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Bio-efficacy of Thermotolerant Strain of *Metarhizium anisopliae* Sorokin against *Galleria mellonella*

Abhirami K. A[1], Deepthy K. B*[1], Haseena Bhaskar[1], Reshmy Vijayaraghavan[2] and Mani Chellappan[1]

[1] Department of Agricultural Entomology, College of Agriculture, Vellanikkara, Kerala Agricultural University, Thrissur - 680 656, Kerala, India

[2] Department of Plant Pathology, College of Agriculture, Vellanikkara, Kerala Agricultural University, Thrissur - 680 656, Kerala, India

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Abstract

The thermotolerant strain EKM2 of *Metarhizium anisopliae* Sorokin was evaluated for its efficacy against third instar larvae of *Galleria mellonella* using contact toxicity bioassay. The results showed that the strain EKM2 exhibited significant mortality against *Galleria mellonella* larvae, with mortality rates ranging from 23.33 per cent to 100 per cent at spore concentrations of 10^5 to 10^9 spores/ml. The LT_{50} values decreased from 7.116 days at 10^5 spores/ml to 4.691 days at 10^9 spores/ml, while the LT_{90} values decreased from 10.904 days to 6.571 days over the same concentration range. The LC_{50} values decreased from $9x10^7$ spores/ml on day five to $1x10^5$ spores/ml on day eight. The findings of this study demonstrate the potential of the EKM2 strain of *Metarhizium anisopliae* as a biological control agent against other lepidopteran pests.

***Key words*:** Bioassay, Entomopathogenic fungi, *Galleria mellonella*, *Metarhizium anisopliae*, Thermotolerant strain

The increasing demand for sustainable and environmentally friendly pest management has increased interest in biocontrol agents. Among the different biocontrol agents, microbial formulations based on entomopathogenic fungi (EPF) are important in reducing crop pests' incidence. The use of EPF as biopesticides provides several benefits, including reduced environmental pollution, lower toxicity to non-target organisms, and increased crop safety. Among them, the green muscardine fungus, *Metarhizium anisopliae* Sorokin, is one of the most promising biopesticides, having a broader host range. It has been reported to infect more than one hundred species of insects, which include wheat grain beetles, termites, crickets, wax moths, cockroaches, locusts, grasshoppers, western flower thrips, etc. [1]. The green muscardine fungus, *M. anisopliae* produces blastospores and appressoria in submerged conditions and in insect cuticles as reproductive units [2]. They also produce various secondary metabolites, including destruxin, a cyclic hexadepsipeptide known for its multiple bioactivities such as insecticidal, phytotoxic, anticancer, and antiviral properties [3]. Beyond its mechanical mode of infection, M. anisopliae also exerts its insecticidal effects through the production of secondary metabolites. One of its most notable metabolites is destruxin, a cyclic hexadepsipeptide with multifaceted bioactivities. Destruxins exhibit potent insecticidal properties, contributing to the fungus's pathogenicity against insect hosts. Additionally, these compounds demonstrate phytotoxic, anticancer, and antiviral activities, expanding their potential applications beyond pest control to include pharmaceutical and agricultural research.

Given its effectiveness, natural occurrence, and environmental safety, *Metarhizium anisopliae* is increasingly being explored as an alternative to chemical pesticides. Its use aligns with sustainable agricultural practices, reducing reliance on synthetic chemicals while offering long-term pest control solutions. Further research into its genetic diversity, formulation techniques, and application methods could enhance its efficacy and commercial viability in pest management programs.

One major factor affecting the performance of entomopathogens in the field is their ability to adapt to a wide temperature range and withstand high temperatures. Tumuhaise *et al.* [4] demonstrated that *M. anisopliae* showed the highest mortality rate to first-instar larvae of *Maruca vitrata* at 25 °C, and the optimum temperature for the growth of fungi was between 25 to 30, with the lowest threshold at 10 °C and the highest threshold at 40 °C.

