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# Assessment of the Biocontrol Potential of Entomopathogenic Fungi against The Red Spider Mite *Tetranychus urticae*

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**Abstract**

Entomopathogenic fungi (EPF) are the most versatile and potential biocontrol agents, due to their adaptability, mode of entry, persistence nature and wide host range. The present study aimed to isolate, identify, and evaluate the virulence of entomopathogenic fungi (EPF) for their potential as biocontrol agents against red spider mites (*Tetranychus urticae*). Out of 81 fungal isolates, 16 (19.25%) showed insecticidal activity under *in vitro* conditions. The results revealed that among 16 isolates, ENPF-8 and ENPF-58 had significantly higher mortality rates (93-95 %) at a concentration of $1x10^8$ conidia/ml after 9 days after treatment (DAT). The virulent fungal isolates were identified as *Isaria fumosorosea* (Gene bank accession number: MT997932) and *Hirsutella thompsonii* (Gene bank accession number: MT997936) by sequencing the ITS region of the 18S rRNA gene. *H. thompsonii* (ENPF-58) was found to be more virulent against test insects, with lower $LC_{50}$ ($7.6\times10^5$ conidia/ml) and $LT_{50}$ (5.7, 5.89 and 5.99 days) values compared to the other EPF. Based on the results, *H. thompsonii* could be a promising biocontrol agent with a wide host range and high virulence as tested in other experiments.

*Keywords* : Biocontrol pofential, Entomopathogenic fungi, Sucking pest, Red spider mite, Leaf disc bioassay, LC50

Sucking pests, also known as sap-sucking insects, include aphids, thrips, whiteflies, mites and leafhoppers. These pests are called 'sucking' pests because of their piercing and sucking mouth parts, which they use to extract sap from host plants. As a result of this sap removal, the affected plants become stunted in growth, distorted in appearance and lose chlorophyll content, leading to reduced strength and premature leaf loss. In addition to transmitting diseases, some of these pests also inject toxic substances into the host plants while feeding, resulting in significant agricultural losses (15-45%) and increased management costs.

The Red Spider Mite (*Tetranychus urticae*) is a severe pest in tropical regions of the world. The pest causes economic damage in most of the crops across the world. The pest shows resurgence to pyrethroid pesticides and it creates resistance quickly when pesticides are frequently used. Integrated pest management is necessary to manage the severity of the pest in different crop ecosystems. In recent times, the need for alternative, sustainable and eco-friendly pest management techniques in agriculture have become increasingly important due to the decline in global crop losses. Despite the use of approximately 2.5 million tonnes of pesticides annually, crop losses due to pests have only decreased from 41.1 per cent during 1988-90 to 32.1 per cent during 2001-03 (Dhaliwal *et al*., 2015). The use of pesticides has led to various problems, including the development of resistance and resurgence of sucking pests (Sharma

*et al*., 2019) 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.

Microbial biocontrol agents (MBCAs) have gained popularity as an alternative to chemical pesticides in controlling insect pests. These natural enemies are effective in reducing pest populations without posing a risk to human and environmental health. This method of biological control has been adopted globally for controlling both field and forest insect pests due to its persistence, mode of action, cost-effectiveness, non-polluting characteristics and compatibility with chemical pesticides (Ramanujam *et al*., 2014). Fungi, viruses and bacteria are the most commonly used microbial agents in field trials, while Rickettsia, protozoa and nematodes have seen limited use due to their dependence on environmental conditions or difficulty in application (Shahid *et al*., 2012).

Entomopathogenic fungi 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 (Nadeau *et al*., 1996). 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 they have a 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). Among the numerous types of fungi. The fungi that can invade dead insects are called saprophagous and fungi that infect living insects are called entomophagous (Butt *et al*., 2006). Of the estimated 1.5 to 5.1 million species of fungi in the world, approximately 750 to 1,000 are considered entomopathogens placed in over 100 genera many of these have great significance in insect pest management (Charnley and Leger, 2010). The most common and extensively studied entomopathogens are *Metarhizium* spp. *Beauveria* spp. *Nomuraea rileyi*, *Lecanicillium* spp. *Paecilomyces* and *Hirsutella* spp. (Kachhawa, 2017, Wraight *et al*., 2007 and Lacey *et al*., 2008). The *Metarhizium* spp. and *Beauveria* spp. have been extensively used to combat different sucking pests under both greenhouse and field conditions. However, the success of biological control depends on the environmental conditions, such as high relative humidity, moderate temperatures and soil organic matter (Fargues *et al.,* 1997; Braga *et al*., 2001; Van, 2007 and Sabbahi *et al.,* 2008).

