Original Research Article

Assessment of Entomopathogenic Fungi for the Biocontrol of Sucking Insect Pests: Pertaining to Red Spider Mites (*Tetranychus urticae*)

ABSTRACT

Aim: The study was undertaken to assess the efficiency and select the virulent entomopathogenic fungal isolates for the biological control of red spider mites (*Tetranychus urticae*) under *in vitro* conditions.

Place and duration of work: The study was carried out in the Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bangalore, India.

Methodology: A leaf disc bioassay was employed to assess the virulence of entomopathogenic fungal isolates isolated from 2 agro-climatic zones of Karnataka, India under *in vitro* conditions. The lethal concentration and lethal time were determined by obtaining the mortality data from various conidial concentrations and the data was analysed using Probit analysis. For ~~the~~ molecular identification the fungal DNA was isolated through Cetyl trimethyl ammonium bromide (CTAB) extraction buffer, followed by purification through phenol/chloroform extraction and precipitation with isopropanol or ethanol, and the ITS regions of the 18S rDNA were amplificated using universal primers.

Results: Out of 81 fungal isolates, 16 (19.25%) showed insecticidal activity under *in vitro* conditions. The results of the *in vitro* studies revealed that two isolates, ENPF-8 and ENPF-58 had ~~the~~ significantly higher mortality rates (93-95 %) against mites at a concentration of $1x10^8$ conidia/ml after 9 days after treatment (DAT). The virulent fungal isolates were identified as *Isaria fumosorosea* (with accession no: MT997932) and *Hirsutella thompsonii* (MT997936) using the sequencing of the ITS region of the 18S rDNA gene. *H. thompsonii* (ENPF-58) was found to be more virulent against mites, with lower LC_{50} (7.6×10^5 conidia/ml) and LT_{50} (5.7, 5.89, and 5.99 days) values compared to the other EPF.

Conclusion: Based on results, it is evidenced that though all isolates caused mortality in the mite population but the percent mortality was varied. Based on these results, *H. thompsonii* (MT997935) and *Isaria fumosorosea* (MT997932) could be a promising biocontrol agent due to their high virulence against mites.

Keywords: Biological control, Sucking pest, Leaf disc bioassay, LC_{50}, *Hirsutella* sp.

1. INTRODUCTION

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.

In recent times, the need for alternative, sustainable, and eco-friendly pest management techniques in agriculture has 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% during 1988-90 to 32.1 % during 2001-03 (Dhaliwal *et al*., 2015). The use of pesticides has led to various problems, ~~includes~~ including the development of resistance and resurgence of sucking pests (Sharma *et al*., 20l9) 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~~ finding a sustainable and environmentally friendly pest management solution is essential. 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 the application (Shahid *et al*., 2012).

Entomopathogenic fungi are potentially the most diverse and versatile biological control agents due to their wide host range, ~~that~~which ~~often~~ resulting ~~s~~ in natural epizootics. An attractive feature of these fungi is that they infect by contact and act through penetration (Nadeau and Boisvert, 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 of 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; Vu *et al.,* 2008; Sabbahi *et al*., 2008).

Several researchers studied and evaluated the different entomopathogenic fungi for the control of different ~~sap sucking~~sap-sucking insect pests of agriculture (Lacey *et al*., 2008) ~~provided~~ providing the most satisfying results and ~~evidences~~ evidence 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~~sap-sucking insects like mites 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.

2. MATERIALS AND METHODS

2.1 Entomopathogenic fungal isolates

The fungal isolates were isolated from two agro-climatic zones (eastern dry zoon and southern dry zone) of Karnataka, India. The spore suspension of isolates was prepared by adding 10ml 0.5 % sterile tween 80 to 10 days old cultures and various concentrations of conidial suspension ~~was~~ were prepared by serial dilution. The conidial count was determined using an improved Neubauer Hemocytometer.

2.2 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 percent alcohol. The leaf discs were separately immersed in fungal spore suspension of concentrations 1×10^8 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. Twenty laboratory-reared red spider mites were placed onto the treated and control leaf discs using a sterile camel brush. The complete setup was then incubated at 25±1 ºC. The plates were observed at two-day intervals from 3 days 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±1 ºC with 90 percent relative humidity to promote fungal development and sporulation to confirm that the death of test insects was caused by infection from individual fungal isolates.

2.3 Determination of lethal concentration (LC_{50}) and Lethal time (LT_{50})

The screened fungal isolates which were showed high virulence against the mites were subjected to further testing to determine their lethal concentration and lethal time. 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 the leaf disc bioassay method (Trinh *et al*., 2020).

2.4 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 us 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 rRNA gene was isolated, amplified, and sequenced from all six isolates that exhibited higher mortality of test insects under *in-vitro* studies.

