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Enhanced entomopathogenic nematode yield and fitness via addition of pulverized insect powder to solid media

Shiyu Zhen,[1] Yang Li,[1] Yanli Hou,[1] Xinghui Gu,[2] Limeng Zhang,[2] Weibin Ruan[1]* and David Shapiro-Ilan[3]

[1]College of Life Sciences, Nankai University, Tianjin 30071, China.

[2]Tobacco Company, Yuxi 653100, Yunnan, China.

[3]USDA-ARS, SEFTNRL, Byron, GA 31008, USA.

*E-mail: ruanweibin2004@hotmail.com.

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Abstract

Beneficial nematodes are used as biological control agents. Low-cost mass production of entomopathogenic nematodes (EPNs) is an important prerequisite toward their successful commercialization. EPNs can be grown via *in vivo* methods or in sold or liquid fermentation. For solid and liquid approaches, media optimization is paramount to maximizing EPN yield and quality. In solid media, the authors investigated the effects of incorporating pulverized insect powder from larvae of three insects (*Galleria mellonella, Tenebrio molitor*, and *Lucillia sericata*) at three dose levels (1, 3, and 5%). The impact of insect powder was assessed on infective juvenile (IJ) yield in solid media. Additionally, IJs produced in solid culture were subsequently assessed for virulence, and progeny production in a target insect, *Spodoptera litura*. The dose level of larval powder had a significant effect on IJ yield in both trials, whereas insect type had significant effect on IJ yield in trial 1 but not in trial 2. The maximum solid culture yield was observed in *T. molitor* powder at the highest dose in both trials. Moreover, the time-to-death in *S. litura* was substantially shortened in trial 1 and in trial 2 when IJs from the *T. molitor* powder treatment were applied. There was no significant effect of combining two insect powders relative to addition of powder from a single insect species. These findings indicate that addition of insect powder to solid media leads to high mass production yields, and the fitness of the IJs produced (e.g., in virulence and reproductive capacity) can be enhanced as well.

Key words

EPN, Soil culture, *Galleria mellonella*, *Tenebrio molitor*, Mortality time.

Agricultural crops have been prone to attack from various pest insects which lead to loss of yield (Shapiro-Ilan et al., 2016; Ángel et al., 2018). Biological control agents are attractive alternatives to chemical pesticides due to increased awareness of the potentially harmful effects of chemical residues in food and in the environment, the increasing resistance of pests to chemical pesticides, and the high cost of developing new compounds. One strategy is to develop entomopathogenic nematodes (EPNs) as control agents for arthropod pests; these organisms are deemed to be safe to humans and the environment to the extent that they are exempt from pesticide registration procedures in many countries (Shapiro-Ilan and Gaugler, 2002).

EPNs in the genera *Steinernema* and *Heterorhabditis* (Rhabditida: Steinernematidae, Heterorhabditidae) are natural enemies of a number of important agricultural pests (Garriga et al., 2017; Nyamwasa et al., 2018), especially soil-dwelling and stem boring insects (Begley, 1990; Shapiro-Ilan et al., 2014). Extensive research over the past four decades has demonstrated both their application in various systems including field crops, orchards, ornamental plants, lawn,

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and turf (Shapiro-Ilan and Gaugler, 2002; Patil et al., 2017; Geisert et al., 2018). Up to now, more than 115 species of *Steinernema* and *Heterorhabditis* have been described, and about one dozen have been commercialized (Lacey et al., 2015; Shapiro-Ilan et al., 2018).

Infective juvenile nematodes (IJs) encounter a susceptible host through natural openings (mouth, anus or spiracles) or occasionally through the cuticle; once inside the host the IJs enter the hemocoel (Dunphy and Webster, 1988; Peters and Ehlers, 1997; Bowen et al., 1998). Once inside the host, the nematodes release symbiotic bacteria (e.g., *Xenorhabdus* spp. or *Photorhabdus* spp. for Steinernematids and Heterorhabditids, respectively) that colonize and kill the host (Simões and Rosa, 1996; Godjo et al., 2017). After completing 1 to 3 generations, IJs leave the cadaver and disperse back into the soil to in a search for new target hosts (Ehlers, 1996; Stock, 2015; Geisert et al., 2018). Nematode growth and reproduction depends upon suitable conditions established in the host cadaver by the bacterium. Conversely, the bacterium lacks invasive power and is dependent upon the nematode vector to locate and penetrate suitable hosts.

EPNs can be mass produced using *in vivo* methods by inoculating living insects, and *in vitro* methods, i.e., solid or liquid fermentation (Ehlers, 2001; Shapiro-Ilan et al., 2014). *In vitro* liquid culture is considered the most cost efficient process for producing EPN (Shapiro-Ilan and Gaugler, 2002; Cho et al., 2011). However, advantages of solid culture lie in it being an intermediate between *in vivo* and liquid culture in terms of labor, capital outlay, and technical expertise required (Gaugler and Georgis, 1991; Shapiro-Ilan and Gaugler, 2002). Solid culture has been successfully implemented among various species of *Steinernema* and *Heterorhabditis* (Bedding, 1990; Bedding et al, 1993; Ehlers et al., 2000; Strauch and Ehlers, 2000; Adams and Nguyen, 2002).

