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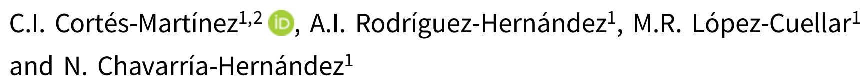
Surface culture of *Steinernema* sp. on two solid media and their pathogenicity against *Galleria mellonella*



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Abstract

The use of native entomopathogenic nematodes as biocontrol agents is a strategy to decrease the environmental impact of insecticides and achieve sustainable agriculture crops. In this study, the effect of the surface culture of *Steinernema* sp. JAP1 over two solid media at 23–27°C on infective juvenile (IJ) production and pathogenicity against *Galleria mellonella* larvae were investigated. First, the bacterial lawn on the surface of the media with egg yolk (P2) or chicken liver (Cl) were incubated in darkness at 30°C for 48 and 72 h, and 100 surface-sterilized IJs were added. Four harvests were conducted within the next 35 days and the mean accumulated production was superior on Cl (210×10^3 IJs) than on P2 (135×10^3 IJs), but the productivity decreased up to 10% when the incubation time of the bacterial lawn was of 72 h. The mean pathogenicity of *in vitro*- and *in vivo*-produced IJs were of 47–64% and 31%, respectively. It is worth noting that none of the two solid media had a statistically significant difference in IJ pathogenicity. Considering that the maximum multiplication factor of IJs on solid media was 2108 and that the pathogenicity against *G. mellonella* was outstanding, *Steinernema* sp. has a good potential for *in vitro* mass production.

Introduction

The infective juvenile (IJ) is the only free-living stage of entomopathogenic nematodes (EPNs), which occupy soil habitats and develop their life cycle by infecting a new insect host (Ehlers, 2001; Goodrich-Blair *et al.*, 2009). Several species of EPNs are commercially available for the biological control of soil insect crop pests in organic farming (Sarwar & Mukhtar, 2021). The application of commercially available EPN products based on native species is a strategy to eliminate the use of synthetic insecticides on organic crops (Ferreira *et al.*, 2016).

The industrial mass production of viable IJs at low cost (Dunn *et al.*, 2021), the maintenance of infectivity and extension of shelf life (Ehlers, 2001) are the main challenges for the successful commercialization of EPNs as a biopesticide product. For scaling up to a profitable industrial mass production of IJs, the submerged monoxenic culture is the most suitable technology because the yield is higher, although it is variable (Shapiro-Ilan *et al.*, 2012; Cortés-Martínez & Chavarría-Hernández, 2020).

However, in the *in vitro* culture, the retention of the bacterial symbiont within the juvenile can be highly variable and the virulence of the produced IJs depends on the bacterial cell load inside them (Akhurst, 1986). Furthermore, the pathogenicity can differ depending on strains within the same species of the symbiotic bacterium, while retention requires a recognition mechanism, finely tuned between the two partners (Sicard *et al.*, 2003). Sharmila & Subramanian (2020) found that reared *Steinernema glaseri* IJs on *Galleria mellonella* larvae were more virulent than *in vitro*-produced IJs. Hang *et al.* (2007) reported that 10,000 cells of *Xenorhabdus poinarii* from *S. glaseri* Dongrae strain caused 100% mortality of *G. mellonella* at 30°C and 35°C in a period of 48 h. This fact raises the challenge of reconciling the productivity of the *in vitro* culture with the virulence of the produced IJs, through proper evaluation of the nematode–bacteria complexes.

The surface culture on solid agar media allows us to study the ability of the symbiotic bacteria to colonize the IJ stage, and monitor the process of the beginning of colonization outside the insect (Akhurst & Boemare, 1988; Goodrich-Blair *et al.*, 2009), as a screening phase of virulent strains that could also be productive in the mass production process (Ravensberg, 2011).

Initially, the nematodes ingest the bacteria lawn on agar and, afterwards, they reproduce and develop. Then, the solid medium provides nutrients for developing both the symbiotic bacteria and the nematodes, which include specific nutrients for the nematode growth – for example, oil and cholesterol (Ravensberg, 2011). If the medium formulation is adequate for

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the growth of the bacterium and nematode, then phase I bacterial cells colonize J3 efficiently, and the productivity and virulence of IJs will be higher (Converse & Miller, 1999).

