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Novel Entomopathogenic Fungi of Tamil Nadu Soils and Their Pathogenicity on Waxmoth Larva (*Galleria mellonella*)

Palle Pravallika[1], M. Muthuswami[1], P.S. Shanmugam[2], V. Rajasree[3], K.K. Kumar[4], Aruna Beemrote[5]

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ABSTRACT

Background: Biological plant protection with entomopathogenic fungi is a vital component of sustainable pest management. The most widely used entomopathogenic fungi (EPF) are *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium* sp. And *Isaria* sp., though there are several other genera of EPF whose potential correlates to that of commonly used fungi for reducing insect pests. The primary goal of this study was to assess the efficiency of a few novel fungal isolates against insect pests.

Methods: Two concentrations of four different entomopathogenic fungi were evaluated for their potency against wax moth larvae, *Galleria mellonella* at Insectary, Tamil Nadu Agricultural University, Coimbatore.

Result: Mortality was observed in both the concentrations of four fungi, among which *Penicillium simplicissimum* performed well on par with *Clonostachys rosea* and *Purpureocillium lilacinum*. Hence, these fungi could be formulated and utilized in biological pest control.

Key words: Entomopathogenic fungi, Novel, *Penicillium simplicissimum*, Pest management, *Talaromyces*.

INTRODUCTION

Pest concerns are a fundamental component of present-day agricultural practices and are typically brought about by agroecosystems that are too simplified in addition to the development of less stable natural ecosystems. Natural enemies, which keep pests in check, are annihilated when broad-spectrum pesticides are used. Because of this, scientists are now urging to focus on developing environmentally beneficial alternatives. Biocontrol is the most effective substitute among them. In a sustainable pest management project, biological plant protection with entomopathogenic fungi is vital. Entomopathogenic fungi offer an indispensable part in the microbial control of insect pests. According to Sinha *et al*. (2016), entomopathogenic fungi play an imperative role in the microbial management of insect pests. Although there are other biological control strategies involving bacteria, viruses and protozoa, EPF is the most significant because of a variety of characteristics, such as simple production processes, the availability of numerous strains that have already been identified and the over-expression of endogenous and exogenous toxins and proteins (St Leger and Wang, 2010). The two basic requirements to successfully employ an entomopathogenic fungus as a myco biocontrol agent are the insect's sensitivity and the fungus's virulence.

However, it is not a novel concept to use microbes to eradicate pests. The first entomopathogenic fungus that brings about the insect disease white muscardine was discovered and described by Agastino Bassi. This fungus was later given the name *Beauveria bassiana*. Entomopathogenic fungi still have many unrealized potential benefits despite being commercialized recently (Mantzoukas *et al.,* 2022). Entomopathogens are preferred to kill insects at different phases of their life cycles due to their biopersistence and eco-friendliness.

Clonostachys rosea is a filamentous fungus found in diverse environments featuring different kinds of soil types and decaying plant material. It was formerly known as *Gliocladium roseum*. The morphology, ecology, teleomorph and DNA sequence data of *Gliocladium roseum* were significantly different from those of other *Gliocladium* species, which led to the reclassification of *Gliocladium roseum* as *Clonostachys rosea* (Sun *et al*., 2020). It is known as a parasite of nematodes and fungal pathogens and

[1]Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India.
[2]Department of Pulses, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India.
[3]Department of Horticulture, Agricultural College and Research Institute, Karur-639 001, Tamil Nadu, India.
[4]Department of Plant Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India.
[5]ICAR Research Complex for North Eastern Hill Region, Manipur Centre, Lamphelpat-795 004, Manipur, India.

Corresponding Author: M. Muthuswami, Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India. Email: mmsagricbe@gmail.com

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invades living plants as an endophyte and a saprophyte, respectively, as well as consuming soil-based substances. *Purpureocillium lilacinum* formerly called *Paecilomyces lilacinus* is an excellent soil fungus for biological control. The effectiveness of this species has been comparable to that of commonly used nematicides and it is also effective at controlling insects.

