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Mass multiplication of entomopathogenic nematodes in artificial media

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Abstract
This study evaluated ten different media of plant origin and four different media of animal origin for *in vitro* mass production of *Heterorhabditis bacteriophora*, *Steinernema aciari* and *Oscheius chongmingensis*. Maximum multiplication of all the tested species was observed in the media of plant origin containing green gram and coconut oil. Maximum multiplication of all the tested species was observed in the media of animal origin supplemented with pork waste homogenate.

Keywords: Entomopathogenic nematodes (EPNs), Mass production, biopesticides, artificial media of plant and animal origin

1. Introduction
Entomopathogenic nematodes have been shown to be lethal pathogenic to a wide range of agriculturally important pests and are useful alternative to chemical insecticides. Low-cost mass production of entomopathogenic nematodes is necessary for large-scale laboratory production and field application. The symbiotic bacteria associated with the nematodes plays a major role in the conversion of substrate into media suitable for the nematode multiplication and development [1]. Many studies have been carried out for mass culture of these nematodes *in vitro* on solid media [2-8] and *in vitro* on liquid fermentation production method [1]. Bedding three-dimensional monoxenic culture [5] is the best method in solid culture and it is presently used for commercial production of *Heterorhabditis* and *Steinernema* species. The present study was carried out to standardize a procedure for *in vitro* mass production of *Heterorhabditis bacteriophora*, *Steinernema aciari* and *Oscheius chongmingensis* by using different media of plant origin and animal origin.

2. Materials and Methods
2.1. Source of entomopathogenic nematodes (EPNs): *Heterorhabditis bacteriophora* and *Oscheius chongmingensis* were isolated from rhizosphere of citrus and coconut respectively from the experimental farm of the Department of Horticulture, Assam Agricultural University, Jorhat, Assam. *Steinernema aciari* was isolated from rhizosphere of mung bean from the Instructional Cum Research Farm, AAU, Jorhat, Assam. The nematodes were extracted using soil baiting technique using *Galleria mellonella* larvae as bait. All of these EPNs were cultured on *G. mellonella* larvae as per the procedure described by Woodring and Kaya [9].

2.2. Isolation of symbiotic bacteria
To isolate symbiotic bacteria, five final instar larvae of wax moth, *Galleria mellonella*, Linnaeus (Lepidoptera: Pyralidae), were inoculated with 500 IJs of each of the three species, viz. *H. bacteriophora*, *O. chongmingensis* and *S. aciari* in 100 µL distilled water placed in a Petri dish (65 mm) lined with double filter paper. Two days after inoculation, mutualistic bacteria (*Xenorhabdus sp.* from *S.aciari*, *Photorhabdus* sp. from *H. bacteriophora* and *Serratia* sp. from *O. chongmingensis*) were isolated from the cadavers according to the procedure of Kaya and Stock [10]. Cadavers were surface sterilized by dipping in absolute ethanol for 30 seconds, and allowing the ethanol to evaporate. The cadaver was held upside down with forceps and the head was removed with sterile scissors. The first drop of haemolymph which oozed out was discarded, but the second drop was deposited onto a plate of NBTA agar medium [11]; 2.3% (w/v) nutrient agar, 0.025% (w/v) bromothymol blue,

0.004% (w/v) 2-3-5 triphenyl-tetrazolium chloride (TTC) and was streaked with an inoculation loop, to make primary, secondary and tertiary streaks to yield isolated colonies [12, 13]. Plates were incubated at 27 °C for 72 h. After confirming colony morphology and colony colour, according to Boemare [14], a single phase I colony was selected and inoculated to 100 mL Nutrient Broth and incubated at 27 °C with shaking at 100 rpm for 48 h.

