Original Research Article

Formulation of Entomopathogenic nematode, *Steinernema kushidai,* their survival and infectivity

Abstract: The commercialization and large-scale use of EPNs are~~is~~ limited by their short shelf life in formulations and in storage, thus leading to poor quality and reduced efficacy against insects in the field. This study explored the effects of some formulations on survival and infectivity of infective juveniles (IJs) of entomopathogenic nematodes *Steinernema* at 5 and 25°C for 6 weeks. In the formulations, the survival and virulence rate differed at different temperature with decrease over the period of time. The alginate retained most of the IJs and can be stored for a longer time at 5^0C followed by sawdust formulation .From the economic point of view, sawdust formulation shows better results.

Key words: Entomopathogenic nematodes, *Steinernema kushidai*, formulation, survival , infectivity

Introduction

Entomopathogenic nematodes (EPNs) have great potential as a biocontrol agent~~-~~ against various insect pests of economic importance. EPN from the Steinernematidae and Heterorhabditidae families are obligate parasites of insects (Shapiro-Ilan et al., 2012). They are associated mutualistically with pathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, which are lethal to insects (Adams et al., 2006). ~~-~~EPN are used against a wide range of insect pests because of their ease of mass culture, high lethality against key pests, and nonexistent safety issues (Kaya and Gaugler, 1993; Lewis et al., 2006). The development of commercialized EPNs is mainly due to growing concerns raised by insect resistance to and unwanted side effects of chemical pesticides on beneficial organisms, the environment, and human health (Gaugler and Kaya, 1990, Ehlers, 2003, Shapiro-Ilan et al., 2012, Shapiro-Ilan et al., 2014). Nevertheless, the most common formulations for EPN application have a limited shelf life in storage and relatively short survival after application, which are obstacles to the commercialization of EPN-based biopesticides (Gaugler, 2002; Georgis et al., 2006; Lacey et al.,2015). Several studies have examined the effects of different substrates on the longevity and infectivity of IJs stored within them. Their results have shown that survival of IJs varied on different substrates; hence, the type of substrate to be used must be taken into consideration

(Grewal, 2000a, Grewal, 2000b, Andalo et al., 2010, Leite et al., 2018). Grewal (2000a) demonstrated that the formulation of IJs in water--dispersible granules consisting of clay, silica, lignin, cellulose, and starch partly desiccated the nematodes and improved their viability for 3 -months more at 25 ºC compared to IJs held in water. Ramakuwela et al. (2015) reported that storage of IJs on sponges had no negative effect on their survival or infectivity even if previously stored in distilled water at 25 ºC. Leite et al. (2018) used seven different substrates and two combinations containing polyacrylamide gel and vermiculite to store -*S. feltiae* -at three temperatures. They noted that IJs survived better in some substrates when stored at certain temperature ranges. The shelf-life of most of the formulated products has been enhanced by reducing nematode activity through physical trapping, by induction of anhydrobiosis and cold storage (Grewal, 2002).

Materials and Methods

Insect rearing

Galleria mellonella -(Lepidoptera: Pyralidae) larvae were used throughout this study. They were reared on an artificial diet ~~i.e.,corn~~i.e., corn flour (400 gm), wheat bran (150 gm), wheat flour (200 gm), wheat germ (50 gm), yeast and milk powder (200 gm)-, Honey and glycerine (200 gm) , vitamin complex (2 ml) and streptomycin sulphate (100 gm) at 29°C and 70% RH in 2liter glass containers. Last instar -*G. mellonella*, prior to use, were immersed in water at 60°C for 5 seconds to prevent them from spinning their silken cocoon and proceeding to the pupa -formation.

