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Characterization and biocontrol potential of a naturally occurring isolate of *Metarhizium pingshaense* infecting *Conogethes punctiferalis*

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ABSTRACT

An entomopathogenic fungus was isolated from an infected larva of *Conogethes punctiferalis* (Guenée) (Crambidae: Lepidoptera), a highly polyphagous pest recorded from more than 120 plants and widely distributed in Asia and Oceanic countries. The fungus was identified as *Metarhizium pingshaense* Q.T. Chen & H.L. Guo (Ascomycota: Hypocreales) based on morphological characteristics and molecular studies. Scanning electron microscopic studies were conducted to study the infection of *C. punctiferalis* by *M. pingshaense*. Bioassay studies with purified conidial suspension proved that the isolate was highly virulent to *C. punctiferalis*, causing more than 86 % mortality to fifth instar larvae at 1×10^8 spores/mL, under laboratory conditions. The median lethal concentration (LC_{50}) of the fungus against late instar larvae was 9.1×10^5 conidia/mL and the median survival time (MST) of late instar larvae tested at the doses of 1×10^8 and 1×10^7 conidia/mL were 4.7 and 6.4 days, respectively. The optimal temperature for fungal growth and sporulation was found to be 25 ± 1 °C. This is the first report of *M. pingshaense* naturally infecting *C. punctiferalis*. Isolation of a highly virulent strain of this fungus holds promise towards development of a potential mycoinsecticide against this pest.

1. Introduction

Microbial bio-pesticides, especially entomopathogenic fungi (EPF) have great potential to be developed as an alternative to chemical pesticides. Among them, EPF occupy a prime position because of their unique mode of action and ability to infect a wide range of sucking and chewing types of insect pests. They are considered as environmentally safe and represent novel tools in pest management (Chandler, 2017). Moreover, the additional ecological roles played by EPF other than being insect pathogens, such as endophytes, plant growth promoters, rhizosphere colonizers, disease antagonists, etc., make them ideal candidates for use in sustainable agriculture (Jaber and Ownley, 2018). Although an estimated 700 species of fungi belonging to about 90 genera have been reported to be entomopathogenic, only 12 species have been developed as commercial products. Among the 171 commercial products of EPF available worldwide, about 34 % of them are based on *Metarhizium* spp. alone (Faria and Wraight, 2007). Success of a mycoinsecticide depends on selection of native isolates occurring on the same host species and adapted to the local environmental conditions with high virulence and sporulation potential (Drummond and Heale, 1988).

Lepidopteran borer pests are emerging as a threat for production and productivity of horticultural crops. Due to their cryptic nature, they remain undetected during the initial stages of infestation posing a great challenge for their effective control. Apart from damaging the stems, they also cause damage to economic parts like inflorescences, fruits, tubers, etc., affecting both the quality and quantity of the produce. The borer pests belonging to the family Crambidae are of worldwide concern because of the huge economic damage inflicted by them to various agricultural and horticultural crops.

The genus *Conogethes* (=*Dichocrocis*) (Crambidae: Lepidoptera) belongs to the complex taxon of Crambids comprising of at least 17 recognized species (Nuss et al., 2003–2020). These insects with their ability for speciation, invasion to new areas and wide host range have emerged as an important pest group causing huge economic loss to many horticultural crops (Shashank et al., 2014). Among them, *C. punctiferalis* (Guenée) commonly known as the shoot borer or yellow peach moth is widely distributed in Eastern Palearctic, Oriental, Oceanic regions (CABI, 2018), Hawaii (Nishida, 2002) and has been intercepted in quarantine on imported fruits in Europe (Koryniska, 2012). Worldwide, *C. punctiferalis* has been reported to attack more than 120 plants including important crops like cotton, cocoa, orange, grapes, mango,

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apple, peach, pear, sunflower, guava, pomegranate, castor, mulberry, banana, sugarcane, maize, sorghum, zingibers, etc., belonging to diverse families and in India alone the insect attacks more than 35 plant species belonging to 19 families (Devasahayam and Koya, 2007; Shashank et al., 2014). *C. punctiferalis* is a serious insect pest of turmeric (*Curcuma longa* L.) and ginger (*Zingiber officinale* Rosc.) in India, which are high-value spice crops of commerce. The larva bores into shoots (pseudostems) of ginger and turmeric and feed the internal tissues resulting in yellowing and drying of infested shoots and cause significant reduction in yield, when more than 50 % of the shoots in a clump are infested (Koya et al., 1986; Devasahayam et al., 2010).

Although several insect parasitoids (Devasahayam, 1996) and other natural enemies including mermithid nematode (Senthil Kumar et al., 2018b) have been reported on *C. punctiferalis*, difficulties in their mass multiplication have impeded their field application. Hence, synthetic insecticide based management strategy is the only available option for efficient management of this pest. Development of alternate strategies for the management of *C. punctiferalis* is important in view of the global concern on overdependence of pesticides for the management of insect pests. We have recorded for the first time the natural incidence of a fungal entomopathogen, *Metarhizium pinghaense* Q.T. Chen & H.L. Guo (Clavicipitaceae: Hypocreales), isolated and characterized from this pest. *M. pingshaense* is reported to have a wide host range (Cito et al., 2014; Kirubakaran et al., 2018; Nishi and Sato, 2017) and has been frequently isolated from rhizosphere soils (Nishi and Sato, 2019, 2017). The objectives of the study were to characterize the fungus based on morphological and molecular tools; to assess the virulence and infection process of the fungus by laboratory bioassays and scanning electron microscopy, and to study the influence of temperature on the growth and sporulation of the fungus under laboratory conditions. The biocontrol potential of the fungus and its suitability for developing it as a mycoinsecticide are discussed.

