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RESEARCH

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Molecular characterization of an *Isaria fumosorosea* (Wize) native strain, and its pathogenicity on *Eublemma amabilis* (Lepidoptera: Noctuidae)

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Abstract

Background: *Eublemma amabilis* Moore (Lepidoptera: Noctuidae) is an important pest of lac insect, *Kerria* spp. (Hemiptera: Tachardiidae: Coccoidea) causing up to 20–25% damage of lac and its encrustation, which has immense industrial utilities. Extensive field monitoring in Regional Lac Insect Field Gene Bank (RLIFGB) resulted in collection of a large number of naturally occurring entomopathogenic fungi (EPF) infected insect cadavers on the lac encrustation of host plant, *Flemingia semialata*. Pathogenicity test under laboratory conditions showed its efficacy on *E. amabilis* larvae. Molecular characterization of this EPF by 18S rDNA identified it as *Isaria fumosorosea* (Wize) (accession number, MH414514.1).

Results: Being a potent EPF, biological parameters viz., conidial density and sporulation were determined and its pathogenicity were evaluated against eggs, larvae and pupae of *E. amabilis* at different spore dilution under laboratory conditions. The highest mortality rates of larvae and pupae (96 & 72%) and (88 & 72%) were recorded at 10^7spore/ml both in dipping and spraying methods, respectively. The egg mortality, however, was recorded as 40 and 35% for both methods, respectively, at 10^7spore/ml, which was significantly lower than other stages of *E. amabilis*. Field study at (10^7 spore/ml) recorded 81.18, 59.41 and 76.36% mean population reductions over control during monsoon 2019, winter 2019–2020 and summer 2020, respectively. Biosafety analysis of the native EPF on productivity linked parameters of lac insect was found to be safe suggesting that the native *Isaria fumosorosea* (MH414514.1) strain, reported first from Assam.

Conclusions: In this study, it was confirmed that the EPF *I. fumosorosea* can be offered as an effective microbial agent, alternate to insecticide against *E. amabilis*, either as a stand-alone strategy or in an integrated approach.

Keywords: Lac insect, *Eublemma amabilis*, Pathogenicity, *Isaria fumosorosea*, Mortality

Background

Lac is a naturally occurring economically important animal resin secreted by *Kerria* spp. (Hemiptera: Tachardiidae: Coccoidea) as a part of its defense strategy (Mohanta et al. 2012), which otherwise provides resin (68–90%), dye (2–10%) and wax (6%) that has been extensively utilized in commerce and industry from time immemorial. About 50–60% of the total world lac production are

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contributed from different states of India viz., Jharkhand, Chhattisgarh, Madhya Pradesh, Maharashtra West Bengal and Assam, where Jharkhand alone contributes 56.92% of lac production (Yogi et al. 2021) contributing on an average of 20,000 tons per annum (Varshney et al. 2020). However, the quantity and quality of lac production is significantly reduced due to variety of abiotic factors like temperature, rainfall, humidity, wind (Bhagat and Mishra 2002) and biotic stresses like: vertebrate, invertebrate predators and microbial diseases (Sharma et al. 2001).

About 20 insect pest species are considered as serious pests of lac cultivation causing 35–45% loss (Shah et al. 2015), out of which, *Eublemma amabilis* Moore (Lepidoptera; Noctuidae), is one of the coccidophagous pests, which feeds specifically on living lac insects and resins. The adult female lays eggs on the surface of lac encrustation; the freshly hatched ones directly enter the lac, either by tunneling through the lac encrustation or through anal or brachial pore of the living lac cell and feed within the encrustation (Chattopadhyay 2001). To minimize yield loss due to pests, various management strategies are attempted, where the significance of agrochemicals like: endosulfan and cartap hydrochloride (Bhattacharya et al. 2005), dichlorvos and ethofenprox (Jaiswal et al. 2004), fipronil (Singh and Jaiswal 2015) in improving lac productivity cannot be denied. But heavy reliance and frequent indiscriminate use of chemical pesticides have a significant negative impact on the environment as well as on other beneficial insects. As an alternative to chemical control, keen interest on use of EPF has been increased worldwide due to its eco-friendly nature, effectiveness, specificity and harmlessness to non-target organisms. In nature, around 1000 species of EPF under phyla Entomopthoromycota, Blastocladiomycota, Mycosporidia, Basidiomycota and Ascomycota regulate insect populations (Das et al. 2019). The most intensively investigated fungi for preparing 687 mycoinsecticide are *Verticillium lecanii* (Zimm), *Isaria fumosorosea* (Wize), *Beauveria bassiana* (Vuill), *Metarhizium anisopliae* (Metschinkoff), *Hirsutella thompsonii* (Fisher) and *Nomuraea rileyi* (Farlow) (Das et al. 2019) for controlling different pest species of Coleoptera, Lepidoptera, Hemiptera, Thysanoptera, Isoptera, and Orthoptera and Acarina (Hussein et al. 2016). Till date, 306 formulations have been registered under section 9(3) in CAB and RC record 2020. In lac cultivation, a strong biological control program using mycoinsecticides was advocated and for that purpose a regular monitoring in the Regional lac insect field gene bank (RLIFGB), Department of Entomology, Assam Agricultural University, Jorhat-13 revealed occurrence of several mycosed larvae, and examination of those cadavers resulted in identification of *I. fumosorosea* (Cordycipitaceae: Hypocreales) for the first time and showed great potential. With an aim to explore the potential of *I. fumosorosea* as a biocontrol agent to manage lac pests, this study presents data on molecular characterization and bio-efficacy of the native EPF strain against different stages of *E. amabilis* as well as bio-safety test on the beneficial Lac insect (*Kerria lacca*).

Methods

Collection of Entomopathogenic fungi

EPF-infected-cadavers of *E. amabilis* larvae were observed naturally occurring in the Regional Lac Insect Field Gene Bank (RLIFGB), Department of Entomology, Assam Agricultural University, Jorhat-13 (Latitude:25°45 N, Longitude: 93°30 E), and were collected for identification in the laboratory.

Isolation and pure culture

Potato Dextrose Agar (PDA, Himedia) was used to culture the fungal isolate. The sporulated cadavers were cut into small pieces around 0.5 to 1 mm size and surface sterilized with 1 percent sodium hypo chlorite solution ($NaOCl_2$) for 30 s. The sterilized pieces were inoculated on fresh PDA plates/ tubes with streptomycin sulfate and incubated at BOD incubator at a temperature of 26 ± 1 °C for 15 days for complete sporulation. After 15 days of incubation, Koch postulate was carried out by standard procedure (Ross and Woodland 2016). Subsequently, a pure culture of the fungal pathogen was obtained and sub-cultured, stored at − 4 °C for further study.

