Indian Journal of Entomology Online published Ref. No. e24138 DoI. No.: 10.55446/IJE.2024.2138

FIRST REPORT OF *METARHIZIUM RILEYI* INFECTING FALL ARMY WORM *SPODOPTERA FRUGIPERDA* (J E SMITH) FROM NEPAL

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ABSTRACT

The fall army worm (FAW) *Spodoptera frugiperda* (Noctuidae: Lepidoptera) is an invasive polyphagous pest that prefers maize crop in the newly invaded regions like Africa, Asia, Australasia, and some Pacific Islands and was recorded in Nepal in the year 2019. Surveys were conducted in the maize fields to collect various natural enemies for utilization in the management of FAW. Several larvae infected with an unidentified fungus were collected and subjected to morphological and molecular analysis for identification as *Metarhizium rileyi*. In bioassay studies we observed percent larval mortality at $1x10^8$ conidia/ml suspension. This is the first report of *M. rileyi* infecting *S. frugiperda* from Nepal.

Key words: *Spodoptera frugiperda*, *Metarhizium rileyi*, maize, entomopathogenic fungus, IPM, natural enemies, pathogenicity, spores, laboratory studies, molecular characterisation

Metarhizium rileyi Kepler, Rehner and Humber (Hypocreales: Clavicipitaceae) is a dimorphic facultative entomopathogenic fungus (Fronza et al., 2017) that displays a high degree of host specificity (Suwannakut et al., 2005) and is a noctuid specialist (Hatting, 2012). Entomopathogenic fungi (EPF) are those fungal bioagents that kill by attacking and infecting their insect hosts (Singkaravanit et al., 2010). They can infect almost every stage of insect lifecycle and can be mass produced in laboratory which make them a unique component in IPM (Rajula et al., 2020). Farmers in Nepal spray highly toxic insecticides like a cocktail formulation of chlorpyriphos and cypermethrin which are available in local markets to control FAW (Bajracharya et al., 2020), an invasive pest recorded for the first time in Nepal on maize from Nawalpur district on 9th May 2019 (Bajracharya et al., 2019). FAW is a polyphagous pest (Montezano et al. 2018) with two strains, rice strain and corn strain (Adamczyk et al., 1997) but the strain that invaded Africa, Asia, Australia, and some Pacific Islands prefers maize (Bankar and Bhamare, 2023). Indiscriminate use of pesticides cause insecticide resistance in pests, pest resurgence, pesticide residue in environment and food and harms beneficial flora and fauna (Dhakal and Singh, 2019). So, the use of entomopathogenic fungus in IPM has its advantage. *Metarhizium rileyi,* an entomopathogenic fungus shows significant pathogenicity and virulence against FAW (Khairnar et al., 2023). To explore the fungal pathogens attacking FAW, surveys were conducted in maize fields in Nepal and the field collected fungal infected insect specimens were subjected to a series of laboratory studies.

MATERIALS AND METHODS

The study was carried out at the Department of Entomology, Agriculture and Forestry University, Rampur, Chitwan, Nepal from January to October, 2023. Larvae mummified with white and green fungus of FAW were collected from the maize fields of the National Maize Research Programme (NMRP), Rampur, Chitwan (l27^0 65'3.02''N, 84^0 34'79" E, 175 masl). The collected samples were kept individually in petri dishes on moistened filter paper for further growth of the fungus as suggested by Mamatha et al. (2023) and kept at 28± 2˚C, relative humidity of 45± 5%, with a photoperiod of 11L: 13D. Those mummified cadavers with green spores were dried to avoid further deterioration and stored in a refrigerator as suggested by Nirmalkar et al. (2020), and were used for further studies. Spores were taken from mummified green cadavers and transferred to a glass slide with a drop of water using a sterile inoculation loop and covered with a cover slip. The slide was observed on a compound microscope and the fungus was identified based on

morphological characters (Mamatha et al., 2023). The fungus was isolated from mummified larvae and cultured on Sabouraud dextrose agar (SDA) medium (10 g meat peptone, 20 g glucose and 18 g agar-agar) diluted in 1l of distilled water and autoclaved at 120^{o} C for 20 min. After cooling the medium to 60^{0}C, 0.6 g streptomycin, 0.05 g tetracycline, and 0.05 g cycloheximide previously mixed in distilled water were added together with 0.1 ml dodine (G C et al., 2008). The green spores were inoculated with the use of flame sterilized loop on the SDA medium in petri dish (9.5 cm* 2 cm) and were incubated for 15 days at 28± 2˚C (Padanad and Krishnaraj, 2009) and at 50± 5%RH until sporulation.

