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Exploiting the Efficacy of Entomopathogenic Fungi against Common Floricultural Pests: A Focus on Aphids (*Myzus persicae*), Whiteflies (*Bemisia tabaci B-biotype*) and Western Flower Thrips (*Frankliniella occidentalis*)

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ABSTRACT

This study evaluated the efficacy of Entomopathogenic Fungi (EPF) as biocontrol agents against aphids, whiteflies, and western flower thrips. The research employed a leaf disc bioassay with various conidia concentrations to determine Lethal Concentration (LC) and Time (LT) for pest eradication. Additionally, the study assessed the activity of cuticle-degrading enzymes produced by EPF (Chitinase, Protease, and Lipase) to understand their pathogenic mechanisms. Molecular identification using ITS region of 18S rDNA identified virulent isolates. Results indicated that four isolates, namely, ENPF-16, 24, 41, and 60 achieved significant mortality rates (95 to 100%) at a concentration of 1×10^8 conidia mL^{-1} after nine days. *Akanthomyces* sp. (ENPF-41) exhibited the highest enzyme activity, followed by *Beauveria* sp. (ENPF-60). The virulent fungal isolates were identified as *Beauveria bassiana* and *Akanthomyces lecanii*. Among EPF, *Akanthomyces lecanii* (MT997935) displayed greater virulence against all three test insects with lower LC_{50} and LT_{50} values compared to the other EPF. In summary, all fungal isolates induced mortality in the tested pests, but their effectiveness varied. *Akanthomyces lecanii* (MT997935) emerged as a promising biocontrol candidate due to its broad host range and strong virulence.

Keywords: *Beauveria*, Biological control, Chitinase, Cuticle degrading enzymes, *Lecanicillium,* Sucking pests.

INTRODUCTION

Entomopathogenic Fungi (EPF) are potentially the most diverse and versatile biological control agents due to their wide host range that often results in natural epizootics. An attractive feature of these fungi is that they infect by contact and act through penetration (Mondal *et al.,* 2016). They have certain advantages in pest control programs over other insect pathogens as they infect all stages of insects and directly infect through the cuticle, while other agents need to be ingested. Mass production techniques for these fungi are simpler, easier, and cheaper, and the persistent nature making them a potential candidate in pest control programs over other insect pathogens. The mode of entry and action of these fungi make them a promising option for combating sucking and piercing insects (Ramanujam *et al.,* 2014). Approximately 750 to 1,000 fungi are considered entomopathogens placed in over 100 genera, many of these have great significance in insect pest management (Kachhawa, 2017; Ranadev *et al.,* 2023). The most common and extensively studied entomopathogens are *Metarhizium* spp. *Beauveria* spp.

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Nomuraea rileyi, *Akanthomyces* spp., *Isaria* and *Hirsutella* spp. (Lacey *et al.,* 2008; Kachhawa, 2017). The *Akanthomyces* spp. and *Beauveria* spp. have been extensively used to combat different sucking pests under both greenhouse and field conditions.

Sucking pests, also known as sap-sucking insects, encompass aphids, thrips, whiteflies, mites, and leafhoppers. Their name stems from their piercing and sucking mouthparts, employed to extract sap from host plants. This sap removal leads to stunted growth, distorted appearance, chlorophyll loss, reduced strength, and premature leaf shedding in affected plants. Beyond transmitting diseases, some of these pests inject toxic substances into host plants during feeding, causing substantial agricultural losses (15-45%) and heightened management costs. Despite the use of approximately 2.5 million tons of pesticides annually, crop losses decreased from 41.1% during 1988-1990 to 32.1% during 2001-2003 (Dhaliwal *et al*., 2015). The use of pesticides has led to various problems, including the development of resistance and resurgence of sucking pests (Vandoorn and Vos, 2013) and residual toxic effects on humans, animals, insect parasites, and predators. Additionally, the use of pesticides increases the cost of production. To address these challenges, it is essential to find a sustainable and environmentally friendly pest management solution.

Despite extensive research on biological control of insect pests, limited information exists on the biocontrol of sucking insect pests. Screening of local fungal isolates for their virulence characteristics is crucial for the success of biocontrol strategies (Sun *et al.,* 2021). Conservation and periodic improvement of the efficacy of these biological control agents will significantly aid in crop protection and help to produce pesticide residue-free agricultural commodities, reducing the usage of pesticides in agriculture.

The present study aimed to isolate and identify the virulent native entomopathogenic fungal strains from soil and insect cadaver samples collected from various flower growing regions of India. Further, the isolates were screened for biocontrol potential against the sap sucking insect pests *viz*. aphids, thrips and whiteflies in flower crops.

MAAERIALS AND METHOTDS

Sample Collection and Isolation of Entomopathogenic Fungi

A systematic survey was conducted to collect soil and mummified insect samples from different locations of agro-climatic zones (eastern and southern dry zones) of Karnataka, India. The EPF isolates were isolated by insect bait method, serial dilution and plating on specific media, and directly placing the surface sterilized insect cadavers on Potato Dextrose Agar (PDA). The spore suspension of isolates was prepared by adding 10 mL 0.5% sterile Tween-80 to ten days old cultures and various concentration of conidial suspension was prepared by serial dilution. The conidial count was determined using an improved Neubauer Haemocytometer.

