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Cordyceps javanica-An indigenous microbial agent for controlling *Helopeltis theivora*

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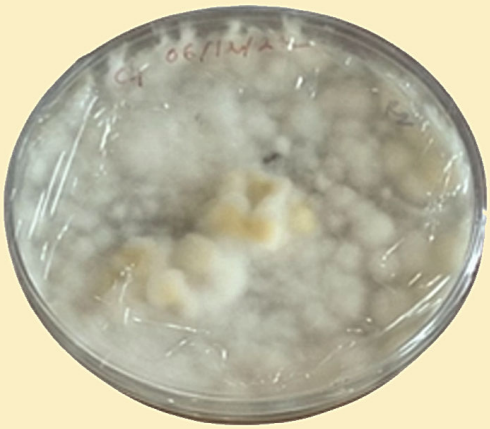

DST- ANRF (SERB) Project- "Exploration and development of bio-formulation from indigenous strain of entomopathogenic fungi, ***Cordyceps javanica*** against tea mosquito bug, *Helopeltis theivora* Waterhouse a devastating pest of tea plantation of North East India"

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Preface

The significance of entomopathogenic fungi in integrated pest management has been brought to light by the hunt for environmentally safe and sustainable substitutes for chemical pesticides. *Cordyceps javanica* has become one of the most promising biological control agents among them, because of its broad host range and pathogenic efficiency. In this study, an indigenous strain (OM321438) that is effective against *Helopeltis theivora*, a significant sucking pest of tea plantations, was characterized.

The characterization of indigenous fungal strain both morphological and molecular level (ITS rRNA sequencing, Gene bank accession number OM321438) and its effectiveness were thoroughly studied in this monograph. Additionally fungal biological parameters like radial growth, conidial density, germination percentage, and colony forming units were observed to characterize the effectiveness of the fungus. To make it easily accessible to public along with its long-term preservation and authentication the culture was deposited at ICAR-National Bureau of Agriculturally Important Microorganisms and deposit number is NAIMCC-F-04639.

The results highlighted- how a native microbial agent can be used to develop environmentally friendly bio formulation in tea pest management.

Authors

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The authors offer deepest sense of gratitude of Dr. Bidyut Chandan Deka, Honorable Vice Chancellor, Assam Agricultural University, Jorhat for offering kind help and cooperation to the project team.

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Contents

INTRODUCTION:

Cordyceps javanica (Frieder. & Bally) (OM321438) a newly identified indigenous strain of entomopathogenic fungi (EPF), effective against major sucking pests of tea, tea mosquito bug (TMB), *Helopetis theivora* Waterhouse (Heteroptera: Miridae). This fungus caused up to 80% mortality of adults TMB at a concentration of 1 × 10 conidia/ml on fifth day after treatment. A total of 680 species of *Cordyceps* have been documented across various climatic regions (Mahadevakumar, & Sridhar 2024) and has been recognized as a noteworthy EPF strain due to its effectiveness against wide host range from numerous orders, including Araneae, Blattodea, Coleoptera, Dermaptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Mantodea, Odonata, Orthoptera, and Phasmatodea (Evans 1982; Wang and Yao 2011). Approximately 60% of *Cordyceps* species are associated with Coleoptera and Lepidoptera, with more than 95% of hosts in these groups being larvae, which makes accurate host identification more challenging (Shrestha et al. 2012, 2016). In contrast, hosts from other insect orders are predominantly adults, including spiders, cockroaches, termites, flies, earwigs, cicadas, bugs, scale insects, coccids, ants, bees, wasps, grasshoppers, mantises, dragonflies, crickets, and stick insects (Evans 1982).

The infection process of *Cordyceps* starts after attachment of conidia or spore with insect cuticle. Under suitable conditions; conidia germinate and produce digestive or cuticle degradative enzymes, enabling the penetration of fungal mycelium to the insect cuticle layer. Inside the insect body cavity the growing mycelium will disrupting the vital systems viz the digestive and circulatory systems and produces toxins that ultimately result in the insect pest's death.

Classification of *Cordyceps javanica:*

Kingdom: Fungi

Phylum: Ascomycota

Class: Sordariomycetes

Order: Hypocereales

Family: Cordycipitaceae

Genus: *Cordyceps*

Species: *javanica*

ISOLATION AND PURE CULTURE:

Isolation of fungal biocontrol agent from insect cadavers

• Naturally occurring cadaver of leaf roller, *Archips* sp. (Lepidoptera: Tortricidae), a pest collected from a Lac host plant (*Flemingia semialata*),

"Regional Lac Insect Field Gene Bank" under Dept. of Entomology, AAU, Jorhat-13.