Morphological characterization of fungal isolate

The fungal isolate was first identified to genus level based on comparison of morphological characteristics of the conidial structures with descriptions available in the literature (Samson 1974). Conventional slide culture was used to study the morphology of the fungus for which the sterile PDA were cut into small square blocks of approximately 1 cm and the blocks were placed on a sterile glass slide inside Petri plates (15 mm) under laid with wetted Whatman1filter paper. Each agar block was then aseptically inoculated with a fungal colony on the four corners using sterile inoculating needles and a cover slip was placed over it. After 3 days of incubation at 26 ± 1 °C, the cover slip was gently taken off and placed on a glass slide containing a drop of lactophenol cotton blue (LPCB) and examined under the Axiostar plus with a 40 × objective lens (Woo et al. 2010). Conidial color, shape, and presence or absence of septation was recorded. In vivo germination of fungal conidia on the cuticle of *E. amabilis* was examined under Scanning Electron Microscope (SEM) and shown in Fig. 1.

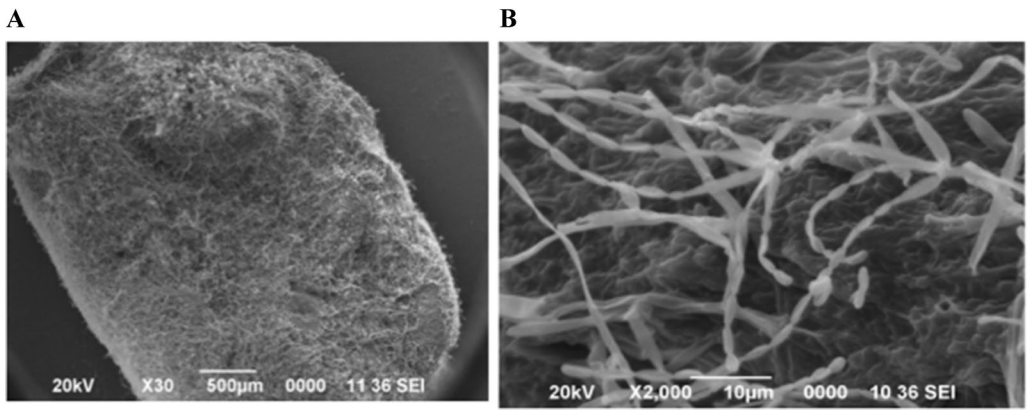


Fig. 1 Scanning electron microscope (SEM) images of *Isaria fumosorosea* infected cadaver of *Eublemma amabilisa*. **a** Dead larvae, **b** chain of conidia

Molecular characterization and phylogenetic analysis

Molecular identification and characterization of the fungal isolate were carried out by 18S rDNA sequencing. The fungal DNA was isolated using a GeneElute genomic DNA extraction kit (Sigma-Aldrich, Mumbai, India) as per the manufacturer instructions. Amplification of 18SrDNA was carried out in a thermal cycler (PerkinElmer, Norwalk, Connecticut, USA) using the universal ITS primers: ITS1 (5′-TCCGTAGGTGAA CCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) (Das et al. 2021; Alfiky 2022). All the PCR reagents were purchased from Promega (San Luis Obispo, California, USA). The PCR program was as follows: initial denaturing step of 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and elongation at 72 °C for 1.5 min; a final extension step at 72 °C for 7 min. The amplified products were sequenced according to the BigDye Terminator sequencing protocol in an ABI 377 automated DNA sequencer (Applied Biosystems, Carlsbad, California, USA). The 18S rDNA sequence reads obtained after sequencing were assembled into contig using Codon Code Aligner (Codon Code Corporation, Centerville, Massachusetts, USA). The sequence similarity tool BLAST was employed to find the similarity of the sequences and the sequences were deposited in GenBank of the National Center for Biotechnology Information (NCBI) to get the accession number. Phylogenetic analysis of the isolates was performed based on the 18S rDNA sequences. A phylogenetic tree was constructed using 18S rDNA sequences of the isolates along with the sequences of the most similar strains retrieved from the NCBI GenBank (Rehner and Buckley 2005). The sequences were aligned with ClustalW, using default parameters, and the phylogenetic tree was constructed using the neighbor-joining method inMEGA6 software (http://www.megasoftware.net/) (Tamura et al. 2013).

Biological parameters of fungal isolate

Radial growth

Inoculums of fungal isolate were cultivated on PDA plates for 7 days in a BOD incubator at 26 ± 1 °C. With the help of a 0.65 cm diameter cork borer, a piece was cut out from the actively growing region of a 7-day-old culture, and the same was placed aseptically in the center of a sterile Petri plate containing PDA medium followed by incubation in BOD incubator at 26 ± 1 °C. Five plates were taken for measuring the radial growth. After 3 days of inoculation (DOI) five orthogonal diameters was measured at an interval of 24 h. up to 15 days (Das et al. 2011).

Conidial density and spore germination

The fungal spores were harvested from the slant culture, and the fungal conidia were suspended in 10 ml distilled water with Tween-80 (0.023%) in 10 ml water. Spore density was calculated using the prior method (Das et al. 2011) under light microscope at a magnification of 400X with Neubauer hemocytometer. The germination test of viable conidia was performed by following previously documented method (Francisco et al. 2006; Das et al. 2011). Only conidia with a germ tube were considered to be germinated. Germination percentage was recorded by direct examination at 400X with a phase contrast microscope.

Collection and rearing of *E. amabilis* larvae and pupae

The larvae of *E. amabilis* were collected by hand picking from the lac encrustation of host plants and reared in plastic container (7.5 cm × 7.5 cm) containing brood lac as food material under laboratory condition at 28 ± 1 °C

and 85–90% relative humidity (RH). After the emergence of adults, the population were collected and then sorted on the basis of their morphology. Pairs of gravid female and male were transferred to a fresh set of plastic container (7.5 cm × 7.5 cm) for mating, supplied with phunki lac (5 cm) for egg laying. The cotton swabs dipped in 10 percent honey solution was provided as food substrate for the adults. The cultures of the insects to be used for the efficacy assays were maintained thereafter.