Laboratory Bioassay to Assess the Virulence of Entomopathogenic Fungal Isolates

The leaf disc bioassay, based on Nazir *et al.* (2018) protocol was used with slight modifications, involved using 8 cm diameter healthy gerbera leaf discs. These discs were surface sterilized with 70% alcohol and immersed in fungal spore suspensions at a concentration of 1×10^8 conidia per mL for 10 seconds. A control group was treated with sterile distilled water. After air drying, the leaf discs were placed on sterile Petri plates with filter paper for humidity control during incubation. Each treatment had three replications. Twenty adult aphids (*Myzus persicae*), 2nd instar thrips larvae (*Frankliniella occidentalis*), and whitefly nymphs (*Bemisia tabaci B-biotype*) were placed on treated and control leaf discs using a sterile brush. The setup was incubated at 25±1ºC. Observation

for insect mortality occurred at two-day intervals from the 3rd to the 9th Day After Treatment (DAT). Dead insects were transferred to PDA plates and incubated at 25±1ºC with 90% humidity to confirm fungal infection as the cause of death.

Cuticle Degrading Enzyme Activity of Entomopathogenic Fungal Isolates

The cuticle degrading enzyme index was determined by measuring the clear zone formed by degradation of specific organic compounds in the medium and the zone of clearance around the well was measured five days after incubation. The enzymatic index was calculated by dividing the diameter of the halo zone by the diameter of the well, using the formula described by Bai *et al*. (2012). The chitinase enzyme index was determined using chitin yeast extract agar. Protease index was determined on skim milk agar and tributyrin agar was used for lipase enzyme index determination.

The chitinolytic activity of fungal isolates was determined by measuring the amount of reducing saccharides released from colloidal chitin using dinitro salicylic acid measuring the absorbance of the reaction mixture at 540 nm. The enzyme activity was expressed as unit per microliter. One unit of enzyme activity was defined as the amount of enzyme that released 1µmol of N-acetyl D-glucosamine per minute under described conditions (Bai *et al.,* 2012). Proteinase activity was estimated by measuring the absorbance at 280 nm. The number of amino acids released was calculated from a standard curve plotted against known concentrations of tyrosine (Hossain *et al*., 2006). Lipase activity was determined by a modified method of Pignede *et al.* (2018).

Determination of Lethal Concentration (LC_{50}) and Lethal Time (LT_{50})

The selected fungal isolates that showed high virulence against the sucking pests of gerbera, including aphids, thrips, mites and whiteflies, were subjected to further testing to determine their lethal concentrations and lethal times. This was done at six different conidia concentrations (1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia per mL) using both the leaf disc method and the whole leaf method (Trinh *et al*., 2020).

Molecular Identification of Virulent Entomopathogenic Fungal Isolates

The DNA was extracted from fungal mycelia by the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer (Doyle and Doyle, 1987) method, followed by purification through phenol/chloroform extraction and precipitation with isopropanol or ethanol (Ashktorab and Cohen, 1992). The ITS1 and ITS2 of regions of 18S rDNA were amplified using universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), each at a concentration of 0.5 µL (Hirata and Takamatsu, 1996). The PCR products were sequenced through Sanger sequencing using outsourcing services Barcode BioSciences, an ISO-certified company based in Bangalore. The sequences were identified by using BLAST at NCBI website. Further, detailed phylogenetic analyses were conducted in MEGA 6.06 based on partial 18S rDNA gene sequences.

Data Analysis

The data obtained from the results was analyzed using ANOVA (analysis of variance) with the help of the software Web Agri Stat Package 2.0 (https://ccari.icar.gov.in/wasp2.0/index.php, accessed on October 22, 2022), and the means were compared using post-hoc test (Duncan's multiple range test) at a 5% level. The LC_{50} and LT_{50} values were calculated using the Probit analysis (Finney, 1971) function in the IBM SPSS Statistics v 20 software (IBM Corp., Armonk, NY, USA).

The LC_{50} values for entomopathogenic fungal isolates were considered significantly different if the 95% Confidence Intervals (CIs) did not overlap with the CIs of other isolates.

RESULTS

Sample Collection and Isolation of Entomopathogenic Fungi

In the present study, total of 81 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, Trichoderma, Fusarium, Isaria* and *Hirsutella* spp. based on macro and microscopic observations (Ranadev *et al*., 2023).

Virulence of Entomopathogenic Fungal Isolates

The results obtained from the virulence assay were diverse in terms of the mortality caused by different isolates over time, with an increase in the rate of mortality. All the entomopathogenic isolates successfully caused mycosis, starting from the Third Day After Treatment (3 DAT) to the Ninth Day After Treatment (9 DAT).

Beauveria isolate ENPF-60 and *Akanthomyces* isolates ENPF-24 and ENPF-41 demonstrated significantly highest aphid mortality, reaching 42.20% on the third day post-treatment. ENPF-16 recorded 33.33% mortality, while *Metarhizium* isolate ENPF-67 had the lowest at 4.44%. *Isaria*, *Aspergillus*, and *Hirsutella* isolates caused mortality ranging from 6% to 25%. Over subsequent days, mortality rates increased for all isolates, with *Akanthomyces* isolates showing pronounced mortality (75-88%) on the fifth to seventh days. *Beauveria* isolate ENPF-60 exhibited significantly higher mortality (80.00 and 91.07%) on the fifth and seventh days compared to other *Beauveria* isolates. *Metarhizium* and *Aspergillus* isolates were less effective, causing 30% to 60% mortality between the fifth and ninth day. *Akanthomyces* (ENPF-24) and *Beauveria* (ENPF-60) isolate achieved the statistically highest mortality (100 and 92.00%, respectively) on the ninth day post-treatment (Table 1; Figure 3).

