International Journal of Research Studies in Zoology (IJRSZ)
Volume 3, Issue 2, 2017, PP 45-50
ISSN 2454-941X
http://dx.doi.org/10.20431/2454-941X.0302003
www.arcjournals.org

Evaluation of Different Solid Media for Mass Production of Native Entomopathogenic Nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* Isolated from Cotton Fields

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Abstract: Thirteen different plant and animal protein media such as nutrient agar medium-I, nutrient agar medium-II, Wouts medium, modified Wouts medium, wheat flour medium, modified wheat flour medium, egg yolk medium-I, egg yolk medium-II, modified egg yolk medium, dog biscuit medium, modified dog biscuit medium, bengal gram medium-I and bengal gram medium-II were tested for mass multiplication of entomopathogenic nematodes such as *Heterorhabditis bacteriophora* strain KKMH1 and *Steinernema carpocapsae* strain APKS2 by i*n vitro* solid culture technique. Results showed that modified Wouts medium and nutrient agar medium-I multiplied significantly higher numbers of 39.02 - 39.14 lakh IJs of *H.*. *bacteriophora* strain KKMH1 per 250 ml flask. The IJs produced from these media were more virulent to cotton pest such as *Helicoverpa armigera*, *Earias vitella* and *Spodoptera litura.* For multiplication of *Steinernema carpocapsae* strain APKS2, modified dog biscuit medium and nutrient agar medium-I got significantly higher IJ populations of 49.69 - 52.31 lakh /250 ml flask. The IJs multiplied on these media caused 100% mortality of *H. armigera*, *E. vitella* and *S. litura*. The results of this study suggest that mass production of the *H. bacteriophora* strains KKMH1 on modified wouts medium or nutrient agar medium-I and *S. carpocapsae* strain APKS2 on modified dog biscuit medium or nutrient agar medium-I are promising option while promotion of these strains as potential bio-pesticide against cotton insect pests *H. armigera*, *E. vitella* and *S. litura*.

Keywords: Entomopathogenic nematodes, mass multiplication, different media composition, Virulence, *Helicoverpa armigera*, *Earias vitella* and *Spodoptera litura*, cotton.

Cotton (*Gossypium* spp.), popularly known as 'white gold', is an important cash crop of India. One of the prime challenges to attain high cotton production is damage caused by insect pests. Among insect pests of cotton, bollworm complex consisting of American bollworm *Helicoverpa armigera* Hub. (Lepidoptera: Noctuidae), spotted bollworm *Earias vitella* Fab. (Lepidopter: Noctuidae) and cotton leafworm *Spodoptera litura* (Fabricius) are most important pests affecting the cotton plants and they can cause yield loss of 20 – 80% [1, 2]. The habit of developing resistance to many insecticides including Bt transgenic cotton necessitate to find out an alternate strategy to manage bollworms. In this situation, exploitation of naturally occurring entomopathogenic nematodes (EPN) from two families *viz*, Heterorhabditidae and Steinernematidae to develop biopesticide for the control of cotton bollworms is an ecologically sound novel approach. These nematodes are characterized by their ability to carry specific pathogenic bacteria, *Photorhabdus* with Heterorhabditidae and *Xenorhabdus* with Steinernematidae, which are released into the insect haemocoel after penetration of the insect hosts by the infective stage of the nematodes. Most biocontrol agents require days or weeks to kill the pest, but entomopathogenic nematodes with their symbiotic bacteria kill insects in 24-48 hr. Many cotton pests like bollworm *Helicoverpa zea*, fall armyworm *Spodoptera frugiperda*, beet armyworm *Spodoptera exigua*, cabbage looper *Trichoplusia ni*, tobacco budworm *Heliothis virescens* and the pink bollworm *Pectinophora gossypiella* are susceptible to entomopathogenic nematodes [3, 4]. The infectivity of EPN species *Steinernema carpocapsae*, *S. riobravus* and *S. feltiae* on *H. arimigera* and *E. insulana* was established earlier [5, 6]. Gassmann et al., [7] demonstrated the successful field control of pink bollworm, *P. gossypiella* using an EPN species *Steinernema riobrave* on cotton.

