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Morphological and molecular identification of the entomopathogenic fungus *Purpureocillium lilacinum* and its virulence against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) larvae and pupae

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Abstract

Background: The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a serious pest of solanaceous plant species, mainly *Solanum lycopersicum* L. The entomopathogenic fungi, an alternative to chemical insecticides, proved to be an efficient biocontrol agent in reducing pest population density. In the present study, an entomopathogenic fungus, *Purpureocillium lilacinum* isolated from soil was identified based on the morphological and molecular characteristics and its pathogenicity was tested against target pest.

Results: The micromorphological characters showed variations in growth pattern, shape and colour on different cultural media. For molecular analysis, a phylogenetic tree based on ITS/LSU and ITS/β-tubulin (*ben*A) gene regions was constructed which revealed the isolate (FC18) as *P. lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones and Samson (Family: Ophiocordycipitaceae). Further, the pathogenicity of *P. lilacinum* was tested using different spore concentrations (1×10^8, 1×10^7, 1×10^6, 1×10^5 and 1×10^4 spores/ml) on larval and pupal stages of *T. absoluta*, which showed a dose-dependent mortality. At the highest concentration (1×10^8 spores/ml), the mean mortality of 92.99, 83.05, 72.0% of second, third and fourth instar was observed, respectively. Pupal mortality also showed significant differences at different spore concentrations.

Conclusion: Obtained results showed that the virulence of the indigenous strain of *P. lilacinum* on *T. absoluta* can be utilized in the field suppression of the pest as a potent biocontrol agent.

Keywords: *Tuta absoluta*, Entomopathogenic fungi, *Purpureocillium lilacinum*, Phylogenetic analysis

Background

Tuta absoluta (Meyrick), (Lepidoptera: Gelechiidae), is known as South American pinworm, is a multivoltine lepidopteran species and one of the most destructive pests of tomato (*Solanum lycopersicum*) and other solanaceous plant species (Öztemiz 2013). In India, this invasive pest was first reported at Pune, Maharashtra, in October 2014 and now is found in almost all tomato growing states of India (Sharma and Gavkare 2017).

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Chemical insecticides are commonly used to control the *T. absoluta*, but frequent use of chemical insecticides has developed pest resistance (Lietti et al. 2005). The chemical insecticides also lead to toxic effects on human health, environment and reduce the population densities of beneficial insects (Roditakis et al. 2015). Therefore, biological control agents are deployed for eco-friendly pest management (Bukhari et al. 2011).

Entomopathogenic fungi (EPF) are considered as important biocontrol agents having potential to cause mycosis and sometimes kill the insect pests (Shah and Pell 2003). Naturally occurring EPF are one of the best alternatives to harmful chemical insecticides. These fungi infect the host by the attachment of conidia (spores) on the external body surface of insect's cuticle. After breaching the cuticle, the germinating hyphae of fungal pathogen perforate into the haemocoel of insect body where it colonizes and proliferates throughout the host to form hyphal bodies after the replication (Wanchoo et al. 2009). Upon colonization of the host, the EPF release toxic compounds that often lead to the death of the host (Trienens and Rohlfs 2012). To exploit these fungi as biocontrol agents, selective isolation, confirmation of the identity by combined approaches of morphological and molecular characterization is highly essential (Dunlap et al. 2017).

Recent studies have revealed the pathogenicity of the EPF, *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones and Samson against nematodes and a few insect pests like thrips, bugs, beetles, aphids and white flies (Goffré and Folgarait 2015). Very little information is available on molecular characterization of the indigenous strain of *P. lilacinum* and its pathogenicity against *T. absoluta*. Therefore, present study aimed to isolate and characterize the indigenous entomopathogen to evaluate its efficacy against larvae and pupae of *T. absoluta* under laboratory conditions.

Methods

Isolation of *P. lilacinum* from soil sample

The EPF were isolated from the soil using bait method of Zimmerman (1969). Two hundred and fifty grams of soil was collected from a depth on 5 cm from the agricultural field of Savitribai Phule Pune University campus, Pune, India (18.5524°N; 73.8267°E), and brought to the laboratory in plastic bags. Five numbers of second instar larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) were released into 50 g of soil placed in plastic bottles as bait for isolation of *P. lilacinum*. The soil was incubated at 25 ± 2 °C, and 75% R.H. After 7 days, larvae (of *G. mellonella*) were examined for infection and the dead larvae were separated from the containers. They were surface sterilized with 1% sodium hypochlorite solution for 2 min and then gently rinsed twice with distilled water (Brownbridge et al. 1993). The cadavers were kept in sterile Petri plates moistened with filter papers and incubated at 25 °C. After seven days of the incubation, cadavers were examined for the appearance of fungal growth. Cadavers with external fungal growth were used for isolation of the entomopathogen and subsequent studies. The pure and identified culture of *P. lilacinum* was accessioned and preserved in the National Fungal Culture Collection of India (NFCCI; WDCM-932), MACS-Agharkar Research Institute, G.G. Agharkar Road, Pune 411,004, India, under accession number NFCCI 5268.

