Pak. J. Nematol., 30 (1): 1-20, 2012

MASS PRODUCTION OF EIGHT PAKISTANI STRAINS OF ENTOMOPATHOGENIC NEMATODES (STEINERNEMATIDAE AND HETERORHABDITIDAE)

J. SALMA AND F. SHAHINA*

National Nematological Research Center, University of Karachi, Karachi, Pakistan

*Corresponding author's e-mail: shahinafayyaz@gmail.com

Abstract

Eight nematode species of the genera *Steinernema* and *Heterorhabditis* viz., *Steinernema pakistanense*, *S. asiaticum*, *S. abbasi*, *S. siamkayai*, *S. carpocapsae*, *S. feltiae*, *Heterorhabditis indica* and *H. bacteriophora* were cultured *in vivo* on three insect species, *in vitro* on soya flour, wheat flour, lipid media, corn flour and on assemblage culture on *Galleria mellonella* larva and lipid modified media for mass scale to assess their production potential. On *in vivo* culture at the highest concentration the production of infective juveniles were 60×10^4 to 87.4×10^5 IJs from each larva of *G. mellonella* 4.2×10^4 to 9.8×10^4 IJs from each adult of *Callosobruchus chinensis* and 0.3×10^5 to 1.7×10^4 IJs from each larva of *Tribolium castaneum*. Soya flour medium gave the highest population as compared to other media. The minimum multiplication was found in corn flour medium. As compared to cultured separately, the production of infective juveniles increased approximately two fold in assemblage medium. *In vivo* production of IJs in *G. mellonella* larvae and *in vitro* soya flour medium were also exposed to four different temperatures. Maximum production of all other species was found at 32 ± 2°C, except *S. feltiae* which gave highest production at 20 ± 2°C. The present investigation can be valuable for selecting strains of entomopathogenic nematodes for mass production on large scale to provide protection to crops against insects.

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are lethal pathogens of insects (Poinar, 1990). These pathogens contribute to the regulation of natural population of insects, but the main interest in them is as inundative applied biocontrol agents. Their success in this role can be attributed to the unique partnership between a host-seeking nematode and lethal insect pathogen bacterium. Because of their biocontrol potential considerable attention has been directed over the past few decades to *Heterorhabditis* and *Steinernema* and their special bacterial partners, *Photorhabdus* and *Xenorhabdus* (Griffin *et al.*, 2005). Since the late 1970s, there has been a tremendous research and commercial interest in entomopathogenic nematodes and their associated bacteria. This interest was sparked in part by (1) lack of adequate tools to control soil-inhabiting insect pests in an effective, environmentally acceptable manner, and (2) the capability of producing these nematodes monoxenically *in vitro* which was followed by scale-up to

commercial production levels (Ehlers, 2001; Klein, 1990). For laboratory use and small scale field application, *in vivo* production of entomopathogenic nematodes is the appropriate method. When it comes to commercial use of EPNs on large scale for international market for *in vitro* production, it is currently the only economically reasonable means to supply EPNs of high quality and at reasonable costs (Ehler & Shapiro- Il., 2005). The most common insect host used for *in vivo* laboratory and commercial EPN production is the last instar of the greater wax moth, *Galleria mellonella* because of the high susceptibility to most nematodes, ease in rearing, wide availability and ability to produce yields (Woodring & Kaya, 1988). Only few EPNs i.e., *S. kushidai*, *S. scarabaei* and *S. scapterisci* exhibit relatively poor reproduction in *G. mellonella* due to extremes in host specificity (Nguyen & Smart, 1990; Kaya & Stock, 1997). Nematode yield is proportional to insect host size but yield per milligram insect and susceptibility to infection is usually inversely proportional to host size or age (Flanders *et al.*, 1996). In addition to yield, ease of culture and infection are important factor when choosing a host (Shapiro-Ilan & Gaugler, 2002). Nematode quality may also need to be considered in choosing a host. Nematode quality appears greater when cultured in hosts that are within the nematode's natural host range (Abu Hatab *et al.*, 1998). *G. mellonella* larvae may often be the most efficient host (Bedding, 1981). In the USA, Glaser (1931) tried to mass-produce *S. glaseri in vitro*. The presence of its symbiotic bacterium was not yet known and efforts to continuously produce EPNs, therefore, failed. Early attempts to grow *Steinernema* spp. in axenic culture were successful (Stoll, 1952), but the yields were too low and the media, containing unsterilized raw rabbit liver, were difficult to produce and too expensive for the system to be exploited for mass production. Although Bovien (1937) first observed bacteria inside the DJ of *S. feltiae*, many years elapsed until Poinar & Thomas (1965) described the significance of the bacterium *X. nematophilus* for the reproduction of *S. carpocapsae*. Solid culture was first accomplished in two-dimensional arenas, e.g., Petri dishes, containing various media such as those based on dog food, pork kidney, cattle blood, and other animal products (Hara *et al.*, 1981). Wouts (1981) developed an improved medium (less expensive and more consistent from batch to batch) that included yeast extract, nutrient broth, vegetable oil, and soy flour. *In vitro* solid culture advanced considerably with the invention of a three-dimensional rearing system involving nematode culture on crumbled polyether polyurethane foam (Bedding, 1981). A liquid medium is mixed with foam and autoclaved. Bacteria are inoculated first followed by the nematodes ca. 3 days later. Nematodes can be harvested within 2–5 weeks by placing the foam onto sieves, which are immersed in water. Infective juveniles migrate out of the foam, settle downward, and are pumped to a collection tank; the product is cleaned through repeated washing with water, i.e., sedimentation and decanting (Bedding, 1984). As in Petri dishes, media for this approach were initially animal product based (e.g., pork kidney or chicken offal) but was later improved (for cost and

consistency) and may include various ingredients including peptone, yeast extract, eggs, soy flour, and lard (Han *et al.*, 1992). Present paper deal with *in vivo* and *in vitro* production potential of eight Pakistani strains of entomopathogenic nematodes at different medium, doses and temperature.

Materials and Methods

***In vivo* mass production on three insect species:** For *in vivo* production of infective juveniles, last larval stage of wax moth *Galleria mellonella* larvae, freshly emerged adult of pulse beetle *Callosobruchus chinensis* and last larval stage of red flour beetle *Tribolium castaneum* were selected for each concentration and tested species were placed in 4.5× 4.5 mm plastic cavity block having sterilized moist sand. Insects were exposed at the rate of 5, 10, 20, 30, 40 and 50 IJs per ml of water suspension of *Steinernema pakistanense* Pak.S.*S.*43 strain (GU130180); *S. asiaticum* Pak.S.*S.*4 strain (RFLP); *S. abbasi* Pak.S.*S.*16 strain (JN571096); *S. siamkayai* Pak.S.*S.*10 strain (JF892544); *S. carpocapsae* Pak.S.*S.*23 strain (GU130182); *S. feltiae* Pak.N.*S.*7 strain (RFLP); *Heterorhabditis indica* Pak. S. *H.* 77 strain (JN157774) and *H. bacteriophora* Pak. S. *H.* 17 (EF469774). After 48 hrs insects were transferred to a white trap for collection of new progeny of IJs. The entire experiment was repeated four times with ten insect for each concentration and replicate. Temperature was maintained at 32± 2°C except for *S. feltiae* (20 ± 2°C).

***In vitro* mass production on four different media:** Mass production of the above mentioned Pakistani strains carried out *in vitro* with four different media (Table 1).

Culture flask: Polyurethane sponge was served as a carrier material. Sponge was cut into small pieces of 1.25 × 1.25 cm size, washed with 70% ethanol and incubated at 50°C for 20 minutes The porous foams afforded an outstanding surface area to volume ratio for growth while providing adequate gas exchange. Components were homogenized in a blender with 200 ml distilled water. The resulting homogenate was evenly smeared on polyurethane sponge at the ratio of 2: 55grams (sponge; medium) separately for each sterilized flask and species. Sponge coated pieces were transferred carefully to 500 ml conical flask. Mouths of flasks were wiped well, plugged with cotton wools and wrapped with brown paper. Medium was autoclaved at 121 °C and 15 psi for 15 min.