Several researchers studied and evaluated the different entomopathogenic fungi for the control of different sap sucking insect pests of agriculture (Lacey *et al*., 2008) provided most satisfying results and evidences in many crops. The present study aimed to isolate and identify the virulent native entomopathogenic fungal strains from soil and insect cadaver samples collected from various regions of South Karnataka, India. This was done to assess their potential for biocontrol against the sap sucking insects red spider mites (*Tetranychus urticae*) in flower crops. Despite extensive research on the topic in India, limited information exists on the biocontrol of these pests. Screening of local fungal isolates for their virulence characteristics is crucial for the success of biocontrol strategies. Conservation and periodic improvement of the efficacy of these biological control agents will significantly aid in crop protection and help produce pesticide residue-free agricultural commodities, reducing the usage of pesticides in agriculture.

## Material and Methods

### Entomopathogenic Fungal Isolates

A systemic survey was conducted to collect soil and mummified insect samples from different locations of agro-climatic zones (eastern dry zoon 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. The fungal isolates were

isolated from insect cadavers as method described by Rani *et al*., 2015 and also from soil sample by serial dilution and plating technique and Insect bait technique. The spore suspension of isolates was prepared by adding 10ml 0.5 per cent sterile tween 80 to 10 days old cultures and various concentration of conidial suspension was prepared by serial dilution. The conidial count was determined using an improved Neubauer Hemocytometer. Further the 81 isolates were tested for their virulence against test insect using the leaf dip bioassay method as described by Sain *et al.* (2019).

**Virulence of Entomopathogenic Fungal Isolates**

The leaf disc bioassay method was performed following the protocol by Nazir *et al*. (2018). Healthy gerbera leaves were used to obtain 8 cm diameter leaf discs, which were later surface sterilized with 70 per cent alcohol. The leaf discs were separately immersed in fungal spore suspension of concentrations $1\times10^8$ conidia per mL for 10 seconds, and a control was maintained by dipping the leaf discs in sterile distilled water. All the leaf discs were air dried to remove excess moisture and transferred onto sterile Petri plates containing filter paper to maintain humidity during incubation. Three replications for each treatment were maintained throughout the experiment (Fig. 1a). Twenty laboratory-reared mites were placed onto the treated and control leaf discs using a sterile camel brush. The complete setup was then incubated at 25±1ºC. The plates were observed at two-day intervals from 3rd day after treatment (DAT) up to 9 DAT for mortality of test insect pests. Dead insects were collected from leaf discs and transferred onto PDA plates, which were then incubated at 25±1ºC with 90 per cent relative humidity to promote fungal development and sporulation to confirm that the death of test insects was caused by infection from individual fungal isolates.

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

The screened fungal isolates which were showed high virulence against the sucking insect pests of gerbera, including mites were subjected to further testing to determine their lethal concentration and lethal time. This was done at six different conidia concentrations ($1\times10^4$, $1\times10^5$, $1\times10^6$, $1\times10^7$, $1\times10^8$ and $1\times10^9$ conidia per mL) using the leaf disc bioassay method (Trinh *et al*., 2020).

**Molecular Identification of Virulent Entomopathogenic Fungal Isolates**

The molecular identification of the virulent entomopathogenic fungal isolates was an important step in the study, as it allowed to accurately identify the fungal species and determine their relationships with other known fungal species. To identify the efficient entomopathogenic fungal isolates at the species and strain level, the 18S rDNA gene was isolated, amplified and sequenced from all six isolates that exhibited higher mortality of test insects under *in-vitro* studies.