Talaromyces was previously considered as the genus *Penicillium*. Relying on their traditional classification in the guild of fungal antagonists, *Talaromyces* sp. (Eurotiales: Trichocomaceae) have merely sometimes been associated with insects. Species such as *T. flavus*, *T. pinophilus* and *T. purpureogenus* are already known to produce bioactive chemicals and play a crucial part in the antagonism against plant diseases (Nicoletti and Becchimanzi, 2022). The genus *Penicillium* contains fungi that have been identified for their entomopathogenic behavior and certain species have been suggested as efficient biocontrol agents. At least 62 *Penicillium* species have been discovered in conjunction with insects (Nicoletti *et al*., 2023).

On the entomopathogenic potential of *Clonostachys rosea* and *Purpureocillium lilacinum*, there is very little research. Whether *Talaromyces pinophilus* and *Penicillium simplicissimum* exhibit an impact on insects, is not yet known. Hence, it is necessary to investigate novel fungal species to use them for pest management. The current investigation's goal was to assess novel native Tamil Nadu entomopathogenic fungus strains pathogenic potential.

MATERIALS AND METHODS

The present study was conducted at Insectary, Tamil Nadu Agricultural University, Coimbatore during 2021-2022. Fungi were isolated from the soils of Tamil Nadu using the soil baiting method described by Zimmermann (Inglis *et al*., 2012). Soil samples were collected from a depth of 15 cm, processed and preserved for further use. The test insect *Galleria mellonella* was cultured at Insectary, Tamil Nadu Agricultural University, Coimbatore, at 25°C. The artificial diet recommended by Gitanjali (2021) was modified and used for rearing *Galleria mellonella*.

Morphological identification of isolated entomopathogenic fungi was conducted by observing the colour of the fungal colony (front and reverse), texture, appearance and shape of the spore. Fungal isolates were confirmed by following DNA extraction by the Ctab method (Zhang *et al.,* 2010). The extracted DNA was amplified using ITS primers ITS1 (forward) and ITS4 (reverse) and sequencing was conducted at Syngenome (OPC) Private Limited, Coimbatore. The sequences were run through BLAST and compared with data already present in NCBI. Sequences were aligned using BIOEDIT 7.2 software and then submitted to NCBI for accession numbers. A phylogenetic tree was constructed using the MEGA 11.0 software. For this, sequences of isolated fungi and other sequences of fungi from Genbank were aligned using MUSCLE. A neighbor-joining tree was constructed using the Tamura-3 model (Tamura *et al*., 2007). Bootstrap analysis with 1000 replications was performed.

Novel fungal isolates were tested for their virulence against *Galleria mellonella*. Fifteen larvae were used for each fungal isolate and were replicated four times. The spore suspension was prepared by scraping the fungi into sterile distilled water, homogenized and the number of spores was counted using a haemocytometer. Spore concentration was fixed to 2.5×10^8 spores/ml, 2.5×10^6 spores/ml and 0.1% tween 80 was added as a surfactant. Wax moth larvae were dipped in spore suspension, air-dried and placed in plastic boxes lined with filter paper at the bottom. Control larvae were treated with 0.1% tween 80. Larvae were observed each day for up to 10 days. Isolation of fungi from infected cadavers was performed to obtain a pure culture of fungi. Percent mortality was corrected using Abbott's formula (Abbott, 1925) and LC_{50} and LT_{50} were calculated using the probit analysis method.

RESULTS AND DISCUSSION

Fungal isolates

Fungal cultures isolated were identified as *Clonostachys rosea*, *Penicillium simplicissimum*, *Talaromyces pinophilus* from the soils of Ooty (The Nilgiris) and *Purpureocillium lilacinum* from the soils of Kondayampalayam (Coimbatore) (Table 1) by comparing their sequences with sequences in NCBI database. They were named as TNAU OTD 1 (Cr), TNAU KDP 1 (Pl), TNAU OTY 1 (Tp) and TNAU OTE 1 (Ps). In the phylogenetic tree (Fig 1), *Clonostachys rosea* split from a common ancestor with *Purpureocillium lilacinum*, *Talaromyces pinophilus* and *Penicillium simplicissimum* at a common node. Subsequently, *Purpureocillium lilacinum* diverged from a common ancestor with *Talaromyces pinophilus* and *Penicillium simplicissimum* at another node. *Talaromyces pinophilus* and *Penicillium simplicissimum* on the other hand, share a close evolutionary relationship as they both originated from a common ancestor.

