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Survival and infectivity of entomopathogenic nematode *Heterorhabditis indica* Poinar, Karunakar & David incubated in solutions containing different spray adjuvants

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Abstract
We examined the survival and infectivity of the entomopathogenic nematode (EPN) *Heterorhabditis indica* incubated in 1, 2 and 3% solutions of three different spray adjuvants: Tween 20, Sticker 99 and Tween 80 under laboratory conditions. Infective juveniles (IJs) of *H. indica* were added to aqueous solutions containing each of the three adjuvants and nematode survival and infectivity were studied over time. Incubation of IJs in solutions containing the Sticker 99 resulted in consistently higher survival and infectivity compared to solutions containing Tween 20 or Tween 80. Findings indicate that tank-mixing of EPNs with Sticker 99 should not pose a significant risk to the nematodes. The influence of these adjuvants on *H. indica* performance in the field remains to be examined.

Keywords: *Heterorhabditis indica*; adjuvant; survival; infectivity

Introduction
Heterorhabditis indica (Rhabditida: Heterorhabditidae) is an environment-friendly and naturally occurring bio-control agent which specifically targets the larval stages of insects belonging to economically important orders Lepidoptera and Coleoptera. In many cases, despite the pests showing good susceptibility in laboratory trials, success is not achieved following the lab to land transfer. In recent years an Indian isolate of *H. indica* has been effectively validated as a potent bio-control agent against a number of insect-pests *viz*., white grubs, termites, diamond back moth, cutworms, semiloopers, aphids, cabbage white butterfly etc. (Mohan *et al.,* 2016) [9]. A few bio-pesticide companies across India have started commercially producing and marketing *H. indica* for insect pest management in a wide range of crops like sugarcane, vegetables, groundnut, grapevine yards, tea, cashew plantations etc. A major obstacle for field level application of *H. indica* under Indian scenario is the lack of information on the delivery system and post-application survival either under open field or protected cultivation conditions.

EPNs are often applied in conjunction with other biological and chemical pesticides, adjuvants, fertilizers and soil amendments and it is usually more economical to 'tank-mix' nematodes with one or more inputs for application. Adjuvants are commonly added to pesticide formulations to enhance pesticide performance. In the application of IJs of EPNs against foliar pests, the major problems are the limited tolerance of the IJS to extremes of temperature, UV radiation and desiccation (Grewal *et al.,* 2005) [5]. UV radiation and desiccation are the most critical and have received considerable attention, with a wide range of adjuvants having been tested in an attempt to reduce the negative impacts of desiccation (Webster and Bronskill, 1968; Mac Vean *et al.,* 1982; Shapiro *et al.,* 1985; Glazer *et al.,* 1992) [19, 6, 18, 4] and UV radiation (Gaugler and Boush, 1979; Nickle and Shapiro, 1992; Nickle and Shapiro, 1994) [3, 11, 12]. However, no single adjuvant is suitable due to a number of interacting factors, including the insect target, host plant and method of application etc., for all situations and there is a need for screening of potential adjuvants for use in a particular situation. When applied in conjunction with chemical additives that prolonged their survival above ground, EPNs might be effective against a wider range of foliar insect pests.

So the present study was carried out to evaluate compatibility of *H. indica* with different adjuvants as it will be useful in mixing the compatible adjuvant along with the suspension of

this nematode to enhance its field efficacy and making novel formulations.

Materials and Methods

a. Nematode culture

Pure population of *H. indica* was obtained from Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi and used in this study. Nematodes were cultured in the laboratory on the greater wax moth larvae, *Galleria mellonella* following the standard procedures (Kaya and Stock, 1997) [7]. Infective juveniles (IJs) emerging from the wax moth larval cadavers were collected in sterilized distilled water using White traps (White, 1927) [20], and stored in BOD incubator (Sandeep Instruments and Chemicals (SANCO), New Delhi, India) at 15°C. The IJs from this suspension were used for subsequent studies within 2-4 weeks.

b. Suspension preparation

The stored suspensions were removed from the BOD incubator and left for 30 min to allow the nematodes to adjust to the room temperature. The suspensions were then examined under the binocular zoom microscope (Olympus SZ-PT) to determine if there were any dead nematodes present requiring the suspension to be cleaned. The nematode suspensions were cleared of dead nematodes by placing approximately 200 ml of the suspension in a 500 ml beaker and then after 20-25 minutes, the liquid from the top containing dead nematodes and debris was decanted. The remaining suspension in the beaker was then slowly poured over a tissue paper, wetted and placed over a mesh screen on top of a 150 x 20-mm plastic Petri dish to separate the living nematodes from the dead nematodes. The living nematodes swim through the tissue paper into the water below in the Petri dish. The filtered suspensions were checked by scanning the Petri dish under the light microscope, and if there were dead nematodes present, the cleaning procedure was repeated again.