2. Materials and methods

2.1. Fungus source and isolation

A naturally dead larva of *C. punctiferalis* showing signs of mycosis observed inside an infested turmeric pseudostem, collected from the research farm of Indian Council of Agricultural Research-Indian Institute of Spices Research (ICAR-IISR), Peruvannamuzhi (11°35′0″ N, 75°49′0″ E), Kozhikode District, Kerala, India was used to isolate the fungus. Bits of cadaver were streaked on Oat Meal Agar (OMA) — CTAB medium (Posadas et al., 2012) in petri dishes (100 × 17 mm) under aseptic conditions in a laminar air flow chamber and kept at 26 ± 1 °C for 10 days in a biochemical oxygen demand (BOD) incubator for growth and sporulation. The fungus was sub-cultured on quarter strength Sabouraud's dextrose agar medium supplemented with yeast extract (SDAY/4). Single spore cultures of the fungus obtained by plating a serially diluted conidial suspension on SDAY/4 plates (Senthil Kumar et al., 2016) were used for further studies. A pure culture of the fungus after confirmation of its identity was deposited in the Entomopathogenic Fungal Repository of ICAR-IISR with Accession Number IISR-EPF-14.

2.2. Insect culture

Laboratory culture of *C. punctiferalis* was established using late instar larvae collected from infested turmeric pseudostems from the research farm of ICAR-IISR, Kozhikode, Kerala. The insects were reared on turmeric pseudostem bits on plastic trays kept inside insect rearing cages (60 × 60 × 60 cm) and maintained at 27 ± 2 °C, 60–70 % relative humidity (RH) with 12:12 h day night photoperiod until pupation. Pupae collected from the pseudostems were transferred to a petri dish (100 × 17 mm) and placed in another rearing cage for adult emergence and mating. The adults were allowed to lay eggs on young turmeric plants grown on pro-trays inside the cages. Larvae on hatching, initially fed on the leaf tissues and once they reached second instar, larvae were collected from the pseudostems and reared on turmeric pseudostems as described earlier for future studies.

2.3. Bioassays

Conidial suspension was prepared by washing 2 weeks old fungal culture grown on petri dishes (100 × 17 mm) with 3 mL of sterile 0.05 % Triton-X 100. The suspension was vortexed and passed through double layered muslin cloth and the final volume was made up to 10 mL with sterile 0.05 % Triton-X 100. For the initial screening bioassay, 10 mL of spore suspension made up to a concentration of 2.5×10^8 conidia/mL was prepared in triplicate through serial dilution. Three groups of fifth instar larvae (n = 10/group) were immersed in 3 mL of conidial suspension (2.5×10^8 conidia/mL) separately for 30 s and then transferred to a petri dish (100 × 17 mm) lined at the bottom with a layer of cotton beneath a circular sterile filter paper (Senthil Kumar et al., 2016). An equal number of insects dipped in sterile 0.05 % Triton-X 100 served as control. Turmeric pseudostem bits were provided to the insects for feeding, which were replaced with fresh pseudostem bits every 3 days. A moist cotton ball was placed inside the petri dish to maintain humidity. The experimental setup was maintained at 28–32 ± 2 °C, 60–70 % relative humidity and a 12:12 h day:night photoperiod under ambient room conditions and replicated three times. The mortality of insects was recorded daily after 24 h of post treatment and the experiment was terminated 15 days post inoculation (p. i.). Dead insects were collected and washed individually in 70 % ethanol for few seconds followed by rinsing twice in sterile distilled water to remove surface contaminants. The cadavers were then immersed in 0.5 % sodium hypochlorite solution for 30 s, rinsed in three changes of sterile distilled water and blot dried on a sterile filter paper (Lacey and Solter, 2012). Surface disinfected cadavers were then transferred to humid chambers (petridishes lined with moist filter paper) for sporulation. Sporulating cadavers were observed under a light microscope to confirm infection by the entomopathogen and the fungus was re-isolated by inoculating on PDA.

The median lethal concentration (LC_{50}) of the fungus was tested with fungal suspensions of 10^5, 10^6, 10^7 and 10^8 conidia/mL and the median survival time (MST) of larvae infected with the fungus was determined at 10^7 and 10^8 conidia/mL of suspension, against fifth instar larvae of *C. punctiferalis*. There were 10 larvae in each test group and three replicates for each group. The experiments were repeated three times during the period of study with freshly prepared conidial suspensions to confirm the results. The larvae in the controls were treated with sterile 0.05 % Triton® X-100 in water. Other treatment details were as described above. Insect mortalities were recorded at 24 and 12 h intervals for LC_{50} and MST studies, respectively up to 15 days p. i.

2.4. Identification of the fungus

2.4.1. Morphological characterization

Two-week old monosporic cultures of the fungus grown on potato dextrose agar (PDA) at 25 ± 1 °C were used to study the macro- and micro-morphological features of the fungus. Macroscopic features such as growth and appearance of the colony, pigmentation on reverse side of the media were recorded. Micro-morphological features such as shape and size of the conidia were recorded using Leica DM 5000 B research microscope fitted with a digital camera.

