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Culture of *Steinernema glaseri* on three solid media and their virulence against *Galleria mellonella* larvae

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Abstract: *Steinernema glaseri* is a potential biocontrol agent against white grubs of the *Phyllophaga* spp. complex; however, its suitability for *in vitro* multiplication has been scarcely investigated. In this study, the effects of the surface culture of NJ-43 strain with its symbiotic bacteria *Xenorhabdus poinarii* on egg-yolk agar (P2), chicken-liver agar and nutrient meat-peptone (MP) agar on infective juvenile (IJ) productivity and their virulence against *Galleria mellonella* larvae were investigated. The bacteria on the surface of the agar were first incubated in darkness at 30 °C for 54–102 h, and then 100 surface-sterilised IJs were added. After two harvests, the accumulated productivity was higher on chicken-liver agar (536 × 10^3 IJs/m^2 day) and P2 agar (534 × 10^3 IJs/m^2 day) than on nutrient MP agar (58 × 10^3 IJs/m^2 day). The mean virulence of the *in vitro* produced IJs was 46–60% and showed no statistically significant difference among the three culture media. In conclusion, the maximum multiplication factor of *S. glaseri* NJ-43 on solid media was 385, and its original virulence was retained.

Keywords: biocontrol; entomopathogenic nematodes; *in vitro* production; one-on-one assay

The infective juvenile (IJ) is the only free-living stage of entomopathogenic nematodes (EPNs) capable of occupying soil habitats and resuming its life cycle by infecting a new insect host (Ehlers 2001). Industrial mass production of viable IJs at low cost, maintenance of infectivity, and extension of shelf life (Ehlers 2001) are the challenges to increasing the commercialisation of EPNs as a biopesticide product. *Steinernema glaseri* is known to be one of the most pathogenic nematodes on coleopteran species, such as white grubs of the *Phyllophaga* spp. complex, an edaphic pest that affects the seed quality and yield in corn production (*Zea mays*).

Studies on the *in vitro* mass production of *S. glaseri* report variable yields. In 500 mL Erlenmeyer

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flask containing a homogenate of 70% pig kidney, 10% beef fat, and 20% tap water in a polyester polyurethane crumb sponge, the yield ranged from 6.5×10^6 to 10.0×10^6 IJs (Bedding 1981). In plastic bags containing 3 kg of medium consisting of chicken offal homogenate (heads, legs, and gall bladders removed) with 20% water on 240 g of crumb sponge, the yield was 700×10^6 IJs (Bedding 1984).

Yang et al. (1997) demonstrates that the type of protein source in the culture media influences the quality of the nematodes, in terms of IJ size, dry weight, motility, fatty acid content and penetration efficiency. When animal protein was made available to *S. carpocapsae*, the size (length and width) of IJs was comparable to IJs reared in *Galleria mellonella* larvae. These morphometric characteristics are also correlated with penetration efficiency. Therefore, length or width can be used as an additional standard to compare the quality of nematodes.

Akhurst (1986) reported that *Xenorhabdus poinarii*, the bacterial symbiont of *S. glaseri*, is weakly pathogenic, its retention is highly variable and the virulence of *in vitro* produced IJs depends on the bacterial cell load within them. Indeed, pathogenicity can also vary between strains within the same species of the symbiotic bacterium, while retention requires a finely tuned recognition mechanism between the two partners (Sicard et al. 2003). Sharmila and Subramanian (2020) found that reared *S. glaseri* IJs in the last instar of the wax moth, *G. mellonella* were more virulent than IJs produced *in vitro*. 10 000 cells of *X. poinarii* from the *S. glaseri* Dongrae strain caused 100% mortality of *G. mellonella* at 30 °C and 35 °C in 48 h (Hang et al. 2007). This fact raises the challenge of reconciling the productivity of the *in vitro* culture with the virulence of the juveniles produced through proper evaluation of *S. glaseri*–*X. poinarii* complexes.

This work researches the effect of surface propagation of *S. glaseri* NJ-43 strain and its symbiotic bacteria, *X. poinarii*, on three solid media and their virulence in a one-on-one assay on *G. mellonella* larvae.