IJs concentration in the cleaned suspension was approximately 10,000 to 20,000 IJs/ ml.

The desired concentration used for tests, unless otherwise indicated in the text, was between 1000 to 2000 EPNs/ml. A suspension was diluted by adding 100 ml of the thoroughly mixed suspension to 900 ml of water in a 2 L flask. This gives 1000 ml of suspension containing 1000 to 2000 IJs/ ml.

The concentration of the dilute suspension was assessed by removing a 100 µl sample with a micro-dispenser from a thoroughly mixed suspension, and adding approximately 10 ml of water in a 55 x 10-mm plastic Petri dish to allow for easy viewing under the light microscope. The number of live IJs counted in the 100 µl sample should be between 100 and 200 to give the desired concentration of1000 to 2000 IJs/ml. If the number of live IJs counted was less than 100, the suspension was too dilute and, after allowing the suspension to settle, a portion of the water was decanted. If the number of live IJs counted was greater than 200, water was added to dilute the suspension further. These steps were repeated until the desired concentration was obtained.

c. Bait insect cultures

The initial culture of *Galleria mellonella* (greater wax moth) was obtained from the Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi. It was subsequently maintained on a semi-synthetic diet which is described as follows-

a. **Artificial diet for *Galleria mellonella*:** Ingredients for one set of 10,000 to 12,000 eggs, distributed in four jars of 2 L capacity to yield approximately 3000 larvae per jar- corn meal 400 g, wheat flour 200 g, wheat bran 200 g, milk powder 200 g, yeast 100 g, honey 350 ml, glycerine 350 ml. All the dry contents were mixed thoroughly in a clean container and then honey and glycerine were added. The mixture was kneaded to obtain semi-solid golden yellow coloured dough.
b. **Planting of eggs in the medium and after care:** The prepared medium was transferred to a wide mouthed jar so that it occupied only one-fourth volume in the jar. Egg masses (each containing around 450-550 eggs) collected from previous cultures were placed on the medium in jar. Normally 2-3 egg masses after hatching will give rise to 500 to 800 larvae. The eggs hatched within a week and hatched larvae became visible in 3-4 days. They were voracious feeders and attained a length of 2-2.5 cm. within a fortnight. If there was a need to arrest the growth of the larvae, they were transferred to a beaker containing wood scrapings and placed at 15°C.

The pupation took place in the jar and adults emerged within 10-12 days. Adults were fed on 20-30% honey solution through a cotton wick. After mating, they laid eggs in clusters around the rim or on the tissue paper at top of the jar. These eggs were further processed for next generation.

d. Standardize additives for tank mixture: Details of adjuvants tested in the study is given in Table no. 1.

Table 1: Trade Names and Chemical Types of Adjuvants Used in the study

Trade name	Chemical type	Manufacturer
Tween 20	Polyoxyethylene sorbitan monolaurate (ester)	Hindustan Crop Science, Nanded, India
Tween 80	Polyoxyethylene sorbitan monooleate (ester)	TCI Chem. (Ind.) Pvt. Ltd., Chennai, India
Sticker 99	Silicone based	Ajay biotech, Pune, India

Solutions of the adjuvants were tested at concentrations of 1%, 2% and3% (v/v) and mortality was recorded up to 72 hrs.

e. Effect of adjuvants on survival of *H. Indica* IJs

The toxicity of the three adjuvants to IJs of *H. indica* was assessed by incubating IJs in 1, 2 and 3% solutions of the adjuvants. Solutions of the IJs (200 IJs/ml) and adjuvants were prepared with distilled water. Five ml of each solution were placed in 25 ml plastic vials. Following incubation at 25° C in a BOD incubator for 24, 48 and 72 hrs, 0.5 ml of test solution was removed and the numbers of dead and live IJs assessed. All IJs that did not respond to mechanical stimulation were considered dead. There were five replicates per adjuvant concentration. Controls consisted of adding IJs to distilled water and incubation as described above.

f. Effect of adjuvants on infectivity of IJs of *H. indica*

The effect of the three adjuvants on the infectivity of IJs of *H. indica* was assessed using late instar larvae of G. *mellonella* as bait. Nematode/adjuvant suspensions were prepared using distilled water to give a final nematode concentration of 200

IJs/ml with adjuvant concentrations of 1, 2 and 3%. Controls consisted nematode suspension with no adjuvants or distilled water only.