Galleria mellonella was chosen as the test insect to examine the pathogenicity and virulence of *M. anisopliae*. The larvae of the greater wax moth, *G. mellonella* (Lepidoptera: Pyralidae), have been employed as a host and model system for numerous bacterial and fungal diseases because of their weak innate immune system [5]. This model is particularly useful for analyzing host-pathogen interactions, especially due to its low cost, quick results, and absence of ethical or legal concerns [6]. This study aimed to investigate the bio-efficacy of the thermotolerant strain of *Metarhizium anisopliae* against *G. mellonella* larvae, with a focus on determining its virulence against lepidopterans.

***Correspondence to:** Deepthy K. B, E-mail: deepthy.kb@kau.in; Tel: +91 9746756988

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MATERIALS AND METHODS

Fungus culture

Thermo tolerant strain EKM2 (OP97534), available in the repository of the Department of Agricultural Entomology, College of Agriculture, Vellanikkara, was used for the study. The fungus mass multiplied on the nutrient-rich rice bran will be utilized (Plate 1). From the well-sporulated rice bran media, spores will be extracted into sterile distilled water with tween 80, and the spore concentration will be adjusted to 10^5-10^9 spores/ml using a Neubauer Haemocytometer [7].

Rearing of Galleria mellonella

Larvae were reared on synthetic media comprising cornflakes (200g), wheat flour (100g), milk powder (100g), wheat bran (100g), honey (100ml), glycerol (50ml), and yeast (30g). The third instar larva was used for the bioassay study.

Bioassay of Metarhizium anisopliae on Galleria mellonella

For the bioassay, third-instar larvae were selected and pre-starved for two hours in rearing boxes. These larvae were then transferred to sterile petri plates lined with sterile tissue paper at the rate of 10 numbers per plate. Then, the prepared spore suspension was poured evenly over the larvae at the rate of 1 ml per plate. Observations for mortality were taken at 24h intervals [8].

Statistical analysis

Per cent, mortality was determined using Abbot's formula after appropriate adjustments [9]. To determine the dose-mortality relationship, LC50 (Lethal Concentration required to kill 50% of test population), LT50 (Lethal Time required to kill 50% of test population), and fiducial limit probit analysis were calculated using Polo Plus software.

RESULTS AND DISCUSSION

The efficacy of the EKM2 strain of *M. anisopliae* multiplied on rice bran was assessed against the third instar larvae of *Galleria mellonella* utilizing the contact toxicity bioassay method. The number of larvae killed at varying spore suspension concentrations was monitored at 24h intervals.

Table 1 Mortality (%) of *Galleria mellonella* larva

Isolate	Dose (Spores/ml)	4th day	5th day	6th day	7th day	8th day
EKM2	10^5	0^c (0.906)	23.33^d	33.33^e	53.33^d	56.66^c
	10^6	0^c (0.906)	33.33^c	46.66^d	63.33^c	73.33^b
	10^7	0^c (0.906)	36.66^c	56.66^c	73.33^b	83.33^b
	10^8	26.66^b (30.996)	50^b	70^b	83.33^a	100^a
	10^9	40^a (39.23)	60^a	80^a	90^a	100^a
	Control	0^c (0.906)	0^e	0^f	3.33^e	6.66^d
CD (P=0.05)		2.787	7.263	7.263	9.37	11.09

*Mean of three observations, treatments followed by the same letter do not differ significantly according to DMRT, figures in parenthesis are subjected to arc sin transformation

Plate 1 EKM2 multiplied on rice bran

The results revealed a significant positive correlation between spore concentration and mortality. Initially, there was no sign of mortality until three days after treatment. Mortality started from the fourth day onwards in treatments with 10^8 and 10^9 spores/ml. From the fifth day onward, larval mortality rates were observed in all treatments. On the eighth day of treatment, cent per cent mortality was shown by both 10^8 and 10^9 spores/ml, followed by 10^7 spores/ml with 83.33 per cent mortality and 10^6 spores/ml with 7333 per cent. The treatment 10^5 spores/ml showed the lowest mortality percentage of 56.66 per cent compared to other suspension concentrations (Table 1) (Plate 2).