2.4.2. Molecular characterization

2.4.2.1. DNA extraction, PCR and sequencing. One-week old fungus grown on PDA at 25 ± 1 °C was used to extract total genomic DNA using fungal genomic DNA extraction Kit (Chromous Biotech, India) from approximately 500 mg of mycelium following the manufacturer's instructions. Polymerase chain reactions (PCR) were performed to amplify

the following partial gene sequences: ITS gene with primers ITS4 and ITS5 (White et al., 1990), translation elongation factor 1α gene (TEF) with primers 983 F and 2218R (Rehner and Buckley, 2005), RNA polymerase II largest subunit (RPB1) with primers RPB1a and RPB1-VH6R, RNA polymerase II second largest subunit (RPB2) with primers RPB2_5 F and f RPB2−7cR and DNA lyase (APN2) with primers APN_clavF2 and APN_clavR8 (Bischoff et al., 2009; Kepler et al., 2014). PCR reactions were carried out with 10–20 ng of template DNA in volumes of 25 μL containing 2.5 μl of 10 × PCR buffer, 2.0 μl of dNTPs (2.5 mM each), 0.75 μl each of (20 μM) forward and reverse primer pairs, 0.2 μl of 5 units/μl *Taq* DNA polymerase (Sigma, USA) after making the remaining volume with PCR grade water (Himedia, India). The PCR conditions for ITS and TEF were as described by (Senthil Kumar et al., 2016) and the PCR conditions for RPB1 & 2 and APN2 were slightly modified from the protocol of Bischoff et al. (2009). Briefly, PCR conditions for RPB1 were: initial denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min; for RPB2: initial denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 57.5 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min; for APN2: initial denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The amplified bands were excised, purified using Sigma GelElute™ Gel Extraction Kit (Sigma, India) as per manufacturer's protocol and the products were sent to SciGenom Labs Private Ltd., Kochi, India and bi-directional sequencing was carried out in ABI 3730 XL DNA Analyzer using the same set of PCR primers. Trimmed sequences were manually edited using BioEdit software, checked and subjected to BLAST (Basic Local Alignment Search Tool) search to identify sequences deposited in GenBank that had significant homology. The ITS sequence was deposited in GenBank with accession number MK537396 and that of TEF, RPB1, RPB2 and APN with accession numbers, MK583958– MK583961.

2.4.2.2. Phylogenetic analysis. The ITS, TEF, RPB1, RPB2 and APN2 sequences along with reference sequences of recognized species of *Metarhizium* (Bischoff et al., 2009; Kepler et al., 2014) retrieved from the GenBank were aligned using ClustalW Multiple alignment tool and the alignments were cured using Gblocks (Dereeper et al., 2008). A partition homogeneity test was performed to assess incongruences among three gene sets (RPB1, RPB2 and TEF genes) with PAUP* version 4.0a149 (Swofford, 2002). The test indicated congruence between RPB1, RPB2 and TEF genes. A concatenated set of these genes were developed and an optimal evolutionary model for phylogenetic analysis was selected based on the lowest score value of Bayesian Information Criterion (BIC) available in MEGA X (Kumar et al., 2018). A maximum likelihood (ML) tree was constructed for these gene sets using the model, Tamura-Nei model plus Gamma (T93 + G). ITS and APN2 genes were analysed separately due to non-availability of sequence information for all the reference taxa. The ITS and APN2 sequences were aligned separately with reference sequences of *Metarhizhium* spp. retrieved from GenBank and ML trees were constructed using Kimura two-parameter model plus Gamma distribution (K2 + G) and Tamura-Nei model plus Gamma (T93 + G) respectively, based on the scores of BIC. Gaps in alignment were treated as missing data and bootstrapping was performed with 1000 replicates. *Pochonia chlamydosporia* var. *chlamydosporia* (Goddard) Zare et Gams (Metacordycipitaceae) was used as outgroup.

2.5. Scanning electron microscopy (SEM)

Sporulated insect cadavers obtained from bioassays studies were prepared for SEM studies following Senthil Kumar et al. (2016). Briefly, the cadavers were dehydrated in a desiccator for 5 days at room temperature, mounted on carbon stubs, gold sputtered for 20 s and directly viewed under a Scanning Electron Microscope (Hitachi SU6600).

2.6. Effect of temperature on growth and sporulation of the fungus

The growth and sporulation efficiency of the fungus under different temperature regimes (18 ± 1 °C, 20 ± 1 °C, 25 ± 1 °C, room temperature (28–32 ± 2 °C) and 35 ± 1 °C) were studied by inoculating agar plugs of 6 mm diameter obtained from 2-week old actively growing colonies onto the centre of PDA plates (100 × 17 mm). The plates were incubated at the above temperatures in BOD incubators except for room temperature and each treatment was replicated five times. Observations on colony growth and total spore production were recorded 20 days after inoculation (DAI). Total spore production was estimated by harvesting the conidia from individual plates by scrapping with a sterile scalpel, washed with 5 mL of sterile 0.05 % Triton® X-100 and enumerated using Neubauer haemocytometer. The experiment was repeated once to confirm the results.

2.7. Statistical analysis

Bioassay data was subjected to arc sine $[(x + 0.5)/100]^{½}$ transformation prior to analysis. Data on bioassay and the influence of temperature on fungal colony growth and spore production were subjected to one-way analysis of variance (ANOVA) and mean comparisons were done by Fisher's least squares difference (LSD) using GraphPad Prism® Version 7.0 for Windows, GraphPad Software, La Jolla, California USA. Insect mortality was not subjected to Abbott's correction as there were no mortality in control. The median lethal concentration (LC_{50}) of the fungus to kill the insects was calculated using IBM SPSS Statistics 25 (IBM Corp., 2017). The web-based programme, Online Application for Survival Analysis (OASIS), developed by Yang et al. (2011) was used to determine median survival time (MST) of the insects under each treatment based on Kaplan-Meier survival distribution function.

