Virulence of *Steinernema ceratophorum* against different pest insects and their potential for in vivo and in vitro culture

Xun Yan¹², Guimei Chen²³, Yuqing Chen², Bingjiao Sun⁴, Xinghui Gu⁵⁶, Weibing Ruan⁴ and Richou Han²*,

¹Innovative Institute for Plant Health, College of Agriculture and Biology, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China.
²Guangdong Key Laboratory of Animal Conservation and Resource Utilization, Guangdong Public Laboratory of Wild Animal Conservation and Utilization, Institute of Zoology, Guangdong Academy of Sciences, Guangzhou 510260, China.
³College of Plant Protection, South China Agriculture University, Guangzhou 510642, China.
⁴College of Life Sciences, Nankai University, Tianjin 300071, China.
⁵Yuxi Tobacco Company, Yuxi 653100, China.
⁶Application Technology of Biological Control for Tobacco Diseases and Insect Pests Engineering Research Center of China Tobacco, Yuxi 653100, China.

*E-mail: hanrc@giabr.gd.cn.

This paper was edited by Raquel Campos-Herrera.

Received for publication December 23, 2020.

Entomopathogenic nematodes (EPN) of the genera *Steinernema* Travassos and *Heterorhabditis* Poinar are natural parasites of many insects (Kaya and Gaugler, 1993). The infective juveniles (IJ) of EPN harbor the symbiotic bacteria *Xenorhabdus* Thomas and Poinar and *Photorhabdus* Boemare in their intestines (Boemare, 2002; Poinar, 1990; Qiu et al., 2009). The EPN-bacteria complex actively searches, infects and kills the host, propagates in the host, and produces progeny to start a new life cycle after leaving the host (Gotz et al., 1981). EPN can actively search for a suitable host and be mass-produced using conventional fermentation technology (Grewal et al., 2002, 2005). Due to its advantages as a biological control agent, EPN have been widely used to control various insect pests in different parts of the

Abstract

Entomopathogenic nematodes (EPN) are biological control agents used to control different insect pests. Indigenous EPN resources are important for the successful control of endemic pests. A native EPN isolate collected in Yunnan, China that showed high virulence against *Spodoptera litura* was identified and studied for virulence and production potential. The EPN isolate was identified as *Steinemema ceratophorum* and showed high virulence against different larval stages of *Spodoptera exigua*, *Ostrinia nubilalis*, *Helicoverpa armigera*, and the 2nd instar larvae of *Spodoptera frugiperda* when applied at 50 IJ/larva under in vitro conditions. In vivo culture of the *S. ceratophorum* isolate showed that *Galleria mellonella* larvae were the most suitable host compared to *Tenebrio molitor* and *S. litura*. The highest yield in in vivo culture was 1.15 × 10⁵ IJ/larva when cultured in *G. mellonella* with inoculum concentration of 50 IJ/larva at 25°C. For in vitro solid culture, the highest yield for *S. ceratophorum* was 2.09 × 10⁶ IJ/g when cultured at optimal temperature of 25°C. Nematodes from the in vivo and in vitro culture, both retained their virulence against the *G. mellonella* larvae. The *S. ceratophorum* showed high potential to be mass produced, which will enable the commercial use of this EPN isolate. This is the first attempt to mass culture the *S. ceratophorum* species. The study explores the usage of *S. ceratophorum* and provides basis for the commercialization of the EPN species.

Keywords

world, especially against subterranean and boring pests (Kaya et al., 2006; Labaude and Griffin, 2018).

Most EPN species have a wide host range and can infect different insect species in the laboratory, but the susceptibility of pests varies depending on EPN species (Labaude and Griffin, 2018). As the introduction of non-native EPN species may have negative effects on non-target organisms (Bathon, 1996), collection of indigenous EPN isolates is the basis for the successful control of endemic pests (Ma et al., 2010). To collect EPN sources for better control of the tobacco (Nicotiana rustica L.) pests, an extensive survey of EPN was conducted in the tobacco planting area in Yunnan province, China. Several EPN isolates were collected and maintained in the laboratory (Yan et al., 2020). In previous research to screen EPN species/isolate against the tobacco cutworm, Spodoptera litura L. (Lepidoptera: Noctuidae), an isolate recovered from the Yunnan survey, Steinernema sp. 64-2, was found to be with high virulence against S. litura (Yan et al., 2020). This indigenous EPN isolates may contribute to successful pest control in the tobacco field in Yunnan.