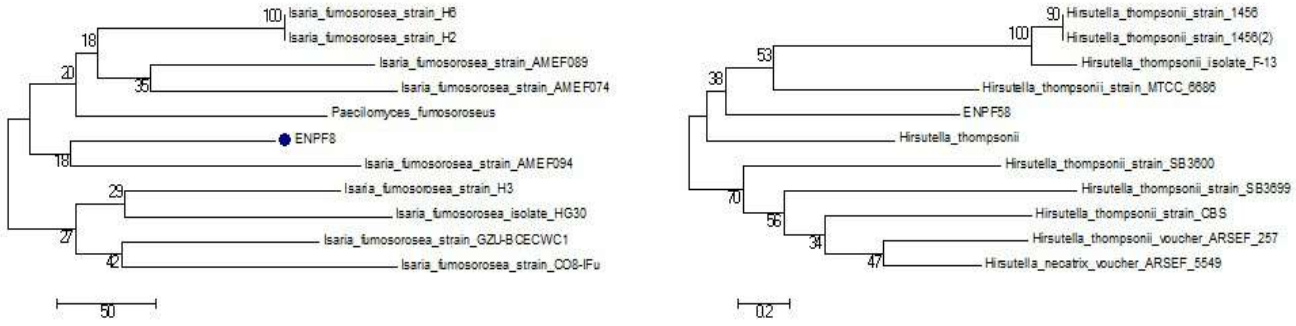


Fig. 1 : Phylogenetic placement of *Isaria* and *Hirsutella* isolate based on nearly full-length 18S rRNA gene sequences. The tree was calculated with the Neighbour-joining method. Bootstrap values are based on 1,000 replicates. Numbers shown above branches are bootstrap percentages for clades supported above the 70 % level. The bar indicates 5 % sequence divergence.

**DNA Extraction**

The mycelia of entomopathogenic fungal cultures were inoculated to potato dextrose agar and incubated for 10-15 days, depending on the growth of the organisms. After incubation, the mycelia were collected by scraping the fungal mat using a sterile glass slide. The DNA extraction method was standardized and certain steps were optimized to obtain a good concentration of DNA using the cetyl trimethyl ammonium bromide (CTAB) extraction buffer (Doyle & Doyle, 1987), followed by purification through phenol / chloroform extraction and precipitation with isopropanol or ethanol (Ashktorab and Cohen, 1992).

The ITS1 and ITS2 rDNA regions were amplified using universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'), each at a concentration of 0.5 µl. The amplification reaction included 5 µl of sample DNA as template, 1 µl of 1X Buffer, 1 µl of dNTP mix, 0.25 µl of DNA polymerase, and 13.25 µl of ultrapure water (Saito *et al*., 2012). The thermocycler was used to perform the reaction. The PCR conditions were: initial denaturation for 5 minutes at 94 °C, followed by 35 cycles of denaturation (30 seconds at 94 °C), annealing (30 seconds at 58 °C) and extension (1 minute at 72 °C). The final extension step was 7 minutes at 72 °C. The amplified DNA products were sequenced through outsourcing. The initial identification of the strain was performed using BLASTN against the EzTaxon-e database, which contains all 18s rRNA gene sequences of type strains with validly published eukaryotic names. Further, detailed phylogenetic analyses were conducted in MEGA 6.06 based on partial 18S rRNA gene sequences. The partial 18S rRNA gene sequence of ENPF isolates was aligned with the type strain sequences of the respective genera. Sequence similarities were calculated using the neighbour-joining tool without applying an evolutionary model. Phylogenetic trees were reconstructed using the maximum-parsimony method, the neighbour-joining method and the Jukes-Cantor correction. All trees were based on partial 18S rRNA gene sequences and were resampled 100 times using bootstrap analysis.

**Statistical Analysis**

The data obtained from the results was analysed 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, 2021) and the means were compared using post-hoc test (Duncan's multiple range test) at a 5 per cent level. The lethal concentration ($LC_{50/90}$) and median lethal time ($LT_{50}$) were used as relative measures of the susceptibility of the host population

a. Leaf disc bioassay

b. *H. thompsonii* infected cadaver of mite

Fig. 2 : a. Laboratory set up of the leaf disc bioassay experimtents under *in vitro*
b. *H. thompsonii* infected cadaver of mite under 4x magnification

and are convenient and commonly used indices for evaluating the efficacy of biological control agents. The $LC_{50}$ 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 per cent confidence intervals (CIs) did not overlap with the CIs of other isolates. $LT_{50}$ values for mortality were estimated by survivorship analysis (Kaplan-Meier survival curves) using IBM SPSS v 20.0 with censored data for insects surviving more than 8 days of incubation period in bioassay studies and survival curves were compared using the log-rank test $\chi 2$ (chi-square) value at $P \leq 0.05$.