MATERIAL AND METHODS

Entomopathogenic nematode. *S. glaseri* NJ-43 strain Access GenBank AF122015.1 (Steiner 1929) was reproduced in *G. mellonella* larvae. The collected IJs were surface-sterilised with benzethonium chloride solution [0.1% (w/v) × 10 min + 3 rinses with sterile distilled water (DW)] and then with sodium hypochlorite solution Cloralex® [0.5% (w/v) × 10 min + 3 rinses with sterile DW].

Culture media. The isolation medium for *X. poinarii* was MacConkey agar 5% (w/v). The liquid media for bacterial culture comprised tryptic soy broth (TSB) 3% (w/v), and yeast extract 0.5% (w/v). The following solid agar media were prepared in sterile 6 cm Petri dishes: (*i*) P2 agar (Chavarría-Hernández & de La Torre 2001), (*ii*) chicken-liver agar (Cortés-Martínez et al. 2021) and (*iii*) nutrient MP agar (Kondo & Ishibashi 1991). All the culture media used were Bioxon®, except for the meat-peptone in nutrient MP, which was DIBICO®.

Isolation of symbiont bacteria. The bacteria *X. poinarii* was extracted from crushed surface-sterilised IJs suspended in 8 mL TSB and incubated at 30 °C in darkness for 44 h. A loopful of the TSB *X. poinarii* broth was streaked on MacConkey agar and incubated at 30 °C for 48 h (model Gl6; Shel Lab™, OR, USA). The primary form of the expected species, *X. poinarii*, was confirmed by macroscopic morphological characteristics and size of red colonies, according to Akhurst (1986), and microscopic morphological characteristics by Gram staining (Thomas & Poinar 1983). The symbiotic bacteria were conserved in 2 mL vials with glycerol 20% (w/v) and stored at –74 °C till use in the experiments. Aliquots of an *X. poinarii* vial were streaked on MacConkey agar and incubated under the conditions described above. A loopful of isolated phase I-bacterium was then inoculated in 50 mL TSB and incubated at 30 °C and 130 rpm (Barnstead/Labline E-class; ThermoFisher Scientific, MA, USA) during 38 h to produce the bacterial inoculum for *in vitro* culture experiments.

***In vitro* culture of IJs.** The following procedure was conducted: (*i*) 0.1 mL aliquots of the 38 h old TSB *X. poinarii* culture broth transferred to Petri dishes of each solid agar medium (i.e., P2, nutrient MP, and chicken liver); (*ii*) plates were incubated in darkness at 30 °C for 54–102 h, (*iii*) after 54, 66, 78, 90 and 102 h, 100 surface-sterilised IJs were inoculated on a Petri dish randomly selected from each solid medium and stored in darkness at 21–25 °C and 12–23% relative humidity (RH). The experiment consisted of fifteen Petri dishes, replicated twice, and monitored daily under a ste-

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reoscopic microscope (model Z30V; Leica®, CA, USA). When IJ production was detected, modified White traps were set up and stored in a desiccator (Scienceware®) at 20–24 °C and 13–22% RH. IJs were harvested and counted at 14 and 35 days post-inoculation. The IJs were rinsed twice with sterile DW, concentrated at a rate of 1 000 IJs/mL, and stored at 4 °C to 8 °C in tissue culture flasks. Suspensions of harvested IJs from each White trap were diluted (10^0–10^2) with sterile DW, and IJ concentrations were determined by counting in five 0.02 mL samples under a stereoscopic microscope (model Z30V; Leica®, CA, USA).

Measurement of IJ length. Photographs of alive IJs harvested from each solid medium were taken at 40× using a digital camera (model DSFi3; Nikon®, Japan) coupled to a light microscope (model 80i; Nikon®, Japan). The length of IJs was measured using the "segmented line" tool of ImageJ software (version 1.52). Twenty IJs per solid culture medium were measured in each of the two harvests carried out.

Assay of virulence. The produced IJs on solid agar media were evaluated according to the mortality caused to *G. mellonella* in the one-on-one assay (Converse & Miller 1999). A filter paper disk (medium pore, 21 mm in diameter) was placed in each well of a 12 well plate, and one IJ was transferred into each well in 5 µL DW, followed by 50 µL DW. A *G. mellonella* larva was then added. Plates were sealed with adhesive tape to minimise evaporation and stored at 21–27 °C and 9–21% RH. The experiment consisted of fifteen treatments with *in vitro* cultured IJs from each solid medium and each incubation time of the bacterial lawn. The controls were: a treatment using IJs reared *in vivo* and an untreated control without IJs but with DW. Each treatment and control were replicated twice, and five insects were used for each replication. Thus, one hundred and sixty *G. mellonella* larvae were used for these assays. Insect mortality was recorded every 24 h for three days.