Table 1. Components of four different media for *in vitro* mass production.

Components/Media	Soya flour	Wheat flour	Lipid	Corn flour
Nutrient broth (g) (Merck)	9.0	9.0	9.0	9.0
Yeast extract (g) (Merck)	3.0	3.0	3.0	3.0
Soya flour (Local market) (g)	150	-	-	-
Wheat flour (Local market) (g)	-	150	-	-
Corn flour (Local market) (g)	-	-	-	150
Corn oil (Rafhan) (g)	99	99	-	99
Cod liver oil (Seven seas) (g)	-	-	0.5	-
Honey (Ebia) (g)	-	-	2.5	-
$MgCl_2 6 H_20$ (Merck) (g)	-	-	0.5	-
Distilled water (ml)	200	200	200	200

Inoculation with bacteria and nematodes: Pure colonies of each symbiotic bacterium based on morphological observation were transferred aseptically to nutrient broth and incubated at 28±2 °C for 24 hrs. Each flask of solid media was inoculated with 0.5 ml of bacterial suspension and incubated at 28°C for 24 hrs to allow multiplication of bacterial colonies, optical density at 600 nm were measured for confirmation growth of bacteria. Freshly harvested infective juveniles of each species were inoculated at the rate of 1000 IJs per flask. Four replicates were maintained for each species and medium.

Harvesting and cleaning: *In vivo* produced nematodes were collected using White traps (White, 1927). Dead cadavers were placed on the filter paper. IJs emerged out and migrated into the water. Once nematodes begin to appear they were harvested daily into the beaker up to 10 days. Infective juveniles from *in vivo* production were harvested after 15 days of the inoculation by adopting Bedding (1984) method. Smeared sponge medium were spread separately on 20 mesh sieve on an aluminum pan filled with distilled water so that the foam was immersed in water. Flasks were thoroughly washed and the washing was also added to sieve. Collected material was kept at 15-20°C in growth chamber for 24 hrs. During this time at least 90% of all nematodes moved in to the water. When all the infective juveniles settled downward, then were pumped to a collection beaker. The product was cleaned thoroughly by repeated washing to obtain nematodes free from debris.

Counting and Storage: Concentrations of the total viable IJs were determined by counts in 10 µl of initial samples diluted in 5ml under the stereomicroscope

(40×), in triplicate. Counting was done on a counting slide. Infective juveniles were stored in growth chamber (15-20°C) in 100 ml flask having distilled water with a drop of Triton X-100.

Effect of temperature on *in vivo* and *in vitro* mass production: Same procedure was followed as mentioned above for *in vivo G. mellonella* and *in vitro* soya flour medium for mass production of Pakistani strain of entomopathogenic nematodes viz., *Steinernema pakistanense*, *S. asiaticum*, *S. abbasi*, *S. siamkayai*, *S. carpocapsae*, *S. feltiae*, *H. indica* and *H. bacteriophora*. Initial inoculums were 50 IJs/ larva and 1000 IJs/ flask for *in vivo* and *in vitro* cultures respectively. Culturing media were exposed at four different temperatures viz., 20 ± 2, 25 ± 2, 32 ± 2 and 38 + 2°C.

Mass production of EPNs on assemblage of *in vitro* and *in vivo* culture: Lipid agar media was modified with the following components: 7g NA + 0.0062g bromothymol blue (0.00 25%) + 0.01g triphenyltetrazolium chloride (0.00 4%), 1.7g Agar Agar, 0.5 g Cod liver oil, $MgCl_2.6H_2O$ and honey; 200 ml distilled water was used for *in vitro* culture.

When all components were thoroughly mixed, the medium was autoclaved at 121°C and 15 psi for 15 min. 18 ml aliquot was transferred aseptically into sterilized 90 mm Petri-dishes. After 2-3 hrs an infected surface sterilized *G. mellonella* larvae with 50 IJs/ larva of each eight species of Pakistani strains were transferred separately on a medium. Plates were incubated at 32+ 2°C except for *S. feltiae* temperature was 20 ± 2°C. New progenies of infective juveniles were harvested up to 15 days.

Data analysis: Data were analyzed by multifactor analysis of variance (ANOVA); if the ANOVA was significant, differences in treatments were elucidated through Duncan's multiple range test ($P < 0.05$) using SPSS statistical software.

Results

***In vivo* mass production:** Six different concentrations of infective juveniles 5, 10, 20, 30, 40 and 50 IJs per insect of eight indigenous species were applied to last larval stage of *G. mellonella*, freshly emerged adults of *C. chinensis* and last larval stage of *T. castaneum*.

Significant difference was found between dose concentration and IJs production ($p < 0.001$). The final estimation of infective juveniles was ended after 10 days. Maximum numbers of infective juveniles were found in each dose from *G. mellonella* larvae as shown in graphical presentation Fig.1 (A-H). It was

observed that at the lowest concentration (5 IJs), the production of infective juveniles of *S. pakistanense* were 26.6×10^4, 3.0×10^4, 0.51×10^4 IJs; *S. asiaticum* 22.3×10^4 2.4×10^4, 0.23×10^4 IJs; *S. siamkayai* 25.5×10^4, 3.9×10^4, 0.48×10^4 IJs; *S. feltiae* 20.2×10^4, 2.4×10^4, 0.36×10^4 IJs; *S. carpocapsae* 15.6×10^4, 1.2×10^4, 0.20×10^4 IJs; *S. abbasi* 24.5×10^4, 3.2×10^4, 0.15×10^4 IJs; *H. bacteriophora* 14×10^4, 3.2×10^4, 0.28×10^4 IJs and *H. indica* 14.1×10^4, 2.9×10^4, 0.32×10^4 IJs from each in *G. mellonella* larva, adult of *C. chinensis* and larval stage of *T. castaneum*, respectively (Fig. 2).

Table 2 illustrates average production at different concentrations of eight indigenous nemotodes species. *S. pakistanense* produced massive yield of infective juveniles as compared to other nematodes species in all the three insect hosts. At the highest concentration (50 IJs), the production of infective juveniles were 60×10^4 to 87.4×10^4 IJs from each larva of *G. mellonella*, 4.2×10^4 to 9.8×10^4 IJs from each adult of *C. chinensis* and 0.3×10^4 to 1.7×10^4 IJs from each larva of *T. castaneum*.

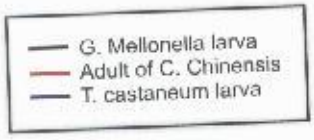
***In vitro* mass production:** For *in vitro* production eight indigenous species were tested on soya flour, wheat flour, lipid medium and corn flour medium. After 15 days of inoculation successful production were observed (Fig. 3). Soya flour medium gave the highest population as compared to other media. The minimum multiplication was found in corn flour medium. Significant difference of production potential was found between four media ($p < 0.001$). When the nutrients were depleted, IJs moved around the flask (Fig.4). This indicated the harvesting period of nematodes. *S. pakistanense* produced 20×10^4, 13×10^4, 18×10^4, 7×10^4 IJs; *S. asiaticum* produced 15×10^4, 10×10^4, 12×10^4, 5×10^4 IJs; *S. siamkayai* produced 20×10^4, 13×10^4, 16×10^4, 4×10^4 IJs; *S. feltiae* produced 13×10^4, 10×10^4, 12×10^4, 6×10^4 IJs; *S. carpocapsae* produced 9×10^4, 8×10^4, 6×10^4, 7×10^4 IJs; *S. abbasi* produced 15×10^4, 11×10^4, 13×10^4, 7×10^4 IJs; *H. bacteriophora* produced 16×10^4, 11×10^4, 15×10^4, 4×10^4 IJs and *H. indica* produced 15×10^4, 10×10^4, 13×10^4, 4×10^4 IJs from each flask of soya flour, wheat flour, lipid medium and corn flour medium, respectively.