Morphological characterization

The isolated pure fungus was grown on 2 different culture media, Sabouraud dextrose yeast agar (SDYA; 40 g dextrose, 10 g mycological peptone, 15 g agar, 0.1gm chloramphenicol (pH: 5.6)) and Malt extract agar (MEA; 20 g malt extract, 20 g dextrose, 6 g peptone, 15 g agar, 0.1 g chloramphenicol (pH: 5.6)). After 14 days of incubation of culture plates at 25 ± 2 °C, observations on colony characters, shape, diameter, etc., were recorded. Methuen Handbook of Color (Kornerup and Wanscher 1978) was used for recording colour of the cultured colonies. For microscopic characters, the slide cultures technique was followed. Small amounts of mycelial inoculum were inoculated on a small block cut out of PDA plate and placed in the centre of the cleaned glass slide overlaid by coverslip. After 5–7 days of incubation, fungal growth on the PDA block was observed directly under microscope and observations noted. Lactic acid and lactophenol-cotton blue mounts were prepared separately for the observations of conidial and other morphological characters using Olympus (Model CX-41, Japan) and Carl Zeiss (AXIO Imager 2, Germany) microscopes.

Extraction of genomic DNA for molecular identification

The genomic DNA was extracted from 7-day-old fungal culture grown on PDA medium and harvested by scrapping the fungal mass using the fine spatula. The fungal mass was placed in a 2 ml tube containing a ceramic pestle and 60-80 mg sterile glass beads (425 µM, sigma). Homogenization of fungal mass was done with lysis buffer (100 mM Tris HCl (pH-7.5); 50 Mm EDTA, 3% SDS) using a FastDNA® spin kit as per manufacturer's instructions (MP Biomedicals GmbH, Germany) at 6 M/S for 60 s. The PCR amplification of fragments containing regions encoding to ITS 1–5.8S nrDNA-ITS 2 (ITS), 28S nrDNA (LSU) and beta-tubulin (*ben*A) was performed by using primers ITS5/ITS4(5′-TCCTCCGCTTATTGATATGC-3′)/(5′GGAAGTAAAAGTCGTAACAAGG-3′) (White et al. 1990), and LROR/LR7 (5′-ACCCGCTGAACTTAAGC-3′)/(5′-TACTACCACCAAGATCT-3′) (Rehner and Samuels 1994) and T10/T22 (5′-ACGATA

GGTTCACCTCCAGAC-3′)/(5′-TCTGGATGTTGT TGGGAATCC-3′) (Glass and Donaldson 1995), respectively. The purification of PCR products was done by an Axygen PCR cleanup kit (Axygen Scientific Inc., CA, USA) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were run on an ABI 3100 automated DNA sequencer (Applied Biosystems, USA).

Phylogenetic analysis and nucleotide sequence submissions

The reference sequences of ITS/LSU and ITS/β-tubulin obtained were combined and aligned manually, using the text editor option of the Molecular Evolutionary Genetics Analysis (MEGA) Version 7.0 (Kumar et al. 2016) for constructing phylogenetic tree (Tables 1 and 2). The isolated nucleotide sequences obtained after sequencing were deposited in GenBank, National Centre for Biotechnology Information, under the accession number (MT67260-ITS, MT672602-LSU, MT792846-*ben*A).

Laboratory bioassay

Rearing of T. absoluta

The culture of *T. absoluta* was obtained from the Germplasm Division Conservation and Utilization, ICAR-National Bureau of Agricultural Insect Resources, Bangalore, and reared under laboratory conditions for bioassay studies. Adults were released into large breeding cages with tomato seedlings under controlled conditions at 27 ± 2 °C and 55% R.H. Moths were provided with 10% honey solution as a source of food. Newly laid eggs were collected and reared in plastic jars on the tomato leaves for their growth and development. The different larval instars were collected from rearing jars and used for bioassay studies.