Pathogenicity assay

All four isolates caused mortality in *Galleria mellonella* larvae at both concentrations (2.5×10^8 spores/ml and 2.5×10^6

Table 1: List of fungal isolates used in the study.

Scientific name	Isolate	Location from which isolated
Clonostachys rosea	TNAU OTD 1 (Cr)	Ooty (The nilgiris)
Penicillium simplicissimum	TNAU OTE 1 (Ps)	Ooty (The nilgiris)
Purpureocillium lilacinum	TNAU KDP 1 (Pl)	Kondayampalayam (Coimbatore)
Talaromyces pinophilus	TNAU OTY 1 (Tp)	Ooty (The nilgiris)

Table 2: LC_{50} value of *Clonostachys rosea*, *Purpureocillium lilacinum*, *Penicilliumsimplicissimum* and *Talaromyces pinophilus* on *Galleria mellonella* larvae.

Fungal isolate	LC_{50}	LT_{50} (days)	UL	LL	Regression equation
TNAU OTD 1 (Cr)	1.47×10^6	4.67	3.62×10^6	6.01×10^5	y = 0.754× + 0.348
TNAU OTE 1 (Ps)	7.69×10^5	3.95	2.07×10^6	2.85×10^5	y = 0.781× + 0.4
TNAU KDP 1 (Pl)	8.5×10^5	4.37	2.63×10^6	2.74×10^5	y = 0.671× + 1.02
TNAU OTY 1 (Tp)	1.56×10^6	4.92	4.34×10^6	5.64×10^5	y = 0.659× + 0.922

UL- Upper limit, LL- Lower limit.

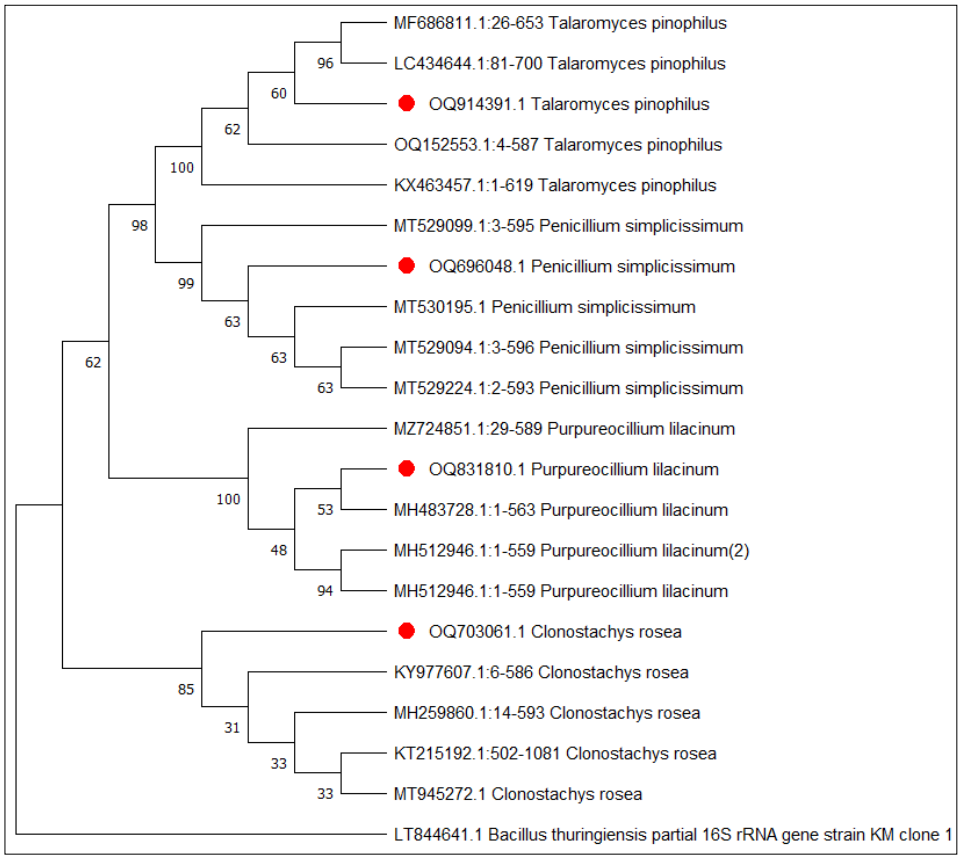

Fig 1: Phylogenetic tree comparing the isolates in the present study (in red circle) with other isolates in the NCBI database with *Bacillus thuringiensis* as an outgroup.

