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Isolation and Molecular Characterization of Entomopathogenic Fungus, *Beauveria bassiana* Isolated from Rice Bug, *Leptocorisa* spp. (Hemiptera : Alydidae)

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ABSTRACT

The rice bug, *Leptocorisa* spp. is one of the most important sap sucking insect pests of rice in the tropics. Both nymphs and adults suck sap from developing rice grains during the milky grain stage, leading to discolored, empty, or half-filled grains. Biological control of rice bug remains hampered by absence of ideal bioagents. Entomopathogenic microbes, particularly fungi, have been receiving increased attention of late as potential biocontrol agents of the rice earhead bug. The entomopathogenic fungus (EPF), *Beauveria bassiana* (Bals.) has been reported as infecting rice bug, from several parts of the world, including India. It can manage various insect pests. They are ideally suited as biopesticides owing to their amenability for mass production and formulation as well as ease of application. A potential fungal pathogen infected rice bug cadaver earlier obtained from Vellanikkara, Thrissur, Kerala, India and maintained at AICRP (All India Co-ordinated Project) on BCCP (Bio-control of crop pests) was used for the fungal isolation and molecular characterization. The above-mentioned fungal isolate was identified through study of colony characters as well as through ITS sequencing and Basic Local Alignment Search Tool (BLAST). The fungal strain isolated from rice bug was identified as *Beauveria bassiana*.

Keywords *Beauveria bassiana*, Rice, Biocontrol, Sequencing BLAST, Entomopathogen.

INTRODUCTION

The rice bug, *Leptocorisa* spp. is one of the most important sap sucking insect pests of rice in the tropics. Both nymphs and adults suck sap from developing rice grains during the milky grain stage, leading to discolored, empty or half-filled grains. Yield loss due to the bug infestation ranges from 10 to 35%.

Management of the bug essentially involves spraying with insecticides, for want of safer options. Biological control of rice bug remains hampered by absence of ideal bioagents. Entomopathogenic microbes, particularly fungi, have been receiving increased attention of late as potential biocontrol agents of the rice earhead bug. The entomopathogenic fungus (EPF), *Beauveria bassiana* (Bals.) has been reported as infecting rice bug, from several parts of the world, including India. It has the ability to manage various insect pests. They are ideally suited

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Fig. 1. Rice bugs infected by *B. bassiana.*

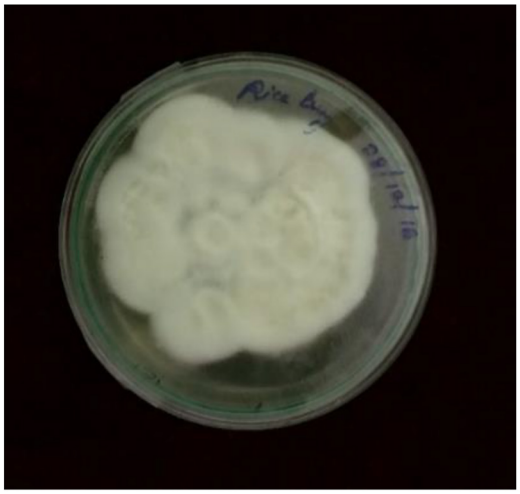

Fig. 2. Colony of Vellanikkara isolate.

as biopesticides owing to their amenability for mass production and formulation as well as ease of application. Steinhaus (1956) reported that *B. bassiana* causes mycosis in 175 insect hosts from Lepidopteran, Coleopteran and Hemipteran orders.

MATERIALS AND METHODS

The present study on "Characterization, evaluation and formulation of *Beauveria bassiana* (Bals.) strains against rice bug *Leptocorisa* spp. (Hemiptera : Alydidae)" was conducted at the Department of Agricultural Entomology, College of Horticulture, Vellanikkara, Thrissur and RARS Pattambi, during the year 2016–2019. The details of the materials used and the methods followed for the study are described below.

Isolation and maintenance of fungi from rice bug cadavers

The rice bug cadavers collected from rice fields were kept separately in moist chambers for the development of fungal growth (Fig. 1). Cadavers with fungal growth were selected for the isolation of fungi as per standard procedures (Zimmermann 1986). They were surface sterilized using one per cent sodium hypochlorite followed by repeated washing in sterile water under aseptic conditions. The cadavers were then covered in sterilized filter paper and air dried. The dried cadavers were subsequently placed in Petri dishes containing potato dextrose agar (PDA) medium for the development of mycelia.

Streptomycin, an antibiotic, was added to PDA medium at the rate of 0.16g per 200 ml to prevent bacterial contamination before pouring the medium into Petri dishes. Subculturing was done after the development of mycelia. The pure cultures of the fungi were maintained on PDA slants.

