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Isolation and Identification of Entomopathogenic Fungi from Soils of Tea Ecosystem in South India

Kammatteri Kunnu Ashif^{1*}, Thattante Parambil Rabeesh¹, Shanmugam Ashokraj², Annet Babu¹ and M. L. Ajith¹

¹Department of Entomology, ²Department of Plant Physiology and Biotechnology, UPASI TRF Tea Research Institute, Valparai - 642127, Coimbatore, Tamil Nadu, India

*Corresponding author

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ABSTRACT

In this section we report the outcomes of our survey to identify native isolates of entomopathogenic fungi from South Indian tea-growing regions. Numerous isolates are from the genera *Metarizhium* sp. and *Beauveria* sp. were retrieved from the four regions of south India where cultivate tea. Among the 180 samples, 50 isolates of an entomopathogenic fungus have been identified. The isolated fungi performed exceptionally well in controlling insects, and they might be mass-produced in the future to manage pests effectively.

Introduction

Tea is an agro-ecosystem comprising tea plants, shade tree and other ancillary crops along with the tea biosphere which includes climate and soil. Natural enemies such as predators, parasitoids, as well as pathogens is having a key role in biological control and integrated pest management (IPM) strategies. Soil is considered an excellent environment shelter for entomopathogenic fungi since it is protected from ultra violet radiation and other adverse abiotic and biotic influences (Keller and Zimmerman, 1989). Entomopathogenic fungus (EPF) effectively infect and eliminate pests, thereby contributing to the control of insect populations through the induction known as epizootics. Mantzoukas *et al.*, (2022) these fungi have certain advantages in pest control is they infect all the stages of insect pests, these even can infect sucking and

piercing pest. This laborious effective method relies on attracting and isolating EPF through the use of insect baits method, (Zimmermann, 1986; Leger *et al.*, 1998; Inglis *et al.*, 2001). Hajek *et al.*, (1994) states that the high susceptibility of *Galleria* larvae to various EPF species enables the detection of even low fungal densities in soil samples. Since EPF possess a unique capacity to infect insects, the baiting strategy reduces contamination from non-pathogenic fungus by concentrating exclusively on species (Vega *et al.*, 2008).

Consequently, it became imperative to transition to sustainable pest management techniques for both human and environmental safety. Reduce the total reliance on chemical pesticides and the amount of pesticides that are released into the environment by using biocontrol agents like EPF. Meyling and Eilenberg (2006) state that effective management of native microorganisms in the

soil is essential for controlling the population of insect pests inside the agro ecosystem without requiring any external intervention. This may be achieved by a thorough understanding of the local composition and distribution of EPF species and strains. We hope to investigate the diversity and existence of EPF in the soils of South India's tea-growing regions. Enhancing its usage as a biological control agent for long-term management of insect pests in tea gardens.

Materials and Methods

The survey for identifying the EPF from the tea ecosystem was conducted during the years from 2021-2023 and samples were collected from various sites across tea growing areas of Valparai and Gudalur of Tamil Nadu along with Vandiperiyar and Wayanad regions of Kerala. The soil samples were collected as a mixture of five different quadrants in the plot. Approximately 200 grams of soil were collected from each site, from a depth of 10-15 cm below the ground. Overall, 180 samples were collected from the tea gardens with low pest infestation. All the soil samples were stored in a refrigerator at 4-5°C until further processing. In the laboratory, each bag containing soil was thoroughly mixed and homogenized manually.

Isolation of entomopathogenic fungi from soil samples

Entomopathogenic fungi were isolated from the collected soil samples by soil baiting method as proposed by [Bharathi et al., \(2022\)](#) by using rice moth, *Corcyra cephalonica*. The assessment of pathogenicity of soil was tested using ten fourth instar larvae. The mortality of the larvae was assessed in every 24 hours interval upto 15 days after inoculation of larvae. The dead larvae were carried out into the humidity chambers. The larvae were allowed for the growth of fungus mycelia. After infestation with the fungal spores, the larvae were sterilized with ethanol and continued washing in distilled water three times. The larvae were then placed on potato dextrose agar media in petri plates to facilitate further growth of entomopathogenic fungi.