Effect of temperature on *in vivo* and *in vitro* mass production: *In vivo* production of IJs in *G. mellonella* larvae and *in vitro* soya flour medium were exposed to four different temperatures. Significant difference was found between temperature and yield multiplication ($p < 0.001$). *Steinernema pakistanense*; *S. asiaticum*; *S. abbasi*; *S. siamkayai* ; *S. carpocapsae*; *Heterorhabditis indica* and *H. bacteriophora* failed to produce IJs at 20 + 2°C and 25 ± 2°C. In *G. mellonella* larvae and soya flour medium production of S. *feltiae* was $60 \pm 2.0 \times 10^4$ and $13 \pm 1.0 \times 10^4$, respectively.

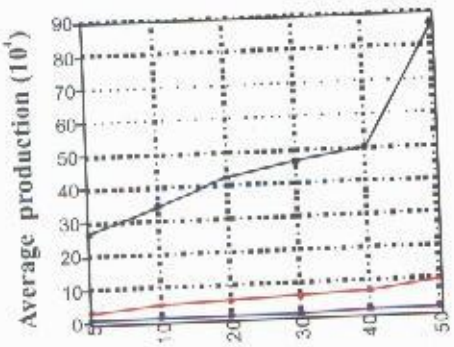
Table 2. *In vivo* mass production of eight Pakistani strains of EPNs in insect species.

Nematodes species	Doses IJs/ml	*G. mellonella*	*C. chinensis* Average yield ($\times 10^4$)	*T. castaneum*
S. pakistanense Pak.S.S.43 strain (GU130180)	5	26.6±5.2 f	3.0±1.0 e	0.51±5.2 e
	10	34.1±3.9 e	4.9±2.8 d	0.82±3.9 b
	20	42.3±4.8 d	5.3±1.8 c	0.89±3.4 b
	30	46.4±2.0 c	6.1±3.2 b	0.92±1.6 b
	40	50.4±3.1 b	6.9±2.3 b	1.30±1.1 a
	50	86.3±1.2 a	9.8±1.2 a	1.50±1.2 a
S. asiaticum Pak. S. S. 4 strain (RFLP)	5	22.3±4.2 d	2.4±4.2 d	0.23±1.2 d
	10	23.2±1.2 d	3.5±2.9 c	0.29±4.1 d
	20	33.0±1.6 c	3.9±1.0 c	0.31±1.0 c
	30	33.9±1.1 c	4.0±4.4 b	0.39±1.9 c
	40	44.5±5.0 b	4.8±2.0 b	0.40±2.0 b
	50	61.9±4.2 a	5.3±2.0 a	0.80±1.1 a
S. ablasi Pak. S. S. 16 strain(JN571096)	5	24.5±1.8 f	3.2±2.7 e	0.15±1.5 c
	10	26.4±1.0 e	3.9±2.0 c	0.18±2.3 c
	20	29.4±4.2 d	4.2±1.2 b	0.20±2.0 b
	30	37.4±1.8 c	4.5±3.0 b	0.25±2.6 b
	40	45.7±5.6 b	6.0±1.0 a	0.28±1.0 b
	50	80.5±2.3 a	6.6±1.0 a	0.30±1.3 a
S. siamkayai Pak S.S. 10 strain (JF892544)	5	25.5±1.2 f	3.9±2.0 e	0.48±1.2 e
	10	33.1±3.4 e	4.2±2.5 d	0.70±2.5 d
	20	41.8±1.0 d	4.8±2.4 d	0.85±1.0 c
	30	53.6±2.5 c	5.5±1.2 c	0.89±2.5 c
	40	63.5±1.7 b	6.9±1.3 b	0.98±1.5 b
	50	87.4±1.5 a	8.6±2.5 a	1.70±2.5 a
S. carpocapsae Pak. S. S. 23 strain (GU130182)	5	15.6±1.0 f	1.2±2.0 d	0.20±2.0 e
	10	25.3±2.3 e	2.4±2.5 c	0.45±1.5 d
	20	30.7±2.5 d	2.8±1.5 c	0.52±2.0 c
	30	42.8±3.2 c	3.2±2.5 b	0.63±1.5 b
	40	45.2±1.2 b	3.5±1.0 b	0.90±1.2 a
	50	65.2±1.0 a	4.2±10 a	0.99±1.4 a
S. feltiae Pak. N. S. 7 strain(RFLP)	5	20.2±2.3 f	2.4±2.0 e	0.36±1.0 f
	10	22.5±1.5 e	3.0±2.5 d	0.52±1.5 e
	20	28.9±1.2 d	3.7±2.4 d	0.63±1.5 d
	30	40.5±1.0 c	4.5±1.2 c	0.72±1.2 c
	40	45.5±2.0 b	5.8±1.3 b	0.88±1.2 b
	50	60.0±2.0 a	7.2±2.5 a	0.99±1.5 a
H. indica Pak. S. *H.* 1 strain (JN157774)	5	14.1±1.0 f	2.9±2.3 f	0.32±1.0 d
	10	22.7±2.5 e	3.2±1.2 e	0.71±2.5 c
	20	25.4±1.5 d	4.8±1.5 d	0.75±1.0 c
	30	41.6±1.2 c	5.5±2.5 c	0.80±1.5 b
	40	48.5±1.0 b	6.2±2.0 b	0.89±2.0 b
	50	85.0±1.5 a	8.7±1.0 a	0.99±1.5 a
H. bacteriophora Pak. S.*H.*17strain(EF469774)	5	14.0±2.0 f	3.2±1.2 d	0.28±1.2 e
	10	18.6±2.5 e	3.8±2.0 d	0.82±2.0 d
	20	20.9±2.0 d	4.2±1.5 c	0.89±1.5 d
	30	31.1±1.2 c	5.2±2.2 b	0.92±2.0 c
	40	49.5±1.0 b	5.7±1.5 b	1.30±1.5 b
	50	80.5±1.2 a	9.0±1.0 a	1.50±2.2 a

Values having same letters in a column are not significantly different.

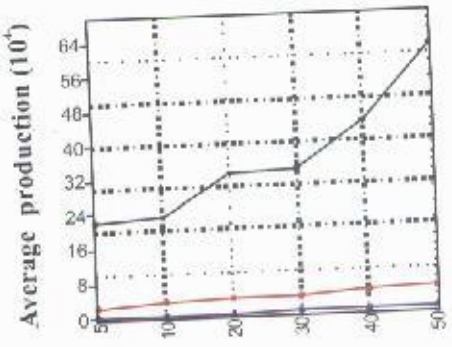


A



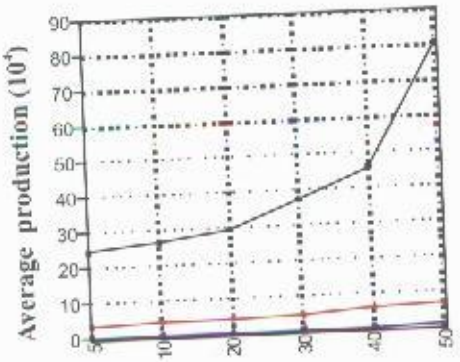
Doses of *S. pakistanense* Pak. S. *S* 43