This aligns with Serebrov's [10] findings, which indicated that isolates of both *B. bassiana* and *M. anisopliae* resulted in 86.67 to 100 per cent mortality in *G. mellonella.* Khalid *et al*. [11] also reported that after 10 days of treatment with 10^8 spore/ml of both *B. bassiana* and *M. anisopliae* against *G. mellonella* larva, they could cause 100 and 98.4 per cent mortality, respectively. The findings of Margy *et al.* [12] supported this observation, as they noted that higher concentrations of spores led to increased mortality in *G. mellonella* larvae. Treatments with higher spore concentrations required a shorter duration to cause 50 per cent mortality (LT_{50}) compared to the longer duration and lower mortality rates observed with lower spore concentrations. As more conidia penetrate, more poisons or enzymes are produced, which raises the insect mortality rate, according to Neves and Alves [13].

The lethal time (LT_{50} and LT_{90}) and lethal concentration (LC_{50}) to kill the third instar larva of *G. mellonella* were determined to understand the virulence of the fungal strain. It was observed that an increase in spore concentration resulted in fewer days required to reach 50 per cent mortality. At the highest dosage of 10^9 spores/ml, the fungus showed lower values of LT_{50} (4.691 days) and LT_{90} (6.571 days) while the LT_{50} value for the lowest concentration, 10^5 spores/ml, was 7.116 days, and LT_{90} was 10.904 days (Table 2). This is consistent with the findings of Bischoff *et al*. [14], where the *B. bassiana* and *M. anisopliae* showed an LT_{50} value ranging from

2.36 to 5.01 days. With the increase in days after treatment, the dosage to kill 50 per cent of test insects gradually decreased (Table 3). On day five, 50 per cent mortality was caused by $9x10^7$ spores/ml. On day six the LC50 value was $2x10^7$ spores/ml. Post seven and eight days after treatment; the LC50 value was $1x10^5$ spores/ml. The study demonstrated a significant positive correlation between spore concentration and insect mortality, with higher concentrations of *Metarhizium anisopliae* leading to faster and greater larval mortality. The LT50 and LC50 values confirmed that increased spore dosages resulted in reduced lethal time. These results highlight the potential of *M. anisopliae* as an effective biocontrol agent, emphasizing the importance of optimizing spore concentration for enhanced pest management efficiency.

Table 2 Lethal time to cause mortality of *Galleria melonella*

Dose (spores/ml)	LT_{50} (Days)	Fiducial limit (95%)	Chi-square	Degrees of freedom	Slope ± SE	LT_{90} (Days)	Fiducial limit (95%)
1×10^5	7.116	6.226 – 9.270	11.142	6	6.915 ± 1.215	10.904	8.657 – 24.448
1×10^6	6.346	5.571 – 7.449	12.244	6	7.823 ± 1.180	9.253	7.764 – 15.193
1×10^7	5.942	5.245 – 6.699	12.145	6	9.026 ± 1.251	8.240	7.178 – 11.481
1×10^8	5.081	4.489 – 5.635	9.8383	6	8.709 ± 1.13	7.130	6.318 – 8.973
1×10^9	4.691	4.099 – 5.222	10.488	6	8.756 ± 1.090	6.571	5.821 – 8.223