The dehydrated egg yolk is a processed component usually used in media for *in vitro* culture of EPNs, which is difficult to acquire in local markets, while fresh Cl is easier to acquire and has proven to be a suitable component for the reproduction of nematodes. Therefore, this work aimed to investigate the effect of surface propagation of *Steinernema* sp. JAP1 on two solid media, the incubation time of bacterial lawn and the harvest time on the production of IJs and its pathogenicity in a one-on-one assay on *G. mellonella*.

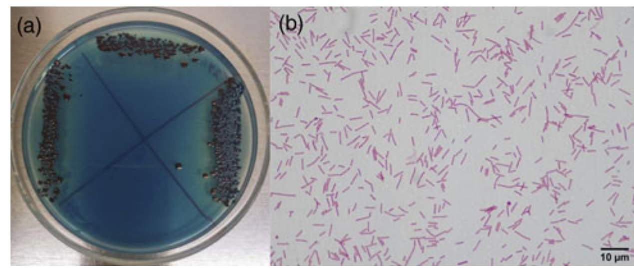

Fig. 1. *Xenorhabdus* sp. isolated from infective juveniles of *Steinernema* sp. JAP1. (a) Blue colonies on Nutrient blue tetrazolium agar plate medium. (b) Light micrograph of phase I bacterial cells at 1000× magnification. Scale bar: 10 μm.

Materials and methods

EPN

Steinernema sp. JAP1, originally isolated in the Asia district of Cañete, Lima, Peru, was kindly provided by the Entomologist J. Alcázar. The IJ pathogenicity was proved by the Koch's postulates. Following the procedure of Stock & Goodrich-Blair (2012), IJs were reproduced in the last instar wax moth larvae (*G. mellonella*) and the harvested IJs were surface-sterilized with benzethonium chloride solution (0.1% (w/v)) for 10 min and rinsed three times with sterile distilled water (DW). After that, the sterilized IJs were concentrated in an aqueous suspension at a rate of 1000 IJs·mL^{-1} and stored at 5–9°C in 250 mL tissue culture flasks, until they were used.

Culture media

The isolation medium of *Xenorhabdus* sp. was Nutrient blue tetrazolium agar (NBTA)-adjusted to a pH of 8.2 (Akhurst, 1980): 2.3% (w/v) nutrient agar, 0.025% (w/v) bromothymol blue (Sigma, Saint Louis, Missouri, USA), 0.004% (w/v) triphenyltetrazolium chloride (Sigma, USA), dissolved in DW. Liquid media for bacterial culture comprised tryptic soy broth (TSB) 3% (w/v) and yeast extract 0.5% (w/v). In the form of solid medium, 15 mL of the following diets were deposited into sterile 6-cm Petri dishes: (1) P2 (Chavarría-Hernández & de la Torre, 2001) – yeast extract 2.3% (w/v), dehydrated egg yolk 1.25% (w/v), sodium chloride 0.5% (w/v), corn oil 4% (v/v) (Altea®, Guadalajara, Jalisco, Mexico) and bacteriological agar 3.6% (w/v); and (2) Cl (Moreno-Salgüero, 2017) – small pieces of fresh Cl 10% (w/v) were placed into a blender with corn oil 4% (v/v), nutrient broth 1.3% (w/v), bacteriological agar 3.6% (w/v) and DW, and processed according to McMullen & Stock (2014). All solid culture media were incubated overnight at 30°C after preparation. All the culture media used were Bioxon® brand (Cuautitlán Izcalli, Estado de México, México).

Isolation of symbiont bacteria

The bacterial cells were extracted from crushed surface-sterilized IJs that were suspended in 8 mL of TSB and incubated at 28°C in darkness for 44 h (Koppenhöfer, 2007). The primary form of the expected species *Xenorhabdus* sp. was confirmed through the macroscopic morphological characteristics and the size of blue colonies onto NBTA plate medium (fig. 1a), according to Akhurst & Boemare (1988), Kaya & Stock (1997) and Gaugler *et al.* (1992). The microscopic morphological characteristics were studied by Gram staining (fig. 1b) (Thomas & Poinar, 1983). A loopful of an isolated phase I bacterium colony was cultured in 50 mL of TSB medium and incubated at 28°C and 130 rpm for 38 h (Barnstead/Labline E-class; ThermoFisher Scientific, Waltham, Massachusetts, USA). The symbiotic bacteria were conserved in 2 mL vials with glycerol 20% (v/v) and cryopreserved at −170°C, till use in the experiments. After a month, samples of a *Xenorhabdus* sp. vial were streaked on NBTA plates and incubated in the conditions previously described. Later on, a loopful of an isolated phase I bacterium was inoculated in 50 mL of TSB and incubated at 28°C and 130 rpm for 35 h to produce the bacterial inoculum for the experiments.