In vitro media composition such as lipid content, which is important in determining nematode survival and virulence, is critical to predicting nematode quality (Friedman et al., 1990; Abu et al., 1998; Abu Hatab and Gaugler, 1999, 2001; Yoo et al., 2000). Most research has focused on identifying essential nutrient sources of media of *in vitro* nematode production such as protein sources and lipid sources (Buecher et al., 1970; Yoo et al., 2001; Shapiro-Ilan et al., 2014; Leite et al., 2016a). Sources of nutrients have included organs of various domestic animals (Bedding, 1981; Hara et al., 1981), extracts of animals (peptone, beef extract, egg yolk, and milk) or plant origin (flour, corn oil, and peanut oil), and yeast extract (Wouts, 1981; Friedman et al., 1990, 1991; Surrey and Davies, 1996; Ehlers and Shapiro-ILan, 2005; Shapiro-Ilan et al., 2014; Leite et al., 2016a). As expected, media with little or no resemblance to the insect host composition can result in an inferior physiological quality in IJs (Womersley, 1993). It was demonstrated that the lipid supplement from natural hosts added to artificial media could give a relatively similar composition to *in vivo*-produced nematodes and could provide a better growth rate and a higher yield than media with other lipid sources (Abu Hatab and Gaugler, 1999). Axenic liquid static culture of *S. carpocapsae* supplemented with nematode-infected insect cadaver largely improved nematode growth and propagation (Fuchi et al., 2016).

Insect quality could affect the relative quality of nematodes produced *in vivo*. For example, *H. bacteriophora* and *S. glaseri* produced in the natural host Japanese beetle, *Popillia japonica*, had a higher lipid content than nematodes produced in the factitious host *Galleria mellonella* or liquid culture (Abu et al., 1998; Abu Hatab and Gaugler, 1999). Lipid content can affect EPN efficacy or quality such as by enhancing persistence (Abu et al., 1998; Abu Hatab and Gaugler, 1999). In another study, *Musca domestica* larvae were directly used for IJ production in solid culture and produced the highest yield (Ramakuwela et al., 2014), indicating insect nutrients might be a useful component for IJ production in solid culture. Accordingly, the need to develop media that mimic the natural host is emphasized (Shapiro-Ilan and Gaugler, 2002). Adding insect host powder to *in vitro* nematode culture medium might be quite attractive to enhance IJ yield and thus increase EPN competitiveness via reduced costs. However, the effect of insect host powder on IJ yield *in vitro* production has rarely been reported. Moreover, the choice of insect species as sources for EPN media may impact the relative impact on EPN quality or yield, but this issue has not been explored. Also, it is conceivable that combinations of insect sources could have the greatest impact.

EPNs have been commercialized for nearly three decades, but their successful implementation in biological control has been limited (Leite et al., 2016b). The greatest barrier to wide application of these nematodes is higher cost of production as compared to synthetic pesticides (Shapiro-Ilan and Gaugler, 2002). Here, we focused on developing less expensive, efficient media for solid culture via the addition of insect larvae powder.

The objectives were (i) to assess the effects of insect powder from three host species from different orders: *Galleria mellonella*, *Tenebrio molitor*, and *Lucillia sericata*, and the effects of larval powder dose on IJ production and virulence; (ii) to evaluate the effects of combining insect powders (from different hosts) on IJ yield and virulence. The three insect larvae tested in this study are already commercially available and therefore it would be straight forward to utilize them for EPN production if their role on IJ yield is positive.

Materials and methods

Insects and nematode

The insect larvae of *Galleria mellonella*, *Tenebrio molitor*, and *Lucillia sericata* were purchased from commercial markets and killed by storing the insects at –20°C and then placed into the frozen-dry machine for 48 hr to get dried cadaver (ALPHA1-4D CHRIST). The cadaver was ground to get the powder for later use.

The entomopathogenic nematode, *Steinernema feltiae* (SN strain) was provided by USDA-ARS, Southeastern Fruit and Tree Nut Research Lab, Byron, GA. For culture propagation, infected last-instar *G. mellonella* cadavers as hosts were placed on White trap at 25°C for IJ collection (White, 1927). Distilled water was used for IJ collection and IJs were stored at 14°C and used within 2 wk.

Bacterial isolation

The symbiotic bacteria *Xenorhabdus bovienii* was isolated from *S. feltiae* (Akhurst, 1980). Briefly, IJs (100 IJs/15 µL) and one last-instar larvae of *G. mellonella* were added to each well of a 24-well culture plate with a filter paper lined in the bottom of each well. Around 30 hr after infection, one drop of haemolymph was obtained from the infected insect by snipping the very end of the second proleg and adding it to nutrient bromothymol agar (NBTA). Phase I bacterial lawns (colored with blue) were distinguished by NBTA. *X. bovienii* was purified by successive streak transfers on selective media of NBTA.

