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Isolation and Identification of Entomopathogenic Fungi from Soils of Manipur (N-E India)

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ABSTRACT

Background: Entomopathogenic fungi (EPF) are natural adversaries of insects, serving a crucial role in the regulation of insect pest populations. In response to the growing demand for sustainable agricultural practices that prioritize environmental protection, human safety and animal welfare, the utilization of bio-control agents like entomopathogenic fungi offers a superior and safe alternative to chemical pesticides. Entomopathogenic fungi effectively infect and eliminate insects, thereby contributing to the control of insect populations through the induction of lethal infections known as epizootics. Isolating EPF from the soil is an effective method as they naturally inhabit soil ecosystems. The north-eastern region of India possesses a forest cover exceeding 80%, with Manipur alone accounting for nearly 75% of forest cover in its total geographical area. This abundant forest cover, along with undisturbed land, contributes to the region's wealth of micro flora and fauna, including a thriving population of entomopathogenic fungi. However, the potential of these fungi in pest population management remains largely unexplored. Therefore, this study was conducted to investigate the diversity of these promising entomopathogenic fungi.

Methods: In this study, we isolated fungi from the soils of ten districts of Manipur and identified several isolates with entomopathogenic properties. Soil bating using *Galleria mellonella* larvae was employed for the isolation of entomopathogenic fungi.

Result: A total of 73 fungal isolates were obtained from 100 soil samples, out of which 54 were identified as entomopathogenic fungi. The genus *Aspergillus* constituted the most commonly isolated entomopathogenic fungi, followed by isolates of *Beauveria, Clonostachys, Talaromyces, Trichoderma, Fusarium, Aspergillus, Candida* and *Meyerozyma* genera. Diversity studies revealed variations in the types and proportions of fungi among different regions of Manipur. Pathogenicity tests confirmed the virulence of the isolated entomopathogenic fungi, with 14 isolates of *Beauveria bassiana* and two isolates of *Talaromyces purpureogenus* causing 100% mortality of the test insects. The isolated fungi exhibited excellent performance in insect control and could be further mass-produced for effective pest management.

Key words: Diversity index, Entomopathogenic fungi, Manipur, Pathogenicity, Soils.

INTRODUCTION

The North-eastern region of India represents one of the biggest biodiversity hotspots, known for very rich and diverse micro flora and fauna due to its unique climatic conditions. It has predominantly sub-tropical climate with high rainfall and relative humidity with extensive forest cover and diverse topography. North east India is having eight states *viz*., Arunachal Pradesh, Assam, Meghalaya, Manipur, Tripura, Mizoram, Nagaland and Sikkim. Manipur, one of the easternmost border states of India, witnesses an annual rainfall ranging from 2100 to 2500 mm, average temperature ranging from -1 to 38°C and relative humidity between 80-96% (Singh *et al*., 2003). These environmental factors are favourable for the growth and establishment of entomopathogenic fungi.

Crop losses due to insect pests in India are accounts for nearly 23% (Dhaliwal *et al.,* 2007). To reduce these losses, chemical insecticides have been extensively used, with over 60% of India's chemical insecticide consumption directed to crop pest management. However, the use of chemical pesticides causes harm to the environment, as well as health hazards to animals and humans. In addition

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to killing the pests, they also harm the beneficial insects and non-target animals with in the ecosystem (Abhilash and Singh, 2009). Thus, switching over to sustainable pest management practices became crucial for environmental protection, human safety. Utilization of bio control agents such as entomopathogenic fungi (EPF) can help to reduce the complete reliance on chemical pesticides and reduce the chemical pesticides load on the environment.

Soil serves as the main reservoir for entomopathogenic fungi (EPF) and healthy soils are identified by high populations of beneficial soil borne organisms. (Magdoff, 2001). Therefore, exploring and harnessing the potential of naturally occurring entomopathogenic fungi is essential for successful and reliable biologically based pest management. Currently, more than 400 species of EPF have been recognized in nature, belonging to 5 subdivisions: Mastigomycotina, Zygomycotina, Ascomycotina, Basidiomycotina and Deuteromycotina. However, limited efforts have been made to study the presence and pathogenicity of entomopathogenic fungi from North Eastern region of India (Puzari *et al*., 2006). Understanding the local composition and distribution of entomopathogenic fungal species and strains is crucial for effective management of indigenous micro organisms in the soil, facilitating the control of insect pest population within the agro ecosystem without any external intervention (Meyling and Eilenberg, 2006). In this study, we aim to explore the presence and diversity of entomopathogenic fungi in the soils of ecologically diverse regions of Manipur in north-eastern India. These EPF can be harnessed for insect pest management without causing harm to the environment and human health, offering a sustainable alternative to the harmful chemical pesticides.