3. Results

3.1. Bioassays

Preliminary screening of the fungus at a dose of 2.5 × 10^8 conidia/mL caused 100 % mortality of the insects within 10 days after

Fig. 1. Infected fifth instar larvae of *C. punctiferalis* showing (**a**) mycosis (**b**) proliferation of mycelia and (**c**) profuse sporulation on the cadaver.

inoculation. Infected insects in the treatment showed sparse signs of white mycosis (Fig. 1a) initially, which gradually spread throughout the body surface (Fig. 1b). The entire body of the insect was covered with densely packed greyish green conidia within 2–4 days after mummification of the cadaver (Fig. 1c). All the larvae in the control treatment survived and pupated normally.

The median lethal concentration (LC_{50}) of the fungus against late instar larvae of *C. punctiferalis* was 9.1×10^5 conidia/mL (probit model: p = -3.312 + 0.556*x*; SE of intercept = 0.756; $\chi^2 = 0.588$, *df* = 2) and the 95 % confidence interval (CI) ranged from 2.3×10^5 to 2.5×10^6 conidia/mL. The larval mortality (mean ± SE) ranged from 30 ± 3.3 to 86.7 ± 3.3 % at different doses tested and there were significant differences in mortality among the doses tested ($F = 109$; $P < 0.0001$; $df = 410$) (Fig. 2). No mortality was observed in control. The median survival time of late instar larvae, tested at two different doses differed significantly from each other (Log-rank test; $P < 0.05$). The MST of the larvae tested at the highest dose of 1×10^8 conidia/mL was 4.7 days (± 0.14 SE) and the 95 % CI ranged from 4.4 to 5.0 days, whereas the MST value at the dose of 1×10^7 conidia/mL was 6.4 days (± 0.36 SE) and the 95 % CI ranged from 5.7 to 6.2 days (Table 1).

3.2. Identification of the fungus

3.2.1. Morphological identification

Colonies of the fungus grown on quarter strength SDAY media initially appeared white and turned greyish-green upon sporulation with yellowish green reverse. Conidial production was denser at the middle of the colony and they appeared in a concentric circular pattern towards the end. Conidia were cylindrical to ovoid in shape and were formed in long chains. The average length and width of the conidia (n = 50) were 6.5 ± 0.9 μm (range 5.0–7.6 μm) and 2.3 ± 0.3 μm (range 1.7–2.9 μm) respectively, which were similar to the earlier reported reference isolates (Table 2). Based on the colony features and micro-morphology, the fungus was identified as *Metarhizium pingshaense* Q.T. Chen & H.L. Guo.

3.2.2. Molecular characterization

Amplification of the partial gene sequences of different conserved gene regions of the present fungus to confirm its morphological identity resulted in amplicons of size: 514 bp, 797 bp, 690 bp, 1041 bp and 987 bp for ITS, TEF, RPB1, RPB2 and APN2 genes, respectively. The partial ITS gene sequence of the fungus was 100 % identical with a *M. pingshaense* strain ARSEF 2162 (HM055447). The TEF sequence was 99 % identical to ARSEF 4342 strain (EU248851), and the partial RPB1 showed 100 % similarity with *M. pingshaense* strains ARSEF 3210

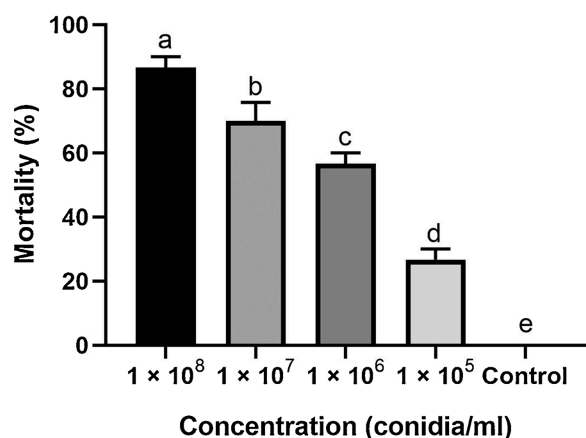

Fig. 2. Per cent mortality (Mean ± SE) of fifth instar *C. punctiferalis* larvae exposed to different concentrations of *M. pinghanese*. Bars represent original data. Data were analysed after arc sine $[(x + 0.5)/100]^{½}$ transformation. Bars represented by the same letter are not significantly different by Fisher's LSD (α = 0.05). Error bar represent standard error (SE) of three replicates.

Table 1
Survival time analysis of fifth instar *C. punctiferalis* larvae treated at different doses of *M. pingshaense.*

Dose	N	MST (days)*	SE	95 % CI
1×10^8	30	4.7^a	0.14	4.4 – 5.0
1×10^7	30	6.4^b	0.36	5.7 – 6.2

* Survival time values with different letters are significantly different at $P < 0.05$ by log-rank test.

Table 2
Morphological features of *M. pingshaense* isolates.

Strain/ Isolate	Host	Origin	Size of conidia (range)		Reference
			Length (μm)	Width (μm)	
ARSEF 4342	Coleoptera	Solomon Islands	4.5–7.0	2.0–3.0	Bischoff et al. (2009)
CBS 257.90 *	Coleoptera	China	4.5–8.0	2.0–3.0	Bischoff et al. (2009)
IISR-EPF-14	Lepidoptera	India	5.0–7.6	1.7–2.9	This study

* Ex-type isolate.