The results of the virulence assay on nymphs of whiteflies showed diverse outcomes in terms of the mortality caused by different isolates over time. Only the *Akanthomyces* isolates (ENPF-24 and ENPF-41) caused significantly higher mortality at all days after treatment (Table 1**)**. The *Akanthomyces* isolate ENPF-41 showed the highest mortality, with 17.78, 73.33, 86.67, and 100% of the whitefly's mortality at 3, 5, 7, and 9 days after treatment, respectively. *Beauveria* isolate ENPF-16 caused 22.22, 66.67, 80, and 91.11% mortality at the same respective time points. The statistically lowest mortality of nymphs, at 33.33%, was reported for the *Hirsutella* isolate ENPF-58. In general, the mortality of whiteflies caused by different isolates ranged from 30 to 100% on the 9^{th} day after treatment.

On the third day after treatment, only half of the isolates (*Beauveria, Akanthomyces and Isaria*) were successful in causing mortality in thrips, while the *Aspergillus, Metarhizium* and *Hirsutella* isolates failed to cause any mortality. The statistically highest percent mortality of thrips, 20% was caused by *Akanthomyces* isolate (ENPF-24), followed by *Beauveria* isolates (13.33%). The mortality rate increased as the days after treatment increased. On the 5th, 7th, and 9th day after treatment, both *Akanthomyces* isolates (ENPF-24 and ENPF-41) and *Beauveria* isolates (ENPF-16 and ENPF-60) showed significance in causing mycosis in thrips. The mortality caused by *Akanthomyces* isolates ranged from 66 to 93%, while it was around 53% to 90% in *Beauveria* isolates. The *Aspergillus*,

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Table 1. Virulence of entomopathogenic fungal isolates on per cent cumulative and corrected mortality under *in vitro* conditions.[a]

