

Mass production of the nematode *Acrobeloides longiuterus* using *Tribolium castaneum* and artificial solid media

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Abstract. Free-living nematode *Acrobeloides longiuterus* (Rhabditida: Cephalobidae) exhibits a potential to kill some insect pests. Mass production of this species is a requirement for use it in pest management programs. *Tribolium castaneum* has been used as a primary host for this nematode as an alternative for *Galleria mellonella*. Use of artificial media is another option for mass culturing and such recipes based on soy flour are available. Production of *A. longiuterus* using cost effective method and easily available insect host is important in setting up of small-scale production unit. Therefore, this study has the objectives of evaluating the production feasibility of *A. longiuterus* on *T. castaneum* larvae, pupae and adults as *in vivo* production method. Further, feasibility of using different solid media such as soy flour, palmyra tuber flour, corn flour, black gram flour and dhal flour with other basic ingredients as *in vitro* conditions system was evaluated. Results revealed that pupa of *T. castaneum* yielded the highest number of infective juveniles (36112 IJs/ pupa) compared to other life stages tested. *In vitro* production of *A. longiuterus* on soy flour and black gram flour media yielded 21530 and 16538 IJs/20g, respectively. Pathogenicity against *T. castaneum* was shown up to 93% by the infective juveniles produced from the *in vitro* cultures. In conclusion, *T. castaneum* is an alternative insect that can be used as a host to produce the *A. longiuterus*. In addition, soy flour and black gram flour can be used as the sources for this nematode production without losing their entomopathogenicity.

Keywords: Entomopathogenic nematodes, free-living nematode, insect host, flour media, red flour beetle.

1 Introduction

Application of entomopathogenic nematodes (EPNs) in pest management is ecologically safe and has a quick response. EPNs have been formulated as bio-

pesticides and are commercially available to be used in pest management programs (Ehlers and Shapiro-Ilan 2005). Free-living nematode *Acrobeloides longiuterus* (Rhabditida: Cephalobidae) which is not a true member of entomopathogenic genera such as *Steinernema* and *Heterorhabditis* has shown a potential to kill economically important insect pests (Thiruchelvan *et al.* 2021). This nematode species causes mortality of the insect host within 48-72 h of application (Thiruchelvan *et al.* 2019, 2021), which is a quick knock down effect similar to the classical EPNs. Therefore, *A. longiuterus* could be used as an additional insect management strategy in integrated pest management. This strategy is environmentally friendly as well as safe for non-target organisms. In order to use this nematode as a biological control agent, it is necessary to produce it in bulk at a reasonable cost. This can be achieved through *in vivo* and *in vitro* production using insect hosts and artificial solid media, respectively. Glaser (1940) introduced a method of large-scale *in vitro* production of EPNs using solid media. Then, Friedman (1990) took it into the next level of *in vitro* liquid fermentation production method from the three-dimensional *in vitro* production by Bedding (1981, 1984). Meanwhile, farmers are benefited from both *in vivo* and *in vitro* production of nematodes if the production process utilizes substrates generated from their farms (Ehlers and Shapiro-Ilan 2005).