Statistical analysis. The productivity (IJs/m^2 day) on each solid media was calculated based on the total number of IJs harvested divided by the internal surface area (2×10^3 m^2) in a 6 cm Petri dish and the longest harvest time (day). The productivity on three solid media, the incubation time (h) of bacterial lawn, the IJ length (µm), and virulence (%) of the IJs on *G. mellonella* larvae were subjected to a one-way ANOVA of means ($P < 0.05$; Tukey's test). A Pearson correlation analysis ($P < 0.05$) was conducted to determine the type of correlation among the bacterial lawn age, the productivity of IJs and virulence. All analyses were performed in Sigmaplot® (version 12).

RESULTS

***In vitro* production of IJs.** New offspring on solid media appeared 5–6 days post-inoculation of IJs. After two harvests of IJs, the mean accumulated production on agar media was: 191 100 from P2, 192 900 from chicken-liver, and 20 890 from nutrient MP. The highest mean productivities of IJs (Figure 1) were obtained in chicken-liver (536×10^3 IJs/m^2 day) and P2 (534×10^3 IJs/m^2 day), while the lower was obtained in nutrient MP (58×10^3 IJs/m^2 day). The IJ-productivities on P2 and chicken liver was statistically significantly different ($P < 0.05$) only versus the productivity on nutrient MP (Table 1). The Pearson correlation analysis shows no statistically significant correlation ($n = 5$, $P > 0.05$) between the number of IJs produced and the incubation time of the bacterial lawn (for 54, 66, 78, 90, and 102 h) for the three solid agar media and two harvest times.

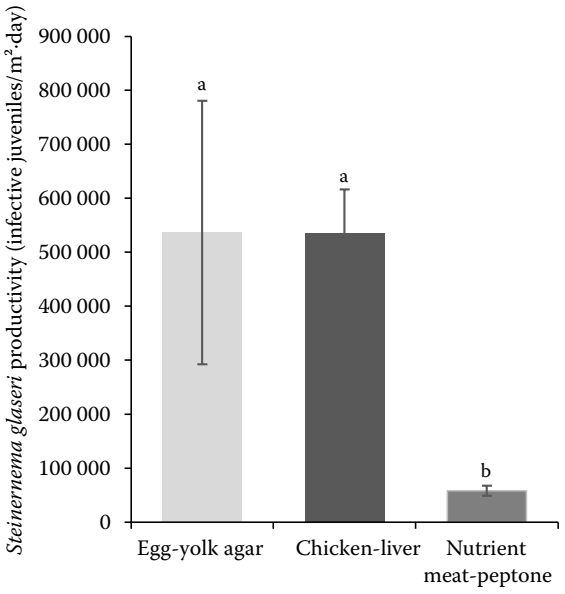

Figure 1. Productivity of *Steinernema glaseri* infective juveniles NJ-43 strain on three solid media in co-culture with *Xenorhabdus poinarii* bacterium stored at 20–25 °C and 12–23% RH

[a,b]Same letters in bars do not show a statistically significant difference ($P > 0.005$)

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Table 1. *In vitro* production of *Steinernema glaseri* infective juveniles NJ-43 strain through the surface culture on three solid media at 21–25 °C with a bacterial lawn of *Xenorhabdus poinarii* previously incubated at 30 °C for 54–102 h, their mean virulence (%) in one-on-one assays on *Galleria mellonella* and comparison with results from other studies