In vitro evaluation

Pathogenicity test of fungal isolate against E. amabilis larvae and pupae

The pathogenicity of fungal isolate at different spore dilutions viz., 10^3, 10^5, 10^7, 10^8 and 10^9 spores/ml was prepared and tested against 3rd instar larvae/pupae of *E. amabilis* under laboratory conditions of 28 ± 1 °C and 85–90% RH. The experiment was conducted by following dipping and spraying method. In case of dipping method, the larvae of *E. amabilis* were dipped in 2 ml of each spore suspensions mixed with 0.023% Tween-80 for 2 min. Subsequently, the dipped larvae were taken out with the help of brush and air dried, prior to release in container (size: 7.5 × 7.5 cm). However, in spraying, 9 ml of different spore suspensions, hand atomizer (Borosil) was used. Control was treated with water mixed with Tween-80. Herein, the lids of the bottle were perforated for proper aeration, and stuffed with coarsely crushed brood lac (5gm/replication) as a feed to the larvae to avoid starvation. Mortality data of both the sets were recorded till 15th days of treatment. Each treatment was replicated seven times having five insect in each replication.

Pathogenicity test of fungal isolate against *E. amabilis* eggs

To evaluate the ovicidal activity of *E. amabilis* eggs, the adults were obtained from laboratory reared culture (28 ± 1° C and 80–90% RH) and a pair of male and female was placed in a plastic container (25 cm × 10 cm) for egg laying. The plastic container was supplied with a 5 cm lac encrustation having live insect for egg laying and 10% honey solution, soaked in cotton as feed. After egg laying, the adults were removed and known numbers (n = 20) of eggs were taken for evaluation at different spore dilutions viz., 10^3, 10^5, 10^7, 10^8 and 10^9 spore/ml. Prior to that, the supplied encrustation was observed under the microscope, to ensure that it did not contain previously laid eggs of *E. amabilis*. The evaluation was done by both dipping and spraying methods. In dipping method, known numbers of two-day old eggs laid in the encrustation were dipped for two min in 2 ml of each conidial suspension. The eggs were then immediately kept on glass Petri dish (9–10 cm) for air drying. In spraying method, the two-day old eggs laid in lac encrustation were sprayed with 9 ml of different spore suspension using hand atomizer. Control set were treated with water mixed with Tween-80 @ 0.001%. Eggs hatchability percent of both the sets were recorded till 15th days of treatment by observing them under Carl Zeiss stereo zoom microscope at 40 × magnification microscope.

In vivo evaluation of larval mortality of *E. amabilis*

Based on laboratory assay on different stages of *E. amabilis*, the best spore dilution of fungal strain was tested under field condition in lac insect during monsoon 2019, winter 2019–2020 and summer 2020 in RLIFGB, Department of Entomology, Assam Agricultural University. For comparison, recommended concentration of Fipronil 5% SC (1.5 ml/l) was sprayed at 30th, 60th and 90th days after inoculation (DAI) and untreated control was maintained. In all the treatments Tween 80 was added @ 0.001%. An established plantation of the lac host plant *Flemingia semialata*, planted at a spacing of 1 m was utilized for the experiment. The evaluation was done following two methods viz., (1) Dipping method and (2) Spraying method. The brood lac was first dipped in EPF solution for 2–5 min and inoculated in the host plants and emergence of *E. amabilis* larvae were recorded subsequently; the 1st spray was done at initial crawler settlement stage, 2nd at sexual differentiation stage, 3rd at after male emergence and 4th at female wax secreting stage with a hand sprayer. The population count of *E. amabilis* was recorded from three randomly selected *Flemingia semialata* plants for each treatment at 15 days interval. Three branches were chosen from each plant, and divided into three parts namely lower, middle and upper part of each branches. The number of hollow encrustations made by *E. amabilis* were then observed and recorded visually, in 30 cm in each part of the branches, in each of the selected plants from the date of inoculation till harvesting.

Bio-safety test of the fungal strain on lac insect

The bio-safety test of best fungal spore dilution at 10^7spore /ml was also tested on lac insects by following two methods viz., (1) Dipping method, (2) Spraying method. The brood lac were dipped in EPF spore suspension for 2–5 min and inoculated in the host plants. Subsequently, the inoculated brood lac in the host plants were sprayed with fungal EPF spore suspension, 1st spray was done at initial crawler settlement stage, 2nd at sexual differentiation stage, 3rd at after male emergence and 4th female wax secreting stage with a hand sprayer. After dipping and spraying, the emergence of crawlers from the treated brood lac, productivity linked parameters and yield were recorded by following the method described by Mohanasundaram et al. (2016).

Statistical analysis

Radial growth rate, conidial density and spore germination of *Isaria fumosorosea* strains with different dilution and each treatment hading seven replications were compared by one-way analysis of variance (ANOVA) with completely randomized design where ANOVA indicated significance ($P < 0.05$). Bio-efficacy of *I. fumosorosea* on *E. amabilis* and productivity linkage parameters of lac insect were subjected toone-way analysis of variance (ANOVA) with randomized block design.

Results

Morphological and molecular characterization of fungal isolate

The fungal isolate obtained from *E. amabilis* was identified through morphological and molecular basis. The result shows that the fungal colony grown in PDA media was white to light gray in color and appeared floccose and powdery (Fig. 2a). The conidiophores were straight with conidia hyaline, 1-celled and ovoid (Fig. 2b). The average diameter of conidia was (4.62–4.82 µm). Molecular characterization of this fungus was carried out using ITS primers which yielded 854 bp fragments. The genomic DNA bands as obtained from the agarose gel electrophoresis having intact single band which indicated that the genomic DNA has good integrity and free of RNA contamination. The isolated DNA was quantified and its purity was estimated with the help of Nano drop. The sequencing results was aligned with Codon code Aligner and analyzed for homology using nBLAST (NCBI), which displayed homology with *I. fumosorosea.* The sequencing data were submitted to Gene Bank, (NCBI), and was assigned accession number MH414514.1. The specific PCR primers will be useful to confirm the identity of cultures or identify cadavers collected in field collections.

A homology blast showed that MH414514.1 sequence was substantially homologous to other *Cordyeceps* isolates and *Isaria* spp. with sequence identity of 95 to 99%. A multi-alignment comparison analysis of the query and the template nucleotide sequences using Clustal W confirmed that the MH414514.1 sequence structure and functional sites were strongly conserved when compared to other related fungal species.