## Results and Discussion

### Virulence of Entomopathogenic Fungal Isolates

The results of the virulence assay on mites exhibited diverse outcomes in terms of the mortality caused by different isolates over time. All the entomopathogenic isolates successfully caused mycosis, starting from the third day after treatment (3 DAT) to the ninth day after treatment (9 DAT). The results of virulence studies on mites revealed that only *Hirsutella* and *Isaria* fungal isolates showed significantly higher mortality, starting from 3 DAT to 9 DAT. *Hirsutella* isolate (ENPF-58) caused significantly higher per cent mortality of mites, 17.78, 46.67, 73.33 and 95.53 per cent at 3, 5, 7 and 9 days after treatment, followed by *Isaria* (ENPF-8) isolate, which caused 15.56, 40, 68.89 and 93.33 per cent mortality at 3rd, 5th, 7th and 9th DAT respectively (Fig. 2b).

On the third day after treatment, many of the fungal isolates failed to cause mortality in mites, among sixteen isolates the significantly the highest mortality of mites is caused by *Hirsutella* sp. (17.78%) followed by *Isaria* sp. (15.56%). On the fifth and seventh day after treatment, the mortality rate of mites was

Table 1

Virulence of entomopathogenic fungal isolates on per cent cumulative mortality of red spider mites (*Tetranychus urticae*) under *in vitro* conditions.

| Treatments | Isolates | Per cent cumulative mortality (DAT*) | | | |
|---|---|---|---|---|---|
| | | 3 DAT | 5 DAT | 9 DAT | 7 DAT |
| $T_1$ | *Beauveria* sp.(ENPF-3) | 0.00 (0.74) $^{c}$ | 6.67 (14.66) $^{e}$ | 15.56 (22.67) $^{ef}$ | 33.33 (35.21) $^{e}$ |
| $T_2$ | *Metarhizium* sp.(ENPF-6) | 0.00 (0.74) $^{c}$ | 10.00 (18.00) $^{d}$ | 24.44 (29.33) $^{cd}$ | 43.33 (41.23) $^{d}$ |
| $T_3$ | *Isaria* sp.(ENPF-8) | 15.56 (22.94) $^{a}$ | 40.00 (38.33) $^{b}$ | 68.89 (55.21) $^{ab}$ | 93.33 (75.23) $^{a}$ |
| $T_4$ | *Metarhizium* sp.(ENPF-9) | 2.22 (5.60) $^{bc}$ | 13.33 (20.67) $^{c}$ | 26.67 (30.67) $^{cd}$ | 40.00 (39.54) $^{d}$ |
| $T_5$ | *Beauveria* sp.(ENPF-16) | 0.00 (0.74) $^{c}$ | 10.00 (18.00) $^{d}$ | 33.33 (33.67) $^{c}$ | 63.67 (49.23) $^{d}$ |
| $T_6$ | *Lecanicillium* sp.(ENPF-24) | 4.44 (10.47) $^{b}$ | 13.33 (20.67) $^{c}$ | 26.67 (30.67) $^{cd}$ | 43.33 (41.23) $^{d}$ |
| $T_7$ | *Aspergillus* sp.(ENPF-26) | 0.00 (0.74) $^{c}$ | 3.33 (10.33) $^{f}$ | 17.78 (24.33) $^{ef}$ | 40.00 (39.54) $^{d}$ |
| $T_8$ | *Aspergillus* sp.(ENPF-33) | 0.00 (0.74) $^{c}$ | 3.33 (10.33) $^{f}$ | 15.56 (22.67) $^{ef}$ | 33.33 (35.21) $^{e}$ |
| $T_9$ | *Lecanicillium* sp.(ENPF-41) | 6.67 (12.40) $^{b}$ | 10.00 (18.00) $^{d}$ | 46.67 (42.33) $^{b}$ | 68.33 (54.54) $^{b}$ |
| $T_{10}$ | *Beauveria* sp.(ENPF-48) | 2.22 (5.60) $^{bc}$ | 6.67 (14.66) $^{e}$ | 15.56 (22.67) $^{ef}$ | 43.33 (41.23) $^{b}$ |
| $T_{11}$ | *Aspergillus* sp.(ENPF-53) | 0.00 (0.74) $^{c}$ | 0.00 (0.74) $^{g}$ | 13.33 (20.67) $^{f}$ | 26.67 (31.27) $^{f}$ |
| $T_{12}$ | *Hirsutella* sp.(ENPF-58) | 17.78 (24.75) $^{a}$ | 46.67 (42.33) $^{a}$ | 73.33 (58.00) $^{a}$ | 95.53 (78.00) $^{a}$ |
| $T_{13}$ | *Beauveria* sp.(ENPF-60) | 2.22 (0.74) $^{d}$ | 6.67 (14.66) $^{e}$ | 15.56 (22.67) $^{ef}$ | 43.33 (41.23) $^{d}$ |
| $T_{14}$ | *Metarhizium* sp.(ENPF-67) | 0.00 (0.74) $^{c}$ | 11.11 (19.32) $^{cd}$ | 28.89 (32.45) $^{cd}$ | 40.00 (39.54) $^{d}$ |
| $T_{15}$ | *Metarhizium* sp.(ENPF-68) | 2.22 (5.60) $^{bc}$ | 13.33 (20.67) $^{c}$ | 31.11 (33.67) $^{c}$ | 43.33 (41.23) $^{d}$ |
| $T_{16}$ | *Aspergillus* sp.(ENPF-79) | 0.00 (0.74) $^{c}$ | 4.44 (12.23) $^{f}$ | 13.33 (20.67) $^{f}$ | 33.33 (35.21) $^{e}$ |