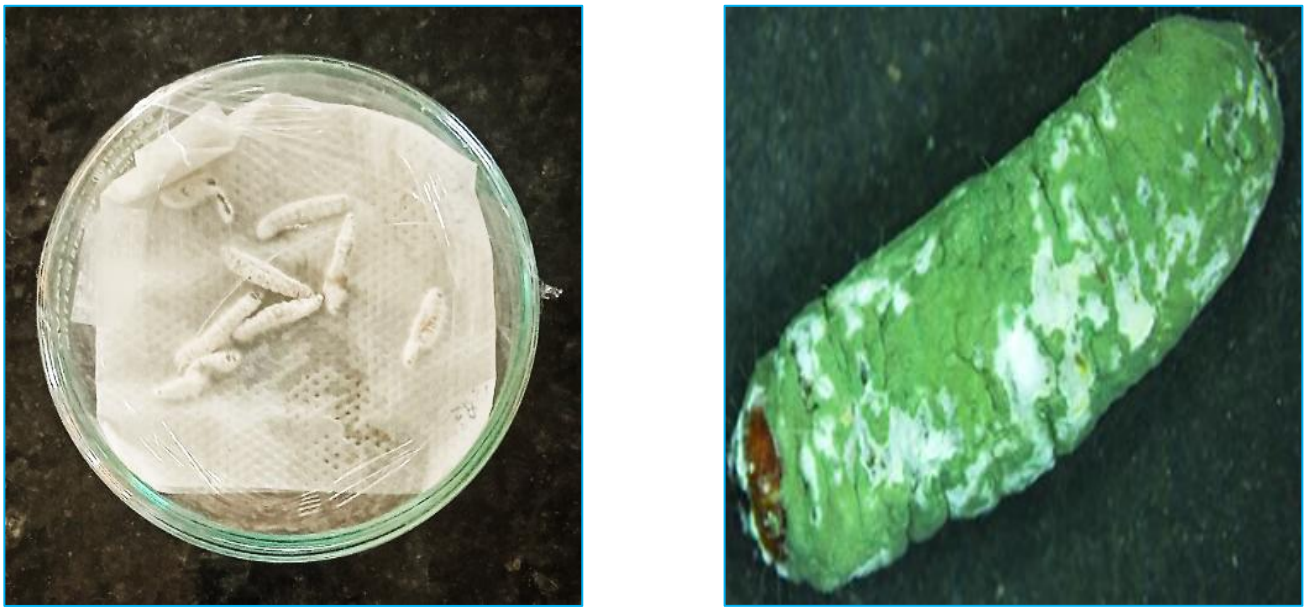

Plate 2 (a) White mycelial growth on larva, (b) Green sporulation on larva

Table 3 Lethal concentration to cause mortality of *Galleria mellonella*

Day	LC_{50} (spores/ml)	Fiducial limit	Chi-square	Degrees of freedom	Slope ± SE
5	9×10^7	$1.27 \times 10^7 – 9.01 \times 10^9$	0.045	3	0.239 ± 0.076
6	2×10^7	$1.04 \times 10^7 – 2.13 \times 10^7$	0.041	3	0.35 ± 0.078
7	1×10^5	$4.50 \times 10^2 – 9.85 \times 10^5$	0.036	3	0.311 ± 0.087
8	1×10^5	$9.77 \times 10^3 – 3.74 \times 10^5$	2	3	0.628 ± 0.137

This was in accordance with the results by Margy *et al*. [12] that an LT_{50} value of 5.18 and 6.37 days was obtained for two isolates of *M. anisopliae* against *Galleria mellonella* and the LC_{50} value of the two isolates was 6.3×10^7 and 1.1×10^8 spores/ml. After screening five *Metarhizium anisopliae* isolates ($1x10^7$ or $1x10^8$ spores/ml) for pathogenicity against *Ceratitis capitate*, revealed LT_{50} values ranged from 4.6 to 6.1 days [15]. Similarly, a value of 4.22 days was obtained when *M. anisopliae* isolate with a concentration of 108 spore/ml was applied against larvae of *Capnodis tenebrionis* [16]. The findings align with previous studies demonstrating the effectiveness of *Metarhizium anisopliae* against various insect pests. The LT50 and LC50 values observed in different studies indicate its strong pathogenicity, making it a promising biocontrol agent. The variation in lethal time across different isolates and target pests highlights the importance of selecting the most virulent strains for optimal pest management.

CONCLUSION

Taking into account the low LT_{50} and LC_{50} values as well as the high mortality percentage, the data obtained demonstrated that this strain of *Metarhizium anisopliae* displayed high pathogenicity on *Galleria mellonella* larvae. Future field research would benefit from these fungal strains. Considering the health of people and the environment, biopesticides derived from entomopathogenic fungi (EPF) can be a good alternative to chemical pesticides. Therefore, more research should be done on using these entomopathogenic fungi (EPFs) to reduce insect pests.

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Statements and Declarations

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Competing interests

The authors declare no competing interests.

Author contributions

Deepthy K. B. contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Abhirami K A. The first draft of the manuscript was written by Abhirami K. A. and Deepthy K. B. All authors read and approved the final manuscript.

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