(DQ468354), 4342 (EU248903), 7929 (EU248899) and CBS 257.90 (EU248902). The partial RPB2 and APN2 gene sequences shared > 99 % similarity with the *M. pingshaense* strain ARSEF 3210 (DQ468369) and ARSEF 4342 (KJ398524), respectively. The ML tree generated with concatenated nucleotide sequences of RPB1, RPB2 and TEF (Fig. 3) showed that the isolate (IISR-EPF-14) clustered with the reference taxa of *M. pingshaense* (ARSEF 4342) with a strong bootstrap support of 98 % confirming its morphological identity. This was also supported by the ML trees generated with the ITS sequences (Fig. 4) and APN2 (Fig. S1).

3.3. SEM studies

Different stages of infection by the fungus on the insect host were observed in SEM studies. Conidia were found adhered to and germinated on the cuticle surface with signs of cuticle degradation at the site of germination of the conidia (Fig. 5a). Ramifying intercalary appresoria and infective pegs were found to descend on the surface for cuticular penetration (Fig. 5b). Upon completing the host colonization, the mycelia were found to emerge through the intersegmental membranes (Fig. 5c). Mycelial ramification was seen throughout the body surface (Fig. 5d) with chains of cylindrical conidia formed on phialides (Fig. 5e-f) mummifying the insect host proving the infective potential of the fungus.

3.4. Effect of temperature on growth and sporulation of the fungus

Temperature had a significant influence on the growth of the fungus ($F = 2463$; $P < 0.0001$; $df = 4, 20$). The highest temperature (35 ± 1 °C) tested retarded the growth of the fungus and the low temperatures slowed down the fungal growth (mean colony diameters were 3.24 ± 0.02 and 2.92 ± 0.04 cm at 20 ± 1 °C and 18 ± 1 °C, respectively, 20 days p. i.). The mean colony diameter of the fungus was significantly higher at 25 ± 1 °C (5.68 ± 0.09 cm dia), followed by room temperature conditions (5.04 ± 0.02 cm dia) (Fig. 6). Similarly, total spore production of the fungus was also influenced significantly by variation in temperatures ($F = 82.93$; $P < 0.0001$; $df = 4, 20$). Maximum spore yield was obtained at 25 ± 1 °C (2.3×10^9 conidia) 20 days p.i. No significant difference in spore yield was observed at room temperature and at 20 ± 1 °C (4.7×10^8 and 4.6×10^8 conidia respectively). However, the lowest and the highest temperatures tested were found to have a negative influence on the spore production by the fungus (Fig. 7).

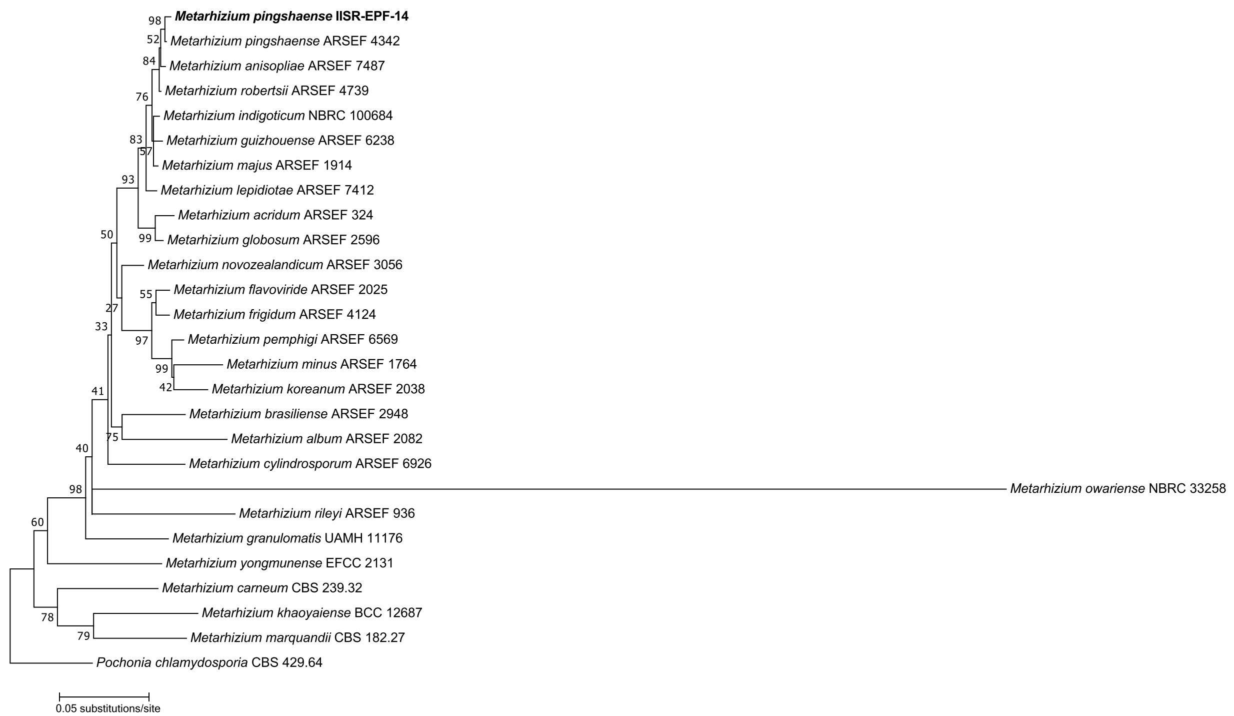

Fig. 3. Maximum likelihood (ML) tree based on analysis of concatenated nucleotide sequences of RPB1, RPB2 & TEF genes of different *Metarhizium* spp. Numbers above or below the nodes indicate bootstrap values generated after 1000 replications using the model, Tamura-Nei model plus Gamma (T93 + G). Reference sequences of different *Metarhizium* spp. obtained from GenBank are shown with their strain numbers. *Pochonia chlamydosporia* was used as outgroup.

