for 2 min, and transferred to dry in a laminar airflow Class II cabinet for 3 min. Cadavers were opened with sterile needles and a drop of haemolymph was streaked on to the NBTA medium (Nouh and Hussein, 2014) in 9 cm Petri-dishes and incubated at 28°C. Single 48 h-colonies of pure 1^{0}colonies were inoculated on agar plates and subcultured continually. The isolates pathogenicity was confirmed by injecting cells of the bacteria into *G. mellonella* larvae. In a 250 ml Erlenmeyer flask, a colony was inoculated into nutrient broth (18 g nutrient broth in 500 ml distilled water) and kept on a shaking incubator at 160 rpm for 48 h at 28°C±2. Stock culture was kept in -20^{0}C till use.

In vitro Production of Egyptian strains of EPNs

Three modified media were used in this study. Table (1) represents the constituents for each media. The components of each medium were mixed homogeneously and smeared on sponge pieces at the ratio of 3 g: 110g (sponge; medium) separately for each flask. Sponge pieces coated with medium were then transferred carefully to the conical flask. The conical flask was cleaned and autoclaved at 121^{o} C, 15 lbs for 15 min.

Table (1). Composition of media used in mass culture of EPNs

Media	Components	Amount
A	Nutrient broth	8 g
	Yeast extract	12 g
	Soy flour	10 g
	Corn oil	40 ml
	Distilled water (ml)	1000 ml
B	Dog biscuit	15 g
	Peptone	10 g
	Beef extract	5 g
	Yeast extract	5 g
	$MgCl_2$6H2O	2g
	Cod liver oil	3 ml
	Distilled water (ml)	1000 ml
C	Dried Egg yolk	12.5 g
	Soy flour	10 g
	Yeast extract	23 g
	Na Cl	5 g
	Corn oil	40 g
	Distilled water (ml)	1000

Later, the cultured flasks containing medium coated sponge pieces were theninoculated with bacterial suspension from nutrient broth. Each flask was inoculated with 0.5 ml of bacterial suspension, aseptically. After inoculation, flasks were incubated at 28^{o}C for 40hrs to allow the spread of the colonies all over the sponge media.

Newly harvested IJs of BA1 and BA2 were surface sterilized in 0.1 % hyamine to prevent the contamination and inoculated at the rate of 3000 IJs per flask. Three replicates were used for each species. Thus in total, (18 flasks for 3 artificial media). Cultured flasks were incubated at 28^{o} C in dark for 25 days. Once the nutrients depleted and IJs emerged, the sponge pieces were transferred to modified White's trap, separately. IJs were harvested daily in 0.1 % formalin and total numbers of IJs per flask were recorded.

RESULTS

The results indicated that among the three media tested, only modified dog biscuit medium recorded the positive results with respect to successful mass production of the Egyptian strain *H. bacteriophora* BA1. The largest amount yield was obtained from dog biscuit medium, the infecive stages number was ranged between 10–12.5 millions with an average 11.345 millions of IJs for *H. bacteriophora* BA1, and ranged between 6.8-8.95 millions with an average 7.78 millions of IJs for *S. carpocapsae* BA2. The smallest amounts of yield of *H. bacteriophora* BA1 was ranged 2.95-5.95 millions with an average 4.7 millions of IJs while, the harvest of *S. carpocapsae* BA2 was ranged 1.7-3.5 millions with an average 2.37 millions of IJs was obtained from dried egg yolk medium (Table 2, Fig 1). The differences between three media, (F value= 7.39, α= 0.05, p=0.0013, p<0.05) are significant, while the differences between two genus BA1, BA2 not significant p>0.05.

Most effective juveniles were that produced from dog biscuits medium, the mortality was reached to 100% of both tow species *H. bacteriophora* BA1 and , *S. carpocapsae* BA2, at 400 IJs / larva , the mortality was 100% of BA1 and 93% of BA2 were produced from egg yolk, at the same conc. And finally morality was 100% of *H. bacteriophora* BA1 and 87% of *S. carpocapsae* BA2 was reared on nutrient broth medium (Table 3, Fig.2 and 3). From factorial analysis (Table 4) the differences between the concentrations of EPNs juveniles are significant (F=98.71, α=0.05, P=0.0001, P<0.05).