- Each cadaver was cut into small pieces, each piece measuring about 0.5 to 1 mm in length and these pieces were then surface sterilized using 1% sodium hypochlorite solution ($NaOCl_2$) for 30 sec.
- The sterilized pieces were then transferred to PDA medium (Potato Dextrose Agar) with streptomycin sulfate (60 µg/ml) and incubated them in BOD at a temperature of 26 ±1°C for 5-7 days.
- Observe the plates constantly for growth and development of the associated microorganisms.
- After 5 days, subculture the microbes for purification by selecting the desired colonies.
- Transfer the pure culture to slants or petri dishes following the techniques of single spore isolation or hyphal tip culture.
- The full growth of the cultures on petri plates developed in 14 days (Fig 1).
- The cultures of Cordyceps javanica were yellowish in centre and whitish on the edge

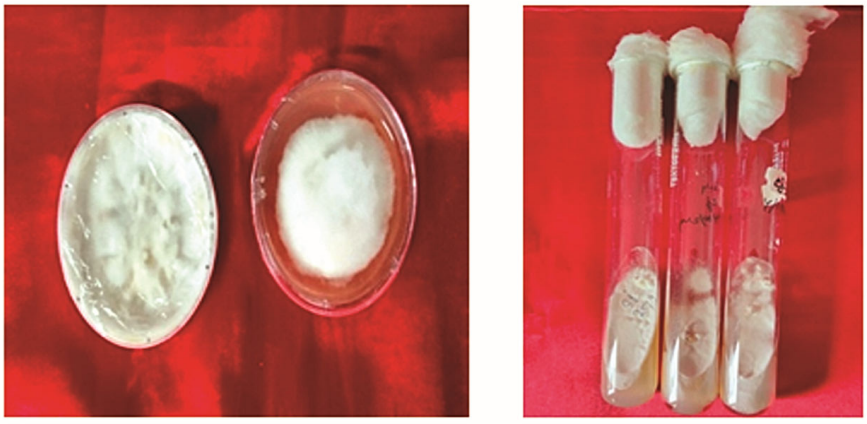

Fig1: Pure culture of *Cordyceps javanica*

MORPHOLOGICAL IDENTIFICATION:

Equipments required visualizing and identifying the structural components of fungi

- Microscope slides and coverslips (Sterile)
- Pure culture plate of fungi (7-10 days old)
- Lactophenol cotton blue stain
- Inoculating needle
- 95% ethanol

Steps in preparation of microscopic slide

- Microscopic slide for the strain was prepared
- A small section of the fungal strain from 15 day old culture plate was taken on a slide with sterilized needle
- Stained the fungal strain with a drop of cotton blue and placed a cover slip over it carefully avoiding any kind of air bubble to make a clear picture of the fungal morphology.
- The prepared slide was observed under the light microscope at 40X (Fig.2).
- Conidial color, shape of conidiophore and presence or absence of septation were recorded

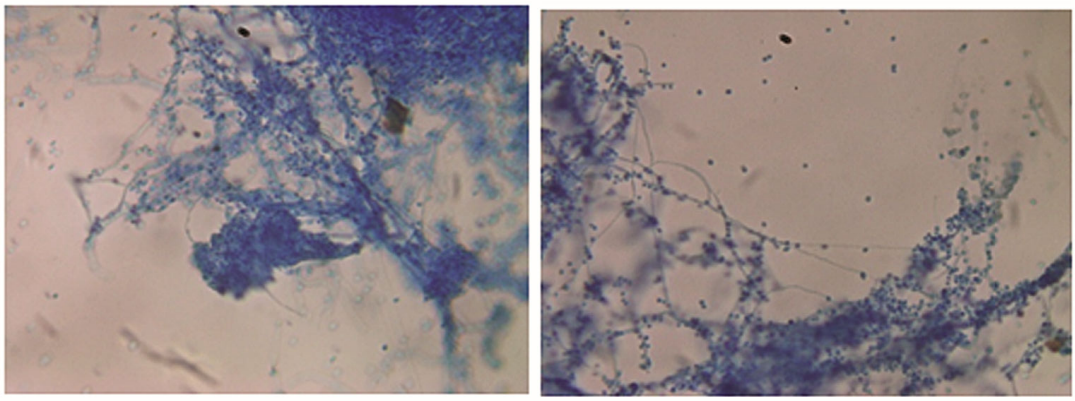

Fig 2: Conidia and conidiogenous cells of *Cordyceps javanica* (under 40X)

BIOLOGICAL PARAMETERS OF EPF

i. Radial growth

Materials required to measuring the colony diameter of the given fungus

- Fungal culture
- Sterile Petri dish (90 mm)
- PDA
- Cork borer

Procedure:

- 0.8 mm diameter circular disc of the EPF is cut using a cork borer, from 14-day old culture onto a fresh PDA medium plate.
- Plates prepared should be sealed with cellophane tapes and Incubated in BOD incubator at 26±1°C temperature.
- The diameter of the colony is measured perpendicularly with scale after 7th, 14th, and 21st day from the day of inoculation of the disc (Fig 3).
- The measurements are done along the same axis each time.
- The full growth of the cultures on petriplates developed in 14 days.