Pathogenicity of P. lilacinum against T. absoluta

Fifteen-day-old fungal cultures were scraped using a sterile loop and transferred into 10 ml of sterile distilled water containing 0.02% Tween 80 (Rombach et al. 1986). The suspension was filtered through sterile muslin cloth, and five different spore concentrations (1×10^8, 1×10^7, 1×10^6, 1×10^5 and 1×10^4 spores/ml) were prepared using a Neubauer haemocytometer. The pathogenicity of different conidial concentrations of *P. lilacinum* was tested on 2nd, 3rd, 4th instar larvae and pupae of *T. absoluta*. For each instar, 10 larvae per replication (total 6 replicates) were dipped in different spore concentrations for 10 s and then air-dried under the laminar flow. After drying, the treated larvae were released on tomato seedlings whose stems were wrapped in wet cotton to maintain turgor pressure. The control larvae were treated with distilled water with 0.02% Tween 80. To determine the pupal mortality, about 250 g of soil was sieved and placed in 500 ml plastic container and autoclaved for 20 min at 15 psi. The sterilized soil was moistened with distilled water. Then, spore concentration (1×10^4 to 1×10^8) was poured in the soil samples and pupae were released in the plastic containers having sterile soil. Each treatment was replicated 6 times with 10 pupae per replicate. The number of larvae and pupae succumbing to fungal infection was recorded daily, and till 7 days. The control larvae and pupae were treated with sterile distilled water containing 0.02% Tween 80 and maintained in similar way. The experiment was repeated twice.

Virulence analysis

Mortality rates were calculated according to Abbott's formula (1952)

$$\text{Corrected mortality} = \frac{T - C}{100 - C} \times 100$$

where T = dead larvae in treatment, C = dead larvae in control.

R studio software (Version 4.1.2; R Core Team, 2020) was used for analysis of data which included factorial CRD and ANOVA.

Results

Isolation of *P. lilacinum* and morphological characterization

The larvae of *G. mellonella* placed in the soil samples to recover the fungus were found covered with fungal growth (Fig. 1a). The colony characters of isolated fungus were recorded on MEA medium: colony diameter 50–56 mm, after 14 days of incubation, circular, margins regular, smooth, slightly raised, cottony, dull white (3B9) and reverse yellowish-white (2B5) (Fig. 2a, b). On SYDA medium, the colonies were reddish grey (2B9), 35–40 mm in diameter after 14 days of incubation, cottony, velvety, slightly raised, circular, margins smooth and entire, conidia *en masse* and reverse greyish yellow (6B4) (Fig. 2c, d).

Mycelium simple to branched, septate, smooth walled, bundles of hyphae present. Conidiophores appeared simple to branched arising from superficial hyphae, mono-verticillate to tertiary or quarter verticillate (22–81 × 1.85–2.0 µm). Phialides variable in shape and size, paeciliform, tapered towards apex, (9.25–20.5 × 2.15–2.5 µm), metulae 1–2 in number, straight to curved with a slender neck; conidia mostly fusoid, sub-globose to globose, 2.5–3.15 × 2.0–2.5 µm (Fig. 2e, f). Based on morphological characters recorded, this fungus was identified as *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones and Samson and the same is deposited and accessioned in NFCCI (5268).

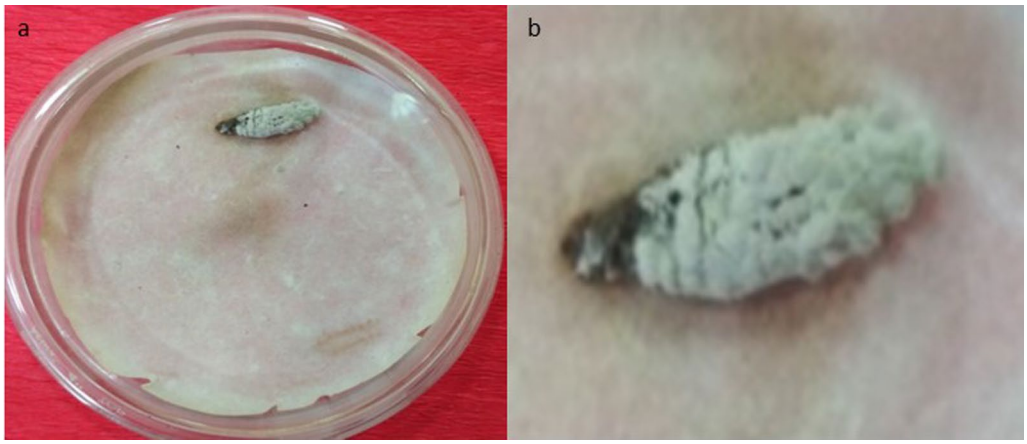

Fig. 1 Isolation of entomopathogenic fungi, *Purpureocillium lilacinum* by Galleria bait method **a** Growth of *P. lilacinum* on *Galleria mellonella* larvae, **b** Magnified view of the *P. lilacinum* covering the dead cadaver

