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Development of biopellet formulation of *Lecanicillium saksenae* for the management of pepper root mealybug (*Formicoccus polysperes*)

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ABSTRACT

Present study was carried out during 2020–23 in the Biocontrol Laboratory for Crop Pest Management, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. An indigenous isolate of *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno, ITCC 7714 was assessed for its effectiveness against the pepper root mealybug (*Formicoccus polysperes* Williams). Peculiar symptoms like sudden dissolution of mealy coating subsequent to the application of conidial suspension and paralysis occurring 12 h post-treatment was observed in mealybugs upon infection. Its high potency to mealybug was established with its low LC_{50} value of 2.2×10^5 conidia/ml and its quick killing ability was proved with a lethal time of 23.13 h. Of the various carriers tested for pelleting, talc + chitin (95:5) was found to be the best as it exhibited highest conidial viability of 78.54%, 5-weeks after storage. Other carriers such as talc, chitin, chitosan and talc + chitosan (90:10) exhibited conidial viability of 33.96–74.58%. Among the different binding agents examined, the combination of talc + chitin (95:5) + carboxymethyl cellulose 6% was the superior one to maintain conidial viability to the tune of 80.63% when compared to microcrystalline cellulose 6% and acacia gum, Arabic 5% which exhibited 30.67–51.50% viability. The investigation therefore concluded that *L. saksenae* is a promising bioagent for the management of root mealybugs with high speed of action unlike the other microbes. Biopellets formulated at 10^9 conidia/ml using talc + chitin + 6% CMC at 15% moisture content were found to retain its viability and virulence during a storage period of three months, under ambient conditions.

Keywords: Biopellet, Entomopathogenic fungi, Formulation, *Formicoccus polysperes, Lecanicillium saksenae,* Pepper root mealybug

Black pepper (*Piper nigrum* L.), the king of spices, graces international market with its distinctive flavour profile. Pest infestation is a major challenge in pepper cultivation. In recent years, root mealy bug (*Formicoccus polysperes* Williams) has emerged as a substantial threat, adversely affecting the growth and productivity of black pepper in Kerala. It also poses serious threat to ginger, elephant foot yam and betel vine. Root mealybugs colonize the roots, and desap the roots leading to drying and weakening of plants with ultimate reduction in yield (Ummer and Kurien 2021). Being a soil inhabiting pest, its infestation often goes unnoticed, demanding a rapid action for curative treatment. Over reliance on chemical pesticides in agricultural practices has become a growing concern. This alarming situation necessitates the advancement of microbial control agents in pest management. Among the various microbials used in pest management, fungi have the potential to be the most adaptable biocontrol agent, because of their unique contact mode of action. An indigenous isolate of *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno, ITCC 7714, isolated and characterised by Rani *et al.* (2015) from the soils of Vellayani, Kerala, India stands as the first report on its entomopathogenicity particularly targeting sap sucking pests of vegetables. Its biosafety was earlier evaluated by Jasmy (2016) in crop plants, predators such as *Chilomenes sexmaculata* and *Coccinella septumpunctata*, parasitoids, viz. *Bracon brevicornis, Goniozus nephantidis* and *Trichogramma* spp., pollinators *Xylocopa* spp. and wasp *Vespula* spp. High selectivity of this entomopathogen to hemipteran pests and safety to other non-target organisms in rice ecosystem was proved by Sankar and Rani (2018). The ability of this fungus to kill the host insect within a short period of time was established through its metabolite profiling. Presence of the wine red pigmented oosporein sets it apart from other fungal pathogens (Sreeja *et al.* 2023).

Importantly, the key challenge in harnessing the full potential of *L. saksenae* lies in its formulation and application methods. Most of the traditional microbial formulations

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often encounter limitations related to stability and efficacy. These limitations often hinder the broad scale adoption of highly promising biopesticides. Innovative formulations like biopellets can offer a targeted and controlled delivery system. The present research was, therefore, aimed to assess the pathogenicity of *L. saksenae* to pepper root mealybug, *F. polysperes* and formulate biopellets based on it, for the soil application.