2.3. Mass production

To determine yield, 30 g of each production media was absorbed in 1 g of sponge cubes (measuring 0.5 cm^2 each) in 100 mL Erlenmeyer flasks and autoclaved at 121 °C (20 lb pressure per square inch) for 15min. The media were inoculated with 2 mL of 48 hour old bacterial suspension and incubated at 27 °C for 72 h. Infective juveniles (IJs) emerged from the *G. mellonella* larvae were surface sterilized with 0.1% Hyamine ® 1622 (Himedia) for 15 min and washed with sterilized distilled water thrice before inoculation. The IJs were inoculated aseptically into the flasks @ 1000 IJs /flask under laminar flow chamber. The sealed flasks were incubated at room temperature (28 $^0C \pm 2\ ^0C$) for 30 days. Care was taken not to shake the flasks after inoculation of nematodes. The colonies of nematodes started appearing on the walls of the flasks after 20 days of post inoculation. The experiment was laid out in Completely Randomized Design (CRD) with ten treatments and three replications under each treatment of plant origin and four treatments and five replications of animal origin media.

2.4. Extraction of infective juveniles from different media

After 30 days of inoculation of nematodes into the media, they were extracted from the media by transferring the foam chips from the flasks into a facial tissue paper which was spread over a 20 mesh aluminum wire mesh support. The flasks were thoroughly washed and washings were also added to the foam chips. The wire mesh support was then kept over a Petri dish filled with water, so that a thin film of water touched the material over the filter paper. The nematodes settled in the Petri dish were collected at frequent intervals and transferred to one liter beaker. The nematode-bacterial suspension in the flasks was allowed to settle and the supernatant was decanted. This process was repeated several times until a clear suspension of nematode was obtained. The total number of nematodes produced per conical flask (30 g media) from each replication was counted and the data recorded was subjected to analysis of variance.

3. Results and Discussion

3.1. Nematode yield in plant origin media

Results indicate the difference in suitability of different media tested for *in vitro* mass production of nematodes. Active third stage infective juveniles were recovered from all the different artificial media comprising of varied sources of proteins and lipids of plant origin (Table 1). The medium IX containing green gram flour and coconut oil recorded highest yield of *H. bacteriophora* (97.92 x 10^5 IJs/ flask), *S. aciari* (114.17 x 10^5 IJs/ flask) and *O. chongmingensis* (104.10 x 10^5 IJs/ flask) which were found to be at par with medium VIII containing green gram flour and soya oil. However, yield of infective juvenile of *H. bacteriophora*, *S. aciari* and *O. chongmingensis* was increased by 41.15%, 290.99% and 287.85% respectively in the medium IX over the Wout's medium and the percentage increase were more than the medium VIII. Besides these two media, medium VI containing Soya flour and coconut oil and medium VII containing green gram flour and sunflower oil were also recorded increase multiplication rate of all the three isolates over the control (Wout's media).Good multiplication of *O. chongmingensis* and *S. aciari* was noticed in medium IV containing soya flour and sunflower oil and medium V containing soya flour and soya oil that were increased multiplication over the Wout's media whereas multiplication of all the tested species was less in the media I, Media II and Media III. Multiplication of *H. bacteriophora* was less than *O. chongmingensis* and *S. aciari* in the Medium V containing soya flour and soya oil. Among the different plant media tested, the medium supplemented with green gram flour and coconut oil recorded the highest nematode yield of all the tested species of entomopathogenic nematodes. The medium supplemented with wheat flour and sunflower oil recorded the least multiplication rate of *O. chongmingensis* and media supplemented with wheat flour and soya oil recorded the least multiplication rate of *H. bacteriophora* and *S. aciari* indicating adverse effects on associated bacterium. Wouts [15] harvested a maximum of I0x10^6 IJs of *H. heliothidis* per 250 ml flask. Dunphy and Webster [16] compared production of *N. carpocapsae* DD136 strain when the lipid agar was supplemented with different concentrations of butter, halibut oil, cod liver oil, olive oil, corn oil and sunflower oil. Production was highest (294,000/plate) when sunflower oil was used at a concentration of 0.1 in 100 mL media. Han *et al*., [17] found that yeast extract, soy flour, egg and lard yielded a maximum of 38x10^6 IJs/flask of *S. carpocapsae* in 20 days at 19 °C. Hussaini *et al*., [18] revealed highest multiplication by rate of *H. indica* in medium containing soya flour and corn oil followed medium containing green gram flour and coconut oil which served as a source for proteins and lipids for the development of nematodes. Increased yield of *S. carpocapsae* and *S. tami* was recorded when corn oil in Wout's media was replaced with soya oil [19]. Media composition had a substantial effect on quality and quantity of IJs produced [20]. Lipids and constitutive fatty acids are the main energy sources that provide 60% of the total energy for the IJs of EPN and their symbiotic bacteria [21-23]. The variation of other media components such as proteins and salts may also have an effect on nematode production [16]. The average mass production of *H.bacteriophora* in the media comprises of 2 x nutrient broths supplemented with a 2% agar and 1% olive oil with 500 IJs/cm^2 of surface areas was 5.5x10^5IJs/gram [24]. The observed differences in our results and that of Wout's could be due to variation in species used and other culture media.

