Pathogenicity of *Heterorhabditis bacteriophora* in Ultraviolet Light–Irradiated Agar Suspension on *Spodoptera frugiperda* (Lepidoptera: Noctuidae) Larvae[1]

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Abstract *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is an economically important pest of variety of crops, including maize (*Zea mays* L.), rice (*Oryza sativa* L.), and cotton (*Gossypium* spp.). Conventional chemical insecticides are usually used for management of this pest; however, a viable alternative is the use of microbial agents or their biological products, such as entomopathogenic nematodes. Their efficacy, however, is affected by abiotic conditions including ultraviolet (UV) light and desiccation. It is therefore necessary to develop formulations that preserve or extend the viability of the agent or product while facilitating ease of application. This laboratory study was conducted to determine the pathogenicity of the nematode *Heterorhabditis bacteriophora* (Poinar) against *S. frugiperda* third-instar larvae. Infective juveniles (IJs) of the nematode were tested at a concentration of 350 IJs/ml in two substrates (water and 0.1% [w/v] agar suspension) with four periods of exposure (0, 5, 10 and 15 min) to UV radiation at 253.7 nm. Each of the eight treatments included 40 larvae, with each treatment replicated three times in a completely randomized design (two factors of type of medium and UV exposure time). We determined that the 0.1% (w/v) agar suspension, compared with the water substrate, protected the IJs from harmful UV light and thus improved the survival and pathogenicity of the IJs against third-instar *S. frugiperda*.

Key Words entomopathogenic nematode, *Spodoptera frugiperda*, biological control, ultraviolet radiation

The larvae of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), the fall armyworm, damage several economically important crops, including maize (*Zea mays* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* [L.] Moench.), and cotton (*Gossypium* spp.). Damage ranges from small holes in the foliage to consumption of floral parts and grain (Jeger et al. 2017). Its host range includes more than 80 recorded host plants, but it prefers grasses such as maize, sorghum, and Bermuda grass (*Cynodon dactylon* [L.] Persoon). Its hosts also include weeds such as doublegrass (*Agrostis* spp.), crab grass (*Digitaria* spp.), Johnsongrass (*Sorghum halepense* [L.] Persoon), bellflower (*Ipomoea* spp.), coquito (*Cyperus*

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spp.), pigweed (*Amaranthus* spp.), and sand spur (*Cenchrus tribuloides* L.) (Prasanna et al. 2018).

The fall armyworm is native to the tropical and subtropical regions of the Americas, where it is endemic. Its life cycle spans 30 d at a daily temperature of ~28°C in summer, but it can be as long as 60–90 d in cooler temperatures. *Spodoptera frugiperda* does not have the capacity for diapause; therefore, mixed generations occur the entire year where the pest is endemic. This pest invades nonendemic areas when environmental conditions allow and may exhibit only one generation before winter kill (Prasanna et al. 2018). It is a highly reproductive pest whose life cycle includes the developmental stages of egg, six larval instars, pupa, and adult. Adult females lay their eggs in masses on plant surfaces, oftentimes near the junction of the base of the leaves with the stem (Adhikari et al. 2020, Prasanna et al. 2018).

Damaging populations of fall armyworm are usually controlled with applications of conventional chemical insecticides; however, these are harmful to the environment and pose risks to nontarget organisms and workers when not used with the appropriate doses and recommendations. An alternative to avoid these impacts, while managing the pest population, is biological control using entomopathogenic microorganisms or their biological products (Sánchez et al. 2019). One of the less-explored but promising strategies in the biological control of *S. frugiperda* is the use of entomopathogenic nematodes (EPNs) such as *Heterorhabditis bacteriophora* (Poinar), *Heterorhabditis indica* Poinar, Karunakar & David, and *Steinernema carpocapsae* (Weiser) (Abbas et al. 2022, García et al. 2008, Prasanna et al. 2018). These EPNs have proven to be environmentally friendly alternatives to chemical pesticides for controlling many soilborne insect pests, including other noctuids. Studies indicate that *S. frugiperda* is very susceptible to these EPNs at a rate of 25,555 nematodes per m^2, attacking both young and mature larvae (Prasanna et al. 2018).