MATERIALS AND METHODS

The experiment was conducted during the 2021-2022 at Tamil Nadu Agricultural University, Coimbatore. Soil samples were collected from Manipur and were processed for further studies at Insectary, Department of Agricultural Entomology, Nadu Agricultural University, Coimbatore.

Soil sample collection

The soil samples were collected from various sites across 10 districts of Manipur (Fig 1), ranging from cultivated lands to forest areas. The geographic coordinates of each sample collection site were recorded (Table 1). From each district, a total of 10 samples were collected, with each sample being a mixture of five different soil samples. Approximately 200 grams of soil were collected from each site, from a depth of 10-15 cm below the ground, in five randomly selected points. These soil samples were mixed together and stored in double zip lock bags. Overall, 10 samples were collected from each district, resulting in 100 samples in total, which were subsequently transported to Tamil Nadu Agricultural University, Coimbatore for further analysis. The transportation process took several days, necessitating the preparation of the samples for transport without compromising the viability of entomopathogenic fungi, as per the methods outlined by Clasen *et al.,* 2020. 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 using '*Galleria* bait method' developed by Zimmermann (1986).

Rearing wax moth

Greater Wax moth (*Galleria mellonella*) larvae were obtained from Insectary at Tamil Nadu Agricultural University, Coimbatore. These larvae were reared in plastic boxes. An artificial diet was prepared for feeding the larvae of the wax moth while the adult moths were fed on 30% honey solution. The diet formulation was prepared following the method provided by Singh *et al.* (2019), with slight modification. The ingredients used for making artificial diet included wheat flour, corn flour, milk powder, yeast, honey, glycerine, wheat bran, bees-wax, vitamin E capsule and Streptomycin sulphate. The diet was stored at 4°C in a refrigerator for further use.

Soil baiting

Prior to baiting, the wax moth larvae were subjected to heat treatment to prevent webbing in soil. Water was heated to 56°C in water bath and then larvae were treated for 10 seconds in the hot water. Afterward, the larvae were placed on a dry tissue paper and kept in dark condition for 4-5 hours, following the method described by Woodring and Kaya (1988). The soil was then transferred from the bag to transparent plastic cups, leaving some air space at the top. If the soil was excessively dried, then it was moistened with water to provide required moisture for the growth of entomopathogenic fungi. Subsequently, five larvae were placed in each box. The boxes were then incubated at a temperature of 25±5°C for a period of 21 days. Initially for seven days, the boxes were inverted once a day for increasing the exposure of more surfaces of larvae and left undisturbed for the remaining days. After 21 days, the larvae that showed signs of fungal infection were treated with 1% sodium hypochlorite solution for 2 minutes, followed by 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.

Preliminary pathogenicity test (*Koch's postulates)*

The isolated fungi were first grown on Potato dextrose agar media for 10 to 15 days until sporulation. The Fourth and fifth instar larvae of *Galleria mellonella* were rolled across a sporulating culture with the help of forceps. The infected larvae were then transferred into 5 cm diameter petridishes containing moistened filter paper to encourage spore germination on the insect cuticle. Lids of the petridishes were kept with moistened filter paper to encourage spore

germination on the insect cuticle. The lids of the petridishes were sealed with parafilm® to maintain a suitable relative humidity and incubated at a temperature of 25°C in darkness. These larvae were inspected daily until larval death or pupation occurred. Fungus recovery was attempted using the afore-mentioned procedures and the experiment was replicated four times to confirm the pathogenicity of the fungi.

Identification and characterization of common entomopathogenic fungal isolates

The isolated and confirmed entomopathogenic fungi were identified morphologically by preparing slides for light microscopy at a magnification of 400x. The molecular characterization was performed by comparing rDNA ITS sequences for similarity.

Morphological identification

Macroscopic characteristics of fungi, including colony colour (front and reverse side), colony texture and colony appearance were recorded. Observations on the microscopic characters were made to examine shape of the spores.

