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IDENTIFICATION AND TOXICITY EVALUATION OF *BEAUVERIA* SP. ASSOCIATED WITH WHITE MUSCARDINE DISEASE IN MUGA SILKWORM *ANTHERAEA ASSAMENSIS* HELFER

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ABSTRACT

The present study is an effort to isolate and identify the *Beauveria* sp. from white muscardine infected *Antheraea assamensis* Helfer (muga silkworm) larvae collected from rearing fields. The isolate was subjected to morphological identification followed by DNA barcoding analysis by sequencing the ITS1-5.8S-ITS2 region. The isolate was identified morphologically up to genus level. For molecular identification, the DNA was first isolated, amplified by using PCR followed by sequencing the ITS region. A phylogenetic tree was also constructed based on the data obtained from sequencing to trace the evolutionary history of the isolated fungus. The fungal species was identified as *Beauveria bassiana* with 98.6% sequence similarity with already documented *B. bassiana* strains. The toxicity of the isolated fungus was also evaluated against *A. assamensis* larvae. The LC_{50} value was determined at 96 hr and recorded at $1.1x10^8$ spore/ ml concentration.

Key words: *Beauveria* sp., *B. bassiana*, *Antheraea assamensis*, morphology, ITS region, BLAST, sequence, phylogenetic analysis, LC_{50}, white muscardine disease, hyphae, conidia, instar

The production of globally popular glittering golden muga silk spun by the muga silkworm (*Antheraea assamensis* Helfer) is the cultural heritage of Assam since ages and Assam contributes about 95% of India's total production of muga silk (Das et al., 2023). This is an agro-based cottage industry in Assam which contributes immensely to the rural economy (Rabha and Saikia, 2022). As *A. assamensis* is semi-domesticated and have to be reared outdoors during their larval development, they are exposed to a hostile microbial environment conducive for incidence of diseases (Borgohain et al., 2015). *A. assamensis* larvae frequently encounter a fungal infection referred to as White Muscardine Disease (WMD) characterized by loss of appetite, reduced elasticity of the integument of the larvae and as time progress, the larval body is covered by heaps of white fungal hyphae resulting in death of the larvae within 72-96 hr (Das and Das, 2017). The disease occurs during rainy winter when temperature is low (<25°C) and humidity is high (90-95%). Although this disease was not widespread in earlier days, the outbreak of WMD has been increasing over the last few years (Choudhury, 1981; Bora and Saikia, 2022). Das et al. (2014) and Subramanyam et al. (2018) isolated the entomopathogenic fungus *Beauveria* sp. from WMD infected *A. assamensis* and reported, it as the causative pathogen. However, there are no published records of molecular characterization of any of these fungi till now which is crucial for species identification. This study is an attempt to identify the fungal species responsible for causing WMD in *A. assamensis* based on internal transcribed spacer sequencing (ITS) data, which is recognized as a standard DNA barcode marker (Schoch et al., 2012; Bellemain et al., 2010).

MATERIALS AND METHODS

The white muscardine infected mummified 5th instar *A. assamensis* cadavers were collected during Jarua crop (December-January) by observing the presence of white powdery conidial layer on the body surface. The infected larvae were collected from rearing field in Udalguri district, Assam (26.75367° N, 92.10215° E) in 2021. For initial characterization, colony morphology and conidia shapes were considered. The fungus was isolated from the dead 5th instar *A. assamensis* larvae and purified by single spore isolation technique as described by Noman et al. (2018). In order to get the fungal culture, autoclaved potato dextrose agar (PDA) media (Himedia laboratories) was used. The plates with the streaked isolate were studied under Labomed Lx400 trinocular digital microscope to observe the conidia and hyphae by staining it with lactophenol cotton blue (Micromaster Laboratories). The identification of the fungus up to genus level was done as per the fungal identifying criteria by Humber, (1997).