B



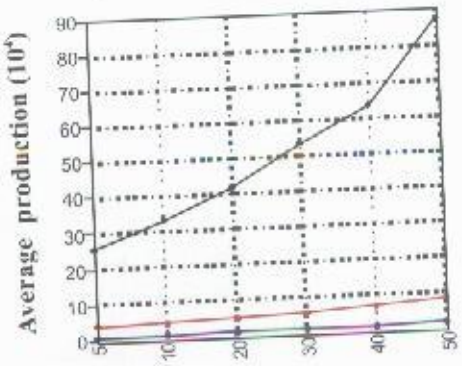
Doses of *S. asiaticum* Pak. S. *S.* 4

C



Doses of *S. abbasi* Pak. S. *S.* 16

D



Doses of *S. siamkayai* Pak. S. *S.* 10

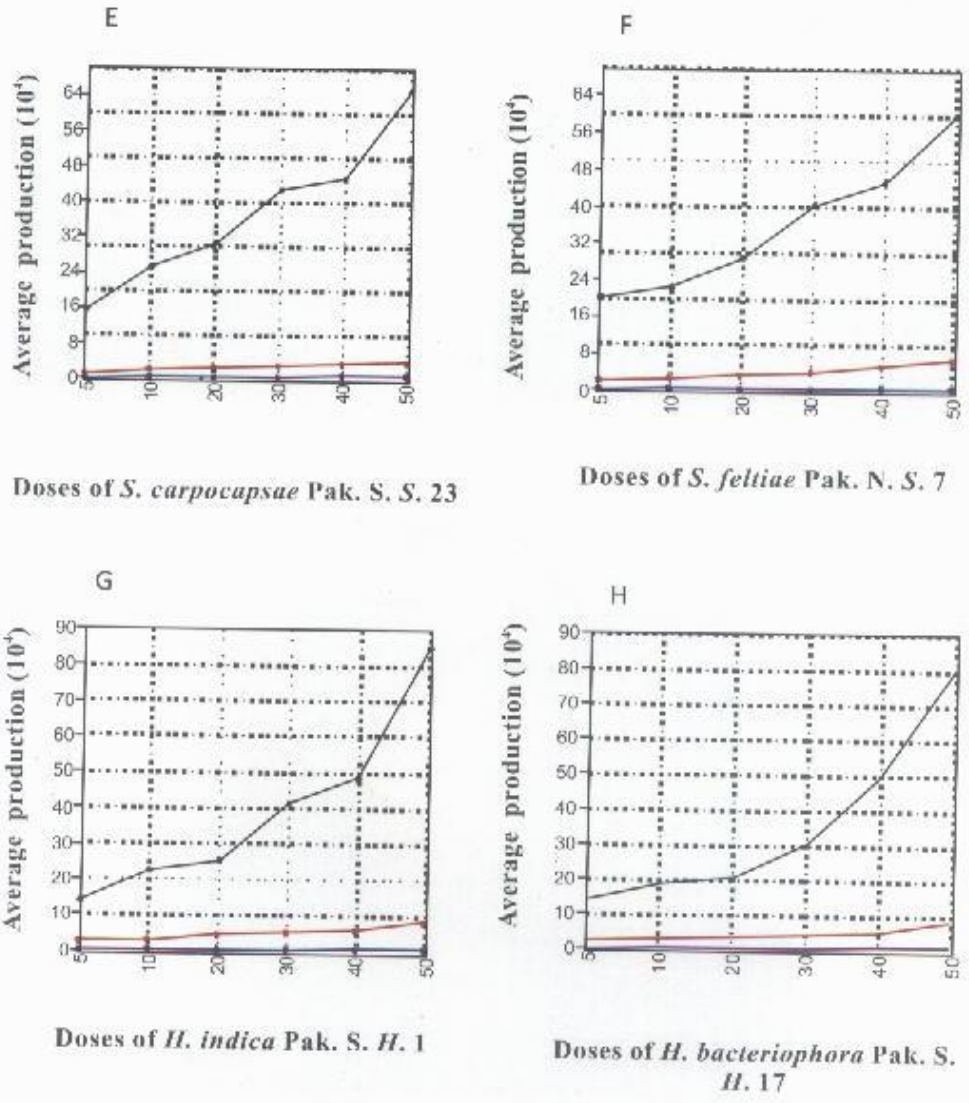


Fig. 1 (A-H). *In vivo* average production of eight Pakistani strains of entomopathogenic nematodes having six different concentrations.

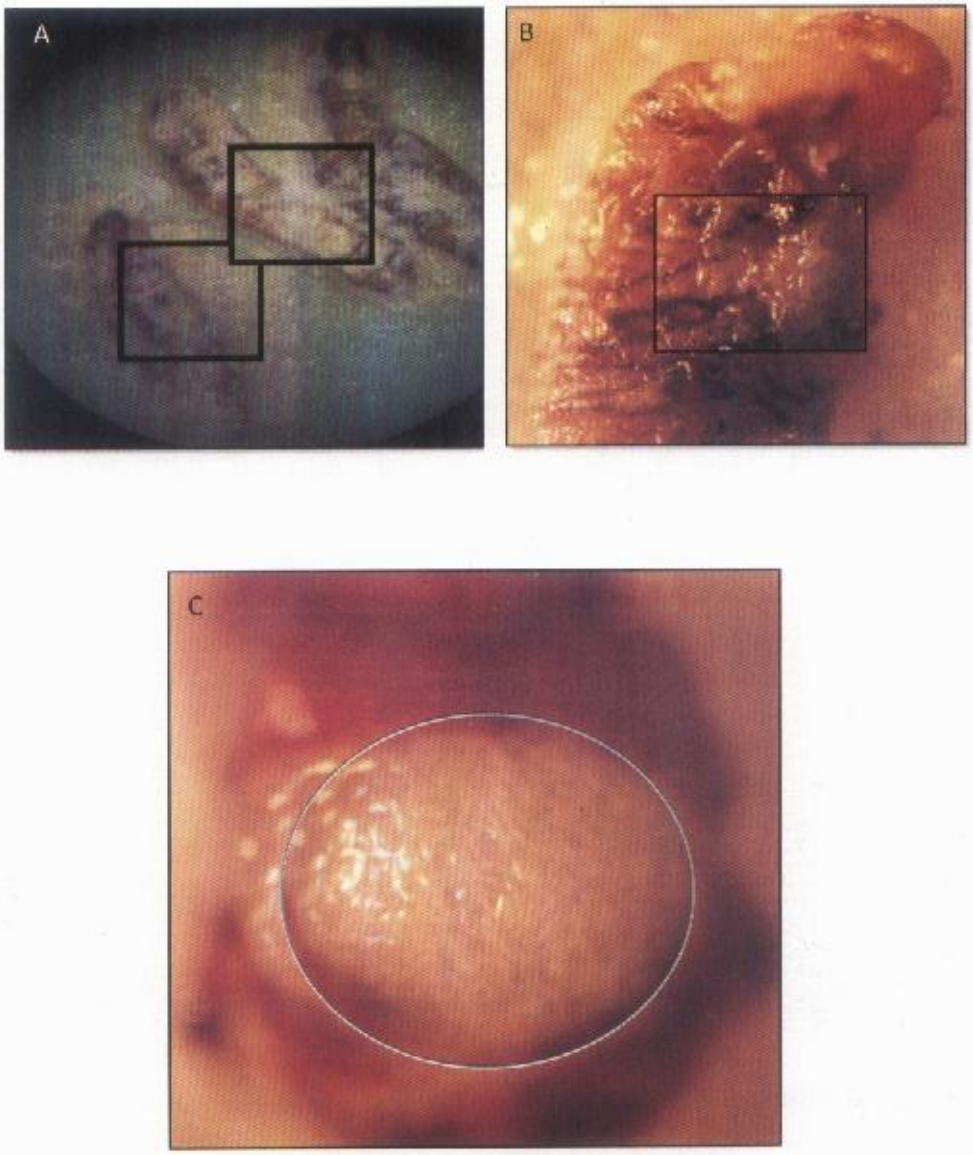


Fig. 2. (A-C). *In vivo* mass production of entomopathogenic nematodes: A. *G. mellonella* larva; B. *T. castanum* larva; C. *C. chinensis* adult.