2.4.1 DNA extraction

The mycelia of entomopathogenic fungal cultures were inoculated onto 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 a 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.

2.5 Statistical analysis~~:~~

The data obtained from the results ~~was~~ were 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, 2022), and the means were compared using a post-hoc test (Duncan's multiple range test) at a 5 percent 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 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 percent 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 $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 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 ~~days~~ 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~~ isolates (ENPF-58) caused significantly higher percent mortality of mites, 17.78, 46.67, 73.33, and 95.53 percent 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 percent mortality at 3, 5, 7 and 9 DAT respectively (Table 1).

On the third day after treatment, many of the fungal isolates ~~were~~ 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~~ days after treatment, the mortality rate of mites was significantly increased in all isolates. *Hirsutella sp.*~~—~~. and *Isaria* sp. caused pronounced mortality ranging from 65-85 percent on the fifth to seventh days after treatment. Among the *Beauveria* isolates, ENPF-16 caused the highest percent mortality (10 and 33.33 percent) on the 5^{th} and 7^{th} days after treatment respectively. Whereas, among 4 *Metarhizium* isolates, 13.33 and 31.11 percent mortality was caused by ENPF-68. Out of 4 *Aspergillus* isolates, ENPF-26 caused 3.33 and 17.78 percent mortality of mites on the 5^{th} and 7^{th} 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 respectively (Table 1). All *Metarhizium* and *Aspergillus* isolates were not effective in causing mortality (30-60 % on the ninth day after treatment).

Although all the fungal isolates were able to cause mortality of mites ~~under~~ *in vitro* 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*. in 2013; Sayed *et al*. in 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 the fungal isolates belonging to the same genera 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*. in 2019; Márquez-Gutiérrez *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 percent, followed by treatment with *H. thompsonii* at $4.6x10^8$ spores /mL + dicofol at 0.025 %. This treatment caused 81.21 percent 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 percent at 15 days after ~~spray~~ spraying in greenhouse conditions against the red spider mite *T. neocaledonicus* on okra in Shimoga. Tamai *et al*. (2002), also confirmed similar results.

3.2 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 3.5×10^6 and 8.3×10^9, and for *Hirsutella* sp. were 7.6×10^5 and 1.3×10^9, 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 2.4×10^5, 8.5×10^4 and 3.6×10^8, 9.1×10^7 respectively (Table 2). The results of lethal time (50 and 90 percent 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) (Table 3).

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~~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 Vu *et al*., 2008, 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 3.5×10^8, 2.9×10^7, and 1.4×10^6 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 5.9×10^7, 5.8×10^9, and 3.3×10^9 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.

3.3 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 us 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 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~~ were 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% to the *I. fumosorosea* strain_AMEP094 and *H. thompsonii* strain MTCC_6686 respectively (Figure 1)**.**

4 CONCLUSION

It can be inferred from the results that the different entomopathogenic fungal isolates caused varied levels of mortality in 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 millilitre 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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COMPETING INTERESTS

Authors have declared that no competing ~~Interests~~ interests exist.

REFERENCES

ASHKTORAB, H. AND COHEN, R. J., 1992, Facile isolation of genomic DNA from filamentous fungi. *BioTechniques,* 13:198-200.

BUTT, T. M., INGLIS, G. D., GOETTEL, M. S. AND STRASSER, H., 2006. Use of hyphomycetous fungi for managing insect pests. In: Fungi as biocontrol agents: progress, problems and potential. Ed. by Butt TM, Jackson C, Magan N, CAB Inter. Wallingford, UK, pp. 23-69.

CHARNLEY, A. K. AND LEGER, R. J., 2010, The role of cuticle degrading enzymes in fungal pathogenesis in insects, In: The Fungal spore and disease initiation in plants and animals, eds. G.T. Cole and H.C. Kock, Plenum Press, New York, USA, pp. 267-286.

DHALIWAL, G. S., VIKAS, J. AND BHARATHI, M., 2015, Crop Losses due to insect pests: Global and Indian Scenario*. Ind. J. Entomol*., 77:165-169.

DOYLE, J. J. AND DOYLE, J. L., 1987, A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bulletin,* 19:11:15.

EL-SHARABASY, H. M., 2015, Laboratory evaluation of the effect of the entomopathogenic fungi, *Hirsutella thompsonii* and *Paecilomyces fumosoroseus*, against the citrus brown mite, Eutetranychus orientalis (Acari: Tetranychidae). Plant Prot. Sci., 51: 39-45.

FARGUES, J., GOETTEL, M. S., SMITS, N., OUEDRAOGO, A. AND ROUGIER, M., 1997. Effect of temperature on vegetative growth of Beauveria bassiana isolates from different origins. Mycologia., 89:383-392.