Identification and characterization of common entomopathogenic fungal isolates

The microscopic features of the isolated entomopathogenic fungus were noted, including the colour (front and reverse) of the colonies, their texture,

and their appearance. By establishing slides for light microscopy at a 40x magnification, observations were done to investigate the morphology of the spores and verified entomopathogenic fungi were detected in terms of appearance. The molecular characterization was performed by comparing rDNAITS Search Tool (BLAST). MEGA 11.0 software was used for the construction of phylogenetic tree, using Neighbor joining tree statistical method and Kimura-2 parameter model. For this, ITS sequences of this study were compared with already published sequences present in NCBI database.

Fungal DNA extraction

Genomic DNA extraction from fungal mycelium was carried out using CTAB method ([Zhang et al., 2010](#)). The 15-day old mycelium was scraped from the fungal plates and crushed in mortar and pestle using 1 ml of CTAB buffer followed by incubated in water bath at 65 °C for 1 hour. Then it was centrifuged at 12,000 rpm for 10 minutes at 4 °C and the supernatant was collected. To this supernatant an equal ([Sun and Liu, 2008](#)) volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and vortexed. The mixture was centrifuged at 12,000 rpm for 10 minutes at 4 °C, and supernatant was collected and an equal volume of ice-cold isopropanol was added. The mixture was incubated overnight at -20 °C and was centrifuged at 13,000 rpm for 15 minutes at 4 °C. Finally, the pellet that contains DNA was washed with 70% ethanol, air dried and dissolved in 50 µl of nuclease free water.

Amplification and sequencing

The ITS1-5.8S-ITS2 region of isolated DNA was PCR amplified and sequenced using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The reaction mixture (30 µl) contains 15 µl of PCR master mix, 12 µl of nuclease free water, 1 µl of ITS1 and ITS4 primers each and 1 µl of DNA. PCR amplification was carried out with specific PCR conditions, described by [Gandarilla-Pacheco et al., \(2021\)](#). After completion of PCR, the products were analyzed on 1% Agarose gel with Ethidium bromide and visualized through the Gel documentation system. The amplified PCR products were then processed at Syngenome (OPC) Private Limited, Coimbatore and sequences were obtained. Sequences were edited using BIOEDIT software 7.2 and compared with sequences in NCBI database using Basic Local Alignment.

Results and Discussion

In order to develop the biopesticides we have surveyed for the virulent strains of entomopathogenic fungus in the tea garden. In this survey, a total of fifty entomopathogenic fungal isolates were isolated from 180 soil samples in which twenty-nine were obtained from the Valparai region, thirteen from the Vandiperiyar region, eight from the Wayanad region, and one from Gudalur. The morphological identification of entomopathogenic fungi was done based on the taxonomic keys given by Palestine (1999). Isolated fungi belonged to seven different genera, including *Beauveria*, *Cordyceps*, *Metarhizium*, *Aspergillus* and *Lecanicillium*. Among these genera, the majority of the isolated fungi belonged to the *Beauveria* sp. and *Metarhizium* sp. with 41 isolates. Fungi included twenty one isolates of *B. bassiana*, twenty isolates of *M. anisopliae*, two isolates of *A.niger*, two isolate of *L. fusisporum* and one isolates of *L. lecanii*. Species confirmation was achieved through sequence comparison with NCBI database.

During the present study we were identified sixty-one isolates, among them eighteen isolates were selected for the further identification of the respective fungus through 18s rRNA sequencing. Based on the NCBI blast, the isolated fungi were TPEPF-5, TPEPF-6, TPEPF-7, TPEPF-9, TPEPF-10, TPEPF-11, TPEPF-12, TPEPF-13, TPEPF-14, TPEPF-15, TPEPF-16, TPEPF-17 and TPEPF-18. This was identified as *Metarhizium anisopliae* (OP269684), *Metarhizium anisopliae* (OP268209), *Beauveria bassiana* (OP269689), *Beauveria bassiana* (OP269691) *Metarhizium anisopliae* (OP269693), *Cordyceps cateniannulata* (OP269695), *Metarhizium anisopliae* (OP269697), *Beauveria bassiana* (OP288145), *Lecanicillium longisporum* (OP269943), *Cordyceps cateniannulata* (OP270020), *Aspergillus niger* (OP270167), *Aspergillus niger* (OP270191), *Lecanicillium lecanii* (OP270192), *Lecanicillium fusisporum* (OP270214) *Aspergillus niger* (OP270167), *Aspergillus niger* (OP270191), *Lecanicillium lecanii* (OP270192) and *Lecanicillium longisporum* (OP270214) respectively. The phylogenetic trees were constructed for the respective organisms (Table 1).