Morphological characters of the fungus grown on the SDA medium were observed after 15 days under compound microscope (Olympus CH20, 100x). The single colony was transferred to a fresh sterilized SDA media on a petri dish with sterile inoculation loop and incubated for at least 15 days at 28± 2˚C and 50± 5%RH. For morphological identification, colony growth and appearance were recorded (Zhou et al., 2020) and the codes and descriptions of genera and species of entomopathogenic fungi as suggested by Samson et al. (1998) and Humber (2005) were used. For molecular characterization, the infected insect samples after the bioassay experiment were sent to the laboratory of Intrepid Nepal, Thapathali, Kathmandu, Nepal where DNA extraction, PCR and sequencing were carried out. Genomic DNA was extracted from a portion of the fungus-infested larvae using GeneAll® ExgeneTM Tissue SV Plus kit. Then, the polymerase chain reaction (PCR) was performed to amplify the ITS region using the primer ITS1: TCCGTAGGTGAACCTGCGG and ITS4: TCCTCCGCTTATTGATATGC yielding an amplicon size of 648 bp. The reaction mixture was prepared with 3 µl of Nuclease free water, 12.5 µl of Qiagen 2X Master mix, 1 µl of primer forward (ITS1F), 1 µl of primer reverse (ITS4R), 5 µl of Q solution, and 2.5 µl of template DNA obtaining a total volume of 25 µl. The PCR thermocycler settings were initial denaturation at 95°C for 15 min followed by 5 cycles of denaturation at 94°C for 30 sec, annealing at 47°C for 30 sec and extension at 72°C for 30 sec, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 5 min and storage at 4°C. PCR amplicons were carried out using Sanger sequencing (ABI310 Genetic Analyzer) in both directions using forward and reverse primers. The sequences were subjected to nucleotide blast at Genbank database Release 241 (December 15, 2020) using Blastn 2.9.0.

Egg masses and different larval instars were collected from the maize fields of the National Maize Research programme (NMRP) at Rampur, Chitwan and a colony was maintained on healthy maize leaves (cv Rampur Composite) in Department of Entomology, Agriculture and Forestry University, Rampur, Chitwan at 25± 1^{0}C and 50± 5%RH. To obtain eggs both male and female moths were enclosed in Bug dorm (40* 40 cm), fed with 10% honey in cotton wads and also provided with potted maize seedlings. The egg masses were further collected and placed in sterile Petri dishes lined with filter paper and third instar larvae were used for bioassay. Fungal growth was scrapped from 15-days-old well-sporulated culture and placed in distilled water with 0.1 ml of 0.1% Tween 80 was added in the solution. Fungal conidia were counted using Neaubeaur haemocytometer under a compound microscope (Dev et al., 2021). Pathogenicity of the isolated fungus was determined using the method of Visalakshi et al. (2020). Laboratory-reared third instar larvae of FAW were sprayed with 2 ml of suspected *M. rileyi* conidial suspension (1x 10^{8} conidia/ ml) on the dorsal body part of larvae and replicated 30 times. The inoculated larvae were kept in petri dishes with filter paper, moist cotton, and pesticide-free maize leaf pieces. Petri dishes were cleaned and filter paper, moist cotton and the maize leaves were also changed daily. This experiment was conducted in a controlled environment of 28± 2°C and 50± 5%RH. Larval mortality was observed at every 24 hr intervals (Liu et al., 2019) for up to 10 days after the treatment.