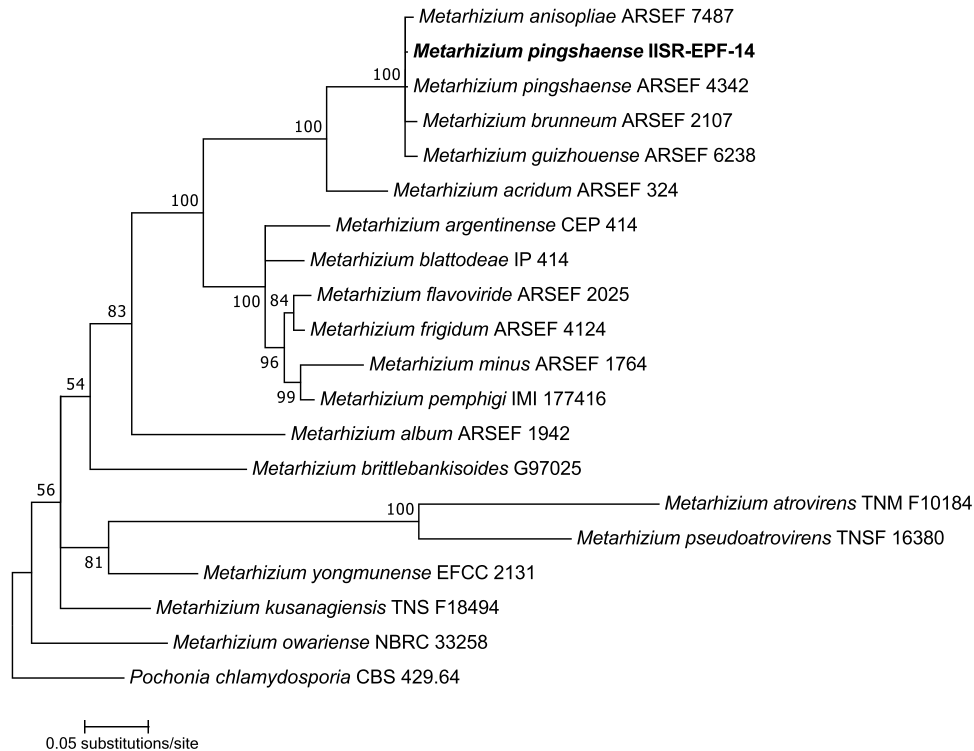

Fig. 4. Maximum likelihood (ML) tree based on analysis of nucleotide sequences of ITS gene of different *Metarhizium* spp. Numbers above or below the nodes indicate bootstrap values generated after 1000 replications using Kimura two-parameter model + gamma distribution (K2 + G). Reference sequences of different *Metarhizium* spp. obtained from GenBank are shown with their strain numbers. *Pochonia chlamydosporia* was used as outgroup.

4. Discussion

Current insect pest management strategies based on synthetic chemical pesticides and their ill effects on environment and human health necessitate intensified search for naturally available environmentally safe alternatives for pest management. Microbial biocontrol agents, especially those belonging to EPF, owing to their several innate advantages such as their ease in mass production, formulation and

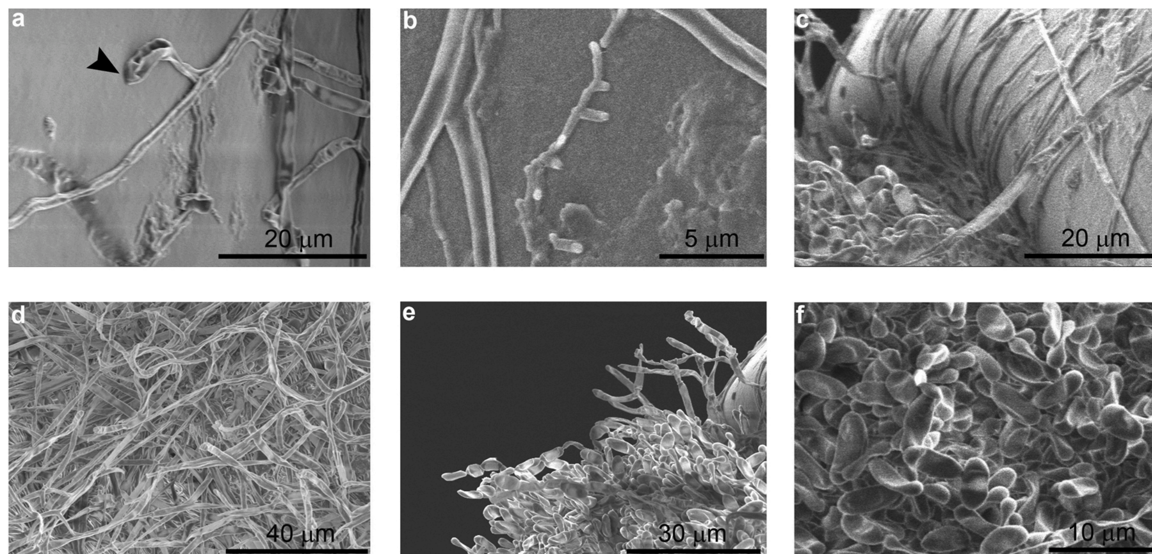

Fig. 5. SEM studies on the infection process of *M. pingshaense* to fifth instar larvae of *C. punctiferalis* showing **(a)** conidia adhered to the host cuticle, germinating and proliferating on the host body (arrow indicates cuticle degradation) **(b)** Intercalary appresoria formation on the host surface **(c)** hyphal emergence through intersegmental membrane **(d)** formation of hyphal network on the body surface **(e)** phialides bearing conidia and **(f)** close-up of chains of conidia.