3.2. Nematode yield in animal origin media

From the data, it is revealed that among the different animal media tested, the medium III supplemented with pork waste homogenate recorded the highest nematode yield of all the tested species of entomopathogenic nematodes (Table 2). Highest yield of *H. bacteriophora*, *S. aciari* and *O. chongmingensis* were 6.54x10^5, 17.24x10^5, 5.99x10^5 IJs/flask respectively, where as the medium supplemented with goat waste homogenate recorded the least multiplication rate of all the tested species viz. *H. bacteriophora* (3.67x10^5 IJs/flask), *S. aciari* (6.75x10^5 IJs/flask) and *O. chongmingensis* (3.47x10^5 IJs/flask) respectively. There is no significant difference in yield of *H. bacteriophora* between the medium III and IV (control), but the per cent increase in yield was more in the medium III containing pork waste homogenate

than the control. The pork homogenate medium recorded highest multiplication rate of *H. bacteriophora* and *O. chongmingensis* which was found to be at par with medium I containing dog biscuit. Among the different animal media tested, the medium supplemented with pork homogenate recorded the highest yield of *S. aciari* where as the medium supplemented with goat waste homogenate recorded the least multiplication rate of *O. chongmingensis.* With Bedding's method, normally 30-50 million infective juveniles (IJ) of *Heterorhabditis* and *Steinernema* can be produced in a single 500 ml flask containing 80-100 g of chicken offal medium, but yields vary from very poor (a few million) to a record high of over 100 million per flask, and both the quality and quantity of nematodes from successive cultures can decrease significantly [25]. The cause for the low yield may be due to presence of complex fatty acid chains in the medium as reported by Fodor *et al*., [26]. Tabassum and Shahina [27] mass multiplied *S. pakistanense*, *S. asiaticum*, *S. feltiae* and *H. indica* using the chicken offal media yielding 5-7, 4-5, 1-2, 5-7 millions of IJs respectively per 500 mL flask. El-Sadawy [28] reported that seven species of EPNs failed to reproduce on dog food agar, but they were all successfully produced on modified Wout's medium containing soy flour as the primary source of protein. Somwong and Petcharat [29] observed $3.04x10^5$, $2.45x10^5$ and $2.98x10^5$ *Steinernema carpocapsae* IJs/g using different media such as dog food, powdered fish and silk warm pupa. The method and the media used for mass culture might also affect the bacterial load carried by the infective juveniles and its activity [30].

Table 1: Yield of Entomopathogenic nematode in different media of plant origin. (Mean of 3 replications)