To explore insect pest control and evaluate the possibility of mass culture of the native EPN isolate, the promising EPN isolate was identified in the present study. The virulence of the isolate against different pests was tested, including the fall armyworm, Spodoptera frugiperda Smith (Lepidoptera: Noctuidae), which invaded China and was first found in Yunnan in 2019. The in vivo using susceptible host insects and in vitro using sponge mass production were developed for the EPN isolate. Factors that affected yield in both in vivo and in vitro culture were also evaluated.

Materials and methods

Nematode identification and inoculum production

The soil collection and isolation of Steinernema sp. 64-2 was conducted by the Nematode Laboratory, Nankai University, China. The taxonomic status of the isolate was determined using the classical characteristics of morphology and morphometrics together with molecular techniques (Hominick et al., 1997; Jian et al., 1997; Vrain et al., 1992). Primer pairs TW81/AB28 and D2F/536 were used to clone the ITS1-5.8S-ITS2 rRNA gene, and D2-D3 expansion segment of the 28S rRNA gene, respectively (Nguyen et al., 2006). The nematode was identified as Steinernema ceratophorum Jian, Reid and Hunt. IJ of S. ceratophorum were reared in last instar larvae of the greater wax moth, Galleria mellonella (L.) (Lepidoptera: Pyralidae), and harvested by using White traps (Kaya and Stock, 1997). IJ suspension was concentrated to 2,000 IJ/ml, stored at 15°C, 100 rpm shaker and used within 2 weeks. Bioassays were repeated at least twice using different batches of nematodes and insects.

Origin of insects

Galleria mellonella were fed on an artificial diet (Vanhaeche and Degheele, 1980) and reared in an environmentally controlled room at 28°C and 60% relative humidity (RH). The yellow mealworm, Tenebrio molitor L. (Coleoptera: Tenebrionidae), were purchased from the local market. The 9th to 11th instar larvae were reared at room temperature prior to being used for nematode in vivo culture. The 5th instar larvae of S. litura, different instar larvae of the beet armyworm Spodoptera exigua Hubner (Lepidoptera: Noctuidae), the fall armyworm S. frugiperda, the European corn borer Ostrinia nubilalis Hubner (Lepidoptera: Crambidae) and the cotton bollworm Helicoverpa armigera Hubner (Lepidoptera: Noctuidae) and their artificial diet were all purchased from Henan Jiyuan Baiyun Industry Co., Ltd. The larvae were reared on the artificial diet in growth chambers at 26±1°C, 60 to 70% RH, and photoperiod of 14:10 (L:D) prior to being used in bioassays.

Virulence of S. ceratophorum against different pest larvae

Two arenas were used to evaluate the pathogenicity of S. ceratophorum against different target pests. For the 2nd and 3rd instar of H. armigera and S. exigua, and different larval stages of O. nubilalis, 9-cm-diam. Petri dishes were used for bioassays (Yan et al., 2020). Briefly, 10 larvae were added in each Petri dish, lined with two layers of 9-cm-diam. filter paper (Xinhua, China). A volume of 1.5 ml IJ suspension containing 250, 500, and 1,000 IJ (equal to 25, 50, and 100 IJ/larva) was added to the filter paper by pipette. Same volume of sterilized water was used as control. For S. frugiperda and other larval stages of H. armigera and S. exigua, 24-well plates were used for the bioassay to avoid cannibalism of the insects (Yan et al., 2014). Briefly, a volume of 100µl nematode suspension containing 25, 50, and 100 IJ (equal to 25, 50, and 100 IJ/larva) was transferred to individual wells of 24-well plates padded with two 1-cm-diam. filter paper discs. Thereafter, one pest larva was placed in each well. The same volume of sterilized water was used as control. Each replicate contained six wells. Four replicates were set up for each treatment and
each experiment was conducted twice in time. Petri dishes and plates were sealed with Parafilm (SPI Supplies, West Chester, USA), placed at 26 ± 1°C, 75% to 80% RH, and 14:10 (L:D). Insect mortality was recorded 24, 48, and 72 h after infection.

