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Pathogenic potential of *Metarhizium anisopliae* and *Lecanicillium longisporum* on tea mosquito bug, *Helopeltis theivora* Waterhouse (Hemiptera: Miridae)

Nagaraj Santhana Bharathi[1*], Pannerselvam Mahendran[2], Krishnaswamy Sujatha[3], Shanmugam Ashokraj[4] and Thattante Parambil Rabeesh[1]

Abstract

Background: The study was focused on identifying the pathogenic potential of native entomopathogenic fungi (EPF) viz., *Metarhizium anisopliae* (Metsch.) and *Lecanicillium longisporum* (Zimm.) against adult tea mosquito bug (TMB), *Helopeltis theivora* (Waterhouse) under in vitro conditions.

Results: Four EPF were isolated and the sequence has deposited to NCBI Genbank with accession numbers of MZ930378 (*Metarhizium anisopliae* isolate UPASI_1), MZ930384 (*Lecanicillium longisporum* isolate UPASI_2), MZ930388 (*Metarhizium anisopliae* isolate UPASI_3) and MZ930389 (*Metarhizium anisopliae* isolate UPASI_4). Isolates were evaluated against adult TMB using dipping and direct spray methods under in vitro conditions. The *M. anisopliae* isolates achieved 100 and 69–81% of adult mortality in dipping and direct spray method, respectively after the 10th day of application with 1×10^9 spores/ml. Among the tested isolates, a significant ($p < 0.001$) and highest mortality were observed in *M. anisopliae* (MZ930388). On the other hand, spraying of *L. longisporum* with 1×10^9, 1×10^7 and 1×10^5 spores/ml caused the adult mortality of 76, 55 and 46% respectively after the 10th day of application.

Conclusions: The study found that the indigenous EPF, *M. anisopliae* (MZ930388) was an effective and promising biocontrol agent against adult TMB under in vitro conditions.

Keywords: Indigenous entomopathogens, Entomopathogenic fungi, Tea mosquito bug, Biological control, IPM, In vitro pathogenicity

Background

Tea, *Camellia sinensis* (L.) Kuntze, is one of the most inexpensive and reasonable beverages in the world. The evergreen, perennial and monoculture crop provides a relatively optimum microclimate and continuous food supply to various insects and mites. Hence, the tea plantations are intensively affected by a wide range of pests. Among the various pests, the tea mosquito bug (TMB), *Helopeltis theivora* Waterhouse (Hemiptera: Miridae), is a major pest in mid-elevation areas of south Indian tea-growing districts such as Vandiperiyar (Kerala) and Valparai (Tamil Nadu) (Sachin et al., 2008). Infestation accounts for up to 17% of crop loss and sometimes it can able to cause 100% in south India (Srikumar et al., 2017; Suganthi et al., 2018). At present, the population has been started to build up in lower elevation areas of Munnar and Neliyampathi (Kerala). TMB infestation leads to a decline in both the quantity and quality of the tea. Both adults

*Correspondence: santhanabharathin@gmail.com

[1] Division of Entomology, UPASI TRF Tea Research Institute, Nirar Dam BPO, Valparai, Coimbatore, Tamil Nadu 642127, India
Full list of author information is available at the end of the article