Identification of entomopathogenic fungi

Morphological identification

Petri dishes containing PDA media were inoculated with the isolated fungi. The plates were maintained at room temperature and observed for mycelial color and growth. The size of the conidia was calculated by measuring the dimensions of ten conidia under a phase contrast microscope equipped with LAS image analysing software and mean was worked out.

Molecular identification

Molecular characterization of fungi was done by sequence analysis of the ribosomal spacer (ITS_1 and ITS_4). DNA isolation was done with the facilities available at AINPAO, Vellanikkara, Thrissur and sequencing was carried out. The protocol for DNA isolation and sequencing was as follows.

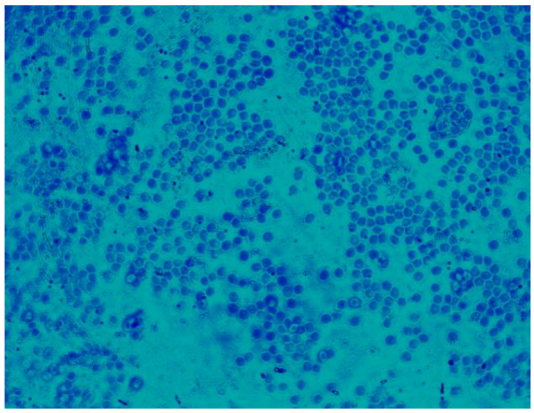

Fig . 3. Spores of Vellanikkara isolate (45X).

DNA isolation

The DNA isolation was done by following CTAB method with suitable modifications. The fungal mycelia were crushed with sterilized micro pestle and 1 ml CTAB extraction buffer was added. After vortexing the mixture was incubated at 65°C for 90 min. Then cooled and equal amount of chloroform: Isoamyl alcohol (24 : 1) was added and mixed gently. The mixture was then centrifuged at 10,000 rpm for 10 min, took the aqueous layer and equal amount of chloroform isoamyl alcohol was again added and centrifuged at 10,000 rpm for 10 min. Again, took the top aqueous layer, add equal amount of chilled isopropanol, and mix gently. Then add 30 µl, 3 M sodium acetate incubate at – 20°C for 1 h and centrifuged at 13,000 rpm for 10 min. supernatant was removed and 1 ml of 70% ethanol was added and centrifuge at 13,000 rpm for 10 min. Then air dried to remove the ethanol and suspended the pellet in 25 µl distilled water.

Analysis of purity of isolated DNA

Nanodrop (JENWAY Genova Nano, ver.1.55.3) was used to evaluate the purity of DNA. The quantity of the isolated DNA was checked using agarose gel electrophoresis. Five µl of DNA sample was mixed with two µl of gel loading buffer and loaded in 0.8% agarose gel for agarose gel electrophoresis.

Preparation of agarose gel and agarose gel electrophoresis.

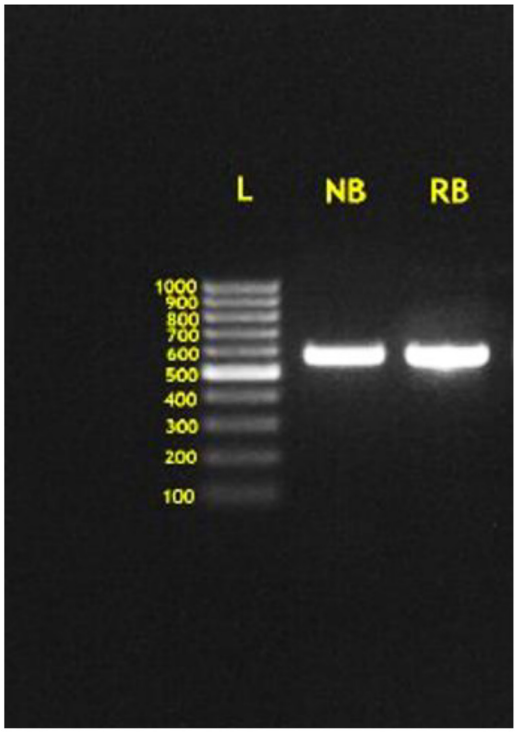

Fig. 4. PCR gel image. (NB – *B. bassiana* NBAIR isolate, RB - Vellanikkara isolate).

The gel was poured to the casting tray to a height of 3 to 5 mm with comb in position in such a way that the teeth are 0.5 to one mm above the plate. Then allowed to solidify, latter the comb and tape was removed and the tray with gel was placed in electrophoresis tank. 1x TAE buffer was added to the tank to cover the gel to a depth of 1 mm. Then mix the DNA sample with gel loading dye in the ratio 5 : 2 µl and load the mixture into the well of gel and run the gel at a voltage of 70 volts.

The gel was visualized under UV transilluminator and the image was documented using gel documentation system.