Insects	DAT	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
Aphids	3rd	26.7 (31.1)^{c}	13.3 (21.4)^{e}	20.0 (26.6)^{d}	13.3 (21.4)^{e}	33.3 (35.3)^{b}	40.0 (39.2)^{a}	13.3 (21.4)^{e}	8.9 (17.3)^{f}	40.0 (39.2)^{a}	20.0 (26.6)^{d}	13.3 (21.4)^{e}	6.7 (15.0)^{g}	42.2 (40.5)^{a}	20.0 (26.6)^{d}	13.3 (21.4)^{e}	11.1 (19.4)^{ef}
	5th	60.0 (50.8)^{d}	33.3 (35.3)^{g}	40.0 (39.2)^{f}	33.3 (35.3)^{g}	66.7 (54.7)^{c}	73.3 (58.9)^{b}	33.3 (35.3)^{g}	31.1 (33.9)^{gh}	73.3 (58.9)^{b}	53.3 (46.9)^{c}	31.1 (33.9)^{gh}	26.7 (31.1)^{h}	80.0 (63.4)^{a}	34.0 (35.7)^{fg}	33.3 (35.3)^{g}	31.1 (33.9)^{gh}
	7th	73.3 (58.9)^{c}	44.4 (41.8)^{e}	53.3 (46.9)^{d}	48.9 (44.4)^{de}	82.2 (65.0)^{b}	86.7 (68.6)^{ab}	46.7 (43.1)^{de}	46.7 (43.1)^{de}	88.9 (70.5)^{a}	66.7 (54.7)^{c}	53.3 (46.9)^{d}	33.3 (35.3)^{f}	91.1 (72.6)^{a}	44.4 (41.8)^{e}	53.3 (46.9)^{d}	48.9 (44.4)^{de}
	9th	80.0 (63.4)^{c}	60.0 (50.8)^{ef}	66.7 (54.7)^{e}	57.8 (49.5)^{ef}	95.6 (77.8)^{b}	100.0 (90.0)^{a}	53.3 (46.9)^{fgh}	55.8 (48.3)^{fg}	95.6 (77.8)^{b}	73.3 (58.9)^{c}	60.0 (50.8)^{ef}	46.7 (42.8)^{h}	100.0 (90.0)^{a}	51.1 (45.6)^{gh}	57.8 (49.5)^{ef}	57.8 (49.5)^{ef}
	X	**60.0**	**37.8**	**45.0**	**38.3**	**69.4**	**75.0**	**36.7**	**35.6**	**74.4**	**53.3**	**39.4**	**28.3**	**78.3**	**37.4**	**39.4**	**37.2**
Whitefly	3rd	17.8 (24.9)^{c}	4.4 (12.2)^{g}	15.6 (23.2)^{d}	8.9 (17.3)^{e}	22.2 (28.1)^{ab}	24.4 (29.6)^{a}	6.7 (15.0)^{f}	6.7 (15.0)^{f}	17.8 (24.9)^{c}	20.0 (26.6)^{bc}	6.7 (15.0)^{f}	8.9 (17.3)^{e}	24.4 (29.6)^{a}	4.4 (12.2)^{g}	6.7 (15.0)^{f}	4.4 (12.2)^{g}
	5th	46.7 (43.1)^{e}	26.7 (31.1)^{g}	53.3 (46.9)^{d}	33.3 (35.3)^{f}	66.7 (54.7)^{b}	63.3 (52.7)^{bc}	26.7 (31.1)^{g}	20.0 (26.6)^{h}	73.3 (58.9)^{a}	53.3 (46.9)^{d}	26.7 (31.1)^{g}	13.3 (21.4)^{i}	60.0 (50.8)^{c}	22.2 (28.1)^{gh}	33.3 (35.3)^{f}	26.7 (31.1)^{g}
	7th	60.0 (50.8)^{d}	40.0 (39.2)^{ef}	66.7 (54.7)^{d}	46.7 (43.1)^{e}	80.0 (63.4)^{b}	80.0 (63.4)^{b}	40.0 (39.2)^{ef}	33.3 (35.3)^{f}	86.7 (68.6)^{a}	73.3 (58.9)^{c}	40.0 (39.2)^{ef}	26.7 (31.1)^{g}	73.3 (58.9)^{c}	40.0 (39.2)^{ef}	40.0 (39.2)^{ef}	33.3 (35.3)^{f}
	9th	68.9 (56.1)^{d}	48.9 (44.4)^{ef}	73.3 (58.9)^{d}	51.1 (45.6)^{ef}	91.1 (72.7)^{b}	93.3 (75.0)^{b}	48.9 (44.4)^{ef}	40.3 (39.4)^{gh}	100.0 (90.0)^{a}	82.2 (65.0)^{c}	44.4 (41.8)^{fg}	33.3 (35.3)^{h}	84.4 (66.8)^{c}	51.1 (45.6)^{ef}	53.3 (46.9)^{e}	46.7 (43.1)^{fg}
	X	**48.3**	**30.0**	**52.2**	**35.0**	**65.0**	**65.3**	**30.6**	**25.1**	**69.4**	**57.2**	**29.4**	**20.6**	**60.6**	**29.4**	**33.3**	**27.8**
Thrips	3rd	13.3 (21.4)^{c}	0.0 (0.28)^{f}	11.1 (19.4)^{d}	0.0 (0.28)^{f}	11.1 (19.4)^{d}	20.0 (26.6)^{a}	0.0 (0.28)^{f}	0.0 (0.28)^{f}	15.6 23.2)^{b}	13.3 (21.4)^{c}	0.0 (0.28)^{f}	0.0 (0.28)^{f}	13.3 (21.4)^{c}	0.0 (0.28)^{f}	2.2 (8.56)^{e}	0.0 (0.28)^{f}
	5th	53.3 (46.9)^{c}	13.3 (21.4)^{fg}	40.0 (39.2)^{d}	15.6 (23.2)^{f}	53.3 (46.9)^{c}	66.7 (54.7)^{b}	20.0 (26.6)^{e}	13.3 (21.4)^{fg}	66.7 (54.7)^{b}	53.3 (46.9)^{c}	20.0 (26.6)^{e}	13.3 (21.4)^{fg}	73.3 (58.9)^{a}	20.0 (26.6)^{e}	20.0 (26.6)^{e}	11.1 (19.5)^{g}
	7th	66.7 (54.7)^{b}	26.7 (31.1)^{de}	53.3 (46.9)^{c}	28.9 (32.5)^{d}	66.7 (54.7)^{b}	80.0 (63.4)^{a}	33.3 (35.3)^{de}	26.7 (31.1)^{de}	82.2 **(65.0)**^{a}	66.7 (54.7)^{b}	28.9 (32.5)^{d}	20.0 (26.6)^{e}	80.0 (63.4)^{a}	26.7 (31.1)^{de}	26.7 (31.1)^{de}	20.0 (26.6)^{f}
	9th	73.3 (58.9)^{d}	36.7 (37.3)^{gd}	60.0 (50.8)^{e}	33.3 (35.3)^{hi}	80.0 (63.4)^{c}	93.3 (75.0)^{a}	30.0 (33.2)^{ij}	33.3 (35.3)^{hi}	91.1 (72.6)^{b}	73.3 (58.9)^{d}	33.3 (35.3)^{hi}	26.7 (31.1)^{j}	90.0 (71.6)^{b}	46.7 (43.1)^{f}	40.0 (39.2)^{g}	26.7 (31.1)^{j}
	X	51.7	19.2	41.1	19.4	52.8	65.0	20.8	18.3	63.9	51.7	20.6	15.0	64.2	23.3	22.2	14.4

[a] Values in the parentheses are ARCSINE transferred values. The values represented by same letters in each row are statistically on par with each other by DMRT mean of three replications at 95% confidence interval (CI). **X:** Mean.