Exploring indigenous EPN in cotton fields of Tamil Nadu state in India, Seenivasan et al., [8] recovered 27 strains belonging to 16 *Steinernema carpocapsae*, 3 *Steinernema siamkayai*, 1 *Steinernema monticolum* and 7 *Heterorhabditis bacteriophora* from cotton ecosystem. Later, Seenivasan and Sivakumar [9] established that all the 27 EPN strains were suitable host for cotton bollworms *H. armigera*, *E. vitella* and *S. litura*. Among them, the strains of KKMH1 (*H. bacteriophora)* and APKS2 (*S. carpocapsae)* showed the advantages such as more virulence against *H. armigera*, *E. vitelli* and *S. litura;* high reproduction potential; tolerant to heat and desiccation [9]. The successful implementation of these EPN strains as potential bio-pesticide against cotton bollworms is possible only after standardization of suitable mass production technique. EPN can be multiplied by either *in vivo* or *in vitro* culture techniques. The *in vivo* production is very simple, but this method is labour intensive and mainly used to maintain EPN culture. The *in vitro* culture technique has more potential to mass produce EPN as it yield very high EPN with reduced cost, labour and equipment when compared to the *in vivo* production methods. Earlier EPN were grown on *in vitro* solid medium axenically, but later it was understood that presence of symbiotic bacterium is essential for multiplication of EPN. Nowadays *in vitro* culturing of EPN involves introduction of infective juveniles (IJs) to a monoculture of their symbiont either *Photorhabdus* or *Xenorhabdus* in the nutrient medium. The solid culture technology using ployether-polyurethane sponge has been proved the most successful method and is now exploited for mass production of *Steinernema* and *Heterorhabditis* species [10]. The nutritional composition of the culture medium is an important factor to determine the yield potential of particular EPN strain [11]. The inappropriate media composition apparently produces less virulent symbiotic bacteria which in turn may result in less pathogenic EPN with reduced storage stability. Hence, the present work was attempted to standardize suitable plant or animal based media to mass produce virulent *H. bacteriophora* (KKMH1) and *S. carpocapsae* (APKS2).

1. MATERIALS AND METHODS

EPN culture

Two EPN strains namely KKMH1 (*H. bacteriophora)* and APKS2 (*S. carpocapsae*), earlier isolated by baiting of soil samples from different cotton fields of Tamil Nadu, India were taken from the Department of Nematology, TNAU, Coimbatore, India. They were cultured in the laboratory at room temperature using last instar larvae of the rice grain moth, *Carcyra cephalonica* (Lepidoptera: Pyralidae) as described by Kaya and Stock [12]. The infective juveniles (IJs) releasing from *C. cephalonica* larval cadavers were collected in sterile distilled water using a modified White's trap [12], sterilized in formalin (0.05%) solution and maintained on aerated sterile water in plastic tissue-culture flasks at 15°C. Before inoculation, IJs were surface sterilized by suspending in 0.1% $HgCl_2$ for 10 min followed by 3-4 time washing with distilled water by the method of centrifugation at 5000 rpm for 5 min.

Symbiotic bacteria culture

The EPN strains KKMH1 (*H. bacteriophora*) and APKS2 (*S. carpocapsae*) were made to infect 4th instar larvae of *C. cephalonica*. Then 24 h later, the infected *C. cephalonica* were first surface sterilized with 70% ethanol, dissected aseptically and the infected hemolymph was streaked on plates containing NBTA medium (2.3 g nutrient agar, 0.025 g bromothymol blue and 0.004 g triphenyl-tetrazolium chloride in 100 ml distilled water). The plates were incubated 25°C for 48 h and then *Xenorhabdus* spp. (bacterial colonies showing dark blue) from APKS2 and *Photorhabdus* spp. (bacterial colonies showing green) from KKMH1 were isolated and grown in nutrient broth. *Xenorhabdus* spp. and *Photorhabdus* spp. grown in nutrient broth (3 g beef extract, 5 g peptone and 8 g NaCl in 1 L of distilled water) was used as inoculum for solid *in vitro* culture. Bacteria containing 1x 10^5 cfu/ml were used as inoculum.

