scientific reports

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Host range and virulence of *Metarhizium pingshaense* against *Chilo* species and expression of fungal virulence genes in *Conogethes punctiferalis*

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The infectivity and virulence of *Metarhizium pingshaense* was tested against three major pests: *Chilo infuscatellus* (sugarcane early shoot borer), *C. sacchariphagus indicus* (sugarcane internode borer) and *C. partellus* (sorghum stem borer). Bioassay studies indicated high pathogenicity of this fungus against all the three species with the highest mortality recorded in *C. sacchariphagus indicus* (96%), followed by *C. infuscatellus* (93%) and *C. partellus* (83%). The median lethal concentrations of *M. pingshaense* against late-instar larvae were 4.6×10^5, 1.7×10^5, and 9.5×10^5 conidia/ml for *C. infuscatellus*, *C. sacchariphagus indicus*, and *C. partellus*, respectively. Median survival times ranged from 5.3 to 6.9 days for *C. infuscatellus*, from 5.4 to 7.9 days for *C. sacchariphagus indicus*, and from 6.9 to 8.3 days for *C. partellus*, at the tested doses of 1×10^8 and 1×10^7 conidia/ml. Qualitative and quantitative analyses of cuticle-degrading enzymes by the fungus, which are critical virulence factors, confirmed the production of chitinases and lipases. Enzyme production significantly increased in media with insect cuticle, indicating substrate-dependent regulation. Genes encoding chitinase and protease were cloned, sequenced, and were found to be closely related to those of *M. anisopliae*. RT-PCR studies confirmed the temporal expression of these two virulence genes, which play a critical role in pathogenesis. There was a gradual upregulation of these genes in the fungus during infection of its original host, *Conogethes punctiferalis* with the progression of time rising up to 3000-fold compared to untreated insects. These findings highlight the potential of *M. pingshaense* as an effective biocontrol agent for a wide range of crambid pests, supporting its development as a broad-spectrum mycoinsecticide.

Keywords Borer pests, Crambidae, Cuticle degrading enzymes, Mycoinsecticide, Pathogenicity, RT-qPCR

Lepidopteran borers, particularly those belonging to the Crambidae family, include some of the most destructive pests of agricultural and horticultural crops worldwide. Due to their cryptic nature, these pests often remain undetected during the early stages of infestation, posing significant challenges to their management[1]. Among the various genera within the Crambidae family, the genus *Chilo* Zincken comprises several species whose larval stages are major agricultural pests, affecting the yield of staple crops such as rice, sugarcane, maize, and sorghum. These pests cause considerable economic losses and are widely distributed across tropical and subtropical regions, including Africa, Asia, Southern Europe, South America, and Oceania[2]. Prominent species of this genus include *Chilo suppressalis* (Asian rice borer), *C. partellus* (sorghum stem borer), *C. infuscatellus* (sugarcane early shoot borer), and *C. sacchariphagus indicus* (sugarcane internode borer)[3–6]. These pests primarily cause damage to crops by boring into plant stems, leading to dead-heart symptoms, resulting in reduction in quality and yield, causing substantial crop losses. Although chemical pest control options are available, their adverse effects on

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the environment and non-target organisms underscore the importance of biological control strategies as a more sustainable and eco-friendly solution for managing these pests.

Among the biological control agents (BCAs) available for the management of insect pests, entomopathogenic fungi (EPF) are considered promising alternatives to chemical pesticides. They are widely distributed across various ecosystems, possess a broad host range, and act as natural regulators of insect pests in the environment. Unlike bacteria and viruses, EPF do not need to be ingested by the insect to initiate infection; the mere attachment of a viable conidium on the cuticle surface of a susceptible host is sufficient to trigger infection, thus acting as contact biopesticides. Strains of various entomopathogenic fungal species belonging to the genera *Metarhizium*, *Beauveria*, *Lecanicillium*, and *Isaria* have been successfully developed as commercial mycoinsecticides worldwide for managing pests of agricultural, veterinary, and medical importance[7]. Among these, the genus *Metarhizium* Sorokīn is a highly diverse and globally distributed group of fungi, comprising over 60 described species. It is a well-known pathogen of several major crop pests and has been successfully utilized in various biocontrol programs[8]. At least 11 species from this genus have been commercially exploited, and more than 34% of the commercially available mycoinsecticides belong to this genus alone, indicating its insecticidal potential and applicability across diverse agro-ecosystems[9,10].

Entomopathogenic fungi exhibit varying degrees of host specificity, ranging from broad-spectrum hyphomycetes pathogens like *B. bassiana, M. anisopliae* and *I. fumosorosea* that can infect hundreds of insect species across multiple orders[11,12], to highly specialized pathogens like entomophthoralean fungi, which are more host-specific and are obligate pathogens[13]. The host range of a fungus is determined by a complex interplay of factors, including fungal genetics, host cuticular properties, environmental conditions, and host immune responses. The molecular basis of host specificity involves specific recognition patterns, enzyme production, the ability to overcome host defences, genome composition, and horizontal gene transfer[14–16]. Fungi with a broader host range have more gene content and a complex gene regulation to degrade diverse cuticle types, detoxify host-specific compounds, and synthesize toxins for different hosts[16]. Understanding host range is crucial for biological control applications, as it influences both the efficacy against target pests and the potential impacts on non-target organisms[17].

The penetration of the insect cuticle by EPF is a critical step in initiating pathogenesis in insects. The insect cuticle is mainly composed of proteins, chitin, and lipids, which act as a protective shield against invading pathogens. Fungal pathogens employ a combination of physical pressure, exerted through the formation of an appressorium on the host's cuticular surface, and an array of hydrolytic enzymes, such as proteases, chitinases, and lipases, to penetrate the host cuticle[18,19]. Proteases play a pivotal role by breaking down proteins in the cuticle, which serve as a primary nutrient source for fungal germination and growth[20]. Chitinases target chitin, a key structural component of the cuticle, to aid fungal invasion while releasing carbohydrates for fungal nutrition[18,21,22]. Lipases contribute by hydrolyzing ester bonds in lipids, fats, and waxes, thereby enhancing fungal attachment, penetration, and development[23]. These extracellular enzymes are expressed sequentially and act synergistically to ensure the successful breach of the insect integument, followed by colonization and proliferation within the hemocoel[22]. The expression of these enzymes underscores the virulence of EPF and highlights their potential use as commercial biopesticides[17,24].

Despite the proven efficacy of EPF in managing pest populations, some limitations hinder their widespread adoption as reliable biocontrol agents. Many EPF strains exhibit a narrow host range, which limits their applicability across different pest species[25]. Moreover, environmental factors such as temperature, humidity, and UV radiation can significantly affect fungal viability and infectivity under field conditions[26,27]. Repeated application of certain biocontrol agents may also lead to resistance development in pest populations, thereby reducing long-term efficacy[28]. The present study investigates the pathogenicity of *Metarhizium pingshaense*, a promising EPF species known for its broader host infectivity and increased tolerance to abiotic stress[1,29] against *Chilo* spp. We report the infective potential of *M. pingshaense*, previously isolated from *Conogethes punctiferalis*[1], against three major crambid stem borers: *Chilo infuscatellus*, *C. sacchariphagus indicus*, and *C. partellus*, which are globally significant agricultural pests.

The objectives of this study were to (1) evaluate the physiological host range of the fungus by testing its pathogenicity and virulence against these pests through laboratory bioassays; (2) investigate the infection mechanism by estimating the cuticle-degrading enzymes (CDEs) produced by the fungus; and (3) analyze the expression of these enzyme associated genes in its original host, *C. punctiferalis* over the course of infection using molecular tools. We hypothesized that confamilial insect species may exhibit susceptibility to a fungal isolate derived from a member of the same family with a similar lifestyle. The findings are discussed in the context of the potential of the fungus as a broad-host-range pathogen and its prospects for development into a biopesticide for use in sustainable agricultural systems.

Materials and methods

Insects

Artificial rearing of Chilo spp.