and nymphs suck the plant sap from buds, young leaves, petioles and tender stems of tea plants with the help of proboscis (Santhana Bharathi et al., 2022). The severe infestation leads to curling up, leaf defoliation and might also encourage the growth of secondary fungal pathogen *Fusarium* sp., which causes dieback disease in tea shoots and results in further loss to the tea plantations (Ekka et al., 2019). The conventional practices have been followed to control TMB as per the instructions by the Tea Board of India through the Plant Protection Code (PPC). The conventional practices lead to various secondary issues such as major pest resurgence, secondary pest outbreak, resistance development, residues in made tea, health hazards to warm-blooded animals, environmental contaminations and increased cost of application (Somnath & Muraleedharan, 2014). Biological control measures are one of the alternate tools to overcome the concern through the integrated pest management (IPM) system (Rachel et al., 2009). Among the various biological controlling agents, entomopathogenic fungi (EPF) are the most effective and promising biocontrol agents over the various insect pests in several agricultural and horticultural crops (Litwin et al., 2020). *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) (Sahayaraj & Borgio Francis, 2010) and *Lecanicillium longisporum* (Ascomycota: Hypocreomycetidae) (Rachel et al., 2009) are commercially used as a biological control agent against various insect pests. Many previous studies have found that *M. anisopliae* was a promising biocontrol agent on various hemipteran pests such as *Bactericera cockerelli* (Hemiptera: Triozidae), *Lygus lineolaris* (Hemiptera: Miridae), *Blissus antillus* (Hemiptera: Lygaeidae) and *Myzus persicae* (Hemiptera: Aphididae) (Lacey et al., 2011; Houping et al., 2002; Samuels et al., 2002; Ullah et al., 2022). Similarly, *L. longisporum* also reported effectiveness against the following hemipterans viz., *Planococcus citri* (Hemiptera: Pseudococcidae) and *Cinara pini* (Hemiptera: Lachnidae) (Ghaffari et al., 2017; Nazemi et al., 2014). Initial EPF infection starts with the adhesion of spores on the insect cuticle and during spore germination, EPF produces lytic enzymes such as proteo-, lipo- and chitinolytic that disintegrate the insect's body shells to reach the insect body cavity through the penetration process. At this stage, it produces some toxic secondary metabolites which cause paralysis and disrupt the insect's metabolic process which leads to insect mortality (Ferron, 1978). In this background, the present study was focused on identifying the effective and promising native biocontrol agent, entomopathogenic fungi to control TMB.

Methods

Soil collection and isolation of native entomopathogen

Soil samples (approx. 500 g) were collected randomly at the depth of 15 cm from each field during the South-West monsoon season (June–July 2020) in Aanaimalais, Coimbatore, Tamil Nadu using sterile plastic bags. The EPF was isolated from the soil samples using the insect bait method (Zimmermann, 1986) with a small modification. *Corcyra cephalonica* larvae were used in the insect bait method instead of *Galleria mellonella*. *C. cephalonica* larval mortality was observed after the 5th day of the inoculation and continuously monitored every 24 h until the 15th day. Dead larvae were carefully examined as per the standard protocols. Larval cadavers were initially washed with sterilized distilled water to remove surface soil then the surface was sterilized with 0.1% mercuric chloride followed by three times sterilized distilled water wash. Washed larvae were incubated in a potato dextrose agar (PDA) plate (9.5 cm diameter) at 27 ± 1 °C with a 12L:12D period for 7 days. After 7 days, the selected colonies were subcultured to isolate the native entomopathogens.

Identification of native isolated entomopathogens through 18s rRNA sequencing

The EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd was used for the DNA isolation from the well-grown fungal colonies. The isolated DNA was quantified using Qubit 3.0 Fluorometer (Thermo Fisher Scientific, United States). Added 5 µL of isolated DNA in 25 µL of PCR reaction solution containing, each 1.5 µL of forward and reverse primers, 5 µL of nucleic acid-free water, and 12 µL of Taq Master Mix followed by performed the PCR. The PCR was carried out in the following condition: initial denaturation at 95 °C for 2 min, 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min followed by the final extension at 72 °C for 10 min. The final PCR product was purified using Montage PCR Clean up kit (Millipore) to remove the unincorporated PCR primers and dNTPs from PCR products. The purified PCR product was sequenced using the ITS1 (5′ TCCGTA GGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCT TATTGATATGC 3′) primers. Sequencing reactions were performed using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The sequence was subjected to the NCBI blast similarity searching tool for the phylogeny analysis with the closely related sequence. The multiple alignments of sequences were performed by MUSCLE 3.7 (Edgar, 2004) and the phylogeny analysis was carried out in PhyML 3.0 aLRT using HKY85 as a

substitution model followed by the phylogenetic tree rendering was completed through Tree Dyn 198.3 (Dereeper et al., 2008).