Table (2). Population density of the Egyptian nematodes, *Steinernema carpocapsae* BA2 and *Heterorhabditis bacteriophora* BA1 cultured on three modified media.

Population density (in million)	*Steinernema carpocapsae* BA2			*Heterorhabditis bacteriophora* BA1		
	Media A	Media B	Media C	Media A	Media B	Media C
	2.95-4.15	6.8-8.95	1.7-3.05	7.81-10.35	10-12.5	2.95-5.65
Mean	3.51	7.78	2.37	9.25	11.35	4.71

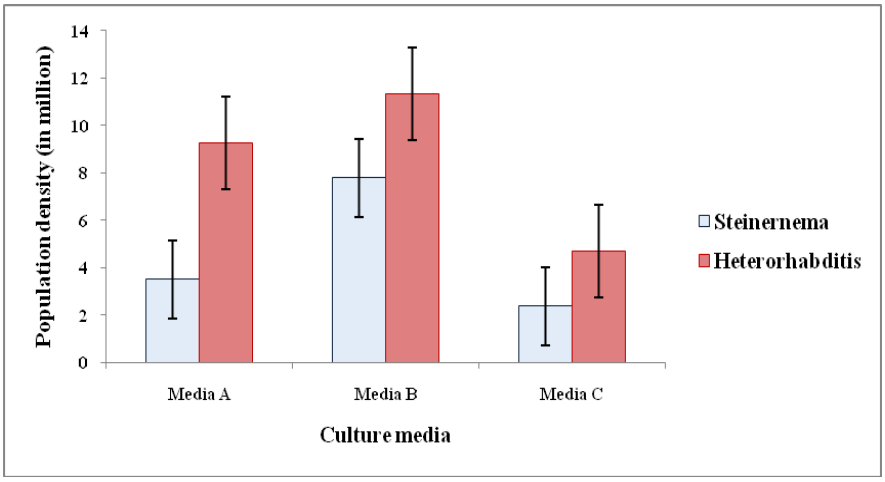

Figure1: The population density of the Egyptian nematodes, *Steinernema carpocapsae* BA2 and *Heterorhabditis bacteriophora* BA1 cultured on three modified media.

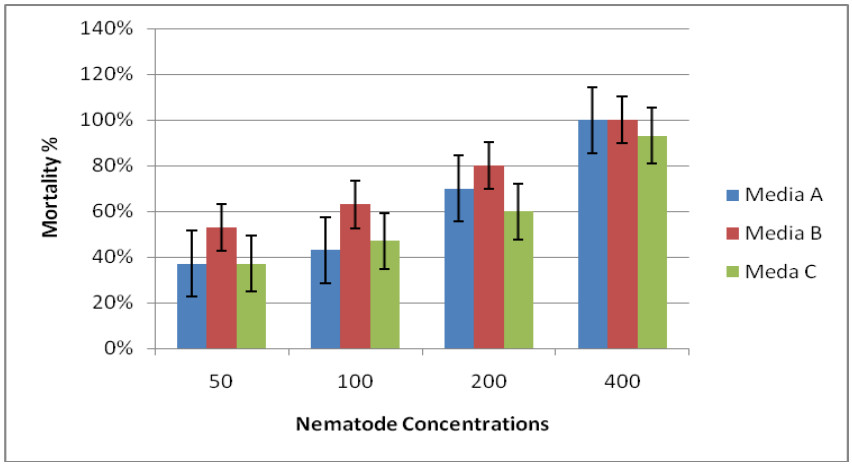

Figure2: Virulence of different concentrations of *Heterorhabditis bacteriophora* BA1 mass cultured on different media on the 6th instar larvae of *Galleria mellonella*.