In vivo production is a simple process of culturing EPNs in live insect hosts. *In vivo* production is based on the White's trap method, which involves the natural migration of infective juveniles (IJs) from the infected host cadaver into a surrounding water layer, from where it can be harvested. White (1927) invented a method, later it was modified by several researchers (Dutky *et al.* 1964, Poinar, 1979, Woodring and Kaya 1988, Lindegren *et al.* 1993, Abdel-Razek and Abd-Elgawad 2007). The selection of the insect host totally depends on the susceptibility of a particular host for the nematode infestation. In addition, the host must be easily culturable using cost effective materials (Shapiro-Ilan and McCoy 2000, Ehlers and Shapiro-Ilan 2005). Diets for the insect host rearing should be carefully selected, as the diet may influence on juvenile yields (Nunchanart 2002). Generally, last instar larva of the wax moth (*Galleria mellonella*) is the conventional host used for *in vivo* multiplication of EPNs (Shapiro-Ilan and McCoy 2000, Shapiro-Ilan *et al.* 2002, Ehlers and Shapiro-Ilan 2005, Costa *et al.* 2007). *Galleria mellonella* is naturally found in beehives and can be reared using artificial diets containing cereals, wax, yeast and glycerol; however, these ingredients are relatively expensive (Costa *et al.* 2007). Use of other insect hosts is an option for *in vivo* production to *G. mellonella* larva. Such hosts are *Tenebrio molitor* (Blinova and Ivanova 1987, Shapiro-Ilan *et al.* 2002, Shapiro-Ilan and Gaugler 2002), *Diaprepes abbreviatus* (Shapiro-Ilan and McCoy 2000), *Corcyra cephalonica* (Blinova and Ivanova 1987, Karunakar *et al.* 1999, Ganguly and Singh 2000, Shapiro-Ilan and Gaugler 2002, Raj Kumar *et al.* 2003, Singh and Gupta 2006, Khan *et al.* 2007, Ali *et al.* 2008, Shapiro-Ilan *et al.* 2012), *Diatraea saccharalis* (Folegatti *et al.* 1988), *Achroia grisella*, *Bombyx mori* (Saenz and Luque 2000, Zaki *et al.* 2000), *Chilo sacchariphagus indicus* (Karunakar

et al. 1999), *Hellicovepa armigera* (Subramanian 2003, Ali *et al.* 2008, Rishi and Prasad 2012), *Spodoptera litura* (Ali *et al.* 2008, Gupta *et al.* 2008), *Plutella xylostella* (Rishi and Prasad 2012), *Odontotermes obesus* (Devi *et al.* 2018) and *Capnodis tenebrionis* (Morton and Gracia-del-Pino 2009).

In vitro EPN production is the practice of culturing nematodes on a non-living, nutritive medium containing pre-cultured symbiotic bacteria. Culture media should be sterilized to avoid any microbial contamination and should facilitate the EPN-specific bacterial growth. Sterilized medium is inoculated with bacteria followed by the inoculation of EPNs. Infective juveniles could be collected from the second week after inoculation of EPNs and collected IJs could be stored in water (Devi 2018). House *et al.* (1965) developed a dog food based medium for commercial scale production of EPNs. Hara *et al.* (1981) reported the production of 125 million IJs/100 g of dog food agar within a week. Animal protein-fat based media were developed by Bedding (1981, 1984) *viz.*, Pig's kidney-beef fat homogenate on shredded polyether polyurethane sponge (Bedding 1981). Then, Bedding (1984) developed another animal protein-fat based media for EPN production, such as homogenate of chicken offal for steinernematids, homogenate of chicken offal with 10% beef fats for heterorhabditids, absorbed on shredded polyether polyurethane sponge. Similar chicken offal medium has been used for EPN production by Tabassum and Shahina (2004) in Pakistan.

Solid culture method is economically feasible up to a production level of approximately 10×10^{12} nematodes/month (Friedman *et al.* 1989, Ramakuwela *et al.* 2016). However, in *in vitro* production of EPNs, solid media compositions and culturing environment of the culture conditions should be considered. In addition, proper selection of the nutrients should be considered because it has an impact on the nematode virulence (Ehlers and Shapiro-Ilan 2005). Therefore, the objective of this study was to evaluate the production efficiency of *Acrobeloides longiuterus* using larva, pupa and adult stages of *Tribolium castaneum* and artificial solid nutrient media under *in vitro* conditions.

2 Materials and Methods

The research was carried out at the Laboratory of Department of Agricultural Biology, Faculty of Agriculture (FoA), University of Jaffna (UoJ), Sri Lanka during 2017 to 2018. The Coleopteran insect species *T. castaneum* and the nematode *A. longiuterus* obtained from the Entomology Laboratory, Department of Agricultural Biology, (FoA, UOJ, Sri Lanka) were used for this study. All the experiments were conducted under the room temperature (27 ± 2 °C).