Fungal DNA extraction

Genomic DNA extraction from fungal mycelium was carried out using CTAB method (Zhang *et al.,* 2010). Fungal mycelium (10 mg) was scraped from 15-day old fungal plates and crushed in mortar and pestle using 1 ml of CTAB buffer. Crushed mycelium was then transferred to an Eppendorf® tube and incubated in water bath at 65°C for 1 hour. Afterwards, the tube was centrifuged at 12000 rpm for 10 minutes at 4°C and the supernatant was collected. An equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the supernatant and vortexed. The mixture was centrifuged at 12000 rpm for 10 minutes at 4°C, resulting in phase separation. The supernatant was collected and an equal volume of ice-cold isopropanol was added. The mixture was incubated overnight at -20°C. The next day, the mixture was centrifuged at 13000 rpm for 15 minutes at 4!. The DNA pellet was washed in 70% ethanol, air dried and dissolved in 50 µl of nuclease free water.

Amplification and sequencing

The ITS1-5.8S-ITS2 region of extracted rDNA was PCR amplified and sequenced using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR mixture (30 µl) consisted of 15 µl of universal master mix, 12 µl of molecular water, 1 µl of primers ITS1 and ITS4 each and 1 µl of extracted DNA. PCR amplification was carried out with specific temperatures, described by Gandarilla-Pacheco *et al*. (2021). After amplification, the PCR products were run on 1% Agarose gel with Ethidium bromide and then bands were visualized under UV light using a Gel documentation unit. 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 Search Tool (BLAST). MEGA 11.0 software was used for the construction of phylogenetic tree, using Neighbour 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.

Evaluation of diversity indices

The diversity of isolated entomopathogenic fungi was determined by using Simpson's index, Shannon-Wiener index and species richness (Yakubu *et al.,* 2022). These indices provide measures of species diversity, abundance and evenness within the fungal community.

$$\text{Simpson index (D)} = \frac{1}{\sum_{i=1}^{S} P_i^2}$$

$$\text{Shannon-wiener index (H')} = \sum_{i=1} P_i \ln P_i$$

Where,
P= No of individual of specific species / Total no of individuals.
□= Sum of calculations.
ln= Natural log.
S = No of species.

$$\text{Species richness (Ma)} = \frac{S-1}{\ln N}$$

Where, (Sun and Liu, 2008)
S= No of species.
N= Total no of isolates.

RESULTS AND DISCUSSION

Isolation of fungi from soil

In this study, a total of 73 fungal isolates were isolated from 100 soil samples, out of which 54 were identified as entomopathogenic fungi. The remaining were either

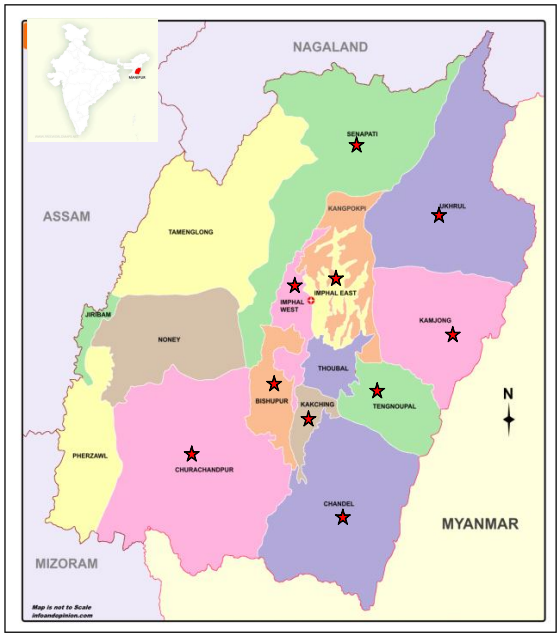

Fig 1: Map of Manipur showing soil sampling sites (districts).