The present study concludes that the tea ecosystem is having a wide range of entomopathogenic fungus which may affect the insect population present in the ecosystem. The present study also conveys that for the development of the EPF naturally there is an impact of

pesticides hence reduction in the application may promotes its growth. According to Abhilash and Singh (2009), pesticides kill pests but also harm beneficial insects and non-target species in the ecosystem. As a result, it is necessary to produce biopesticides that are safe for beneficial insects. The diversity of fungi and rate of isolation in the soil varied amongst sites in the south Indian tea ecosystem. Ramanuj (2015) stated that the *B. bassiana* is described by the feature of this species as the colonies appear white mycelium bearing masses of powdery spores. *M. anisopliae* can be identified using the spore structure. The spore germinate on the insect body and the hyphae that emerge penetrate the cuticle eventually killing the insects a white mold, then grows on the cadaver that soon turns green as spores of the fungus are produced. *A. lecanii* were characterized by forming yellowish-white, fluffy, branched mycelium in a centered cycle pattern, typically short-ellipsoidal conidia (Zare and Gams, 2001).

The EPF are one of the natural control measures, serving a crucial role in the regulation of pest. These fungi have been considered as a source of biocontrol agents (Goettel et al., 2000). The present study also shows similar results of Asensio et al., (2003) utilized *Galleria* larvae as bait to isolate EPF from soils and isolated *B.bassiana* and *M.anisopliae*. In the study conducted by Sharma et al., (2012), the fungi with the highest occurrence was *M.anisopliae* (30.12%), followed by *A. flavus* (23%), *F.oxysporum* (18.66%) and *B.bassiana* (10.2%).

In the study conducted by Safaryan and Tkaczuk (2021), EPF were isolated from soil and identified four fungal genera viz., *Beauveria* spp., *Cordyceps* spp., *Metarhizium* spp. and *Lecanicillium* spp. Abdullah et al., (2020) discovered that the diversity of fungi from rice field ecosystems includes genera such as *Fusarium* species, *Aspergillus* species, *Rhizopus* species, *Trichoderma* species, *Penicillium* species, *Rhizoctonia* species and *Metharizium* species.

Afandhi et al., (2022) conducted research on soil-inhabiting EPF the findings revealed a greater occurrence in organic farms compared to conventional farms, specifically *Aspergillus* sp., *Beauveria* sp. and *Gliocladium* sp. exclusively in organic soils. Ranadev et al., (2023) experiment on isolation of EPF from different zones of Karnataka revealed that, among 81 fungal isolates, they were belonging to genera *Beauveria* & *Metarhizium* 25 % each, *Aspergillus* 18.75 %, *Lecanicillium* 12.5 %, *Paecilomyces* 6.25 and *Hirsutella* 6.25 %.

Table.1 Details of soil sampling sites.

Location	Latitude and longitude	Altitude(MSL)	Number of soil samples collected	Number of fungal isolates
Valparai	10.3270° N, 76.9554° E	1,059 m	91	29
Gudalur	11.5012° N, 76.4922° E	1300 m	4	1
Vandiperiyar	9.5721° N, 77.0896° E	836m	53	13
Meppadi	11.7032° N, 76.0834° E	2100 m	8	4

Table.2 Details of entomopathogenic fungus isolated from the tea soil ecosystem.