Mycelial mass weighing between 50-100 mg were scraped from the growing edges of the culture and transferred to zymo bead bashing tubes. DNA extraction was carried out using the Quick-DNA™ Fungal Miniprep Kit from Zymo Research, following the manufacturer's instructions. The extracted DNA was quantified spectrophotometrically using Nanodrop (Thermo Nanodrop 1000), with a quality purity assessment performed to ensure a 260/ 280 nm ratio of ≤1.8. For amplification of the rRNA ITS gene region, the ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 primer (5'TCCTCCGCTTATTGATATGC3') pairs were used (White et al., 1990). The polymerase chain reaction (PCR) was performed in a total volume of 50 µℓ, consisting of 1 µℓ of each primer (10 µM), 1 µℓ (50 ng) of genomic DNA, 10 µℓ of Taq-&GO™ mastermix for PCR polymerase, and the remaining volume filled with nuclease-free water. The PCR amplification conditions were set as follows: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 90 sec, primer annealing at 52°C, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The amplified PCR products were visualized on a 1% agarose gel to assess their size and quality. Subsequently, the PCR products were purified from the gel using the Hi Yield Plus Gel/PCR DNA Mini Kit, following the manufacturer's protocol. The purified PCR products were then subjected to sequencing using universal primers (ITS-1 and ITS-4) in 10 µℓ reaction volumes. The sequencing of the ITS region was performed at the laboratory of Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. The quality of sequenced raw data was evaluated, and a consensus sequence was generated from raw data using Bioedit software. The final sequence was subjected to NCBI sequence similarities search by BLASTn in GenBank, at the National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed to understand the evolutionary history. Multiple sequence alignment for the top 50 hits from NCBI blast was carried out using MUSCLE (Multiple Sequence Comparison by Log-Expectation) and MAFFT (Multiple Alignment using Fast Fourier Transform) and refined using the BMGE (Block Mapping and Gathering with Entropy) tool and phylogenetic analysis was reconstructed using iTOL (Interactive Tree of Life) 3.0. (Criscuolo and Gribaldo, 2010; Rozewicki et al., 2019).

For toxicity test, the fungal spore suspension was prepared as described by Bamisile et al. (2020). The number of spores were counted using an improved Neubauer's haemocytometer and serially diluted to get the suspensions from 10^9 to 10^2 spores/ ml. Immediately after fourth moult, the larvae were subjected to topical inoculation through immersion in fungal spore suspensions of nine concentrations at 50 ml/ 100 worms. Larvae dipped in distilled water were used as control. The inoculated larvae were then reared on host plant (som plant). Three replicates with 10 larvae each for each concentration were taken to determine the LC_{50} value. Frequent monitoring was done to notice mortality up to 96 hr after which mean mortality from a particular dose was calculated. For acute toxicity testing, the LC_{50} value was calculated by probit analysis (Finney, 1964) statistical method using IBM SPSS 25.0 version and the linear regression curve with probit mortality plotted in Y-axis and log concentration plotted in X-axis was drawn by using MS Excel 2010.

RESULTS AND DISCUSSION

After seven days, yellowish white coloured round colonies were produced with numerous smooth dry powdery aerial conidia. The most notable characteristic of the isolate observed under microscope is the presence of single celled globose or ellipsoid shaped conidia and branched hyphae. The hyphae are septate and extended into zig zag apical extension, the rachis. The condia exhibited sympodial arrangement at rachis. By noting these characteristics, the isolated strain is recognized as *Beauveria* sp. (Fig. 1). For identification purpose, the isolate was initially subjected to morphological examination both macroscopically and microscopically. Although a few studies have identified *B. bassiana* as WMD causing fungus in *A. assamensis* morphologically, sometimes it can lead to misidentification (Sullivan and Neigel, 2017). Hence, molecular techniques are required (Liu et al., 2000).