Laboratory populations of three *Chilo* species: *Chilo infuscatellus* (Fig. 1A), *C. sacchariphagus indicus* (Fig. 1B), and *C. partellus* (Fig. 1C) were established from field-collected late instar larvae of each species, and the subsequent generation reared on an artificial diet was used for bioassays. Field-collected *C. infuscatellus* larvae were reared in plastic boxes (6.5 × 9.0 cm), fed with tender shoots of sugarcane, and maintained at 27 ± 2 °C, 60–70% relative humidity (RH), with a 12:12 h day-night photoperiod until pupation. Pupae were sexed and kept separately until adult emergence. Upon emergence, 10 pairs of adults were released into oviposition cages (50 cm × 50 cm × 75 cm) for mating and egg-laying. The insects were provided with sugarcane midribs for egg-laying and fed with 10% honey solution. Leaf bits with eggs were collected daily, surface-sterilized with 0.5% sodium hypochlorite for 60 s, and kept for larval emergence[6]. After emergence, neonate larvae were transferred

Fig. 1. Healthy late instar larvae of (**A**) *Chilo infuscatellus*, (**B**) *C. sacchariphagus indicus* and, (**C**) *C. partellus*.

to 15 ml glass vials (three neonates per vial) half-filled with artificial diet[30] and maintained until they reached the fifth instar for further studies. Similarly, *C. sacchariphagus indicus* and *C. partellus* were field-collected and reared on their respective hosts, and the second-generation fifth instar larvae reared on artificial diet were used for bioassay studies.

Fungus source

Metarhizium pingshaense strain IISR-EPF-14 was originally isolated from naturally infected *C. punctiferalis* cadavers found in turmeric plants, as detailed elsewhere[1]. For bioassay experiments, a pure culture of this fungus, available in the Entomopathogenic Repository of ICAR-IISR, was utilized. Sequence data for various conserved gene regions of the fungus are accessible in the NCBI database, with the GenBank accession numbers MK537396 for the partial internal transcribed spacer (ITS) and MK583958 – MK583961 for the translation elongation factor (TEF), RNA polymerase II largest subunits (RPB1, RPB2), and DNA lyase (APN) respectively.

Preparation of fungal spore suspension

The fungus was grown on petri dishes (100 × 17 mm) containing potato dextrose agar (PDA) media at 26 ± 1 °C for 2 weeks. Conidia were harvested by scrapping the surface with a sterile scalpel and suspended in 10 mL of sterile 0.05% Triton-X 100. The spore suspension was passed through a double-layered muslin cloth to remove fungal debris, and the final spore concentration was adjusted to 10^9 conidia/ml using an improved Neubauer hemocytometer. Spore viability was checked prior to use, and it was found to be more than 95%. Bioassays for different insects were conducted each time with freshly prepared spore suspension.

Bioassays

Two sets of bioassay experiments were conducted for each insect species at a time (i) to determine the median lethal concentration (LC_{50}) of the fungus, and (ii) to assess the median survival time (MST) of the insects infected by the fungus. The LC_{50} studies were conducted with four conidial concentrations: 10^5, 10^6, 10^7 and 10^8 conidia/ml, while two concentrations, 10^7 and 10^8 conidia/ml were tested for MST studies against early fifth instar of three *Chilo* spp. Groups of 10 insects of each *Chilo* spp. were dipped in each concentration of the fungus for 30 s and then transferred to plastic containers (500 ml) lined with a filter paper at the bottom placed above a layer of cotton. A similar number of insects treated with sterile 0.05% Triton® X-100 solution served as control. Each treatment was replicated three times, and the insects were treated with conidial suspensions prepared separately for each replication[1]. Treated insects were fed with natural diet, i.e., sugarcane shoots for *C. infuscatellus* and *C. sacchariphagus indicus* and sorghum shoots for *C. partellus*, and maintained at 28–32 ± 2 °C, 60–70% relative humidity, and a 12:12 h day: night photoperiod under ambient room conditions. Insect mortality in the experiments was recorded at 24 h intervals for LC_{50} studies and at 12 h intervals for MST studies, up to 15 days post-inoculation (p. i.). Dead insects were surface sterilized and re-isolated by inoculating them on PDA[1]. The experiments were repeated with freshly prepared fungal spore suspension to confirm the results.

Assay for cuticle degrading enzymes

The enzymatic activity of *M. pingshaense* was assessed using both qualitative (plate) and quantitative (broth) assays for chitinase, protease, and lipase. All experiments were conducted in triplicate unless stated otherwise.

Plate assays

To screen for extracellular enzyme activity, 6 mm agar plugs from 15-day-old fungal cultures grown on PDA were inoculated onto specific agar media and incubated at 26 ± 1 °C in a BOD incubator.

- **Chitinase**: Chitin agar (pH 6.0) consisted of (g/L): 10 g colloidal chitin, 5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 2.4 g KH_2PO_4, 0.6 g $K_2HPO_4{\cdot}3H_2O$, and 15 g agar per litre[31]. Media without colloidal chitin served as control. Chitinolytic activity was indicated by a clear zone surrounding the fungal colony at 7 DAI.
- **Protease**: Casein agar contained (g/l): 10 g casein, 1 g glucose, 1 g yeast extract, 1 g K_2HPO_4, 0.5 g KH_2PO_4, 0.1 g $MgSO_4$, and 20 g agar[32]. Control plates lacked casein. Proteolytic activity was indicated by the formation of a hydrolysis ring 24 h after incubation.
- **Lipase**: Tributyrin agar (pH 8.0) was composed of 1% tributyrin, 10 g tryptone, 5 g yeast extract, 10 g NaCl, 0.1 g phenol red, and 20 g agar[33]. Lipolytic activity was identified by the presence of a clear halo zone 15 DAI.

Broth assays

For quantitative enzyme estimation, 6 mm mycelial discs were inoculated into 50 ml of specific broth media, and incubated at 120 rpm. Culture filtrates were collected at 3, 5, 7, 10, and 15 days after inoculation (DAI), filtered, and centrifuged to obtain crude enzyme. Protein content was determined by Lowry's method using bovine serum albumin (BSA) as standard.

- **Chitinase**: Cultures were grown in chitin broth (pH 5.5) containing 15 g colloidal chitin, 0.5 g yeast extract, 1 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4{\cdot}7H_2O$, and 1.36 g KH_2PO_4 at 26 ± 1 °C. Chitinase activity was measured using a modified method of Yanai et al.[34]. The reaction mixture, comprising 250 µl of 0.5% colloidal chitin, 0.2 M sodium acetate buffer (pH 4.0), and 500 µl of enzyme solution, was incubated at 37 °C for 2 h. After brief centrifugation (2460 ×g, 2 min, room temperature), 500 µl of the supernatant was mixed with 100 µl of 0.8 M boric acid. The pH was adjusted to 10.2 with KOH, followed by heating for 3 min in boiling water. After cooling, 3 ml of DMAB reagent (1 g *p*-dimethylaminobenzaldehyde in 100 ml glacial acetic acid with 1% v/v HCl) was added and incubated at 37 °C for 20 min. Absorbance was read at 585 nm against a water blank. One unit of chitinase activity was defined as the amount of enzyme that releases 1 µmol of N-acetylglucosamine per minute under the assay conditions.
- **Protease**: Fungal discs were inoculated into basal salts medium containing 1 g/L casein[35] and incubated at 28 ± 1 °C. Total protease activity was assessed using casein as a substrate and quantified by Folin–Ciocâlteu reaction[36]. Subtilisin-like (Pr1) and trypsin-like (Pr2) activities were determined using N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and N-Benzoyl-Phe-Val-Arg-p-nitroanilide, respectively[37]. Absorbance was recorded at 660 nm for total protease and at 410 nm for Pr1/Pr2 activity. One unit of protease or Pr1/Pr2 activity was defined as the amount of enzyme that released 1 mM tyrosine or *p*-nitroanilide per minute.
- **Lipase**: Mycelial discs were inoculated in minimal broth (pH 5.0) containing 4 g $NaNO_3$, 2 g KH_2PO_4, 1 g $MgSO_4{\cdot}7H_2O$, and 4% (v/v) coconut oil or olive oil (in separate experiments) and incubated at 26 ± 1 °C. On day 4, 0.25% Triton X-100 was added and filtrates were collected on 6, 8, 10, 12, and 15 DAI. Enzyme activity was measured using *p*-nitrophenyl palmitate (pNPP) as the substrate[38]. The release of *p*-nitrophenol was quantified spectrophotometrically at 420 nm. One unit of lipase activity was defined as the amount of enzyme that liberates 1 µmol of *p*-nitrophenol per minute.

Assay for cuticle degrading enzymes in insect cuticle amended media

Extracellular enzyme production in media supplemented with insect cuticle of its original host, *C. puntiferalis* was conducted using the following composition (g/l): 0.02 g KH_2PO_4, 0.01 g $CaCl_2$, 0.01 g $MgSO_4$, 0.02 g Na_2HPO_4, 0.01 g $ZnCl_2$ and 0.01 g yeast extract[5]. The media was supplemented with 1% cuticle extract from *C. punctiferalis* larvae, inoculated with mycelial disc (6 mm) of *M. pingshaense,* and maintained in an incubator at 26 ± 1 °C with shaking at 120 rpm. Media without cuticle extract served as the control. After 5 days of incubation, the culture filtrates were centrifuged at 9,838 g for 10 min, and the resulting supernatant was used as crude enzyme for the estimation of various CDEs as described above.