2.1 *In vivo* production of *Acrobeloides longiuterus* using *Tribolium castaneum*

Red flour beetle *Tribolium castaneum* (Herbst) larvae, pupae and adults were assessed for their suitability to be used in mass culturing of nematodes following the procedure described by Shapiro-Ilan *et al.* (2002). For each life stage, ten specimens were put in a moisture chamber (9 cm diameter Petri dish containing moistened filter paper). Infective juveniles (IJs) were pipetted directly onto the insects at the concentrations of 50, 100 and 150 IJs/1 mL water in moisture chamber. There were four replicate dishes per each concentration and hence, 40 insects were in total per treatment. The moisture chambers were maintained at 27 ± 2 °C in darkness for 10 days. Insects were deemed dead if they did not move following gentle prodding with a needle. Dead insects were transferred into the White's trap separately and IJs were collected 10 days after inoculation (DAI) in water, and the final volume of the IJs suspension was adjusted to 100 mL. Nematodes were counted using a counting dish under a stereo microscope (X 40 magnification). This was done by taking 1 mL nematode sample using a pipette and placing it on a counting dish. Total nematode counts were taken five times.

2.2 *In vitro* production of *Acrobeloides longiuterus* using flour media

Acrobeloides longiuterus culture was produced *in vitro* using artificial nutrient media containing different flour with other common ingredients. Five culture media were prepared using following ratios. Soy flour medium was prepared using 75 g of soy flour, 4.5 g of nutrient broth, 1.5 g of yeast extract, and 49.5 mL of corn oil mixed with 100 mL of distilled water (Salma and Shahina 2012). Four other media were prepared replacing soy flour by 75 g of either palmyra tuber flour, corn flour, black gram flour or dhal flour.

All the ingredients of a medium were mixed thoroughly and the 20 g of each was made absorbed to 5 pieces of polyurethane sponge (1.5 mm^3). Five sponge pieces were added into a polyethylene bag ($10 \times 15 \text{ cm}^2$), plugged with cotton, and was replicated five times. All the bags were sterilized using an autoclave at 121°C, 1.054 kg/cm^3 for 20 min. Thereafter, 2 mL of bacterial suspension isolated from the nematodes was added per bag and incubated over three days under the room temperature at 27 ± 2 °C. Subsequently, 100 IJs were released into each bag. Produced IJs were collected using White's trap technique 21 DAI.

Bacterial isolation was done as described by Upadhyay *et al.* (2015); *A. longiutres* (400-500 IJs/mL) were pipetted directly onto the larvae of *T. castaneum* in a moisture chamber and incubated at 27 ± 2 °C for 72 hours in the dark. Then, infected dead larva were surface sterilized by dipping them in 70% ethanol for 1-2 seconds and the cadavers of dead larvae were aseptically dissected and a loop full of haemolymph was streaked on nutrient agar plates. Bacterial culture plates were incubated in an

incubator at the temperature of 27 ± 1 °C for 48 hours. Bacterial colony was washed off into a 100 mL volumetric flask and final volume of bacterial suspension was adjusted to a 100 mL suspension.

2.3 Quality testing of *Acrobeloides longiuterus* produced from the *in vitro* culture media against *Tribolium castaneum* larvae and pupae

A moisture chamber assay was used to test the quality of *A. longiuterus* from *in vitro* production method against *T. castaneum* larvae and pupae as described by Kaya and Stock (1997). For each life stage, 10 specimens were put into a moisture chamber. Infective juveniles were pipetted directly onto the insects at concentrations of 50, 100, 150 and 200 IJs/dish in 1 mL water. Control insects were only treated with 1 mL of double distilled water. There were four dishes per treatment; hence, there were 40 insects in total per treatment. The dishes were maintained at 27 ± 2 °C in darkness. Mortality of pupae and larvae stages was recorded 48 and 72 hours after inoculation, respectively. LC_{50} and LC_{90} values were calculated based on the mortality data obtained by this study. The experiment was arranged in a completely randomized design.