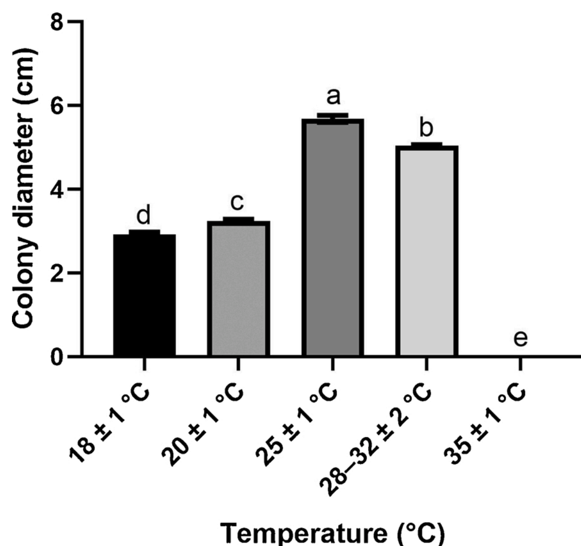

Fig. 6. Effect of different temperatures on the *in vitro* growth (Mean ± SE) of *M. pinghanese* grown at different temperatures (20 DAI). Error bars represent standard error (SE) of five replicates. Bars represented by different letters are significantly different by Fisher's LSD (α = 0.05).

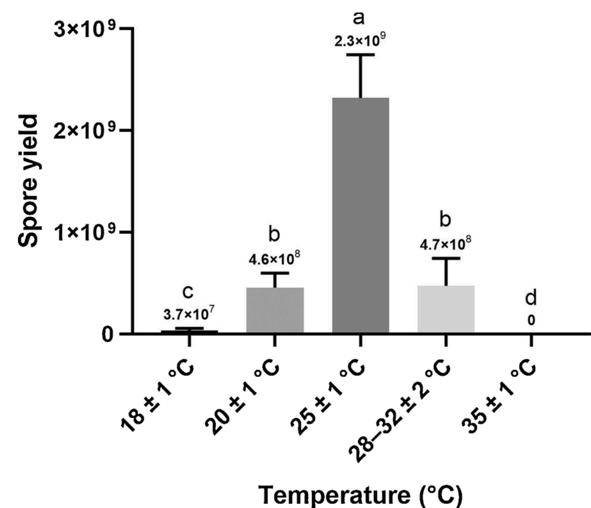

Fig. 7. Effect of different temperatures on the total spore yield (mean ± SE) of *M. pingshaense* grown at different temperatures (20 DAI). Error bars represent standard error (SE) of five replicates. Bars represented by different letters are significantly different by Fisher's LSD (α = 0.05).

application, safety to non-target organisms, rhizosphere competency, etc. offers great scope for replacement of synthetic chemical pesticides. Among them, EPF of the genus *Metarhizium* are important natural regulators of insect pests belonging to different orders across the environment and have been successfully developed as commercial biocontrol agents (Faria and Wraight, 2007). Identification of naturally occurring virulent strains of fungal entomopathogens of an insect pest is the first step towards their use in biological control programmes and subsequent incorporation into integrated pest management (IPM) strategies. In this study, we report for the first time the natural occurrence of *M. pingshaense* on *C. punctiferalis*, a serious pest of several horticultural crops including spice crops. Earlier studies with *M. pinshaense* isolates had demonstrated their ability to infect and cause significant mortalities to red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Cito et al., 2014), rice leaf folder, *Cnaphalocrocis medinalis* (Guenée) (Kirubakaran et al., 2018), subterranean termite, *Odontotermes obesus* (Rambur) (Francis, 2019), mosquitoes (Lovett et al., 2019), etc.

In our laboratory bioassays, the fungus was found to cause above 86 % mortality in fifth instar larvae of *C. punctiferalis,* when tested at 1×10^8 conidia/mL within 15 DAI proving its infective potential and virulence against late instars. The mortality of the insects differed with varying concentration of the fungus, which was consistent with earlier findings (Han et al., 2014; Kirubakaran et al., 2018; Wang et al., 2020). Infected insects showed signs of mycosis and profuse sporulation within 2–4 days of death. Profuse sporulation on cadavers is an important trait of a successful biocontrol agent, which will help in spreading the fungus under field conditions resulting in epizootics (Shan and Feng, 2010). In general, early instars of insects are highly susceptible to EPF than the late instars (Kirubakaran et al., 2018), because late instar larvae have thicker body wall and higher disease resistance compared to early instars (Pu and Li, 1996). However, highly virulent EPF may overcome the host defence with the aid of cuticle degrading enzymes and by

production of toxins.

In our studies, the median lethal concentration of *M. pingshaense* against fifth instar larvae of *C. punctiferalis* was 9.1 × 10^5 conidia/mL and the MST values ranged from 4.7 to 6.4 days. Earlier studies using *M. pingshaense* had reported LC_{50} values in the range of 8.35 × 10^4 to 4.45 × 10^5 conidia/mL against third instar larvae of *C. medinalis* for different isolates of *M. pingshaense* and MST values in the range of 3.9–4.4 days against fourth instar larvae of the insect (Kirubakaran et al., 2018) and 7.0 days against adult beetles of *R. ferrugineus* (Cito et al., 2014). However, it is difficult to make a comparison across these findings because of the variation in isolates, insect hosts, and the stages tested.