Neighbor-Joining method was used for inferring the evolutionary history. The phylogenetic tree was drawn to scale with same units of branch lengths as the evolutionary distances, which were computed using the Maximum Composite Likelihood method and were presented with the units of the number of base substitutions per phylogenetic analysis. The MH414514.1 sequence had clustered with the *I. fumosorosea* and *Cordyceps javanica* subgroup (Fig. 3). A BLASTn search against non-redundant (nr) protein sequence showed that sequence had 95–99% identity with other *I. fumosorosea* and *C. javanica* species. The multi-alignment comparison analysis and phylogenetic tree showed the presence of conserved regions among the selected sequences and the evolutionary close homologs.

Radial growth, conidial density and spore germination of strain *I. fumosorosea* (MH414514.1)

The results for colony radial growth, conidial density and percent spore germination of *I. fumosorosea* alone are presented in Table 1. The maximum radial growth of *I. fumosorosea* strain ranged from 66–67 mm with an average of 66.5 ± 0.58 mm after 15 days of incubation. Table 1 shows the conidial density of *I. fumosorosea*

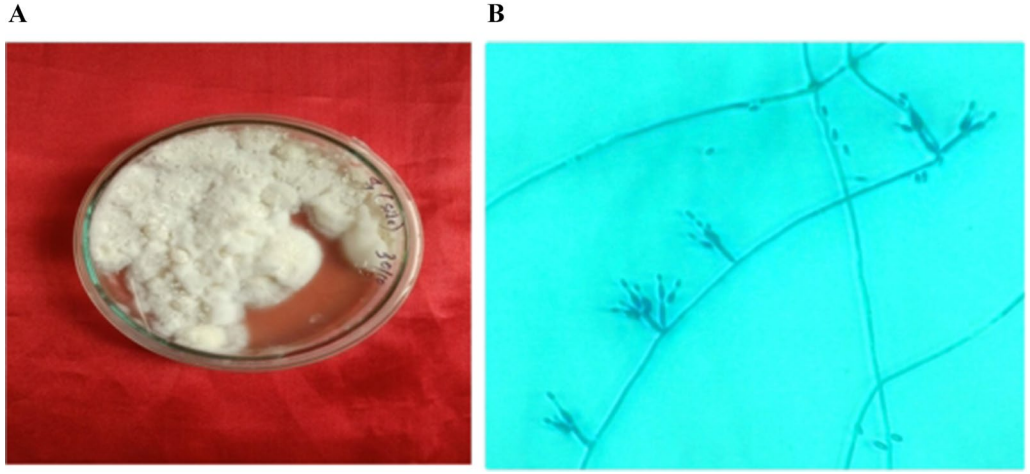


Fig. 2 *Isaria fumosorosea* **a** fungal culture on PDA media, **b** conidia and conidiophores cells (under 40×)

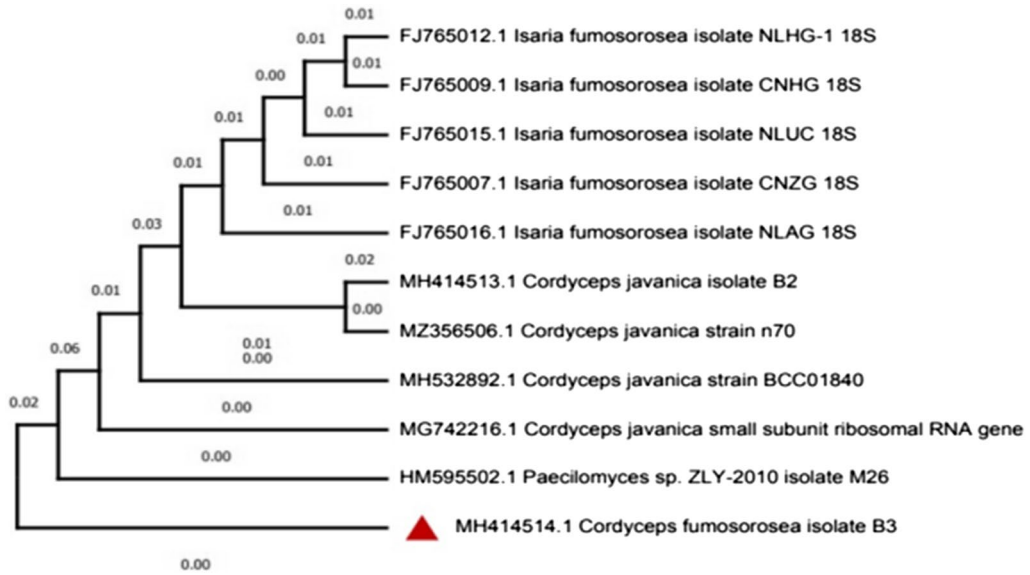


Fig. 3 Phylogenetic tree of MH414514.1 sequence and its closest homologs inferred using Neighbor-Joining method in MEGA v6. 1.0. The numbers of nodes represent the percentage of boot strap value obtained from 1000 sampling. Bar 0.01 shows the substitutions per nucleotide position

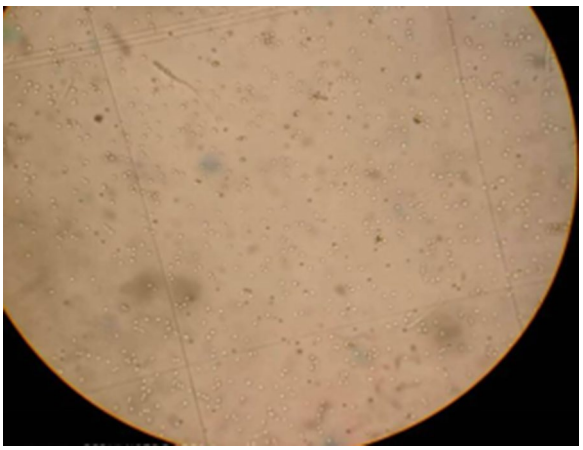


Fig. 4 Conidial density of *Isaria fumosorosea* (MH414514.1) under neubauer hemocytometer (40×)

strains at different dilutions. The highest conidial density (17.81×10^7 spore/ml) was recorded at a spore dilution of 10^7spore /ml; whereas, the lowest conidial density (4.88×10^7spore /ml) was obtained in spore dilution of 10^9spore /ml after 15 days of incubation (Fig. 4). Viable propagules/sporulation was important in any infection process by EPF. The highest spore germination/sporulation (90.49%) was recorded at 10^7spore /ml, followed by (82.53%) at 10^5spore /ml and the lowest (49.60%) was recorded at 10^3 spore /ml (Table 1, Fig. 5).