In vitro culture of EPNs

The productivity of IJs was determined through the surface culture on P2 and Cl media and for two different incubation times post-inoculation of bacterial broth, using the following experimental procedure: (1) 0.1 mL aliquots of the 38 h-old TSB-*Xenorhabdus* sp. culture broth were transferred to 6 cm plates of each solid medium; (2) plates were incubated in darkness at 28°C over 48 and 72 h (model Gl6; Shel Lab, Cornelius, Oregon, USA); and (3) 100 surface-sterilized IJs were inoculated on the surface of the media and stored in darkness at 23–27°C and 20–38% relative humidity (RH). Three plates of the solid medium by treatment were established and the experiment was repeated twice.

Plates were monitored daily under a stereoscope (model Z30 V; Leica®, Rockleigh, New Jersey, USA). When IJ production was detected, modified White traps were established and stored at 23–27°C and 20–38% RH in a desiccator (Scienceware®, Pequamoek, New Jersey, USA). The harvest and counting of IJs were conducted at 14, 21, 28 and 35 days post-inoculation (dpi). The IJs were rinsed three times with sterile DW, concentrated at a rate of 1000 IJs·mL^{-1} and stored at 5–9°C in tissue culture flasks. The suspensions of harvested IJs from each White trap were diluted (10^0–10^2) with sterile DW and the IJ concentrations were determined by counting in five 0.02 mL samples under a stereoscope (model Z30 V; Leica®), following the procedure described by Stock & Goodrich-Blair (2012).

Assay of pathogenicity

The *in vitro*-produced IJs on solid media were evaluated according to the mortality caused to *G. mellonella* in the one-on-one assay (Converse & Miller, 1999; Kazimierczak *et al.*, 2018). A filter paper disk was placed (medium pore, 21 mm in diameter) in each well of a 12-well plate and one IJ was transferred into each well in

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10 µL DW, followed by 50 µL DW. Immediately, one *G. mellonella* larva (previously surface-sterilized with sodium hypochlorite solution 0.5% (v/v) and rinsed twice) was added. The plates were sealed with adhesive tape to minimize evaporation and stored at 23–27°C and 20–38% RH.

The IJs evaluated in this experiment were obtained from four treatments of solid culture that combined two solid media and two incubation times of the bacterial lawn (P2-48 h, P2-72 h, Cl-48 h and Cl-72 h), one treatment using IJs reared *in vivo* as the positive control and a negative control without IJs but with sterile DW. The experiment was repeated three times and conducted by duplicate. This experimental procedure was carried out to evaluate IJs harvested at 14, 21, 28 and 35 dpi of 100 IJs. Thus, 1152 *G. mellonella* larvae were used in this bioassay. The insect mortality was recorded as 3 dpi.

Measurement of IJ length

Micrographs of alive IJs harvested from each solid medium were taken at 40× using a digital camera (model DSFi3; Nikon®, Tokyo, Japan) coupled to a light microscope (model 80i; Nikon®, Japan). The length of IJs was measured with the 'segmented line' tool in the software ImageJ 1.53j (https://imagej.nih.gov/ij/index.html), using the following procedure: (1) individuals with an elongated or slightly curved body were selected, (2) the initial measurement point was placed in the anterior end of the body, (3) the dimension line was extended at an approximate distance to the inflection point of the imaginary central body line (as many times as necessary until reaching the tail) and (4) the length was registered in µm. Sixteen IJs per treatment were measured in each of the four harvests carried out, for a total amount of 960 individuals.

Statistical analysis

Data are presented as the mean value ± standard error (SE). The productivity of IJs in each solid media was reported as $IJs \cdot m^{-2} \cdot day^{-1}$, which represent the accumulated number of IJs produced by unit area in a 6-cm Petri dish (2×10^{-3} m^2 of internal area) by each day of the harvest time. The Shapiro–Wilk normality test ($P > 0.05$) was applied to the dataset of IJ production and pathogenicity to prove the assumption of normality. The treatment effects on the production of IJs by solid media (P2 and Cl), incubation time (48 and 72 h post-inoculation (hpi) of bacterial broth) and harvest time (14, 21, 28 and 35 dpi of IJs) were assessed via Kruskall–Wallis one-way analysis of variance (ANOVA) on ranks at $P < 0.05$, given the non-normal distribution of the data. The treatment effects on pathogenicity of IJs on *G. mellonella* by solid media, incubation time of bacterial lawn and harvest time were assessed via three-way ANOVA at $P < 0.05$, given the normal distribution of the data ($P = 0.62$). A Pearson correlation analysis ($P < 0.05$) was conducted to determine the correlation between the IJs' length and pathogenicity. All analyses were conducted in SigmaPlot® 12 (Systat Software, Inc., San Jose, California, USA).