A single colony was inoculated to a 50 mL flask of sterilized TSB+Y medium (tryptic soy broth (4%) + yeast extract (0.5%)). Flasks were placed on the shaker at 25°C, 200 rpm for 24 hr. After being stored at 4°C for at least 1 wk, the bacteria stock was stored in 15% sterile glycerol (1 mL bacterial stock and 1 mL 30% sterile glycerol) at –80°C.

Nematode axenization

IJs were surface sterilized with 0.1% benzoxonium chloride (1622) solution in a 50-mL sterilized centrifuge tube, then rinsed three times with sterile distilled-water. IJs were inoculated onto the 90-mm petri dish of nutrient agar medium where 200 µL *X. bovienii* was previously inoculated and cultured at 25°C for 1 d. In order to obtain eggs, after 62 to 72 hr, gravid females were rinsed by 1.2% sterile saline solution and lysed by immersing in a solution (5 ml 1.2% sterile saline solution and 5 mL alkaline lysis solution (0.4 M NaOH (1.6% w/v) +10% NaClO (1.34% w/v)). The mixture of eggs juveniles, males, and residue of females was rinsed three times in sterile distilled-water. Then the mixture suspension (1-2 drops) was transferred to wells of a 24-well culture plate with 0.3 mL of TSB+Y medium in each well and incubated at 25°C for 2 d and then used for inoculum (Leite et al., 2016b).

Experimental design and setup

The experiment included three treatments of the three freeze-dried larval powders (G: *G. mellonella,* T: *T. molitor*, and L: *L. sericata*) at three dose levels (Low: 1%, Middle: 3%, and High: 5%) each. Additionally, the three combinations of insect powders were included as treatments; the combinations consisted of each dose level in equal parts (m/m) (1:1, e.g., low dose of one insect powder was mixed with low dose of another powder and different dose levels were not mixed together). G+T (1:1 mixed powder of *G. mellonella* and *T. molitor*); G+L (1:1 mixed powder of *G. mellonella* and *L. sericata*); and T+L (1:1 mixed powder of *T. molitor* and *L. sericata*). Basic medium without insect powder was included as control. The basic culture medium was prepared according to Leite et al. (2016b) with 25 g glucose, 23 g yeast extract, 6.25 g egg yolk, 6.25 g egg white, 5 g NaCl, 2 g agar, and 40 g peanut oil, and distilled water in one litter. Each treatment had four replications. Totally, there were 76 flasks with 19 treatments (6 insect powder × 3 dose + 1 basic medium) with four replications for each. The experiment was performed twice.

After adding 50 mL of basic medium to each flask, the corresponding insect larval powders were added to the corresponding treatments flasks. Finally, 2.4 g sponge (about 0.5-1 cm^3) as three-dimensional growth medium for solid culture (Akhurst, 1980) was added to each flask and thoroughly stirred with glass rod (changed every time). One milliliter 1-d-old *X. bovienii* (approximately 1 × 10^9cells)

which was previously prepared was added to each autoclaved flask (121°C, 15 min), which was subsequently cultured in the incubator at 25°C for 2 d. Then, five thousand 2-d first instars (100 juveniles/mL) were added to each flask. The flasks were incubated at 25°C for 2 wk.

Measurements

Yield

Two weeks after the experiment was initiated, the nematodes were harvested with a modified squeezing method. Briefly, the device was constructed with two ply steel pen-baskets with holes and a piece of wood placed between two baskets; the bottom of top basket is closely in contact with the wood. IJs harvesting was performed in a barrel. The sponge is placed onto the wood in the bottom basket with 1,075 mL tap water, and then another basket placed on the sponge. We pushed the top basket with a handle to squeeze the sponge 60 times, and then the water was poured off to a big beaker. This step was carried out four times, and 4,300 mL water was used. A subsample (30 mL) was taken from the 4,300 mL nematode suspension and added to a flask and stored at 14°C for yield counting with 24 h and virulence testing 2 wk later. IJs were counted under a stereomicroscope.

IJ virulence and in vivo progeny production

Spodoptera litura (Lepidoptera: Noctuidae) artificially reared in our laboratory were used for virulence bioassay (Singh et al., 1983). A total of 100 IJs in 40 µL and one *S. litura* larva were added to each well of a 24-well tissue culture plate lined with a filter paper. Only five larvae were added to each 24-well tissue culture plate and regarded as one replicate. Each treatment had five replicates. In total, 380 *S. litura* larvae were used here. The 24-well culture plates were placed into an incubator at 25°C, and the number of dead larvae was recorded at 8 hr intervals for 96 hr. Meanwhile, each dead larva of *S. litura* was placed on a White trap for IJ collection, and the initial time of IJ emergence from the insect cadaver was recorded. IJs released from *S. litura* in the first 2 wk were counted under a stereomicroscope.

