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Diversity of entomopathogenic fungi across Agro-climatic zones of Karnataka, India

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Abstract: Entomopathogenic fungi are the most versatile and potential biocontrol agents, due to their adaptability, mode of entry, persistent nature and wide host range. The present study aimed to isolate and identify the native entomopathogenic fungi for the biocontrol of major sucking insect pests of floriculture. Eighty-one fungal isolates were isolated from 55 soil samples and 26 infected insect cadavers were collected from the eastern and southern dry zones of Karnataka, India. Serial dilution and plating techniques, and insect bait techniques were followed. The insecticidal activity of fungal isolates against aphids, mites, 2nd instar larvae of thrips and whitefly nymphs was determined by leaf disc bioassay under *in vitro* conditions. The results of the experiment revealed that among 81 fungal isolates, sixteen (19.25 %) exhibited insecticidal activity against test insects and further, a distribution study revealed that 50 per cent of EPF isolates were belonging to genera *Beauveria* and *Metarhizium* (25% each), 18.75% *Aspergillus,* 12.50% were *Lecanicillium* spp. and 13% were *Paecilomyces* spp. (6.25%) and *Hirsutella* spp. (6.25 %) respectively. It was concluded that the recovery rate of EPF is lower with 19.5% and the dominant genera of EPFs are *Beauveria* and *Metarhizium*. This show EPFs with broader host rage will have more chances of survival and the same criteria can be used for the selection of efficient EPFs.

Key words – *Beauveria,* biocontrol, bioassay, insect bait method, sucking pests.

INTRODUCTION

In modern agriculture, there is a decline in global crop losses due to various insect pests from 41.1 % during 1988-90 to 32.1 % during 2001-03 (Dhaliwal *et al*., 2015) due to the intensive use of chemical pesticides (approximately, 2.5 million tons of pesticides are applying annually, Sharma et al. 2019) despite the alarming problems like the development of resistance and resurgence of sucking pests (Vandoorn and Vos, 2013), residual toxic effects on humans, insect parasites, predators, animals and also the use will increase the cost of production. Because of these problems, it is largely felt that, it is necessary to find an alternative, sustainable and eco-friendly pest management technique i.e. biological control or biocontrol.

Entomopathogenic fungi (EPF) are potentially the most versatile biological control agents, due to their wide host range that often results in natural epizootics. These fungi have certain advantages in pest control programs over other insect pathogens because they infect all stages of insect pests, they directly infect insect pests through cuticle as other agents need ingestion hence these can even infect sucking and piercing pests also (Hajek and Leger, 1994; Mantzoukas *et al*., 2022) mass production techniques are simpler, easier and cheaper compared to the other microbial agents and their persistence nature. Over 1000 species of fungi belonging to 100 genera are known to be pathogenic to insects and many of these have great significance in pest management (Rabindra & Ramanujam, 2007). The most important and extensively studied fungal pathogens are *Lecanicillium lecanii, Beauveria* spp., *Metarhizium* spp., *Nomuraea rileyi*, *Paecilomyces* spp. *Aschersonia* spp. (Ascomycota: Hypocreales) and *Hirsutella* spp. (Wraight *et al*., 2007; Lacey et al. 2008; Kachhawa, 2017; Shaurub, 2023). *Lecanicillium* and *Beauveria* are used to combat different sucking pests under both greenhouses as well as field conditions but the success of biological control depends on environmental conditions like high relative humidity, moderate temperatures and soil organic matter (Namasivayam *et al*., 2015; Abdul Qayyum *et al*., 2021). Several researchers studied and evaluated the different entomopathogenic fungi for the biocontrol of various insect pests in agriculture, horticulture and forestry (Lacey et al. 2008) providing the most satisfying results and pieces of evidence. Despite considerable research on this topic in India, little information exists on the biocontrol of sucking pests of flower crops and screening of local fungal isolates for virulence characteristics is of predominant importance for the success of biocontrol strategies towards major insect pests (Faria and Wraight,

2001). Hence, the objective of the present study was to isolate and identify virulent native EPF strains from the soil as well as insect cadaver samples collected from different regions of south Karnataka, India.