2.4 Statistical analysis

All the experiments were arranged in a complete randomized design (CRD) and the data of *in vivo* experiment were analyzed using the ANOVA- General Linear Model (GLM) with two factor and comparison among the means were done using the Fisher test at 95% confidence interval (CI). Data from the *in vitro* production and quality testing were analyzed using one way-ANOVA and mean separation were done as per the Fisher test at 95% CI and the Probit analysis was used to calculate the LC_{50} and LC_{90} using Minitab ver. 17.

3 Results

3.1 *In vivo* production of *Acrobeloides longiuterus* using *Tribolium castaneum* life stages

Figure 1 illustrates *in vivo* production of *A. longiuterus* using different life stages of *T. castaneum* at three different concentrations of infective juveniles (IJs). Number of IJs was significantly different among the concentrations ($F_{(2,54)} = 422.48$, $P < 0.05$), different life stages ($F_{(5,54)} = 87.52$; $P < 0.05$) and the interaction of concentration x life stage ($F_{(10,54)} = 10.30$, $P < 0.05$). Significantly highest yield of IJ ($36,112.5 \pm 14.5$ IJs/pupa) was obtained with the pupal stage inoculated with 150 IJs/mL. This was followed by seventh and sixth instars larvae of *T. castaneum* with 150 IJs/mL

inoculation producing $31,031 \pm 9.1$ and $25,425 \pm 19.2$ IJs/larva, respectively. The use of *T. castaneum* adults as the host yielded $21,031 \pm 47$ IJs/adult. Generally, pupa yielded the highest number of IJs/mL at each concentration compared to the other insect stages tested.

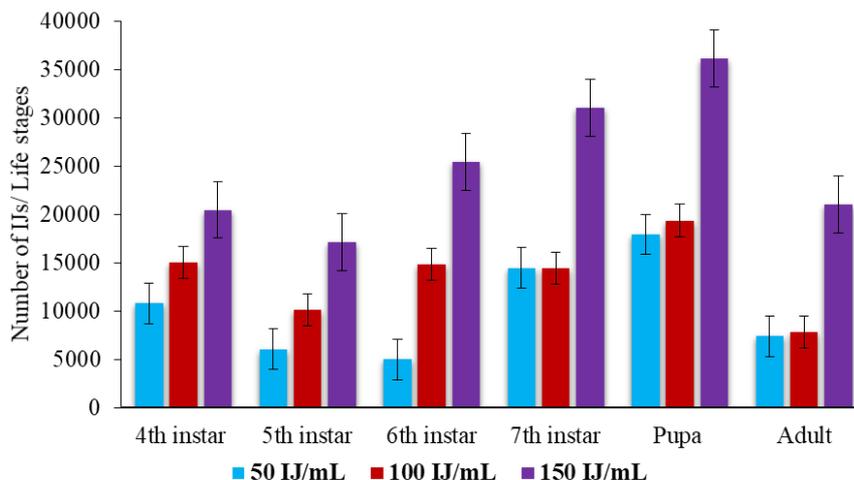


Fig.1: Production of *Acrobeloides longiuterus* with respect to six life stages of *Tribolium castaneum* at three different concentrations of IJs.

3.2 *In vitro* production of *Acrobeloides longiuterus* using flour media

Production of *A. longiuterus* from the different types of flour-based media is shown in Table 1. Nematode production was significantly different among the media ($F_{(4,20)} = 111.51$, $P < 0.05$). Soy flour medium yielded the highest IJs as 21530/20 g of medium whereas the lowest yield was observed in corn flour medium (977/20 g of medium).

Table 1: Mean number of *Acrobeloides longiuterus* produced under *in vitro* conditions.