Results

In vitro production of IJs on solid media

New offspring of IJs were observed 10–11 dpi (fig. 2a), and fig. 2b shows the IJs produced. The culture of nematodes *Steinernema* sp. JAP1 at each harvest time showed a mean production by plate of Cl medium of 46,112 IJs ± 6203 SE, while the culture on P2 medium produced 32,217 IJs ± 5595 SE. Thus, P2 produces a significantly lower amount of IJs than Cl ($P = 0.016$). Regarding the incubation at 30°C, the mean production was not significantly different ($P = 0.7$) when the bacterial lawn was incubated for 48 h (38,863 IJs ± 6410 SE) or 72 h (39,466 IJs ± 5544 SE).

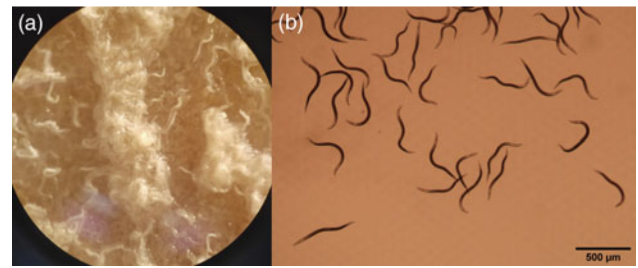

Fig. 2. *In vitro* culture of *Steinernema* sp. JAP1 on solid medium at 23–27°C and 20–38% relative humidity. (a) Development of entomopathogenic nematodes with their corresponding phase I symbiotic bacteria *Xenorhabdus* sp. on an egg yolk medium at 3× magnification (10-day-old culture). (b) Light micrograph captured at 40× magnification of several infective juveniles harvested. Scale bar: 500 µm.

When the bacterial lawn was incubated for 48 h, the mean production in P2 was not significantly different from the production in Cl; however, the production at 14 days (8064 IJs ± 1698 SE) was significantly lower ($P < 0.05$) than the production at 21 days (63,625 IJs ± 12,812 SE), 28 days (54,866 IJs ± 17,911 SE) and 35 days (28,900 IJs ± 6340 SE). The production on the bacterial lawn incubated for 72 h was not significantly different when the culture was carried out on P2 or Cl, while the mean production at 14 days (12,600 IJs ± 2214 SE) was significantly lower ($P < 0.05$) than the production at 21 days (55,200 IJs ± 11 864 SE) and 28 days (54,733 IJs ± 12,166 SE). Table 1 shows the production data of IJs from solid media at each harvest time.

After four harvests of IJs, from solid media incubated 48 hpi of TSB-*Xenorhabdus* sp., the mean accumulated productivities were higher in Cl (294×10^4 IJs m^{-2} day^{-1}) than in P2 (189×10^4 IJs m^{-2} day^{-1}), and the same tendency occurred when the incubation time was for 72 h. However, the productivity decreases up to 10% on both solid media. Statistically significant differences ($P < 0.05$) were observed in IJs' productivities on P2 and Cl.

Pathogenicity of IJs produced

The mortality of *G. mellonella* by single IJs *in vitro*-produced and the positive control using single IJs *in vivo*-reared is shown in fig. 3. The maximum mortality of *G. mellonella* was achieved using IJs harvested at 14 days from the P2 medium (77.77% ± 10.01 SE), followed by IJs harvested at 35 days from the Cl medium (75% ± 8.33 SE); both media were incubated for 72 h. There was no mortality of *G. mellonella* in the negative control.