MATERIALS AND METHODS

Sample collection

A systemic survey was conducted to collect soil and mummified insect samples from different flower growing locations of two agro-climatic zones (Eastern dry zone and Southern dry zone) of Karnataka, India. Soil samples were stored at 4±1°C whereas the insect cadavers samples were used within 24hr after collection for the isolation of entomopathogenic fungi.

Isolation of entomopathogenic fungi from insect cadavers

The insect cadavers were surface sterilized with 1 per cent sodium hypochlorite for one minute and then rinsed 3 times in sterilized water. The excess moisture from the surface-sterilized cadavers was removed by using tissue paper and transferred to a culture plate containing potato dextrose agar medium (PDA). The plates were incubated at 25±1 °C under dark conditions for the growth and development of fungi. After 5 days of incubation, the different fungal colonies were selected and purified by subculturing on PDA. Slants of each culture was prepared from purified fungal isolate and stored at 4±1°C for further studies (Reji Rani *et al*., 2015).

Isolation of entomopathogenic fungi from soil samples.

Serial dilution and plating method: Ten grams of soil from each soil sample was taken in a conical flask containing 100 ml sterile water saline and serially diluted up to 10^{-4}. Spread plating was done by transferring 0.1ml of aliquate from 10^{-2}, 10^{-3} and 10^{-4} dilutions on PDA and on selective media for *Metarhizium* spp. and *Beauveria* spp. given by Vestergaard and Eilenberg, 2000, and Meyling and Eilenberg, 2006 respectively. The Petri plates were incubated at 25±1 °C for the growth and development of fungi for 10 days. The colonies showing different characters on plates were picked up and transferred to PDA slants for further study.

Insect bait method: Insect-associating fungi were isolated from soil samples by using 'the insect bait method given by Zimmermann, 1986; Lui et al. 2021, with little modifications. Three 2nd instar *Spodoptera* larvae were released into a plastic box containing 100gm moistened soil sample and the boxes were incubated at 25±1°C for two weeks under the dark condition to promote the infection of pathogenic fungi. The larvae were examined at 7 and 14 days after incubation and dead larvae were surface sterilized with 1 per cent sodium hypochlorite for one min and then rinsed trice with sterile distilled water. After removing the free water from the larval surface, they were transferred onto PDA plates and incubated at 25±1°C for the growth and development of fungi for 10 days.

Insecticidal activity of fungal isolates

To screen the entomopathogens from non-entomopathogenic fungal isolates, preliminary screening was conducted using the leaf dip bioassay method as described by Sain et al. (2019) with few modifications. The experiment was carried out by cutting healthy gerbera leaves into 8 cm diameter leaf discs and surface sterilizing them with 70 per cent alcohol. The surface sterilized leaf discs were immersed in fungal spore suspension for 10s and the control was maintained by dipping the leaf discs in sterile distilled water. Further, all the leaf discs were air-dried and transferred onto sterile Petri plates containing filter paper to maintain humidity during incubation. Approximately Twenty laboratory-reared aphids, mites, second instar larvae of thrips and nymphs of whitefly were transferred onto the treated and control leaf discs by using sterile camel brush. The plates were kept for incubation at 25±1 °C for 5 days. After incubation leaf discs were observed for mycosis of test insects. The isolates successfully caused mycosis were confirmed as entomopathogenic fungi and selected for future study.

Generic identification of entomopathogenic fungal isolates

Initial characterization of entomopathogenic fungal isolates was done by using the Atlas of entomopathogenic fungi (Robert et al. 1988). Macroscopic colony characteristics like colour, growth pattern, shape and elevation of the colony were observed on PDA plates. Microscopic observation of isolates was done by fungal slide culture technique (Rosana et al. 2014) with lactophenol blue staining. The slides were observed for the arrangement of conidia, phialide and type of mycelium under a Labomed iVu3000 binocular light microscope at 400X magnification.

RESULTS AND DISCUSSION

Different techniques were employed to isolate entomopathogenic fungi from both soil as well as from insect cadaver samples *viz*., serial dilution and plating technique on selective and non-selective media, insect bait technique and directly placing insect cadavers

on potato dextrose agar plates. Previously, Jaber *et al.* (2016) isolated 42 entomogenous fungal isolates from 17 different arthropod cadavers collected from pesticide-free areas. Similarly, Gurlek *et al.* (2018) from walnut fields in Turkey, Mar et al. 2012 isolated from Chiang Mai, Thailand isolated EPF. In the present study, total of eighty-one fungal isolates were isolated from 26 insect cadavers and 55 soil samples and coded serially as ENPF. Most of the isolates were isolated from soil samples and few isolates were isolated from insect cadavers. The isolates were identified as *Aspergillus*, *Penicillium, Metarhizium, Beauveria* spp. *Trichoderma*, *Fusarium*, *Paecilomyces* and *Hirsutella* spp. based on macro and microscopic observation.