Table 1 Effect of different dilutions of *Isaria fumosorosea* strain (MH414514) on conidial density and germination at 26 ± 1 °C after 15 days of incubation

Spore dilution	Conidial density	Spore germination %
Stock Solution	29.23 ± 2.33 (21.65)	61.93 (51.90)
1×10^3	12.56 ± 4.77 (9.67)	49.60 (44.77)
1×10^5	14.56 ± 2.88 (10.34)	82.53 (65.29)
1×10^7	17.81 ± 2.51 (9.56)	90.49 (72.04)
1×10^8	7.68 + 6.50 (8.89)	72.78 (58.45)
1×10^9	4.88 ± 3.21 (2.33)	60.07 (50.81)
Control	0.00 ± 0.00 (0.00)	0.00 (0.00)
S.ED (±)	1.49	1.30
CD (P = 0.005)	3.08	2.84

Data are mean of 7 replications. Data within the parentheses are angular transformed value

Pathogenicity of *I. fumosorosea* against different stages of *E. amabilis* in in vitro

Larval mortality

The mortality of 3rd instar larval stage of *E. amabilis* due to infection of *I. fumosorosea* (MH414514.1) was evaluated at spore dilutions viz. 10^3, 10^5, 10^7, 10^8 and 10^9

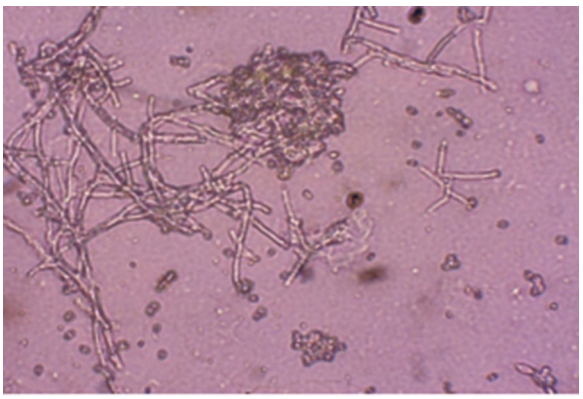
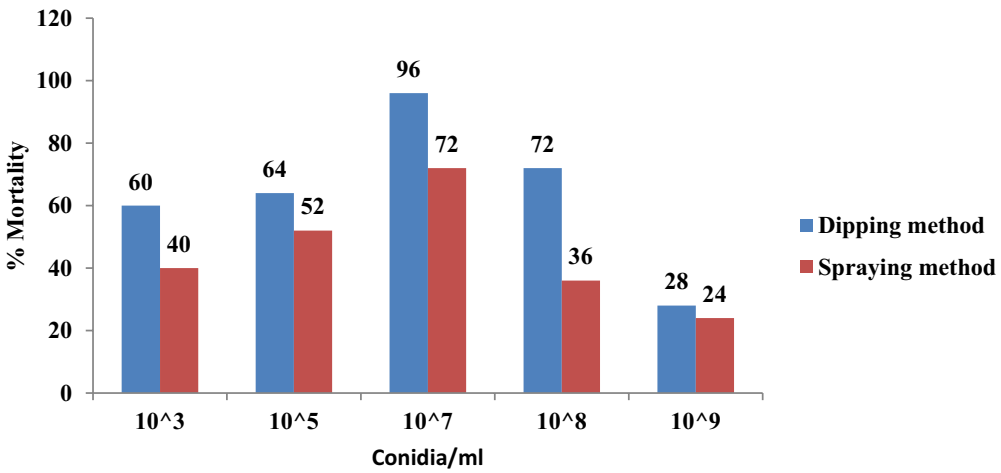


Fig. 5 Germinating conidia of *Isaria fumosorosea* (MH414514.1) under microscope (40×)

spores/ml by dipping and spraying methods. In the initial stage, morphological changes in the body of *E. amabilis* larvae were observed after application of the fungus, i.e., the larval body color changed from creamy white to blackish brown and later became stiff. The mortality of *E. amabilis* larvae was found to be significantly different among the spore dilutions at different days after treatment (DAT). On 15 DAT, the mortality was recorded as 60.00, 64.00, 96.00, 72.00, 28.00% at 10^3, 10^5, 10^7, 10^8 and 10^9 spore/ml, respectively, in dipping method whereas in spraying method, the mortality percent of *E. amabilis* larvae varied from 40.00, 52.00, 72.00, 36.00, 24.00% at 10^3, 10^5, 10^7, 10^8 and 10^9spore/ml, respectively (Fig. 6a, b).

A. Fluffy growth and deformed larvae of *Eublemma amabilis* treated with *Isaria fumosorosea* strain



B: Effect of conidial dilution of *Isaria fumosorosea* strain on *Eublemma amabilis* larvae by dipping and spraying method

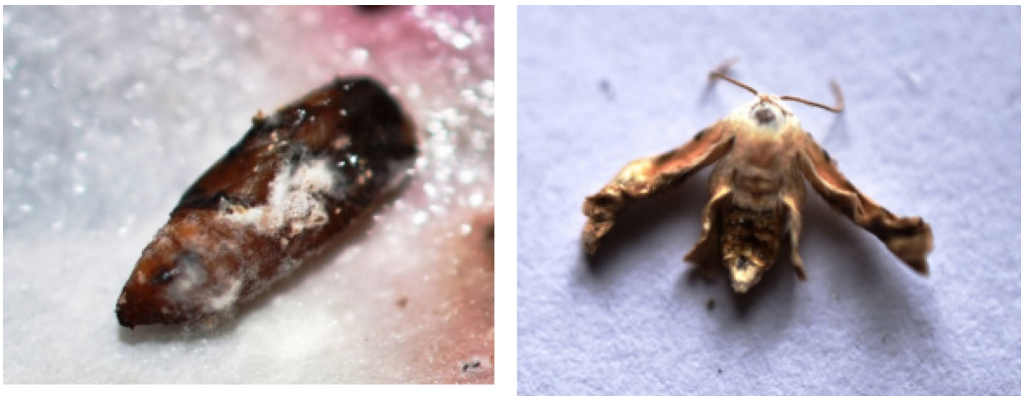
Fig. 6 **a** Fluffy growth and deformed larvae of *Eublemma amabilis* treated with *Isaria fumosorosea* strain. **b** Effect of conidial dilution of *Isaria fumosorosea* strain on *Eublemma amabilis* larvae by dipping and spraying method