saprophytic or non-pathogenic to insects. Isolated fungi belonged to seven different genera, including *Beauveria, Clonostachys, Talaromyces, Trichoderma, Fusarium, Aspergillus, Candida* and *Meyerozyma*. Among these genera, the majority of the isolated fungi belonged to the *Aspergillus* genus, with 21 isolates. Four species of *Aspergillus* were identified, namely *A. oryzae, A. flavus, A. tamarii and A. nomius and* there were few unidentified *Aspergillus* Species. Other identified fungi included 14 isolates of *Beauveria bassiana,* seven isolates of *Clonostachys rosea,* two isolates of *Talaromyces purpureogenus*, one isolate of each *Talaromyces muroii, Trichoderma hamatum, Trichoderma spirale, Trichoderma koningiopsis.* Remaining isolates were different species of *Fusarium, Candida* and *Meyerozyma genus.* Furthermore, the colony color, texture and spore shape of *Beauveria, Talaromyces* and *Clonostachys* were reported to be similar to previous findings of Norjmaa *et al*., 2019; Anwar *et al*., 2018; and Yilmaz *et al*., 2014. Raja *et al*., in 2017 had the similar findings. During their studies, the most fungi isolated from soil were *Aspergillus* genus.

Preliminary pathogenicity test (Koch's postulates)

To assess the pathogenicity of the EPF isolates, a preliminary pathogenicity test based on Koch's postulates was conducted using *Galleria* larvae. The test isolates exhibited varying degrees of pathogenicity, with mortality rates ranging from 15% to 100%.the results of the pathogenicity test (Koch's postulates) are summarized in Table 2. Among the entomopathogenic isolates, *Beauveria bassiana*was found to be the most virulent, causing mortality rates between 80% and 100%. Other isolates that showed significant pathogenicity included *Talaromyces purpureogenus* (75-100%), *Clonostachys rosea* (60-90%) and *Trichoderma hamatum* (60%). Isolates belonging to the genus *Aspergillus* showed mortality rates ranging from 20% to 60%. However, these isolates were not further cultured due to their negative health effects on humans.

A previous study by Tamta *et al*, (2022) reported the pathogenicicty of *Clonostachys rosea* against the mango hopper, *Amritodus atkinsoni,* with a mortality rate of 96.67%. Overall, the results indicate that *Beauveria bassiana, Talaromyces purpureogenus, Clonostachys rosea and Trichoderma hamatum* isolates exhibited high pathogenicity against the tested organisms, while some isolates of *Aspergillus* showed moderate pathogenicity but were not pursued due to their potential health risks to humans. In the study done by Gebremarium *et al*., 2021, the mortality of *Galleria* larvae were 86-100% in various *Beauvaria basssiana* isolates.

Table 1: Details of soil sampling sites.

Location	Latitude and longitude	Altitude (MSL)	Forest/cultivated land	Hill/valley
Kakching	24.4969°N, 93.9831°E	776 m	Forest and cultivated land	Valley
Kamjong	24.8570°N, 94.5135°E	913 m	Forest land	Hill
Senapati	25.2677°N, 94.0210°E	1,500 m	Forest land	Hill
Ukhrul	25.0968°N, 94.3617°E	1,662 m	Forest land	Hill
Bishnupur	24.5245°N, 93.7842°E	901 m	Forest and cultivated land	Valley
Churachandpur	24.3427°N, 93.6978°E	914 m	Forest land	Hill
Chandel	24.3262°N, 94.0006°E	960 m	Forest land	Hill
Imphal East	24.8027°N, 94.0287°E	790 m	Forest and cultivated land	Valley
Imphal West	24.7828°N, 93.8859°E	790 m	Cultivated land	Valley
Tengnoupal	24.3838°N, 94.1482°E	2000 m	Forest land	Hill

Table 2: Pathogenicity test of entomopathogenic fungi and their mortality (%).

Species	No of isolates	Time after infection (days)	Mortality* (%)
Beauveriabassiana	14	3-7	80-100
Clonostachysrosea	7	6-8	60-90
Talaromycespurpureogenus	2	5-7	75-100
Talaromycesmuroii	1	6	50
Trichodermahamatum	1	7	60
Trichodermaspirale	1	6	35
Trichodermakoningiopsis	1	9	45
Aspergillus oryzae	3	5-7	20-60
Aspergillus tamarii	1	5	45
Fusariumkeratoplasticum	2	5-10	35-40
Aspergillus nomius	1	6	15

*Mean mortality of 4 replications *i.e*., 20 larvae.

Morphological and molecular characterization of Entomopathogenic fungi

The morphological identification of entomopathogenic fungi was done based on the taxonomic keys given by Palestine, 1999 and Humber, 2012 (Table 3). Species confirmation was achieved through sequence comparison with NCBI database (Fig 2,3,4).