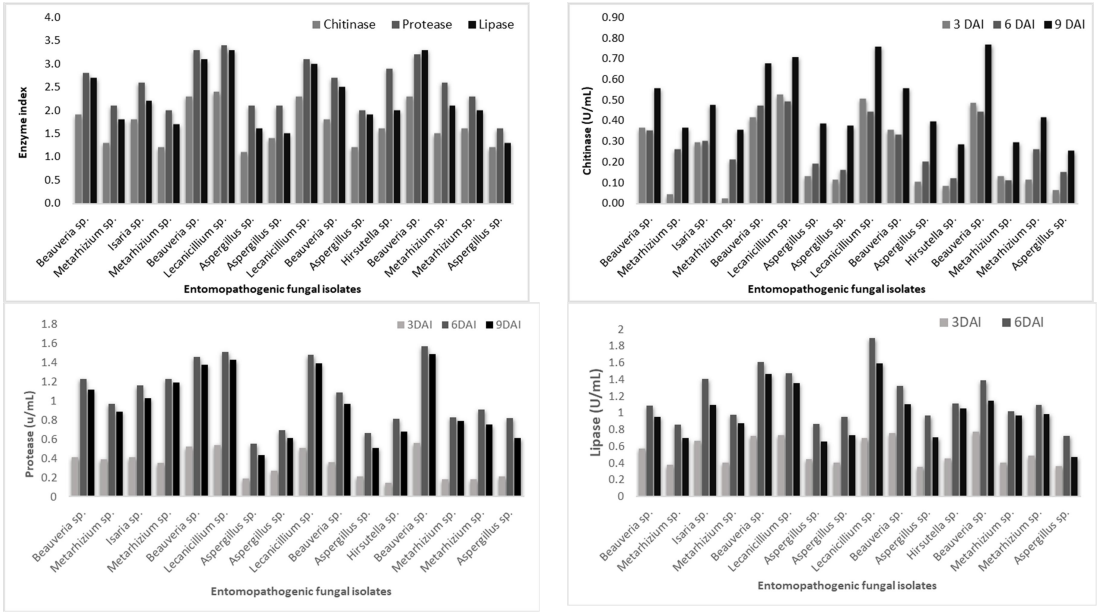

Figure1: Cuticle degrading enzyme of entomopathogenic fungal isolates (Days after incubation).

Metarhizium and *Hirsutella* isolates caused significantly lower mortality, ranging from 13 to 33.66% between the 5th to the 9th day after treatment. All the results related to the mortality of thrips caused by entomopathogenic fungal isolates are given in Table-1.

Cuticle Degrading Enzyme Activity of Entomopathogenic Fungal Isolates

Enzyme activity analysis revealed that among the sixteen EPF isolates, *Beauveria* and *Akanthomyces* isolates displayed the highest chitinase index (1.9 to 2.5), while *Hirsutella* and *Isaria* isolates showed lower chitinase indices of 1.8 and 1.4, respectively. For the proteolytic index, *Akanthomyces* spp. (ENPF-24) and *Beauveria* spp. (ENPF-16) exhibited significantly higher values of 3.4 and 3.3, followed by ENPF-60 and ENPF-41, with the lowest (1.6) recorded for *Aspergillus* spp. (ENPF-79). *Akanthomyces* spp. (ENPF-24) and *Beauveria* spp. (ENPF-16 & ENPF-16) showed similar lipolytic indices (3.3), followed by ENPF-41, while the lowest was observed in *Aspergillus* (ENPF-79) isolate. The enzyme activity index results highlighted variations in enzyme production among different isolates, even within the same genera (Figure 1).

The chitinase activity varied significantly among the sixteen isolates, with *Akanthomyces* (ENPF-24) showing the highest activity (0.53, 0.71, and 0.49 U/mL at 3rd, 6th, and 9th Days After Incubation (DAI), respectively), followed by *Beauveria* (ENPF-60). The lowest chitinolytic activity was recorded in *Aspergillus* spp. (ENPF-79). In terms of protease activity, all sixteen isolates exhibited activities in the range of 0.55 to 1.21 U mL^{-1}. *Beauveria* (ENPF-60) showed increased protease production (0.56, 1.57, and 1.49 U mL^{-1} at 3rd, 6th, and 9th DAI) followed by *Akanthomyces* (ENPF-24), while *Aspergillus* isolates had comparatively lower protease production (0.39-0.55 U/mL) than the other screened isolates. The same trend was observed for lipase activity, with *Beauveria* spp. (ENPF-16) producing the highest lipase activity

(1.27 U mL^{-1}), followed by ENPF-24 (1.20 U mL^{-1}) (Figure 1).

Lethal Concentrations and Lethal Times for EPF Isolates against Sucking Pests

The results of the probit analysis of dose mortality of aphids 7 days after treatment showed that the LC_{50} value for *Beauveria* isolate ENPF-16 was 1.8×10^6 and for *Beauveria* isolate ENPF-60 was 1.6×10^6. Meanwhile, the LC_{50} values for *Akanthomyces* isolates ENPF-24 and ENPF-41 were 6.2×10^6 and 9.4×10^5, respectively. Both *Beauveria* and *Akanthomyces* isolates showed high efficiency against aphids with low lethal concentrations. The lethal time to cause 50% mortality in aphids was determined at 1×10^7 conidia per mL, and the results uncovered that *Beauveria* isolate ENPF-60 had the shortest lethal time, causing 50% mortality in 4.21 days. *Akanthomyces lecanii* isolate ENPF-24 had a lethal time of 4.58 days. The highest lethal time was observed in *Beauveria* isolate ENPF-16, causing 50% mortality in 5.64 days (Table 2). The results revealed that higher conidial spore concentrations (10^7 to 10^{10} conidia mL^{-1}) were required to achieve 50 and 90% mortality in early days, compared to prolonged days of more than 10 days.

Probit analysis of whiteflies' lethal concentration at 7 days post-treatment showed no significant variation in LC_{50} values for both *Akanthomyces* and *Beauveria* isolates, with all four isolates falling within the $\times10^5$ range. However, on the 9th day, differences emerged, notably with *Akanthomyces* isolate ENPF-41 displaying a lower LC_{50} value (2.2×10^4). This suggests varying dose requirements (spore concentration) for mortality among entomopathogenic fungi of the same species. Survivorship analysis indicated that both *Akanthomyces* isolates achieved 50% mortality in approximately 5.5 days, faster than *Beauveria* isolates at 6.2 days (Table 2).