The results of bioassay studies exhibited that, all the fungal isolates from the soil as well as from insect cadavers may not be entomopathogenic because soil harbors different microbial communities including fungi, among them some will be opportunistic pathogens, some will be saprophytes and merely some are true entomogenous fungi. Among eighty-one fungal isolates, sixteen isolates like ENPF-3, ENPF-6, ENPF-8, ENPF-9, ENPF-16, ENPF-24, ENPF-32, ENPF-33, ENPF-41, ENPF-48, ENPF-53, ENPF-58, ENPF-60, ENPF-67, ENPF-68 and ENPF-79 were successful in exhibiting insecticidal activity against test insect pests (**Table 1**). The majority of the isolates shown insecticidal activity was isolated from either insect cadavers or from insect bait method.

Table 1: Insecticidal activity of entomopathogenic fungal isolates against sucking pest

Isolate code	Insecticidal activity against test insects			
	Aphids	Thrips	Mites	whitefly
ENPF-3	+	+	-	+
ENPF-6	+	-	-	-
ENPF-8	-	-	+	-
ENPF-9	+	-	-	-
ENPF-16	+	+	-	+
ENPF-24	+	+	-	+
ENPF-26	+	-	-	-
ENPF-33	+	-	-	-
ENPF-41	+	+	-	+
ENPF-48	+	+	-	-
ENPF-53	+	-	-	-
ENPF-58	-	-	+	-
ENPF-60	+	+	-	+
ENPF-67	+	-	-	-
ENPF-68	+	+	-	-
ENPF-79	+	-	-	-

Note: +: Positive -: Negative

Initial characterization of entomopathogenic fungal isolates was carried out by referring to the Atlas of entomopathogenic fungi (Robert et al.1988). From the observations, the isolates were identified at the generic level as *Metarhizium* spp. (ENPF-6, ENPF-9, ENPF-60 and ENPF-68), *Beauveria* spp. (ENPF-3, ENPF-16, ENPF-48 and ENPF-67), *Aspergillus* spp. (ENPF-26, ENPF-33 and ENPF-53), *Lecanicillium* spp. (ENPF-24 and ENPF-41), *Isaria* (formally known as *Paecilomyces* spp.) (ENPF-8) and *Hirsutella* sp. (ENPF-58).

Morphologically, all the *Metarhizium* isolates exhibited typical characteristics of *Metarhizium* like fast growth, green to light green colony. Whereas each isolate was slightly different concerning the size of the conidia which were between 4.1-5.7 µm. All the *Beauveria* isolates were white or creamy white colonies with dense or dispersed growth patterns and conidia were found in dense with round or spherical in shape and the size varied between 1.5 to 3.5 µm. *Aspergillus* isolates exhibited different shades of colony colour and

Table 2: Generic identification of entomopathogenic fungal isolates based on Macroscopic and Microscopic characteristics