Evaluation of different media

In vitro multiplication of *H. bacteriophora* KKMH1 and *S. carpocapsae* APKS2 were tried in 13 different plant and animal protein media *viz*., nutrient agar medium-I, nutrient agar medium-II, Wouts medium, modified Wouts medium, wheat flour medium, modified wheat flour medium, egg yolk medium-I, egg yolk medium-II, modified egg yolk medium, dog biscuit medium, modified dog biscuit medium, bengal gram medium-I and bengal gram medium-II. The composition of the medium per 100 ml distilled water was detailed here; *Nutrient agar medium I* - beef extract 0.3 g, peptone 0.5

g, agar 0.2 g, NaCl 0.8 g and chicken fat 10 g; *Nutrient agar medium II* - beef extract 0.3 g, peptone 0.5 g, agar 0.2 g, NaCl 0.8 g and soybean oil 5 g; *Wout's medium* - nutrient broth 0.88 g, yeast extract 0.32 g, soyflour 14.40 g and groundnut oil 10.40 g; *Modified Wout's medium* - nutrient broth 0.88 g, yeast extract 0.32 g; soyflour 14.40 g, groundnut oil 10.40 g and beef extract 5 g; *Wheat flour medium* - wheat flour 15.0 g, kabuligram flour 5 g, beef extract 5 g, yeast extract 6 g, agar 1 g and coconut oil 6.0 g; *Modified wheat flour medium* - wheat flour 15.0 g, soyflour 5.0 g, beef extract 5.0 g, yeast extract 1 g, and groundnut oil 10.0 g; *Egg yolk medium I* – spray dried egg yolk powder (SDEY) 7.0 g, yeast extract 2.0 g, NaCl 0.80 g and oil 15.0 g; *Egg yolk medium II* - SDEY 10.0 g, yeast extract 5.0 g, NaCl 0.80 g and oil 12.0 g; *Modified egg yolk medium* - egg yolk 7.0 g,soyflour 20 g, yeast extract 2.0 g, NaCl 0.80 g and oil 15 g: *Dog biscuit medium* - dog biscuit 15.0 g, yeast extract 1.0 g, peptone 3.0 g, agar 2.0 g and oil 10.0 g; *Modified Dog biscuit medium* - dog biscuit 20.0 g, pepton 0.5 g, yeast extract 1.0 g, beef extract 5 g and oil 7.0 g; *Bengal gram medium I* - nutrient broth 1.5 g; yeast extract 0.7 g, bengal gram flour 20 g and ground nut oil 20 g ; and *Bengal gram medium II* - nutrient broth 1.75 g, yeast extract 0.5 g, bengal gram flour 20 g and gingelly oil 32.5 g. Each media were poured into 250 ml volumetric flasks containing sterile 6 pieces of 1.5 cm^3 polyurethane sponge. Each media were replicated 5 times (one flask = one replicate). Then the flasks were sterilized at 10 psi for 10 min in autoclave. Concerned symbiont bacterial culture were inoculated in each flask, shacked well and incubated 25°C for 48 h to assure bacterial growth. Then concerned EPN strain IJs were inoculated at 5000 per flasks and incubated for 20 days at 25°C. Then IJs produced from different media were washed several times with distilled water and harvested using 625 mesh (20 µm) sieve. The number of IJs were counted under steriozoom microscope after appropriate dilutions. Then IJs were store in 0.05% formalin suspension at refrigerator and aerated once in two days.

Virulence test

The IJs of *H. bacteriophora* KKMH1 and *S. carpocapsae* APKS2 multiplied form different media were then tested for their virulence against cotton bollworms *H. armigera, E. vitella* and *S. litura*. The test insect larvae (*H. armigera, E. vittella* and *S. litura*) were collected from a standing cotton crop on TNAU farm and from the farmer's fields at Thondamuthur village in Coimbatore district of Tamil Nadu. They were sorted out and the fourth instar larvae of uniform size were used in the experiments. The test were conducted in 6-cm-diam petri dishes lined with moist filter paper disc. Five forth instar larvae of *H. armigera, E. vittella* and *S. litura* were put in petri dishes lined with filter paper. Then 1 ml of appropriate EPN strain multiplied in different media containing 1000 IJs were applied to the petri dishes. Control plates received only 1 ml distilled water. The dishes were sealed with parafilm, arranged in a completely randomized design (CRD) and incubated at room temperature. Each treatment consisted of five replicates (One Petri dish = one replicate). After 4 days, larval mortality was recorded. The dead insects were dissected in Ringers solution to confirm the death by EPN. The experiments were repeated once.