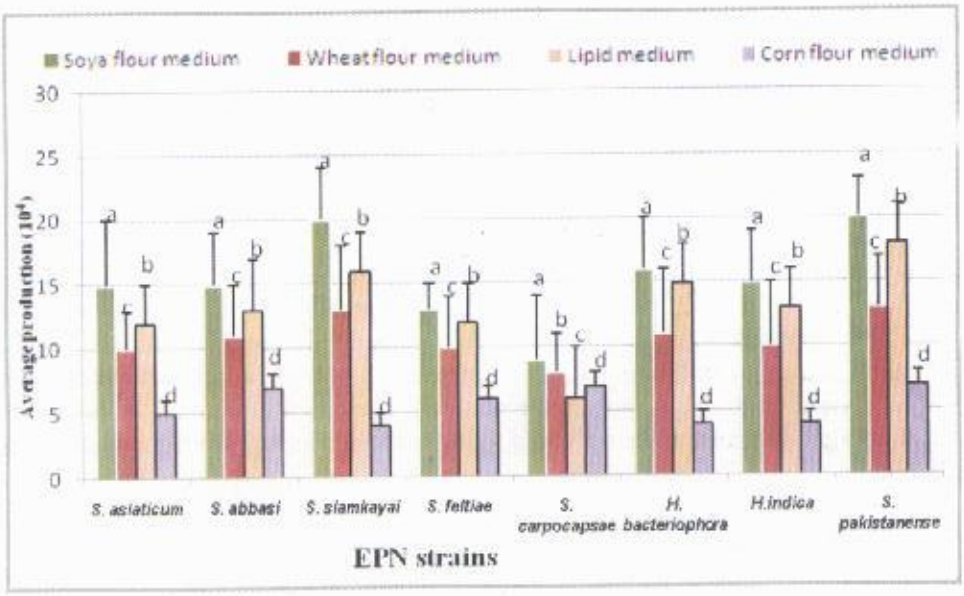


Fig. 3. *In vitro* production of entomopathogenic nematodes in four different artificial media.

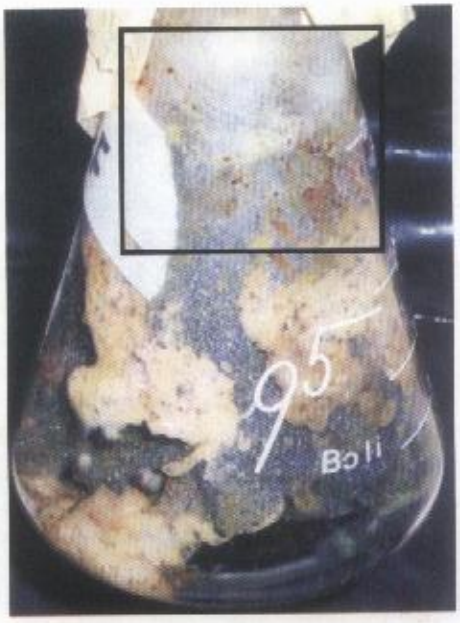


Fig. 4. *In vitro* production of entomopathogenic nematodes.

At 32 ± 2°C and 38 ± 2°C there is no significant difference between productions of *S. pakistanense* in *G. mellonella* larvae. Maximum production of all other species was found at 32 ± 2°C, except *S. feltiae* which failed to reproduce at this temperature (Table 3). Analysis clearly showed that production of infective juveniles was maximum in *G. mellonella* larvae as compared to soya flour medium (Fig. 5 A-D).

Mass production of EPNs on assemblage of *in vivo* and *in vitro* cultures: When *in vivo* culture *G. mellonella* larvae in assemblage with *in vitro* modified lipid agar medium was used, the production of infective juveniles of eight Pakistani strains of entomopathogenic nematodes increased approximately two fold as compared to when these were cultured separately in *G. mellonella* larvae and lipid agar medium. (Fig. 6). Lipid agar medium adopted from Kaya & Stock (1997).

Significant difference of production was found among the three media compared ($p < 0.001$). *S. pakistanense* produced 86.3×10^4, 177×10^4, 38×10^4 IJs; *S. asiaticum* produced 61×10^4, 112×10^4, 32×10^4 IJs; *S. siamkayai* produced 87×10^4, 181×10^4, 36×10^4 IJs; *S. feltiae* produced 60×10^4, 122×10^4, 32×10^4 IJs; *S. carpocapsae* produced 65.5×10^4, 123×10^4, 38×10^4 IJs; *S. abbasi* produced 80×10^4, 148×10^4, 33×10^4 IJs; *H. bacteriophora* produced 80×10^4, 164×10^4, 35×10^4 IJs; and *H. indica* produced 85×10^4, 155×10^4, 33×10^4 IJs from each *G. mellonella* larva, Petri plate of assemblage culture and lipid medium, respectively (Fig.7).

