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## ISOLATION OF POTENTIAL ENTOMOPATHOGENIC FUNGI FROM TEA SOIL OF DIBRUGARH AND TINSUKIA DISTRICT OF ASSAM

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#### ABSTRACT

Tea garden soil extensively supports monoculture plantation of tea plant and there is comparatively little information about the distribution of spores of entomopathogenic fungi within the studied site. The entomopathogenic fungi are promising natural agents that can parasitize a wide range of insect species and most entomopathogenic fungi have several variants and each strain is adapted to various hosts, causing natural infections among insects of many different species. Hence, soil samples from selected tea gardens were qualitatively analysed to find the occurrences of entomogenous fungi in the tea plantation areas which can be used as a bio-control agent against tea pests.

Keywords: Entomopathogenic fungi, Saprophyte, Occurrence.

#### 1. INTRODUCTION

The entomopathogenic fungi are promising natural agents that can parasitize a wide range of insect species and primarily belong to the order Hypocreales of the family Ascomycota (Doberski and Tribe 1980 and Bidochka et al. 1998). The pathogenic nature of such fungus was first identified in the 19th century and gradually entomopathogens like Aspergillus spp., Beauveria spp., Entomopthora spp., and Metarhizium spp., became important part of integrated pest management of many crops (Roberts and St. Leger 2004; Bidochka et al. 1994, Bidochka et al. 1998 and Vey and Butt 2010). These entomopathogenic fungi are found in India belong to the sub-division Zygomycotina. Most entomopathogenic fungi have several variants and each strain is adapted to various hosts, causing infections among insects of different species (Doberski and Tribe 1980, St. Leger et Bidochka al. 1992 and et al. 1994). Most entomopathogenic fungi can exist as saprophytes in the and isolation and establishment of soil such entomopathogenic fungi is important for agricultural research and biotechnology. The fungus stock maintenance is necessary to support research on fungi for effective bio-control of insects. The survivability of the conidial spore and its competitive saprophytic ability can profoundly influence its virulence. Several studies showed that the strains of a fungus can be distinguished by their different levels of enzymatic action to invade and affect the host body (Bridge *et al.* 1990 and Varela). Entomopathogenic fungi are cosmopolitan in nature, but there is comparatively little information about the distribution of spores of these fungi within the soil of tea plantation areas. Thus, this paper aims to survey garden soil samples in search of occurrences of entomogenous fungi in the tea plantation areas. The soil-inhabiting entomopathogenic fungi isolated could be used as a biocontrol agent against tea pests.

#### 2. MATERIAL AND METHODS

#### 2.1. Collection of soil sample

Soil samples were collected from two districts of Assam, viz. Tinsukia  $(27.4886^{\circ}N, 95.3558^{\circ}E)$  and Dibrugarh district  $(27.4728^{\circ}N, 94.9120^{\circ}E)$  of Assam. The list of the selected tea gardens is presented in Table 1. The soil from 0-15 cm was taken using a soil auger. Ten samples were taken in a garden and were mixed into a composite sample then stored in a sterilized polythene bag for analysis.

### 2.2. Preparation of culture media

Potato dextrose agar media was prepared in liquid form which later solidifies and five replicates were made. After distribution, the flask was plugged in and sterilized in the autoclave at 15 PSI for 15 minutes. Then the medium

containing the flask was kept for cooling (at room temperature) for utilization in further use.

Table 1: List	of the	tea gardens	from 7	Finsukia	and	Dibrugarh	districts	where s	soil sample	s were	taken
from		-				-			-		

Code	Tea Garden of Tinsukia district, Assam	Code	Tea Garden of Dibrugarh district, Assam
T1	Powai Tea Estate	D1	Manohari Tea Estate
T2	Namdang Tea Estate	D2	Greenwood Tea Estate
T3	Raidang Tea Estate	D3	Khanikor Tea Estate
T4	Dehing Tea Estate	D4	Tea garden of Dibrugarh University
T5	Sri Krishna Tea Estate	D5	Bokel Tea Estate
T6	Bogapani Tea Estate	D6	Muttuck Tea Estate
Τ7	Bordubi Tea Estate	D7	Mohanbari Tea Estate
Т8	Beesakopie Tea Estate	D8	Jalan South Tea Estate
		D9	Lepetkata Tea Estate
		D10	Mokalbari Tea Estate
		D11	Maijan Tea Estate
		D12	Sessa Tea Estate