*Note* : *DAT : Days After Treatment; Values in the parentheses are ARCSINE transferred values; The values represented by same letters in each column are statistically on par with each other by DMRT mean of three replications at 95 % confidence interval (CI)

significantly increased in all isolates. *Hirsutella* sp. and *Isaria* sp. caused pronounced mortality ranging from 65-85 per cent on the fifth to seventh day after treatment. Among the *Beauveria* isolates, ENPF-16 caused the highest per cent mortality (10 and 33.33 per cent) on 5th and 7th days after treatment. Whereas, among 4 *Metarhizium* isolates, 13.33 and 31.11 per cent mortality was caused by ENPF-68. Out of 4 *Aspergillus* isolates, ENPF-26 caused 3.33 and 17.78 per cent mortality of mites on 5th and 7th day after treatment respectively. The statistically highest (95.33 and 93.33%) mortality was observed in the leaf discs dipped in the spore suspension of *Hirsutella* isolate ENPF-58 and *Isaria* isolate ENPF-8 on the ninth day after treatment (Table 1). All *Metarhizium* and *Aspergillus* isolates were not effective in causing mortality (30-60% on nine days after treatment).

Although all the fungal isolates were able to cause mortality of mites under *in vitro* the per cent 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*. in 2013; Sayed *et al*., 2019). Other factors may include genetic virulence, conidia production and germination (Sani *et al*., 2020). The only two isolates caused significantly high mortality, which may be because mites, although arthropods, do not belong to the insect class and specific secondary metabolites such as Hirsutellin-A produced by *Hirsutella* spp. inhibit ribosomal activity and fumosorinone produced by *I. fumosorosea* inhibits calmodulins activity.