S. No.	NCBI Accn. No	Code	Species	Location
1.	OP269684	TPEPF 5	<i>M. anisopliae</i>	Vandiperiyar
2.	OP268209	TPEPF 6	<i>M. anisopliae</i>	Vandiperiyar
3.	OP269689	TPEPF 7	<i>B. bassiana</i>	Vandiperiyar
4.	OP269693	TPEPF 9	<i>M. anisopliae</i>	Vandiperiyar
5.	OP269695	TPEPF 10	<i>C. cateniannulata</i>	Vandiperiyar
6.	OP269697	TPEPF 11	<i>M. anisopliae</i>	Vandiperiyar
7.	OP288145	TPEPF 12	<i>B. bassiana</i>	Anamallais
8.	OP269943	TPEPF 13	<i>L. longisporum</i>	Anamallais
9.	OP270020	TPEPF 14	<i>C. cateniannulata</i>	Anamallais
10.	OP270167	TPEPF 15	<i>L. lecanii</i>	Anamallais
11.	OP270191	TPEPF 16	<i>A. niger</i>	Anamallais
12.	OP270192	TPEPF 17	<i>A. niger</i>	Anamallais
13.		TPEPF 19	<i>M. acridium</i>	Anamallais
14.		TPEPF 20	<i>B. bassiana</i>	Anamallais
15.		TPEPF 21	<i>M. anisopliae</i>	Anamallais
16.		TPEPF 22	<i>M. anisopliae</i>	Vandiperiyar
17.		TPEPF 23	<i>M. anisopliae</i>	Anamallais
18.		TPEPF 24	<i>M. anisopliae</i>	Anamallais
19.		TPEPF 25	<i>L. longisporum</i>	Anamallais
20.		TPEPF 26	<i>M. anisopliae</i>	Anamallais
21.		TPEPF 27	<i>M. anisopliae</i>	Anamallais
22.		TPEPF 28	<i>M. anisopliae</i>	Anamallais
23.		TPEPF 29	<i>M. anisopliae</i>	Anamallais
24.		TPEPF 30	<i>B. bassiana</i>	Anamallais
25.		TPEPF 31	<i>M. anisopliae</i>	Anamallais
26.		TPEPF 32	<i>M. anisopliae</i>	Anamallais
27.		TPEPF 33	<i>M. anisopliae</i>	Anamallais
28.		TPEPF 34	<i>M. anisopliae</i>	Anamallais
29.		TPEPF 35	<i>C. cateniannulata</i>	Vandiperiyar
30.		TPEPF 36	<i>C. cateniannulata</i>	Vandiperiyar
31.		TPEPF 37	<i>M. anisopliae</i>	Vandiperiyar
32.		TPEPF 38	<i>M. anisopliae</i>	Vandiperiyar
33.		TPEPF 39	<i>M. anisopliae</i>	Vandiperiyar
34.		TPEPF 40	<i>M. anisopliae</i>	Anamallais

35.		TPEPF 41	<i>B. bassiana</i>	Anamallais
36.		TPEPF 42	<i>B. bassiana</i>	Anamallais
37.		TPEPF 43	<i>B. bassiana</i>	Wayanad
38.		TPEPF 44	<i>B. bassiana</i>	Anamallais
39.		TPEPF 45	<i>B. bassiana</i>	Anamallais
40.		TPEPF 46	<i>B. bassiana</i>	Anamallais
41.		TPEPF 47	<i>B. bassiana</i>	Wayanad
42.		TPEPF 48	<i>B. bassiana</i>	Wayanad
43.		TPEPF 49	<i>B. bassiana</i>	Wayanad
44.		TPEPF 50	<i>B. bassiana</i>	Wayanad
45.		TPEPF51	<i>B. bassiana</i>	Gudallur
46.		TPEPF 52	<i>B. bassiana</i>	Wayanad
47.		TPEPF53	<i>B. bassiana</i>	Wayanad
48.		TPEPF 54	<i>B. bassiana</i>	Wayanad
49.		TPEPF55	<i>B. bassiana</i>	Wayanad
50.		TPEPF56	<i>B. bassiana</i>	Anamallais
51.		TPEPF 57	<i>B. bassiana</i>	Anamallais