Fig. 1 (A-B): Collection of soil sample

## 2.3. Sample analysis for presence of desirable fungus

#### 2.3.1. Inoculation and Incubation

One gm of soil sample was weighed and mixed with 10ml of Sterilized neutralized distilled water. A 1/10 dilution series was prepared accordingly by transferring 1 ml from  $10^{-1}$  dilution to 9ml neutralized distilled water and so on up to  $10^{-5}$  dilution. Since the soils may have high microbial population, the following dilutions of  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$ , g soil per plate was considered. 1 ml from each dilution was poured in each plate. The different media; Potato Dextrose Agar, Sabouraud Dextrose Agar and Rose Bengal Agar (Bordoloi *et al.*)

2012 and Martin 1950) was sterilized in Autoclave at 121°C for 15 min. When the media substantially cooled, streptomycin sulphate was added to each media before pouring on plates. Antibiotics inhibit the bacterial growth. Traditional SDA have acidic pH (5.6) that inhibits the growth of bacterial colony. RBC is already loaded with chloramphenicol so adding of streptomycin sulphate was avoided. The media was then poured and evenly distributed in the plate and allowed to solidify. The sealed plates were placed in the incubator at  $28\pm2°$ C and accordingly the fungal density was determined.

#### 2.4. Identification of fungi based on morphological characteristics

The pure culture of fungal isolates was maintained on a petri-plate with sterilized Potato Dextrose Agar (PDA) and sporulated fungal isolates were subjected to lactophenol cotton blue (LPCB) for identification. Preliminary identification was made by comparing the morphological characteristics mentioned in the manual of soil fungi (Ali-Shtayeh *et al.* 1998). The microscopic characteristics like mycelium, shape and length of hyphae, condiospore structure and shape, diameter, size and pattern of spore attachments were observed under binocular microscope at 10X and 45 X magnifications.

### 2.5. Identification

The entomopathogenic fungi culture re-isolated from cadavers was distinguished by the morphological characteristics and the isolates were sent to Indian Type Culture Collection (ITCC), Delhi for identification and sequencing of 16S RNA.

# 2.6. Morphological Identification of fungal isolates

Fungal pathogens isolated and grown on PDA plates for about ten days were used for morphological identification. The cultures were identified based on their colony features and microscopic observations were made. Initial identification was done based on the shape of the colony as well as the spores and the size of the spores. The growth parameters like radial growth, spore count and days taken to cover up the full plate were scrutinized.

### 2.6.1. Colony appearance

The shape, color and type of mycelia of fungal isolates were recorded using 20 days old plate culture (PDA medium).

### 2.6.2. Shape and length of conidia

Fifteen days old fungal culture was used to prepare the conidial suspension. The shape and size of the conidia was measured using the Mag Vision software.

### 2.6.3. Mycelial growth

The mycelia growth was assessed on sterilized PDA, PDAY, SBD, SBDY and RBC. A 10-day-old unsporulated mycelia mat of BPA/B7 was cut by day with a 5cm diameter and gently placed on the respective media in inverted position. The plates were incubating at  $28\pm2^{\circ}C$  and the measurements were recorded at intervals. All the observations were replicated 5 times.

## 2.7. Mortality test

The bioassay was conducted against both the adults and the nymphs' stage of *Helopeltis theivora*. Ten adults per treatment with three replications were taken with CRD under laboratory conditions. The insect were kept for starvation for 2 hours before the application. TV1 shoots were kept in the vile; sprayed with fungal strains and placed inside the glass chimney. The insect were transferred immediately. The percentage of insect mortality was calculated by the formula (Indriyanti *et.al.*2017) given below:

The percentage of insect mortality was calculated by the following formula:

## Percentage of deaths = $\Sigma M / (\Sigma M + \Sigma H) \ge 100\%$

 $\mathbf{M} = \text{dead } Helopeltis sp.$ 

 $\mathbf{H} =$ alive *Helopeltis sp.* 

#### 3. RESULTS AND DISCUSSION

### 3.1. Isolation and Identification of entomopathogenic fungus

T1, T3, T5, T6 and T7 have a percentage of fungal cycle's occurrence (12.50). Minimum numbers of fungal colonies were recorded from T2 followed by T4 with 10.65 and 12.01 numbers of colonies. From the total fungal isolates recorded in soil samples of the selected gardens of Tinsukia region, 47.51% were unidentified and from the known fungal isolates, 20.85%, 14.05%, 12.94%, 1.91, 3.27 and 0.09 were *Aspergillus sp., Tricoderma sp., Fusarium sp., P.lilacinus* and, *B. bassiana* respectively. *Penicillium sp* and *Phytopthora sp* were not found in the samples collected from the selected gardens of Tinsukia.