Pupal hatchability and deformities

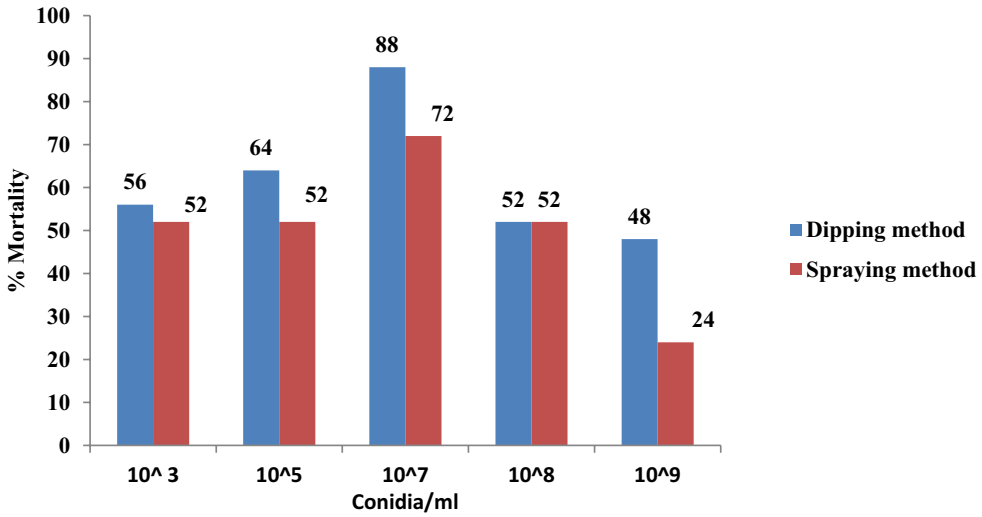
The *I. fumosorosea* (MH414514.1) strain was also potent against the pupae of *E. amabilis*. It was found that in dipping method the infection with fluffy white growth of EPF on *E. amabilis* pupae varied from 56.00, 64.00, 88.00, 52.00 and 48.00% at 10^3, 10^5, 10^7, 10^8 and 10^9spore/ml, respectively, at 15 days of treatment. In this method the highest infection was found at 10^7 and lowest at10^3spore/ml. In spraying method, the fluffy white growth of the fungi on *E. amabilis* pupae varied from 52.00, 52.00, 72.00, 52.00 and 24.00%, at different dilutions, respectively. Adult emergence from the pupae was found to be the highest in the untreated control and a delay of adults' emergence with some deformities in their physical appearance was noticed in treatment with high dilution (Fig. 7a, b). Application of *I. fumosorosea* caused infection with fluffy growth on *E. amabilis* pupae at 10^7 spore/ml and reduced emergence of adults significantly in both spraying and dipping methods.

Ovicidal property

Results of the present bioassay proved that the eggs of *E. amabilis* were also susceptible to the tested fungal strain resulting in 40.00 and 31.00% egg mortality due to infection at $10^7$and 10^8spores/ml, respectively, and the minimum mortality 15.00% was observed at 10^9spores/ ml in dipping method. In case of spraying method, 35.00 and 32.00% egg mortality was observed at $10^7$and 10^5spores/



A : Fluffy growth and deformed adults of *Eublemma amabilis* emerged from treated pupae.



B: Effect of conidial dilution of *Isaria fumosorosea* strain on *Eublemma amabilis* pupae by dipping and spraying method

Fig. 7 **a** Fluffy growth and deformed adults of *Eublemma amabilis* emerged from treated pupae. **b** Effect of conidial dilution of *Isaria fumosorosea* strain on *Eublemma amabilis* pupae by dipping and spraying method

ml, respectively, and the minimum mortality 12.00% was observed at 10^9spores/ ml, while the control did not show any infection and mortality of eggs of *E. amabilis* (Fig. 8).

In vivo condition

Percent reduction in E. amabilis larvae

The results presented in Tables 2, 3, 4 revealed that the application of *I. fumosorosea* best fungal dilution at 10^7spore/ml of water during monsoon 2019, winter 2019–2020 and summer 2020 was found to be effective in reducing mean percent population of predators, i.e., *E. amabilis* 81.18, 59.41 and 76.36% over control with the minimum mean population of 2.67, 6.71 and 4.33 per 90 cm lac stick, respectively. Fipronil 5% SC at recommended concentration (1.5 ml/lit) recorded as the most effective treatment with 92.94, 82.67 and 89.09 mean % reduction in *E. amabilis* over control. The highest mean population of *E. amabilis* of 14.17, 16.54 and 18.33 per 90 cm lac stick were recorded in control during monsoon 2019, winter 2019–2020 and summer 2020, respectively (Tables 2, 3, 4).

Bio safety analysis of lac insects after spraying conidial suspension

Study conducted during August, 2019 to January, 2020 revealed that spraying of *I. fumosorosea* at 10^7spore/ml on lac insects recorded appreciably good results in terms of duration of life cycle as well as productivity linked parameters. The mean values for initial and final density of crawler's settlement, mortality percent, and density of female cell at maturity on the plants are presented in Table 5. The results revealed that the final density of crawler settlement of both the treatment, i.e., EPF and Fipronil were *at par* with each other.