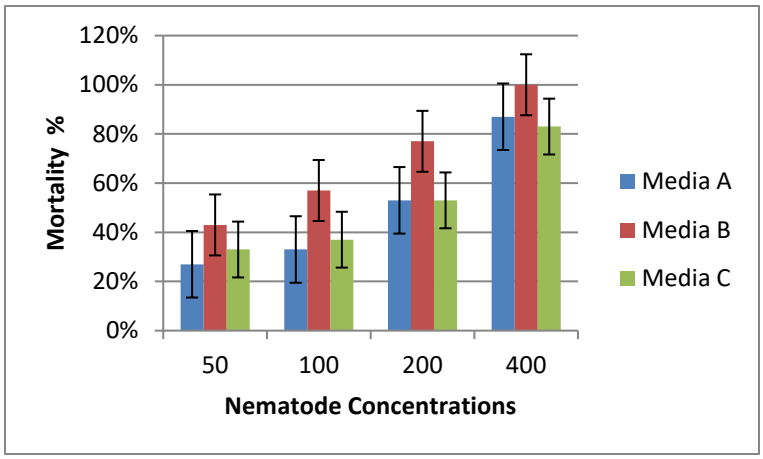

Figure 3: Virulence of different concentrations of S*teinernema carpocapsae* BA2 mass cultured on different media on the 6th instar larvae of *Galleria mellonella*.

Table (3). Virulence of different concentrations of *Heterorhabditis bacteriophora* BA1and*Steinernema carpocapsae* BA2 mass cultured on different media.

Concentration (IJs/larva)	Media A		Media B		Media C	
	BA1%	BA2%	BA1%	BA2%	BA1%	BA2%
50	37	27	53	43	43	37
100	43	33	63	57	57	47
200	70	53	80	77	77	60
400	100	87	100	100	100	93

Table (4).Factorial analysis for obtained data.

Factor	Level	Mean	
Media	A	6.583	a
	B	5.458	b
	C	6.292	c
F value		7.39	
P value		0.0013	
LSD		0.607	
BA	1	6.222	a
	2	6.000	a
F value		0.800	
P value		0.3736	
LSD		0.495	
Conc.	50	3.833	d
	100	4.667	c
	200	6.556	b
	400	9.390	a
F value		98.71	
P value		0.0001	
LSD		0.7005	

DISCUSSION

This is the first successful attempt to demonstrate the ability of the native Egyptian strains of entomopathogenic nematodes to be mass produced on artificial media. Similar successful attempts were also made by House et al. (1965) using *H. minutes* and *S. glaseri*. However, *H. indicus* failed to multiply on other artificial media *viz.,* modified Wout's medium, modified egg yolk medium and modified wheat flour medium. The results are contradictory with the study conducted by Hussaini *et al.* (2003) who successfully mass produced *H. indica* on modified Wout's medium but failed on modified dog biscuit medium. The variation might be due to difference in the strain and quantity of the medium used.

Advances in the production of EPNs in artificial media were a major step toward their commercialization as biological insecticides. The most economical method for large scale commercial production is liquid culture. However, the quality of some EPNs juveniles produced in artificial media may be less than that of nematodes produced *in vivo* (Gaugler and Georgis, 1992). Moreover, the cost of liquid culture for a developing country like Egypt consider unaffordable.

Although liquid culture provides economy of scale, labor saving, enables large batch size, but it requires high initial capital costs, only restricted to a few nematode species, the requirements for advanced expertise and the possibility of large scale contamination. Solid culture, in contrast, has the advantages that it can be used for most nematode species, has flexibility of production, can be used by both cottage industries or for large scale production and contamination is localized. In addition, capital costs are low and limited expertise is required (Han and Ehlers, 2002).

CONCLUSION

According to the results from our investigations we can conclude that the native isolates of the Egyptian entomopathogenic nematodes, *Heterorhabditis bacteriophora* BA1 and *Steinernema carpocapsae* BA2 are qualified to be mass produced on different synthetic media. Moreover, both nematode species were found to be excellent agents for the biological control of insect pests.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

MAH designed and performed the experiments and also wrote the manuscript. IFEE conducted data analysis and reviewed the manuscript. All authors read and approved the final version.

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