Variable	Solid medium	Incubation time before inoculation of infective juveniles (h)					Mean* ± standard error of the mean	Multiplication factor	Reference
		54	66	78	90	102			
Production of infective juveniles	wouts (modified)	–	–	–	–	–	60 0001	15.0	El-Sadawy (2011)
	nutrient peptone	–	–	–	–	–	24 0002	48.0	Kondo and Ishibashi (1991)
	nutrient meat-peptone	4 700	3 550	1 950	5 800	4 850	4 170[b] ± 660	41.7	This study
	chicken-liver	36 750	27 350	29 200	60 300	39 300	38 580[a] ± 5 877	385.8	
	egg-yolk agar	32 950	104 800	3 950	18 000	31 400	38 220[a] ± 17 450	382.2	
Virulence (%)	nutrient meat-peptone	50	50	50	40	40	46[a] ± 2.4	N/A	This study
	chicken-liver	60	50	40	90	0	60[a] ± 10.8	N/A	
	egg-yolk agar	60	60	60	70	50	60[a] ± 3.1	N/A	

N/A – not applicable

*Different letters indicate significant differences between different solid media ($P < 0.05$)

[1]An initial inoculum of C_0 = 4 000 infective juveniles (IJs) was placed on a bacterial lawn in five 9–10 cm Petri dishes previously incubated at 25 °C for 72 h; [2]an initial inoculum of C_0 = 500 IJs was placed in five Petri dishes (5.5 cm in inner diam.) containing 15 mL of nutrient agar and incubated at 25 °C

Virulence of IJs produced. The accumulated mortality of *G. mellonella* by *S. glaseri* was in the range of 40–90% (Table 1). The highest values achieved by using IJs of each solid agar medium were: (*i*) 90% with IJs cultured on chicken-liver with 90 h of incubation, (*ii*) 70% with juveniles cultured on P2 with 90 h of incubation and (*iii*) 50% with IJs cultured on nutrient MP with 54–78 h of incubation. These values are superior to the mortality of 50% caused by IJs reared *in vivo,* while no mortality was observed in control. Non statistically significant differences were found among the mean virulence of IJs from all surface cultures on solid agar media and *in vivo* rearing ($P > 0.05$), while a statistically significant difference was found with respect to the control without IJs ($P < 0.05$). The incubation time of the bacterial lawn on three solid agar media did not show a statistically significant Pearson correlation ($P > 0.05$) with the virulence of the produced IJs.

Length of IJs produced. The mean length and standard error of the *S. glaseri* IJs cultured on solid media were: (*i*) 963 ± 44.6 µm from chicken liver agar, (*ii*) 1 062.31 ± 61.39 µm from nutrient MP agar and (*iii*) 1 021 ± 28.35 µm from P2 agar. The ANOVA shows no statistically significant difference ($P > 0.05$) among them.

DISCUSSION

This study showed that better productivity of *S. glaseri* IJs was achieved on chicken-liver and P2 than on modified Wouts agar (El-Sadawy 2011) and nutrient peptone agar (Kondo & Ishibashi 1991) at a harvest conducted at 15 days post-inoculation of IJs (Table 1). Two relevant experimental differences between our study and previous reports listed in Table 1 were: (*i*) the second harvest of IJs at 35 days and (*ii*) an initial inoculum (C_0) of 100 IJs applied on the surface of solid media, which is minor compared to the 500–4 000 IJs used in previous reports. Therefore, concerning the final concentration of IJs (C) obtained in each solid media agar shown in Table 1, the multiplication factors of IJs (C/C_0) were in descending order, 385, 382, and 41 for chicken-liver, P2, and nutrient MP, respectively. These values were up to nine times higher than those obtained by El-Sadawy (2011) and Kondo and Ishibashi (1991). In addition, the incubation temperature for producing IJs was 21–25 °C, which is reported as adequate for *in vitro* multiplication of *S. glaseri*, which does not survive above 30 °C (Yamanaka et al. 2000).

The composition of agar media influences the physiological quality (Yang et al. 1997), the effectiveness of IJs (Yoo et al. 2000), and yield production (Zhen et al. 2018). Our experiments show