Concerning the solid medium composition factor, the higher mortality of *G. mellonella* was observed using IJs collected from P2 (54.16% ± 4.1 SE), followed by those harvested from Cl (51.38% ± 3.5 SE). The mortality by IJs reared *in vivo* was the lowest observed (31.25% ± 2.84 SE). The pathogenicity of the cultured IJs from the two treatments on solid medium (P2 and Cl) only showed a statistically significant difference ($P < 0.001$) when they were compared with the treatment of IJs reared *in vivo*. The analysis of the incubation time factor showed a minor positive effect in the pathogenicity caused by the harvested IJs, since a statistically significant difference of $P = 0.043$ was found in the

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Table 1. *In vitro* production of *Steinernema* sp. JAP1 infective juveniles (IJs) through the surface culture on two solid media at 21–25°C with a bacterial lawn of *Xenorhabdus* sp. previously incubated at 30°C for 48 and 72 h, and comparison with results from other studies. Our data are present as the mean value per 6-cm Petri dish.

Solid medium	Incubation time of bacterial lawn (h)	Harvest time (dpi of IJs)				Mean accumulated ± SE (C)	Multiplication factor (C/C_0)
		14	21	28	35		
Egg yolk	48	9925**aA**	88,416**bA**	21,800**abA**	11,200**acAB**	135,275**A** ± 22,585	1352
	72	10,825**aA**	83,841**bA**	24,400**abAB**	7333**acA**	126,400**A** ± 19,010	1264
Chicken liver	48	6203**aA**	38,833**abAB**	87,933**bBC**	46,600**bBC**	210,820**A** ± 67,568	2108
	72	14,375**acA**	26,558**bdB**	85,066**cdC**	63,333**dC**	189,333**A** ± 28,642	1893
Wouts (modified)	72	-	-	-	-	60,000[a]	15
Nutrient peptone	NR	-	-	-	-	24,000[b]	48
Soy oil (IV)	48	-	-	-	-	470,000[c]	979

Different bold lowercase letters across rows and bold uppercase letters in columns indicate a significant difference ($P > 0.05$). Abbreviations: SE, standard error; NR, not reported; dpi, days post-inoculation.

[a]According to El-Sadawy (2011), an initial inoculum of $C_0 = 4000$ IJs was placed on a bacterial lawn into five 9–10-cm Petri dishes previously incubated at 25°C for 72 h.

[b]According to Kondo & Ishibashi (1991), an initial inoculum of $C_0 = 500$ IJs was placed in five Petri dishes (5.5 cm in inner diameter) containing 15 mL of nutrient agar and incubated at 25°C.

[c]According to Neira-Monsalve *et al.* (2019), an initial inoculum of $C_0 = 480$ IJs was placed on a bacterial lawn into a 5-cm Petri dish previously incubated at 28°C for 48 h. Harvesting occurred at 26–27 dpi of IJs.

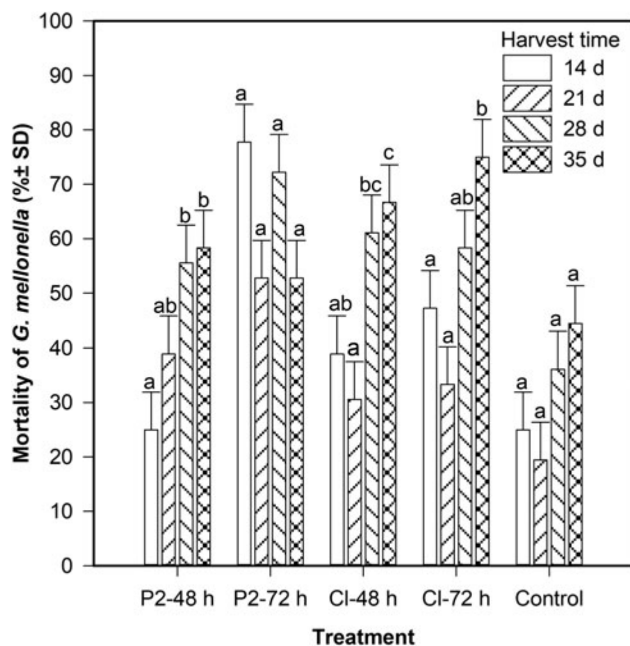

Fig. 3. Mortality of *Galleria mellonella* by the confrontation of single infective juveniles harvested at 14, 21, 28 and 35 days from solid media (egg yolk (P2) or chicken liver (Cl)) stored at 23–27°C. Different letters on the bars within the same treatment indicate significant differences ($P < 0.05$). SD, standard deviation.

bacterial lawn incubated 72 h instead of 48 h, of 58.68% ± 3.63 SE and 46.87% ± 3.64 SE, respectively.