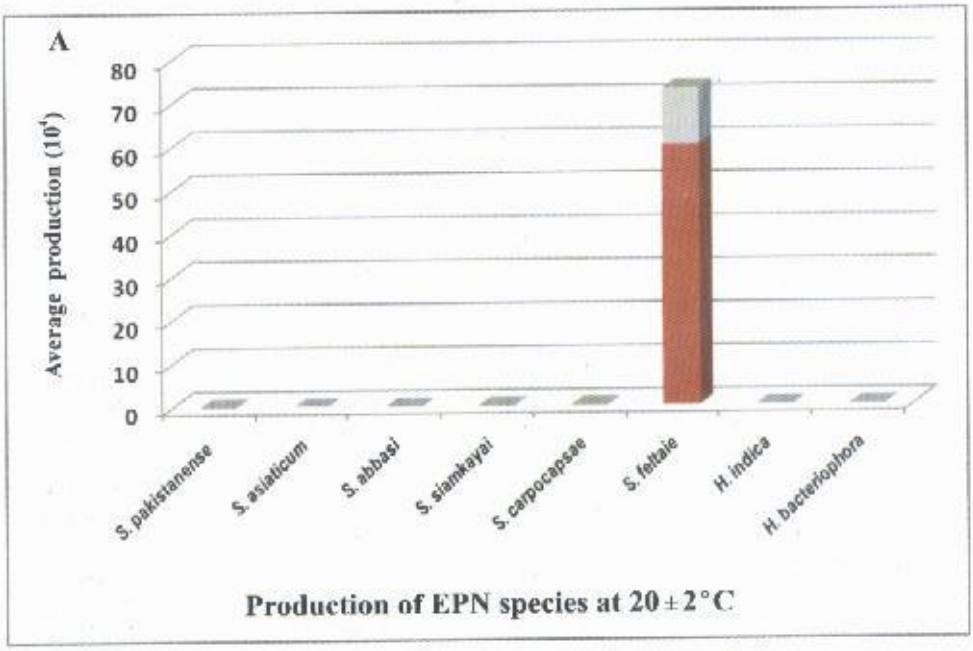
Discussion

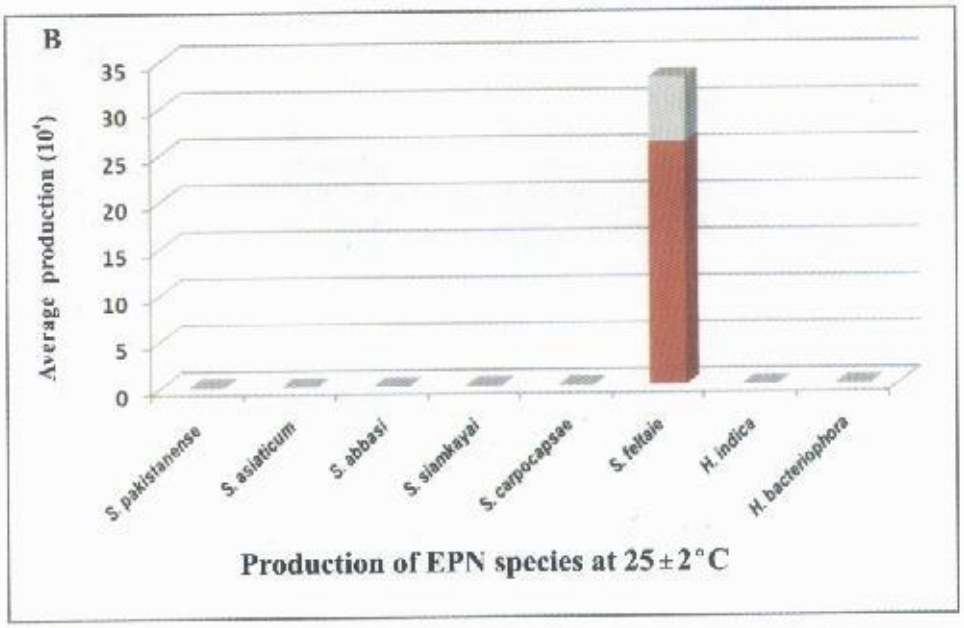
Steinernematids and Heterorhabditis infect and reproduce in a broad spectrum of insects; they may be readily reared *in vivo* in the laboratory. *Galleria mellonella* larvae is used as a host because it is widely available, easily reared, very susceptible and an excellent host for nematode reproduction. In the present study six different concentrations of infective juveniles of eight species were tested for mass production. At the lowest concentration production of eight indigenous species was 14×10^4- 26×10^4, 1.2×10^4- 3.2×10^4, 0.20×10^4- 0.51×10^4 IJs from each larva of *G. mellonella*, adult of *C. chinensis* and larva of *T. castaneum* respectively. According to Woodring & Kaya (1988) when high concentration of nematodes was applied few progenies were produced due to competition or contamination with foreign bacteria. But sometime dosage that is too low results in low host mortality, this reduces production efficiency. The number of nematodes that invade a host is proportional to the exposure concentration. Optimization of initial nematode density within the host maximizes nematode survival and fecundity.

Table 3. Effect of temperature on mass production of EPN in *G. mellonella* larvae and soya flour medium.

Nematodes species	Temperature	*G. mellonella* larvae average yield ($\times 10^3$)	Soya flour medium average yield ($\times 10^3$)
S. pakistanense PAK. S. *S.*43 strain (GU130180)	20 ± 2°C	0.0±0.0 b	0.0+0.0c
	25 ± 2°C	0.0±0.0 b	0.0±0.0 c
	32 ± 2°C	86.3±1.2 a	5.3±1.8 b
	38 ± 2 °C	87.5+1.5 a	7.1±1.8 a
S. asiaticum Pak. S. *S.* 4 strain (RFLP)	20 ± 2°C	0.0+0.0 c	0.0±0.0 c
	25 ± 2°C	0.0±0.0 c	0.0+0.0c
	32 ± 2°C	61.9+4.2 a	15±2.0 a
	38 ± 2 °C	28.9±1.1 b	8.0±2.5 b
S. abbasi Pak. S. *S.* 16 strain (JN571096)	20 ± 2°C	0.0+0.0 c	0.0±0.0 c
	25 ± 2°C	0.0±0.0 c	0.0±0.0 c
	32 ± 2°C	80.5±2.3 a	15+1.5 a
	38 + 2 °C	53.5±1.0 b	9.3±1.0 b
S. siamkayai Pak. S. *S.* 10 strain (JF892544)	20 ± 2°C	0.0±0.0 c	0.0+0.0 c
	25 ± 2°C	0.0+0.0 c	0.0±0.0 c
	32 ± 2°C	87.4±1.5 a	20 ±2.0 a
	38 ± 2 °C	62.9±2.0 b	12±1.0 b
S. carpocapsae Pak. S. *S.* 23 strain (GU130182)	20 ± 2°C	0.0±0.0 c	0.0+0.0c
	25 ± 2°C	0.0+0.0 c	0.0±0.0c
	32 ± 2°C	65.2±1.0 a	9.0±2.5 a
	38 ± 2 °C	45.8±1.5 b	4.0±2.0 b
S. feltiae Pak. N. *S.* 7 strain (RFLP)	20 + 2°C	60±2.0 a	13±1.0 a
	25 ± 2°C	26±1.5 b	7.0+1.0 b
	32 ± 2°C	0.0+0.0c	3.7±2.4 c
	38 ± 2 °C	0.0±0.0c	0.0+0.0c
H. indica Pak. S. *H.* 1 strain (JN157774)	20 ± 2°C	0.0+0.0 c	0.0±0.0c
	25 ± 2°C	0.0±0.0 c	0.0±0.0c
	32 ± 2°C	85.0±1.5 a	15±2.5 a
	38 + 2 °C	63.2±1.0 b	5.0±2.0 b
H. bacteriophora Pak. S. *H.* 17 strain (EF469774)	20 ± 2°C	0.0+0.0 c	0.0±0.0c
	25 ± 2°C	0.0±0.0 c	0.0+0.0c
	32 ± 2°C	80.5+1.2 a	16 .7±1.5 a
	38 + 2 °C	75.1±1.0 b	13.2 ±2.0 b

Values having same letters in a column are not significantly different.


A
Average production (10^4)
80
70
60
50
40
30
20
10
0
S. pakistanense
S. asiaticum
S. abbasi
S. siamkayai
S. carpocapsae
S. feltiae
H. indica
H. bacteriophora
Production of EPN species at 20±2°C


B
Average production (10^4)
35
30
25
20
15
10
5
0
S. pakistanense
S. asiaticum
S. abbasi
S. siamkayai
S. carpocapsae
S. feltiae
H. indica
H. bacteriophora
Production of EPN species at 25±2°C