Preparation of spore suspension

Identified fungal cultures were continuously reared on PDA plates at 27 °C and stored on PDA slants at 4 °C for further studies. The spore suspensions were prepared using 15 days old fungal cultures from the PDA plates. Fungal spore mate was scraped from the PDA plate with the help of a sterile glass slide and the plate was washed thrice using sterile distilled water which contains 0.05% of Tween 80 as a surfactant. Washed fungal spores were kept in a shaker for an hour for a uniform distribution, after that suspension was filtered through sterile double layered muslin cloth to remove the solid debris. Three different spore concentrations of 1×10^5, 1×10^7 and 1×10^9 spores/mL were fixed using a new improved hemocytometer.

Laboratory bioassays

The bioassays were conducted using direct spray and dipping methods to identify the pathogenic potential of isolated native entomopathogens on adult TMB under laboratory conditions. In the direct spray method, the prepared spore suspensions were directly sprayed on the tea shoots (three-leaf and bud) and then transferred to the experimental container (38 cm height × 15 cm width) which contains experimental adults. New shoots were added at the interval of alternate days with initially sprayed tea shoots for 5 days.

In the dipping method, each adult insect was socked in a spore suspension of isolated native entomopathogens for 5 s and then released into the experimental container which contains tea shoots (three-leaf and bud) as a feeding source and tea shoots were changed on alternative days. The mortality was observed on the 5th, 7th and 10th days after the application. All insect cadavers were surface sterilized with 0.1% mercuric chloride and kept on the sterilized moisture tissue paper in a sterilized Petri plate for the conformation of fungal growth over the insect cadaver. Fungal growth was conformed using WESWOX Optic *BXL* CCTV Microscope. Each treatment contains 20 numbers of the mixed adult population with five replicates and the bioassays were performed at 25 ± 1 °C and 75 ± 5% RH (12D:12L).

Statistical analysis

The data were subjected to one way ANOVA and means were compared by Duncan multiple range test (DMRT). Probit analysis was performed to identify the LC_{50} and LC_{90} values of the respective fungal species. All statistical analysis was done using SPSS V16.0 software.

Results

In the present study, a total of 43 soil samples were collected from UPASI experimental farm and Sirukundra Estate, Valparai, Tamil Nadu. Among 43 samples, four soil samples (F. Nos. 65, 39, 24 and 27) were harboured entomopathogenic fungi. The isolated strains from F. No. 65 (10° 15′ 56.0″ N 76° 57′ 59.8″ E), Field No. 39 (10° 18′ 53.9″ N 76° 57′ 26.4″ E), Field No. 24 (10° 17′ 41.6″ N 76° 57′ 27.3″ E) and Field No.27 (10° 17′ 57.6″ N 76° 57′ 51.9″ E) were identified as *Metarhizium anisopliae* (UPASI-1), *Lecanicillium longisporum* (UPASI-2), *Metarhizium anisopliae* (UPASI-3) and *Metarhizium anisopliae* (UPASI-4) respectively using 18S rRNA sequencing. The sequences were submitted to NCBI GenBank and obtained the accession numbers of MZ930378 (*M. anisopliae* isolate UPASI_1), MZ930384 (*L. longisporum* isolate UPASI_2), MZ930388 (*M. anisopliae* isolate UPASI_3) and MZ930389 (*M. anisopliae* isolate UPASI_4). The constricted phylogenic trees are given in Figs. 1, 2, 3 and 4.

During the isolation process, *M. anisopliae* (MZ930388) from F. No. 24 has more virulent than other

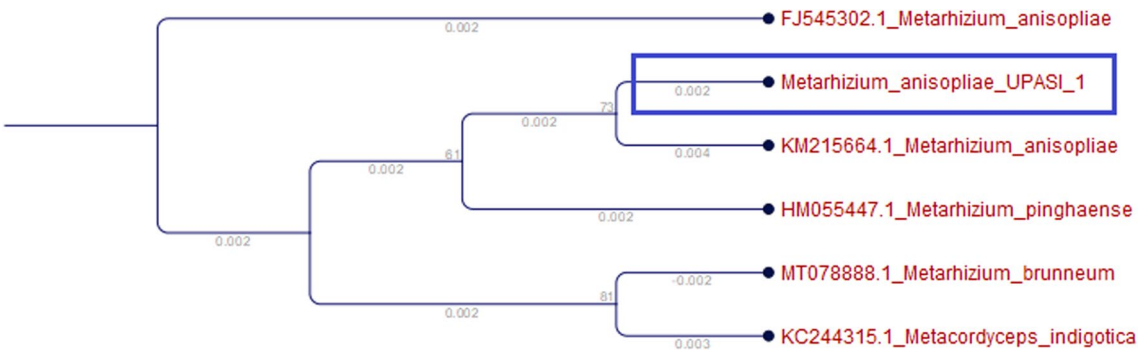

Fig. 1 Phylogenic tree of *Metarhizium anisopliae* isolate UPASI_1 (MZ930378)