MATERIALS AND METHODS

Bioassay: The study was carried out during 2020–23 in the Biocontrol Laboratory for Crop Pest Management, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The isolate utilized for the study, *L. saksenae* (NCBI accession No. MN545844.2 and ITCC accession No. LsVs 1-7714) was the one maintained in the Biocontrol laboratory, Department of Entomology, College of Agriculture, Vellayani, Kerala. Pure and subcultures of this fungus were maintained in Potato Dextrose Agar (PDA) slants. Bioassay on *F. polysperes* was carried out using conidial suspension prepared by blending 21-day old culture in a blender and filtering through a double layered muslin cloth. The concentration was adjusted to 10^5–10^8 conidia/ml using a Naubauer haemocytometer.

The test insect used for the study was root mealybug *F. polysperes*, collected from a pepper plantation at Paliyodu in Thiruvananthapuram district, Kerala, India, which was reared in the laboratory using mature pumpkin fruits, disinfected with carbendazim 50% WP. These fruits were confined in a rearing cage to protect mealybugs from parasitoids. Uniform sized mealybugs collected from the laboratory culture were observed for few days to ensure they are devoid of any latent infection. Single noded pepper cuttings taken in plastic containers lined with moist tissue paper, were inoculated with ten mealybugs per container. Conidial suspension of the fungus was sprayed on the mealybugs using a hand atomizer (average droplet size is 400 microns). Water spray was given as control. Experiment was carried out in completely randomized design (CRD) and each treatment was replicated four times. The treated mealybugs were observed for symptoms of mycosis and for mortality at 12 h interval until 100% mortality was noted in any one of the treatment for assessing effective dose, lethal concentrations (LC_{50} and LC_{90}) and lethal time (LT_{50} and LT_{90}). Probit analysis was carried out using OPSTAT to work out the $LC_{50,}$ $LC_{90,}$ LT_{50} and $LT_{90,}$ with confidential limit fixed at 95%.

Development of biopellets: In order to formulate *L. saksenae* at its effective dose as biopellets for soil application, the carrier material and binding agent were standardized, in separate experiments.

For the preparation of spore pellet of *L. saksenae,* conidial suspension was centrifuged at 4000 rpm for 20 min. The supernatant was decanted to get spore pellets. The spore pellet was suspended at the rate of 1.0 g spore pellet in 1.0 ml of sterile water to form the primary stock for formulation. The concentration was adjusted to 10^9 conidia/ml.

Standardization of carrier material and binding agent: Carrier materials of natural origin which were tested for pelleting were talc, chitin, chitosan and the combinations of talc + chitin (95:5) and talc + chitosan (90:10). These sterilized carrier materials were mixed with the concentrated spore pellet of fungus at 10^9 conidia/8 ml in 75:25 ratios and 15% water was added. The experiment was carried out in CRD with 5 treatments and 4 replications. Viability of conidia in the carrier material was assessed in terms of germination% at weekly intervals for a period of one month. The selected carrier material was mixed with each of the binding agents, viz. Carboxymethyl cellulose (CMC) 6%, Acacia gum arabic (AG) 5% and Microcrystalline cellulose (MCC) 6%. It was mixed with the concentrated spore pellet in 75:25 ratios, with 15% water. The process of biopelleting was done using a pellet extruder fabricated for this purpose. Control was prepared without using any binding agents. Each treatment was replicated four times. Viability of conidia was recorded at weekly intervals for a period of one month. The experiment was carried out at room temperature (28 ± 2°C).

Assessment of viability: Viability test was carried out by ESALQ method suggested by Oliveira *et al*. (2015). From each treatment, 1.0 g was taken and added to sterile test tubes containing 9.0 ml of sterile water and the surfactant tween 80 (0.05%). The mixture was vortexed for three min. The suspension was serially diluted up to 10^5 conidia/ml. The petri plates containing 5.0 ml of PDA were inoculated with 0.3 ml of fungal suspension drop wise, randomly on 9.0 spots. Thereafter, the plates were closed and sealed with cling film and incubated at 27°C for 24 h. The conidial viability was assessed based on the direct count of germinated and non-germinated conidia, as observed under a compound microscope at 400X magnification. The conidia were considered as viable if they had germ tubes longer than their diameter. Viability was estimated as:

$$\text{Germination \%} = \frac{\text{No. of germinated conidia}}{\text{Total no. of conidia counted}} \times 100$$

Shelf life of the pellets at varying moisture level: The biopellets of *L. saksenae* @10^9 spores/ml using the ideal carrier and binding agent standardised as above was prepared at varying moisture levels (8, 10, 12 and 15%) by adding desired amount of the culture filtrate of the fungus from which the conidia were extracted. Pellets from each lot were powdered and the moisture content was determined using a moisture analyser AXIS model ATS60. Each treatment was replicated five times. The biopellets were stored under ambient conditions in air tight containers for a period of three months and were subjected to shelf-life studies, which was determined based on the conidial viability and mortality of test insects at fixed intervals.