Statistical analysis

All yield data in solid media including the control treatment (no insect powder addition) and insect powder treatments were subjected to one-way analysis of variance (ANOVA) and post hoc Duncan's test ($p \leq 0.05$) (SPSS19.0). To evaluate the effect of insect type and dose level on yield and virulence of IJs in solid culture, the time-to-death of insects and initial release time of IJ from insect cadaver in IJ virulence assay were statistically analyzed with two-way ANOVA and post hoc Duncan's test ($p \leq 0.05$).

Results

IJ yield

Data from two trials showed significant differences, therefore, data of the two trials were analyzed separately. As shown in Figure 1, the IJ yield in two trials showed a similar trend. For each insect powder added to the medium, the yield increased significantly as the insect powder dose increased (trial 1, $F = 44.0$, df = 2.54, $p \leq 0.001$; trial 2, $F = 27.7$, df = 2.55, $p \leq 0.001$). In trial 1, IJ yield was influenced significantly by insect type ($F = 3.8$, df = 5.54, $p = 0.005$) but not in trial 2 ($F = 1.124$, df = 5.55, $p = 0.358$). The results of one-way ANOVA showed solid production medium supplemented with insect powder had a significant impact on IJs production relative to the control treatment without insect powder (for trial 1, $F = 7.50$, df = 18.57, $p \leq 0.001$; for trial 2, $F = 4.60$, df = 18.55, $p \leq 0.001$). In trial 1, IJ yield in media supplemented with all the six kinds of insects larvae powder (three insect larvae alone or combination pairs) at the middle and the *T. molitor* at the low dose were significantly higher than control treatment, reaching 1.44 to 2.54 fold as much as that in control treatment. In particular, IJ yield in the treatment of *T. molitor* at the high dose was 2.54 times that of control and was the highest among the treatments. In trial 2, IJ yield in media supplemented with six kinds of insect larvae powder at middle and high doses except the middle doses of G, G+L, and T+L were significantly higher in relative to the control.

Time-to-death

Both trials showed similar results, indicating that for most treatments there was no significant effect of insect type, dose level, and their interaction on the mortality time of *S. litura* larvae (Fig. 2). However, the IJs from the high dose treatment of *T. molitor* significantly shortened the time-to-kill *S. litura* compared to that of the control treatment; the reduction was by 5.2 hr in trial 1 and 7.2 hr in trial 2. The middle dose