Nematode mass culture

Steinernema kushidai were isolated from the rhizosphere of pumpkin from village Allengmora, Jorhat, Assam (26°72’28.05”N 94°19’66.33”E). The nematodes were maintained on the 5th instar larvae of *G. mellonella* (Woodring and Kaya, 1988). Newly emerged IJs from *G. mellonella* cadavers were collected in a White trap (White, 1927) and stored using sponges with 2×1×2 cm dimensions for a maximum of 5 days at 10°C. They were adapted to 21-23°C for 24 h before being subjected to the assays. The IJs were suspended in water and prepared in formulations as indicated below.

Talc formulation

Talc powder (250 g) was added to 25 ml of distilled water in a 500 ml beaker and mixed thoroughly. Fifty ml of freshly harvested IJs of EPNs (2000 IJs / ml) were added in the above

moisten talc and then the contents were thoroughly mixed till the nematode suspension spread over evenly into the talc. Such kinds of ten replications were prepared in ~~a~~ polythene envelope and sealed ~~them~~ individually for further survival and infectivity observation and stored at a temperature of 5°C and 30°C.

Saw-dust formulation

The saw-dust material was grou~~i~~nd~~ed~~ separately to get fine dust with the help of a mixer and grinder and sieved with fine mesh and then sterilized under sunlight for 1 hr. Two hundred and fifty grams of saw-dust ~~were~~was moistened by adding 50 ml of distilled water separately. IJs suspension of 50 ml (2000 IJs/ ml) were added evenly and mixed them gently till nematodes spread over into the saw-dust in ~~a~~ polythene envelope. Such kinds of ten replications were prepared in ~~a~~ polythene envelope and sealed ~~them~~ individually for further survival and infectivity observation and stored at a temperature of 5°C and 30°C.

Alginate gel

A matrix of nematode gel was prepared as per Kaya and Nelsen (1985). Two gm of sodium alginate in 150 ml of water and blend~~ed~~ for 4-5 minutes. Fifty milliliters of nematode suspension (2000 IJs/ml) were placed into the solution of Sodium alginate which is a water-insoluble, gelatinous, cream colo~~u~~red substance and then dripped into the complexing solution which was continuously stirred. An antifungal agent (0.05mg Streptomycin sulphate) was added to prevent the growth of microbes and pH was adjusted to 7.0. Drops of this solution when placed into a 100 mM solution of $CaCl_2.2H_2O$ (the complexing solution) formed a discrete capsule of calcium alginate. Capsules were allowed to complex for 20-30 minutes and then separated from the complexing solution by sieving, rinsed in demonized (deionized?) water and stored at a temperature of 5°C and 30°C in polythene bags for further survival and infectivity observations. The actual number of nematodes was determined by dissolving five capsules in 9.5ml of 0.5M sodium citrate containing 0.1% Triton X-100.The capsules were stirred with magnetic spin bar until dissolution (about 30 minutes)~~-~~, and the nematode in 1 ml of suspension were counted using a Hawksley counting slide.

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Water dispersible granule (WDG)

Clay, aloe gel and starch were mixed at the ratio of 1:1:1. IJs suspension of 50 ml (2000IJs/ ml) were added evenly and mixed them gently till nematodes spread over into the above mixture.

Granules of 10-20 mm diameter wasere prepared and packed in polythene envelop and stored at a temperature of 5°C and 30°C for further survival and infectivity observation.

Compost and charcoal powder mixture

Vermicompost and charcoal powder were mixed at athe ratio of 1:1. IJs suspension of 50 ml (2000 IJs/ ml) were added evenly and mixed them gently till nematodes spread over into the above mixture. The prepared formulation wasere packed in a polythene envelope and stored at a temperature of 5°C and 30°C for further survival and infectivity observation.

Control (water)

Freshly harvested infective juveniles were washed twice in distilled water and 50 ml (2000 IJs/ ml) of suspension was stored in a 250 ml conical flask. Flask wasere closed with non–absorbent cotton and stored at a temperature of 5°C and 30°C.