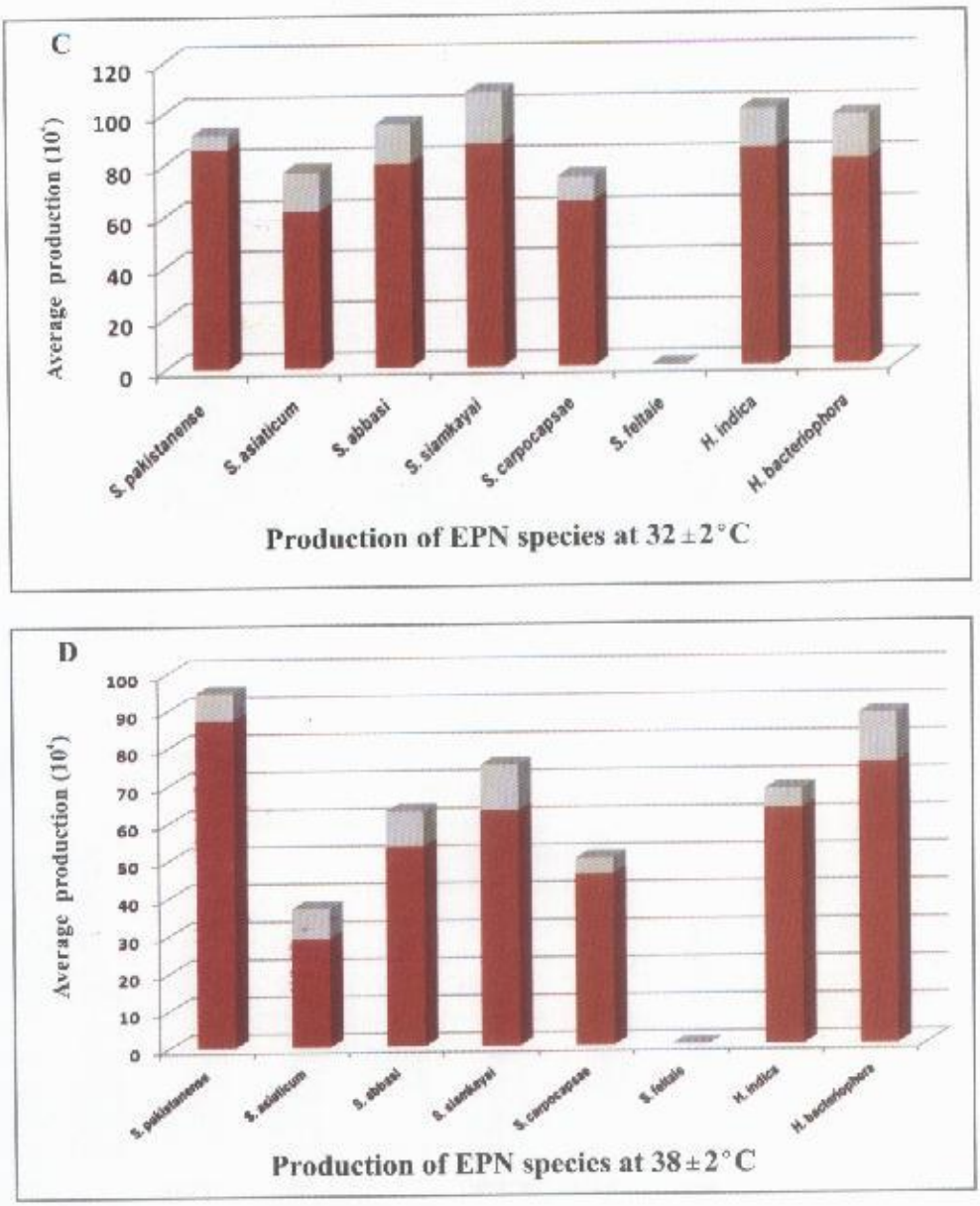


Fig. 5 (A-D). Average production of eight Pakistani strains of entomopathogenic nematodes at four different temperatures, cultured in *G. mellonella* larvae and soya flour medium.

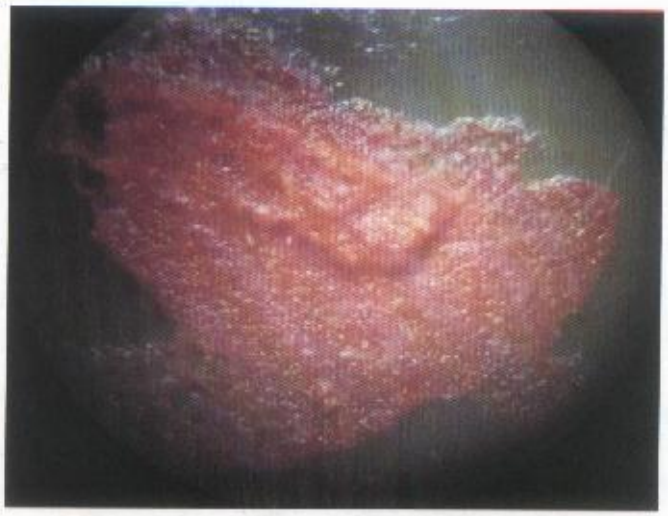


Fig. 6. Production of IJs on assemblage of *in vivo* and *in vitro* cultures.

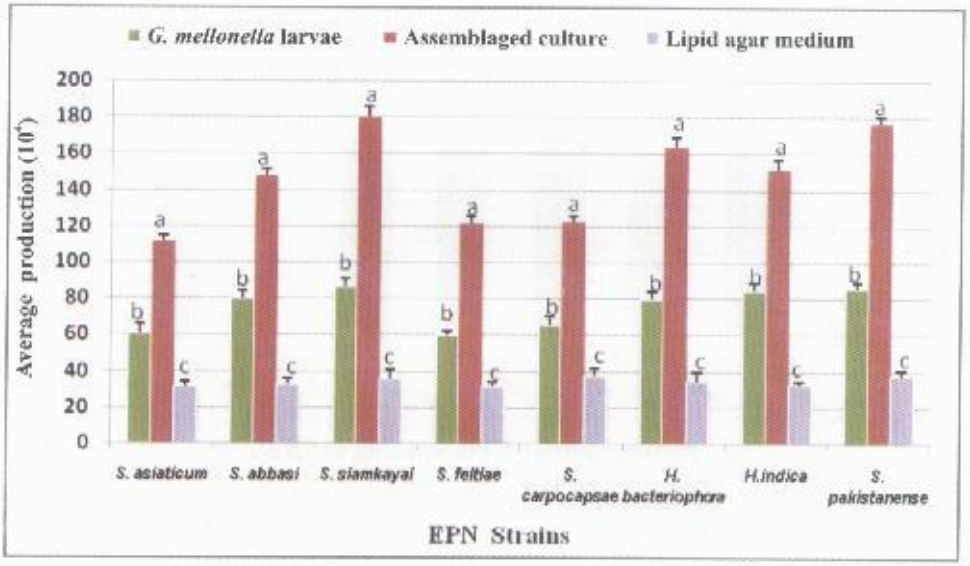


Fig. 7. Average production of IJs in *G. mellonella* larvae, assemblage culture and lipid agar medium.

Thus, intermediate dosage maximize yield (Boff *et al.*, 2000). According to Epsky & Capiner (1993) host density per unit area affects nematode invasion. In a current experiment at 50 IJs the production potential ranged up to 60 thousand to 87 thousands infective juvenile. Dutky *et al.*, (1964) and Milstead & Poinar (1978) reported that 200,000 *S. feltiae* and 350,000 *H. bacteriophora* infective juvenile have been harvested from one last instar *Galleria*. Hazir *et al.*, (2001) reported 80,000 IJs and Shapiro-Ilan *et al.*, (2001) reported up to 300,000 IJs from one *G. mellonella* larvae. *In vitro* production nematodes have been cultured on a variety of substrates: potato mash, ground veal pulp, dog food and chicken offal (McCoy & Glaser, 1936; Hara *et al.*, 1981). In Pakistan Tabassum & Shahina (2004) first time reported *in vitro* method for culturing entomopathogenic nematodes on chicken offal medium. With this method yield of *Steinernema pakistanense* and *H. indica* reached up to 5-7 millions. *S. asiaticum* and *S. feltiae* reproduction potential reached up to 4-5 millions and 1-2 millions, respectively per 500 ml flask. In present research work eight indigenous species of Pakistan were mass cultured on soya, wheat flour, lipid agar and corn flour medium. Soya flour medium gave highest population as compared to other medium. Minimum multiplication was found in corn flour medium. *In vivo* production of IJs in *G. mellonella* larvae and *in vivo* soya flour medium were also exposed to four different temperatures. Maximum production of seven species was found at 32 + 2°C except *S. feltiae* which fail to reproduce at this temperature. The poor production of *S. feltiae* may be due to the fact that this nematode is a naturally cold tolerant species with optimum infectivity closer to 20°C (Hominick & Briscoe, 1990; Wright, 1992; Grewal *et al.*, 1994). When S. *feltiae* Pak.N.S.7 strain (RFLP) was exposed to 20 ± 2°C production increased up to $60\pm2.0 \times 10^{4}$ and $13\pm1.0\times 10^{4}$ in *G. mellonella* larvae and in soya flour medium, respectively. Important factor for moxenically *in vitro* culture is the use of primary form bacteria, a large surface area on which the nematodes may grow a sterol source for the nematode and a food source base for the bacterium (Woodring & Kaya, 1988). Primary phase is most conducive to growth and infective juvenile tends to retain only the primary phase symbionts (Akhurst, 1982). A decrease in production could indicate contamination, a reversion to the secondary phase, unsuitable incubation temperatures, improper moisture contents or many other problems. In a present investigation first time *in vivo* culture *G. mellonella* larvae assemblage with *in vitro* modified lipid agar medium, it was found that nematodes yield increased approximately two fold as compared when it was cultured separately with *G. mellonella* larvae and lipid agar medium. The reason was that in assembled culture as the bacterial colonies grow in modified lipid agar simultaneously the production of IJs raised faster.