Medium	Number of IJs \pm SD/20 g*
Soy flour 75 g + Ing.	21530 \pm 143 ^a
Corn flour 75 g + Ing.	977 \pm 51 ^e
Black gram flour 75 g + Ing.	16538 \pm 380 ^b
Dhal flour 75 g + Ing.	12531 \pm 148 ^c
Palmyra flour 75 g + Ing.	1533.8 \pm 122 ^d

Ing. - Nutrient broth 4.5 g, Yeast extract 1.5g, Corn oil 49.5 mL and Distilled water 100 mL

*Values having the same letter were not significantly different (Fisher test at 95% confidence level)

3.3 Quality testing of *Acrobeloides longiuterus* produced from the *in vitro* culture media against *Tribolium castaneum* larvae and pupae

Mortality of the larvae and pupae stages of *T. castaneum* at different concentrations are given in Table 2. Mortality of larvae at all concentrations of IJs/mL were significantly different from the untreated control ($F_{(4,15)} = 149.25$, $P < 0.05$). The highest mortalities of larvae and pupae were recorded as 92.5 % (9.25 ± 0.50 mean mortality) at the concentration of 200 IJs/mL. LC_{50} and LC_{90} of larvae were calculated as 48.55 and 210.42 IJs/mL, respectively.

Mortalities of *T. castaneum* pupa were significantly different from the untreated control at all four concentrations (IJs/mL) tested ($F_{(4,15)} = 203.2$, $P < 0.05$). Mortalities of pupa were recorded as 65, 75 and 90 % at the concentrations of 50, 100, 150 IJs/mL, respectively. LC_{50} and LC_{90} of the pupae were 32.94 and 173.97 IJs/mL, respectively.

Table 2: Bio-efficacy of *Acrobeloides longiuterus* produced from the *in vitro* culture media against *Tribolium castaneum* larvae and pupae.

Concentrations IJs/ mL	Mean \pm SE Mortality*	
	Larva	Pupa
0	0.25 \pm 0.500 ^d	0.5 \pm 0.577 ^d
50	5.25 \pm 0.500 ^c	6.5 \pm 0.577 ^c
100	7.25 \pm 0.500 ^b	7.5 \pm 0.577 ^b
150	8.00 \pm 0.816 ^b	9.0 \pm 0.0 ^a
200	9.25 \pm 0.500 ^a	9.25 \pm 0.5 ^a
LC_{50}	48.55	32.94
LC_{90}	210.42	173.97

* Values having the same letter in a column were not significantly different according to the Fisher test at 95% confidence level

4. Discussion

Determination of multiplication and production potential of any new nematode isolate using *in vivo* production in live insect hosts, as well as using *in vitro* production in solid culture media are the most important steps before either initiating mass production or formulation and commercialization of EPNs (Shapiro-Ilan and Ehlers 2002, Ehlers and Shapiro-Ilan 2005). Thiruchchelvan *et al.* (2021) reported first that *Acrobeloides longiutreus* isolated from Sri Lanka showed entomopathogenic characteristics. Therefore, it is important to study the mass production ability for the future studies and experiments. Shapiro-Ilan *et al.* (2002) stated that *in vivo* production of EPNs could be used for the laboratory use and small-scale field experiments or applications. *In vivo* production of EPNs appears to be the suitable

method for niche markets and small growers. In addition, it requires a minimum investment cost and technical skills for the initial start-up. However, labour cost and availability on production of the insect hosts are the difficulties for *in vivo* production (Ehlers and Shapiro-Ilan 2005).