Statistical analysis: The data obtained from the experiments were subjected to analysis of variance (ANOVA) using WASP1 software and the treatment differences were compared.

RESULTS AND DISCUSSION

Symptoms of mycosis: Conidial suspension of *L. saksenae* @10^9 conidia/ml applied topically on *F. polysperes* resulted in sudden dissolution of the mealy coating (Fig. 1A). Infected mealybugs turned lethargic and morbid with total paralysis or death 12 h post treatment, with oozing of body fluids from cadavers (Fig. 1B). Sporulation was noted 72 h after death (Fig. 1C).

Quick kill action of *L. saksenae* on *F. polysperes* that was distinctly evident in the present study is also in consonance with the findings of Sankar and Rani (2018). They reported similar effect of *L. saksenae* in rice bug, (*Leptocorisa acuta* Thunberg). Their investigation revealed complete mortality of bugs treated with conidial suspension @10^7 conidia/ml within a remarkably brief time span of three days. They suggested that rapid lethality could be attributed to the presence of secondary metabolite, dipicolinic acid. Altinok *et al.* (2019) also stated that fungal toxins are capable of inducing mortality of insects even before the conidia invade the insect body. Cuticular dissolution of the mealy coating was earlier established by Sreeja *et al*. (2023) wherein they reported the abundance of cuticle degrading enzymes, chitinase, lipase, protease and chitosanase of which protease (μg/ml) was the dominant one. Paralysis of the treated mealybugs noticed on the same day of treatment may be attributed to the acetyl cholinesterase inhibiting property of this fungus, in combination with action of toxic secondary metabolites characterised by them, with copious presence of the wine-red pigment, oosporein (mg/litre).

Dose-mortality response: Dose-mortality response of *L. saksenae* to *F. polysperes* carried out in this study (Table 1), unveiled that the mortality increased with increase in spore concentration. The concentrations of 10^7conidia/ml and 10^8 conidia/ml resulted in 92.5 and 100% mortality, within four days, demonstrating equal effectiveness of these two concentrations, in contrast to the lower doses 10^6 and 10^5 conidia/ml. Consequently, 10^7conidia/ml was determined to be the effective dose for *L. saksenae,* with a view to save the inoculum quantity. LC_{50} value estimated on the fourth day was 2.2×10^5 conidia/mL with 95% confidential limit of 5.2×10^4–9.3×10^5 conidia/ml and the corresponding LC_{90} value was 2.6×10^7 conidia/ml with confidential limit of 1.4×10^6–6.2×10^7 conidia/ml. The LT_{50} and LT_{90} value for 10^8 conidia/ml was 20.70 h (confidential limit of 15.88–26.99 h) and 60.66 h (confidential limit of 46. 52–79.09 h), while for 10^7 conidia/ml corresponding values were 23.13 h (confidential limit of 17.29–30.92 h) and 83.76 h (confidential limit of 62.64–111.99 h), respectively.

These findings are in consonance with the findings of Sankar and Rani (2018) who worked with the pathogenicity of this fungus to *L. acuta*. Their study disclosed that as the concentration increased from 10^5–10^8 conidia/ml, death rate also increased. At the higher dose of 10^8 conidia/ml, complete mortality was observed on the second day itself. In their investigation, LC_{50} value computed was very low to the tune of 10^4 conidia/ml, indicating that *L. acuta* is the most preferred host for *L. saksenae*. Further, a low lethal time noted in this study was also in agreement with their observations, highlighting the quick killing ability of *L. saksenae*.

Effect of carrier materials on conidial viability: Influence of carrier materials on conidial viability of *L. saksenae* evaluated at weekly intervals for a period of one month (Table 2) revealed that the conidia formulated in a combination of talc + chitin (95:5) displayed a remarkable performance with 78.54% germination, five weeks after storage. The corresponding viability data was 74.58% for the carrier material talc + chitosan (90:10). When talc, chitosan and chitin were used singly the performance was less. They exhibited 69.79, 50.62 and 33.96% germination, respectively.