2. RESULTS AND DISCUSSION

The number of IJs of *H. bacteriophora* strain KKMH1 harvested from different test media and their virulence on *H armigera, E vitella* and *S. litura* are presented in table 1. The multiplication of *H. bacteriophora* strain KKMH1 on different media ranged from 6.32 – 39.17 lakh IJs/250 ml flasks. The Modified wout's medium and Nutrient agar medium-I were recorded significantly higher multiplication of 39.02 - 39.14 lakh IJs/250 ml flask which were statistically on par. The moderate multiplication of 25.67 - 26.73 lakh IJs/250 ml flask was observed in Nutrient agar medium-II and Wout's Medium. The multiplication was significantly least in Bengal gram medium-I and Bengal gram medium-II. The wheat flour medium, modified wheat flour medium, egg yolk I, egg yolk II, modified egg yolk medium, dog biscuit medium did not produce any *H. bacteriophora* IJs and hence these media were considered not suitable for mass production of *H. bacteriophora* strain KKMH1. Hatab and Gaugler [11] reported that the composition of *in vitro* solid culture media have the great influence on multiplication of *H. bacteriophora* and increasing the lipid quantity and quality of media lead to tremendous increase in IJs yield. Similarly, in this study the Modified wout's medium containing beef extract and Nutrient agar medium-II having chicken fat were yielded more IJs of *H. bacteriophora* strain KKMH1. Earlier Hussaini et al., [13] observed the highest multiplication of *Heterorhabditis indica* on Wout's medium. In this

study the Wout's medium yielded relatively less IJs of *H. bacteriophora*, but addition of beef extract improved the IJs yield. Many studies proved the importance of media composition, which should satisfy growth conditions of both nematode and its symbiont [14]. This study indicate that media composition of wheat flour medium, modified wheat flour medium, egg yolk I, egg yolk II, modified egg yolk medium, dog biscuit medium are not suitable for growth of *Photorhabdus* spp associated with *H. bacteriophora* strain KKMH1.

Table1. Multiplication of *Heterorhabditis bacteriophora* strain KKMH1 by *in vitro* solid culture on different artificial media and the virulence of IJs harvested from different media on 4th instar larvae of *Helicoverpa armigera*, *Earias vitella* and *Spodoptera litura*.

Test media	Number of IJs harvested per 250 ml/flask (in lakhs)	Larval mortality (%)		
		Helicoverpa armigera	*Earias vitella*	*Spodoptera litura*
Nutrient agar medium I	39.02 a	100.0 a	100.0 a	100.0 a
Nutrient agar medium II	25.67 b	94.3 a	92.5 b	94.0 a
Wout's Medium	26.73 b	95.4 a	94.6 a	96.3 a
Modified wout's medium	39.14 a	100.0 a	100.0 a	100.0 a
Wheat flour medium	X	-	-	-
Modified Wheat flour medium	X	-	-	-
Egg yolk I	X	-	-	-
Egg yolk II	X	-	-	-
Modified egg yolk medium	X	-	-	-
Dog biscuit medium	X	-	-	-
Modified Dog biscuit medium	X	-	-	-
Bengal gram medium I	6.32 c	72.6 b	64.5 c	78.4 b
Bengal gram medium II	9.41 c	78.6 b	70.2 c	84.6 b
CD at 5%	5.82	7.32 b	6.95	8.42
SED	2.61	3.28	3.16	3.86

X – No multiplication; Means followed by the same letter in columns are not significantly different at P<0.05 according to Duncan's multiple rang test.

The significant variation in the virulence of *H. bacteriophora* IJs produced from different media was observed (Table 1). The IJs produced from Modified wout's medium and Nutrient agar medium-I caused 100% mortality of *H. armigera*, *E. vitella* and *S. litura* where as IJs multiplied on Bengal gram medium-I and Bengal gram medium-II caused significantly lease mortality of *H. armigera* (72.6 – 78.6%), *E. vitella* (64.5 – 70.2%) and *S. litura* (78.4 – 84.6%). The IJs produced from Nutrient agar medium-II and Wout's Medium caused 94.3 -95.4% mortality of *H. armigera*, 92.5 - 94.6% mortality of *E. vitella* and 94.0 – 96.3% mortality of *S. litura*. The pathogenicity of EPN species *H. bacteriophora* against *H. armigera*, *E. vitella* and *S. litura* was reported earlier [9]. This study confirmed the earlier findings and established the lethal effect of IJs multiplied on different media. In addition, this study showed that the virulence of *H. bacteriophora* IJs varied when multiplied on different media. Similar results of the differences in the quality of *H. bacteriophora* IJs produced from different culture media was reported by Hatab and Gaugler [11]. They observed that the sterol and oleic acid contents of IJs is highly variable when they multiplied on different media and virulent populations are rich in sterol and oleic acid contents. Susurluk et al., [15] also observed 76 – 100% mortality of *Galleria mellonella* larvae due to different batches of *H. bacteriophora* IJs multiplied on different media which is in line with our results.