Conventionally, macro- and micro-morphological characters such as colony characteristics, colour, shape and size of the conidia are used for delineation of entomopathogenic fungal species (Mayerhofer et al., 2019). The morphological features of the present fungus agreed with the earlier descriptions for *M. pingshaense* (Bischoff et al., 2009; Cito et al., 2014). Though conidial morphology is useful in distinguishing several *Metarhizium* spp., the morphological features, especially the shape and size of the conidia of the *M. anisopliae* complex overlap among each other making it difficult to delimit them based on morphology alone (Bischoff et al., 2009; Mayerhofer et al., 2019). Nevertheless, molecular approaches by sequencing certain key gene sequences has been found to be the most reliable method to differentiate species within the *M. anisopliae* complex. Multi-locus phylogenetic approach using partial gene sequences of ITS, TEF, RPB1, RPB2 and APN2 has been successfully used to delineate species within the *M. anisopliae* complex, which has nine recognized species including *M. pingshaense* (Bischoff et al., 2009; Kepler et al., 2014).

We sequenced the partial gene regions of ITS, TEF, RPB1, RPB2 and APN2 genes of the present isolate for BLAST homology search in GenBank and for phylogenetic analyses to confirm its morphological identity. BLAST homology search for the gene sequences of the present fungus showed high similarity with the *M. pingshaense* strains reported earlier. Phylogenetic trees constructed using the concatenated nucleotide sequences of TEF, RPB1 and RPB2, ITS and APN2 separately along with the reference taxa for *Metarhizium* spp. (Bischoff et al., 2009; Kepler et al., 2014) placed the present fungus in the *M. anisopliae* clade clustering together with *M. pingshaense* reference strain with strong bootstrap support confirming its morphological identity as *M. pingshaense*.

SEM is an useful tool to study the infection process of EPF on their insect hosts (Senthil Kumar et al., 2016, 2015; Vestergaard et al., 1999). The fungus followed the typical infection process of an EPF such as adhesion and germination of conidia on the host surface, appresorium formation and penetration of the host cuticle, mycelial colonization of the body and then emerging through the intersegmental membranes and sporulating on the cadavers completing it life cycle on the host (Aw and Hue, 2017; Leemon and Jonsson, 2008; Senthil Kumar et al., 2015). Conidial adhesion to a host insect body is usually facilitated by hydrophobins (Aw and Hue, 2017) to overcome the hydrophobic epicuticle barrier. A combination of mechanical pressure and cuticle degrading enzymes such as chitinases, proteases and lipases aid the fungus to penetrate the host cuticle (Santi et al., 2010). We observed signs of depression on the site of germination of conidia, which could be due to mechanical pressure and by secretion of cuticle degrading enzymes by the pathogen (Senthil Kumar et al., 2015; Vestergaard et al., 1999). The insect might have been killed by a combination of mycelial proliferation and production of fungal toxins inside the host as reported for other insects (Aw and Hue, 2017).

Temperature greatly influences the biology of EPF (Ekesi et al., 1999; Masoudi et al., 2018; Yeo et al., 2003) and is a key determining factor for its further development as a mycoinsecticide for field applications (Jaronski, 2010). Studies have shown that the optimal temperature for the growth of EPF is between 25 and 35 °C (Leemon and Jonsson, 2008; Masoudi et al., 2018). Our studies also indicated that temperature significantly influenced the *in vitro* growth and sporulation of the present fungal isolate. Maximum growth and sporulation of the fungus was observed at 25 ± 1 °C, which is in concordance with the earlier findings for *Metarhizium* spp. (Arthurs and Thomas, 2001; Ekesi et al., 1999; Keppanan et al., 2018; Masoudi et al., 2018; Orduño-Cruz et al., 2011; Yeo et al., 2003). Though, Moorhouse et al. (1994) reported that the optimal condition for axenic growth of a fungus is ideal to kill the host rapidly, the results of another study by Thomas and Jenkins (1997), showed that the optimal temperature for infection and proliferation in the host was higher than that for *in vitro* growth. Our findings agree with that of Thomas and Jenkins (1997) as the fungus showed high infectivity in the bioassays, which were conducted under natural room temperature conditions (28–32 ± 2 °C). Any fungal entomopathogen that grow and sporulate at the conditions in which the pest occurs would be an ideal candidate for further development as a biopesticide (Yeo et al., 2003).

Our studies have identified a potential biological control agent for *C. punctiferalis,* a serious pest of many crops worldwide. The fungus was found to possess important attributes of a potential mycoinsecticide such as high virulence against the target pest at the conditions in which the fungus will be used and exhibited rapid growth and sporulation under *in vitro* conditions. Isolation of the fungus from an insect cadaver retrieved from a pseudostem of turmeric indicate that the fungus may have an endophytic association with the plants. In general, *M. pingshaense* are found to be associated with both rhizospheric and non-rhizospheric soils (Nishi and Sato, 2019) and are better root colonizers (Nishi and Sato, 2017). Apart from this, EPF are now found to have several new found traits such as disease antagonism, plant growth promotion (Jaber and Ownley, 2018; Senthil Kumar et al., 2018a), etc. Further studies will aim at exploring these traits and also in developing suitable targeted formulations of this fungus for field application and inclusion in IPM schedules.

CRediT authorship contribution statement

C.M. Senthil Kumar: Conceptualization, Funding acquisition, Methodology, Data curation, Writing - original draft, Writing - review & editing, Validation. **T.K. Jacob:** Methodology, Writing - original draft, Writing - review & editing, Visualization. **S. Devasahayam:** Conceptualization, Writing - review & editing, Supervision. **C. Geethu:** Investigation, Validation. **V. Hariharan:** Investigation.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126645.

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