g. Quantification of *H. Indica* infectivity

Infectivity profile of IJs of *H. indica* was developed following a filter paper technique reported by Miller (1989) [8]. Twenty five randomly picked live EPNs were transferred using a micro-dispenser into each plate well lined with double Whatman No.1 filter paper containing one larva of *G. mellonella* (12-well sterile polystyrene tissue culture plates with 15.6 mm diameter wells was used). The plates were sealed and incubated at 25 °C. After 24, 48, and 72 and hours, dead larvae were collected to determine nematode infectivity. The dead larvae were dissected under stereomicroscope in order to prove whether the larva has been killed by nematodes. For each replication, a plate with water but no EPNs was included as a control for *G. mellonella*. The average percent infectivity of EPNs against *G. mellonella* for each treatment was determined by taking the average number of dead *G. mellonella* larvae for the treatment, subtracting the average dead *G. mellonella* larvae for the control, and dividing by 12 (the number of wells per plate).

h. Statistical Analysis

Data were arcsine transformed and analyzed by a completely randomized factorial ANOVA. Means were compared at the P= 0.05 level.

Results

a. Standardize additives for tank mixture for their compatibility with *H. indica* IJs

The data obtained on the effect of exposure to three concentrations (1, 2 and 3%) of three adjuvants (Tween 20, Tween 80 and Sticker 99) on mortality and infectivity IJs of *H. indica* at different hours (24, 48 and 72 h) are presented in Tables 2 and 3, respectively.

Table 2: Effect of different concentrations of various adjuvants on per cent mortality of infective juveniles of *Heterorhabditis indica in vitro* at 27 °C ±1 °C

(Mean of 3 replications)

Adjuvant (A)	Concentration (C)	Per cent nematode mortality				Pooled mean A
		Time (T)				
		24 HAI[a]	48 HAI	72 HAI	Mean (A×C)	
Tween 20	1%	7.8 (16.02)	11.24 (19.09)	19.35 (25.96)	12.8 (20.36)	10.40 (16.06)
	2%	8 (16.4)	9.73 (18.17)	20.71 (27.07)	12.81 (20.54)	
	3%	11.22 (19.51)	14.34 (22.23)	22.39 (28.23)	15.98 (23.32)	
	Control	0 (0)	0 (0)	0 (0)	0 (0)	
	Mean (A×T)	6.76 (12.98)	8.83 (14.87)	15.61 (20.32)	10.40 (16.06)	
Sticker 99	1%	3.14 (10.18)	6.28 (14.46)	9.95 (18.39)	6.46 (14.35)	7.64 (13.45)
	2%	4.78 (12.56)	9.5 (17.9)	17.17 (24.45)	10.48 (18.3)	
	3%	6.91 (12.98)	13.98 (21.92)	19.99 (26.51)	13.63 (21.15)	
	Control	0 (0)	0 (0)	0 (0)	0 (0)	
	Mean (A×T)	3.71 (9.44)	7.44 (13.57)	11.78 (17.34)		
Tween 80	1%	10.6 (18.95)	15.64 (23.21)	26.42 (30.92)	17.55 (24.36)	18.16 (21.58)
	2%	9.38 (17.79)	16.25 (23.72)	22.12 (28.06)	15.92 (23.19)	
	3%	31.13 (32.7)	38.72 (38.78)	47.61 (44.78)	39.15 (38.75)	
	Control	0 (0)	0 (0)	0 (0)	0 (0)	
	Mean (A×T)	12.78 (17.36)	17.65 (21.43)	24.04 (25.94)		

Mean (C×T)				
	24 HAI[a]	48 HAI	72 HAI	**Pooled Mean C**
1%	3.59 (7.53)	5.53 (9.46)	9.29 (12.55)	6.13 (9.84)
2%	3.69 (7.79)	5.91 (9.97)	10.00 (13.26)	6.54 (10.34)
3%	8.21 (11.21)	11.17 (13.82)	15.00 (16.59)	11.46 (13.87)
Control	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0 (0)
Pooled Mean T	3.87 (6.63)	5.65 (8.31)	8.57 (10.60)	

Factors	SE (m)	C.D. at 5%
Adjuvant (A)	0.289	0.810
Concentration (B)	0.289	0.810
A × B	0.500	1.402
Incubation time (C)	0.333	0.935
A × C	0.577	1.619
B × C	0.577	1.619
A × B × C	1.000	2.804

[a]HAI: Hours after incubation

Values in parentheses are *arc sine* transformed.