The varying mortality rate among *Beauveria* and *Lecanicillium* 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. 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 (Sayed *et al*., 2019; Marquez-Gutierrez *et al*., 2022). Rachana *et al*. (2009) reported that the treatment with *H. thompsonii* at $4.6x10^8$ spores /mL resulted in the highest recorded mortality of 78.20 per cent, followed by treatment with *H. thompsonii* at $4.6x10^8$ spores / mL + dicofol at 0.025 %. This treatment caused 81.21 per cent mortality. *F. semitectum* at $2.1x10^9$ spores / mL + *H. thompsonii* at $4.6x10^8$ spores /mL also resulted in high mortality, with a recorded rate of 81.48 per cent at 15 days after spray in greenhouse conditions against the red spider mite *T. neocaledonicus* on okra in Shimoga. Tamai *et al*. (2002), Seiedy *et al.* (2015) also confirmed similar results.

TABLE 2

Dose mortality responses of mites (*Tetranychus urticae*) to entomopathogenic fungal isolates under *in vitro* conditions.

| DAT | Isolates | $\chi^2$ | $LC_{50}$ [1] (Conidia/mL) | 95% CI[2] | | $LC_{90}$ (Conidia/mL) | 95% CI | |
|---|---|---|---|---|---|---|---|---|
| | | | | Lower | Upper | | Lower | Upper |
| 7 | *Isaria* sp. (ENPF-8) | 1.20 | $3.5\times10^{6}$ | $2.9\times10^{5}$ | $6.7\times10^{7}$ | $8.3\times10^{8}$ | $1.6\times10^{7}$ | $2.6\times10^{9}$ |
| | *Hirsutella* sp. (ENPF-58) | 1.54 | $7.6\times10^{5}$ | $6.3\times10^{4}$ | $1.4\times10^{7}$ | $1.3\times10^{8}$ | $9.6\times10^{6}$ | $9.6\times10^{8}$ |
| 9 | *Isaria* sp. (ENPF-8) | 0.68 | $2.4\times10^{5}$ | $1.3\times10^{4}$ | $3.6\times10^{6}$ | $3.6\times10^{7}$ | $5.4\times10^{6}$ | $6.7\times10^{8}$ |
| | *Hirsutella* sp. (ENPF-58) | 1.24 | $8.5\times10^{4}$ | $8.3\times10^{3}$ | $1.1\times10^{6}$ | $9.1\times10^{6}$ | $1.1\times10^{6}$ | $1.1\times10^{8}$ |

*Note* : The $LT_{50}$[1] 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. [2]95 % Confidence intervals that did not overlap indicate differences between $LC_{50}$ and $LT_{50}$ values

**Mortality Responses of Sucking Pests to Lethal Concentration ($LC_{50}$) and Lethal Time ($LT_{50}$)**

The results of the virulence studies under *in vitro* conditions showed that *Isaria* sp. (ENPF-8) and *Hirsutella* sp. (ENPF-58) showed significant mortality of mites in virulence studies under *in vitro* conditions. The $LC_{50}$ and $LC_{90}$ values for *Isaria* sp. were 3.5×106 and 8.3×109 and for *Hirsutella* sp. were $7.6\times10^5$ and $1.3\times10^9$, respectively, on the 7th day after treatment. The $LC_{50}$ and $LC_{90}$ values were lower on the 9$^{th}$ day after treatment. The $LC_{50}$ and $LC_{90}$ for Isaria and *Hirsutella* isolates were $2.4\times10^5$, $8.5\times10^4$ and $3.6\times10^8$, $9.1\times10^7$, respectively. The results of lethal time (50 and 90 per cent mortality) showed that the $LT_{50}$ and $LT_{90}$ values for *Isaria* isolate were higher (6.19 days and 11.03 days, respectively) compared with *Hirsutella* isolate (5.99 days and 11.12 days, respectively).