The infective stages of the EPN families Heterorhabditidae and Steinernematidae are found in the soil and parasitize mostly immature-stage arthropods (Molina et al. 2006), taking advantage of their symbiotic relationship with gram-negative bacteria of the genera *Phothorhabdus* and *Xenorhabdus*, respectively (López-Llano and Soto-Giraldo 2016). When the infective juveniles (IJs) of the nematodes enter the host, they release the bacteria into the insect's hemocoel. These bacteria produce toxins that kill the insect (Shankhu et al. 2020) 24–72 h after the IJs have entered the host through its natural openings, such as the mouth, anus, or spiracles (López-Llano and Soto-Giraldo 2016).

Formulated EPNs are used to control pest insects (Porras and Sáenz-Aponte 2019), replacing insecticides given their ability to parasitize and kill pest insects (Neira-Monsalve et al. 2020), their relatively low production cost compared with agrochemicals, and their ease of application as well as safety for humans and domestic animals (Candanedo-Lay et al. 2020).

Persistence is an important component of the efficacy of these biological agents in the field. Viability and persistence are affected by ultraviolet (UV) light, which is considered one of the most destructive abiotic factors of entomopathogenic bacteria, fungi, and nematodes (Gaugler et al. 1992, Griffin et al. 2005, Shapiro-Ilan et al. 2015). The UV radiation may decrease the viability of IJs (Labaude and Griffin 2018), and UV radiation and desiccation are known to decrease the effectiveness of foliar-applied EPNs (Noosidum et al. 2016). In general, EPNs are

recommended to be applied early in the morning or late at night when the larvae of the fall armyworm remain actively feeding and can be easily found by the IJs (Shapiro-Ilan et al. 2006). EPNs are usually applied with water, without using protectors against unfavorable environmental factors; hence, the need to develop formulations that facilitate their application and extend their persistence as biological control agents (Bogantes et al. 2018).

The adverse effects of abiotic factors on EPNs have been studied in the laboratory by using soil or artificial substrates in an effort to their understand impacts (Stuart et al. 2015). Storage of IJ strains within liquid nitrogen also has been evaluated for prolonging persistence (Wang and Grewal 2002), and certain UV protectants have been used to reduce the negative effects of UV radiation (Labaude and Griffin 2018). For example, agar has been formulated with EPN suspensions for foliar application and has proven effective in increasing efficiency and survival of nematodes by reducing desiccation (Hussein et al. 2012). Adjuvants have proven to increase the persistence of EPN activity (Schroer and Ehlers 2005), increase the ability of IJs to enter the host (Portman et al. 2016), and delay sedimentation of IJs in aqueous suspension (Beck et al. 2013).

Our objective in this research was to determine the pathogenicity of *H. bacteriophora* against fall armyworm third-instar larvae when formulated in a 0.1% (w/v) agar suspension. A single concentration of 350 IJs/ml was tested with four periods of exposure (0, 5, 10, and 15 min) to UV radiation at 253.7 nm.

Materials and Methods

Production of IJs. The trap insect method designed by Bedding and Akhurst (1975) and modified by López-Llano and Soto-Giraldo (2016) was used to obtain IJs from *H. bacteriophora*. Twenty soil samples were taken to the laboratory and placed in 500-ml plastic containers. In each sample, 10 greater wax moth (*Galleria mellonella* [L.]) larvae were placed as bait for the recovery of the IJs. After 1 week, the containers were checked and the larvae that had changed color to a reddish brown, a characteristic of EPN parasitization, were removed. Subsequently, these larvae were placed in a modified white trap to collect IJs from EPNs, identified as *H. bacteriophora* by Steven Edgington (CABI International, Wallingford, Oxfordshire, U.K.).