Fig. 2 Phylogenic tree of *Lecanicillium longisporum* isolate UPASI_2 (MZ930384)

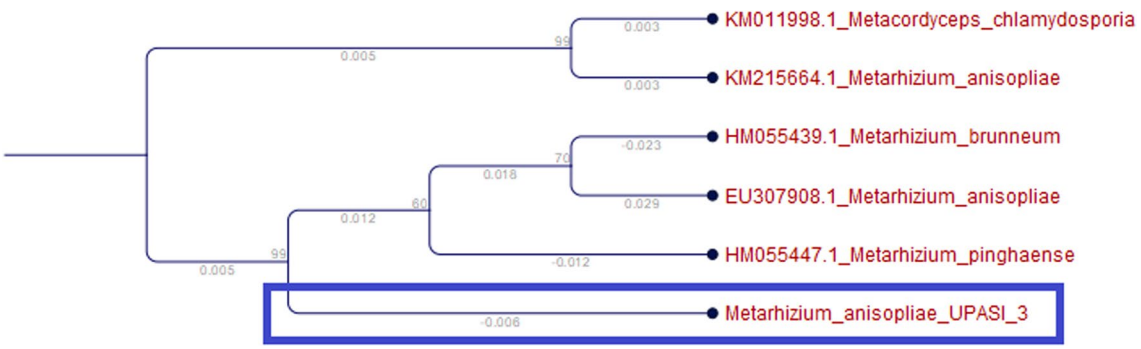

Fig. 3 Phylogenic tree of *Metarhizium anisopliae* isolate UPASI_3 (MZ930388)

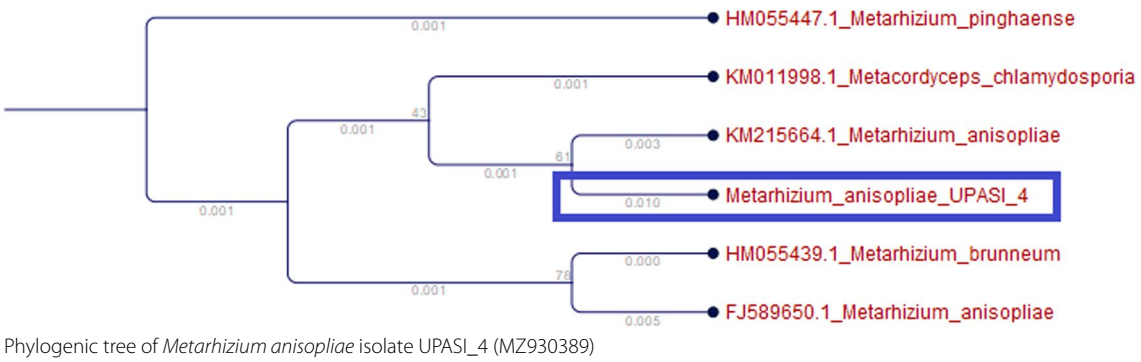

Fig. 4 Phylogenic tree of *Metarhizium anisopliae* isolate UPASI_4 (MZ930389)

isolates. It took 3 and 8 days to kill *C. cephalonica* and spore production respectively. Meanwhile, other isolates such as *M. anisopliae* (MZ930378), *L. longisporum* (MZ930384) and *M. anisopliae* (MZ930389) took on an average of 8 and 13 days to kill the host and spore production, respectively (Figs. 5, 6).