Phylogenetic analysis

Amplification and sequencing of 2 nuclear and one protein-encoding gene regions (ITS/LSU and ITS/beta-tubulin) were carried out to confirm the isolate's genotypic characters. The phylogram were generated by combining ITS/LSU and ITS/beta-tubulin with MEGA 7, which includes 31 and 18 nucleotide sequences, respectively. The out-group taxon in both phylograms was *Metarhizium marquandii* (CBS 182.27) from Clavicipitaceae family (Tables 1, 2). Both ITS/LSU and ITS/beta-tubulin were based on the Tamura 2-parameter model having 917 and 849 sequences positions in the final dataset. Our isolate FC18 clustered with *P. lilacinum* in both ITS/LSU and ITS/beta-tubulin combined phylogenetic tree with 99 and 97% bootstrap values, respectively. Newly generated sequences of our species were deposited in GenBank. The sequence of 2 genes was aligned and analysed separately by Bayesian and Maximum Likelihood analyses, and the resulting trees were compared (Figs. 3 and 4). There were no conflicts between single gene phylogenies, so the datasets were combined to get phylogram. Nevertheless, both Bayesian and maximum likelihood analyses were useful for discriminating at species level.

Pathogenicity of *P. lilacinum* against *T. absoluta* larvae and pupae

Different spore concentrations showed dose-dependent mortality in *T. absoluta* larvae and pupae. At the highest concentration (1×10^8), the mean mortality was 92.99, 83.05 and 72.0% in second, third and fourth instar larvae, respectively. The corrected mean mortality ranged from 37.53 to 92.83% in second instar, 23.78 to 83.05% in third instar and 11.20 to 72.0% in fourth instar larvae with different conidial concentrations from 1×10^4 to 1×10^8 spores/ml (Table 3). Except to the lower concentration (1×10^4), all the concentrations induced significantly higher mortality ($F = 278.23$; df = 4; $P < 0.001$). The mean mortality in instars was observed from 68.15, 56.79 and 41.98% in second, third and fourth instar larvae, respectively ($F = 148.75$; df = 4; $P < 0.001$). It was evident from data that with an increase in concentration, the mortality rate was increased. There were significant differences in mortality within a larval instar with increasing conidial concentration ($F = 9.52$; df = 4; $P < 0.001$). The LC_{50} values for the EPF, *P. lilacinum,* were 5.2×10^7, 4.5×10^6, 3.9×10^5 spores/ml against second, third, and fourth instar larvae, respectively (Table 5).

The corrected mean mortality of pupae was 74.17% at highest concentration 1×10^8 spores/ml, and lowest pupal mortality 11.67% was observed at 1×10^4 spores/ml (Table 4). There was significant difference in all the treatments ($F = 23.4$; df = 4; $P < 0.001$). With an increase in concentration, the mortality rate also increased. The pupal mortality from *P. lilacinum* was dose-dependent. The LC_{50} value of *P. lilacinum* for pupae was 3.9×10^5 spores/ml (Table 5). The larvae treated with fungal preparations fed much as lesser than to control larvae. The treated larvae became stiff and hard after death. The white mycelial growth was appeared after 48 h at the highest concentration and after 72 h at the lowest concentration.

Discussion

The phenotypic and genetic variability of EPF plays a crucial role in developing biopesticide because this variability impacts the efficacy of the fungi against many arthropod species (Du et al. 2019). The EPF, *P.*