The probit analysis results on virulence of entomopathogenic fungi against 2nd instar larvae of thrips uncovered that *Akanthomyces* isolates were more effective in causing mortality of larvae of thrips at relatively lower conidial concentrations, with LC_{50} value $\times10^5$ at 7 days after treatment. Meanwhile, the LC_{50} values for *Beauveria* isolates were in the range of $\times10^6$ conidia per mL at 7 DAT. This trend was observed again at 9 days after treatment, with lower LC_{50}. The results of Kaplan-Meier survival curves analysis of lethal time (50% mortality) revealed that there was no significant difference between both *Akanthomyces lecanii* isolates at 7th day after treatment. The LT_{50} values for the *Akanthomyces* isolates were 5.51 and 5.95 days at a concentration of 1×10^7 conidia per mL. Meanwhile, *Beauveria* isolates took around 6 days to cause 50% mortality in thrips (Table 2).

Molecular Identification of Selected Entomopathogenic Fungal Isolates

The results of the phylogenetic tree analysis showed two broad distinct clusters for *Beauveria bassiana* isolates. The relationships obtained through pairwise sequence similarities were confirmed by the phylogenetic trees generated using different treeing methods. The partial 18S rDNA gene sequences of entomopathogenic fungal isolates ENPF-16 and ENPF-60 were identical to those of the species *Beauveria bassiana* with accession numbers MT997933 and MT997937, respectively. On the other hand, ENPF-24 and ENPF-41 were identified as *Akanthomyces lecanii* with accession numbers MT997934 and MT997935, respectively. The ENPF-16 and ENPF-60 isolates showed a similarity of nearly 93 and 98% to the *B. bassiana* strains Bb-9016 and IHBF14, respectively. Meanwhile, ENPF-24 and ENPF-41 isolates were found to be identical to *Akanthomyces lecanii* strains 6514 and 6543 with a similarity of over 90 % (Figure 2).

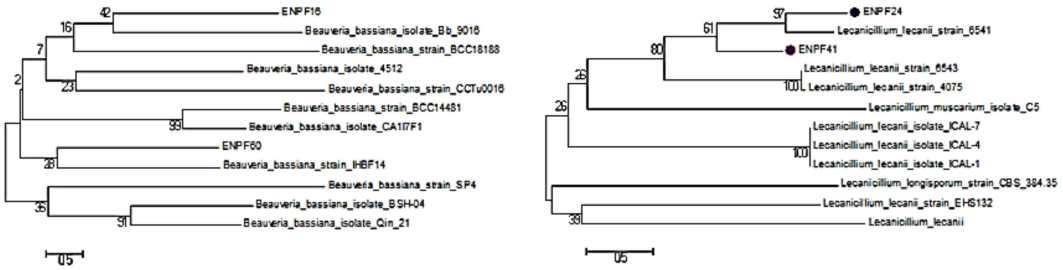

Figure 2. A. Phylogenetic placement of *Beauveria* isolates **B**. Phylogenetic placement of *Lecanicillium* isolates, based on nearly full-length 18S rRNA gene sequences. The tree was calculated with the Neighbour-joining method. Bootstrap values are based on 1,000 replicates. Numbers shown above branches are bootstrap percentages for clades supported above the 70% level. The bar indicates 5% sequence divergence.

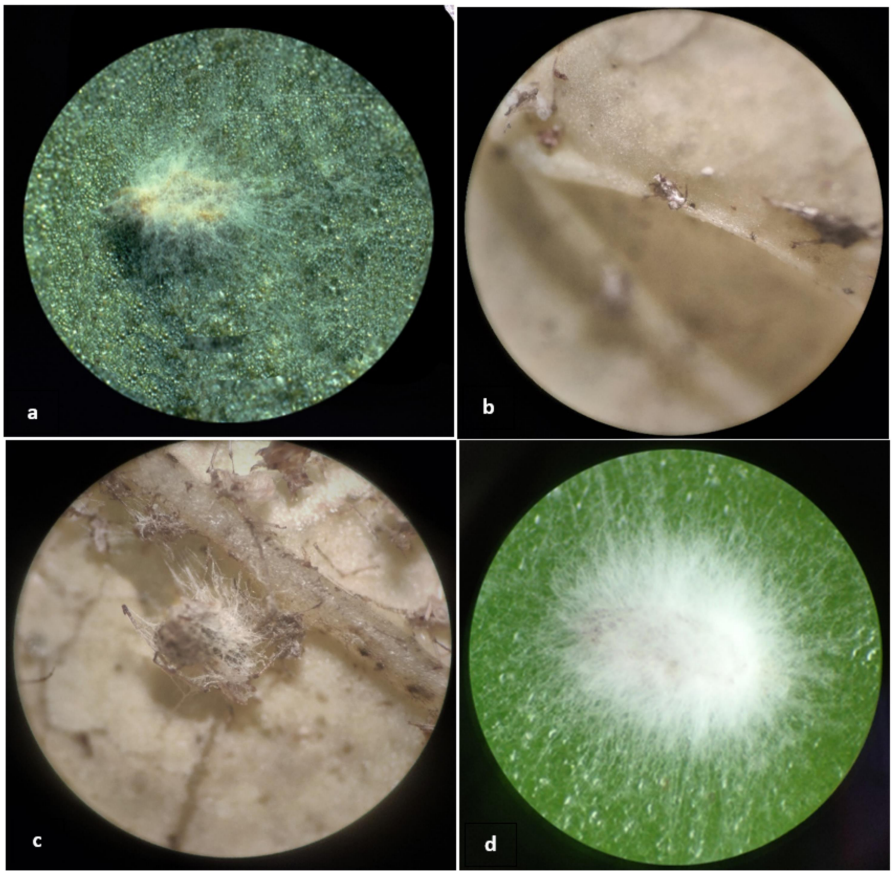

Figure 3. Mortality of various insect sucking insect pests caused by EPF isolates. ***a****: Akanthomyces sp.* infected cadaver of aphid. ***b****: Beauveria sp.* infected cadaver of thrips. ***c****: Akanthomyces sp.* infected cadaver of thrips. ***d****: Akanthomyces sp*. infected cadaver of whitefly.