The overall in vitro bioassay showed that the isolated EPF were able to produce 69 to 81% and 76 to 100% of adult mortality in the direct spray method and dipping method, respectively on the 10th day after the application (Fig. 9). The tested treatments such as 1×10^5, 1×10^7 and 1×10^9 spores/mL were produced highly significant ($df = 2$, $f = 1.54$, $p < 0.0001$) adult mortality in both bioassay methods ($df = 1$, $F = 3.64$, $p < 0.0001$). Similarly, significant mortality was observed after the application viz., on the 5th day, on the 7th day and 10th day ($df = 2$, $f = 452.61$, $p < 0.0001$). The mycelium growth and spore production were noted on the 7th and 10th day respectively for *M. anisopliae* over adult TMB. Well-grown spores were observed on TMB after 15 and 21 days for

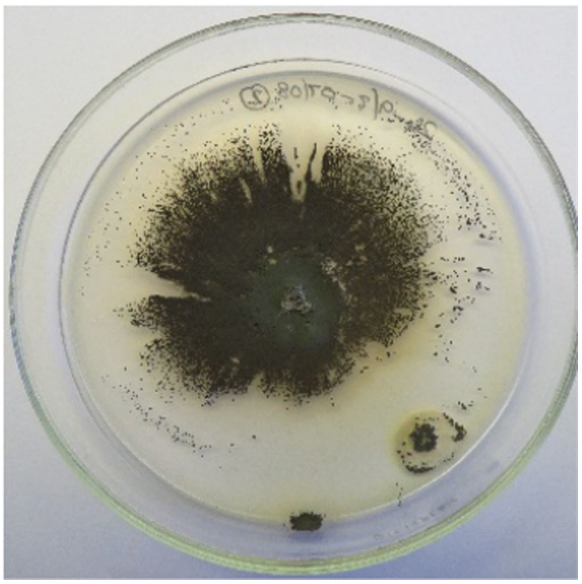

Fig. 5 *Metarhizium anisopliae* isolated from soil

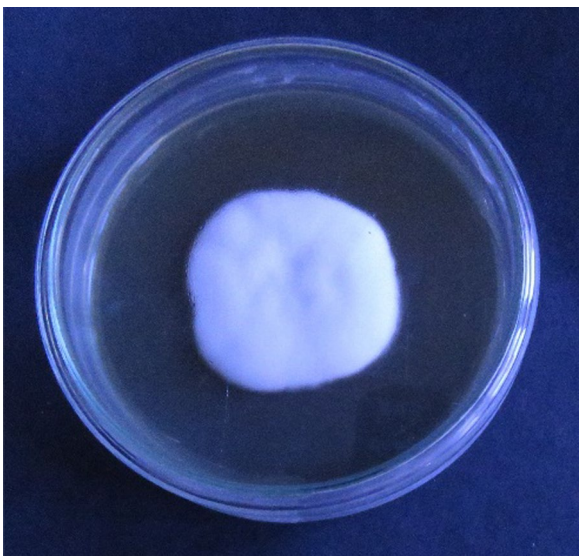

Fig. 6 *Lecanicillium longisporum* isolated from soil

Fig. 7 Well-grown *Metarhizium anisopliae* on adult TMB

Fig. 8 Well-grown *Lecanicillium longisporum* on adult TMB

M. anisopliae and *L. longisporum*, respectively (Figs. 7, 8).

In the dipping method, all four isolates produced significant mortality ($p < 0.05$) while spraying with 1×10^7 and 1×10^5 spores/mL. However, UPASI-1, UPASI-3 and UPASI-4 were achieved on par mortality within them at the concentration of 1×10^9 spores/mL on the 10th day of the after application. All isolated *M. anisopliae* achieved 100% mortality in 1×10^9 spores/mL. However, UPASI-3, UPASI-4 and UPASI-1 isolates were acquired 5, 7 and 10 days respectively to cause 100% mortality. In addition, the other two concentrations such as 1×10^7 and 1×10^5 spores/mL also produced notable mortality with 98 and 90% respectively on the 10th day. Among the all tested isolates, UPASI-3 produced 77 to 100% mortality of TMB on the 5th day. On the other hand, UPASI-2 strain was achieved at about 76% of mortality even on the 10th day. When compared with other strains, UPASI-2 has produced significantly less mortality ($p > 0.05$) than others in all tested concentrations (Fig. 9).