The mechanism behind increased conidiation observed in this study may be due to the binding property of chitin to the plasma membranes of these fungi, stimulating a signal cascade that leads to an increase in conidiation as suggested by Palma-Guerrero *et al*. (2010) who worked with *M. anisopliae*, *B. bassiana* and *Paecilomyces lilacinus*. While developing oil formulation of *L. lecanii,* Nithya and Rani (2017) observed that the preference of chitin 0.1% in its colloidal form as additive to the carrier groundnut oil, wherein they could maintain a viability of 2.2×10^6 conidia/ml, three months after storage compared to the other carriers talc + chitin and talc + chitosan.

Effect of binding agents on conidial viability: The conidial viability of *L. saksenae* in the formulation

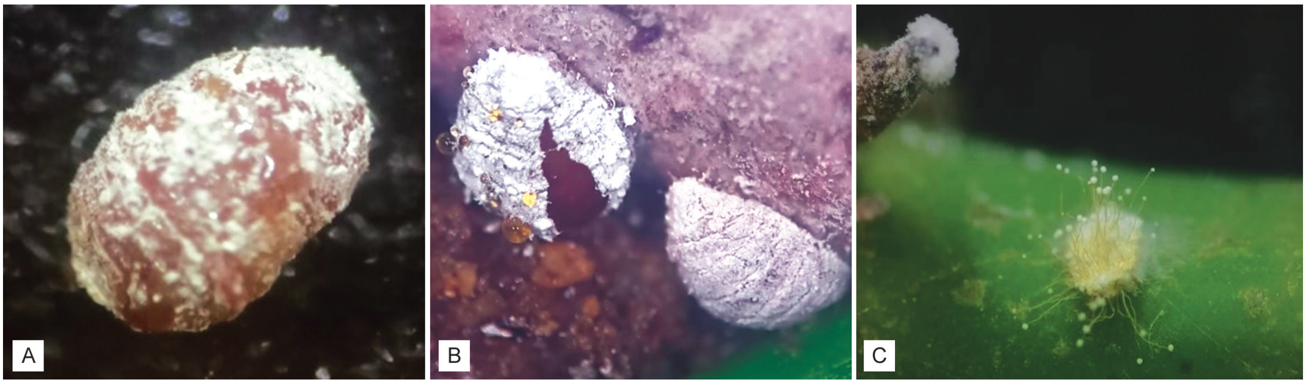

Fig. 1 (A) Dissolution of mealycoating; (B) Oozing of body fluids from cadaver; (C) Sporulation on *Formicoccus polysperes.*

Table 1 Dose-mortality response of *Lecanicillium saksenae* to *Formicoccus polysperes*

Treatment (spores/ml)	Cumulative mortality (%)						
	12 HAT	24 HAT	36 HAT	48 HAT	60 HAT	72 HAT	84 HAT
10^5	0.00 (0.64)^{c}	0.00 (0.64)^{c}	2.50 (5.09)^{c}	5.00 (9.54)^{c}	12.50 (20.47)^{c}	20.00 (26.19)^{c}	25.00 (29.36)^{d}
10^6	12.50 (20.47)^{b}	22.50 (28.23)^{b}	35.00 (36.22)^{b}	47.50 (43.56)^{b}	57.50 (49.33)^{b}	67.50 (55.44)^{b}	75.00 (60.11)^{c}
10^7	27.50 (31.55)^{a}	52.50 (46.44)^{a}	62.50 (52.34)^{a}	75.00 (60.11)^{a}	82.50 (65.47)^{a}	87.50 (71.95)^{a}	92.50 (76.01)^{b}
10^8	30.00 (33.05)^{a}	57.50 (49.39)^{a}	65.00 (53.99)^{a}	82.50 (65.47)^{a}	90.00 (73.98)^{a}	95.00 (82.88)^{a}	100.00 (89.36)^{a}
Control	0.00 (0)^{c}	0.00 (0.64)^{c}	0.00 (0.64)^{c}	0.00 (0.64)^{d}	0.00 (0.64)^{d}	0.00 (0.64)^{d}	0.00 (0.64)^{e}
CD (P=0.05)	(4.968)	(4.817)	(9.190)	(8.716)	(8.555)	(13.316)	(8.881)
Lethal time	*Treatment (spores/ ml)*	*LT_{50} (h)*	*95% Confidential limit (h)*		*LT_{90} (h)*	*95% Confidential limit (h)*	
			Upper	*Lower*		*Upper*	*Lower*
	10^7	23.13	30.92	17.29	83.76	111.99	62.64
	10^8	20.70	26.99	15.88	60.66	79.09	46.52
Lethal concentration		*LC_{50} (spores/ml)*	*95% Confidential limit (spores/ml)*		*LC_{90} (spores/ml)*	*95% Confidential limit (spores/ml)*	
			Upper	*Lower*		*Upper*	*Lower*
		2.2×10^5	9.3×10^5	5.2×10^4	2.6×10^7	6.2×10^7	1.4×10^6