Cloning and sequencing of chitinase *and* protease *genes*

DNA extraction, PCR and sequencing

Genomic DNA of the fungus was extracted from approximately 500 mg of 1-week old culture using fungal genomic DNA extraction Kit (Chromous Biotech, India) following manufacturer's instructions. Gene specific primers were used to amplify partial sequences *chitinase* and *protease* genes. *Chitinase* gene region was amplified using Meta_Chit1_comp forward (5´-TCCCATGTTCTGTACTCGTTC-3´) and Meta_Chit1_comp reverse (5´-CCC TTGCTCTTGAGGTAGGTAAC-3´)[21]. Protease gene was amplified using a degenerate primer set, Prot-IISR-F (5´-GACTTCGTTTACGAGCACRCCWY-3´) and Prot-IISR-R (5´-RWAGTCCATGCCACTGAKGATRC-3´) designed from the conserved gene region of *Metarhizium* spp. *protease* genes available in NCBI database. PCR reactions were carried out with 10–20 ng of template DNA in volumes of 25 µl containing 12.5 µl of 2× PCR TaqMixture (HiMedia®, India), 0.75 µl each of (20 µM) forward and reverse primer pairs, after making the remaining volume with PCR grade water (HiMedia®, India). The PCR conditions for *chitinase* and *protease* genes were: initial denaturation at 95 °C for 4 min, followed by 35 cycles at 95 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min, and a final extension step at 72 °C for 10 min.

The amplified bands were excised, purified using QIAquick® gel extraction kit (Qiagen) and ligated into TA cloning vector pTZ57R/T, and transformed into competent *Escherichia coli* strain DH5α using Thermo Scientific™ InsTAclone™ PCR Cloning Kit. The selected recombinant plasmids after DNA extraction using QIAprep Spin Miniprep Kit (Qiagen) was confirmed for inserts by PCR (M13 F & R primers) and double restriction endonuclease digestion (Eco RI & Hind III). Positive clones were sent to Eurofins Genomics, India, Pvt. Ltd., for bi-directional sequencing using the same set of PCR primers. Sequences were manually trimmed using BioEdit software and subjected to BLAST (Basic Local Alignment Search Tool) search to identify matching sequences deposited in GenBank database. The partial chitinase and protease gene sequences of *M. pingshaense* strain IISR-EPF-14 were submitted in GenBank with accession numbers, PP109068 and PP109069, respectively. The conserved domain of the ORF was analyzed using the Conserved Domain Search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) available from NCBI.

Phylogenetic analysis

The amino acid sequences of *chitinase* and *protease* genes of *M. pingshaense* strain IISR-EPF-14 and relevant sequences of other *Metarhizium* spp. from the GenBank database were aligned in Clustal W Multiple alignment tool separately prior to phylogenetic analysis. Neighbor-joining trees were constructed using Jones-Taylor-Thornton (JTT) model and Dayhoff plus Gamma (G) for *chitinase* and *protease* genes respectively, based on

the lowest score values of Bayesian Information Criterion (BIC) available in MEGA X[39]. Bootstrapping was performed with 1000 replicates and the gaps in alignment were treated as missing data. *Pochonia chlamydosporia* var. *chlamydosporia* (Goddard) Zare et W. Gams (Metacordycipitaceae) was used as outgroup.

In vivo *expression of* Metarhizium pingshaense *virulence genes in infected insects*

The relative expression of two pathogenesis-associated genes, *chitinase* and *protease*, in *C. punctiferalis* infected by *M. pingshaense* strain IISR-EPF-14 was evaluated using reverse transcription quantitative real-time PCR (RT-qPCR). For this study, laboratory-reared late fourth instar larvae of *C. punctiferalis* (n = 40) were treated with *M. pingshaense* spore suspension obtained from a 2-week-old culture grown on PDA, as described earlier in Section"Bioassays". The gene expression was estimated at different time intervals: 72, 96, and 120 h after treatment. Groups of 10 insects were dipped in 3 ml of spore suspension (1×10^8 conidia/ml) for 30 s and transferred to plastic containers (500 ml) containing turmeric pseudostem pieces placed on a filter paper layer above cotton. Insects treated with sterile 0.05% Triton® X-100 solution served as control and were used as the 0-h sample. Insects collected at different time intervals were individually immersed in RNA later™ stabilization solution (Invitrogen), stored at 4 °C overnight, and subsequently transferred to −20 °C for long-term storage. Total RNA was isolated from three infected and control samples using the RNeasy Plant Mini Kit (Qiagen), following the manufacturer's protocol. RNA purity and concentration were assessed spectrophotometrically using a Nanodrop Microvolume Spectrophotometer (ThermoFisher Scientific), and integrity was confirmed on a 1% agarose gel.

Reverse transcription quantitative real-time PCR (RT-qPCR) reactions were performed in a Rotor-Gene Q platform (QIAGEN) with 250 ng of total RNA, 12.5 µl QuantiFast SYBR Green PCR Master Mix (Qiagen), 1.0 µl each of gene-specific forward and reverse primers designed using Primer3web version 4.1.0 (https://primer3.ut.ee) from *M. pingshaense* protease and chitinase genes, 0.5 µl RevertAid Reverse Transcriptase (Thermo Scientific™), and the remaining volume made up with PCR-grade water (HiMedia®, India). The qPCR primers used for *protease* were q-Prot-IISR-F1 (5´-CGTAAGTTGTGCCGCCAAAA-3´) and q-Prot-IISR-R1 (5´-CCGTGGCCATCAGTTTGTTG-3´), while those used for *chitinase* were q-Chit-IISR-F2 (5´-ATTCTCTGGTGTTGGCGACG-3´) and q-Chit-IISR-R2 (5´-ACCCTTTGCCACGTCATCAT-3´). The endogenous control, the *actin* gene, was detected using the primer pair q-Actin F2-CP (5´-TCCATCATGAAGTGCGACGT-3´) and q-Actin R2-CP (5´-CTCCTTCTGCATCCTGTCGG-3´). The 3-step cycling conditions for RT-qPCR were: reverse transcription for 30 min at 50 °C, polymerase activation for 15 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. Each target gene was analyzed with three biological replicates and two technical replicates. Primer efficiency was averaged across all PCR reactions for each primer, and the cycle threshold (Ct) value was averaged from the two technical replicates. The relative expression of each target gene was calculated using the ΔΔCT method[40], in comparison with the control treatment.

Statistical analysis

Data from insect bioassays were subjected to one-way analysis of variance (ANOVA) using the general linear model (GLM). Mean comparisons were carried by Tukey's honest significant difference (HSD) test ($\alpha = 0.05$) in GraphPad Prism® Version 7.0 for Windows, GraphPad Software, La Jolla California, USA. As there was no control mortality, Abbott's correction was not applied for treatments. The median lethal concentration (LC_{50}) of the fungus to kill the insects was done by probit analysis using IBM® SPSS® (Statistical Package for Social Science, IBM, Armonk New York, USA) Statistics Version 25.0 for windows. The web-based programme, Online Application for Survival Analysis 2 (OASIS 2), developed by Han et al.[41] was used to ascertain the median survival time (ST_{50}) of the insects in each treatment based on Kaplan–Meier survival distribution function. All the experiments were repeated to confirm the results.

Results

Laboratory bioassays

The fungus caused significant mortality in all the three tested *Chilo* species. The infected insects exhibited signs of mycosis typical of *M. pingshaense* (Fig. 2 A, B, C). No mortality was observed in the control group, where the insects pupated normally. In *C. infuscatellus*, larval mortality was significant across the tested doses (F = 116;

Fig. 2. Infected early fifth instar larvae larvae of (**A**) *Chilo infuscatellus*, (**B**) *C. sacchariphagus indicus* and, (**C**) *C. partellus*.