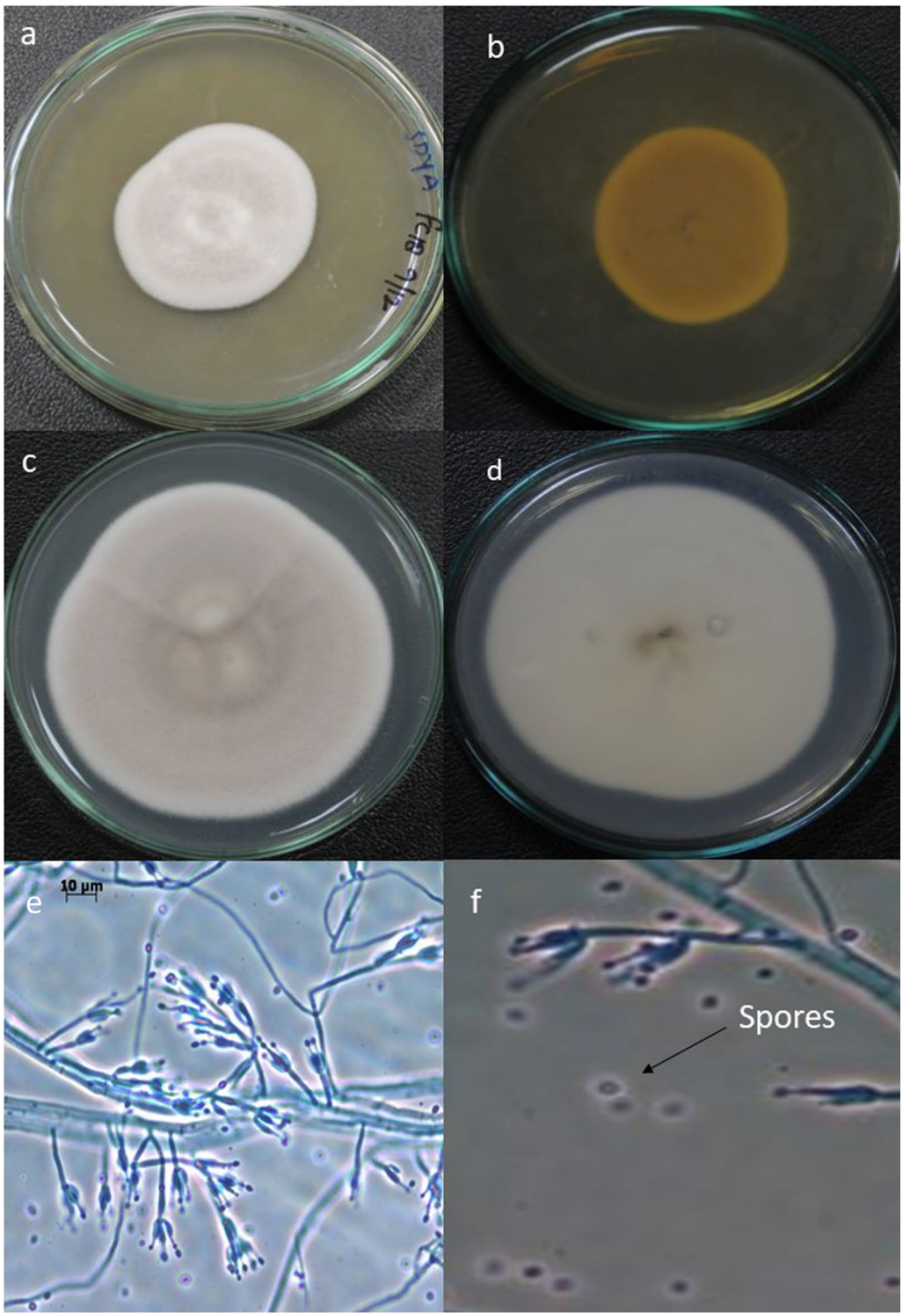

Fig. 2 Colony growth of *Purpureocillium lilacinum* on different culture media after 14 days of incubation at 25 °C **a** Frontal view of *P. lilacinum* on SDYA medium, **b** Rear view of *P. lilacinum* on SDYA medium, **c** Frontal view of *P. lilacinum* on MEA medium, **d** Rear view of *P. lilacinum* on SDYA medium, **e**, **f** Microscopic pictures showing phenotypic characteristics, viz. conidiophores arising from mycelial hyphae, phialides, phialospores (conidia) of *P. lilacinum*

Table 1 Species, isolates and accession number included in the study

Species	Isolate Number	ITS	LSU
Purpureocillium lavendulum	FMR 10376	FR734106	FR775489
P. lavendulum	FMR 10452	FR734107	FR775490
P. lavendulum	FMR 10376	FR734106	FR775489
Purpureocillium lilacinum	CBS 284.36[T]	FR734101	FR775484
P. lilacinum	CBS 431.87	AY624188	EF468844
P. lilacinum	FMR 7231	FR734085	FR775468
P. lilacinum	FMR 8250	FR734086	FR775469
P. lilacinum	FMR 8251	FR734087	FR775470
P. lilacinum	FMR 8253	FR734088	FR775471
P. lilacinum	FMR 8648	FR734089	FR775472
P. lilacinum	FMR 8652	FR734090	FR775473
P. lilacinum	FMR 8747	FR734091	FR775474
P. lilacinum	FMR 10040	FR734092	FR775475
P. lilacinum	FMR 10041	FR734093	FR775476
P. lilacinum	FMR 10068	FR734094	FR775477
P. lilacinum	FMR 10069	FR734095	FR775478
P. lilacinum	FMR 10096	FR734096	FR775479
P. lilacinum	FMR 10380	FR734102	FR775485
P. lilacinum	FMR 10374	FR734097	FR775480
P. lilacinum	JCM 8437	FR734103	FR775486
P. lilacinum	JCM 8438	FR734105	FR775488
P. lilacinum	JCM 8372	FR734104	FR775487
P. lilacinum	UTHSC 08-3504	FR734098	FR775481
P. lilacinum	UTHSC 95-1315	FR734099	FR775482
P. lilacinum	UTHSC 95-736	FR734100	FR775483
P. lilacinum	CBS 284.36	FR734101	FR775484
P. lilacinum	CBS 432.87	HQ842819	MH873778
P. lilacinum	M3748	KC157748	KC157814
Paecilomyces lilacinus strain	ATCC 10114	AY213665	AY213717
Metarhizium marquandii strain (Out-group)	CBS 182.27	AY624193	EF468845