Diversity of entomopathogenic fungi

In Manipur, the soils from different districts exhibited varying numbers of fungi and isolation rates. The mean isolation rate for fungi was 73%, while for entomopathogenic fungi it was 54%. Senapati District had the highest isolation rate, with 130% for fungi and 110% for entomopathogenic fungi, followed by Churachandpur and Kakching with fungi isolation rate of 100% and 90% and 70% respectively for entomopathogenic fungi. Comparing the fungal biodiversity using Shannon-Weiner Index (SWI), Ukhrul had an SWI of 1.79, indicating the highest fungal diversity among the other locations studied. In contrast, Senapati had SWI of 1.64, Churachandpur had 1.58, Imphal west had 1.33 and Kakching had SWI of 1.27. Kakching had highest Simpson Index value of 0.19 suggesting good diversity of fungal species, where as Kamjong, Ukhrul and Chandel had a Simpson index of 0, indicating lower diversity. Species richness was highest in Ukhrul, followed by Senapati and the lowest was in Imphal East. The distribution and diversity indices of fungi are summarized in Table 4.

Asencio *et al.,* (2003) utilized *Galleria* larvae as bait to isolate EPF from soils in Alicante, SE Spain. The most frequently encountered EPF species were *Beauveria bassiana* (found in 21% of soil samples) and the most

Table 3: Morphological characters of isolated entomopathogenic fungi.

No. of Isolate	Fungal species	Growth pattern	Colony colour		Appearance	Texture	Shape of spore
			Front	Back			
14	*Beauveria bassiana*	Disperse	White	White/Light yellow	Umbonate and rugose	Cottony/Powdery/ velvety	Ovoid
7	*Clonostachys rosea*	Disperse	White	White	Rugose	Cottony	Round
2	*Talaromyces purpureogenus*	Disperse	White	White	Umbonate	Velvety/ cottony	Oval
1	*Talaromyces muroii*	Disperse	Pinkish Yellow	Yellow	Umbonate	Velvety	Ellipsoidal
1	*Trichoderma hamatum*	Disperse	White	White	Umbonate	Fuzzy	-
1	*Trichoderma spirale*	Disperse	Creamy white	White	Umbonate	Fuzzy	-
1	*Trichoderma koningiopsis*	Disperse	White	Light yellow	Umbonate	Cottony	-
3	*Aspergillus oryzae*	Disperse	White	White	Umbonate	Powdery	Round
1	*Aspergillus tamarii*	Disperse	White	Brown	Umbonate	Cottony	Spherical
2	*Fusarium keratoplasticum*	Disperse	White	Pale white	Umbonate	Cottony hairy	Canoe-shaped
1	*Aspergillus nomius*	Disperse	Creamy	Brown	Umbonate	Powdery	Spherical