• The cultures of Cordyceps javanica were yellowish in centre and whitish on the edge.

Fig 3: Radial growth

ii) Conidial Density

Steps in to calculate the spore count using Neubauer haemocytometer

- Preparation of a spore suspension.
- 10 µl of spore suspension was pipetted into the haemocytometer.
- Spore count under 10x and 40x magnification.

Fig 4: Serial dilution of EPF

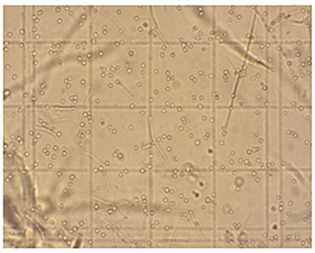

Fig 5: Conidial density of C. javanica under Neubauer haemocytometer (40X)

Results:

Sample	Average number of spores	Spore Concentration
Cordyceps javanica	47.75	19.1 x 10^7 spores/ml =1.91x10^8 spores/ml

The spore concentration of *Cordyceps javanica* at the 10 dilution was determined to be 1.91 × 10 spores/ml. Based on the assessment of total and viable spore counts in the fungal samples, the 10 dilution was found to be suitable for conducting efficacy tests against pests. At higher concentrations (lower dilutions), the spores were too densely packed for accurate counting.

iii) Germination Percentage

Steps to calculate the spore viability in the given samples

• The germination test to evaluate viable conidia was conducted according to the method described by Francisco *et al*. 2006.

- PDA medium was evenly spread onto microscopic slides that had been sterilized with 100% ethyl alcohol.
- The medium on the slides was allowed to dry completely (Fig 6).
- Then, 0.05 ml of the prepared conidial suspension was uniformly distributed over the medium-coated slides.
- The slides were incubated at 26 ±1°C for 24 hours to facilitate maximum germination.
- The germination percentage was determined through direct observation using a phase-contrast microscope at 40x magnification (Fig 7)

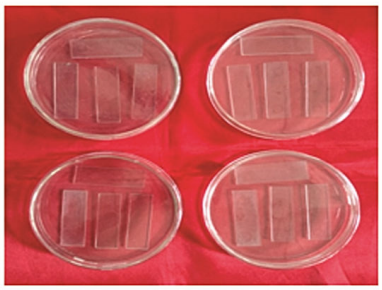

Fig 6: Germination test of EPF

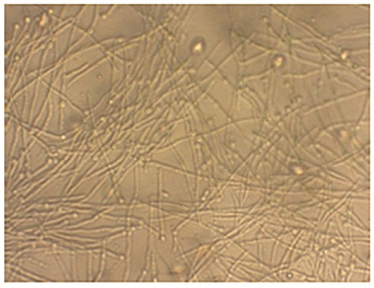

Fig 7: Germinating conidia of *C. javanica* (OM321438) under microscope (40X)

iv) Colony Forming Unit (CFU)

Steps to calculate the number of viable spores in the samples

- A 10 ml stock of spore suspension is prepared from a fully grown (1-month-old) culture of the fungus.
- Sterile test tubes are prepared, each containing 9 ml of Millipore water.
- A 10-fold serial dilution of the spore culture is prepared, starting from 10-1 to 10-9, by adding 1 ml of spore culture to 9 ml of double-sterilized water and mixing thoroughly.
- 10 ml of semi-cool (50-55ºC) molten PDA is poured onto the petri plates.
- 1 ml of spore suspension from each dilution is pipetted on sterile petri plates and spread evenly.
- The petri plates are incubated in a BOD incubator at a temperature of 28±2°C.
- The number of Cordyceps javanica colonies that appear is counted after 48 and 72 hours of plating (Fig 8 & 9).

The results are measured in terms of CFU/ml with the following formula:

$$\text{CFU/ml} = \frac{\text{(Mean plate count x dilution factor)}}{\text{(Volume of culture plated (ml))}}$$

RESULTS:

Name of the fungus	Mean Plate Count	CFU/ml
Cordyceps javanica	24.8	24.8x 10^7cfu/ml =2.48x 10^8cfu/ml