In the direct spray method, a maximum of 81% of adult mortality was observed in UPASI-3 isolate with a

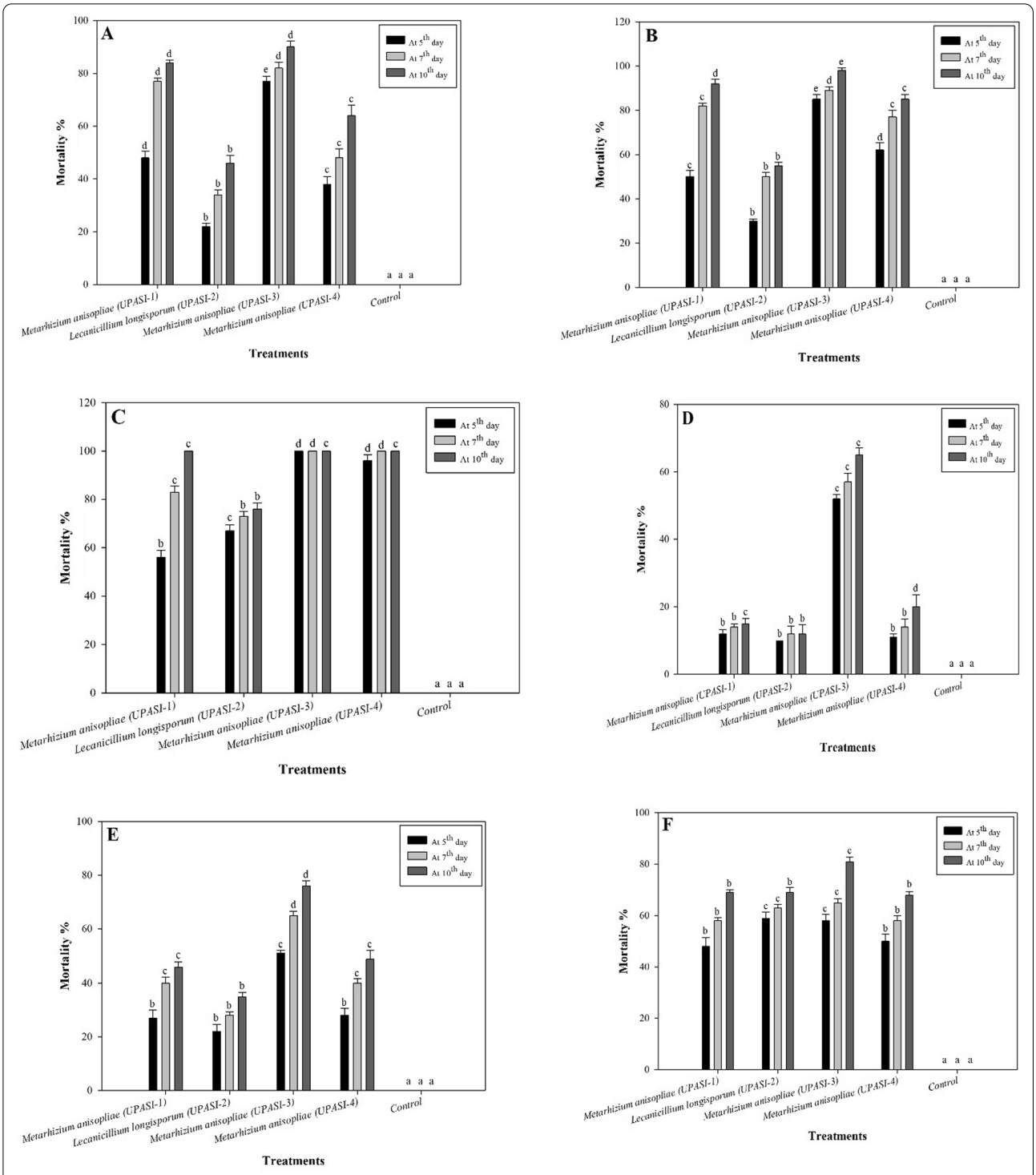

Fig. 9 Mortality percentage (means ± SD) of four isolated entomopathogens on adult TMB at the concentrations of 1×10^5 (**A**), 1×10^7 (**B**) & 1×10^9 (**C**) spores/mL by dipping method and the concentrations of 1×10^5 (**D**), 1×10^7 (**E**) and 1×10^9 (**F**) spores/mL by direct spray method. Treatments followed by the same letter(s) in day(s) after spray (5th, 7th and 10th) is(are) not significant at 5% level by DMRT