FIEDLER, Z., SOSNOWSKA, D. AND BARANOWSKI, T., 2002, The fungal pathogens and active substance of fitoverm, abamectin in biological control against greenhouse pests. Progress Plant Prot., 42(2): 420-423.

FINNEY, D. J., 1971, Probit Analysis. *3rd Edition, Cambridge University Press, Cambridge.*

KACHHAWA, D., 2017, Microorganisms as a biopesticides. *J. Entomol. Zool. Studies*. **5**(3): 468-473.

KIM, R., GAYOUNG, J., HEE HAN, J. AND SANGYEOB LEE., 2013, Biological Control of Aphid Using Fungal Culture and Culture Filtrates of *Beauveria bassiana*. Mycobiol.,17:1443-1449.

LACEY, L. A., WRIGHT, S. P. AND KIRK, A. A., 2008. Entomopathogenic fungi for control of *Bemisia tabaci* biotype B: foreign exploration, research and implementation. In: Gould, J., Hoelmer, K., *Goolsby, J.* (Eds.), Classical Biological Control of *Bemisia tabaci* in the United States – A Review of Interagency Research and Implementation. *Pro. Bio. Control*, 4:33-69.

MÁRQUEZ-GUTIÉRREZ, M. E., PÉREZ-LEZCANO, E., BARO-ROBAINA, Y., VÁZQUEZ, F. P. G., AGUIRRE-MANCILLA, C. AND SANTACRUZ, G. A. A., 2022, Effect of Temperature and Ultraviolet Radiation on Growth and Pathogenicity of Metarhizium anisopliae. Biores. Technol., 99(6): 1530-1537.

NADEAU, M. P. AND BOISVERT, J. L., 1996, Larvicidal activity of the entomopathogenic fungus *Tolypocladium cylindrosporum* (Deuteromycotina: Hyphomycetes) on the mosquito *Aedes triseriatus* and the black fly *Simulium vittatum* (Diptera: Simuliidae). *J. Ame. Mosquito Control Asso.*, **10** :487-491.

NAZIR, T., BASIT, A., HANAN, A., MAJEED, M, Z. AND DEWEN, Q., 2018, In Vitro Pathogenicity of Some Entomopathogenic Fungal Strains against Green Peach Aphid *Myzus persicae* (Homoptera: Aphididae). *Agronomy*, 9(**7**):1-12.

RACHANA, R. R., MANJUNATHA M., NAIK, M. I. AND GAYATHRIDEVI, S., 2009, Evaluation of fungal pathogens, *Fusarium semitectum* Berk. and Hirsutella thompsonii Fisher against red spider mite *Tetranychus neocaledonicus* (Andre) (Acarina: Tetranychidae). Kar. J. Agric. Sci., 21(1): 210-212.

RAMANUJAM, B. R., RANGESHWARAN, G., SIVAKMAR, M., MOHAN, M. AND YANDIGERI, M. S., 2014, Management of Insect Pests by Microorganisms. *Proc. Ind. Nat. Sci. Acad*., 2: 455-471.

SABBAHI, R., MERZOUKI, A. AND GUERTIN, C., 2008, Efficacy of Beauveria bassiana (Bals.) Vuill. against the tarnished plant bug, *Lygus lineolaris* L., in strawberries. J. Appl. Entomol., 132(2):124–134.

SAITO, T., TAKATSUKA, J. AND SHIMAZU, M., 2012, Characterization of *Paecilomyces* cinnamomeus from the camellia whitefly, *Aleurocanthus camelliae* (Hemiptera: Aleyrodidae), infesting tea in Japan. *J. Invertebr. Pathol.* 110:14-23.

SANI, M., ISMAIL, S. I., SUMAIYA, A., JAMIAN, S. AND SAAD, N., 2020, A Review of the Biology and Control of Whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), with Special Reference to Biological Control Using Entomopathogenic Fungi. *Insects,* 11: 619-638.

SAYED, A., ESMAT, F. A. AND SAQER, S. T., 2019, Efficacy of indigenous entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, isolates against the rose aphid, *Macrosiphum rosae* L. (Hemiptera: Aphididae) in rose production. *Egy. J. Biological Pest Control*., 29:19-24.

SHAHID, A. A., RAO, A., BAKHSH, R. AND HUSNAIN, T., 2012, Entomopathogenic fungi as biological controllers: new insights into their virulence and pathogenicity. Arch. Biol. Sci., 64(1): 21-42.