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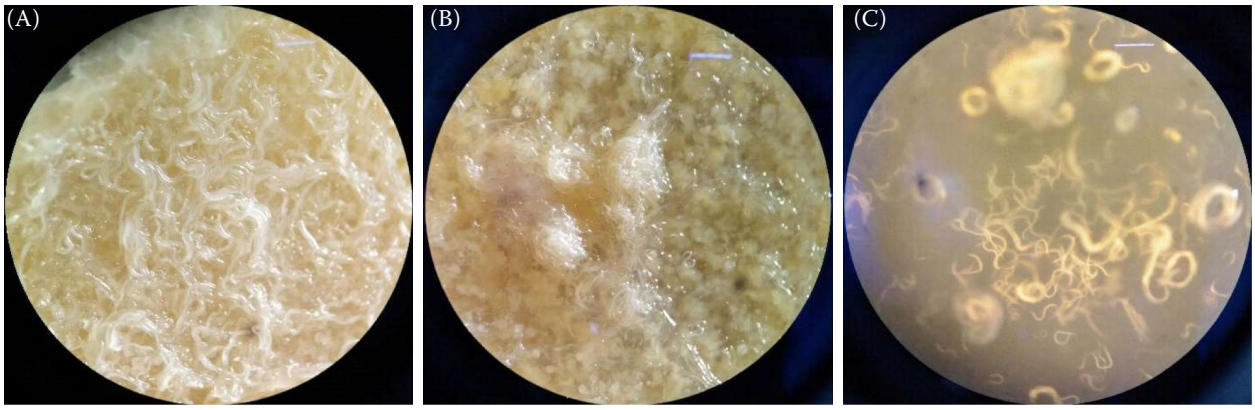

Figure 2. *In vitro* produced nematodes *Steinernema glaseri* NJ-43 strain of different developmental stage on three solid agar media stored at 21–25 °C and 12–23% RH, at 14 days post-inoculation of 100 infective juveniles
(A) Egg-yolk agar, (B) chicken-liver, and (C) nutrient meat-peptone with embedded nematodes

lower productivity of *S. glaseri* IJs on nutrient MP agar not supplemented with corn oil, and it was observed that numerous first and second-generation nematodes were embedded in the nutrient MP agar, which stopped their development (Figure 2). This result was probably due to the low content of bacteriological agar (1.5%) compared to P2 agar and chicken-liver agar. Therefore, the nutrient MP agar is inadequate for the solid culture of EPNs.

The virulence of the *S. glaseri* NJ-43 strain cultured on each of the three solid media evaluated is slightly higher than the value of 48.4 ± 1% reported by Converse and Miller (1999) for the same strain produced in liquid culture and higher than the 36% reported by Dunphy et al. (1985) using IJs produced on lipid agar.

The length values of the IJs produced are within the range reported (Adams & Nguyen 2002), and the ANOVA does not show a statistically significant difference ($P > 0.05$) among them. Therefore, it is impossible to associate the IJ size with the viability by lipid gain or the pathogenicity of this nematode species, as the opposite association has been demonstrated for *S. carpocapsae* (Yang et al. 1997). Although the produced IJs can be considered small (< 1 mm) according to Akhurst (1986), it seems probable that a large number of bacterial cells of this *X. poinarii* strain colonise the IJ nematodes and then support a sufficient virulence of *S. glaseri*. Also, as the virulence of *X. poinarii* in its primary form varies between the strains and bacterial loads (Hang et al. 2007; Ogier et al. 2014), a complementary study is needed to determine the potential virulence of the sole symbiotic bacteria of *S. glaseri* NJ-43 strain.

Considering the price of ingredients purchased from local markets and specialised suppliers in Tulancingo de Bravo, Hidalgo, Mexico, the cost of 15 g of solid diets in each 6 cm Petri dish is the same (i.e. 0.42 USD) for P2 agar and chicken-liver agar and minor (0.26 USD) for nutrient MP agar. Then, based on the mean accumulated production per Petri dish after two harvests, the highest yields were observed for chicken-liver agar (2 572 IJs/g) and P2 agar (2 548 IJs/g) while the lowest was for nutrient MP agar (278 IJs/g). Therefore, the analysis showed that the nominal cost of producing one million *S. glaseri* IJs using Cl and P2 agar was 10.89 USD, while it was about 62.35 USD using the nutrient MP medium.

The main contributions of this study were: (*i*) the achieved productivity of IJs of *S. glaseri* NJ-43 strain cultured on P2 agar or chicken-liver agar at 21–25 °C and ambient humidity; which ninefold increases the multiplication factor achieved using Wouts agar (modified) and nutrient peptone agar; and (*ii*) the retention of virulence of *S. glaseri* against *G. mellonella*.

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