Survival of entomopathogenic nematodes in different formulation

Survival of infective juveniles in different formulations wwasere evaluated by weekly interval up to 6 weeks at 5°C and 30°C temperature, by diluting 0.5 g of formulated IJs in 5 ml distilled water from each and the per-cent IJs survival was counted and the per-cent mean data of survived IJs –was recorded. Four replicates for each treatment were done. Data obtained in a per–cent survival of IJs were transformed to arcsine for statistical analysis. Data were statistically analyzed using Two factorial Completely Randomized Block Design.

Infectivity of entomopathogenic nematodes against *Galleria mellonella*

Soil bioassay

The experiment was conducted in 250 ml capacity beaker. Two fifty grams of sterilized soil were kept in each beaker and 15% moisture was maintained. Five grams of EPN formulations each with 5 replications were tested against 10 larvae of the greater wax moth, *G. Mellonella*. Observations on mortality were done at 24 h intervals for three days. The data from percent larval mortality induced by EPNs were subjected to transformed to arcsine for statistical analysis. Data were statistically analyzed using Two factorial Completely Randomized Block Design.

Results and Discussion

The data on effect of different formulations at different storage period on survival of *Steinernema kushidai* at 5°C are presented in table1. Irrespective of storage time, the formulation (T) of Alginate gel and Sawdust were found to be significantly effective in survival of *S. kushidai* when compared with control (water). Between the two formulations, Alginate gel

was found to be more effective in survival (95.56%) than Sawdust (93.73%). Similarly, irrespective of formulation (T), the storage period (t) was also significantly effective for survival of *S. kushidai*. Survival was more (98.56%) in 1st week of storage followed by 96.10% in 2nd week, 92.33% in 3rd week, 87.06% in 4th week, 81.03%, in 5th week and 75.46% in 6th week of storage. Following the significant interaction of formulation and storage time (T x t), up to 3rd week of storage at 5^0C, Alginate gel and sawdust formulation, *S. kushidai* survival was 100%. After 4th week storage at 5°C it was observed that, survival of *S. kushidai* in sawdust formulation, though significantly higher as compared to control, yet it was lower as compared to Alginate gel formulation (95.80%). During 5th week of storage, survival % of *S. kushidai* was significantly higher in Alginate gel formulation as compared to sawdust (87.40%) and control (82.40%). During 6th week of storage, survival % of *S. kushidai* was significantly higher in Alginate gel formulation as compared to sawdust (79.20%) and control (77.80%).

The data on effect of different formulations at different storage period on survival of *Steinernema kushidai* at 30°C are presented in table 2. Between the two formulations, Alginate gel was found to be more effective in survival (93.80%) at 30°C than Sawdust (91.53%). Similarly, irrespective of formulation (T), the storage period (t) was also significantly effective for survival of *S. kushidai*. Survival was more (97.73%) in 1st week of storage followed by 95.13% in 2nd week, 89.70% in 3rd week, 82.83% in 4th week, 78.10%, in 5th week and 69.83% in 6th week of storage. Following the significant interaction of formulation and storage time (T x t), up to 3rd week of storage Alginate gel and sawdust formulation, *S. kushidai* survival was 100%. After 4th week storage at 30°C it was observed that, survival of *S. kushidai* in sawdust formulation, though significantly higher as compared to control, yet it was lower as compared to Alginate gel formulation (88.80%). During 5th week of storage, survival % of *S. kushidai* at 30°C was significantly higher in Alginate gel formulation as compared to sawdust (83.60%) and control (77.20%). During 6th week of storage, survival % of *S. kushidai* at 30°C was significantly higher in Alginate gel formulation as compared to sawdust (76.80%) and control (58.60%).