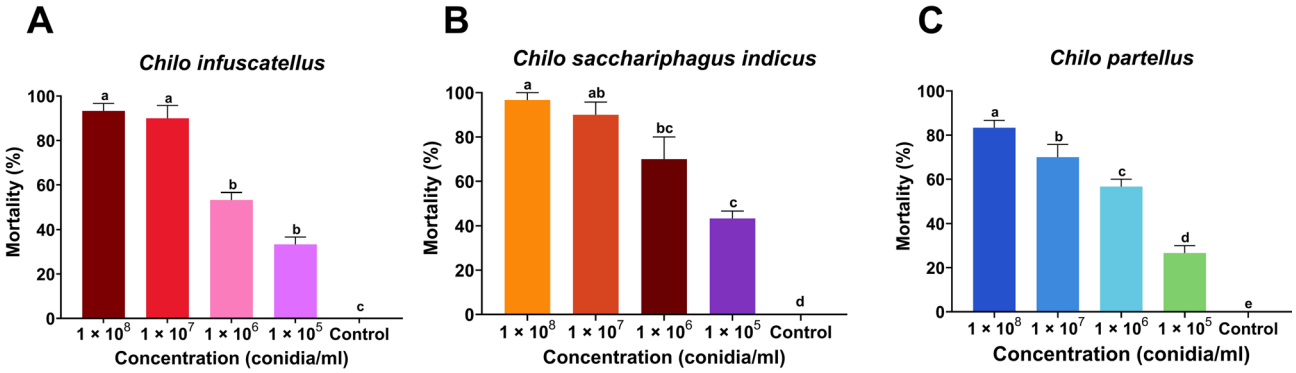

Fig. 3. Mean mortality of early fifth instar larvae of (**A**) *Chilo infuscatellus*, (**B**) *C. sacchariphagus indicus* and, (**C**) *C. partellus* infected by *Metarhizium pingshaense* strain IISR-EPF-14. Bars with the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

Host	N	χ^{2*}	Slope (intercept ± SE)	LC_{50} (conidia/ml)	95% confidence interval
Chilo infuscatellus	150	1.808	−4.088 ± 0.832	4.6×10^5	$1.4 \times 10^5 - 1.1 \times 10^6$
Chilo sacchariphagus indicus	150	0.071	−3.616 ± 0.878	1.7×10^5	$3.1 \times 10^4 - 4.5 \times 10^5$
Chilo partellus	150	0.771	−3.083 ± 0.743	9.5×10^5	$2.1 \times 10^5 - 2.8 \times 10^6$

Table 1. Median lethal concentration (LC_{50}) of early fifth instar larvae of *Chilo* spp. treated with different doses of *Metarhizium pingshaense* strain IISR-EPF-14. *All lines are significantly a good fit ($P < 0.05$).

Dose	N	MST (days)*	SE	95% confidence interval
Chilo infuscatellus				
1×10^8	30	5.3^a	0.35	4.7–6.0
1×10^7	30	6.9^b	0.35	6.2–7.6
Chilo sacchariphagus indicus				
1×10^8	30	5.4^a	0.28	4.8–5.9
1×10^7	30	7.9^b	0.49	6.9–8.9
Chilo partellus				
1×10^8	30	6.9^a	0.37	6.2–8.0
1×10^7	30	8.3^b	0.40	7.5–9.0

Table 2. Median survival time (MST) analysis of early fifth instar larvae of *Chilo* spp. treated with different doses of *Metarhizium pingshaense* strain IISR-EPF-14. *Survival time values with different letters are significantly different at $P < 0.05$ by log-rank test.

$P < 0.0001$; df = 4, 10), ranging from 33.3 ± 3.3% to 93.3 ± 3.3% (mean ± SE) (Fig. 3A). For *C. sacchariphagus indicus*, larval mortality (mean ± SE) ranged from 43.3 ± 3.3% to 96.7 ± 3.3% at different doses, with significant differences observed among the doses tested (F = 50; $P < 0.0001$; df = 4, 10) (Fig. 3B). Similarly, in *C. partellus*, mortality varied significantly (F = 85.6; $P < 0.0001$; df = 4, 10), with mean mortality ranging from 26.7 ± 3.3% to 83.3 ± 3.3% (Fig. 3C).

The median lethal concentration (LC_{50}) of the fungus against late instar larvae of three *Chilo* species was 4.6×10^5, 1.7×10^5, and 9.5×10^5 conidia/ml for *C. infuscatellus*, *C. sacchariphagus indicus*, and *C. partellus*, respectively. The 95% confidence intervals (CI) ranged from 1.4×10^5 to 1.1×10^6, 3.1×10^4 to 4.5×10^5, and 2.1×10^5 to 2.8×10^6 conidia/ml, respectively, for these three *Chilo* species (Table 1).

The median survival time (MST) of late instar larvae of three *Chilo* species differed significantly between the two tested doses (Log-rank test; $P < 0.05$). For *C. infuscatellus*, the MST at the highest dose of 1×10^8 conidia/ml was 5.3 days (± 0.38 SE), with a 95% confidence interval (CI) of 4.7 to 6.0 days, while at the dose of 1×10^7 conidia/ml, the MST was 6.9 days (± 0.35 SE), with a 95% CI of 6.2 to 7.6 days (Table 2 and Fig. 4A). Similarly, for *C. sacchariphagus*, the MST at the highest dose of 1×10^8 conidia/ml was 5.4 days (± 0.28 SE), with a 95% CI of 4.8 to 5.9 days, whereas at the dose of 1×10^7 conidia/ml, the MST was 7.9 days (± 0.49 SE), with a 95% CI of 6.9 to 8.9 days (Table 2 and Fig. 4B). In the case of *C. partellus*, the MST at the highest dose of 1×10^8 conidia/ml was 6.9 days (± 0.37 SE), with a 95% CI of 6.2 to 8.0 days, whereas at the dose of 1×10^7 conidia/ml, the MST was 8.3 days (± 0.40 SE), with a 95% CI of 7.5 to 9.0 days (Table 2 and Fig. 4C).

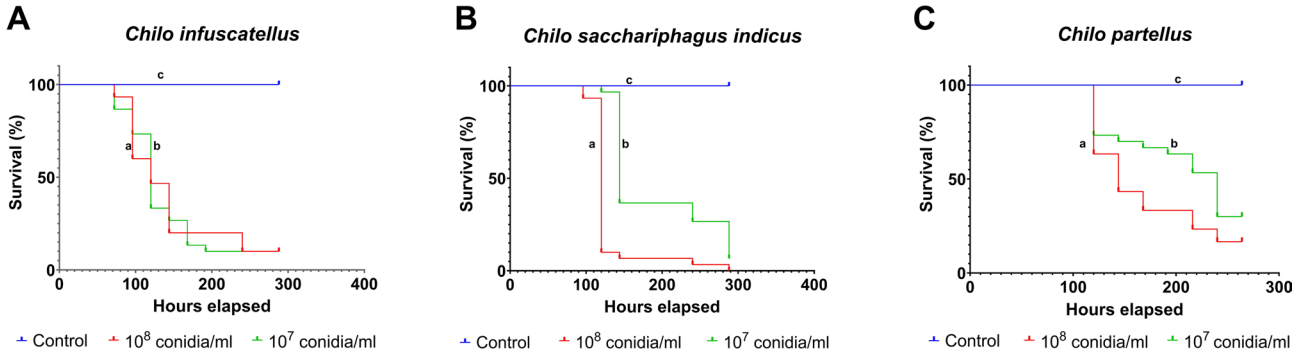

Fig. 4. Kaplan–Meier survival curves for early fifth instar larvae of three *Chilo* species: (**A**) *C. infuscatellus*, (**B**) *C. sacchariphagus indicus*, and (**C**) *C. partellus* treated with *Metarhizium pingshaense* strain IISR-EPF-14 at concentrations of 1×10^7 and 1×10^8 conidia/mL. Different letters indicate significant differences between treatments ($P < 0.05$) according to the log-rank test.

Assay for cuticle degrading enzymes

Production of cuticle degrading enzymes in substrate specific media

In the preliminary chitinase plate assay, the fungus produced a clear zone around the colony 24 h after incubation by degrading the colloidal chitin exhibiting its potential to produce chitinases (Fig. 5A). Chitinase activity in liquid media amended with colloidal chitin gradually increased from 0.02 ± 0.003 U/mg at 3 DAI, reached its peak activity on 10 DAI (0.03 ± 0.003 U/mg) and then decreased afterward (Fig. 5B).

Initial screening for protease activity by the fungus on casein agar plates showed rapid hydrolysis of casein, evidenced by the formation of clear hydrolysis ring around the colony within 24 h after inoculation (Fig. 5C). Total protease activity in liquid media amended with casein increased from 1.5 ± 0.17 U/mg on 3 DAI, and reached its peak activity on 5 DAI (11.1 ± 0.46), followed by a rapid decline in the activity over time (Fig. 5D). The mean peak activities of specific proteases, including Subtilisin-like (Pr1) and trypsin-like (Pr2) enzymes, from culture filtrates were 8.4 ± 1.21 and 3.1 ± 0.50 U/mg, respectively, on 3 DAI, which rapidly decreased with the progress of time (Fig. 5D).