spore concentration of 1×10^9 spores/ml on the 10 day (Fig. 9). Similarly, it achieves about 76 and 65% of adult mortality while spraying with 1×10^7 and 1×10^5 spores/mL respectively. The other isolates such as UPASI-1, UPASI-2 and UPASI-4 were produced on par adult mortality within them on the 10th day while spraying 1×10^9 spores/mL. UPASI-2 isolate produced very low mortality viz., 12 and 35% in 1×10^5 and 1×10^7 spores/mL respectively. It failed to achieve prominent results in the direct spray method. Probit analysis showed that *M. anisopliae* (UPASI-3) isolate requires 1×10^5 and 1×10^9 spores/ml to produce 90% of adult mortality in dipping and direct spray method respectively on the 10th day after the application (Table 1).

Discussion

During the study, three *M. anisopliae* and one *L. longisporum* were identified from the soil ecosystem of tea plantations. Tropical and subtropical regions of tea plantations provide excellent moisture weather conditions and an undisturbed soil ecosystem which plays a major role in the diversity of the entomopathogenic fungi in the tea soil ecosystem (Debnath, 1996; Meyling & Eilenberg, 2006; Ye et al., 2013). In addition, temperature (Inglis et al., 2001), soil texture and pH values are also playing a vital role in privileging the fungal diversity in the soil ecosystem (Groden & Lockwood, 1991).

The in vitro studies show that a significant pathogenic property (90–100%) was noted with native *M. anisopliae* in the dipping method. Moreover, the adhesion of fungal spores is highly facilitated in the dipping method than in the direct spray method. This indicates that the adhesion of EPF spores over TMB is very high in the dipping process and adhesion spores will germinate, penetrate to the haemocoel then rapidly cause insect mortality (Ghaffari et al., 2017; Aw & Hue , 2017). Kumhar et al. (2020) reported that the mortality was significantly dependent on spore concentration. A similar observation was made in the present study too. High adult mortality (81%) was observed in high spore concentration (1×10^9 spores/mL).

During the study, the native EPF produced a promising control over TMB. The isolate, *M. anisopliae* (MZ930388) has achieved a maximum of 81% of adult mortality in the direct spray method. Native soil isolates could be able to adopt the biotic and abiotic factors of the native environment. This might be a reason for the promising mortality of EPF over the various pests. Babu and Kumhar (2014) reported that the native entomopathogen, *Beaveria bassiana* was highly efficient against TMB and they also reported that a biological organism from the same ecosystem is more effective than commercial formulations. Similarly, Ekka et al. (2019) also found that the native soil isolate of *B. bassiana* (BPA/B7) from Tinsukia (Assam) has produced 90% of mortality over TMB within 6 days of treatment at the concentration of 10 ml/L under laboratory conditions. Likewise, Kumar and Chaudhary (2021) too found that the native *M. anisopliae* caused 83.57% of mortality on *Helicoverpa armigera* under laboratory conditions and it found that highly virulent than other isolated EPFs such as *B. bassiana*, *Lecanicillium lecanii* and *Nomuraea rileyi*. Apart from the adult mortality, Navik et al. (2015) reported that *M. anisopliae* is also capable to cause 66% of nymphal mortality on *Helopeltis antonii* Signoret after the 10th day of the application in cashew plantation.