In vivo culture of *S. ceratophorum*

*Galleria mellonella*, *S. litura* and *T. molitor* were used as hosts to culture the nematodes. Larvae of similar size were used for the culture. The average weight of each larva of *G. mellonella*, *S. litura*, and *T. molitor* was of 0.131 ± 0.003, 0.064 ± 0.003, and 0.129 ± 0.007 g, respectively. Ten host larvae were added in each Petri dish lined with two layers of 9-cm-diam. filter paper. A volume of 1.5 ml of IJ suspension containing 500, 1,000 and 2,000 IJ (equal to 50, 100, 200 IJ/larva) was added on the filter paper by pipette. Same volume of sterilized water was used as control. The dishes were randomly divided into three batches and separately incubated at 20, 25, and 30°C. Host mortality was recorded 48 and 72 h after infection. A White trap was set up for each Petri dish 7 d after infection and the dishes were incubated at the corresponding temperature. IJ were harvested from each collection dish every week for 4 weeks after the White trap. The yield was calculated from the batch with higher yield were used to compare the effect of different factors. The yield of *S. ceratophorum* cultured in vivo was expressed as IJ per larvae, and IJ per gram was used to describe the yield of IJ was calculated by sampling and counting IJ amounts from each flask. Infectivity to *G. mellonella* of the harvested IJ was evaluated as described above. The experiment was conducted twice using a different batch of IJ stock.

In vitro sponge culture of *S. ceratophorum*

In vitro sponge culture of *S. ceratophorum* was conducted under sterile conditions. Symbiotic bacteria of *S. ceratophorum* were isolated from freshly infected *Galleria* cadaver. The bacteria were purified and stored at 15% glycerol at −80°C before being used for in vitro culture. Bacteria were cultured in lysogeny broth (LB) and used for nematode culture. Eggs of *S. ceratophorum* were collected from gravid females, separated by sieving and surface-sterilized in a disinfectant (2.5 ml of 4 mol/l NaOH, 0.5 ml of 12% NaOCl, and 21.5 ml of distilled water) before rinsing twice in sterile Ringer’s solution. The sterilized eggs were transferred to sterile LB and incubated until the first-stage juveniles (J1) hatched. The J1 were then inoculated onto agar plate with pure symbiotic bacteria lawn to establish the monoxenic culture system (Peters et al., 2017). Sponge media (15% soy flour, 5% wheat flour, 1% yeast extract, 1% egg yolk powder, 5% corn oil, and 10% crumbled polyurethane foam sponge) were prepared and used for EPN culture (Yan et al., 2018). Symbiotic bacteria of *S. ceratophorum* were cultured at 28°C. 3 ml broth of symbiotic bacteria was then inoculated into 30 g sponge media in each 250 ml flask and cultured at 20, 25, and 30°C for 3 days. After that, 1.5 ml monoxenic IJ suspension at the concentration of 4 × 10⁴ IJ/ml was inoculated into each flask. The flasks were covered with cotton plugs and sealed with brown paper to maintain a monoxenic environment for IJ growth, and incubated at 20, 25, and 30°C, respectively. The nematodes were harvested every week until 7 weeks after being cultured for 3 weeks. Three flasks were randomly sampled for yield calculation at each culture period for each culture temperature. Sponges in each flask were taken out and washed with tap water. IJ were squeezed from the sponge and collected together, to a final total volume of 5,000 ml. The yield of IJ was calculated by sampling and counting IJ amounts from each flask. Infectivity to *G. mellonella* of the harvested IJ was evaluated as described above. The experiment was conducted twice using a different batch of IJ stock.

Statistical analysis

Insect mortality data were corrected according to Abbott (1925) and arcsine transformed before being subjected to statistical analyses. The yield data from the batch with higher yield were used to compare the effect of different factors. The yield of *S. ceratophorum* cultured in vivo was expressed as IJ per larvae, and IJ per gram was used to describe the yield of IJ stock. Means were separated using one-way ANOVA and Univariate General Linear Model (SPSS 16.0). Means were separated using Tukey’s test. Differences among means were considered significant at *P* < 0.05.