Isolate Code	Macroscopic characteristics				Microscopic characteristics				Other key characters	Probable genera
	Growth pattern	Colour	Shape	Elevation	hyphae	Colour of conidia	Length of conidia (µm)	Shape of conidia		
ENPF-3	Disperse and slow growing	White	Round	Raised	Hyaline and septate	Hyaline to creamy spores	1.6-3.2	Globose or ovoid	Conidia found in dense clusters or whorls with characteristic denticulate rachis	*Beauveria*
ENPF-6	Uniform and fast-growing	Dark green	Circular	Flattened	Septate	Green	4.5-5.7	Long, cylindrical with round edges	Conidia arranged in parallel chains	*Metarhizium*
ENPF-8	Uniform and fast-growing	Pinkish white	Round	Flattened	Hyaline and septate	Pinkish white	3.5-4.0	Ovoid or round	Conidiophore smooth and colorless; long slender divergent phialides; conidial chains are often long	*Isaria*
ENPF-9	Uniform and fast-growing	Light or yellowish green	Circular	Flattened	Septate	Green or dull green	4.1-5.4	Long, cylindrical with round edges	Conidia arranged in parallel chains	*Metarhizium*
ENPF-16	Dense and slow growing	White	Round	Raised	Hyaline and septate	White to creamy spores	1.5-3.5	Globose or ovoid	Conidia found in dense clusters or whorls with characteristic denticulate rachis	*Beauveria*
ENPF-24	Dense and fast growing	White	Circular	Flattened with raised mycelium	Hyaline and septet	Hyaline to creamy spores	1.6-3.0	Spherical to ovoid	++	*Lecanicillium*
ENPF-26	Uniform and fast growing	Dark green	Circular	Flattened with raised mycelium	Septate	Light green	1.8-3.5	Spherical to ovoid with think wall	Short conidiophores with long conidial chains	*Aspergillus*
ENPF-33	Uniform and fast-growing	Dark green	Circular	Flattened with raised mycelium	Septate	Light green	1.6-3.2	Spherical to ovoid with think wall	Short conidiophores with long conidial chains	*Aspergillus*
ENPF-41	Dense and fast-growing	White	Circular	Flattened with raised mycelium	Hyaline and septet	Hyaline to creamy spores	1.5-3.2	Spherical to ovoid	++	*Lecanicillium*

Isolate Code	Macroscopic characteristics				Microscopic characteristics				Other key characters	Probable genera
	Growth pattern	Colour	Shape	Elevation	hyphae	Colour of conidia	Length of conidia (µm)	Shape of conidia		
ENPF-48	Dense and slow growing	White	Round	Raised	Hyaline and septate	White to creamy spores	1.5-3.5	Globose or ovoid	Conidia found in dense clusters or whorls with characteristic denticulate rachis	*Beauveria*
ENPF-53	Uniform and fast-growing	Dark green	Circular	Flattened with raised mycelium	Septate	Light green	1.7-3.3	Spherical to ovoid with think wall	Short conidiophores with long conidial chains	*Aspergillus*
ENPF-58	Uniform and slow growing	Brown	Round	Flattened	Septate	Brown	2.5-5.0	Boat-shaped (naviculoid) to cylindrical	Conidia borne in chains	*Hirsutella*
ENPF-60	Dense and slow growing	White	Round	Raised	Hyaline and septate	White to creamy spores	1.5-3.5	Globose or ovoid	Conidia found in dense clusters or whorls with characteristic denticulate rachis	*Beauveria*
ENPF-67	Uniform and fast-growing	Light or yellowish green	Circular	Flattened	Septate	Green or dull green	4.3-5.5	Long, cylindrical with round edges	Conidia arranged in parallel chains	*Metarhizium*
ENPF-68	Uniform and fast-growing	Dark green	Circular	Flattened	Septate	Green	4.5-5.7	Long, cylindrical with round edges	Conidia arranged in parallel chains	*Metarhizium*
ENPF-79	Uniform and fast-growing	Dark green	Circular	Flattened with raised mycelium	Septate	Light green	1.7-3.3	Spherical to ovoid with think wall	Short conidiophores with long conidial chains	*Aspergillus*

Note: ENPF – Isolate code

varied conidia sizes (Fig. 2). *Paecilomyces* produced pinkish-coloured, dense colonies with ovoid or round conidia. Colony characteristics of both the isolates of *Lecanicillium* have not varied much, the dense, white, fast-growing colony and spherical to ovoid conidia. *Hirsutella* produced a typical brown-coloured, thick colony with white strips and conidia that were boat-shaped or naviculoid. A detailed description of macroscopic and microscopic characteristics of EPF is given in table-2. In the present study, it was observed that the recovery rate of entomogenous fungi was 19.5 per cent, in India it varies between 15-38 per cent from the various surveys conducted by research workers all over the country and it is true with other countries.

Kassam *et al*. (2022), studied the morphology of *Metarhizium* sp. which were isolated from Migratory locust, *Locusta migratoria* L. and reported that the length of the conidia of the different *M. anisopliae* isolates varied from 6.1 to 7.4 µm and the width ranged from 2.2 um to 3.1µm. Similarly, Varela and Mornles, (1996), carried out the characterization of some *B. bassiana* isolates and reported that the colony colour of different isolates showed wide variations between white and yellow. In conidial morphology, they observed three sizes and two types of conidial shapes <2.5 µm (globose), 2.6-3.75 µm (globose) and 4.0-5.0×2.5-3.0 µm (Ellipsoidal).