SE (m): Standard Error of Mean; CD: Critical difference

A perusal of data in Table 2 indicated that the tested adjuvants had significant effect on the mortality of IJs of *Heterorhabditis indica* at all concentrations and exposure times. The per cent mortality increased as the concentration and time of exposure increased.

Irrespective of concentrations and incubation time, considering the effect of adjuvants alone, significantly minimum mortality was recorded in case of sticker 99

(7.64%). This was followed by Tween 20 (10.4%). Considering the effect of concentration alone (irrespective of adjuvants and incubation times), minimum mortality was recorded in case of 1% concentration, which was at par with 2% concentration. Incubation time alone resulted in significantly less mortality at 24 h after incubation (3.87%) compared to 48 h and 72 h. The interaction of all the three factors with each other and among themselves were also significant. It was noted that IJs following 24, 48 and 72 h incubation in Sticker 99 showed least mortality at all the three concentrations compared to other adjuvants.

Table 3: Infectivity of *Galleria mellonella* after inoculation with *Heterorhabditis indica* exposed to various adjuvants for different time periods *in vitro* (at 27°C ±1°C)
(Mean of 3 replications)

Incubation time (T)	Adjuvant (A)	Per cent mortality of *Galleria mellonella*					Pooled mean (T)
		Concentration of adjuvant (C)				Mean (T×A)	
		1%	2%	3%	Control		
24 h	Tween 20	100.00 (90.05)	87.50 (69.43)	77.50 (61.75)	100.00 (90.05)	91.25 (77.82)	93.33 (80.54)
	Sticker 99	100.00 (90.05)	95.00 (77.12)	100.00 (90.05)	100.00 (90.05)	98.75 (86.82)	
	Tween 80	100.00 (90.05)	87.50 (69.43)	72.50 (58.43)	100.00 (90.05)	90.00 (76.99)	
	Mean (T×C)	100.00 (90.05)	90.00 (71.99)	83.33 (70.08)	100.00 (90.05)		
48 h	Tween 20	82.50 (65.36)	72.50 (58.43)	62.50 (52.28)	100.00 (90.05)	79.38 (66.53)	80.21 (68.45)
	Sticker 99	100.00 (90.05)	92.50 (74.36)	60.00 (50.79)	100.00 (90.05)	88.13 (76.31)	
	Tween 80	62.50 (52.28)	72.50 (58.43)	57.50 (49.34)	100.00 (90.05)	73.13 (62.52)	
	Mean (T×C)	81.67 (69.23)	79.17 (63.74)	60.00 (50.80)	100.00 (90.05)		
72 h	Tween 20	42.50 (40.70)	40.00 (39.25)	37.50 (37.77)	100.00 (90.05)	55.00 (51.94)	57.71 (53.36)
	Sticker 99	67.50 (55.29)	52.50 (46.46)	37.50 (37.77)	100.00 (90.05)	64.38 (57.39)	
	Tween 80	40.00 (39.25)	42.50 (40.70)	32.50 (34.76)	100.00 (90.05)	53.75 (51.19)	
	Mean (T×C)	50.00 (45.08)	45.00 (42.14)	35.83 (36.77)	100.00 (90.05)		

	Mean (A×C)				
	1%	2%	3%	Control	**Pooled mean A**
Tween 20	75 (65.37)	66.67 (55.70)	59.17 (50.6)	100 (90.05)	75.21 (65.431)
Sticker 99	89.17 (78.467)	80 (71.94)	65.83 (59.54)	100 (90.05)	83.75 (75.00)
Tween 80	67.5 (60.53)	64.04 (62.21)	54.17 (47.51)	100 (90.05)	71.43 (65.07)
Pooled mean C	77.22 (68.12)	70.24 (63.28)	59.72 (52.55)	100 (90.05)	

Factors	SE (m)	C.D. at 5%
Incubation time (T)	0.371	1.07
Adjuvant (A)	0.371	1.07
T × A	0.643	1.84
Concentration of adjuvant (C)	0.428	1.23
T × C	0.742	2.13
A × C	0.742	2.13
T × B × C	1.285	3.69

Values in parentheses are *arc sine* transformed.
SE (m): Standard Error of Mean; CD: Critical difference

Data in table 3 shows the mean infectivity of infective juveniles of *Heterorhabditis indica* following 24, 48 and 72 h incubation in tested adjuvants against *G. mellonella* larvae after 72 h. Irrespective of incubation time and concentration, pooled means (A) revealed that Sticker 99 caused significantly maximum mortality of *Galleria* (83.75%) compared to Tween 20 and Tween 80. Considering the effect of concentrations alone (irrespective of adjuvant and incubation time), significantly maximum mortality of *Galleria* was recorded at 1% concentration (77.22%) which decreased with an increase in concentration. Incubation time alone had a negative correlation with *Galleria* mortality. The interactions of all the three factors with each other and among themselves were also significant. The bioassay provided 90% mortality of *G. mellonella* within 72 h for *H. indica* IJs which were exposed to 1% concentration of all the tested adjuvants for 24 h. Maximum infectivity of IJs was recorded in case of Sticker 99 at all the tested concentrations and incubation times compared to other adjuvants.