The data on effect of different formulation at different exposure period on larval mortality of *Galleria mellonella* by *Steinernema kushidai* stored at 5^0C in soil bioassay are presented in table 3. Irrespective of exposure time, among all the formulations (T) of *S. kushidai*, sawdust, alginate gel and compost charcoal mixture showed higher mortality of *Galleria* larva as compared to control (water). Alginate gel formulation was found to be most effective on larval mortality of

Galleria (82.00%) than sawdust (81.33%). Similarly, irrespective of formulation treatment (T), the exposure time (t) showed significant effect on *Galleria* larval mortality. During 24h of exposure time larval mortality of *Galleria* by *S. kushidai* was highest in Alginate formulation (60.00%). During 48 h of exposure time, 88% mortality of *Galleria* was recorded in case of Alginate gel formulation followed by sawdust formulation (88%) and compost charcoal mixture (66%). Alginate formulation showed larval mortality which is 83.33 % and 96% increased over control during 48 h and 72 h exposure time, respectively.

The data on effect of different formulation at different exposure period on larval mortality of *Galleria mellonella* by *Steinernema kushidai* stored at 30°C in soil bioassay ~~are~~is presented in table4. Irrespective of exposure time, among all the formulations (T) of *S. kushidai*, sawdust, alginate gel and compost charcoal mixture showed higher mortality of *Galleria* larva as compared to control (water). Alginate gel formulation was found to be most effective on larval mortality of *Galleria* (78.66%). Similarly, irrespective of formulation treatment (T), the exposure time (t) showed significant effect on *Galleria* larval mortality. During 24h of exposure time larval mortality of *Galleria* by *S. kushidai* was highest in Alginate formulation (56.00%). During 48 h of exposure time, 84% mortality of *Galleria* was recorded in case of Alginate gel formulation followed by sawdust formulation (84%) and compost charcoal mixture (64%). Alginate formulation showed larval mortality which is 86.66 % and 96% increased over control during 48 h and 72 h exposure time, respectively.

This investigation provides data on the influence of the storage stability of *S. kushidai* and compares the survival and infectivity of IJs in different formulation. Per–cent survival of *S. kushidai* infective juveniles (IJs) was 95.56% in alginate gel, and 93.73% in sawdust, respectively at 5°C, whereas per–cent survival was less 93.80% and 91.53% respectively at 30°C. The results have confirmed observations by Fan and Hominick (1991) who recorded a positive influence of cold storage on the survival of nematode IJs *S. carpocapsae*. Energy reserves usually exhausted much faster as storage time increase (Jung, 1996; Georgis and Kaya, 1998). *S. feltiae* with alginate capsules with 99.8% survival up to 6 months at 23°C has been reached (Chen and Glazer 2005). Divya *et al*. (2011) developed five different formulations of *H. indica*, sawdust, hydrogel, coir dust, talc and sponge and were evaluated its survival at 27±2°C. Sawdust and hydrogel formulations were enhanced highest survival (95%) and (85%) followed by coir dust (80%), talc (75%) and sponge (65%) till 5th week period. A maximum shelf-life of

more than ~~11 week~~11-week periods achieved in hydrogel formulation with 65% of survival than sawdust formulation. Hussein and Abdel-Aty (2012) observed that among the three formulations viz., hydrogel, kaolinite and calcium alginate, storage potential of *S. carpocapsae* juveniles was more (more than 50% in 40 days) than that of calcium alginate formulation of *H. bacteriophora* at room temperature (25±2°C). Navon *et al.*, (1998, 2002) encapsulated *S. carpocapsae* in an edible-to-insects gel to control *Helicoverpa armigera* and *S. littoralis*, at a concentration of 1000 *S. carpocapsae* IJs/g, which caused 95% mortality in *H. armigera* and 100% in *S. littoralis* larvae. Andalo *et al.* (2010) achieved 89.3 and 57.5 % survival in sponge formulation (3000 IJs/ml) after 90 days and 180 days storage, respectively, at 16°C for *Steinernema carpocapsae*. Though alginate gel formulation of *S. kushidai* shows better performance than sawdust formulation in respect of survival and pathogen city, from economic point of view the sawdust formulation is cost effective (Table 5).

Conclusion

Alginate gel formulation and sawdust formulation of *S. kushidai* enhanced storage stability up to six weeks by physically trapping them in gels or through reduced water activity of the substrates. Further research is necessary to use *S. kushidai* more effectively as a potential biocontrol agent of soil insect pest.