SHARMA, S., KUMAR, V., BABAR, S., TANVEER, M., GAGAN, P. S. S., NEHA, H., KOHLI, S. K., YADAV, P., BALI, S. R., RIPU, P. D., OWIAS, I. B., KIRPAL, S., SHIVAM, J., BHARDWAJ, R. AND THUKRAL, A. K., 2019, Worldwide pesticide usage and its impacts on ecosystem. *S. N. App. Sci*. 1: 1446-1453.

TAMAI, M. A., ALVES, S. B., ALMEIDA, J. E. AND FAION, M., 2002, Evaluation of entomopathogenic fungi for control of *Tetranychus urticae* Koch (Acari: Tetranychidae). Arquivos. do. Instituto. Biologico. Sao. Paulo., 69(3): 77-84.

TRINH, D. N., HA, T. K. L. AND QIU, D., 2020, Biocontrol Potential of Some entomopathogenic Fungal Strains Against Bean Aphid *Megoura japonica* (Matsumura). *Agri.,* **2020**, 10*:*114-126.

VAN, H. V., SUK IL HONG, L. AND KEUN KIM., 2007, Selection of Entomopathogenic Fungi for Aphid Control. *J. BIOSCI. BIOENGINEENERING*., 104(**6**) 498-505.

VU, V. H., HONG, S. AND KIM, K., 2008, Selection of Entomopathogenic Fungi for Aphid Control. *J. Biosci. Bioeng*., 104: 498-505.

WRAIGHT, S. P., JACKSON, M. A. AND DE LOCK, S. L., 2007, Fungi as biocontrol agents. CABI Publishing, Wallingford, pp. 253-287.

Table 1: Virulence of entomopathogenic fungal isolates on percent cumulative mortality of red spider mites (*Tetranychus urticae*) under *in vitro* conditions.

Treat -ments	Isolates	Percent cumulative Mortality (DAT*)			
		3 DAT	5 DAT	7 DAT	9 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).

Table 2: Dose mortality responses of mites (*Tetranychus urticae*) to entomopathogenic fungal isolates under *in vitro* conditions.

DAT	Isolates	χ^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	3.5×10^{6}	2.9×10^{5}	6.7×10^{7}	8.3×10^{8}	1.6×10^{7}	2.6×10^{9}
	Hirsutella sp. (ENPF-58)	1.54	7.6×10^{5}	6.3×10^{4}	1.4×10^{7}	1.3×10^{8}	9.6×10^{6}	9.6×10^{8}
9	*Isaria* sp. (ENPF-8)	0.68	2.4×10^{5}	1.3×10^{4}	3.6×10^{6}	3.6×10^{7}	5.4×10^{6}	6.7×10^{8}
	Hirsutella sp. (ENPF-58)	1.24	8.5×10^{4}	8.3×10^{3}	1.1×10^{6}	9.1×10^{6}	1.1×10^{6}	1.1×10^{8}

Note: [1]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. [2]95% Confidence intervals that did not overlap indicate differences between LC50 and LT50 values.

Table 3: Estimation of lethal time mortality responses of entomopathogenic fungal isolates against mites (*Tetranychus urticae*) under *in vitro* conditions.

Isolates	χ^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 $\chi 2$ (chi-square) value at P=0.05. [2]95% Confidence intervals that did not overlap indicate differences between LC50 and LT50 values.

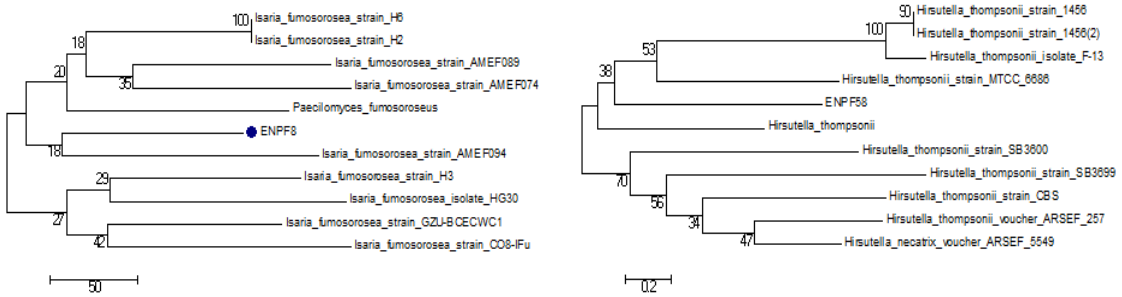

Figure 1: Phylogenetic placement of *Isaria* and *Hirsutella* isolate based on nearly full-length 18S rRNA gene sequences. The tree was calculated with the Neighbour-joining method. Bootstrap values are based on 1,000 replicates. Numbers shown above branches are bootstrap percentages for clades supported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