The fungus was also positive for the production of lipase, as indicated by the clear halo zone around the colony on tributyrin agar plates (Fig. 5E). Spectrophotometric analyses of the basal media amended with vegetable oils showed that the highest lipase production was recorded on 10 DAI, which was 0.97 ± 0.05 and 1.0 ± 0.28 U/mg, for coconut and olive oils, respectively. The production of lipases in the basal media declined gradually with the progress of time (Fig. 5F).

Production of cuticle-degrading enzymes in cuticle amended media

Experiments conducted to assay the production of various CDEs in basal media amended with 1% cuticle extract of *C. punctiferalis* confirmed the secretion of enzymes such as chitinases, proteases, and lipases by the fungus. The production of chitinase and lipase in the media reached 0.89 ± 0.05 and 0.39 ± 0.08 U/mg, respectively, on 5 DAI. The total protease produced was 51.4 ± 3.05 U/mg, while specific proteases like subtilisin-like (Pr1) and trypsin-like (Pr2) enzymes, in the culture filtrates were 9.13 ± 0.52 and 3.76 ± 0.19 U/mg, respectively (Table 3).

Sequencing and characterization of chitinase *and* protease *genes*

Sequencing of *chitinase* gene of *M. pingshaense* using degenerate primers yielded an amplicon of size 870 bp, encoding for 290 amino acid residues. Analysis of the ORFs for conserved domain indicated that the sequenced chitinase gene belongs to the glycoside hydrolase family 18 protein (domain architecture ID 12,217,520), similar to chitinase, which catalyzes the random endo-hydrolysis of the 1, 4-beta-linkages of N-acetylglucosamine in chitin and chitodextrins. Protein homology search with the translated protein sequence of the of the partial chitinase gene showed highest similarity (97.6%) with *M. anisopliae* isolates, BRIP 53,293 and 53,284 (KJK82108 and KJK95695), respectively. Moreover, the sequence shared more than 90% similarity with chitinase genes of other *Metarhizium* species such as *M. humberi* (KAH0599693), *M. robertsii* (XP_007818874), *M. brunneum* (KAK9445903), *M. majus* (KIE02515), *M. guizhouense* (KID88770), *M. acridum* (XP_007815094) etc. The Neighbor-joining tree generated using deduced amino acid sequences of chitinase genes of various entomopathogenic fungi showed that the *M. pingshaense* chitinase sequence clustered with chitinase genes of various *Metarhizium* species with strong boot strap support, while being distinctly different from *M. rileyi* and the outgroup, *P. chlamydosporia* (Fig. 6).

The subtilisin-like *protease* gene of *M. pingshaense* was amplified using gene-specific primers, resulting in an amplicon of 579 bp that encodes 149 amino acid residues. The amplified protein belongs to the conserved protein domain families S8 (subtilisin and kexin) and S53 (sedolisin) superfamilies, which include endopeptidases and exopeptidases. Protein homology search for the deduced amino acid sequence of the partial protease gene showed 97.99% similarity with Pr1 proteins of *M. anisopliae* (Accession nos. BAB70704, ACT66137, AAR26030, ACV71840, ACV71832, and XP_066817309) and *M. humberi* (KAH0601022). The protein sequence also shared more than 90% similarity with other *Metarhizium* species, such as *M. robertsii* (XP_007821864), *M. brunneum* (XP_014549317), *M. acridum* (AAR26026), *M. majus* (ACV71847), and *M. guizhouense* (KID91211),

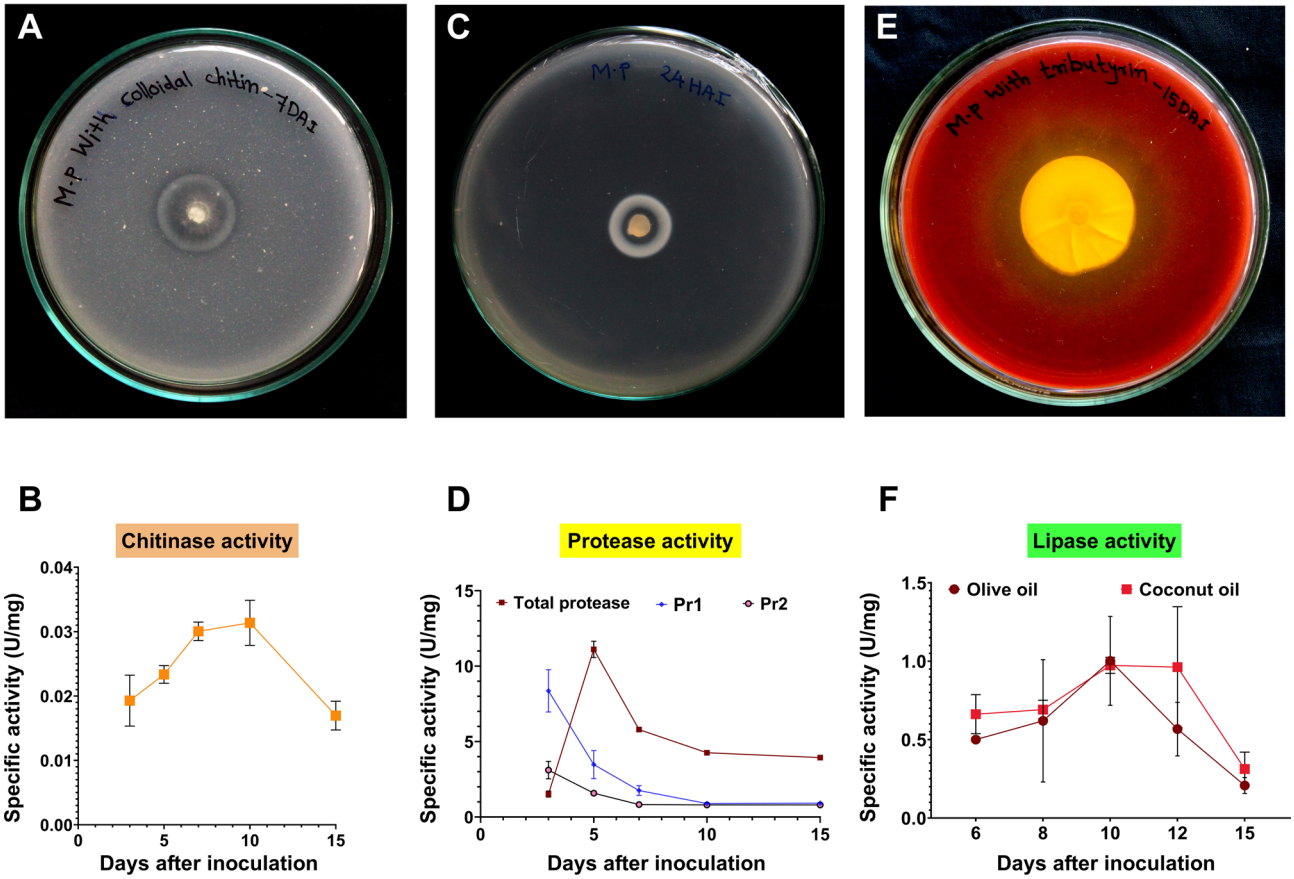

Fig. 5. Qualitative and quantitative estimation of various cuticle-degrading enzymes produced by *Metarhizium pingshaense* strain IISR-EPF-14 on solid media and in broth amended with specific substrates. Chitinase activity assay showing (**A**) formation of a clear zone around the fungal colony on solid media amended with 0.1% colloidal chitin 7 days after inoculation (DAI) and (**B**) specific activity (U/mg) of chitinase in liquid media amended with 0.15% colloidal chitin at 3, 5, 7, 10, and 15 DAI. Protease activity assay showing (**C**) formation of a hydrolysis ring around the fungal colony on solid media amended with 1% casein 24 h after incubation and (**D**) specific activity (U/mg) of total protease, subtilisin-like (Pr1), and trypsin-like (Pr2) enzyme activities in basal salts medium amended with 0.1% casein at 3, 5, 7, 10, and 15 DAI. Lipase activity assay showing (**E**) appearance of a clear zone around the fungal colony on 1% tributyrin agar media 15 DAI and (**F**) specific activity (U/mg) of lipase in minimal broth media amended with 4% (v/v) olive or coconut oil at 6, 8, 10, 12, and 15 DAI.