Overall, the *Hirsutella* isolate was found to be more effective than the *Isaria* isolate in causing mite mortality. A higher concentration of conidial spores ($10^{10}$ spores/mL) was required to cause mortality of mites at early stages compared to later stages (more than 9 days). 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 table 2 and 3 present the results of lethal concentration and lethal time (LC and LT). Variation in lethal concentration and time by isolates may be because of specificity, growth rate, enzyme production rate and even conidial germination. Similar results were obtained by Van *et al*., 2007, Nazir *et al.,* 2018 and Trinh *et al.,* 2020. In 2015, El-Sharabasy conducted a laboratory study to assess the effectiveness of entomopathogenic fungi *Hirsutella thompsonii* (Fisher) and *Paecilomyces fumosoroseus* against all stages of citrus mites. Leaf discs containing larvae, nymphs, adults and eggs were sprayed with different concentrations of conidia. The results showed that all stages were susceptible to both fungal pathogens, but *H. thompsonii* exhibited greater virulence, with $LC_{50}$ values of $3.5\times10^8$, $2.9\times10^7$ and $1.4\times10^6$ conidia/mL and $LT_{50}$ values of 7.78, 7.11 and 6.92 days for larvae, nymphs and adults, respectively. *P. fumosoroseus* had $LC_{50}$ values of $5.9\times10^7$, $5.8\times10^9$, and $3.3\times10^9$ conidia/mL and $LT_{50}$ values of 7.49, 7.14 and 4.31 days for the same stages. *H. thompsonii* was more effective against eggs at its $LC_{50}$ value. Similar results were obtained by Fiedler *et al*. (2002), Tamai *et al*. (2002) and Rachana *et al*. (2009) during their studies on mites.

**Molecular Identification of Selected Entomopathogenic Fungal Isolates**

The molecular identification of the virulent entomopathogenic fungal isolates was an important step in the study, as it allowed to accurately identify the fungal species and determine their relationships with other known fungal species. The results of partially sequencing the 18S rRNA gene of fungal isolates contained more than 530 nucleotide bases, which is sufficient to identify the organisms by blasting the sequence in the NCBI database. The results of the phylogenetic analysis indicate the presence of two major distinct clusters of isolates. The

TABLE 3

Estimation of lethal time mortality responses of entomopathogenic fungal isolates against mites (*Tetranychus urticae*) under *in vitro* conditions

| Isolates | $\chi^2$ | $LT_{50}$[1] (±SE) (Days) | 95% CI[2] | | $LT_{90}$ (±SE) (Days) | 95% CI | |
|---|---|---|---|---|---|---|---|
| | | | Lower | Upper | | Lower | Upper |
| *Isaria* sp. (ENPF-8) | 1.10 | 6.19±0.2 | 5.06 | 7.21 | 11.83±0.4 | 10.65 | 12.93 |
| *Hirsutella* sp. (ENPF-58) | 1.47 | 5.99±0.1 | 5.01 | 7.16 | 11.12±0.6 | 10.48 | 12.72 |

*Note* : [1]LT50 values for mortality were estimated by survivorship analysis (Kaplan-Meier survival curves) using IBM SPSS v 20.0 with censored data for insects surviving >8d incubation period in bioassay studies and survival curves were compared using the log-rank test $\chi2$ (chi-square) value at P=0.05. [2]95 % Confidence intervals that did not overlap indicate differences between $LC_{50}$ and $LT_{50}$ values



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relationships obtained through pairwise sequence similarities were confirmed by the phylogenetic trees generated using different treeing methods.

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 rRNA gene sequences of entomopathogenic fungal isolates ENPF-8 was identical to those of the species *Isaria fumosorosea* with accession numbers MT997932. On the other hand, ENPF-58 was identified as *Hirsutella thompsonii* with accession numbers MT997936. The ENPF-8 and ENPF-58 isolates showed a similarity of nearly 93 and 97 per cent to the *I. fumosorosea* strain_AMEP094 & *H. thompsonii* strain MTCC_6686 respectively (Fig. 1).

It can be inferred from the results that the different entomopathogenic fungal isolates caused varied levels of mortality red spider mites (*Tetranychus urticae*). *Hirsutella thompsonii* and *Isaria fumosorosea* were found to be the most virulent and were successful in causing the significant mortality in insect pests. *Beauveria* and *Lecanicillium* isolates showed moderate mortality, while *Aspergillus* and *Metarhizium* isolates were not as effective in causing mortality. The concentration of conidial spores per milliliter has a significant impact on the lethal concentration and time of the fungi, with higher concentrations resulting in higher mortality rates in a shorter time frame. The results of these studies are consistent with previous research and highlight the potential of *Hirsutella* and *Isaria* as biological control agents for mites. However, more research is needed to fully understand the factors that contribute to the variation in lethal concentration and time among different fungal isolates.

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