There are many lepidopteran, coleopteran and dipteran insect hosts that have been used for *in vivo* production of EPNs (Devi 2018). The host used in this study was *T. castaneum*, a member of the order coleoptera and the same family of *Tenebrio molitor* (Tenibrionidae), which were used by many researchers previously (Ehlers and Shapiro-Ilan 2005, Devi 2018) for *in vivo* EPNs production. The size of the insect host plays a major role in EPNs production (Flanders *et al.* 1996, Kaya and Stock 1997). This has been confirmed with the use of *G. mellonella* in EPN production. Larger larvae give a better yield. For instance, *G. mellonella* larvae (20-22 mm length) yielded the highest of EPN/larva (Flanders *et al.* 1996). In this study, availability of insect host, cost effective and convenient rearing of the host insect are considerations in selecting the host insect, *T. castaneum*. The efficiency of *in vivo* production correspondingly depends on the quality of insect hosts and their life stages used for the production (Morales-Ramos *et al.* 2011). However, the quality of insect hosts and their nutritional characteristics depend on the media where they are reared and the life stages of insects (Shapiro-Ilan 2008). In this study *T. castaneum* was reared in wheat flour, where they are normally crowding and completing their life cycle. Therefore, the IJs production differences with respect to different stages mainly depend on the nutritional properties in the particular life stage, anatomical features and the movement of the insect host. Another factor that influences on IJs yield is the inoculum doses (Shapiro-Ilan *et al.* 2002). Nematode concentrations in this study had a positive effect on the IJs production, and Boff *et al.* (2000) and Shapiro-Ilan *et al.* (2002) obtained similar results. Moreover, we selected intermediate IJs doses (50, 100 and 150 IJ/mL) since the best yields are achieved with intermediate inoculum dosage because higher doses create lower yield due to competition for nutrients (Shapiro-Ilan *et al.* 2002). Environmental factors including temperature, aeration, and moisture can affect yield of EPNs (Grewal *et al.* 1994, Dolinski *et al.* 2007, Shapiro-Ilan *et al.* 2012). Optimum production temperatures lie between 18 and 28°C for different EPN species (Karagoz *et al.* 2009, Morton and Gracia-del-Pino 2009).

Production of EPNs for the commercial use or for international markets, *in vitro* liquid culture is considered the most cost-effective process while *in vitro* solid culture is generally considered intermediate between *in vivo* and liquid culture (Shapiro-Ilan *et al.* 2012). Soya flour-based medium yielded the highest production of IJs (21500 IJs/20 g) compared to the other four flour-based media. Similar results were reported by Banu and Meena (2015) who recorded the yield of the nematodes ranging from 3.22 to 3.87 x 10³ with highest multiplication rate of 3.87 x 10³ in medium-I which has soya flour and corn oil as important ingredients. In addition, Cao *et al.* (2013) stated that soya flour has high protein content which is important to build new tissue

in EPNs population that were cultivated in various *in vitro* media. Results of this study also showed a higher nematode production with increasing protein and fat contents. Soybean powder contained 40.5% of protein, 20.5% of fat, and 22.2% of carbohydrate (Indriyanti and Muharromah 2016). While other flours contain lower amount of protein and fat contents, such as black gram and dhal (protein 25% Fat 6% carbohydrate 53%) (Mazmany 2020), palmyra flour (protein 4% and 1% fat) (Vengaiah *et al.* 2013) and Corn flour (14% protein and 5% fat) (Anonymous 2021). Thus, nematode production has a positive correlation with protein and fat contents of the media or the nutrition content of ingredients. However further experiments with other flour types along with their proximate analysis will provide more information.

5 Conclusions

Acrobeloides longiuterus was successfully cultured *in vivo* using different life stages: larva, pupa and adult of *T. castaneum*. Of them, *T. castaneum* pupa was found as the best stage to produce IJs. Among the *in vitro* media tested, the soy flour-based medium produced the highest yield of IJs compared to black gram, dhal, palmyra and corn flour media. The results show that mass production of *A. longiuterus* is feasible using the tested *in vivo* and *in vitro* methods.

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Author contributions:

All authors equally contributed to the paper. NT and GM conceived and designed the study; NT conducted the research work and wrote the initial draft; GM and GTK supervised the work, reviewed and edited the manuscript.

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