Table 2. Virulence (LC_{50} and LT_{50}) of entomopathogenic fungal isolates to *M. persicae*, *B. tabaci* and *Frankliniella occidentalis*.[a]

Insect pest	Fungus	χ^2	LC_{50} (Conidia/mL)	95% CI		χ^2	LT_{50} (±SE) (Days)	95% CI	
				Lower	Upper			Lower	Upper
Aphids (*Myzus persicae*)	*B. bassiana* (ENPF-16)	1.20	1.8×10^6	2.6×10^5	3.7×10^7	0.78	5.47±0.1	4.47	6.57
	B. bassiana (ENPF 60)	2.54	1.6×10^6	3.3×10^5	4.9×10^7	1.28	5.06±0.2	3.98	6.02
	A. lecanii (ENPF-24)	0.68	9.0×10^6	1.5×10^6	7.8×10^7	0.69	4.59±0.1	3.17	5.76
	A. lecanii (ENPF-41)	1.24	9.4×10^5	1.0×10^5	2.3×10^7	0.20	4.83±0.1	3.59	5.83
Whitefly (*Bemisia tabaci*)	*B. bassiana* (ENPF-16)	3.23	6.5×10^5	4.8×10^4	7.3×10^6	2.26	6.46±0.2	5.12	7.56
	B. bassiana (ENPF 60)	2.46	8.4×10^5	6.2×10^4	1.2×10^7	1.55	6.18±0.1	5.06	7.38
	A. lecanii (ENPF-24)	1.67	6.3×10^5	4.4×10^4	7.4×10^6	0.87	5.78±0.1	4.57	6.69
	A. lecanii (ENPF-41)	2.14	1.5×10^5	9.3×10^3	6.3×10^6	0.95	5.89±0.1	4.71	6.83
Thrips (*Frankliniella occidentalis*)	*B. bassiana* (ENPF-16)	0.32	4.9×10^6	3.5×10^5	1.0×10^8	1.03	6.76±0.2	5.68	7.71
	B. bassiana (ENPF 60)	0.09	3.8×10^6	3.0×10^5	6.0×10^7	1.63	6.17±0.2	4.87	7.22
	A. lecanii (ENPF-24)	1.76	2.3×10^5	1.4×10^4	6.2×10^6	2.60	5.51±0.1	4.48	6.46
	A. lecanii (ENPF-41)	1.49	1.5×10^5	8.9×10^3	7.9×10^6	0.93	5.94±0.1	4.75	6.89

[a] The LC_{50} values were calculated by Probit analysis using IBM SPSS v 20.0 from the mortality data collected from 7 various conidia concentration at 7 days after treatment. LT_{50} values for mortality were estimated by survivorship analysis (Kaplan-Meier survival curves) with censored data for insects surviving >8d incubation period in bioassay studies and survival curves were compared using the log-rank test χ^2 (chi-square) value at $P \leq 0.05$. 95% CI: 95% Confidence Intervals that did not overlap indicate differences between LC_{50} and LT_{50} values.

DISCUSSION

Although all the fungal isolates were able to cause mortality in *Myzus persicae*, *Bemisia tabaci* and *Frankliniella occidentalis* the percent mortality varied significantly among the isolates. This might be due to various factors such as the higher efficiency or virulence of strains isolated from closely related hosts against the same insects (Vu *et al*., 2008), variations in the production of extracellular enzymes such as protease, chitinase, lipase, endoprotease, esterase, and carboxypeptidase, and the role of mycotoxins like beuvericin, bassianolidae, aphidiocolin (specific to aphids), and beauverolide (anti-immune activity) in causing mycosis (Kim *et al*., 2013; Safavi, 2013; Sayed *et al*., 2019). Other factors may include genetic virulence, conidia production and germination (Sani *et al*., 2020). The varying mortality rate among *Beauveria* and *Akanthomyces* isolates is intriguing. This variation could be due to the host specificity of the species, even though all four *Beauveria* isolates belong to the same genus there will be variation in the strain type. The host preference of the isolates might vary based on the species, or it could be attributed to differences in the production of extracellular enzymes is analyzed and published by several researchers like Senthil Kumar *et al*. (2015), Raheem and Keridis, (2017), Sayed *et al*. (2019), Zhang *et al*. (2019), Trinh *et al*. (2020), Arthurs *et al*. (2020), and Eski *et al*. (2022).

Insect cuticle is the foremost obstacle to pathogenesis. The entomopathogenic fungi must invade the cuticle barrier to successfully cause mycosis of insect pests (Mondal *et al*., 2016). Fascinatingly, results of exocellular enzymes activity by all six virulent entomopathogenic native isolates revealed that there was a variation in enzyme production of EPF isolates and even isolates belonging to the same species showed variation in enzyme production (Chitinase, Protease and lipase). The isolate ENPF-24 (*Akanthomyces lecanii*) has significantly high chitinase activity with lower protease and lipase activity, whereas ENPF-60 (*B. bassiana*) has significantly highest protease activity. The variation of enzyme activity of an isolate reveals that enzyme activity varies based on their native host, virulence and gene expression (Nahar *et al*., 2004). The quantitative studies on enzyme production of sixteen isolates revealed that the production of the enzymes was more on the 6th day after inoculation compared with the results on the 3rd and 9th days after inoculation. This was noticed by several researchers like De Moraes *et al*. (2003) and Bai *et al*. (2012). This might be because of degradation and denaturation of enzymes produced during later stages of growth, lack of nutrients, and accumulation of other secondary metabolites (Bai *et al*., 2012; Demir *et al*., 2013).