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Table1. Per-cent survival of infective juveniles of *Steinernema kushidai* in different formulations stored at 5°C (Mean of five replications)

Formulations	Survival (%)						Mean
	1st week	2nd week	3rd week	4th week	5th week	6th week	
T1 :Talc	100 (89.78)	100 (89.78)	92.00 (75.20)	83.60 (66.29)	76.40 (61.08)	73.20 (58.88)	87.53 (73.50)
T2 :Sawdust	100 (89.78)	100 (89.78)	100 (89.78)	95.80 (82.31)	87.40 (69.32)	79.20 (63.11)	93.73 (80.54)
T3 :Alginate gel	100 (89.78)	100 (89.78)	100 (89.78)	97.80 (84.45)	92.80 (74.62)	82.80 (65.54)	95.56 (82.32)
T4 :Water Dispersable granule	91.40 (74.74)	79.60 (63.27)	75.20 (60.19)	70.40 (57.08)	66.40 (54.60)	60.60 (51.12)	73.93 (60.17)
T5 : Compost:Charcole powder mixture	100 (89.78)	97.00 (83.60)	90.80 (72.64)	86.20 (68.36)	82.40 (65.23)	79.20 (62.87)	89.26 (73.15)
T6:(Control): Water	100 (89.78)	100 (89.78)	96.00 (81.05)	88.60 (70.56)	82.40 (65.36)	77.80 (61.94)	90.80 (76.41)
Mean	98.56 (87.27)	96.10 (84.33)	92.33 (78.11)	87.06 (71.51)	81.03 (65.03)	75.46 (60.43)	
CD(P=0.05)	Formulation (T) : (1.70) Storage Time (t):(1.70) Formulation (T) x Storage Time (t): (4.17)						

Figures in parentheses are arc sin transformed values

Table 2. Per-cent survival of infective juveniles of *Steinernema kushidai* in different formulations stored at 30 ^{0}C (Mean of five replications)

Formulations	Survival (%)						Mean
	1st week	2nd week	3rd week	4th week	5th week	6th week	
T1 :Talc	100 (89.78)	100 (89.78)	83.00 (65.70)	82.80 (65.68)	79.20 (62.96)	73.00 (68.76)	86.33 (72.11)
T2 :Sawdust	100 (89.78)	100 (89.78)	100 (89.78)	88.80 (70.50)	83.60 (66.20)	76.80 (61.32)	91.53 (77.90)
T3 :Alginate gel	100 (89.78)	100 (89.78)	100 (89.78)	92.80 (74.62)	88.60 (70.32)	81.40 (64.48)	93.80 (79.79)
T4 :Water Dispersable granule	86.40 (68.59)	77.80 (91.93)	74.60 (59.80)	68.60 (55.95)	63.60 (52.92)	59.40 (50.42)	71.73 (58.27)
T5 : Compost:Charcole powder mixture	100 (89.78)	93.00 (77.98)	87.60 (69.80)	81.00 (64.27)	76.40 (61.05)	69.80 (56.73)	84.63 (69.93)
T6:(Control): Water	100 (89.78)	100 (89.78)	93.00 (76.31)	83.00 (65.81)	77.20 (65.81)	58.60 (61.76)	85.30 (73.57)
Mean	97.73 (86.25)	95.13 (83.17)	89.70 (75.20)	82.83 (66.14)	78.10 (62.39)	69.83 (58.42)	
CD(P=0.05)	Formulation (T) : (1.60) Storage Time (t):(1.60) Formulation (T) x Storage Time (t): (4.32)						

Figures in parentheses are arc sin transformed values

Table3: Per-cent larval mortality of *Galleria mellonella* by *Steinernema kushidai* in different formulations stored at 5^{0}C in soil bioassay (Mean of five replications)