Results

Verification of nematode species

The nematode was identified as *S. ceratophorum*. The sequences of ITS1-5.8S-ITS2 rRNA gene and D2-D3 expansion segment of the 28S rRNA gene of *S. ceratophorum* are available on GenBank (Accessions...
Virulence and mass culture of *Steinernema ceratophorum*: Yan et al.

Nos MW024057 and MW029452. The ITS1-5.8S-ITS2 rRNA gene sequence was identity 99.76% and coverage 100% with the type specimen. The D2-D3 expansion segment of the 28S rRNA gene sequence was identity 99.65% and coverage 94% with the type specimen. The nematode belongs to the Bicornutum-glade with a pair of lateral hornlike structures. The lengths of the IJ are 790.22 ± 7.75 μm.

**Virulence of *S. ceratophorum* against different insect pests**

Corrected mortality rates of four instar larval stages of different pests treated by *S. ceratophorum* are shown in Fig. 1. *Spodoptera exigua* larvae are sensitive to *S. ceratophorum*, with mortality rates of 100% for the 2nd and 3rd instar after treated for 48h. For the 4th and 5th instar of *S. exigua*, although mortality did not reach 100% after treated for 48h when nematode was applied at lower rates of 25 and 50 IJ/larva, mortality reached 100% after treated for 72h. No significant difference in the mortality rate was found among different nematode application rates for different larval stages of *S. exigua*, except that for the 4th instar larvae (*F* = 0.084, df = 2, 21, *P* = 0.042).

For *S. frugiperda*, mortality increased with the nematode application rate and decreased with the larval stage. *Spodoptera ceratophorum* could cause 100% mortality of the 2nd instar larvae within 48h when applied at three rates. For the other three instar larvae, mortality increased significantly when nematode rate increased (*F*≥5.623, df = 2, 21, *P* ≤ 0.011). Mortality of the 3rd, 4th, and 5th instar of *S. frugiperda* treated by nematode at 100 IJ/larva was 97.92 ± 2.08, 91.67 ± 4.45, and 77.08 ± 3.05% after 48h, respectively. Prolonging treated time to 72h could not increase mortality of the 5th instar of *S. frugiperda* to 100%.

For *O. nubilalis*, the 2nd instar larvae were sensitive to *S. ceratophorum* and mortality reached 100% after 48h even when nematode application rate was 25 IJ/larva. For the older larvae, nematode application rate of 25 IJ/larva could only cause 79.5 to 90.9% mortality of the larvae, which was significantly lower than that caused by nematodes at the rate of 50 and 100 IJ/larva (*F* ≥ 3.827, df = 2, 21, *P* ≤ 0.038).

For *H. armigera*, *S. ceratophorum* was able to kill 100% of the 2nd and 3rd instar larvae after 48h when nematode was applied at low rate of 25 IJ/larva. But for the 4th and 5th instar larvae, mortality caused by nematode at 25 IJ/larva was significantly lower than that caused by nematodes at higher rates of 50 and 100 IJ/larva (*F* = 24.782 and 11.932 for the 4th and 5th instar larvae, respectively, df = 2, 21, *P* < 0.001). For the 5th instar larvae, mortality caused by nematode application rates of 25, 50, and 100 IJ/larva for 48h was shown in Fig. 1.

![Figure 1: Corrected mortality rates of different larval stages of Spodoptera exigua, S. frugiperda, Ostrinia nubilalis and Helicoverpa armigera after treated by Steinernema ceratophorum at 25, 50, and 100 IJ/larva for 48h. Bars with different letters indicate significant difference among different nematode rates of the same larval stage of each insect pest according to Tukey’s test at \( P = 0.05 \).](image-url)
at 100 IJ/larva was 91.67 ± 3.15% at 48 h and reached 100% at 72 h after treatment.