The agarose gel electrophoresis yielded approximately 550 bp single DNA band of PCR amplified ITS region of the isolated strain of the fungus. For molecular identification highly conserved rDNA ITS1-5.8S-ITS2 domain was selected for sequencing as it is the most reliable unique DNA barcoding method in case of fungi. The analysis of rDNA ITS1-5.8S-ITS2 sequences by using sequence alignment software resulted in 522 bp consensus sequence. In order to identify the isolated *Beauveria* strain, BLAST search analysis was done based on the ITS sequence data which revealed a 98.6% similarity between our strain and previously documented *B. bassiana* strains confirming it as *B. bassiana.* The sequences

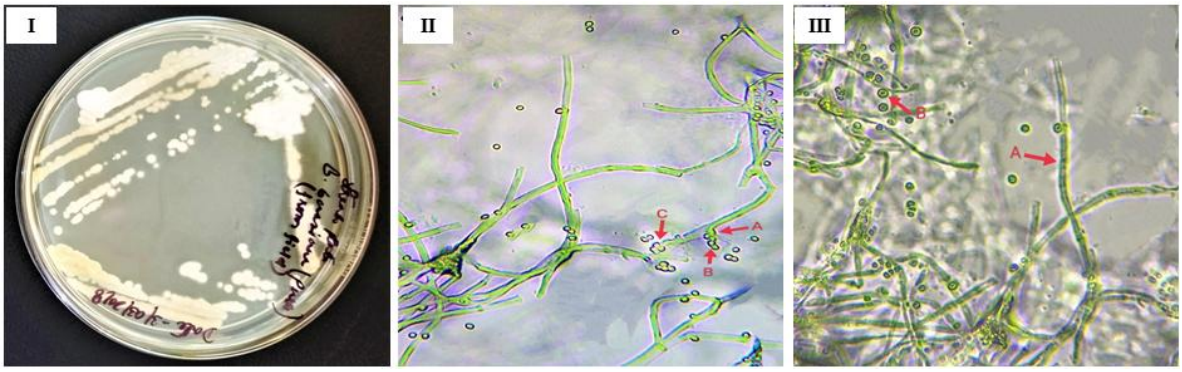

Fig. 1. Morphological characterization of *B. bassiana* isolate (400x)
I. Pure culture of *B. bassiana* on PDA media; II. A. Branched hyphae, B. Rachis,
C. Sympodial arrangement of conidia; III. A. septate hyphae, B. single celled globose conidia

thus obtained were submitted to NCBI (National Center for Biotechnology Information) GenBank and accession number OQ954799 is assigned to the isolate. To illustrate the evolutionary relationship of the isolated *Beauveria* species, phylogenetic analysis was done based on the ITS1-5.8S-ITS2 sequence homology and presented in Fig. 2. The homology search was explored based on BLAST analysis with 50 other closely related fungal species from NCBI genbank with 1000 bootstrap runs. Fig. 2 depicted a rooted bootstrap consensus phylogenetic tree with origin from branch holding the accession number MN544934.1. Strain MZ374514.1 was aligned with our isolated strain (OQ954799.1) forming a separate clade, having the same branch length and can have similar evolutionary origin with strong bootstrap support. Strain having Accession no MT529046.1, KU363833.1, OW988164.1, MT528756.1, MN727364.1 also fall under same clade providing strong evidence towards the same lineage. Although the tree topology analysed phylogeny by aligning homology search with 50 different isolates which are all *B. bassiana* and originated from the same root, the low bootstrap supports suggest intraspecific variations which could be due to the differences in geographical locations from where the strain has been isolated (Bidochka et al., 2002; Gasmi et al., 2021). Previous studies have stated that the ambient environmental factors can act as driving force that can lead to the genetic variations in *B. bassiana* (Ghikas et al., 2010; McGuire et al., 2020). Other factors may include differences in sequencing algorithm or due to the sequence differences probably because of the accumulation of mutation over time (Kimura, 1983). However further investigations are required for identification of different *B. bassiana* ecotypes and to dig out the relationships among them. Thus in the present study, one fungal strain has been isolated from WMD infected *A. assamensis* larvae and identified as entomopathogenic fungus *B. bassiana* that belongs to class: Sordariomycetes, order: Hypocreales and family: Cordycipitaceae.