Formulations	Larval mortality (%)						Mean
	24 hrs	% increase/ decrease over control	48 hrs	% increase/ decrease over control	72 hrs	% increase/ decrease over control	
T1 :Talc	38.00 (37.97)	0.00	56.00 (48.51)	16.66 (+ve)	82.00 (65.35)	64.00 (+ve)	58.66 (50.61)
T2 :Sawdust	60.00 (50.89)	57.89 (+ve)	88.00 (71.94)	83.33 (+ve)	96.00 (82.44)	92.00 (+ve)	81.33 (68.41)
T3 :Alginate gel	60.00 (50.81)	57.89 (+ve)	88.00 (71.94)	83.33 (+ve)	98.00 (86.07)	96.00 (+ve)	82.00 (69.61)
T4 :Water Dispersable granule	28.00 (31.88)	26.31 (-ve)	38.00 (38.02)	20.83 (-ve)	62.00 (50.81)	24.00 (+ve)	42.66 (40.64)
T5 : Compost:Charcole powder mixture	48.00 (43.84)	26.31 (+ve)	66.00 (54.55)	37.50 (+ve)	88.00 (71.94)	76.00 (+ve)	67.33 (56.78)
T6:(Control): Water	38.00 (37.97)		48.00 (43.84)		50.00 (45.00)		45.33 (42.24)
Mean	45.33 (42.22)		64.00 (54.80)		79.33 (67.13)		
CD(P=0.05)	Formulation (T) : (4.87) Exposure period (t):(3.45) Formulation (T) x Exposure period (t): (8.45)						

Figures in parentheses are arc sin transformed values

Table 4. Per-cent larval mortality of *Galleria mellonella* by *Steinernema kushidai* in different formulations stored at 30^{0}C in soil bioassay (Mean of five replications)

Formulations	Larval mortality (%)						Mean
	24 hrs	% increase/ decrease over control	48 hrs	% increase/ decrease over control	72 hrs	% increase/ decrease over control	
T1 :Talc	36.00 (36.82)	0.00	52.00 (46.15)	15.55 (+ve)	78.00 (62.40)	56.00 (+ve)	55.33 (48.46)
T2 :Sawdust	58.00 (49.66)	61.11 (+ve)	84.00 (66.68)	86.66 (+ve)	94.00 (78.81)	88.00 (+ve)	78.66 (65.05)
T3 :Alginate gel	56.00 (48.6)	55.55 (+ve)	84.00 (68.68)	86.66 (+ve)	98.00 (82.44)	96.00 (+ve)	78.66 (66.53)
T4 :Water Dispersable granule	26.00 (30.55)	27.77 (-ve)	36.00 (36.82)	20.00 (-ve)	58.00 (49.66)	16.00 (+ve)	40.00 (39.01)
T5 : Compost:Charcole powder mixture	46.00 (42.69)	27.77 (+ve)	64.00 (53.35)	42.22 (+ve)	84.00 (68.98)	68.00 (+ve)	64.66 (55.01)
T6:(Control): Water	36.00 (36.82)		46.00 (42.69)		50.00 (45.00)		43.66 (41.50)
Mean	43.00 (40.83)		60.83 (52.39)		77.00 (64.55)		
CD(P=0.05)	Formulation (T) : (4.85) Exposure period (t):(3.43) Formulation (T) x Exposure period (t): (8.40)						

Figures in parentheses are arc sin transformed values

Table 5. Ingredient composition and cost per Kg of formulation

Formulation	Ingredients	Amount	Cost(Rs.)
Talc	Talc fine powder	250 g	45.00
Sawdust	Sawdust	250 g	10.00
Alginate gel	Sodium alginate Calcium chloride	2g/50ml suspension 1.1 g/100ml	20.00 0.30
WDG (Clay:Aloe gel:Starch)	Clay Aloe gel Starch	84 g 84 g 84g	10.00 56.00 218.00
Compost: charcoal powder mixture	Vermicompost Charcoal powder	125g 125g	1.25 387.50
Water	Water	-	-