[T] Type strain

lilacinum, is a ubiquitous filamentous fungus isolated from soil, decaying vegetation, insects and nematodes (Quandt et al. 2014). In the present study, the morphological characters of *P. lilacinum*, viz. colony diameter, shape, size, texture and colour, varied on SDYA and MEA media. Other studies have also shown the variation in morphology of *P. lilacinum* when grown on various media (Nawar et al. 2018), which showed variation in the morphology of the fungus. From present study, it is evident that *P. lilacinum* may exhibit variations in colour in response to different nutrient media, as shown by Samson (1974). The mycelial structure, conidiophores, phialides and size of conidia are similar to that found in the previous studies (Dyaranthe et al. 2020).

Fungi from the order Hypocreales are complex and difficult to identify by morphological characters and require genomics to decode the complex nature of species identification (Dornburg et al. 2017). Molecular identification by the sequencing of the ITS region has some limitations, so the other genes are necessary to be sequenced for species delimitation (Kredics et al. 2015). In this context, our studies revealed the phylogenetic description of *P. lilacinum* by amplification of 3 gene sequences including one protein-encoding regions (ITS, LSU and beta -tubulin). The combined ITS/LSU showed that the isolate FC18 belongs to the *P. lilacinum* of Ophiocordycipitaceae. Similarly, the combined tree of ITS/beta-tubulin revealed the same analysis. Similar studies on phylogenetic analysis were done by Sun et al. (2021).

Many workers reported that EPF have the ability to infect different insect orders (Majeed et al. 2017). The rate of virulence of EPF depends on host range, pattern of virulence factors and level of their expression (Khanday et al. 2018). The pathogenicity of *P. lilacinum* was tested against different insect orders (Johny et al. 2012). In the present studies, *P. lilacinum* was tested against second, third and fourth instar *T. absoluta* larvae and it proved to be pathogenic to all instars at different concentrations. The present data also revealed dose-dependent mortality. It was evident that younger instars were more prone to the fungal infection than the older ones and were in accordance with *Spodoptera litura* (L.) (Lepidoptera: Noctuidae) (Purwar and Sachan 2005). Similar findings of *P. lilacinum* were observed in *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) and *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) when treated with fungal concentration of 10^8 spores/ml (Kepenecki et al. 2015). Moreover, the infectivity of *P. lilacinum* was also observed against the cotton aphid, *Aphis gossypii* (Glover) (Hemiptera: Aphididae) under greenhouse and field conditions (Lopez et al. 2014). The dose-dependent mortality was documented for *Acromyrmex lundii* (Hymenoptera: Formicidae) (Guerin Meneville) with different *P. lilacinum* concentrations which reveals the competitive capability of the fungus (Goffré and Folgarait 2015). Highest virulence of *P. lilacinum* in third and fourth instar larvae of *S. litura* and *Plutella xylostella* L. (Lepidoptera: Plutellidae) was demonstrated by Nguyen et al. (2017) which were in concordance with present data. Moreover, the present findings documented the pathogenicity of *P. lilacinum* concentration against *T. absoluta* pupae. Schemmer et al. (2016) also reported that EPF were virulent and induced infection to leaf-miner pupae of *Cameraria ohridella* (Deschka and Dimic) (Lepidoptera: Gracillariidae) under laboratory conditions. Among all isolates tested, *Isaria fumosorosea* (CO10-IFu) from the same fungal family,

Table 2 Species, isolates and accession numbers included in the study

Species	Isolate Number	ITS	β-tubulin
Isaria takamizusanensis	F1724	GU980039	GU980010
I. takamizusanensis	F896	GU980040	GU980011
P. lilacinum	NRRL13872	GU980020	GU979997
P. lilacinum	DTO 63E5	GU968666	GU968702
P. lilacinum	CBS 432.87	HQ842819.1	AY624228.1
P. lilacinum	M3748	KC157748.1	KC157849.1
P. lilacinum	B3A	HM242262	HM242265
P. lilacinum	B59A	HM242263	HM242266
P. lilacinum	SY45B-a	HM242264	HM242267
P. lilacinum	CBS 284.36	AY624189	AY624227
P. lilacinum	DTO 63F2	GU968671	GU968703
P. lilacinum	DTO 63E5	GU968666	GU968702
P lilacinum	DTO 63E1	GU968662	GU968701
Purpureocillium lavendulum	NRRL 22958	GU980033	GU980007
Drechmeria gunnii (Out-group)	OSC 76404	JN049822	DQ522488
Metarhizium marquandii (Out-group)	CBS 182.27	AY624193	AY624229