Mean of four replications; HAT, Hours after treatment; Figures in parentheses are values after arc sin transformation; Values sharing same alphabets in superscript are statistically on par based on ANOVA.

containing talc + chitin (95:5) as carrier material along with various binding agents recorded at weekly intervals (Table 2) unveiled that 6% CMC was the best, compared to 6% MCC and 6% AG. CMC exhibited highest viability of 80.63%, after five weeks compared to 6% MCC (51.5%) and 5% AG (30.67%). On the other hand, pellets formulated without any binding agents exhibited a lower germination percentage (68.83).

Dileep (2022) while testing various binding agents to formulate tablet formulations of *M. anisopliae* for the management of *Culex* wrigglers revealed that 7% CMC was the superior binding agent at it exhibited highest germination rate of 62.66% compared to 7% MCC and 5% AG. Similar findings were obvious in the studies conducted by Veerwal *et al.* (2022) who reported substantial impact of 5% CMC in formulating *B. bassiana* for the management

Table 2 Effect of carrier materials and binding agents on conidial viability of *Lecanicillium saksenae*

Carrier material	Conidial germination at weekly interval (%)				
	1	2	3	4	5
Talc	99.38 (85.92)^{b}	92.92 (74.59)^{b}	87.50 (69.32)^{b}	78.12 (62.12)^{c}	69.79 (56.67)^{c}
Chitin	94.79 (77.06)^{d}	84.17 (66.56)^{c}	64.79 (53.61)^{d}	45.21 (42.25)^{e}	33.96 (35.64)^{e}
Chitosan	98.33 (82.71)^{c}	87.50 (69.51)^{c}	80.21 (63.62)^{c}	68.33 (55.76)^{d}	50.62 (45.36)^{d}
Talc + chitin (95:5)	100.00 (89.36)^{a}	97.50 (80.97)^{a}	91.46 (73.03)^{a}	83.33 (65.91)^{a}	78.54 (62.43)^{a}
Talc + chitosan (90:10)	99.79 (88.21)^{ab}	97.29 (80.65)^{a}	89.17 (70.79)^{b}	80.83 (64.04)^{b}	74.58 (59.72)^{b}
CD (P=0.05)	(3.210)	(3.061)	(1.935)	(1.520)	(1.690)
Binding agents (L. saksenae + talc + chitin (95:5))	*Conidial germination at weekly intervals (%)*				
	1	*2*	*3*	*4*	*5*
CMC 6%	100.00 (89.36)	97.83 (81.57)^{a}	90.83 (72.40)^{a}	85.17 (65.79)^{a}	80.63 (59.77)^{a}
AG 5%	99.66 (87.51)	84.00 (66.43)^{c}	65.00 (53.74)^{d}	44.00 (41.55)^{d}	30.67 (33.62)^{d}
MCC 6%	100.00 (89.36)	93.67 (75.92)^{b}	81.33 (64.42)^{c}	69.17 (56.29)^{c}	51.50 (45.86)^{c}
Control (Without binding agent)	99.83 (88.44)	93.33 (75.12)^{b}	86.33 (68.34)^{b}	77.50 (61.69)^{b}	68.83 (56.07)^{b}
CD (P=0.05)	NS	(3.275)	(1.907)	(1.921)	(1.430)

Mean of four replications; Figures in parentheses are values after arc sin transformation.