Cuticle degrading enzyme	Activity (U/mg of protein)
Chitinase	0.89 ± 0.05
Total protease	51.4 ± 3.05
Pr1	9.13 ± 0.52
Pr2	3.76 ± 0.19
Lipase	0.39 ± 0.08

Table 3. Production of cuticle degrading enzymes (mean ± SE) in liquid media amended with cuticle of *Conogethes punctiferalis* 5 days after treatment.

etc. Phylogenetic analysis of the protease genes indicated clustering of *M. pingshaense* with *M. anisopliae* and *M. humberii* with strong bootstrap support, while being distinctly different from *M. album*, *M. rileyi*, and the outgroup *P. chlamydosporia* (Fig. 7).

In vivo *expression of* Metarhizium pingshaense *virulence genes in infected insects*

The relative expression of the *chitinase* and *protease* genes in *C. punctiferalis* infected by *M. pingshaense* was analyzed over time, at 72, 96, and 120 h post-infection (hpi). It was observed that the expression of virulence and pathogenesis-associated genes was significantly upregulated as the infection progressed in the insects. At 72 hpi,

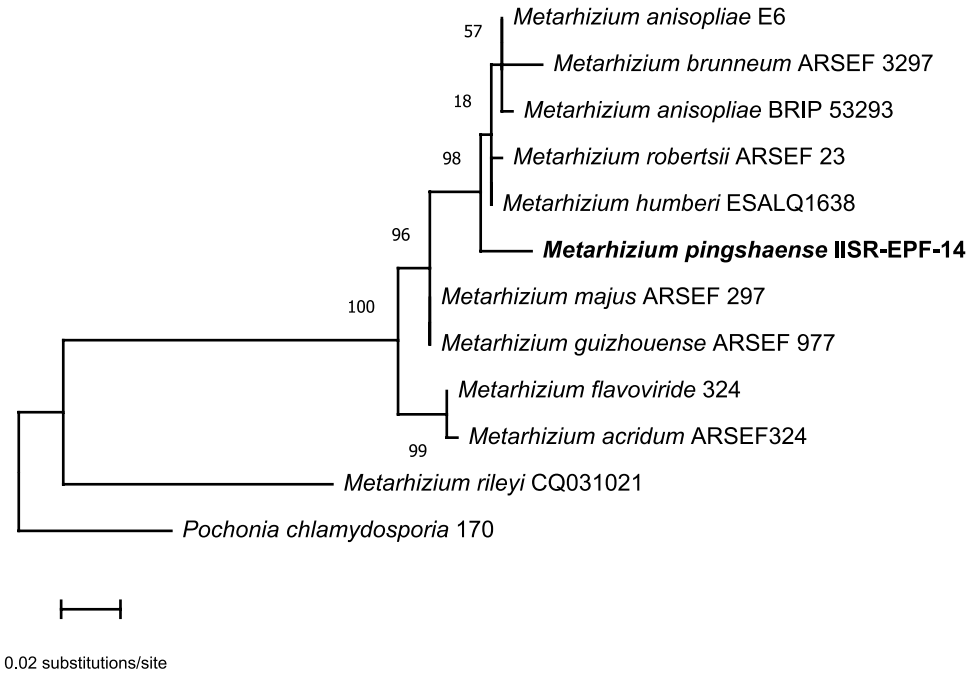

Fig. 6. Neighbor-joining tree based on amino acid sequences of the partial chitinase gene from different *Metarhizium* species, illustrating the relationship with the *M. pingshaense* strain IISR-EPF-14 chitinase gene. Numbers above or below the nodes represent bootstrap values generated from 1000 replications using the Jones-Taylor-Thornton (JTT) model, based on the lowest Bayesian Information Criterion (BIC) scores in MEGA X. *Pochonia chlamydosporia* was used as the outgroup.

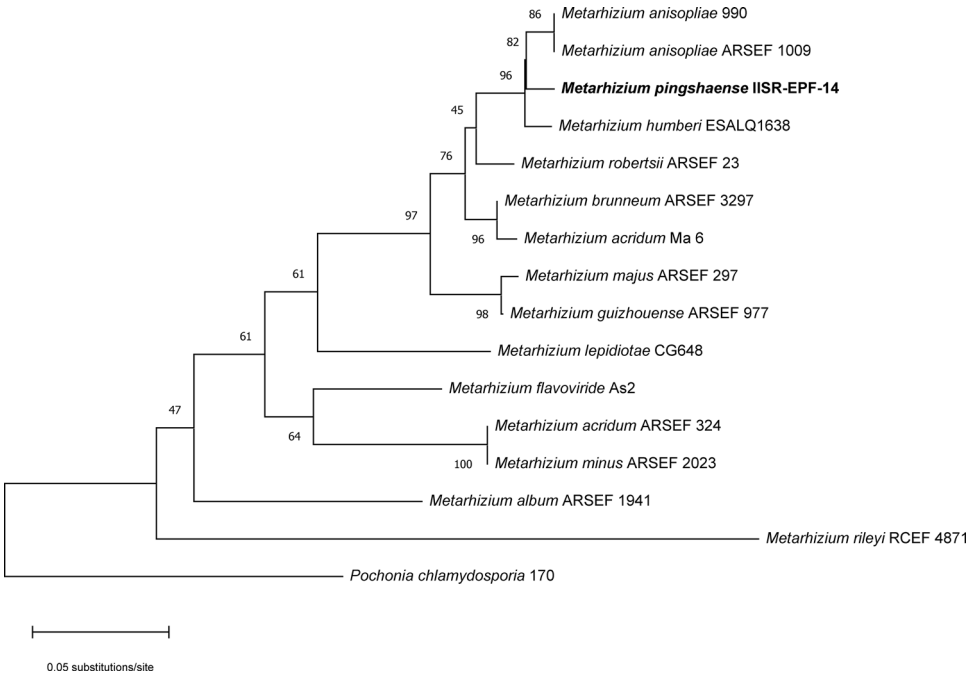

Fig. 7. Neighbor-joining tree based on amino acid sequences of the partial subtilisin-like protease gene from different *Metarhizium* species, illustrating the relationship with the *M. pingshaense* strain IISR-EPF-14 protease gene. Numbers above or below the nodes represent bootstrap values generated from 1000 replications using the Dayhoff plus Gamma (G) model, based on the lowest Bayesian Information Criterion (BIC) scores in MEGA X. *Pochonia chlamydosporia* was used as the outgroup.

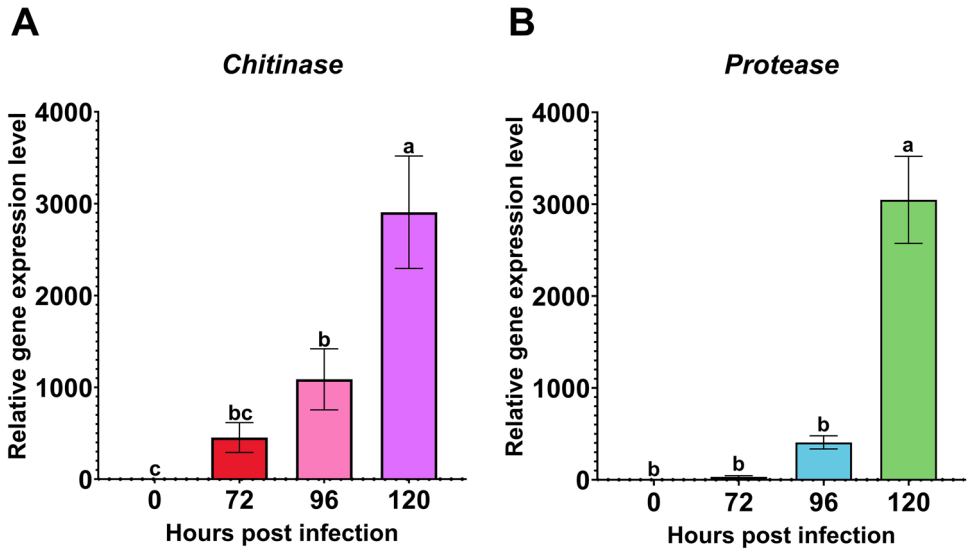

Fig. 8. Relative expression levels of (**A**) *chitinase* and (**B**) *protease* genes in *Conogethes punctiferalis* infected by *Metarhizium pingshaense* strain IISR-EPF-14 at 72, 96, and 120 h post-infection. The relative expression of each target gene was calculated according the ΔΔCT method using *actin* gene as endogenous control. Values represent the mean of two technical and three biological replicates. Bars with the same letter are not significantly different according to Tukey's HSD test (α = 0.05).

the relative expression of the chitinase gene remained low, showing no significant change compared to untreated control. By 96 hpi, an increase in gene expression compared to 72 hpi was observed. A significant up-regulation of *chitinase* gene was observed at 120 hpi, with levels rising more than 2900-fold compared to untreated control ($F = 38.23$; $P < 0.0001$; df = 3, 8) (Fig. 8A).