PCR analysis

Polymerized Chain Reaction (PCR) was carried out in a 20 µl reaction volume which contained 10 µl PCR mix, H_2O – 8 µl, DNA 0.8 µl and 0.6 µl reverse and forward primer each. Details of the primers used

Fig . 5. DNA barcode of Vellanikkara isolate generated by BOLD systems v4.

for amplification are given below.

Target	Primer name	Direction	Sequence (5'→ 3')
ITS	ITS-1F	Forward	T C C G T A G G T G A A T GAACCTTGCGG
ITS-4R	Reverse		TCCTCCGCTTATTGATATGC

The polymerized chain reaction was carried out in a PCR thermal cycler (Veriti Thermocycler (Applied Biosystems)) as per the following details.

Initial denaturation - 94°C for 4 minutes
Denaturation - 94°C for 45 minutes
Primer annealing - 54°C for 1 minute
Primer extension - 72°C for 2 minutes
Final extension - 72°C for 8 minutes
Incubation - 4°C for infinity
Number of cycles—36

Agarose gel electrophoresis of PCR product

The PCR products were analyzed in 1.2% agarose gel containing 0.5 µg/ml ethidium bromide. Five µl PCR product mixed with 2µl loading dye was loaded into the wells of the gel and electrophoresis was done at 75v power supply.

RESULTS AND DISCUSSION

Identification of entomopathogenic fungi

Morphological characters

Observations on initial and final color of the colonies and their growth pattern were recorded. The fungal isolate obtained from AICRP on BCCP Vellanikkara (VKA 01 strain) produced white colored uniform growth with cottony appearance in PDA media (Fig. 2).

Size and shape of spores

Length and width of the spores of each isolate were measured using a microscope equipped with LAS image analyzing software (Table 1). The spores of Vellanikkara isolate were globose in shape with mean length and width of 1.90 µm and 1.91 µm respectively (Fig. 3). The morphological characters showed similarity with *B. bassiana.*

Molecular characterization of fungal isolates from *Leptocorisa* spp.

The fungi isolated from rice bug cadavers obtained from Vellanikkara (VKA 01 strain) were subjected to molecular characterization. The ITS region was amplified using the ITS primers (F: TCCGTAGGTGAACCTGCGG, R: TCCTCCGCTTATTGATATGC) and the PCR product had one intact band at 600 bp when resolved in one per cent agarose gel (Fig . 4). ITS sequence of isolate of entomopathogenic fugus

Table 1. Evaluation of colony characters of fungal isolate from *Leptocorisa* spp.

Sl. No.	Fungal isolates	Initial color of hypha	Final color of hypha	Spore dimension (µm)	Conidial shape	Growth pattern
1	Vellanikkara	White	White	Length : 1.91 Width : 1.90	Globose	Cottony appearance

from Vellanikkara

The ITS sequencing of the *B. bassiana* Vellanikkara isolate yielded a 520 base pair (bp) sequence as given below.

ACCCTTCTGTGACCTACCTATCGTTGCTTC-
GGCGGACTCGCCCCAGCCCGGACGCG-
GACTGGACCAGCCCCGCCGGGGACCT-
CAAACTCTTGTATTCCAGCATCTTCT-
GAATACGCCGCAAGGCAAAACAAATGAATA-
ACTTTCAACAACGGATCTCTTGGCTCTGG-
CATCGATGAAGAACGCAGCGAAATGCGA-
TAAGTAATGAATTGCAGAATCCAGTGAAT-
CATCGAATCTTTGAACGCACATTGCGCCCG-
CCAGCATTCTGGCGGGCTGCCGTTCGAGC-
GTCATTTCAACCCTCGACCTCCCCTGGGG-
GAGGTCGGCGTTGGGGACCGGCAGCACAC-
CGCGGCCCTGAAATGGAGTGGCGGCCCGTC-
CGCGGCGACCTCTGCGTAGTAATACAGCTC-
GCACCGGAACCCCGACGCGGCCACGCCG-
TAAAACACCCAACTTCTGAACGTTGACCTC-
GAATCAGGTAGGACTACCCGCTGAACTTA-
AGCATATCAATAAGCGGAGGAA

The above sequence when subjected to BLAST (Basic Local Alignment Search tool) analysis showed cent per cent similarity with *B. bassiana* isolate Bb2 having accession number KX376474.1 and was assigned accession number MN062772 by NCBI.

An account was opened in BOLD systems v4 database and new projects were created for the two fungal samples isolated from rice bug cadavers. The sample from Vellanikkara was named "RBVKA" (Fig. 5). Specimen data viz., specimen taxonomy, specimen identifier, specimen details, collection details were given and an auto generated process ID 'RBVKA001-18' was obtained. Then primer details, fasta sequence and trace files (.ab1) were uploaded to database and corresponding barcode generated.

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