IJs were then mass produced for our testing by using the last-instar *G. mellonella* as hosts, at 25 ± 4°C and 70 ± 5% relative humidity (RH), according to the protocol described by Sánchez et al. (2019). The concentration of IJs in a suspension was determined according to the modified procedure of Cagnolo and Gonzales (2017) in which a concentrated suspension of IJs was diluted with water up to a total of 10 ml. One milliliter of this solution was pipetted into a counting chamber for counting the number of IJs in that volume of suspension. The process was repeated three times to obtain an average, and suspensions were diluted accordingly.

Rearing fall armyworm. A colony of fall armyworm was reared in the laboratory at 25 ± 4°C and 70 ± 5% RH according to the protocol of Sánchez et al. (2019). Fall armyworm egg masses were initially collected from corn crops and transported to the laboratory where they were prepared for larval emergence. The larvae were fed on castor bean (*Ricinus communis* L.) leaves until pupation. Pupae were placed on moistened cotton in plastic containers until the adults emerged. Adults were fed with 10% (v/v) honey in 0.5-L plastic containers, whose

Table 1. Treatments to determine the pathogenicity of *Heterorhabditis bacteriophora* against *Spodoptera frugiperda* third instars when infective juveniles (IJs) are suspended in different media and irradiated with ultraviolet (UV) radiation different durations.

Treatment No.	Medium for Suspension of IJs	Duration of Exposure to UV Radiation (min)
1	Water	0
2	Water	5
3	Water	10
4	Water	15
5	0.1% agar	0
6	0.1% agar	5
7	0.1% agar	10
8	0.1% agar	15

All treatments had 1.0 ml of the same concentration of *H. bacteriophora* IJs (350 IJs/ml). Wavelength of UV radiation was 253.7 nm.

internal walls were lined with wax paper as an oviposition surface. Larvae used in our assays were obtained from this colony.

Test procedure. All tests were conducted in the Biological Control Laboratory of the Vista Florida Agrarian Experimental Station, National Institute of Agrarian Innovation of Peru (INIA), located in the district of Picsi, province of Chiclayo, Department of Lambayeque. Environmental conditions of the laboratory were 28 $\pm$ 0.82°C and 65 $\pm$ 2.23% RH.

The concentration of IJs was 350 IJs/ml for the eight treatments of the study (Table 1). Each treatment contained 1.0 ml of the EPN concentration. Four treatments were diluted in water, and four treatments were diluted in a 0.1% (w/v) agar suspension. Each of the water and agar treatments were exposed to UV radiation of 253.7 nm for one of four times—0, 5, 10, and 15 min—inside a horizontal laminar flow chamber. The two treatments without exposure to radiation were used as controls (Table 1).

Third-instar larvae (40 per treatment) from the colony were exposed to the respective treatment solutions in individual 120-ml plastic containers lined with filter paper that had been treated with the appropriate IJ treatment suspension. After 48 h of exposure to the treated filter paper, larval mortality caused by EPN parasitization was determined by the characteristic coloration of parasitized cadavers. These were subsequently dissected to determine the presence of EPNs.

In addition, the effect of UV radiation on IJ mortality was determined using the concentration-setting procedure of Cagnolo and Gonzales (2017), but modified to 350 UV–irradiated IJs/ml. Water was used as a substrate in cylindrical plastic containers (12.5 $\times$ 6 cm) for the application of UV radiation, and the mortality of the IJs was verified by determining their immobility when touched with a stylet under a microscope (Fig. 1).