Similarly, the relative expression of the *protease* gene in infected insects remained significantly low during the initial time points. A gradual increase in gene expression was observed up to 96 hpi but was not statistically significant. However, by 120 hpi, a substantial upregulation of the *protease* gene was observed, with expression levels rising more than 3000-fold compared to control, which was significantly higher than the earlier time points ($F = 37.2$; $P < 0.0001$; df = 3, 8) (Fig. 8B).

Discussion

Knowledge about the host range of EPF is essential for evaluating their economic potential and ensuring safety for non-target organisms[17]. While an organism's ecological host range is assessed under field conditions, the physiological host range of EPF is primarily determined through laboratory assays. These assays assess pathogenicity and virulence based on host mortality, mean survival time, and mycosis[17,42,43]. Bioassays, in particular, are the most reliable way to test EPF's virulence and host range[35]. In our studies, *M. pingshaense* demonstrated significant pathogenicity against all tested *Chilo* species, with mortality rates among late-instar larvae ranging from 83.0% to 96.0% at the highest tested doses. The highest mortality was observed in *C. sacchariphagus indicus* (96%), followed by *C. infuscatellus* (93%) and *C. partellus* (83%). Previously, this strain caused 86% mortality in its originally isolated host, *C. punctiferalis*[1]. In India, a strain of *M. pingshaense* has been reported to infect another significant crambid pest of rice, *Cnaphalocrocis medinalis*[44]. This fungus has also been reported to infect several economically important pests, including *Ectropis obliqua* and *Myllocerinus aurolineatus* on tea[45,46], *Anomala cincta* on maize[47], *Rhynchophorus ferrugineus* on palms[48], termites[49], and mosquitoes[50]. The ability of *M. pingshaense* to infect a diverse array of insect pests across multiple insect orders and agro-ecosystems underscores its broad host range, adaptability, significant commercial potential and suitability for inclusion in integrated pest management (IPM) strategies.

The virulence of EPF can be directly assessed through time-mortality and dose-mortality assays[51]. Requirement of lesser concentration of conidia and shorter time to kill the host has been attributed as key indicators to ascertain the virulence of an entomopathogen[5,24]. In our studies, the median lethal concentrations (LC_{50}) of *M. pingshaense* against late-instar larvae of *C. infuscatellus*, *C. sacchariphagus indicus*, and *C. partellus* were 4.6×10^5, 1.7×10^5, and 9.5×10^5 conidia/ml, respectively. The median survival time (MST) for *C. infuscatellus* ranged from 5.3 to 6.9 days, for *C. sacchariphagus indicus* from 5.4 to 7.9 days, and for *C. partellus* from 6.9 to 8.3 days at the tested doses of 1×10^8 and 1×10^7 conidia/ml. Previous studies using the same strain of *M. pingshaense* against late-instar larvae of *C. punctiferalis* reported an LC_{50} of 9.1×10^5 conidia/ml and MST values between 4.7 and 6.4 days[1], comparable to the current findings. These results suggest that the virulence of this fungus remains consistent across different host insects. Previous studies have demonstrated the efficacy of *B. bassiana* and *M. anisopliae* against *C. infuscatellus* and *C. sacchariphagus indicus* infesting sugarcane[52] and *C. partellus* infesting maize[53]. However, our study is the first to establish the infectivity and virulence of *M. pingshaense* against multiple *Chilo* species infesting various economically important crops.

Indirect assessment of virulence of EPF is usually done by measuring the activity of key enzymes involved in infection pathways, such as chitinases, total proteases and more specific proteases like subtilisin-like (Pr1) and trypsin-like (Pr2) enzymes[54], and lipases[5,22,38]. These enzymes are essential for penetrating the insect cuticle, a fundamental step towards successful pathogenesis[55]. Preliminary screening for the production of CDEs by EPF can be conducted using plate assays on solid media containing cuticular substrates. Plate assays provide a rapid and effective method to assess the production of CDEs by the fungus, which are essential for insect infection and colonization. The presence of clear halo zones around fungal colonies indicates substrate hydrolysis by the fungus, suggesting CDE production. In our plate assays, we observed clear zones around the fungal colony within 24 h, confirming the ability of the fungus to produce key enzymes essential for insect cuticle degradation through its enzymatic arsenal[56].

In our quantitative assays, we observed higher production of proteases, followed by lipases and chitinases. In general, these CDEs are critical for the pathogenicity of EPF, playing a pivotal role in the invasion process[57]. Among these, proteases are particularly significant during the early phase of infection, facilitating pathogen penetration through the insect cuticle[37,58] followed by the activity of chitinases and lipases[24,57]. In our study, the total protease activity steadily increased and reached its peak activity at 5 DAI, and declined sharply over time, which is in concordance with the earlier reports[5,22,24,57]. Whereas, specific proteases like subtilisin-like (Pr1) and trypsin-like (Pr2) enzymes, exhibited peak activities at 3 DAI, followed by a gradual decline over time. Interestingly, the peak Pr1 enzyme activity was 2.7 times higher than that of Pr2. Similar findings have been reported in *I. fumosorosea*[22], *B. bassiana*[35], *M. anisopliae*[37,59], etc. Pr1 and Pr2 are the primary enzymes produced by EPF during the early stages of infection, aiding fungal hyphae in breaching the insect cuticle[5]. While Pr1 is the dominant protease, Pr2 acts as its promoter during the initial stages of cuticle colonization, playing a key role in insect penetration and significantly contributing to the pathogen's virulence[60,61].

Fungal hyphae penetrate the insect cuticle with the aid of proteases, followed by the secretion of chitinases, which enhances its penetration efficiency by degrading the host's cuticular chitin. Chitin, an important component of the insect cuticle, acts as a primary barrier against pathogen invasion[18]. Therefore, the secretion of chitinases by fungi not only facilitates fungal invasion but also aids in assimilating the released carbohydrate nutrients from the host, promoting fungal growth and development[21,22]. Chitinases are generally believed to be induced later in the infection process, following the action of proteases, due to the sequential solubilization of cuticular components[55]. This sequential pattern was also evident in our studies, where differences in the peak activity and production levels of chitinases were observed. Unlike proteases, which peaked in activity at 3–5 DAI in synthetic media, chitinase activity peaked at 10 DAI, with comparatively lower production levels. Similar findings have been reported in other EPFs, such as *I. japonica*, *I. fumosorosea*, *A. nominus*, *B. bassiana*, and *M. anisopliae*[22,24,55,62,63]. The lower production and delayed peak activity of chitinases can be attributed to their role in degrading pro-cuticular chitin after the outer cuticular layers have been dissolved by proteases, reflecting their secondary role in the infection process[55,57].

In addition to proteases and chitinases, lipases secreted by EPF have been demonstrated to play a significant role in their entomopathogenicity against various insect pests[5,22,38,64]. Since lipids are an integral part of insect cuticle, the production of lipolytic enzymes by EPF is essential for its successful penetration, growth, and development[23]. Unlike proteases and chitinases, lipases hydrolyze the ester bonds in the waxy cuticular components of the insect integument, as well as the lipoproteins and fats within the insect body, facilitating penetration and proliferation in the host[23,64]. In our study, we observed lipase production in synthetic media amended with vegetable oils, olive and coconut oil respectively, with the peak activity occurring at 10 DAI, similar to the timeline observed for chitinase activity. Notably, the production of lipases was 50 times higher than that of chitinases in our experiments. Higher lipase production by the fungus signifies its virulence potential, confirming the findings of the previous studies that demonstrated the direct role of lipases in EPF virulence[23,38,65]. Furthermore, lipase activity is not only essential during the initial phase of host cuticle penetration by EPF, but also plays a significant role in later stages, supporting fungal growth and proliferation within the host post cuticle degradation[66].

The observed increase and subsequent decline in CDEs activity of *M. pingshaense* in our study may be attributed to multiple interconnected factors. The initial increase in enzyme activity may be attributed to the availability of specific substrates such as chitin, proteins, or lipids in the media that induce enzyme production by the fungus. With the depletion of the available substrates and accumulation of hydrolysis products such as N-acetylglucosamine, peptides, and fatty acids in the media, the enzyme activity declines triggering a feedback inhibition. This decline also coincides with fungal developmental transition from active proliferation phase to vegetative growth and reproduction, typically associated with reduced secondary metabolism[67]. Additionally, culture conditions such as pH shifts and nutrient imbalances may impair enzyme stability or induce autolysis, especially proteases[68].