Figure.1 Isolated entomopathogenic fungus from the soil

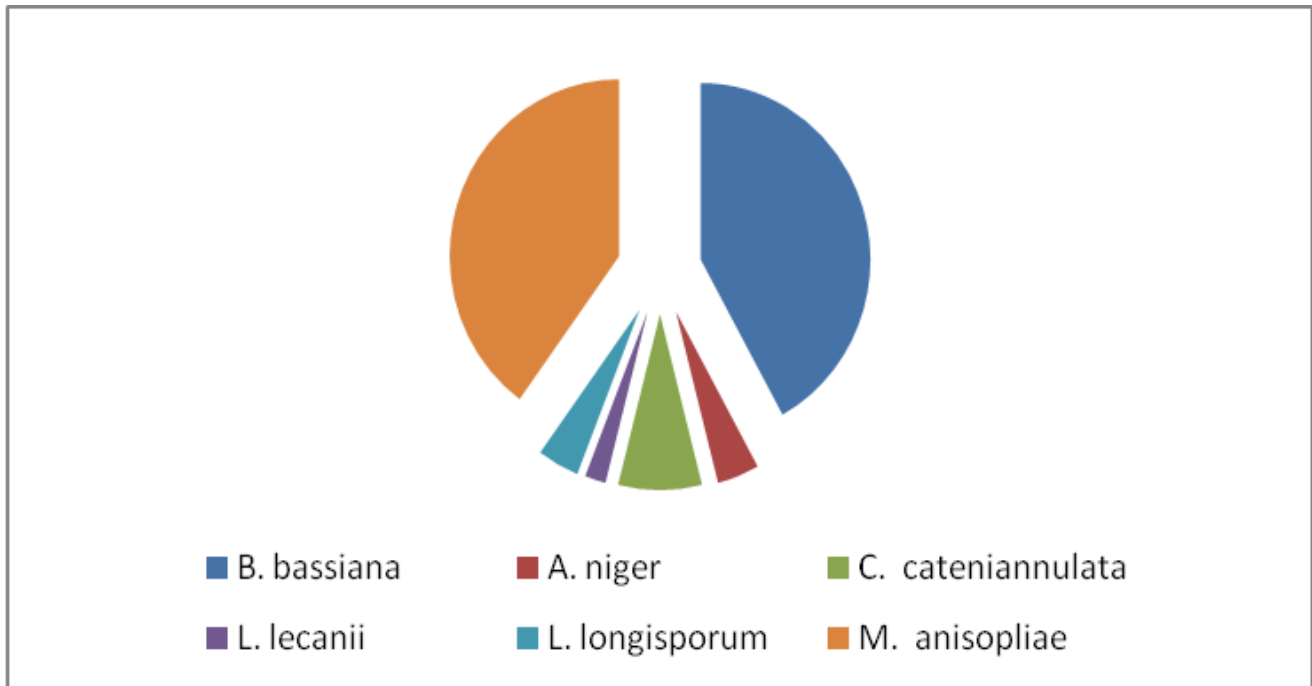


Figure.2 Rice moth larvae infected with a) *M. anisopliea* b) *L. lecanii* c) *B. bassiana*