The mean total number of fungal colonies found in the soil samples collected from Dibrugarh is 12.14. The mean number of fungal colonies is found to maximum in D12 with 13.35 colonies in the soil and a minimum mean of 8.62 followed by 11.90 isolates in D10 and D9 respectively. Soil samples D2, D3, D4, D5 and D11 have similar mean numbers  $(12.50\pm2)$  of colonies. Out of the total fungal isolates recorded from the collected samples of the selected gardens of Dibrugarh, 41.26% were unidentified and from the known fungal isolates, 22.21%, 17.95%, 7.21%, 5.19%, 3.27% and 0.09% were Tricoderma sp., Aspergillus sp. Fusarium sp., Phytopthora sp., P.lilacinus and Penicillium sp.

respectively. B. bassiana was not found in the samples collected from the selected gardens of Dibrugarh.

The fungi shown in Fig. 2 and Fig. 3 were identified from the soil samples studied. The suspects were further observed and the isolates showed morphophological characteristics similar to entomopathogenic fungi. The fungi were sent to Indian Type Culture and Collection (ITCC), New Delhi for identification. The fungal isolates were identified at ITCC, New Delhi by Dr. Pameela Devi and Dr. Deeba Kamil. We found two beneficial fungal isolates isolated from D1, D9 and T5 soil samples which are listed in the table 4.



Tricoderma sp.

Paecilomyces lilacinus

#### Fig. 2: The fungi identified from the soil samples

Table 2: Occurrence% of fung	al isolate recorded fr	om the collected soil sam	ples of Tsoil

	Fungal isolate											
Soil	Beauveria	Paecilomyces	Aspergillus	Tricoderma	Fusarium	Phytophthora	Penicillium	Unknown				
	bassiana	lilacinus	sp.	sp.	sp.	sp.	sp.	UIIKIIOWII				
T1	0	0	44	24	10	0	0	22				
T2	0	0	35.19	5.56	12.96	0	0	31.48				
T3	0	0	22.22	14.58	11.11	0	0	52.08				
T4	0	0	5.88	19.61	19.61	0	0	50.98				
T5	0.64	0	34.39	15.29	7.64	0	0	42.04				
T6	0	13.33	0	8.33	16.67	0	0	61.67				
T7	0	0	4.2	10.92	12.61	0	0	72.27				
T7	0	0	20	0	4	0	0	18				
Mean	0.0796	1.6667	20.736	12.286	11.825	0	0	43.82				

Table 3: The occurrence % of fungal isolate recorded from the collected soil samples of Dsoil

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_	Fungal isolate										
Soil	Beauveria	Paecilomyces	Asparaillus sp	Tricoderma	Fusarium	Phytophthora	Penicillium	Unknown			
	bassiana	lilacinus	Asperginus sp.	sp	sp.	sp.	sp.	unknown			
D1	0	39.13	21.74	29	0	0	0	8.7			
D2	0	0	34.86	45.45	19.68	0	0	0			
D3	0	0	38.35	0	0	0	0	61.65			
D4	0	0	4.32	5.36	2.83	0	0	87.35			
D5	0	0	0	16.64	16.64	2.36	0	64.29			
D6	0	0	15.38	43.62	5.15	0	0	35.92			
D7	0	0	17.65	5.88	0	0	0	74.53			
D8	0	0	20.62	0	0	0	0	77.76			
D9	0	0.024	2.33	6.98	0	0	0.97	84.88			
D10	0	0	26.67	23.07	19.24	0	0	0			
D11	0	0	0	90.48	9.52	0	0	0			
D12	0	0	33.4	0	13.4	60	0	0			
Mean	0	3.27	17.95	22.21	7.21	5.19	0.09	41.26			

Table 4: List of entomopathogenic fungi isolated from various tea pests from Tinsukia and Dibrugarh district of Assam

Sl. No.	Tea garden	Culture No.	Pest species	Fungus identified
1	D1	BPA/B1	Aphis gossypii	Paecilomyces lilacinus
2	D9	BPA/B2	Aphis gossypii	Paecilomyces lilacinus
3	Т5	BPA/B7	Wide range of insect pests like <i>H. armigera</i> , <i>P.brassicae</i> , <i>C.sordidus</i> , etc.	Beauveria bassiana



Paecilomyces lilacinus is a pathogenic fungi mainly used in the crop protection as a nematocide.