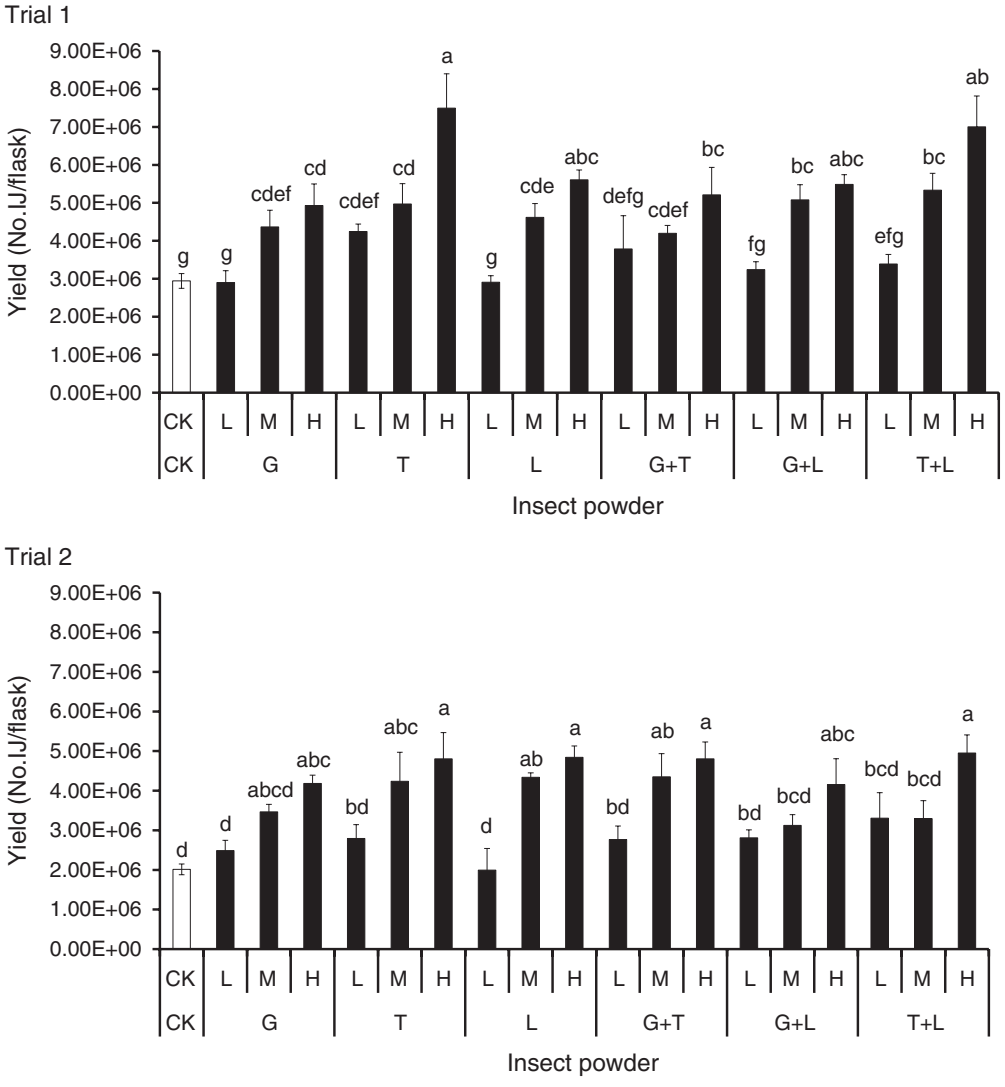

Figure 1: Effect of insect powder and dose level on infective juveniles yield (h) in solid culture media. G: *Galleria mellonella*; T: *Tenebrio molitor*; L: *Lucillia sericata*; G+T:1:1 mixed powder of *G. mellonella* and *T. molitor*; G+L, 1:1 mixed powder of *G. mellonella* and *L. sericata;* T+L: 1:1 mixed powder of *T. molitor* and *L. sericata.* L, M, H: three dose levels of insect powder 1, 3, and 5% (m/m), respectively. Bars with different letters represent significantly different means (SE) at $p \leq 0.05$ among all treatments via the post hoc Duncan's test.

of G+T treatment in trial 1 also had similar results but not in trial 2.

Initial emergence time of IJ from tested Spodoptera litura

We did not detect any significant effects of insect type and dose level on initial release time of IJs from *S. litura* in both trials. No difference was observed in insect powder treatments relative to the control (Fig. S1).

In vivo progeny yield in the tested insect *Spodoptera litura*

Data from the two trials were analyzed separately since there were significant differences between them (Fig. 3). IJ yield in *S. litura* larvae increased in certain insect powder treatments in trial 1 but not in trial 2 (trial 1: insect: $F = 1.98$, df = 5.54, $p = 0.096$; dose: $F = 4.0$, df = 2.54, $p = 0.024$; trial 2: insect: $F = 1.28$, df = 5.55, $p = 0.284$; dose: $F = 0.27$ df