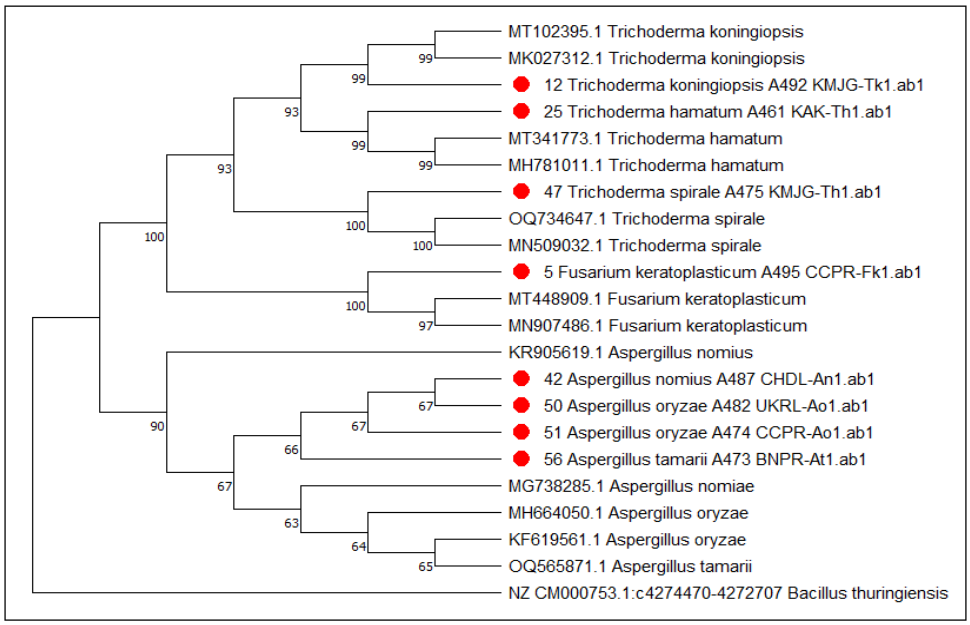

Fig 2: Phylogenetic tree comparing the ITS sequences of isolated entomopathogenic fungal genus *Trichoderma, Fusarium* and *Aspergillus* (mentioned in round shape) with other ITS sequences from NCBI database.

abundant species was *Metarrhizium anisopliae*. In the study conducted by Sharma *et al*. (2012), the fungi with the highest occurrence was *Metarrhizium anisopliae* (30.12%), followed by *Aspergillus flavus* (23%), *Fusarium oxysporum*(18.66%) and *Beauveria bassiana* (10.2%). In the study conducted by Safaryan and Tkaczuk (2021), entomopathogenic fungi were isolated from soil and four fungal genera were identified: *Beauveria spp., Cordyceps spp., Metarrhizium spp. and Lecanicillium spp.* Abdullah *et al*. (2019) discovered that the diversity of fungi in rice field ecosystems exceeded that of dryland ecosystems. The identified genera included *Fusarium species, Aspergillus species, Rhizopus species, Trichoderma species, Penicillium species, Rhizoctonia species* and *Metharizium species. Fusarium* species and *Rhizopus* species were the most commonly isolated genera in rice field ecosystem, whereas *Metharrizium* species was more frequent than *Fusarium* species and *Aspergillus* species in dryland ecosystems. Afandhi *et al.* (2022) conducted research on soil-inhabiting entomopathogenic fungi (EPF) in a conventional and organic farm. The findings

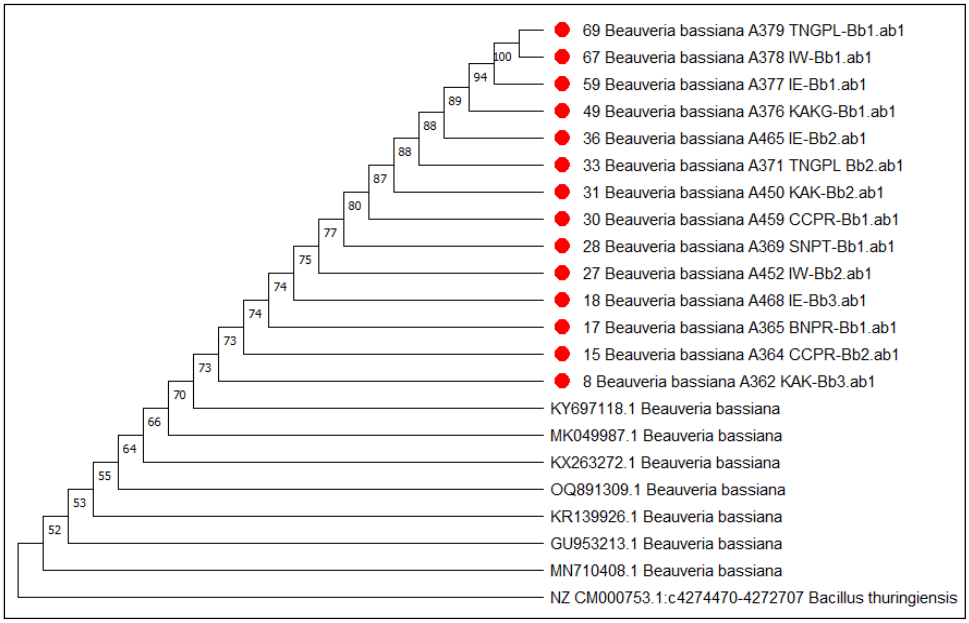

Fig 3: Phylogenetic tree comparing the ITS sequences of isolated *Beauveria bassiana* (mentioned in Round shape) with other ITS sequences from NCBI database.