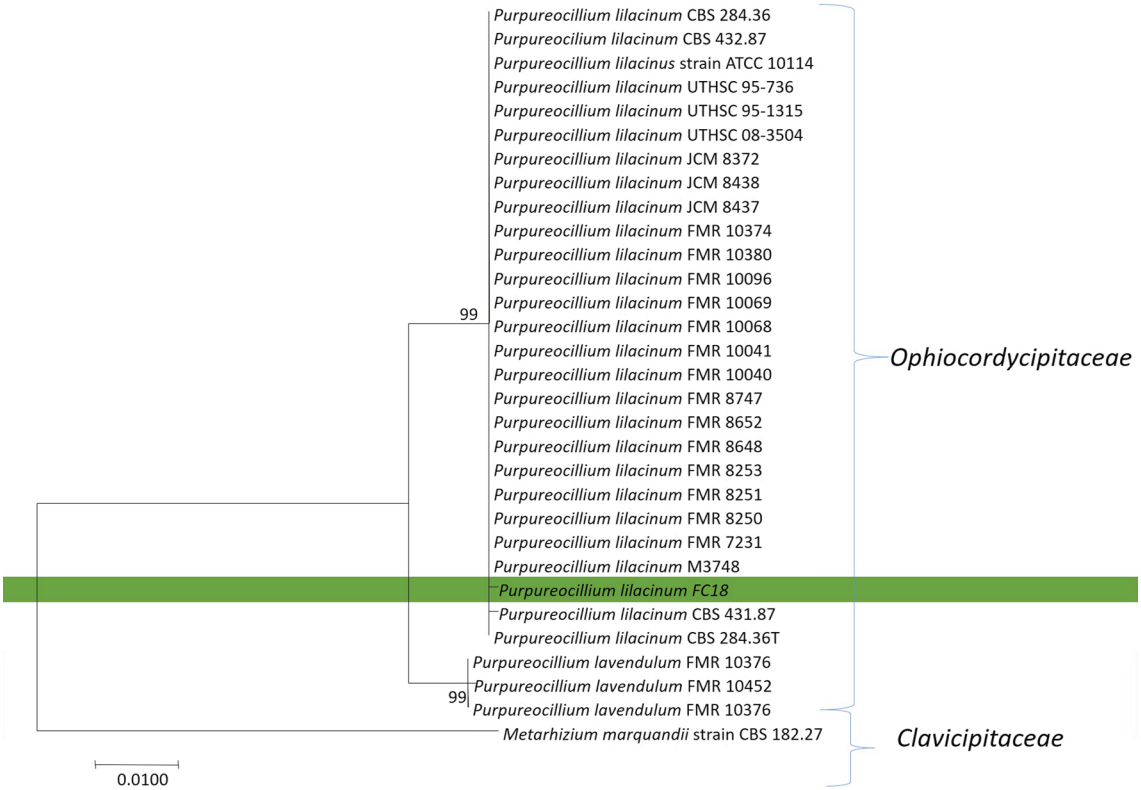

Fig. 3 Phylogenetic tree generated with maximum likelihood from concatenated ITS and LSU sequences of *Purpureocillium lilacinum* using Tamura 2-parameter model having 31 taxa and 917 characters. The numbers at the nodes indicate bootstrap percentages. The isolates retrieved in this study are in bold