Enzyme production on the 6th day was compared to the highest insect pest mortality during *in vitro* virulence studies on the 7th day. The parallel relationship between enzyme production and insect mortality indicates that the enzyme activity of isolates significantly contributes to insect mycosis, along with other factors. Cuticle-degrading enzymes from EPF, specifically chitinolytic enzymes, play a crucial role in the pathogenicity of isolates (Dhar and Kaur, 2009). This correlation is consistent with previous studies on various insect pests, such as *Plagiodera versicolora* (Demir *et al*., 2013), *Pieris brassicae* (Dhawan and Joshi, 2017) and *Myzus persicae* (Khan *et al*., 2012), confirming the importance of cuticle-degrading enzyme production in causing insect mortality.

The low lethal concentration and lethal time observed in *Beauveria* and *Akanthomyces* isolates may be due to their specificity, growth rate, enzyme production rate, and conidial germination (Nazir *et al*., 2018; Trinh *et al*., 2020). This is because as the spore concentration increases, the number of conidia per square area also increases, increasing the likelihood of causing mycosis in the insect pest.

The results of the phylogenetic analysis indicate the presence of two major distinct clusters of *B. bassiana* isolates. The molecular characterization of entomopathogenic fungal isolates showed that ENPF-16 and ENPF-60 were identified as *Beauveria bassiana*, while ENPF-24 and ENPF-41 were identified as *Akanthomyces lecanii*. The results are consistent with the findings of previous studies (Ozcelik and Guven, 2015), which also identified *B. bassiana* and *Akanthomyces lecanii* as common entomopathogenic fungi in different regions. These findings provide valuable information on the distribution and identification of entomopathogenic fungi in different ecosystems, which is essential for the development of effective biocontrol strategies for insect pests.

CONCLUSIONS

The results imply that various entomopathogenic fungal isolates exhibit distinct levels of mortality in *Myzus persicae*, *Bemisia tabaci B-biotype,* and *Frankliniella occidentalis*. *Akanthomyces* isolates prove most virulent, causing significant mortality across all the three insect pests, particularly whiteflies. *Beauveria* isolates also demonstrate effectiveness against thrips and aphids. The correlation between enzyme production and insect mortality underscores the pivotal role of enzyme activity in fungal virulence. The concentration of conidial spores per milliliter significantly influences lethal concentration and time, with higher concentrations leading to increased mortality within a shorter timeframe. These findings align with prior research publications, underscoring the potential of *Akanthomyces* and *Beauveria* as biological control agents for whiteflies, thrips and aphids. Nonetheless, further research is necessary to fully comprehend the factors contributing to the variation in lethal concentration and time among different fungal isolates.

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بهره‌برداری از اثربخشی قارچ‌های بیمارگر حشرات علیه آفات رایج گلکاری: تمرکز بر شته‌ها (*Myzus persicae*)، مگس‌های سفید (*Bemisia tabaci* B-biotype) و تریپس گل غربی (*Frankliniella occidentalis*)

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چکیده

این مطالعه اثربخشی قارچ‌های بیمارگر حشرات (EPF) را به عنوان عوامل کنترل زیستی علیه شته‌ها، مگس‌های سفید و تریپس گل غربی ارزیابی کرد. در این تحقیق از روش زیست‌سنجی دیسک برگ با غلظت‌های مختلف کونیدی برای تعیین غلظت کشنده (LC) و زمان (LT) برای ریشه‌کنی آفات استفاده شد. علاوه بر این، این مطالعه فعالیت آنزیم‌های تجزیه‌کننده کوتیکول تولید شده توسط EPF (کیتیناز، پروتئاز و

لپیاز) را برای درک مکانیسم‌های بیماری‌زایی آنها ارزیابی کرد. شناسایی مولکولی با استفاده از ناحیه ITS از rDNA 18S، جدایه‌های بیماری‌زا را شناسایی کرد. نتایج نشان داد که چهار جدایه، یعنی ENPF-16، ۲٤، ٤١ و ٦٠، پس از نه روز، میزان مرگ و میر قابل توجهی (۹۵ تا ۱۰۰٪) در غلظت ۱ x108کونیدی در میلی‌لیتر داشتند. گونه Akanthomyces (ENPF-41) بالاترین فعالیت آنزیمی را نشان داد و پس از آن گونه Beauveria (ENPF-60)قرار گرفت. جدایه‌های قارچی بیماری‌زا به عنوان Beauveria bassiana و Akanthomyces lecaniiشناسایی شدند. در میانEPF ها، Akanthomyces lecanii (MT997935) در مقایسه با سایرEPF ها، بیماری‌زایی بیشتری علیه هر سه حشره مورد آزمایش با مقادیر LC50 و LT50 پایین‌تر نشان داد. به طور خلاصه، همه جدایه‌های قارچی باعث مرگ و میر در آفات مورد آزمایش شدند، اما اثربخشی آنها متفاوت بود. Akanthomyces lecanii (MT997935) به دلیل طیف میزبانی وسیع و بیماری‌زایی قوی، به عنوان یک کاندیدای امیدوارکننده برای کنترل زیستی پدیدارشد.