The highest initial mortality (%) of lac insects was recorded on the untreated plant. Within 40–47 days

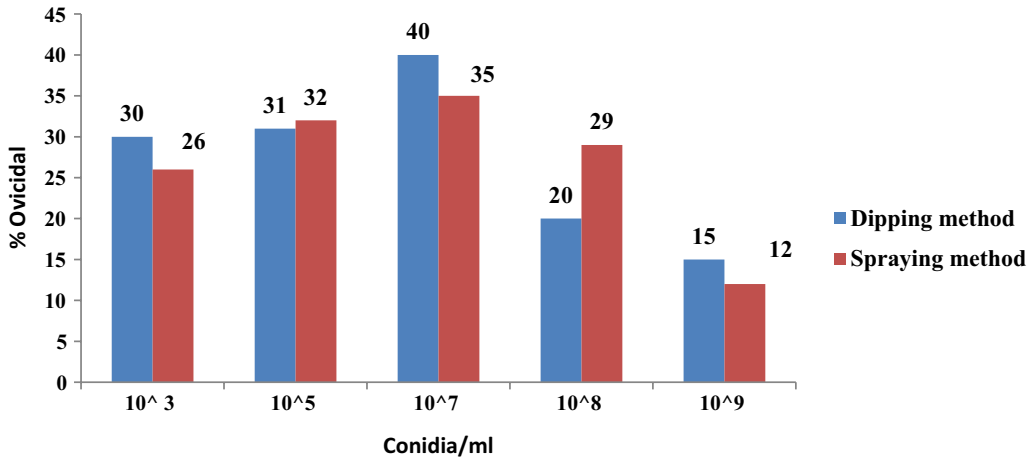


Fig. 8 Effect of conidial dilution of *Isaria fumosorosea* strain on *Eublemma amabilis* eggs by dipping and spraying method

Table 2 Bio-efficacy of *Isaria fumosorosea* (at dilution of 10^7 spore/ml) on *Eublemma amabilis* during June, 2019 to September, 2019 (Monsoon season)

Strains	Mean ± SD (number of infested hollow encrustation/90 cm of stick lac)						
	30 DAI	45 DAI	60 DAI	75 DAI	90 DAI	Mean	% reduction over control
MH414514.1	0.67 ± 0.67 (4.68)	0.67 ± 0.58 (4.68)	0.33 ± 0.52 (3.30)	0.50 ± 0.84 (4.05)	0.50 ± 0.84 (4.05)	2.67	81.18
Fipronil	0.50 ± 0.55 (4.05)	0.33 ± 0.58 (3.30)	0.00 ± 0.00 (0.00)	0.17 ± 0.41 (2.33)	0.00 ± 0.00 (0.00)	1.00	92.94
Control	2.33 ± 1.37 (8.78)	4.17 ± 0.82 (11.77)	2.33 ± 1.21 (8.78)	2.00 ± 0.89 (8.13)	3.33 ± 1.63 (10.51)	14.17	
S.Ed (±)	0.555	0.489	0.433	0.521	0.543		
CD (P = 0.05)	1.143	1.008	0.893	1.07	1.11		

Data are mean of 7 replications. Data within the parentheses are angular transformed value

DAI days after inoculation of broodlac

Table 3 Bio-efficacy of *Isaria fumosorosea* (at dilution of 10^7 spore/ml) on *Eublemma amabilis* during September, 2019 to January, 2020 (Winter season)

Strains	Mean ± SD (number of infested hollow encrustation/90 cm of stick lac)									
	30 DAI	45 DAI	60 DAI	75 DAI	90 DAI	105 DAI	120 DAI	135 DAI	Mean	% reduction over control
MH414514.1	1.00 ± 0.63 (5.74)	0.67 ± 0.52 (5.74)	0.67 ± 0.52 (5.24)	0.83 ± 1.17 (1.17)	1.17 ± 0.98 (2.33)	1.00 ± 0.89 (5.74)	1.17 ± 0.75 (2.33)	0.83 ± 0.98 (0.98)	6.71	59.41
Fipronil	0.33 ± 0.52 (3.31)	0.33 ± 0.52 (3.31)	0.17 ± 0.41 (2.74)	0.33 ± 0.52 (3.31)	0.33 ± 0.52 (3.31)	0.33 ± 0.52 (3.31)	0.50 ± 0.84 (4.05)	0.17 ± 0.41 (3.31)	2.87	82.67
Control	2.67 ± 1.75 (9.39)	2.00 ± 0.63 (9.69)	2.17 ± 0.98 (8.78)	2.50 ± 0.55 (9.09)	2.67 ± 1.03 (9.39)	4.17 ± 1.17 (11.77)	2.50 ± 1.05 (9.09)	1.67 ± 1.39 (7.41)	16.54	
S.Ed (±)	0.683	0.500	0.558	0.534	0.674	0.516	0.567	0.657		
CD (P = 0.05)	1.407	1.030	1.149	1.099	1.388	1.064	1.165	1.354		

Data are mean of 7 replications. Data within the parentheses are angular transformed value

DAI days after inoculation of broodlac

Table 4 Bio-efficacy of *Isaria fumosorosea* (at dilution of 10^7 conidia/ml) on *Eublemma amabilis* during Jan, 2020 to June, 2020 (Summer season)

Strains	Mean ± SD (number of infested hollow encrustation/90 cm of stick lac)										% reduction over control
	30 DAI	45 DAI	60 DAI	75 DAI	90 DAI	105 DAI	120 DAI	135 DAI	150 DAI	Mean	
MH414514.1	0.67 ± 0.82 (4.68)	0.67 ± 0.52 (4.68)	0.67 ± 0.52 (4.68)	0.50 ± 1.22 (4.05)	0.33 ± 0.52 (3.31)	0.67 ± 0.82 (4.68)	0.33 ± 0.82 (3.31)	0.50 ± 0.84 (4.05)	0.00 ± 0.00 (0.00)	4.33	76.36
Fipronil	0.67 ± 0.82 (4.68)	0.33 ± 0.52 (3.30)	0.17 ± 0.41 (2.33)	0.17 ± 0.41 (2.33)	0.00 ± 0.00 (0.00)	0.17 ± 0.41 (2.34)	0.17 ± 0.41 (2.34)	0.33 ± 0.82 (3.31)	0.00 ± 0.00 (0.00)	2.00	89.09
Control	2.83 ± 0.75 (9.69)	2.00 ± 0.63 (8.13)	2.17 ± 0.98 (8.46)	2.17 ± 0.75 (8.44)	1.50 ± 0.55 (7.03)	2.00 ± 1.41 (8.13)	2.00 ± 1.10 (8.13)	2.17 ± 1.83 (8.46)	1.50 ± 1.05 (7.03)	18.33	
S.Ed (±)	0.532	0.500	0.558	0.522	0.371	0.516	0.530	0.551	0.453		
CD (P = 0.05)	1.096	1.030	1.149	1.075	0.764	1.064	1.092	1.135	0.936		

Data are mean of 7 replications. Data within the parentheses are angular transformed value

DAI days after inoculation of broodlac

of settlement of lac crawlers, the sexual differentiation stages become more pronounced. Pre-sexual maturity period, duration of male emergence and complete life cycle duration in all the three treatments were *at par* with each other.