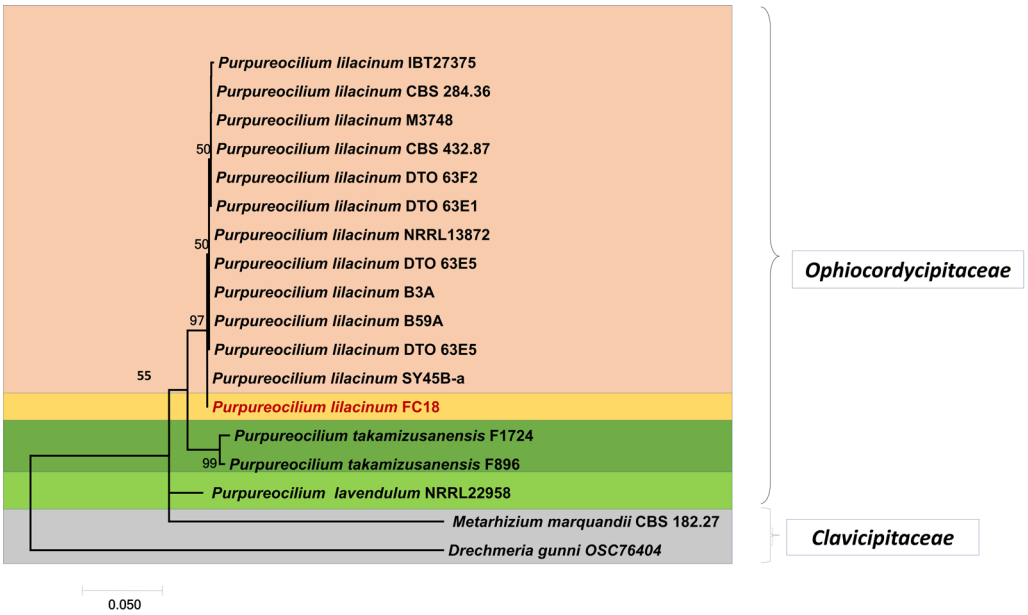

Fig. 4 Phylogenetic tree generated with maximum likelihood from concatenated ITS and β-tubulin sequences of *Purpureocillium lilacinum* using Tamura 2-parameter model having 18 taxa and 849 characters. The numbers at the nodes indicate bootstrap percentages. The isolate retrieved in this study is in bold

Table 3 Corrected mortality of *Tuta absoluta* larvae with different conidial concentrations in response to entomopathogenic fungi, *Purpureocillium lilacinum*, after seven days of treatment

Concentrations (spores/ml)	Instars of *Tuta absoluta*			
	Second	Third	Fourth	Mean
1.0×10^8	92.99	83.05	72.0	82.68a
1.0×10^7	82.15	70.95	57.16	70.08b
1.0×10^6	70.81	59.85	40.71	57.12c
1.0×10^5	57.3	46.34	28.87	44.17d
1.0×10^4	37.53	23.78	11.20	24.17e
Mean	68.15a	56.79b	41.98c	55.64

Pairwise comparison among treatments was done using LSD (Least significant difference) test. Treatment means with different superscript differ significantly ($P < 0.05$)

Table 4 Corrected mortality of *Tuta absoluta* pupae with different conidial concentrations in response to entomopathogenic fungi, *Purpureocillium lilacinum*, after seven days of fungal treatments

Concentrations (spores/ml)	Mean
1.0×10^8	74.17a
1.0×10^7	55.0b
1.0×10^6	39.17c
1.0×10^5	32.50c
1.0×10^4	11.67d
Mean	42.50

Pairwise comparison among treatments was done using LSD (Least significant difference) test. Treatment means with different superscript differ significantly ($P < 0.05$)

Cordycipitaceae, demonstrated promising pathogenic attributes against *C. ohridella* pupae. The present study is the first report of infection of *T. absoluta* pupae with *P. lilacinum*. Obtained data indicate the efficiency of *P. lilacinum* against pupal stage, but future trials on semi-field and field testing are needed.

In this study, as the concentration of spores increased, the mortality rate of larvae and pupae of *T. absoluta* increased significantly. The virulence difference of *P. lilacinum* depends on the application method and the conidial concentrations attached to the cuticle of the studied insect (Rambadan et al. 2011). Further, the previous studies revealed that the range of inoculum has a great significance in causing the infection in the host (Pujol et al. 2011). Other important factors like temperature and humidity, hosts fitness and size also play vital role in causing infection.

Conclusions

In present study, *P. lilacinum* had shown the potential to infect and kill different larval instars and pupae of *T. absoluta* with a wide range of variations against different stages of *T. absoluta*. Morphological and molecular

Table 5 Lethal concentration (LC_{50}) of *Purpureocillium lilacinum* against larval and pupal stages of *Tuta absoluta* after seven days of fungal treatments

Stages	LC_{50} (Conidia/ml)
Second larval instar	5.2×10^7
Third larval instar	4.5×10^6
Fourth larval instar	3.9×10^5
Pupae	2.9×10^5

characterizations are useful tools for distinguishing the complexity of EPF. Future investigations are required to evaluate the efficacy of this fungus under field conditions and to determine the most viable route for its application.

Abbreviations

MEA: Malt extract agar; SYDA: Sabouraud dextrose yeast agar; ITS: Internal transcribed sequence; LSU: Large subunit; *ben*A: β-Tubulin; MEGA 7.0: The Molecular Evolutionary Genetics Analysis Version 7.0; LC_{50}: Lethal concentration.; ANOVA: Analysis of Variance.

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Author contributions

GKB, RSP and SKS designed the study; RSP contributed to the project coordination. GKB and DKM collected the sample and maintained in the laboratory under optimal conditions and submitted to NFCCI; GKB performed the experiments. GKB and FJW analysed and evaluated the data. GKB wrote the paper. The manuscript was critically evaluated and edited by RSP and SKS. All the authors read and approved the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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