Table 3 Shelf-life of *Lecanicillium saksenae* pellets based on viability and virulence

Moisture content	1 MAS		2 MAS		3 MAS	
	Viability	Virulence	Viability	Virulence	Viability	Virulence
8%	80.33 (63.68)^{c}	72.50 (58.45)^{b}	47.02 (43.29)^{d}	60.00 (50.83)^{b}	19.34 (26.08)^{d}	30.00 (33.05)^{bc}
10%	83.86 (66.34)^{b}	77.50 (61.77)^{b}	55.72 (48.29)^{c}	65.00 (53.78)^{b}	28.53 (32.29)^{c}	37.50 (37.73)^{bc}
12%	84.99 (67.21)^{b}	85.00 (67.50)^{a}	61.21 (51.48)^{b}	72.50 (58.45)^{ab}	32.32 (34.64)^{b}	45.00 (42.12)^{b}
15%	87.25 (69.10)^{a}	90.00 (71.56)^{a}	67.44 (55.21)^{a}	80.00 (63.81)^{a}	38.74 (38.49)^{a}	60.00 (50.77)^{a}
CD (*P*=0.05)	(1.748)	(7.480)	(1.432)	(9.250)	(1.254)	(7.567)

Mean of five replications; MAS, Months after storage. Figures in parentheses are values after arc sin transformation.

of *Anopheles stephensi* (L.) in outdoor trials. Moreover, Liang *et al.* (2023) reported that 2% CMC incorporated in the conidial suspension of *Metarhizium rileyi* (Farlow) Samson exhibited better adherence on the surface of corn leaves, with very less inhibition in spore germination and mycelial growth (1.51 and 3.13%). Likewise, the inferiority of MCC as a binding agent in pelleting *L. saksenae* noted in this investigation is in corroboration with the findings of Chamsai and Sriamornsak (2013) who observed that MCC pellets exhibited a notable resistance to disintegration and dissolution.

Shelf life of the biopellets: Shelf-life studies (Table 3) revealed *L. saksenae* biopellets (Fig. 2) formulated with 15% moisture was suitable in maintaining the conidial viability up to 87.25% and virulence up to 90%, after one month of storage. Even at the end of three months of storage, there was 55% germination of conidia and 60% mortality of test insect. Spore pellets with 8, 10 and 12% moisture recorded lesser germination rates of 19.34, 28.53 and 32.32%, respectively and a death rate of 30, 37.50 and 45%, respectively. Therefore, pellets containing 15% moisture content was found to be ideal for storing the biopellets under ambient conditions.

Lopes and Faria (2019) have emphasized that stability of a formulation is strongly influenced by its moisture content. The finding of Dileep (2022) is in consonance with this study that states, *M. anisopliae* tablets formulated at 15% moisture content retained highest viability of 33.66% and highest virulence of 72% to *Culex* larvae, three MAS. According to Remya and Rani (2020), a moisture content of 10% was found to be suitable for chitosan-based capsules to preserve viability and stability of *M. anisopliae* and *B. bassiana*, which was lesser than that observed in this study. This variation may be attributed to the fact that in a capsule formulation, there is no loss of moisture as the conidia is enveloped by the capsule envelop, unlike in a pellet formulation which is bare where the chances of moisture loss can happen. This principle is further substantiated in the formulation studies carried out by Sarma *et al.* (2023), where a very low moisture level of 6% was found to be the ideal in formulating alginate-based microcapsules of *M. anisopliae*.

Fig. 2 *Lecanicillium saksenae* biopellets.

The present investigation concludes that *Lecanicillium saksenae* is a promising bioagent for the management of root mealybugs with high speed of action unlike the other microbes. The bioplellets formulated using a spore concentration of 10^9 conidia/ml using talc + chitin (95:5) as the carrier and carboxy methyl cellulose 6% as the binder with 15% moisture can be stored under ambient conditions for a period of three months. The formulation developed by us is 100% biodegradable as the constituents are purely of natural origin. Therefore, it is suggested that these biopellets would be a safe and ecofriendly alternative for the management of pepper mealybug which is presently managed using highly persistent chemical insecticides that contaminate the soil and water. Its efficacy under field conditions and adoptability in other crops for the management of soil inhabiting pests need to be explored.

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