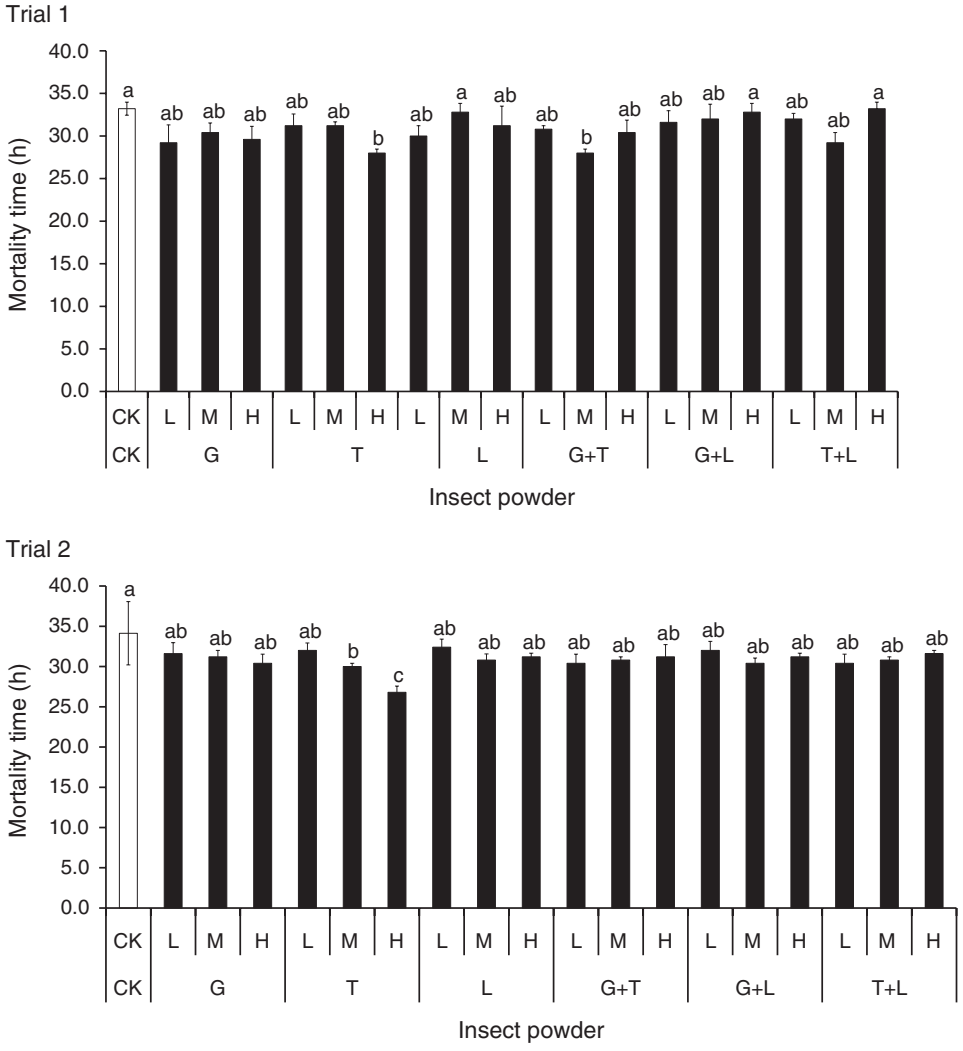

Figure 2: Effect of insect powder and dose level on the time-to-death (h) of *Spodoptera litura* larvae in two trials. Each larva was exposed to 100 *Steinernema feltiae* infective juveniles. Five larvae in five wells were regarded as a replication of each treatment. G: *Galleria mellonella*; T: *Tenebrio molitor*; L: *Lucillia sericata*; G+T:1:1 mixed powder of *G. mellonella* and *T. molitor*; G+L, 1:1 mixed powder of *G. mellonella* and *L. sericata;* T+L: 1:1 mixed powder of T. molitor and *L. sericata.* : L, M, H: three dose levels of insect powder 1, 3, and 5% (m/m), respectively. Bars with different letters represent significantly different means (SE) at $p \leq 0.05$ among all treatments via the post hoc Duncan's test.

= 2.55, p = 0.763). In particular, the high dose of *T. molitor* and G+L caused significantly higher yield in *S. litura* larvae relative to that of the control (in trial 1).

Discussion

Due to the need for a high level of technology input and large capital investment for EPN liquid production, in vitro solid culturing system is still superior to liquid culture technology in developing countries (Strauch and Ehlers, 2000). The nutritional composition of the production medium can have a substantial effect on nematode production in terms of yield and quality (Friedman et al., 1990). The present study found that the addition of insect powder (*L. sericata*, *T. molitor* or *G. mellonella*) could significantly increase the yield of IJs under in vitro solid culture. Additionally, virulence (time-to-kill) was improved in certain insect powder treatments, and there was some evidence of subse-