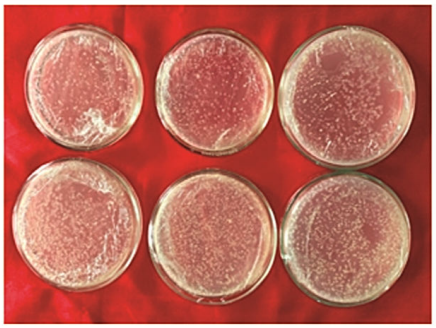

Fig 8: C. javanica in different concentration after 48 hours of plating

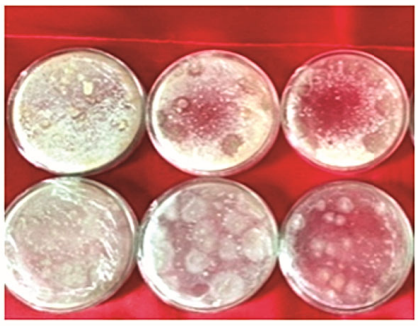

Fig 9: *C. javanica* in different concentration after 72 hours of plating

MOLECULAR CHARACTERIZATION OF EPF

- Isolation of genomic DNA by mini kit protocol
- Polymerase Chain Reaction (PCR)
- Gene sequencing
- Universal primer: ITS rRNA

The PCR amplified products were sequenced and the sequencing results were successfully obtained for one ITS rRNA PCR products. The resultant sequences were aligned with Codon code Aligner and then were analyzed for their homology using nBLAST (NCBI) to obtain any possible identities.

OM321438.1 [organism=Cordyceps javanica] isolate, ITS gene, partial sequence
GGGGGGAAGAGTTTTTCAACTCCCTAACCCTTTGTGAACATACCTATCG
TTGCTTCGGCGGACTCGCCCCGGCGTCCGGACGGCCCTGCGCCGCCCG
CGACCCGGACCCAGGCGGCCGCCGGAAGACCCACAAATTCTGTTTCTA
TCAGTCTTTCTGAATCCGCCGCAAGGCAAAACAAATGAATCAAAACTT
TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGC
GTCATTTCAACCCTCGACACCC

BLAST results of Archips sp. isolate displayed 99.43 per cent homology with Cordyceps javanica strain BCC01857. We received the accession number for Archips sp. isolate on submission of DNA profiling data and it was assigned as OM321438.

SEQUENCE ANALYSIS: BASIC LOCAL ALIGNMENT SEARCH TOOL

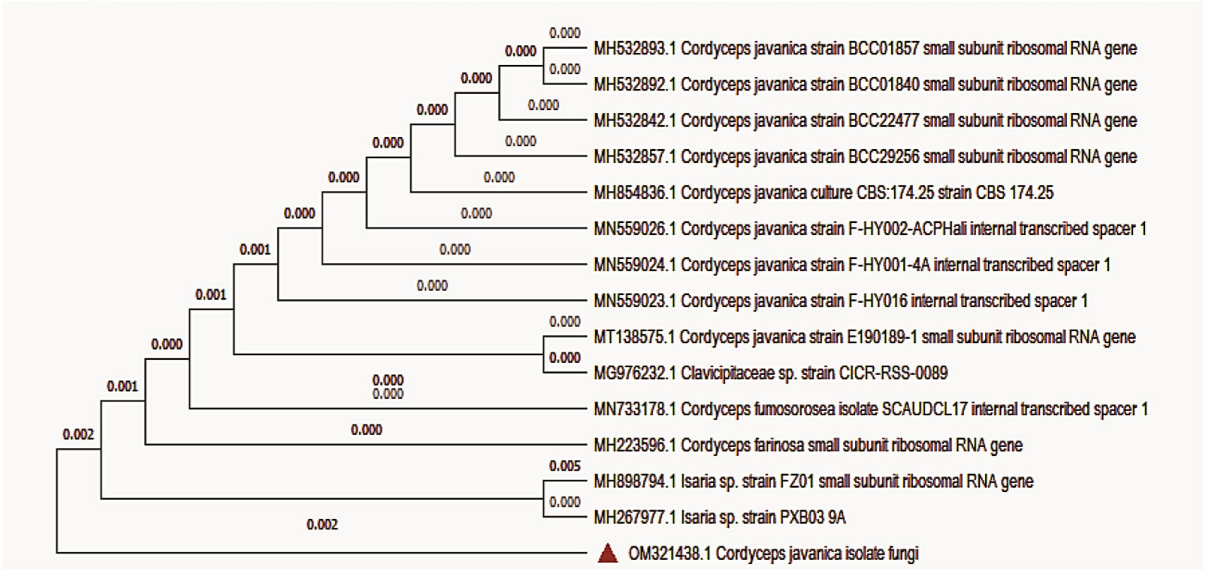

Fig 10: Phylogenetic tree of OM321438.1 sequence and its closest homologs inferred using Neighbor-Joining method in MEGA 11.

The fungus *Cordyceps javanica* (OM321438) was deposited at the National Agriculturally Important Microbial Culture Collection (NAIMCC), ICAR-National Bureau of Agriculturally Important Microorganisms, Mau, for preservation and authentication and was assigned the accession number NAIMCC-F-04639.

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