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

Abstract: The commercialization and large-scale use of EPNs are 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 kushidai* 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°C 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 soil as well as foliar insect pests of economic importance. EPN are obligatory parasitic insects belonging to the Steinernematidae and Heterorhabditidae families [1]. They have a mutualistic relationship with pathogenic bacteria from the genera Xenorhabdus and Photorhabdus, which are both fatal to insects [2]. Because of their simplicity of mass culture, great lethality against important pests, and lack of safety concerns, EPN are utilised against a wide spectrum of insect pests[3,4]. The development of commercialised EPNs is primarily motivated by rising concerns about insect resistance to chemical pesticides and their unfavourable side effects on beneficial creatures, the environment, and human health [5-7]. Nonetheless, the most common formulations for EPN application have a short shelf life in storage and a short survival time after application, which are barriers to the commercialization of EPN-based biopesticides [8-10]. Several research have looked at how different substrates affect the longevity and infectiousness of IJs stored within them. Their findings revealed that IJ survival varied depending on the substrate utilised; thus, the type of substrate to be employed must be considered. [11-14] Grewal [12] showed that IJs formulated in water dispersible granules including clay, silica, lignin, cellulose, and starch partially dehydrated the nematodes and increased their vitality for 3 months longer at 25°C than IJs maintained in water. To store S. feltiae at three temperatures, Leite et al. [14] employed seven different substrates and two combinations combining polyacrylamide gel and vermiculite. They discovered that IJs fared better on certain substrates when stored at specific temperatures. Even when IJs were previously maintained in distilled water at 25°C, Ramakuwela et al. [15] found

that storing them on sponges had no negative effect on their survival or infectivity. Reduced nematode activity through physical trapping, induction of anhydrobiosis, and cold storage have all been shown to extend the shelf life of most manufactured products [16-18]. **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 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* [19]. Newly emerging IJs from *G. mellonella* cadavers were collected in a White trap [20] and kept for a maximum of 5 days at 10°C using sponges with dimensions of 212 cm. They were exposed to the assays after being accustomed to 21-23°C for 24 hours. The IJs were suspended in water and made in the following formulas.

Talc formulation

In a 500 mL beaker, 250 g of talc powder was mixed well with 25 mL distilled water. 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 envelop and sealed 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 ground 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 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 polythene envelope. Such kinds of ten replications were prepared in polythene

envelope and sealed individually for further survival and infectivity observation and stored at a temperature of 5°C and 30°C.

Alginate gel

According to Kaya and Nelsen(1985), a nematode gel matrix was created. Blend two gm sodium alginate in 150 ml water for 4-5 minutes. Fifty millilitres of nematode suspension (2000 IJs/ml) were dropped into a solution of sodium alginate, a water-insoluble, gelatinous, cream-colored material, and then constantly mixed into the complexing solution. To prevent the growth of bacteria, an antifungal drug (0.05 mg Streptomycin sulphate) was added, and the 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 left to compound for 20-30 minutes before being sieved out of the complexing solution, rinsed in deionized water, and stored in polythene bags at 5°C and 30°C for subsequent survival and infectivity testing. Five capsules were dissolved in 9.5ml of 0.5M sodium citrate containing 0.1 percent Triton X-100 to ascertain the actual number of nematodes. The capsules were mixed with a magnetic spin bar until they disintegrated (approximately 30 minutes), and the nematodes in 1 ml of suspension were counted with a Hawksley counting slide. **Water dispersible granule (WDG)**

Clay, aloe gel, and starch were mixed in a 1:1:1 ratio. 50 mL IJs suspension (2000 IJs/ mL) was added evenly and gently stirred until the nematodes distributed throughout the mixture. Granules with a diameter of 10-20 mm were made, packaged in a polythene envelope, and stored at 5°C and 30°C for subsequent survival and infectivity testing. **Compost and charcoal powder mixture**

A 1:1 mixture of vermicompost and charcoal powder was used. 50 mL IJs suspension (2000 IJs/ mL) was added evenly and gently stirred until the nematodes distributed throughout the mixture. The prepared formulation was packed in a polythene envelop 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 was closed with non absorbent cotton and stored at a temperature of 5°C and 30°C.

Survival of entomopathogenic nematodes in different formulation

The survival of infective juveniles in varying concentrations was assessed at weekly intervals up to 6 weeks at temperatures of 5°C and 30°C, by diluting 0.5 g of formulated IJs in 5 ml distilled water from each and counting the percent IJs survival and recording the percent mean data of survived IJs. Each treatment was replicated four times. For statistical analysis, data collected in a percent survival of IJs were translated to arcsine. Two factorial Completely Randomized Block Design was used to statistically evaluate the data. **Infectivity of entomopathogenic nematodes against *Galleria mellonella***

Soil bioassay

The experiment was carried out in a beaker with a volume of 250 mL. Each beaker was filled with 250 grammes of sterilised soil and held at a moisture level of 15%. Ten larvae of the larger wax moth, G. Mellonella, were tested with five grammes of EPN formulations, each with five replications. For three days, observations on mortality were made at 24 hour intervals. For statistical analysis, the data from % larval mortality caused by EPNs was translated to arcsine. Two factorial Completely Randomized Block Design was used to statistically evaluate the data.

Results and Discussion

The data on effect of different formulations at different storage period on survival of *Steinernema kushidai* at 5°C is 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°C, 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 is 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 30^0C 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°C in soil bioassay is 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 is presented in table 4. 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 [21] 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 [22, 23]. *S.feltiae* with alginate capsules with 99.8% survival up to 6 months at 23°C has been reached [24]. Divya *et al*.[25] developed five different formulations of *H.indica*, sawdust, hydrogel, coirdust, 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 coirdust (80%), talc (75%) and sponge (65%) till 5th week period. A maximum shelf-life of more than 11-week periods achieved in hydrogel formulation with 65% of survival than sawdust formulation. Hussein and Abdel-Aty [26] observed that among the three formulation 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., [27,28] (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. [13] 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 °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°C in soil bioassay (Mean of five replications)

Formulations	Larval mortality (%)						Mean
	24 hrs	% increase/ decrease over	48 hrs	% increase/ decrease over	72 hrs	% increase/ decrease over control	

		control		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°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	-	-

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