Distributions of entomopathogenic fungi were studied based on the number of isolates exhibited insecticidal activity against sucking pests used in the study. Among 81 fungal isolates, sixteen isolates (19.25 %) were shown positive for pathogenicity against test insects. Among *Beauveria.* spp. (25 %), *Metarhizium* spp. (25%), *Aspergillus* spp. (18.75%), *Lecanicillium* spp. (12.50 %), *Paecilomyces* spp. (6.25%) and *Hirsutella* spp. (6.25 %) were identified (Fig. 1). The results of distribution explain that the fungi which were having a broader host range and adaptability will survive the most under natural conditions. Previously, Franco *et al.* (2011), were examined 142 soil samples from different states of Mexico for the isolation of entomopathogenic fungi using the insect bait method (*Galleria mellonella* L.). Around 23 per cent of samples were shown positive for the presence of entomopathogenic fungi in that 12 % (17 isolates) were *B. bassiana,* 1 % (2 isolates) were *M. anisopliae* and 10 % (14 isolates) were *Isaria fumosorosea.* This was further confirmed by González-Baca *et al*. (2019); Chen *et al*. (2021); Qayyum *et al*. (2021).

In the present study, it was observed that the recovery rate of entomogenous fungi was 19.5 per cent, in India it varies between 15-38 per cent from the various surveys conducted by research workers all over the country (Reji Rani *et al*., 2015; Maryam *et al*., 2014) and it is even true with other parts of the globe (Franco *et al*., 2011 from Mexico, Jaber *et al*., 2016 from Mexico, Niu et al. 2019 from Brazil, Gurlek *et al*. 2018 from Turkey, Maryam *et al*., 2014 from Iran). The low recovery rate from the various surveys conducted worldwide due to low soil organic matter content, high pesticide usage and influence of environmental factors *viz*., high temperature and low humidity were affecting the existence and survival of entomopathogenic fungi in soil (Márquez-Gutiérrez *et al*., 2022; Namasivayam *et al*., 2015; Abdul Qayyum *et al*., 2021). Since these fungi are heterotopic, they use soil as a habitat and organic matter as a source of nutrients for long-term persistence when crops are not present in the field. Meyling et al. in 2011, in a study on the diversity and distribution of entomopathogenic fungi revealed that the population of entomopathogenic fungi was significantly higher in the field under organic cropping systems than in the fields of the conventional cropping system. This was further confirmed by Uzman et al. 2019; Afandhi *et al*. (2022). There is a considerable effect of farming systems, field margins, land-use type and bait-insect on the occurrence of insect pathogenic fungi in soils (Klingen *et al*., 2002; Fernández-Bravo *et al*., 2021).

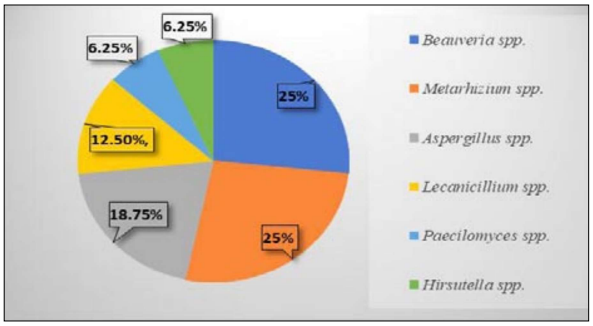

Fig 1: Diversity of entomopathogenic fungal isolates in soils of two agroclimatic zones of Karnataka, India.

CONCLUSION

In conclusion, the isolation and screening of efficient biocontrol agents plays an important role in the sustainable management of pests in agriculture. The native isolates will perform better due to adaptability in comparison to the isolates isolated from other locations having varied environmental, soil conditions and other factors. The presence of entomopathogenic fungal isolates will be largely affected by the use of agrochemicals and the low organic content of the soil as evidenced by the low recovery rate during the study.

Conflict of Interest

All the authors of the manuscript declare that they do not have any conflict of interest.

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