In the condition where the bacterial lawn of *Xenorhabdus* sp. on the solid medium was incubated for 48 h before the inoculation of IJs, statistically significant differences in the pathogenicity of the IJs harvested from the P2 (44.44% ± 5.46 SE) and Cl (49.30% ± 4.96 SE) were found, only for the IJs reared *in vivo* (31.24% ± 4.11 SE), of $P = 0.025$ and $P = 0.003$, respectively. Regarding the pathogenicity about the harvest time, there were statistically significant differences ($P < 0.05$) using IJs harvested at 28 days (58.33 ± 3.72 SE) compared to those collected at 14 days (31.94% ± 3.34 SE) and 21 days (34.72% ± 7.58 SE), and similarly using IJs collected at 35 days (62.50 ± 3.56 SE) to those at 14 days ($P = 0.003$) and 21 days ($P = 0.005$). There were no statistically significant interactions between harvest time and *in vivo* rearing or solid medium.

Regarding the condition in which the bacterial lawn of *Xenorhabdus* sp. on the two solid media was incubated for 72 h before the inoculation of IJs, statistically significant differences ($P < 0.05$) were observed between the pathogenicities of the IJs harvested from P2 (63.88% ± 4.84 SE) and Cl (53.47% ± 5.17 SE), and among these two treatments, with the positive control of IJs reared *in vivo* (31.24% ± 4.11 SE). Concerning the pathogenicity by the harvest time, statistically significant differences were observed among the IJs harvested at 21 days (43.05% ± 6.6 SE), for those collected at 28 days (65.27% ± 3.97 SE) and 35 days (63.88% ± 7.02 SE), of $P = 0.033$ and $P = 0.042$, respectively.

At 72 h of incubation time, some statistically significant interactions between solid media and harvest time were observed ($P = 0.02$). Using IJs collected at 14 days, the statistical difference ($P = 0.007$) was between P2 (77.77% ± 10.01 SE) and Cl (47.22% ± 2.77 SE). Using IJs collected at 35 days, the statistical difference value was $P = 0.038$, between P2 (52.77% ± 7.34 SE) and Cl (75% ± 8.33 SE).

Length of IJs produced

The lengths of IJs *in vitro*-produced from all treatments by harvest time are shown in fig. 4. The range of IJ lengths was 464–628 µm. The mean length and SE of the IJs produced on solid media were (1) 564 µm ± 4.87 from Cl and (2) 551 µm ± 5.03 from P2, and the one-way ANOVA does not show a statistically significant difference ($P = 0.079$) among them. However, when the bacterial lawn on solid media was incubated for 72 h instead of 48 h, the produced IJs were significantly shorter in length ($P < 0.001$) – around 51–53 µm less – and regardless of whether they were produced on P2 (48 h: 578 µm ± 3.5 SE; 72 h: 525 µm ± 5.48 SE) or Cl (48 h: 589 µm ± 3.91 SE; 72 h: 538 µm ± 4.93 SE), the tendency is the same. Therefore, IJs were statistically significant longer ($P < 0.05$) when they were harvested from P2-72 h at 14

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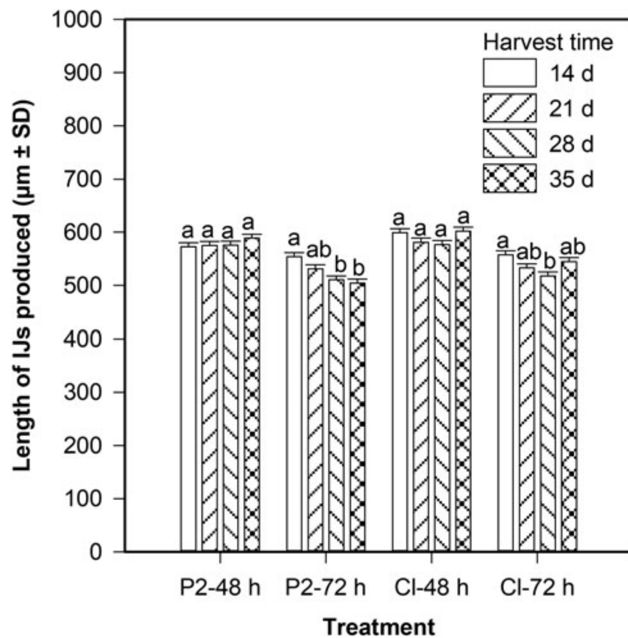

Fig. 4. Mean length of *Steinernema* sp. JAP1 infective juveniles (IJs) produced on the solid media chicken liver (Cl) and egg yolk (P2) with a lawn of its symbiotic bacterium incubated at 30°C for 48 and 72 h before the inoculation of 100 IJs. Different letters on the bars within the same treatment indicate a statistically significant difference ($P < 0.05$). SD, standard deviation.

days compared with 38 days and 35 days, and from Cl-72 h at 14 days compared to 28 days. The length of IJs produced on P2 or Cl was not correlated to their pathogenicity on *G. mellonella* ($N = 16$, $P > 0.05$).