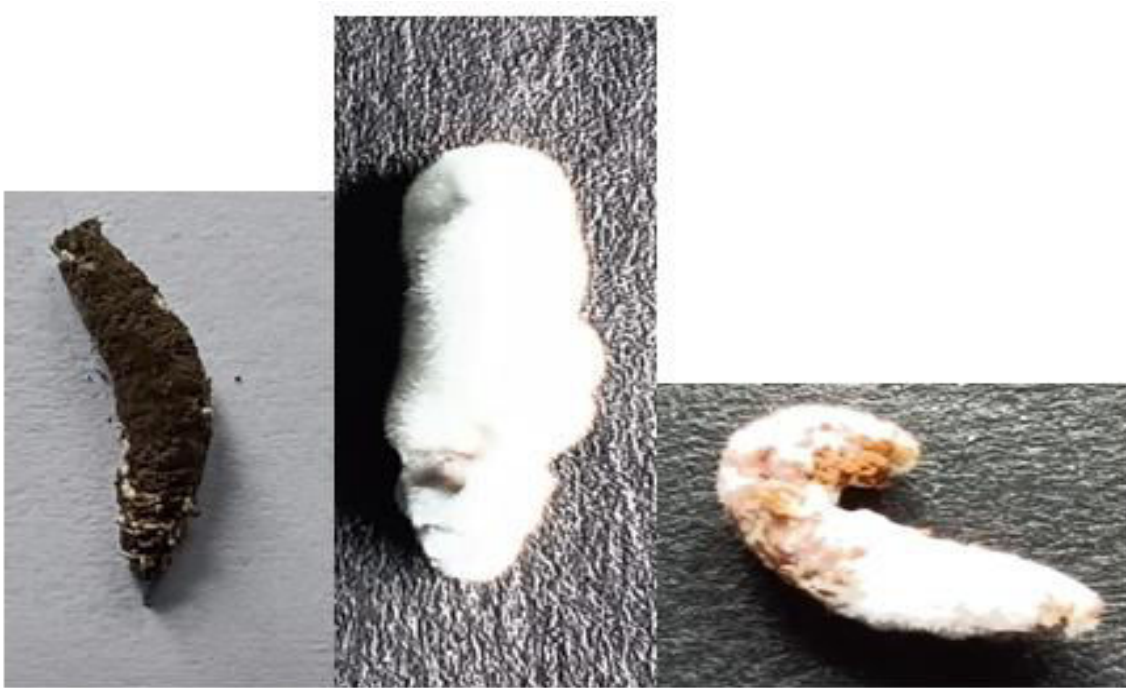
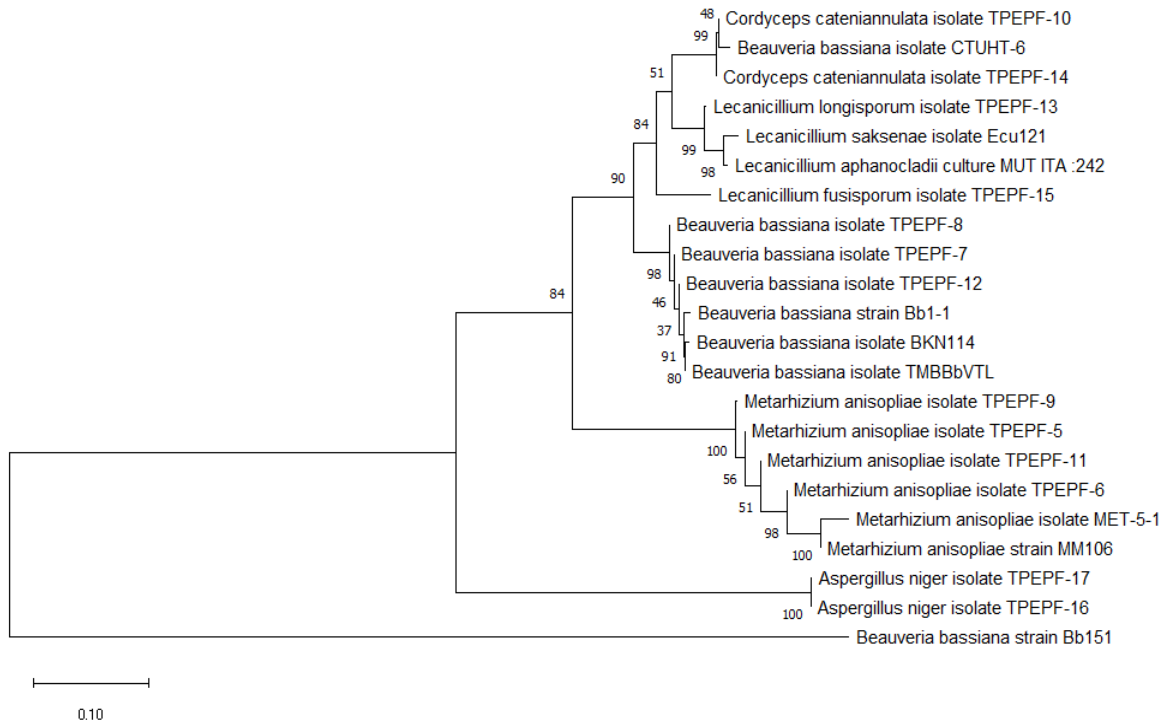


Figure.3 Rice moth Larvae on the soil collected from the tea growing area.



Figure.4 Phylogenetic tree depicting the similarity of neighboring isolates



Beemrote *et al.*, (2003) states that among the different isolates of entomopathogens isolated from the soil, the same species among the isolated strains were having different levels of pathogenicity towards the galleria larvae. The present also revealed that most commonly the same species were isolated from the soil ecosystem and also proved that the native isolates is having higher efficacy against the native pest of the tea ecosystem.

In summary, this study examined the entomopathogenic fungi that were isolated from a variety of soil samples in the South Indian tea ecosystem. The results showed that the fungi in the soils of the tea ecosystem were moderately diverse, and some of the isolated fungi demonstrated excellent performance in terms of pathogenicity against major tea pests.

Additionally, different isolates of the same fungi showed varying levels of performance in the preliminary pathogenicity test, suggesting that each isolate possesses a unique potential for controlling insect pests. As a result, it is advised to isolate local strains of entomopathogenic fungi for the purpose of effectively managing tea pests in the affected area. In summary, this study highlights the

significance of entomopathogenic fungi in the tea ecosystem.

Abbreviations

UPASI: United Planters' Association of Southern India; TRF: Tea Research Foundation; EPF: Entomopathogenic Fungi; TMB: Tea Mosquito Bug; IPM:-Integrated pest Management; RSM:- red spider mite;

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Author contributions

AKK and TPR: designed the experiment; AKK and AB: conducted all the experiments and drafted the manuscript; AKK, AB, MA and TPR: reviewed the manuscript; AKK and AB: executed statistical analysis; SA: executed the Molecular analysis. All authors read and approved the final manuscript.

Data Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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