RESULTS AND DISCUSSION

Metarhizium rileyi-infected FAW larvae were collected from the maize fields at the National Maize Research programme, Rampur, Chitwan, Nepal. From the infected insect cadavers, entomopathogenic fungus *M. rileyi* was isolated. This is similar to Alvarez et al. (2018) who isolated *M. rileyi* from FAW larvae collected from maize fields in Quivican municipality, Mayabeque province, Cuba. Similarly, *M. rileyi*-infected larvae of FAW were collected from the maize fields of the research farm of Regional Agricultural Research Station, Anakapalli and the *M. rileyi* was isolated from the cadavers (Visalakshi et al., 2020). Acharya et al. (2022) also confirmed fungus-infected FAW larvae collected from corn fields in Yeongcheon, Korea as *M. rileyi*. Green color development was observed on spores in SDA media after 15 days of incubation. Initially, colonization by the white mycelium of the fungus were

seen, which turned into olive-green aerial conidia. Fungal colony was seen as pale yellow to orange brown at the bottom of the Petri dish. Septate hyaline mycelium with erect conidiophores were observed. Conidia were found to be hyaline, aseptate, and ellipsoidal forming conidial chains. Visalakshi et al. (2020) and Montecalvo et al. (2022a) reported that the colony color of *M. rileyi* ranges from white to olive green and septate hyaline mycelium with erect conidiophores. The colonies were velvety, dense, and dusty, and the undersides of the fungal colony were pale yellow to orange-brown (Zhou et al., 2020). Conidia shape was reported to be ellipsoidal by Padanad and Krishnaraj (2009), Álvarez et al. (2018), and Sartiami et al. (2020). Zhou et al. (2020) also reported the arrangement of conidia in chains. Isolated fungus was identified as *M. rileyi* based on morphology and further subjected to molecular identification. The NCBI-BLAST analysis of partial genome sequence amplified by ITS1 and ITS4 has confirmed the EPF as *M. rileyi*.

Sequence information:

> FAMF01:

CGGAGGGATCATTACCGAGTTTACAACTCC
CAAACCCCATGTGAACTTATACCCTTT

TCCTGTTGCCTCGGCGGGTCATTTGCCCC
GGACCGGGCTCGTCCAGAGCCCGCCCGG

AAACAGGCGCCCGCCGCGGGACCGAAAC
TCTGTATCTCTTAGCCTTTGGCACGTCTG

AGTGGAATCATACAAAAATGAATCAAAAC
TTTCAACAACGGATCTCTTGGTTCTGGC

ATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGA

ATCATCGAATCTTTGAACGCACATTGCGCC
CGCCAGTATTCTGGCGGGCATGCCTGT

TCGAGCGTCATTTCAACCCTCAAGCCCCC
GCGGTTTGGTGTTGGGGGCCGGCGATTG

TCAGCTGGGCCGCTCAGGCGGTTCCCTGC
GGCGCCGCCCCCGAAATGAATTGGCGG

CCCCGTCGCGGCCTCCTCTGCGTAGTAGC
ACAACCTCGCAACAGGAGCGCGGCGCG

GCCACTGCCGTAAAACGCACAAACTTCT
CCAAGAGTTGACCTCGAATCAGGTAGGA

ATACCCGCTGAACTTAAGCATATCAT.

The ITS-generated sequence of the fungus (Lab ID FAMF01) was deposited in NCBI Gene Bank database and obtained with an accession number (PP091973.1). Further, Nucleotide BLAST was performed on NCBI by depositing the obtained sequence data information and a phylogenetic tree was constructed from MEGA software (Sartiami et al., 2020) version 11 using maximum parsimony method (Fig. 1). The isolate FAMF01 in this study might have evolved from China isolate having accession number (KX641194.1) as it shows 99% similarity between them. Larvae treated with *M. rileyi* started to die from the sixth day post treatment. By the 10th day, all treated larvae died while none died in control treated with 0.1% of Tween- 80.