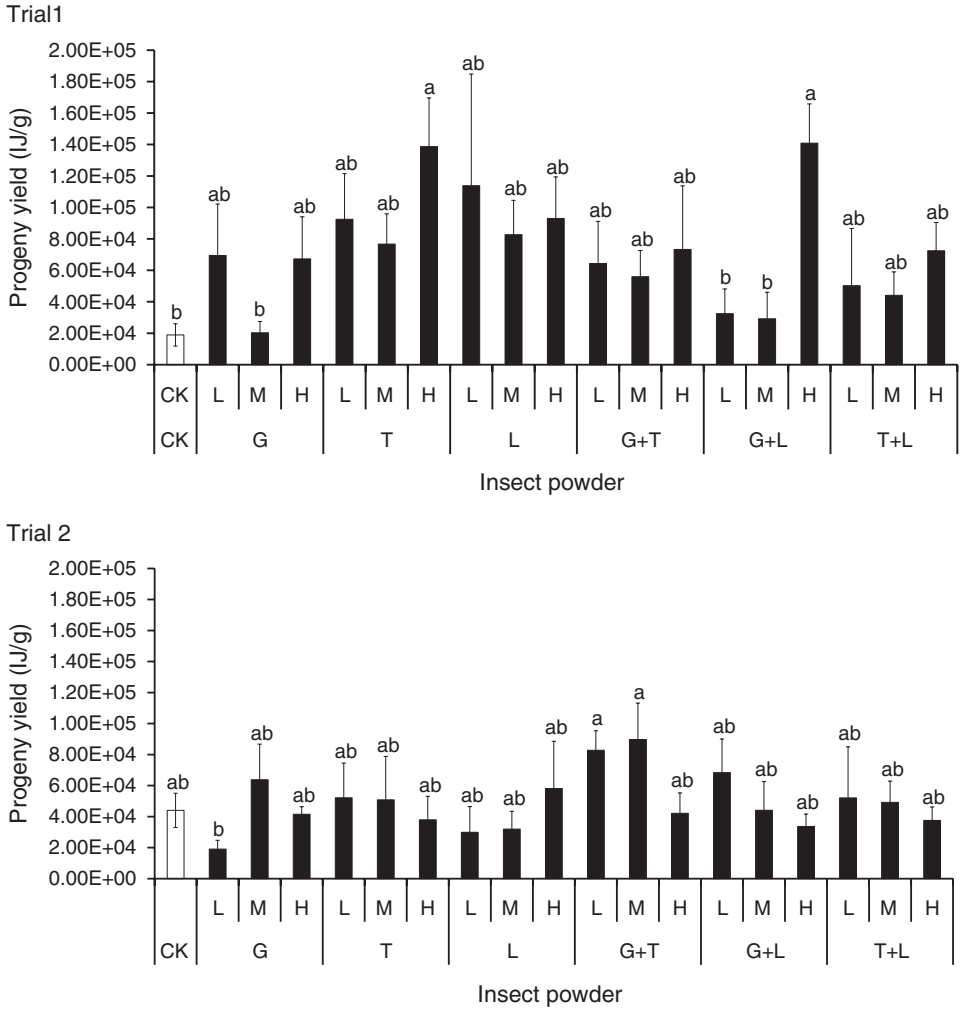

Figure 3: Effect of insect powder and dose level on the progeny yield (h) of *Spodoptera litura* larvae in two trials. Each larva was exposed to 100 *Steinernema feltiae* infective juveniles. Five larvae in five wells were regarded as a replication of each treatment. G: *Galleria mellonella*; T: *Tenebrio molitor*; L: *Lucillia sericata*; G+T:1:1 mixed powder of *G. mellonella* and *T. molitor*; G+L, 1:1 mixed powder of *G. mellonella* and *L. sericata;* T+L: 1:1 mixed powder of *T. molitor* and *L. sericata.* L, M, H: three dose levels of insect powder 1, 3, and 5% (m/m), respectively. Bars with different letters represent significantly different means (SE) at $p \leq 0.05$ among all treatments via the post hoc Duncan's test.

quent *in vivo* progeny production stemming from certain treatments. Taken together, the increased IJ yield, shortened mortality time, and higher progeny production indicate substantial advantages for adding insect powder to in vitro solid culture media.

In both trials, IJ yield in the treatment at high dose was over two-fold more than that in the control treatment without insect larvae powder addition, indicating the positive effects of *T. molitor* on IJ production. Ramakuwela et al. (2016) reported that the highest IJ yield (1.56×10^5 IJs/g medium) among treatments was obtained with the addition of *M. domestica* larval puree (5 g) plus 0.15 g canola oil in solid culture. In our study, the highest concentration of IJs was 9.5×10^5 IJs/g medium excluding the weight of water and thus the value was really higher than that reported by Ramakuwela et al. (2016). This result was close to the value reported when using basic medium in liquid culture with an agitation speed 280 rpm (Leite et al., 2016b). In another study, 9.7×10^5 IJs/g medium (after 15 d incubation) was achieved using chicken offal medium (Tabassum and Shahina,

2004). Hara et al. (1981) reported 10^5 IJs/g (after 30 d incubation) when dog food agar medium was used. Supplementation of media with autoclaved insect cadavers infected by *S. carpocapsae* was a useful method to improve axenic culture and thus the autoclaved nematode-infected insect might contain important heat-stable nutritional factors for the growth and reproduction of EPNs (Fuchi et al., 2016). Therefore previous studies and the present study indicate that adding infected or non-infected insects to growth medium can significantly increase in vitro yields.

The basis for the positive effect of adding insect powder to media on IJ yield probably lies in the addition of valuable nutrients important to EPN growth, and likely candidate nutrients for the outcome are lipids. In reference to lipids, some studies showed that the quantity and quality of fats in the media increased IJ yield during *in vitro* culture (Dunphy and Webster, 1989; Han et al., 1992; Abu et al., 1998; Gil et al., 2002). Leite (2016a) assessed different lipid source on IJ yield and found that pork lard provided the lowest yield. Nematodes grown in media supplemented with insect lipids accumulated significantly higher lipid proportion per dry biomass than media supplemented with other lipid sources (Abu Hatab and Gaugler, 2001).

Protein sources may also have a direct effect on nematode production. Leite (2016a) tested the effects of six of nitrogen source on IJ yield and observed the lowest IJ yield in yeast extract treatment. El-Sadawy (2011) reported that seven species of EPNs failed to reproduce on dog food agar, but they were all successfully produced on modified medium containing soy flour as the primary source of protein (El-Sadawy, 2011). We speculate that the protein from insects might provide certain intrinsic characters to facilitate nematode production.

Another possible explanation for the observed impact of the insect powder is that certain properties contained therein lead to a priming effect for the IJs and their production. When IJs invade suitable host insects, unknown signals such as food signals in the insect's haemolymph induce the development of IJs, which is called "recovery" (Strauch and Ehlers, 1998). The percentage of IJ recovery varies between 0 and 81% and takes 3 to 5 d on artificial media, while IJ recovery is approximately 100% and occurs within 24 hr in insect haemolymph with *Heterorhabditis* (Strauch and Ehlers, 2000). Poor and unsynchronized recovery is the major reason for inconsistent yields (Ehlers et al., 1998). In addition, the fact that, in a prior study, the stimulation was caused by addition of the autoclaved insect powder suggests that the enhancing factor is heat stable (Fuchi et al., 2016). Microbe-derived small molecules (i.e., antibiotics and quorum sensing molecules, etc.) have been shown to regulate transcription in microbes within the same environmental niche, views as elicitors (Soliman and Raizada, 2013; Adnani et al., 2017). Therefore, we predicted certain active compounds inside insect larval powder might play a role as elicitors for recovery and the bacteria-nematode production system in solid culture (in addition to enhancing nutrient composition). However, details related to this prediction need to be clarified further in future research.