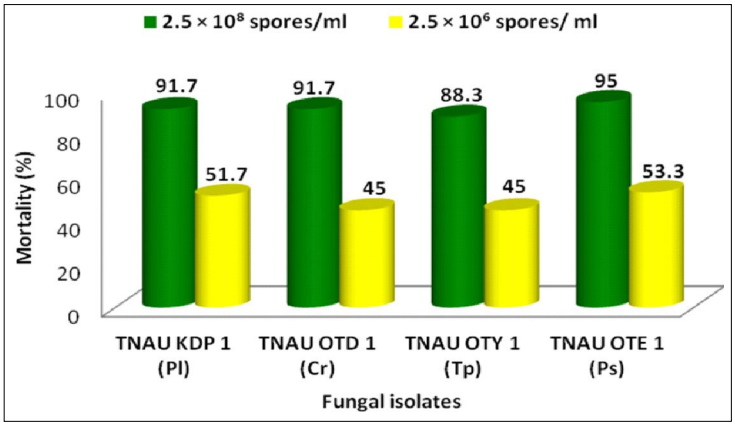

Fig 2: Mortality (%) of *Clonostachys rosea*, *Purpureocillium lilacinum*, *Penicillium simplicissimum* and *Talaromyces pinophilus* at different concentrations.

spores/ ml) and no death was observed in the control (Fig 2). All isolates performed equally well, while TNAU OTE 1 (Ps) caused 95% mortality at higher concentration compared to TNAU OTD 1 (Cr), TNAU KDP 1 (Pl) (91.7%) and TNAU OTY 1 (Tp) (88.3%). The variation in the pathogenic ability of these entomopathogenic fungi against *Galleria mellonella* may be attributed to variances in the toxins or enzymes released by the fungi, which play a crucial role in the infection process. Additionally, the fungi's capability to evade the host's immune response and the impact of environmental factors such as temperature, humidity and p^H on their pathogenicity may also contribute to these differences. These fungi also performed well at low concentration by causing nearly 50% mortality in wax moth larvae. These results prove that these fungi were efficient in insect control. Toledo *et al*. (2006) observed that *Oncometopia tucumana* experienced a mortality rate of 82.5% within 14 days when exposed to 6×10^5 spores/ml of *Clonostachys rosea*. Anwar *et al*. (2018) documented that *Clonostachys rosea* exhibited virulence against *Bemisia tabaci* resulting in 50.42% mortality in nymphs and 23.54% mortality in adults after 6 days. Mohammed *et al*. (2021) reported *Clonostachys rosea*'s efficacy in controlling coleopteran storage pests, showing mortality rates ranging from 70.7 to 75.7%. Our study's results complement these findings, demonstrating a mortality rate 45% at a concentration of 2.5×10^6 spores/ ml of *Clonostachys rosea*, with an LT_{50} of 4.67 days. *Penicillium simplicissimum* and *Purpureocilium lilacinum* exhibited LC_{50} values of 7.69×10^5 and 8.5×10^5 spores/ml respectively, resulting in 50% population mortality within 3.95 days and 4.37 days (Table 2). A similar finding was reported by Sun *et al.* (2021) on the pathogenicity of *Purpureocillium lilacinum* against *Bemisia tabaci.*

CONCLUSION

Although studies were being conducted in recent years on the efficacy of *Talaromyces* sp and *Penicillium simplicissimum*, they were found effective as a mycoparasite and their potential as an entomopathogen is being explored. In the current research, pathogenicity of *Talaromyces pinophilus* and *Penicillium simplicissimum* against *Galleria mellonella* was evaluated and at low concentration (2.5×10^6 spores/ml), they showed 45% and 53.3% mortality against wax moth larvae. Notably, *Clonostachys rosea* (45%) and *Purpureocillium lilacinum* (51.7%) exhibited remarkable mortality rate. These fungal species hold significant potential for managing insect pests, thus broadening the selection of entomopathogenic fungi for biocontrol purposes. Consequently, the identification of novel fungal strains opens up promising opportunities for enhanced and effective pest control measures.

Conflict of interest: None.

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