We also investigated the production of CDEs in synthetic media supplemented with the insect cuticle of *C. punctiferalis* as the sole carbon source to understand the role of cuticular components in enzyme production. Interestingly, we observed significantly higher levels of total proteases, specific proteases (Pr1 and Pr2), and chitinases in the insect cuticle-amended media 5 DAI. The production levels of these enzymes were 4.6, 2.6, 2.4, and 44.5 times higher, respectively, compared to in vitro studies using specific substrates such as casein and chitin. Previous research has also reported an increased production of CDEs by EPF like *I. fumosorosea*, *B. bassiana*, and *M. anisopliae,* when insect cuticular components from susceptible hosts were added to the growth medium[5,22,64,69,70]. A plausible explanation for this observation is the immediate recognition of the insect cuticular components by the EPF[64,] likely due to the stimulatory effect of the cuticular substrates, resulting in rapid conidial germination and development triggering the enzyme machinery into action[71]. Further, it is also to be noted that the higher secretion of these enzymes in media with cuticular components indicate the synergistic interaction between these CDEs during infection, proliferation, disintegration, and nutrient

assimilation by the EPF[72]. Interestingly, we did not observe discernible differences in lipase production between media amended with vegetable oils and insect cuticle. This may be due to the estimation of the enzyme activity at an earlier time interval in cuticle amended media in our studies. Nevertheless, it has been well-documented that lipases play a limited role during the early stages of cuticle penetration, but are crucial during the later stages of fungal development, aiding in the utilization of the insect's lipid reserves within the hemocoel[5,73]. Further, earlier studies have also reported the late production of lipases in media supplemented with insect cuticular components compared to proteases and chitinases, confirming the findings of our study[24,55].

We sequenced and characterized the genes encoding two key CDEs, *chitinase* and *protease*, in *M. pingshaense* to understand their similarity and evolutionary relationships with other EPF. Based on the amino acid sequence similarity, the *chitinase* gene of *M. pingshaense* was found to be closely related to the glycoside hydrolase family 18 protein. Previous studies have shown that the *chitinase* gene of *M. anisopliae* also shares similarity with this protein family[21,74]. This was also evident from the BLAST homology search, which revealed high similarity between the deduced amino acid sequence of the *chitinase* gene of *M. pingshaense* and those of *M. anisopliae* and other *Metarhizium* species. Furthermore, it suggests that *chitinase* genes of *Metarhizium* species are highly similar to the glycoside hydrolase family 18 protein. Phylogenetic analysis further confirmed these findings, revealing a close evolutionary relationship among *Metarhizium* species[75].

The characterized subtilisin-like *protease* gene of *M. pingshaense* was found to be closely related to subtilases derived from various entomopathogenic and nematophagous fungi. These subtilases belong to two families: the subtilisin-like protease S8 and the serine-carboxyl protease S53[76], and plays a significant role in disrupting the physiological integrity of insect and nematode cuticles during penetration and colonization[77–79]. At least 11 subtilisins have been identified as being secreted by the broad host range pathogen *M. anisopliae* during its growth on insect cuticle, as revealed by expressed tag analyses[80]. In our study, the subtilisin-like protease gene of *M. pingshaense* shared a high degree of sequence similarity with the corresponding genes from *M. anisopliae* and *M. humberi*, which was further supported by phylogenetic analysis. The strong sequence similarity between the subtilisin-like *protease* gene of *M. pingshaense* and that of the generalist pathogen *M. anisopliae* may explain its ability to infect hosts beyond its original host species. However, this requires detailed molecular studies to identify the array of subtilisins secreted by *M. pingshaense* to compare them with those of *M. anisopliae* and other broad host range pathogens. Such studies will provide insights into the molecular and biochemical pathways that contribute to its wide host range.

Quantitative real-time PCR (qRT-PCR) is a powerful tool for studying the gene expression of EPF within an infected insect host. Its high sensitivity enables the detection of fungal transcripts in mixed cDNA samples containing both insect and fungal material. Additionally, the ability to analyze insect gene expression from the same sample enhances its utility in tracking the progression of infection over time[81]. Entomopathogenic fungi secrete chitinases and proteases to hydrolyze chitin and proteins, which are major components of the insect cuticle, thereby paving way for the successful infection of the host. Therefore, the regulation of these enzymes is considered vital for the infection of a susceptible host[19]. In our in vivo studies, we observed a gradual increase in the expression of these two pathogenesis-related genes, *chitinase* and *protease*, during the course of infection. The expression of these genes was initially low during the early phase (0–72 h) but increased over time, peaking at 120 hpi. These findings are consistent with our biochemical assays, which also showed a steady increase in enzyme activity over time. The high expression of the *protease* gene during the later phase of infection suggests that it not only facilitates fungal penetration during the initial phase but also supports conidiation upon completion of the fungal life cycle within the host[82,83]. Similarly, the role of the *chitinase* enzyme in the later phase of infection is significant, as it helps create perforations in the peritrophic membrane of the gut, allowing the pathogen to penetrate host tissues[22]. Collectively, our findings demonstrate that the *Pr1* gene, along with the *chitinase* gene of entomopathogenic fungi, serves as critical markers of fungal virulence against target pests[84–86].

Overall, our studies indicate that the enzyme production profile of *M. pingshaense* aligns with that of other well-studied EPF, such as *B. bassiana* and *M. anisopliae*, which also produce CDEs like chitinases, proteases, and lipases as essential virulence factors[19,37,55]. However, gene expression analysis during infection of *C. punctiferalis* revealed a unique temporal pattern in *M. pingshaense*, where both chitinase and protease genes showed minimal expression at 72 hpi, followed by a moderate increase at 96 hpi and a dramatic upregulation by 120 hpi. In contrast, studies on *M. anisopliae* and *B. bassiana* have demonstrated that CDE gene expression and production is often rapidly induced upon contact with insect cuticle, with their expression occurring as early as 24 h post-infection of the host[55,77,87]. Conversely, studies also indicate late expression of virulence genes in *B. bassiana*, indicating strain-specific variations in gene expression[83]. The delayed but intensified expression in *M. pingshaense* suggests a tightly regulated, host-responsive activation of virulence genes, possibly reflecting a stealth-phase strategy early in infection to evade host immunity, followed by an aggressive degradation phase for cuticle penetration and nutrient acquisition. These findings collectively highlight the adaptive infection strategy of *M. pingshaense* and underscore its potential as a highly effective biocontrol agent.

In conclusion, our studies highlight *M. pingshaense* strain IISR-EPF-14 as a potential biocontrol agent against *Chilo* species, which are major pests of various crops, including sugarcane, rice, maize, sorghum, etc. Additionally, the studies have elucidated the underlying virulence mechanism of this pathogen, including the production of CDEs and the upregulation of related genes during host infection. The high expression levels of these virulence genes explain the high mortality rate caused by this fungus, even against late-instar larvae of *Chilo* spp. Previous studies have demonstrated the potential use of this pathogen against *C. punctiferalis*, a polyphagous pest of various crops[1]. Further, the additional ecological role played by this fungus in the environment by means of organic acid production and mineral solubilization thus promoting plant growth was recently reported[88]. These traits, typical of a promising biocontrol agent, make *M. pingshaense* an ideal candidate for biopesticide development and for use in sustainable agriculture. Future studies should focus on testing the physiological host range of this organism against insects from different orders, evaluating its ecological host

range under field conditions, and assessing its impact on natural enemies. Moreover, it is essential to identify other genes and toxins involved in determining the pathogen's host range and virulence through comprehensive molecular and biochemical studies.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 22 January 2025; Accepted: 21 May 2025
Published online: 27 May 2025

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Acknowledgements

CMSK acknowledges the funding support by Kerala State Council for Science, Technology and Environment (KSCSTE) project, Development of a *Metarhizium* sp. based bio-pesticide formulation for the control of shoot borer, *Conogethes punctiferalis* infesting cardamom, ginger and turmeric (No. 466/2020/KSCSTE dated 23.06.2020) under the"Young Investigators Programme in Biotechnology"of Kerala Biotechnology Commission. The authors thank the Director, ICAR-IISR, Kozhikode, for necessary facilities. We also thank the anonymous reviewers for their constructive comments, which helped to improve the manuscript.

Author contributions

CMSK: Conceptualization, Funding acquisition, Methodology, Data curation, Writing—original draft preparation, Writing—review & editing, Validation. MS: Investigation, Data curation. MBR: Investigation; MP: Investigation. SDS: Investigation. CG: Investigation. PA: Investigation. TKJ: Writing—original draft, Validation. SD: Editing, Validation, Supervision. AIB: Validation, Supervision. All authors reviewed the manuscript.

Funding

Kerala Biotechnology Commission,No. 466/2020/KSCSTE dated 23.06.2020.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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