**Yield of S. ceratophorum in in vivo culture**

Larval mortality caused by *S. ceratophorum* was significantly affected by host insect, IJ inoculum concentration, culture temperature and the interaction of these factors (*P* ≤ 0.041), except for the interaction of temperature and inoculum concentration at 48 h after treatment (*F* = 1.614, df = 4, 189, *P* = 0.172). Corrected mortality rates of different hosts treated by *S. ceratophorum* for 48 and 72 h are given in Fig. 2. *Galleria mellonella* and *S. litura* were more sensitive to *S. ceratophorum*. *Steinernema ceratophorum* could cause 100% mortality of the *G. mellonella* and *S. litura* larvae after treated for 72 h. In the case of *T. molitor*, the highest mortality rates after treated for 72 h were 96.25, 97.50, and 98.75% when treated at 20, 25, and 35°C, respectively. Temperature affected mortality of the three hosts when treated by *S. ceratophorum* for 48 h. Mortality rates of the hosts were lower at 20°C than at 25 and 30°C after treated for 48 h. When treated time extended to 72 h, the difference among mortality rates of the same host at different temperatures were not obvious. For *T. molitor*, the differences among different inoculum concentrations were significant after treated for both 48 and 72 h (*F* ≥ 4.158, df = 2, 21, *P* ≤ 0.030), except for treatment at 20°C for 48 h. Only dead host larvae were collected for White trap to calculate IJ yield. Numbers of dead larvae used for white trap for different hosts are shown in Table 1. Because *S. ceratophorum* could cause 100% mortality of the *G. mellonella* and *S. litura* larvae, almost all the host cadavers could be used for white trap, except for 1–2 rotten cadaver(s). Less number of *T. molitor* cadavers were used for white trap, because mortality of *T. molitor* larvae could not reach 100% even after treated for 7 days.

IJ yield of *S. ceratophorum* was significantly affected by host insect, IJ inoculum concentration, culture temperature and the interaction of these factors (*P* < 0.001). Total yields of *S. ceratophorum* per host in different hosts for 4 weeks are shown in Fig. 3. *Steinernema ceratophorum* cultured in *G. mellonella* gained the highest IJ yield, followed by *T. molitor* and *S. litura*. The optimal temperature for *S. ceratophorum* culture was 25°C, when compared with 20 and 30°C. Nematodes cultured at 20°C yielded more IJ than at 30°C. When cultured in *G. mellonella* and *T. molitor*
at 25°C, the highest IJ yield was gained at inoculum concentration of 50 IJ/larva, followed by 100 and 200 IJ/larva. Increase of the inoculum concentration did not increase the yield of *S. ceratophorum*. While when being cultured using *S. litura*, no significant effect on the IJ yield was found among different inoculum concentration at 25°C ($F=0.766$, df = 2, 9, $P=0.493$). The highest IJ yield was $7.73 \times 10^3$ IJ/larva when cultured in *S. litura*. The highest IJ yield of *S. ceratophorum* was $1.15 \times 10^4$ IJ/larva, which was gained when cultured in *G. mellonella* with inoculum concentration of 50 IJ/larva at 25°C.

Infectivity of the progeny IJ to *G. mellonella* was tested only in the week that yield of the progeny IJ was higher than 100 IJ/larva. No IJ were collected at 20°C 1 week after White trap for all the three hosts. For all the three hosts, the progeny IJ cultured at 20 and 25°C caused 85 to 100% mortality of the *G. mellonella* larvae after treated for 48h, and 100% after 72h. The harvested IJ cultured at the two temperatures retained the infectivity against *G. mellonella* larvae at 25°C. Mortality rates of the *G. mellonella* larvae treated by the progeny IJ cultured at 30°C from different hosts are shown in Table 2. When cultured at 30°C,
mortality rates of the \textit{G. mellonella} larvae caused by the progeny IJ cultured from \textit{G. mellonella} and \textit{T. molitor} were 70 to 100\% after treated for 48h, and reached 90 to 100\% after 72h. Mortality rates of the \textit{G. mellonella} larvae caused by the progeny IJ cultured from \textit{S. litura} were only 5 to 70\% after 48h, which were lower than that from \textit{G. mellonella} and \textit{T. molitor}. IJ invasion was confirm for all the dead \textit{G. mellonella} larvae treated by \textit{S. ceratophorum} harvested from different hosts.