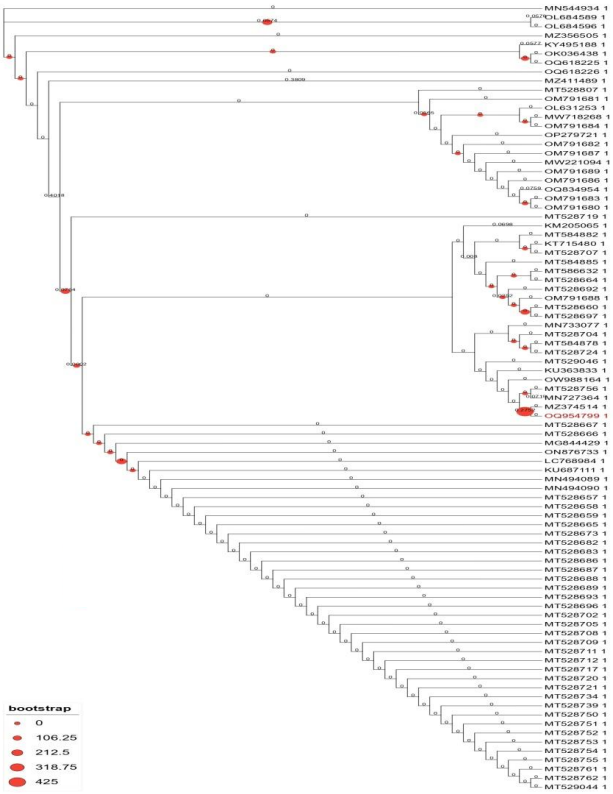

Fig. 2. Molecular phylogeny of *B. bassiana* based on ITS region sequence homology.
(Multiple sequence alignment for the top 50 hits from NCBI genBank with 1000 bootstrap runs was carried out)

The mortality response of the silkworm larvae were increased with the increase in time and concentration of *B. bassiana* spores indicating a direct proportional relationship of mortality with time and concentration.

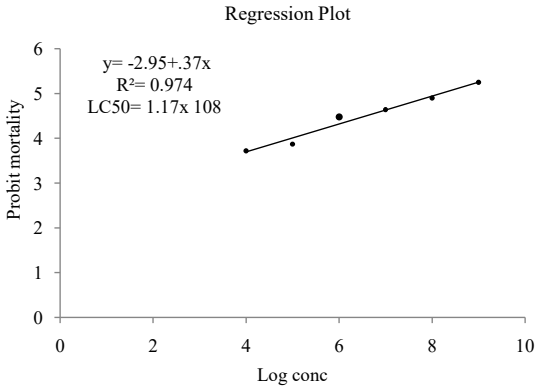

Fig. 3. Regression plot of probit mortality and log concentration for *B. bassiana* spore to *A. assamensis* 5th instar larvae

No mortality was recorded in control group. Among the *B. bassiana* spore treated groups, no mortality was recorded at 1 x 10^2 spore/ ml and 1 x 10^3 spore/ml concentrations. The LC50 value of *B. bassiana* spore on the silkworm larvae was noticed at 1.1 x 10^8 spore/ ml concentration (Fig. 3). Toxicity analysis of *B. bassiana* has not been carried out in *A. assamensis* so far. Results obtained in this study are quite synonymous with that of the previously conducted toxicity test in *B. mori* (Chavan et al., 2015). Earlier workers found out LC_{50} values for *B. bassiana* against *B. mori* 5th instar larvae. Chavan et al. (2015) and Rajitha and Savithri (2015), onserved the LC_{50} values at 1x10^5 and 2.15x10^6 spore/ ml, respectively. Another study revealed this as 1x10^6 spore/ ml (Devi, 2015). The present study recoded the LC_{50} value as 1.17x10^8 spore/ ml which indicated that 5th instar *A. assamensis* larvae are somewhat less susceptible to *B. bassiana* than *B. mori* in 5th instar larvae.

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AUTHOR CONTRIBUTION STATEMENT

SS and MN conducted the experiments and analysed data. SK and DK assisted in preparing the manuscript and SB designed the research work.

CONFLICT OF INTEREST

No conflict of interest.

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