In order to record the emergence of crawlers from the gravid female cell, the cells were treated with *I. fumosorosea* condial dilution under laboratory conditions at 29 ± 1 °C and RH 85–90%. The study recorded that these EPF was extremely safe for lac insects and their emergence were recorded up to 19 days of treatment. From a single cell around 250–350 crawlers were recorded, which was *at par* with the untreated control.

Discussion

In today's environmentally conscious world, bio-control methods of pest management are gaining importance due to its eco-friendly, target specific and self-multiplicative capacity. Once applied, if environmental condition is congenial, they can grow profusely on the living body of insects and penetrate by enzymatic degradation, killing the organism both by mechanical blockage of the organs and toxins. For formulating an effective management tool, proper identification of any bio-control agent and its efficacy against the target pest is of utmost importance.

Based on colony morphology on nutrient agar and molecular characterization, the fungal propagules were

Table 5 Effect of *Isaria fumosorosea* strain on productivity linkage parameters of lac insect in field condition

Treatments	Initial density of settlement (crawlers per cm sq.)			Final density of settlement (crawlers per cm sq.)			Initial mortality %			Density of female cell no. at maturity			Yield (kg)/ plant	Fecundity/cell
Isaria fumosorosea	L	M	U	L	M	U	L	M	U	L	M	U		
	92.02 ± 8.37	91.32 ± 4.52	86.69 ± 5.24	85.29 ± 4.02	83.24 ± 2.24	74.42 ± 3.50	7.31	8.84	10.69	11.20 ± 0.05	11.20 ± 0.96	11.20 ± 1.79	0.28 ± 1.75	279.67 ± 2.75
Fipronil	L	M	U	L	M	U	L	M	U	L	M	U		
	105.35 ± 6.07	100.07 ± 5.71	89.35 ± 4.19	98.54 + 1.73	93.02 + 3.79	79.43 ± 3.41	6.46	7.04	11.10	10.40 ± 1.73	10.60 ± 1.73	10.20 ± 1.30	0.29 ± 1.53	273.66 ± 3.96
Control	L	M	U	L	M	U	L	M	U	L	M	U		
	89.10 ± 4.02	79.27 ± 4.87	74.49 + 4.59	77.81 + 5.73	69.31 + 3.79	59.61 ± 5.29	12.67	12.56	19.97	9.40 ± 0.58	8.40 ± 0.50	8.4 ± 1.14	0.23 ± 2.75	285.66 ± 2.76

Data are mean of 7 replications

identified as *Isaria fumosorosea* strain (MH414514.1). The mean radial growth of *I. fumosorosea* cultures was found to be 66.5 ± 0.58 mm, similar results of 45.2 mm of radial growth of *I. fumosorosea* (ifr strain) were recorded in study conducted by Meyer et al. (2008). While studying the effect of suspension dilutions on conidial density and sporulation, the conidial count (17.81×10^7 spore/ml and sporulation (90.49%) was found to be the highest at a concentration of 1×10^7 spore/ml), which is in agreement with previous studies (Fergani and Rafaei 2021). The lowest conidial count (4.88×10^7spore/ml) was found in dilution of 1×10^9 spore/ml and lowest sporulation (49.60%) was found at a dilution of 1×10^3spore/ml. In the initial stage, morphological changes in the body of *E. amabilis* larvae was observed after application of the fungus, i.e., the larval body color to blackish brown and later become stiff. It was observed that at 15th days of treatment, *I. fumosorosea* at 1×10^7conidia/ml caused 96% mortality of *E. amabilis* larvae in dipping method and 72% mortality in spraying method, which concluded that dipping method was more preferable than spraying method. Testing of *I. fumosorosea* on the pest has been attempted for the first time showing great potentiality as a biocontrol agent for the lac industry. Application of *I. fumosorosea* caused infection with fluffy growth on *E. amabilis* pupae at 1×10^7 spore/ml and reduced emergence of adults significantly in both spraying and dipping methods, which further reinforced our hypothesis of using the EPF as a good agent for pest management as was reported against many pests in agriculture and horticulture. Abnormal changes in emerged adults having crippled wings was observed for the first time in *E. amabilis* when treated with *I. fumosorosea* strain, which was a significant finding. As a result, the deformed adults fail to perform normal flight behavior and mating, which was earlier reported in *Cydalima perspectalis* and *Spodoptera litura* (Zemek et al. 2020). The egg mortality was found the highest (40.00%) at 1×10^7 spore/ml upon infection, which was significantly lower as compare to other stages of *E. amabilis,* which proposed that the egg stage was the most resistant stage to fungal infections (Mochi et al. 2009). It is clear from the mentioned results that the comparison between total mortality percentages recorded at the five different spore dilutions (10^3, 10^5, 10^7, 10^8 and 10^9 spore/ml), of *I. fumosorosea* strain toward eggs, larval instars and pupae indicated that 10^7spore/ ml was found to be most effective. Most of the times, fungal isolates performed well in laboratory bioassays, by exhibiting higher mortality rates within 1–2 weeks, but they may not exhibit similar results under field conditions. Therefore prior to field use, laboratory screening is vital step to identify the potentiality of EPF against insect pests (Cherry et al. 2005).The application of *I. fumosorosea* stain at 1×10^7 spore/ml against *E. amabilis* in field condition was found to be effective with up to 81.18% reduction in the mean population of pests of lac insect over control. The bio safety analysis of lac insect revealed that the application of EPF was totally safe and significantly enhanced the quality of brood lac by reducing the pest population, which is one of the major sources of pest infestation in subsequent crop.

Conclusions

It may be concluded that *I. fumosorosea* strain (MH414514.1) is virulent to eggs, larvae and pupae of *E. amabilis* which may be considered a good option to develop a fungal bio pesticide and also can be suitably integrated as a component of pest management programs in lac production system. Since lac is the only resin of animal origin hence for controlling lac insect pests, EPF or EPF based mycoinsecticide is one of the best strategies under organic cultivation especially in lac cultivation. Moreover, the region specific strain of EPF is more effective in any insect pest control program.

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Author contributions
PD guide and supervises the experiment. PD, BB, PS collaborated in the creation of the manuscript. BB carried out the experiment and recorded data. BB and PS interpreted the results and wrote the manuscript. TG analyses some part of the experiment. RB and RK provided laboratory facilities and help in analysis. LKH, KKS, AM guided and checked the manuscript. All authors read and approved the final manuscript.

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