### Yield of \textit{S. ceratophorum} in solid sponge culture

Yields of \textit{S. ceratophorum} produced in sponge media are shown in Fig. 4. Yields in in vitro culture were

![Figure 4: Yield of infective juveniles (IJ) of \textit{Steinernema ceratophorum} cultured in sponge media at 20, 25, and 30°C for different culture period. Bars with different uppercase letters indicate significant differences in IJ yield cultured at different temperatures at the same culture period, and lowercase letters indicate significant differences in IJ yield cultured for different period at the same temperature, according to Tukey’s test at \( P=0.05 \).]
Virulence and mass culture of *Steinernema ceratophorum*: Yan et al.

Significantly affected by culture period ($F=142.278$, $df=4, 30, P<0.001$), temperature ($F=212.941$, $df=2, 30, P<0.001$), and the interaction between the two factors ($F=14.168$, $df=8, 30, P<0.001$). Higher IJ yield was gained when cultured at 25°C, followed by 20°C and then 30°C, which was the same as that when cultured in vivo. For culture period, 5–6 weeks are optimal culture time for *S. ceratophorum*. Shorter culture period gained lower yield, while IJ died when culture period extended to 7 weeks. The highest IJ yield at each temperature was recorded as $1.92 \times 10^6$ IJ/g at 20°C, $2.09 \times 10^6$ IJ/g at 25°C and $9.59 \times 10^5$ IJ/g at 30°C.

Progeny IJ produced from the in vitro sponge media retained the infectivity to the *Galleria mellonella* larvae. Mortality rates of the *G. mellonella* larvae treated by IJ produced from different culture period at different temperatures after 48 h are shown in Fig. 5. IJ produced at different temperatures could cause 100% mortality of the *G. mellonella* larvae at 25°C. No significant difference was found in mortality of the tested *G. mellonella* larvae caused by IJ harvested from different culture period at different temperatures ($F \leq 3.000$, $df=4, 15, P \geq 0.053$).

**Discussion**

The present study expanded the insecticidal spectrum of *S. ceratophorum*, which provides basis for the application of this EPN species against various insect pests. Efficacy of EPN against the four pests tested in this study has been previously studied. *Steinernema carpocapsae* and *S. feltiae* showed high virulence against the *S. exigua* larvae (Ansari et al., 2007; Mahmoud, 2014). *Steinernema riobravis*, *S. arenarium* and *H. amazonesis* were found to be effective against *S. frugiperda* larvae both in the lab and in the field (Andaló et al., 2010; Andaló et al., 2012; de Souza et al., 2012; Leyva-Hernandez et al., 2018). *Steinernema feltiae* could infect and kill *O. ubilalis* larvae at low temperature of 7°C (Eculica et al., 1997), and *S. carpocapsae* was able to reduce the rate of economic ear damage from 20 to 5% when applied on sweet corn plants to control *O. ubilalis* (Ben-Yakir et al., 1998). *Helicoverpa armigera* larvae were sensitive to *S. hermaphroditum* and *H. bacteriophora* isolated in India (Bhat et al., 2019; Vashisth et al., 2019). Addition of antidesiccants with *S. carpocapsae* resulted in a four-fold increase (to 85–95%) of mortality of *H. armigera* (Glazer et al., 1992), and *H. indica* also showed potential for deployment in IPM program for management of *H. armigera* (Gokte-Narkhedkar et al., 2019). In the present study, *S. ceratophorum* were also found to be effective against different instar larvae of *S. exigua*, *O. ubilalis*, *H. armigera* and young larvae of *S. frugiperda* when applied at 50-200 IJ/larva in the laboratory, when compared to the published EPN isolates. *Steinernema ceratophorum* was first reported and identified in north-east China (Jian et al., 1997) and later isolated in north and south-west China (Ma et al., 2010; Yan et al., 2020). *Steinernema ceratophorum* caused high mortality of larvae of the bark beetle *Dendroctonus valens LeConte* (Coleoptera: Scolytidae) (Jian et al., 2002), *Haemaphysalis longicornis* Neumann (Acar: Haemaphysalinae) (Gao et al., 2004) and *Bradyisia odoriphaga* Yang & Zhang (Diptera: Sciaridae) (Ma et al., 2013a, b). *Steinernema ceratophorum* is an intermediate foraging EPN species, which has both ambush and cruise foraging behaviors (Kaya and Campbell, 2000). *Steinernema ceratophorum* will have superiority when applied on plant foliage to control pests that burrow into the whorl or tunnel into the ear of