## Fig. 3: A and B showing Paecilomyces lilacinus and Beauveria bassiana



Fig. 4: Beauveria bassiana of (a) Dooars (West Bengal) and (b) Tinsukia (Assam). (c) Paecilomyces lilacinus growing on media

## 3.2. Morphological identification of entomopathogenic fungi

Two isolates of *Paecilomyces lilacinus* and two isolates of *B.bassiana* which has been isolated from monoculture and polyculture technique in the Plant Pathology Laboratory, TRA, NBRRDC, West Bengal. Macroscopic characters of the colonies that observed were colony color, spore shape, spore size, time required to cover the 5 cm petri dish and mycelium characters. Macroscopic characters of the isolates have been mentioned in the Table 5.

Examinations of the morphological characteristics showed no significant difference between the two isolates of B. bassiana isolated from the soil of Tinsukia (Assam) and Dooars (West Bengal). The color of the isolate was white, smooth and powdery, however difference was noticed on spore size and time required covering the petri-plate, BPA/B7 has smaller spore size and required more time to cover the petri-plate than BKN-20. When growth parameters were compared, it was noted colony formed by local strain is more whitish and has faster generation rate that that of the commercial one. The characteristics of these two isolates of B. bassiana are related to Kulu et al. 2015. P. lilacinus is white and smooth texture with round spore ranging from  $3.52 \pm 0.22 \ \mu m$  in size. It is fast growing fungus which covers the petri-plate of 5cm diameter within 7 days.

The mean mycelial dry weight was observed to increase between the days of incubation with 6.94 mg/50ml, 7.94 mg/50ml and 10.94 mg/50ml between 7th, 14th and 21th days of incubation. The maximum mean mycelial dry weight was recorded in *Paecilomyces lilacinus* 

(9.54 mg/50ml) followed by BKN 20 (8.33 mg/50ml) and the minimum mean mycelial dry weight was recorded in BPA/ B7 (7.95 mg/50ml). On the 7th day of incubation, the maximum mean mycelial dry weight was recorded in Paecilomyces lilacinus (7.21 mg/50ml) followed by BKN 20 (6.97 mg/50ml) and the minimum mycelial dry weight was recorded in BPA/B7 (6.66 mg/50ml). On the 14th day of incubation, the maximum mycelial dry weight was recorded in Paecilomyces lilacinus (8.41 mg/50ml) followed by BKN 20 (8.02 mg/50ml) and the minimum mean mycelial dry weight was recorded at BPA/ B7 (7.41mg/50ml). Similar trend was followed by the end of 21 days of incubation with Paecilomyces lilacinus (13.02 mg/50ml) having the highest mycelial dry weight, followed by BKN 20 (10.01 mg/50ml) and the minimum was noted at BPA/ B7 (9.80 mg/50ml).

Dry mycelial weight and mycelial growth were observed to be significantly different with maximum mycelial growth of 37.8 mm followed by 30.0 mm in BKN 20 and BPA/B7 respectively. The mycelial growth of *Paecilomyces lilacinus* was recorded as a minimum of 29.3mm only though, the highest dry weight, suggesting less water content compared to BKN 20 and BPA/B7. Similarly, differences were observed between the dry mycelial weight and the conidial germination rate only, and *Paecilomyces lilacinus* was recorded to have only 78.2% germination rate followed by BPA/B7 with 74. A germination rate and a maximum germination rate of 81.6%. However, a significant difference was observed in isolated mycelial growth and conidial production and *Paecilomyces lilacinus*. The conidial production was found to be inversely proportionate to that of mycelial dry weight. The BPA/B7 has a low mycelial dryweight and the average mycelial diameter showed a highest production of 2.68 g conidia/100gm of fresh mycelial weight. However, *Paecilomyces lilacinus* with the maximum mycelial dry weight had a minimum conidial production of 1.04 g conidia/100gm of fresh mycelial weight.