The approach of adding insect powder to in vitro media promises to increase EPN production efficiency. The order of the cost per unit was *L. sericata* > *G. mellonella* > *T. molitor*. Adding 2.5 g *T. molitor* insect powder (5%, high dose) to 50 mL basic medium can double the yield and increase the insecticidal efficiency, and the cost of 2.5 g *T. molitor* insect powder was only about 5 cents of USD. The increased yield of nematode was 4.55×10^6 IJs, which was worth USD 2.27. Thus, the gains in yield outweigh the costs of adding insect powder, and additional gains are made in nematode fitness; therefore the approach enhances the competiveness of EPN production.

In conclusion, insect powder appears to be quite suitable for in vitro mass production of EPNs using solid culture. The advantages of adding *T. molitor* insect powder include enhancement of IJ yield, virulence, and increased progeny yield in subsequently infected hosts. The addition of insect powder as indicated in the present study offers a highly competitive alternative method to production of EPN products in vitro solid culture. Future research will focus on the potential positive effects of insect powder on IJ yield under liquid culture and their application efficiency in field.

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Appendix

Figure S1

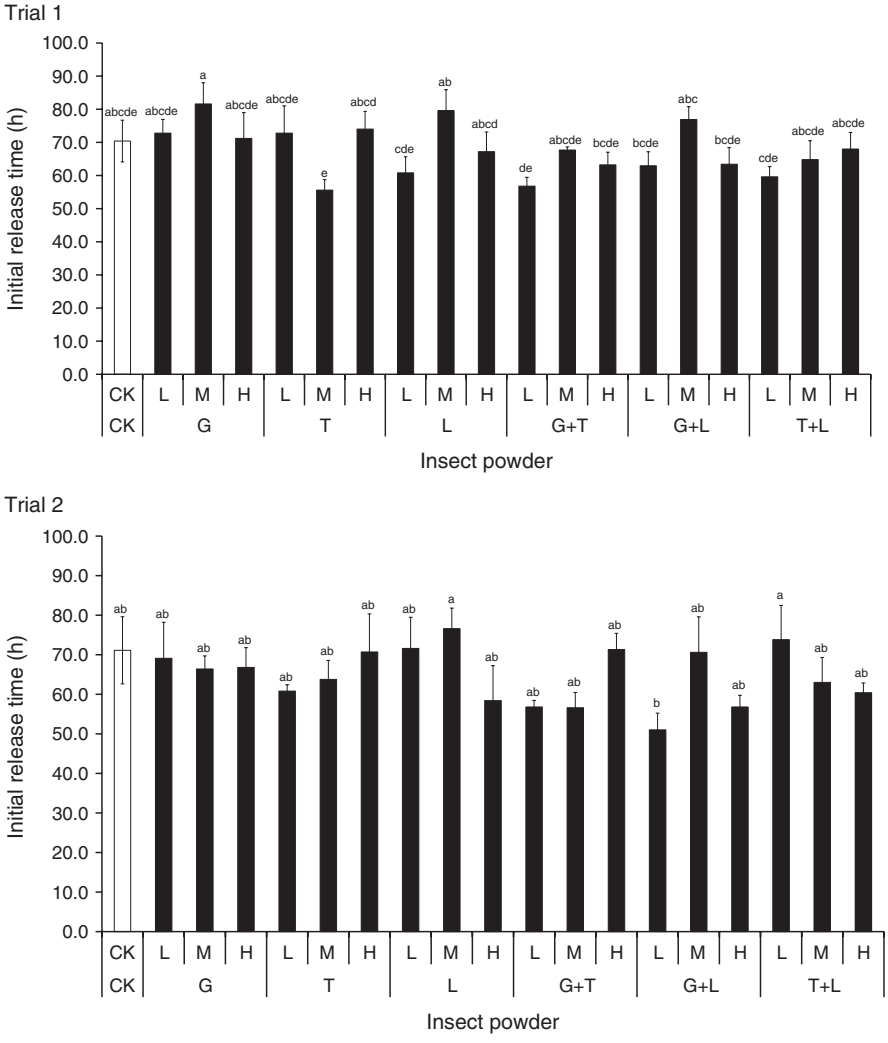

Figure S1: Effect of insect powder and dose level on the initial emergence time of infective juveniles from *Spodoptera litura* larvae in two trials. Each larva was exposed to 100 *Steinernema feltiae* infective juveniles. Five larvae in five wells were regarded as a replication of each treatment. G: *Galleria mellonella*; T: *Tenebrio molitor*; L: *Lucillia sericata*; G+T:1:1 mixed powder of *G. mellonella* and T. molitor; G+L, 1:1 mixed powder of *G. mellonella* and *L. sericata*; T+L: 1:1 mixed powder of *T. molitor* and *L. sericata*. : L, M, H: three dose levels of insect powder 1, 3, and 5% (m/m), respectively. Bars with different letters represent significantly different means (SE) at $p \leq 0.05$ among all treatments via the post hoc Duncan's test.