Yang et al. (2024) reported 98.33% mortality of third instar larvae at 1x10^8 conidia/ml. Similarly, Peng et al. (2024) reported 30-100% mortality of third instar larvae when treated with 1x 10^8 conidia/ml of *M. rileyi* and the mortality varies as per strains from where they were collected. Zhou et al. (2020) also reported 100% mortality of larvae when treated with *M. rileyi*. Montecalvo et al. (2022a) reported larval mortality starting from the sixth day of treatment and also first to fourth larval instars treated with *M. rileyi* had similar degree of mortality ranging from 89-98% at ten days after the treatment which is similar to present findings. Similarly, *M. rileyi* pathogenicity against 3rd larval

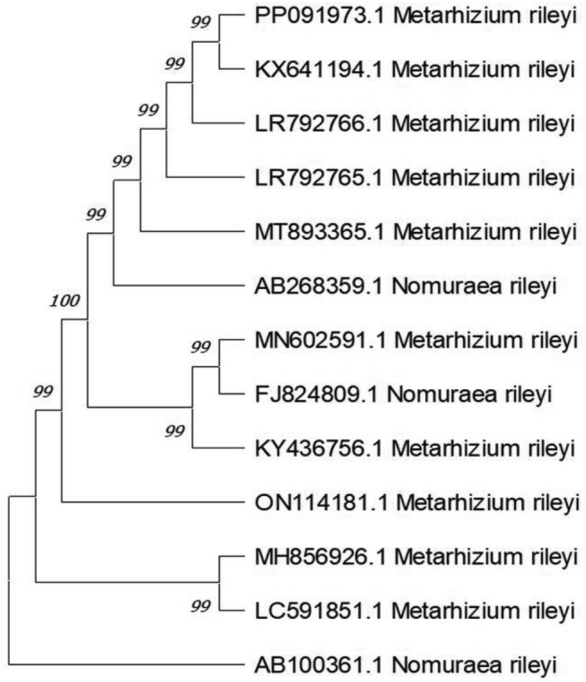

Fig. 1. Phylogenetic tree based on the nucleotide sequences of ITS region of isolated fungus, *M. rileyi*, PP091973.1 isolate FAMF01 with relevant sequences from NCBI database

instar of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) caused 73-100% mortality (Montecalvo and Navasero, 2020). According to Montecalvo et al. (2022a), mortality of third instar larvae of *S. exigua* started from six days. Conidial concentrations of 1 x 10^8 conidia/ml induced mortality up to 100% in all larval instars of *S. exigua*. Virulence of *M. rileyi* depends upon concentration of biopesticides (Grewal et al., 2021, Montecalvo and Navasero, 2021), temperature and humidity (Visalakshi et al., 2020), insect life stages (Montecalvo et al., 2022a), storage time (Grijalba et al., 2018), and the genetic diversity and characteristics of insect cuticle (Fronza et al., 2017).

Symptoms observed in *M. rileyi*-treated larvae after their death were the body of the dead larva were stiffened and the growth of white fungus was observed 2 to 3 hr after the death of insect. White fungal growth completely covered insect body within 1 day then white fungal growth changed to light green spores. The symptomatology observed in our experiment was similar to the experiment conducted by Montecalvo et al. (2022b) and Montecalvo and Navasero (2021), where FAW larvae stiffened after death and were subsequently covered with white fungal growth and later with olive green sporulation. These findings match the observations of the above-mentioned authors with the identity of the fungus isolated from FAW larvae in the maize fields of Nepal as *Metarhizium rileyi*. The present results provide an opportunity to field test *M. rileyi* against FAW in maize fields of Nepal and to develop IPM program.

ACKNOWLEDGEMENTS

The authors acknowledge the Department of Entomology, Agriculture and Forestry University, Chitwan, Nepal for providing laboratory facilities.

FINANCIAL SUPPORT

The researcher is greatly indebted to USAID/ Feed the Future Nepal Integrated Pest Management for funding the project (Cooperative Agreement no. 72036720LA00001).

AUTHOR CONTRIBUTION STATEMENT

Conceptualization, investigation, methodology design, data collection by NS and ST. Original draft preparation by NS. RM, ST and MRP supported on funding acquisition, supervision, manuscript review and editing. NS: Nisha Subedi, ST: Sundar Tiwari, MRP: Min Raj Pokhrel, RM: Rangaswamy Muniappan

CONFLICT OF INTEREST

No conflict of interest.

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(Manuscript Received: April, 2024; Revised: April, 2024;
Accepted: April, 2024; Online Published: April, 2024)
Online First in www.entosocindia.org and indianentomology.org Ref. No. e24138