Discussion

This study reveals a better-accumulated production of *Steinernema* sp. JAP1 IJs on solid media than previous studies, reporting the production of 60,000 *S. glaseri* IJs on modified Wouts agar (El-Sadawy, 2011) and 24,000 IJs on nutrient peptone agar (Kondo & Ishibashi, 1991), but minor to 470,000 IJs of *H. indica,* harvested at 26–27 days from medium IV with soy oil (Neira-Monsalve *et al.*, 2019). Two relevant experimental differences between our study and those previous reports were: (1) we performed three additional harvests of IJs at 21, 28 and 35 days; and (2) the initial inoculum (C_0) of 100 IJs applied on the surface of solid media was less than 480–4000 IJs used.

Based on the production data reported by Neira-Monsalve *et al.* (2019), maximum accumulated productivity of 4.7×10^5 *H. indica* SL0708 IJs per 5-cm Petri dish can be obtained using solid medium IV at 27 dpi of 480 IJs; hence, the maximum multiplication factor in this study was 979. Our results show a maximum multiplication factor of 2108 because the accumulated productivity at 35 days was 2.1×10^5, with an inoculum of 100 IJs on Cl medium, incubated 48 h after inoculation of an 0.1 mL aliquot of the 38 h-old TSB-*Xenorhabus* sp. culture broth. Hence, concerning the final concentration of IJs (C) obtained in each solid media shown in table 1, the best multiplication factors of IJs (C/C_0) in descending order were 2108 and 1352, for Cl and P2, respectively. Therefore, we were able to surpass the values obtained by El-Sadawy (2011), Kondo & Ishibashi (1991) and Neira-Monsalve *et al.* (2019).

The size of inoculum for IJs is certainly a preponderant factor in the production since, when media were inoculated with greater concentrations of IJs, it would be expected to produce more IJs in less time, as Shapiro-Ilan *et al.* (2012) suggest. However, Neira-Monsalve *et al.* (2019) argue that the number of IJs produced depends on the nutritional quality of the media and the efficiency of the bacteria–nematode complex to assimilate the nutrients and bacteria signalling IJ recovery. The optimization of the inoculum amount for *Steinernema* IJs remains an objective for future study.

Media composition influences the physiological quality of EPNs (Yang *et al.*, 1997), the effectiveness of IJs (Yoo *et al.*, 2000) and yield production (Zhen *et al.*, 2018). Concerning the corn oil as a source of lipids in P2 and Cl, Neira-Monsalve *et al.* (2019) suggest that nematodes cannot modify the fatty acid composition of lipids that are supplemented in culture media, and if the highest proportion of polyunsaturated fatty acids are present in corn oil (Zambiazi *et al.*, 2007), low production of IJs from both media could be expected. However, our experiments reveal higher productivity of *Steinernema* sp. JAP1 IJs on Cl than on P2.

In our study, the protein source to support the growth and development of the nematodes on the diet composition could be the most critical factor in the production of IJs, since the nutritional value of fresh Cl (Xiong *et al.*, 2017) is more significant than egg yolk in the P2 medium (Rannou *et al.*, 2015). Cl contributes with a better source of lipids than P2 and its phospholipid components are highly unsaturated (Damien Dorman *et al.*, 1995).

Another important result of our study is the significant difference in IJs' pathogenicity between *in vitro* cultured and *in vivo* reared. A necessary condition to cause the death of *G. mellonella* is that bacterial cells of the symbiont *Xenorhabdus* sp. colonize the IJ stage. This may be explained because numerous bacterial cells in phase I were available to metabolize the nutrients of solid media, and nematodes feed on the symbiont biomass at the beginning of the multiplication process, ensuring that new generations also presented the symbiont bacteria housed in their intestinal vesicle, as Boemare (2002) suggests. For example, when the incubation time was 72 h instead of 48 h, a more significant number of bacterial cells were available to new IJs and support the pathogenicity observed, as our data suggest.