Discussion

Since EPNs are sensitive to desiccation and UV radiation, use

of adjuvants in the spray solution assists in their application in the field, particularly on the foliage. Anti-dessicants like sticker 99, Tween 20, Glycerol etc. reduce evaporation and increase the longevity of a droplet; while adjuvants like Congo Red etc. protect IJs from UV radiation during foliar applications. The choice and appropriate use of adjuvant requires its compatibility with specific nematode species. In the present study, minimum mortality was recorded in case of sticker 99 (7.64%), followed by Tween 20 (10.4%). Considering the effect of concentration alone, minimum mortality was recorded in case of 1 and 2% concentrations. Also, there was a positive correlation between incubation time and mortality. Significantly less mortality was observed at 24 h after incubation (3.87%) compared to 48 h and 72 h. Sticker 99 showed least mortality at all the three concentrations and incubation times compared to other tested adjuvants.

The IJs exposed to adjuvants for a period of three days when inoculated on 4th instar larvae of *G. mellonella* recorded least larval mortality (57.71%) compared to exposure period of two days (80.21% mortality) or one day (93.33% mortality). This observation confirmed the loss of virulence in IJs exposed to the tested adjuvants. So it is inferred that these adjuvants are compatible with *H. indica* without any significant loss of either viability or virulence at 1% concentration and exposure for one day only.

Previous studies have also established that the efficacy of EPNs can be enhanced through addition of adjuvants (Moreira *et al*., 2013; Noosidum *et al.*, 2016; Portman *et al.*, 2016; Shapiro-Ilan *et al*,. 2010) [10, 13, 14, 16], this information could be useful in the better use of *H. indica* against insect pests. However, Baur *et al.* (1997) [1] studied the effects of several spray additives on the persistence and efficacy of EPN against *Plutella xylostella* and found no adjuvant toxic to the nematodes or *P. xylostella*. Further, the additives generally improved nematode persistence and efficacy. Addition of adjuvants (arabic and *guar* gum, alginate and xanthan in concentrations between 0.05 and 0.3%) prevented *S. carpocapsae* from settling in the tank mix of backpack sprayers and significantly improved deposition of nematodes on the cabbage leaves (Schroer *et al.*, 2005) [15]. Above-ground applications of *S. carpocapsae* were enhanced using a sprayable fire gel (Barricade® gel) @ 2% against *Synanthedon pictipes*. The gel can be applied in a single spray mixed with nematodes or separately. The nematode + gel combination controlled the pest as well as the standard chemical (Shapiro-Ilan *et al.*, 2016) [17]. Dito *et al.* (2016) [2] demonstrated that *S. carpocapsae*, combined with a protective gel, and anti-UV ingredients, have potential for above-ground pest management. UV protection provided by titanium dioxide (TD) and octyl methoxycinnamate with 1% protective gel solution resulted in higher insect mortality than other treatments and 0.25% protective gel solution had the most live EPNs on leaves after 8 h in the greenhouse. Addition of gel at low concentration protects EPNs, and addition of TD enhanced the UV protective properties of the formulation.

Hence it can be concluded that adjuvants Tween 20, Sticker 99 and Tween 80 at 1 or 2% concentrations have no adverse effect on the survival or infectivity of IJs of *H. indica* up to a period of 24 h and can be tank mixed at recommended concentrations to enhance the field efficacy of *H. indica*.

Conclusion

Effect of exposure to three concentrations (1, 2 and 3%) of three adjuvants (Tween 20, Tween 80 and Sticker 99) on the mortality and infectivity IJs of *H. indica* at different periods after incubation (24, 48 and 72 h) was evaluated. The tested adjuvants had significant effect on the mortality of IJs of *H. indica* at all concentrations and exposure times. The per cent mortality of IJs increased as the concentration and time of exposure increased. Minimum mortality of IJs and maximum infectivity of *Galleria mellonella* larvae was recorded in case of Sticker 99 at all the tested concentrations and incubation times. There was a positive correlation between mortality and negative correlation between infectivity of IJs with incubation time and concentration of adjuvant solutions, respectively.

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