Medium	*H. bacteriophora*		*S. aciari*		*O. chongmingensis*	
	IJs ($x10^5$) /100 ml flask	**% increase or decrease over control**	**IJs ($x10^5$) /100 ml flask**	**% increase or decrease over control**	**IJs ($x10^5$) /100 ml flask**	**% increase or decrease over control**
M I: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Wheat flour 7.2 g + Sunflower oil 5.2 ml + sponge 1.0 g + Water 27 ml)	6.36^g	90.79 (-ve)	2.77^f	90.51 (-ve)	3.68^g	86.28 (-ve)
M II: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Wheat flour 7.2 g + Soya oil 5.2 ml + sponge 1.0 g + Water 27 ml)	5.19^g	92.49 (-ve)	2.67^f	90.85 (-ve)	4.48^g	83.30 (-ve)
M III: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Wheat flour 7.2 g + Coconut oil 5.2 ml + sponge 1.0 g + Water 27 ml)	49.57^e	28.33 (-ve)	24.74^e	15.57 (-ve)	19.07^f	28.94 (-ve)
M IV: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Soyaflour 7.2 g + Sunflower oil 5.2 ml + sponge 1.0 g + Water 27 ml)	56.66^d	18.08 (-ve)	67.35^c	130.65 (+ve)	35.47^d	32.15 (+ve)
M V: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Soya flour 7.2 g + Soya oil 5.2 ml + sponge 1.0 g + Water 27 ml)	10.18^f	82.28 (-ve)	69.83^c	139.14 (+ve)	63.98^c	138.37 (+ve)
M VI: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Soya flour 7.2 g + Coconut oil 5.2 ml + sponge 1.0 g + Water 27 ml)	71.17^c	2.89 (+ve)	53.54^d	83.35 (+ve)	34.79^d	29.61 (+ve)
M VII: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Greengram flour 7.2 g + Sunflower oil 5.2 ml + sponge 1.0 g + Water 27 ml)	89.30^b	29.10 (+ve)	93.11^b	218.86 (+ve)	90.20^b	236.06 (+ve)
M VIII: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Greengram flour 7.2 g + Soya oil 5.2 ml + sponge 1.0 g + Water 27 ml)	95.20^a	37.63 (+ve)	111.39^a	281.47 (+ve)	100.60^a	274.81 (+ve)
M IX: (Nutrient broth 0.44 g + Yeast extract 0.16 g + Greemgram flour7.2 g + Coconut oil 5.2 ml + sponge 1.0 g + Water 27 ml)	97.92^a	41.15 (+ve)	114.17^a	290.99 (+ve)	104.10^a	287.85 (+ve)
M X: (Control: Wout's medium) (Nutrient broth 0.44 g + Yeast extract 0.16 g + Soya flour 7.2 g + Corn oil 5.2 ml + sponge 1.0 g + Water 27 ml)	69.17^c		29.2^e		26.84^e	
CD:(P=0.05)	4.60		4.69		4.67	
CV%	8.50		8.39		9.84	

Means followed by the same letter are not significantly different

Table 2: Yield of Entomopathogenic nematode in different media of animal origin (Mean of 5 replications)

Medium	*H. bacteriophora*		*S. aciari*		*O. chongmingensis*	
	IJs(x10^5) /100 ml flask	% increase or decrease over control	IJs(x10^5) /100 ml flask	% increase or decrease over control	IJs(x10^5) /100 ml flask	% increase or decrease over control
MI: (Nutrient broth 0.5 ml + Dog biscuit 4.0 g + sponge 1.0 g + water 25 ml)	6.14^{ab}	6.78 (+ve)	10.69^{b}	56.28 (+ve)	5.45^{ab}	15.95 (+ve)
MI: (Nutrient broth 0.5 ml + Goat waste homogenate 4.0 g + sponge 1.0 g + water 25 ml)	3.67^{c}	36.17 (-ve)	6.75^{c}	1.31 (-ve)	3.47^{c}	26.17 (-ve)
MIII: (Nutrient broth 0.5 ml + Pork waste homogenate 4.0 g + sponge 1.0 g + water 25 ml)	6.54^{a}	13.73 (+ve)	17.24^{a}	152.04 (+ve)	5.99^{a}	27.44 (+ve)
MIV: (Control: Bedding's media) (Nutrient broth 0.5 ml + Chicken offal homogenate 4.0 g + sponge 1.0 g + water 25 ml)	5.75^{ab}		6.84^{c}		4.70^{b}	
CD(P=0.05)	0.89		1.17		0.75	
CV%	18.82		10.3		20.84	

Means followed by the same letter are not significantly different

4. Conclusion

Further manipulation of the media may be necessary for initiating multiplication. Future studies may focus on the use of natural raw products as solid media components, making production cheaper.

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