In our studies, the pathogenicity of single *Steinernema* IJs surface cultured on any of the two solid media evaluated was superior to the value 48.4 ± 1% reported by Converse & Miller (1999) using the single *S. glaseri* NJ43 strain produced in submerged liquid culture and higher than the 36% pathogenicity using IJs obtained on lipid agar by Dunphy *et al.* (1985). In the studies of Kazimierczak *et al.* (2018), the mortality of wax moth larvae exposed to single IJs of *Steinernema carpocapsae*, *Steinernema feltiae* and *Steinernema arenarium* were 50, 43 and 41%, respectively. These results are by ∼4–13% lower than those achieved in our study for *Steinernema* sp. JAP1 IJs.

Although our produced IJs can be considered small (<1 mm), according to Akhurst (1986), it seems probable that numerous bacterial cells of its symbiont *Xenorhabdus* sp. were able to colonize the IJ stage during the solid culture and then support the higher pathogenicity, when compared to *in vivo*-reared IJs. Also, since the pathogenicity of *Xenorhabdus* in primary form varies between the different strains and bacterial load (Hang *et al.*, 2007; Ogier *et al.*, 2014), a complementary study is needed to determine the potential pathogenicity of the sole symbiotic bacterial strain.

The length values of our produced steonernematid IJs are within the range reported for *S. carpocapsae* IJs (Adams &

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Nguyen, 2002); however, this clearly requires confirmation by molecular biological techniques. Based on our correlation analysis, it is impossible to associate the IJ length to the viability by lipid gain or virulence, as the opposite association was demonstrated for *S. carpocapsae* (Yang *et al.*, 1997).

Considering the price of ingredients purchased in local markets and specialized suppliers in Tulancingo de Bravo, Hidalgo, Mexico, the cost of 15 g of solid diets in every 6-cm Petri dish is the same (i.e. 0.29 USD). However, based on the mean accumulated production per Petri dish after four harvests, the highest yields were observed in Cl (14,000 IJs/g) compared with P2 (9000 IJs/g). Hence, the analysis showed that using Cl for the production of one million *Steinernema* sp. JAP1 IJs had a nominal cost of around 21.15 USD, while it was about 32.63 USD when using the P2 medium. The cost of 21.28 g of the medium VI with soy oil (Neira-Monsalve *et al.*, 2019) to produce one million *H. indica* IJs is very cheap – around 2.53 USD. Nevertheless, at the industrial production scale, the costs of raw materials can be quite different.

Steinernema sp. JAP1 was well cultured on a solid medium with Cl, and the IJs produced showed better pathogenicity compared with IJs reared in *G. mellonella*. Its potential application as a biocontrol agent remains conditioned to evaluate efficacy and susceptibility tests against insect pests of economic importance.

In conclusion, the main contributions of this study are as follows: (1) the achieved productivity of IJs of *Steinernema* sp. JAP1 strain cultured on P2 agar or Cl agar at 23–27°C and ambient humidity increased the multiplication factor achieved using Wouts agar (modified), nutrient Peptone agar and IV medium; and (2) a high pathogenicity of the produced IJs against *G. mellonella*.

The P2 medium allowed a higher production of IJs than the Cl medium, and an additional 24 h after 48 h of incubation of the bacterial lawn did not significantly increase the production; however, higher productions were obtained from the intermediate harvest (21 days and 28 days), both from P2 and Cl.

The pathogenicity of IJs produced *in vitro* was higher than those reared *in vivo*, and increased when they were grown on a bacterial lawn with 72 h of incubation at 30°C. If the bacterial grass on the agar medium was incubated at 30°C for 48 h, the pathogenicity of the IJ harvested late (28 days and 35 days) was higher than those harvested early (14 days and 21 days), regardless of the solid medium used for the cultivation of EPNs.

When the bacterial lawn on the solid medium (P2 or Cl) was incubated at 30°C for 72 h, the IJs harvested at 28 days and 35 days were more pathogenic than those collected at 21 days. However, if the harvest of IJs is performed at 14 days in P2, the pathogenicity is higher than that from Cl, while the opposite was observed at 35 days of harvest in these same solid media.

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Conflicts of interest. None.

Ethical standards. This article does not contain any studies with human or animal subjects performed by any of the authors.

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