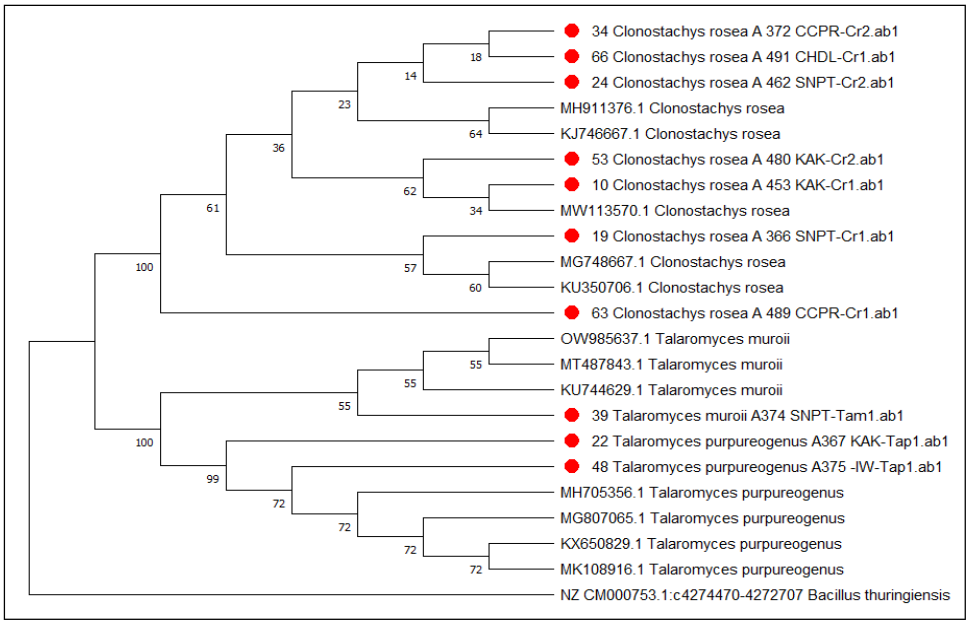

Fig 4: Phylogenetic tree comparing the ITS sequences of isolated entomopathogenic fungi for genus *Talaromyces* and *Clonostachys* (mentioned in Round shape) with other ITS sequences from NCBI database.

Table 4: Diversity indices of fungi in different Districts.

Location	No of sample	Number of fungi		Isolation rate (%)		Simpson index	Shannon-Wiener index	Margalef species richness index
		Fungi	EPF	Fungi	EPF			
Kakching	10	10	7	100	70	0.1905	1.27	1.542
Kamjong	10	3	2	30	20	0	0.693	1.443
Senapati	10	13	11	130	110	0.1455	1.642	2.085
Ukhrul	10	6	6	60	60	0	1.792	2.791
Bishnupur	10	7	4	70	40	0.1667	1.04	1.443
Churachandpur	10	10	9	100	90	0.1111	1.581	1.82
Chandel	10	4	3	40	30	0	1.099	1.82
Imphal East	10	5	5	50	50	0.4	0.673	0.6213
Imphal West	10	8	5	80	50	0.1	1.332	1.864
Tengnoupal	10	7	2	70	20	-	-	-
Total	100	73	54	-	-			
Average				73*	54*	0.1236	1.236	1.714

revealed a greater occurrence of EPF in organic farms compared to conventional farms, specifically *Aspergillus sp., Beauveria sp. and Gliocladium sp*. exclusively in organic soils.

CONCLUSION

In conclusion, this study focused on the isolation and characterization of fungi, particularly entomopathogenic fungi, from various soil samples of Manipur. The findings revealed a moderate diversity of fungi in the soils of Manipur. Among the isolated fungi, certain strains exhibited excellent performance in terms of pathogenicity against wax moth larvae, resulting in 60-100%. It was also observed that different isolates of the same fungi displayed varying levels of performance in the preliminary pathogenicity test. This suggests that each isolate possesses its own unique potential in controlling insect pests. Therefore, the isolation of local strains of entomopathogenic fungi is recommended for effective pest control in the specific region. Overall, this study highlights the importance of understanding the diversity and potential of entomopathogenic fungi in the context of microbial pesticides for insect pest management. Further research and exploration of local strains can contribute to the development of more efficient and region-specific pest control strategies.

Conflict of interest: None.

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