![Figure 5: Mortality of Galleria mellonella larvae 48h after treated by infective juveniles of Steinernema ceratophorum produced in sponge media at 20, 25, and 30°C for different culture period.](image-url)
cereal plants base on its intermediate foraging behavior, because the nematodes can actively search for host insects and withstand certain degree of desiccation (Ma et al., 2013a). Steinernema ceratophorum was isolated in a corn field. The high virulence of S. ceratophorum against different corn pests makes it a promising EPN isolate to be used in corn protection.

Mass production of EPN of reliable quality is the backbone of the EPN industry (Peters et al., 2017). EPN are capable of being mass-produced by in vitro or in vivo methods. Their suitability for industrial mass production is a prerequisite for their successful commercialization. Production of S. ceratophorum in vivo and in vitro solid culture was evaluated in the present study. For in vivo culture, three insects were evaluated on their superiority as a host of S. ceratophorum. Galleria mellonella is a best choice of host because the larvae are highly reliable, exceptionally susceptible to most EPN species and relatively inexpensive (Peters et al., 2017). The results in our study further confirmed the suitability of G. mellonella as a host for S. ceratophorum culture. The highest yield of IJ was 1.15 × 10^5 IJ/larva when cultured in G. mellonella, which was significantly higher than IJ yield cultured in T. molitor and S. litura under the same conditions. EPN yields cultured in G. mellonella ranged from 1 to 3.5 × 10^4 IJ/larva (Peters et al., 2017). The IJ yield of S. ceratophorum cultured in G. mellonella in this study proved that G. mellonella was an appropriate host for S. ceratophorum. The attempts to optimize the mass rearing of G. mellonella has helped to reduce the cost for EPN culture in G. mellonella (Kotchofaand Baimey, 2019). Tenebrio molitor larvae are substantially cheaper, but mortality of T. molitor caused by S. ceratophorum was lower than the other two insects and the cadavers used for white trap were relatively fewer. IJ yield from T. molitor was much lower, even when inoculum concentration was as high as 200 IJ/larva. The operating time also increased when doing White trap because alive T. molitor larvae were required to be removed. Spodoptera litura larvae are available in the market and could be purchased as experimental insects for research. The price of S. litura larva was 15 times of that of G. mellonella, so the cost for production of S. ceratophorum by using S. litura was 90 times of that by using G. mellonella. In previous study, only one out of 10 H. indica isolates was able to produce IJ on S. litura (Boff et al., 2000). In our study, S. ceratophorum could have a yield of 7.73 × 10^4 IJ/larva in S. litura. When using S. litura for S. ceratophorum culture, very few residues were remained because the nematodes consumed almost the whole body of the host. Spodoptera litura could be a choice as host for S. ceratophorum culture in small scale in case that no other host insects are available and the S. litura larvae can be easily collected from infested field.

Inoculation concentration is another factor which affects IJ yield in EPN in vivo production. Usually, intermediate dosages can be used to maximize yield (Shapiro-Ilan et al., 2014). Rates of approximately 25–200 IJ per insect are usually sufficient for infecting G. mellonella, whereas higher rates of 100–600 IJ per insect are generally needed to infect T. molitor (Peters et al., 2017). The results in our study showed that IJ yields of S. ceratophorum were the highest at inoculation concentration of 50 IJ/larva when cultured in G. mellonella and T. molitor at 25°C. Increase of inoculation concentration did not increase IJ yield accordingly, although increase the inoculation concentration did increase mortality of the T. molitor larvae. Inoculation concentration of 50 IJ/larva was optimal for in vivo culture of S. ceratophorum in G. mellonella and T. molitor.