Table 1 LD_{50} and LD_{90} values of evaluated entomopathogens

Species	DAS*	Direct spray method			Dipping method		
		LD_{50}**	LD_{90}**	Chi-square value	LD_{50}**	LD_{90}**	Chi-square value
Metarhizium anisopliae (UPASI-1)	5	1.2×10^9	1.0×10^{10}	54.06	5.2×10^7	8.0×10^9	3.73
	7	3.4×10^8	4.0×10^9	42.07	1.3×10^8	1.2×10^9	51.74
	10	9.5×10^7	2.4×10^9	44.78	2.4×10^7	7.0×10^6	2.47
Lecanicillium longisporum (UPASI-2)	5	3.2×10^8	3.5×10^9	72.94	5.0×10^8	2.3×10^9	11.29
	7	5.3×10^8	2.4×10^9	29.48	8.8×10^7	2.0×10^9	9.95
	10	3.0×10^8	2.2×10^9	39.58	9.4×10^6	1.8×10^9	3.47
Metarhizium anisopliae (UPASI-3)	5	1.2×10^7	6.3×10^9	1.639	2.4×10^7	1.8×10^7	2.47
	7	1.4×10^8	3.9×10^9	9.76	2.9×10^7	1.2×10^7	1.83
	10	1.0×10^9	4.0×10^9	19.408	1.6×10^7	1.0×10^5	5.28
Metarhizium anisopliae (UPASI-4)	5	8.5×10^8	8.6×10^9	58.78	3.5×10^6	7.3×10^8	20.52
	7	3.4×10^8	4.0×10^9	42.88	7.3×10^5	1.7×10^7	3.87
	10	2.3×10^8	2.4×10^9	28.84	5.1×10^6	1.4×10^7	4.34

* DAS = Days after spray; **Spores/mL; LD_{50} = Lethal Dose required to kill 50% of the test insect; LD_{90} = Lethal Dose required to kill 90% of the test insect

In this study, *L. longisporum* almost achieves on par mortality of *M. anisopliae* at higher concentration in the direct spray method and contrast, it achieves low mortality in the dipping method while all *M. anisopliae* achieves 100% mortality. *L. longisporum* performed very well over various pests such as *Macrosiphum euphorbiae* (Alavo et al., 2002), *Aphis craccivora* (El-Salam & El-Hawary, 2011), *Planococcus citri* (Ghaffari et al., 2017), *Trialeurodes vaporariorum* (Fazeli-Dinan et al., 2016). In addition, Nazemi et al. (2014) have found that *L. longisporum* produced 90% of adult mortality on the aphid, *Cinara pini* (Hemiptera: Lachnidae) while spraying the concentration of 10^8 spores/ml after the 7th day of the application under laboratory conditions. Besides the *M. anisopliae* and *L. longisporum*, many fungi viz., *Fusarium* sp., *Aspergillus flavus, Aspergillus niger, Cladosporium* sp., *Curvularia* sp., *Acremonium, Trichoderma* (Bordoloi et al., 2012), *Beauveria bassiana* (Deka et al., 2021; Kishor et al., 2020) were reported as capable of causing mortality on adult TMB.

Conclusions

From the above study, *M. anisopliae* isolates produced 81–100% of adult mortality under in vitro conditions. However, field evaluations of these indigenously isolated promising fungal strains against these target insect pests and their natural enemies constitute the future perspectives of this work.

Abbreviations

UPASI: United Planters' Association of Southern India; TRF: Tea Research Foundation; PPC: Plant Protection Code; EPF: Entomopathogenic Fungi; NCBI: National Center for Biotechnology Information; F. Nos.: Field Numbers; F. No.: Field Number; TMB: Tea Mosquito Bug.

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Author contributions

PM: designed the experiment; NSB: conducted all the experiments and drafted the manuscript; PM, SA and TPR: reviewed the manuscript; KJ and NSB: executed statistical analysis; SA and KJ: performed molecular identification of the isolates. All authors read and approved the final manuscript.

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Availability of data and materials

All generated data for the current study are presented in this research article and the corresponding author has no objection to the availability of data.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they do not have a competing interests.

Author details

[1]Division of Entomology, UPASI TRF Tea Research Institute, Nirar Dam BPO, Valparai, Coimbatore, Tamil Nadu 642127, India. [2]ATGC Biotech Pvt. Ltd., Hyderabad, Telangana 500078, India. [3]PG and Research Department of Zoology, Govt. Arts and Science College, Coimbatore, Tamil Nadu 641018, India. [4]Division of Plant Physiology and Biotechnology, UPASI TRF Tea Research Institute, Nirar Dam BPO, Valparai, Coimbatore, Tamil Nadu 642127, India.

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