The *S. carpocapsae* APKS2 was multiplied on 11 media out of 13 tried (Table 2). The Wheat flour medium and Dog biscuit medium did not multiply *S. carpocapsae*. There was significant variation exist among 11 media to produce *S. carpocapsae* IJs. The Modified Dog biscuit medium and Nutrient agar medium-I got significantly higher number of IJs than other test media. The multiplication in these media was 49.69 - 52.31 lakh IJs/flask. The Modified Wheat flour medium, Bengal gram medium-I and Bengal gram medium-II recorded the significantly least production of 9.62 - 13.26 lakh IJs/flask. The results are in accordance with Hussaini et al., [13] who observed that Wout's medium, modified egg yolk, soyflour cholesterol media and modified dog biscuit yielded highest number of *S. carpocapsae* IJs. The potential of Nutrient agar medium-I to

get highest number of EPN IJs also demonstrated by Rabhaji [16]. Somwong and Petcharat [17] tried different media composition and produced 2.44 - 5.54 lakh IJs *S. carpocapsae*. This result is similar to that of present study.

Table 2. Multiplication of *S. carpocapsae* APKS2 by *in vitro* solid culture on different artificial media and the virulence of IJs harvested from different media on 4th instar larvae of *Helicoverpa armigera*, *Earias vitella* and *Spodoptera litura*.

Test media	Number of IJs harvested per 250 ml/flask (in lakhs)	Larval mortality (%)		
		Helicoverpa armigera	*Earias vitella*	*Spodoptera litura*
Nutrient agar medium I	49.69 a	100.0 a	100.0 a	100.0 a
Nutrient agar medium II	38.72 b	96.2 a	94.3 a	97.6 a
Wout's Medium	23.61 c	94.5 a	92.8 a	96.3 a
Modified wout's medium	18.52 c	84.3 b	82.6 b	83.6 b
Wheat flour medium	X	-	-	-
Modified Wheat flour medium	9.62 d	80.7 b	79.2 b	80.3 b
Egg yolk I	34.36 b	100.0 a	100.0 a	100.0 a
Egg yolk II	19.64 c	78.5 b	76.4 b	77.4 b
Modified egg yolk medium	43.13 b	100.0 a	100.0 a	100.0 a
Dog biscuit medium	X	-	-	-
Modified Dog biscuit medium	52.31 a	100.0 a	100.0 a	100.0 a
Bengal gram medium I	11.64 d	84.2 b	81.3 b	82.4 b
Bengal gram medium II	13.26 dc	79.5 b	78.2 b	80.5 b
CD at 5%	6.84	8.63	8.73	8.82
SED	3.38	4.12	3.95	4.26

X – No multiplication; Means followed by the same letter in columns are not significantly different at $P<0.05$ according to Duncan's multiple rang test.

The pathogenicity of EPN species *S. carpocapsae* against *H. armigera*, *E. vitella* and *S. litura* was established by Seenivasan and Sivakumar [18]. This study showed that the virulence of *S. carpocapsae* APKS2 varied significantly when multiplied on different media (Table 2). The IJs multiplied on Nutrient agar medium-I, Egg yolk-I, Modified egg yolk medium and Modified Dog biscuit medium were highly virulent to *H. armigera*, *E. vitella* and *S. litura* which caused 100% mortality. The IJs grown in other media caused < 100% mortality. The IJs developed on Modified wout's medium, Modified wheat flour medium, Egg yolk-II, Bengal gram medium-I and Bengal gram medium-II were significantly least virulent which caused 78.5 – 84.3% mortality of *H. armigera*, 76.4 – 82.6% mortality of *E. vitella* and 77.4 – 83.6% mortality of *S. litura*. Somwong and Petcharat [17] found that mortality of *S. litura* ranged from 40 – 100% due to IJs of *S. carpocapsae* depending on the media from which it was multiplied. This result is comparable to that of present study.

In conclusion, the mass multiplication of *H. bacteriophora* strain KKMH1in Modified wout's medium or Nutrient agar medium-I and *S. carpocapsae* strain APKS2 on Modified dog biscuit medium or Nutrient agar medium-I can be recommended for commercial exploitation as they generated more IJs with high virulence against cotton pests *H. armigera*, *E. vitella* and *S. litura*.

ACKNOWLEDGEMENT

The author is grateful to the Life Science Research Board, Defense Research and Development Organization, New Delhi, India for the financial support through a grant (No. DLS/81/48222/LSRB-136/FSB/2007).

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