In vivo production of EPN requires the least capital outlay but is with high costs of labor. In vitro culture enables mass production of EPN outside insect hosts based on the support of their symbiotic bacteria. In vitro production of EPN includes liquid and solid culture. Liquid culture of EPN accounts for the bulk of commercial production and offers the greatest economic efficiency but requires the largest capital outlay. In vitro solid culture is intermediate between the other two methods in most aspects (Han and Ehlers, 2001). In vitro solid culture does not require high technology inputs and large investments. The effect of phase variation of the symbiotic bacteria on the yields is less than in liquid culture (Ehlers et al., 2000). The in vitro solid culturing system is superior to liquid culture technology in the developing countries (Han, 1995). The in vitro solid culture of S. ceratophorum were studied using the technique developed in our institute (Han, 1995; Qiu et al., 2016). The maximum IJ yield of S. ceratophorum could reach 2.09 × 10^6 IJ/g when cultured at optimal temperature of 25°C, which was high when compared to the two commercialized EPN isolates, H. indica LN2 (IJ yield of 9.3 × 10^4 IJ/g) and H. bacteriophora H06 (IJ yield of 5.1 × 10^4 IJ/g) (Han et al., 1992). This suggested that S. ceratophorum is with high reproductive capacity and is highly potential to be mass produced in solid media. Other developmental stages of S. ceratophorum were observed when calculating IJ yield after harvest. The inconsistency of developmental stages can be improved by optimization of media composition and the proportion of different components (Ramakuwela et al., 2016; Seenivasan, 2017). Inoculum concentration, age, and type might also affect IJ yield.
and IJ consistency (Shapiro-Ilan et al., 2014). The optimization of solid culture of *S. ceratophorum* can be further studied before commercialization of the nematode.

Temperature is a crucial factor affecting survival, infectivity, culture, and storage of EPN. The *S. ceratophorum* 64-2 isolate was recovered in August 2017 at Pingyuan Town (N: 23°48’3”, E: 103°38’54”) in Yanshan County, Wenshan Zhuang, and Miao Autonomous Prefecture, Yunnan Province. The average temperature in the area was 18–26°C of the period. The highest IJ yield was gained at 25°C in both in vivo and solid sponge culture when compared with the IJ yield cultured at 20 and 30°C. IJ yield was obviously lower when cultured at 30°C, suggesting that *S. ceratophorum* 64-2 has adapted itself to a mild warm temperature of 20–25°C, rather than a hot temperature of 30°C. Besides yield, quality of the produced IJ is another important indicator assessing the mass production technique. The performance of EPN was used to assess quality of the progeny IJ, which was influenced by mobility, lipid reserves, retention of symbiotic bacteria, and the most important trait of virulence against the host (Peters et al., 2017). In the present study, the progeny IJ cultured at different temperatures were able to cause almost 100% mortality of the tested *G. mellonella* larvae in 72h, suggesting that the progeny IJ produced from both in vivo and in vitro culture retained their infectivity against *G. mellonella*. Before using *S. ceratophorum* to control target pest(s), virulence of the produced IJ against the target pest(s) should be assessed after mass production.

The indigenous EPN isolate collected in Yunnan, China, which showed high virulence against *S. litura*, was identified as *S. ceratophorum* in this study. The *S. ceratophorum* isolate was found to show high virulence against different larval stages of *S. exigua*, *O. nubilalis*, *H. armigera* and the 2nd instar larvae of *S. frugiperda* when applied at 50 IJ/larva in the laboratory. High yield of the *S. ceratophorum* isolate gained in both in vivo and in vitro culture proved the capability of mass culture of the EPN isolate. The progeny nematodes retained the virulence against *G. mellonella*, confirming the quality of the produced nematodes. It’s the first attempt to mass culture the *S. ceratophorum* species. The present study explores the usage of *S. ceratophorum* and provides basis for the commercialization of this EPN species.

**Acknowledgments**

This research was funded by the Key-Area Research and Development Program of Guangdong Province (2020B020224002), Science & Technology Project of Yunnan Tobacco Company (110202001034(LS-03)), Guangzhou Science and Technology Project (201904020002), and National Key Research and Development Program of China (2017YFE0130400).

**References**


Ma, J., Chen, S., Moens, M., De Clercq, P., Li, X. and Han, R. 2013a. Characterization in biological traits of entomopathogenic nematodes isolated from North China. Journal of Invertebrate Pathology 114:288–76.


Nguyen, K. B., Qiu, L. H., Zhou, Y. and Pang, Y. 2006. Steinernema leizhouense sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode