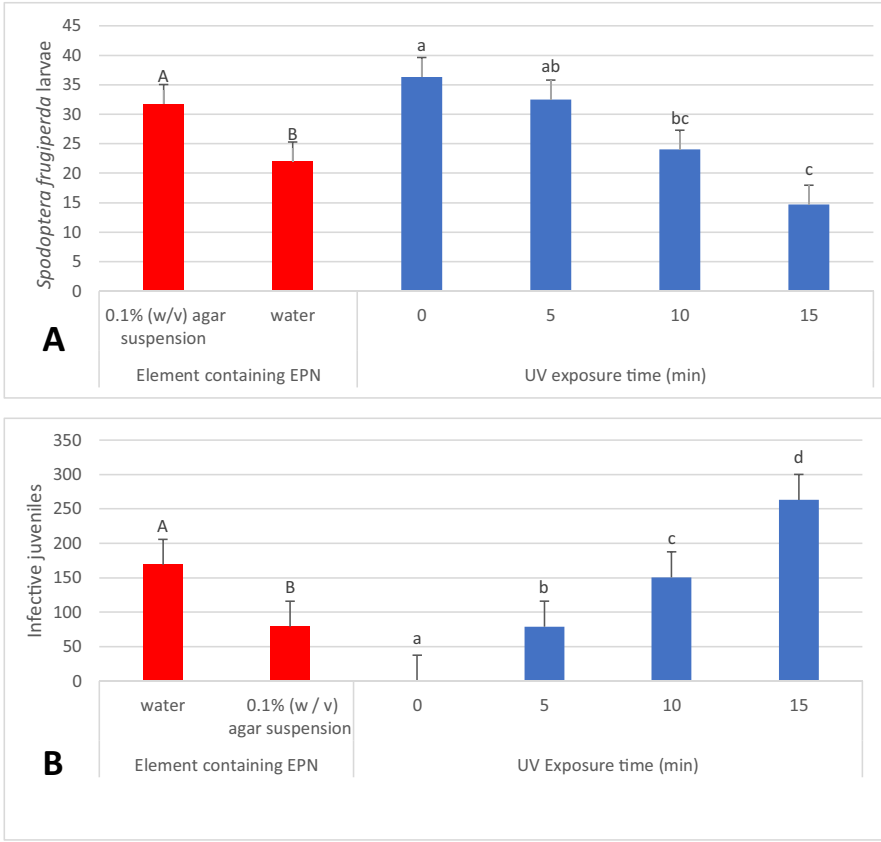

Fig. 1. Larval mortality of *Spodoptera frugiperda* third instars (A) and *Heterorhabditis bacteriophora* infective juvenile (IJ) mortality (B) in water versus agar suspensions containing *H. bacteriophora* IJs and exposed to ultraviolet radiation for four different durations.

Statistical design and analysis. The study was conducted using a completely random design with two factors (EPN and UV exposure time) with three repetitions. Larval and IJ mortalities were determined. The Shapiro–Wilk test was performed to demonstrate that the data followed a normal distribution ($P > 0.05$), allowing for use of a two-factor analysis of variance. In addition, treatment means were statistically separated Tukey's honestly significant difference test ($P < 0.05$). To determine the relationship between dead IJs and dead fall armyworm larvae, Spearman's correlation analysis was performed. The data were processed with the Excel and InfoStat software.

Results

Mortality of third-instar *S. frugiperda* larvae caused by exposure to *H. bacteriophora* IJs decreased with increasing exposure time to UV radiation ($F = 12.95$; df $= 3$,

Table 2. Mean (±SD) number of dead *Heterorhabditis bacteriophora* infective juveniles (IJs) in two types of suspensions and exposed to ultraviolet (UV) radiation for four exposure times.

Treatment No.	Suspension Medium Containing IJs	UV Exposure Duration (min)	No. of Dead IJs*
1	Water	0	0.5 ± 0.5e
2	Water	5	132.9 ± 54.0cd
3	Water	10	223.8 ± 16.8b
4	Water	15	317.4 ± 5.8a
5	0.1% agar	0	0.25 ± 0.25e
6	0.1% agar	5	24.8 ± 6.7e
7	0.1% agar	10	77.4 ± 19.98de
8	0.1% agar	15	208.8 ± 57.4bc

* Means followed by the same letter are not significantly different (Tukey's honestly significant difference test, $P > 0.05$).

16; $P = 0.0002$). The number of dead larvae decreased from a mean of 36.3 (90.8%) following exposure to IJs that were exposed to 0 min of UV radiation to 14.7 (36.7%) following exposure of larvae to IJs that were exposed to 15 min of UV radiation (Fig. 1A). Mortality also was significantly higher in those larvae exposed to IJs in the 0.1% agar suspension than in water alone ($F = 13.26$; df = 1, 16; $P = 0.0022$), regardless of the duration of the UV exposure. The interaction between the main factors of water versus agar and duration of UV exposure was not statistically significant ($F = 1.25$; df = 3, 16; $P = 0.3233$).

Conversely, mortality of *H. bacteriophora* IJs increased with increasing time of exposure to UV radiation ($F = 86.19$; df = 3, 16; $P < 0.0001$), with a mean mortality of 0.38 (0.12%) when not exposed to UV radiation and 263.04 (87.7%) when exposed for 15 min. Regardless of UV exposure, IJ mortality was higher in water than in the agar suspension ($F = 57.16$; df = 1, 16; $P < 0.0001$), with 168.7 (48.2%) in water and 77.8 (22.2%) in agar (Fig. 1B).

The highest IJ mortality (317.4) occurred among those suspended in water and exposed to UV radiation for 15 min, followed by those suspended in water with 10 min of UV exposure (223.83) (Table 2). There was no statistical difference between that latter treatment and the treatment with agar suspension plus 15 min of exposure (208.75). The significantly lowest IJ mortality was seen with the treatments of either water or agar and no UV exposure and the agar suspensions with either 5- or 10-min exposures to UV (Table 2). This clearly demonstrates that IJ survival is compromised with increasing exposure time to UV radiation and that a 0.1% agar suspension can help protect the IJs from harmful UV radiation.

Spearman's correlation analysis showed an r-coefficient of −0.929 ($P = 0.014$), indicating a strong negative correlation between *S. frugiperda* larval mortality and *H. bacteriophora* IJ mortality. Thus, as IJ mortality increases, larval *S. frugiperda* mortality decreases (Fig. 2).

Discussion

Our results indicate that exposure to UV radiation of 253.7 nm negatively affects the viability and pathogenicity of *H. bacteriophora* IJs; therefore, decreasing exposure by either reducing the exposure time or mixing the IJs in a suspension with an ingredient that blocks the UV radiation guarantees higher mortality of targeted pest species, including *S. frugiperda* larvae. The 0.1% agar suspension that we tested herein helped preserve the activity of the IJs exposed to UV radiation for different durations.

UV wavelength type and the species of the EPN are important in determining the pathogenicity of the IJs. Gaugler and Boush (1978) reported that a wavelength of 366 nm had no adverse effects on *S. carpocapsae* IJs despite an exposure of 8 h, whereas a shorter wavelength of 254 nm caused high mortality. Gaugler et al. (1992) found a maximum mortality of 17.5% in *G. mellonella* larvae caused by *H. bacteriophora* IJs exposed for 5 min to 302-nm UV radiation at a distance of 31.75 cm without a shield; the same wavelength that did not affect *S. carpocapsae* IJs. This parasitism is lower, compared with the result of our investigation where the same species of EPN was used, but the target was fall armyworm, a species that is less susceptible to EPN parasitism than *G. mellonella* and at a shorter wavelength (253.7 versus 302 nm). Our response is definitely due to the type of EPN used, the distance from the UV light source (54.5 cm), and the addition of 0.1% agar to the water containing the IJs.

Fujiie and Yokoyama (1998), in their study of three wavelengths of radiation (350, 310, and 240 nm) with the EPN *Steinernema kushidai* Hamakita, found that IJ mortality increased significantly after exposure to the three UV wavelengths and sunlight for 40 min, which reduced the insecticidal activity of the nematode. UV rays of 350 nm were not harmful to IJs during exposure up to 40 min. By contrast, all IJs died from exposure to 310 nm for 5 min and 240 nm for 30 s, confirming that the impact of UV radiation is highly wavelength dependent. On the other hand, Ganpati and Parwinder (2007) reported that, after a 3-h exposure to UV radiation at 340 nm, the mortality of *Steinernema feltiae* Filipjev, *S. carpocapsae*, and *Steinernema riobrave* Cabanillas, Poinar & Raulston was not affected, but the virulence of the latter two species was affected. By contrast, we observed a higher pathogenicity when using a shorter wavelength of 253.7 nm. According to reported studies, this type of wavelength generates a greater effect on mortality and virulence in certain species of nematodes, so the factors of EPN type, distance from the light source, and medium used are important for maintenance of persistence and pathogenicity of EPNs.

The wavelength used in this experiment is very similar to that used by Shapiro-Ilan et al. (2015), who compared UV tolerance among 15 strains of nine EPN species (five *Heterorhabditis* and four *Steinernema*) and determined the relationship between the reduction in nematode viability (after exposure to UV rays) and the virulence against *G. mellonella* larvae. Those nematodes were exposed to 254-nm UV radiation for 10 to 20 min, resulting in a reduction in the viability of all five *Heterorhabditis* species after 20 min of exposure, although the viability of several *S. carpocapsae* strains was not affected. They further found that the viability of seven strains did not differ from their unexposed controls after 10–20 min of exposure. However, unlike viability measurements, all nematodes experienced reduced

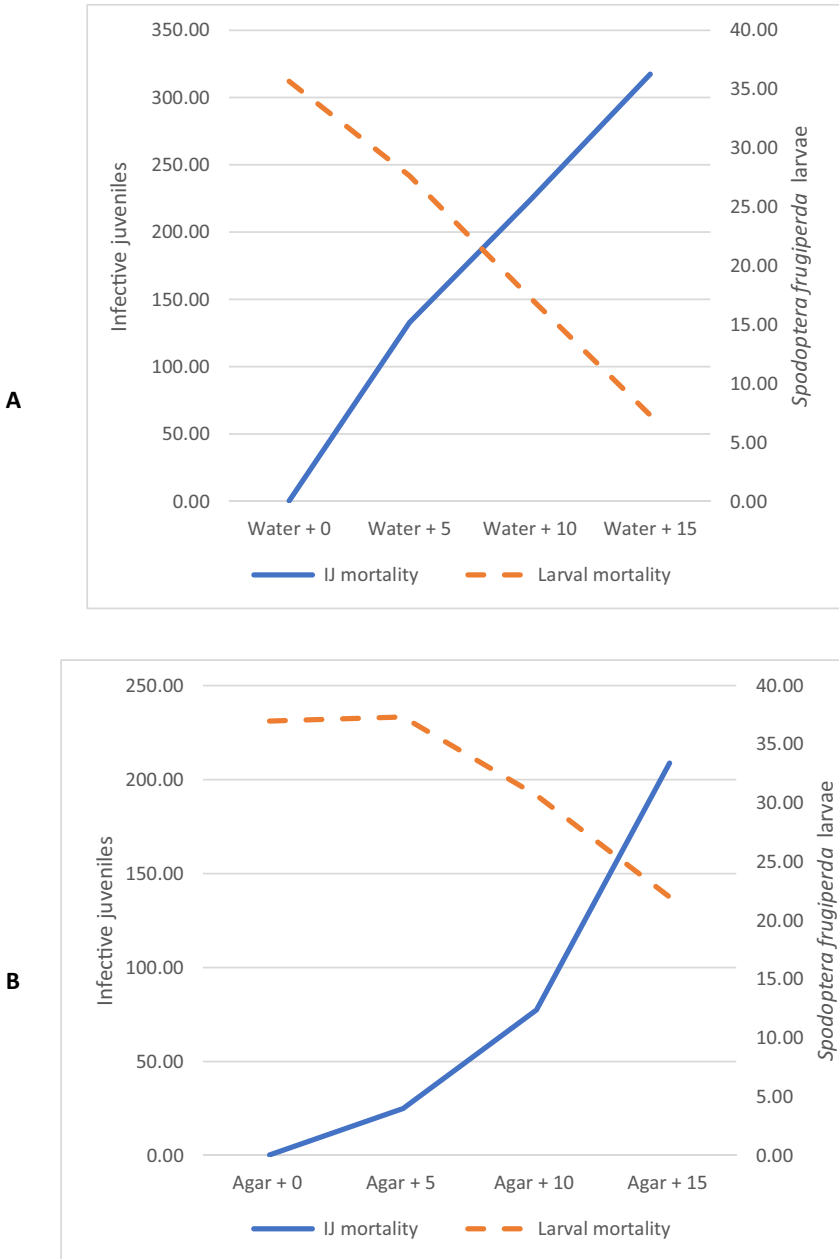

Fig. 2. Correlation of number of dead third-instar larvae of *Spodoptera frugiperda* with the number of dead *Heterorhabditis bacteriophora* infective juveniles (IJs) following exposure of IJs in water (A) or agar suspension (B) and exposed to UV radiation for four different durations.

virulence relative to their controls. In our study, the viability of *H. bacteriophora* IJs showed significant differences with the control treatments of 0.1% agar suspension with 0-UV exposure time and water with 0-UV exposure time and the viability of the IJs exposed for 5 min to UV radiation with a 0.1% agar protector is not significantly different from the control treatments (Table 2).

Results such as those of Shapiro-Ilan et al. (2015) indicate that tolerance to UV radiation depends on the EPN strain used. However, the addition of 0.1% agar to the medium containing the IJs helps maintain the virulence or pathogenicity of the *H. bacteriophora* strain. In this study, the virulence of the strain used is corroborated by comparing treatments with 0.1% agar suspension with the water treatments (Fig. 1; Table 2). The differences between the results of Shapiro-Ilan et al. (2015) and our study are due to factors such as the type of strain used, the type of host, the dilution medium, and the IJ concentration.

The inclusion of agar significantly improves efficacy by increasing nematode survival by reducing desiccation compared with the water-based nematode formulation. Hussein et al. (2012) studied the effect of agar on potato foliage by applying nematodes with agar formulations as a protector at 4, 2, 1, and 0.5% and detected a mortality of only 8% at a concentration of 1%. Suspension of nematodes in 1% agar gel was shown to be effective in both laboratory and greenhouse tests in prolonging nematode survival. The agar formulation improved IJ survival by providing a suitable environment, thus delaying drying and increasing the possibility of nematodes invading their host on the foliage.

Our Spearman correlation result differs from those of a similar analysis by Shapiro-Ilan et al. (2015). In their study, a group of nematodes formed by *H. bacteriophora* (strains fl11 and Oswego), *S. carpocapsae* (strains All, Cxrd, DD136, and Sal), *S. riobrave* (strain 355), and *Steinernema rarum* Doucet (strain 17C and E), yielded an r-coefficient of -0.714 ($P = 0.0465$) for 10 min of exposure and an r-coefficient of -7.62 ($P = 0.028$) for 20 min of exposure. Yet, for the group formed by *H. bacteriophora* (Baine and Baine strains), *Heterorhabditis floridensis* Nguyen, Gozei, Köppenhöfer & Adams (strain 332), *Heterorhabditis georgiana* Nguyen, Shapiro-Ilan & Mbata (strain Kesha), *H. indica* (strain Hom 1), *Heterorhabditis megidis* Poinar, Jackson & Klein (strain UK211), *S. carpocapsae* (strain All), and *S. feltiae* (strain SN), the result was not significant as that correlation yielded an r-coefficient of -0.167 ($P = 0.6932$) for 10 min of exposure and an r of -0.366 ($P = 0.373$) for 20 min of exposure. These results indicate that the correlation of the two variables is determined by the type of strain of the EPN.

In summary, our results suggest that EPN parasitization of fall armyworm can be achieved with the third instar as the target of the *H. bacteriophora* IJs contained in an agar suspension at 0.1%. The agar in the suspension likely acts as a protector against UV radiation, improving IJ survival and preserving the pathogenicity of the entomopathogen and thus its activity against *S. frugiperda*.

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