In our present research successful results were obtained for mass production of eight Pakistani strains, which can be applied for biological pest suppression. The problems faced in solid culture methods for harvesting infective juveniles

and microbial contamination. In this method of production it requires time and labors for harvesting juveniles and repeatedly cleaning which increases its cost while production in *G. mellonella* larvae minimized time, labor and contamination because infective progenies easily moved from dead cadaver through White trap method. Concerning about availability of *G. mellonella* larvae in Pakistan, it is only available in bulk amount from National Nematological Research Centre, University of Karachi (NNRC); but due to continuous rearing *G. mellonella* larvae in artificial diet, it loses its useful genetic traits. Hence, therefore, availability of this important biopesticides in Pakistan, in order to minimize impact of chemicals, we have to adopt advance technology (in pipe line of NNRC) for production of these valuable infective juveniles which can make these available to local markets. These findings would be useful for further extension of production from small scale to commercialization of Pakistani strains of EPNs.

Acknowledgement

We would like to thank Pakistan Science foundation (PSF) for financially supporting this work through project No. PSF/NSLP/S-KU (65).

References

Abu Hatab, M., Gaugler, R. & Ehlers, R.U. (1998). Influence of culture method on *Steinernema glaseri* lipids. *J. Parasitol.*, 84: 215-221.

Akhurst, R.J. (1982). Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. *J. Gen. Microbiol.*, 128: 3061-3066.

Bedding, R.A. (1981). Low cost *in vitro* mass production of *Neoaplectana* and *Heterorhabditis* species (Nematoda) for field control of insect pests. *Nematologica*, 27: 109-114.

Bovien, P. (1937). Some types of association between nematodes and insects. *Vidensk Medd Dan Naturhist Foren Khobenhavn*, 101: 1-114.

Bedding, R.A. (1984). Large-scale production storage and transport of the insect parasitic nematodes *Neoaplectana* spp. and *Heterorhabditis* spp. *A. Appl. Biol.*, 104: 117-120.

Boff, M.I.C., Wiegers, G.I., Gerritsen, L.J.M. & Smits, P.H. (2000). Development of the entomopathogenic nematode *Heterorhabditis megidis* strain NLH- E 87, 3 *in G. mellonella*. *Nematology*, 2: 303-308.

Dutky, S.R., Thompson, J.V. & Cantwell, G.E. (1964). A technique for mass production of the DD-136 nematode. *J. Insect. Pathol.*, 6: 417-422.

Epsky, N.D. & Capinera, J.L. (1993). Quantification of invasionof two strains of *S. carpocapsae* (Weiser) into three lepidopteran larvae. *J. Nematol.*, 25: 173-180.

Ehlers, R.U. (2001). Mass production of entomopathogenic nematodes for plant protection. *App. Microbiol. Biotechnol.*, 56: 623-633.

Ehlers, R.U. & Shapirollan, D.I. (2005). Mass production 65-78pp. In: *Nematode as biological control agent*. (Eds.) P.S Grewal, R. U. Ehler & D.I. Shapiro-Ilan. CAB International, Wallingford, UK.

Flanders, K.L., Miller, J.M. & Shields, E.J. (1996). *In vivo* production of *Heterorhabditis bacteriophora* 'Oswego' (Rhabditida: Heterorhabditidae), a potential biological control agent for soil inhabiting insects in temperate regions. *J. Econo. Entomol.*, 89: 373-380.

Grewal, P.S., Lewis, E.E., Gaugler R. & Campbell, J.F. (1994). Host finding behavior as a predictor of foraging strategy in entomopathogenic nematodes. *Parasitol.*, 108: 207-215.

Griffin, C.T., Boemare, N.E. & Lewis, E.E. (2005). Biology and Behaviour 47-64 pp. In: *Nematode as biological control agent*. (Eds.) P.S Grewal, R.U. Ehler & D.I. Shapiro-Ilan. CABI Publishing Willingford, UK.

Glaser, R.W. (1931). The cultivation of a nematode parasite of an insect. *Science*, 73: 614-615.

Han, R.C., Cao, L. & Liu, X. (1992). Relationship between medium composition, inoculum size, temperature and culture time in the yields of *Steinernema* and *Heterorhabditis* nematodes. *Fundam. Appl. Nematol.*, 15: 223-229.

Hara, A.H., Lindegren, J.E. & Kaya, H.K. (1981). Monoxenic mass production of the entomopathogenic nematode, *Neoaplectana carpocapsae* Weiser, on dog food / agar medium. *Adv. Agric. Technol., AAT-W-16 June 1981*: 1-8.

Hazir, S., Stock, S.P., Kaya, H.K., Koppenhöfer, A.M. & Keskin, N. (2001) Developmental temperature effects on five geographic isolates of the entomopathogenic nematodes *Steinernema feltiae* (Nematoda: Steinernematidae). *J. Inver. Pathol.*, 77: 243-250.

Hominick, W.M. & Briscoe, B.R. (1990). Occurrence of entomopathogenic nematode (Rhabditida: Steinernematidae and Heterorhabtidae) in British soil. *Parasitol.*, 100: 295-302.

Kaya, H.K. & Stock, S.P. (1997).Techniques in insect Nematology. In: *Manual of Techniques in Insect Pathology*. Academic Press Limited, NY.

Klein, M.G. (1990). Efficacy against soil-inhabiting insect pests. pp. 195–214. In: *Entomopathogenic Nematodes in Biological Control*. (Eds.) R. Gaugler & H.K. Kaya, CRC Press, Boca Raton, FL.

Mc Coy, E.E. & Glaser, R.W. (1936). *Nematode culture for Japanese Beetle Control.* New Jers. Dept. Agric. Cire, 265, 10p.

Milstead, J. E. & Poinar, G. O. (1978). A new entomogenous nematode for pest management for management system. *Calif. Agric.*, 32: 12.

Nguyen, K.B. & Smart, J.r. (1990). *Steinernema scapterisci* n. sp. (Rhabditida: Steinernematidae). *J. Nematol.*, 22: 187-199.

Poinar, G.O.Jr. & Thomas, G.M. (1965). A new bacterium, *Achromobacter nematophilus* Poinar and Thomas (Achromobacteriaceae: Eubacteriales) in the development of the nematode, DD-136 (Neoaplectana sp., Steinernematidae). *Parasitol.*, 56: 385-390.

Poinar, Jr.G.O. (1990). Taxonomy and biology of Steinernematidae and Heterorhabditidae. 23-61 pp. In: *Entomopathogenic nematodes in biological control* (Eds.) R. Gauglar and H.K. Kaya, CRC Press, Boca Raton, Boston.

Shapiro-Ilan, D.I. & Gaugler, R. (2002). Production technology for entomopathogenic nematodes and their bacterial symbionts. *J. Indust. Microbiol. Biotech.*, 28: 137-146.

Shapiro-Ilan, D.I, Lewis, E.E., Behle, R.W. & McGuire, M.R. (2001) Formulation of entomopathogenic nematode-infected cadavers. *J. Invert. Pathol.*, 78: 17-23.

Stoll, N.R. (1952). Axenic cultivation of the parasitic nematode *Neoaplectana glaseri* in a fluid medium containing raw liver extract. *J. Parasitol.*, 39: 422-444.

Tabassum, K.A. & Shahina, F. (2004). *In vitro* mass rearing of different species of entomopathogenic nematodes in monoxenic solid culture. *Pak. J. Nematol.*, 22: 167-175.

Woodring, J.L. & Kaya, H.K. (1988). *Steinernematid* and *Heterorhabditid nematodes: A handbook of techniques.* Southern Cooperative Series Bulletin 331. Arkansas Agricultural Experiments Station, Fayetteville, Arkansas. 28 pp.

Wouts, W.M. (1981). Mass production of the entomogenous nematode *Heterorhabditis heliothidis* (Nematoda: Heterorhabditidae) on artificial media. *J. Nematol.*, 13: 467-469.

White, G.F. (1927). A method for obtaining infective nematode larvae from cultures. *Science*, 66: 302-303

Wright, P.J. (1992). Cool temperature reproduction of Steinernematids and Heterorhabditis. *J. Invert. Pathol.*, 60: 148-151.

(Received for publication on 30th November, 2011)