Table 5: Morphology an	d Characterisation	of entomopatho	genic fungi (	$(* Mean value \pm SE of mean)$

Isolate	Colony color	Spore shape	Texture	Spore size	Age (Day)	Mycelium characteristics
(a) BKN-20	White	Ovoid	Smooth	$2.4\pm0.12~\mu m$	10	Hyphae hyaline (clear), smooth walled bearing groups of swollen lateral cell and growth pattern is dispersed and dense with raised elevation.
(b) BPA/B7	White	Ovoid	Smooth	3.63 ± 0.68 μm	15	Hyphae hyaline (clear), smooth walled bearing groups of swollen lateral cell and growth pattern is dispersed and dense with low elevation.
(c) Paecilomyces lilacinus	White	Round	Smooth	$3.52\pm0.22~\mu\mathrm{m}$	7	Phialides are swollen at their bases, gradually tapering towards apices. Hyphae hyaline, conidia are produced in basipetal succession from Phialides and conidia are unicellular.

Table 6: Determination of dry and fresh weight, germination rate and mycelial growth and conidia production

	Dry	weight (mg/	′50ml)	Germinat	Mycelial	Conidia	
Sample	7 DAI	14 DAI	21 DAI	Mean	ion rate (9 DAI)	Growth (mm)	Production (g conidia/ 100gm)
(a) BKN-20	6.97±1.23	$8.02 \pm 0.5$	$10.01 \pm 2.02$	8.33	81.6±21.0	37.8±0.19	1.78
(b) BPA/B7	6.66±1.00	7.41±1.4	9.80±1.98	7.95	74.1±1.5	$30.0\pm0.06$	2.68
(c) Paecilomyces lilacinus	7.21±0.97	8.41±1.35	13.02±1.55	9.54	78.2±2.0	29.3±0.23	1.04
Mean	6.94	7.94	10.94	-	-	-	-

\* Mean value  $\pm$  SE of mean

3.3. Mortality trials of entomopathogenic fungi The efficacy trials of different isolates of B. bassiana showed that BKN-20 and commercial formulation started to kill the insect by the 3<sup>rd</sup> day of application causing 20% and 16.67% mortality of host insect. BPA/B7 has been reported to cause 33.33% mortality by the 4<sup>th</sup> day of application. The mean mortality percent was maximum on BPA/B7 followed by BKN-20 with 30.56% and 24.44% mortality respectively and commercial formulation was recorded with 17.22% mean mortality percent. The mean mortality percent was minimum on 72h with 12.22% mortality and maximum on 144h with 63.33% mortality. M. anisoplae cause a mortality of 3.33% after 5 days of treatment and P. lilacinus was observed to be non-virulent to H. theivora.



Fig. 5: Bio-efficacy and screening of fungal isolates against *H. theivora*.



## Fig. 6: Mycelial growth of pathogenic fungi on insect cadaver

#### 4. CONCLUSION

Soil pH is an important factor in a healthy and fertile soil and all samples' pH were within the optimum range of 4.5-5.5 with slight variation in some samples which is considered ideal for the growth of soil fungi and pH lower than 4 is inappropriate for fungal growth. Several fungi were isolated of which two were identified as pathogenic fungi, Beauveria bassiana and Paecilomyces lilacinus The entomopathogenic fungi that are normal habitants of soil have greatly reduced and low occurrence of entomopathogenic fungi has been observed in the present study. Similar result was reported by researchers like Bidochka et al. 1998 that B. bassiana occurs abundantly in natural habitats than in agriculture and *M. anisopliae* is more abundant in cultivated area. There can be several factors affecting the survival of entomopathogenic fungi in soil, like the presence of pesticides or competitive soil microbes (Daoust and Pereira 1986 and Lingg and Donaldson 1981). Tea gardens used different synthetic chemicals for the management of different pest and pathogens and often indiscriminately used and some chemicals like quinalphos, fenzaquin or hexaconazole are fungistatic to the growth of B. Bassiana. Such sensitivity of the

respective entomopathogenic fungi and its compatibility with other fungi, organic oils and synthetic pesticides affects the occurrence of entomopathogenic fungi in the soil.

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## Conflict of interest

None declared

## Source of funding

None declared

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