

Evaluating the virulence and endophytic colonization of *Metarhizium anisopliae* and *Beauveria bassiana* in *Dalbergia sissoo* seedlings for the biological control of subterranean termites

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Received: 22 August 2024

Revised: 19 December 2024

Accepted: 14 February 2025

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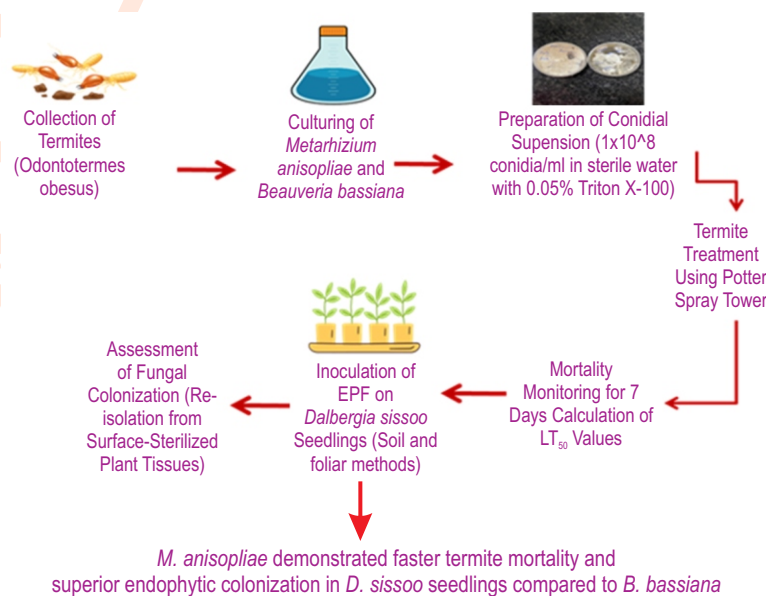
Abstract

Aim: To evaluate the virulence and endophytic colonization efficacy of *Metarhizium anisopliae* and *Beauveria bassiana* in *Dalbergia sissoo* seedlings against *Odontotermes obesus*.

Methodology: Termites (*O. obesus*) were collected from infested wood and acclimatized at $26 \pm 2^\circ\text{C}$ and $75 \pm 5\%$ RH. Active, uninjured workers were used for bioassays. *M. anisopliae* and *B. bassiana* cultures from ICAR-NBAIR were grown on SDA and PDA media, respectively, at $25 \pm 1^\circ\text{C}$. Conidia (1×10^8 conidia ml^{-1}) were suspended in sterile water with 0.05% Triton X-100. Termites were treated using a Potter spray tower, with controls receiving sterile water. Mortality was observed over seven days, and LT_{50} values were calculated. *D. sissoo* seedlings were inoculated with fungal suspensions via soil drench and foliar spray. Colonization was assessed by re-isolating fungi from surface-sterilized plant tissues.

Results: *M. anisopliae* showed greater virulence against *O. obesus*, with a lower LT_{50} (32.84 hr) compared to *B. bassiana* (42.48 hr) and a steeper dose-response slope (4.88). Colonization studies revealed the *M. anisopliae* achieved higher rates in foliage (84.31%), roots (76.36%), and leaves (80.94%) compared to *B. bassiana* (74.32%, 60.52%, 69.98%, respectively), with significant differences noted in root and leaf colonization ($P < 0.001$).

Interpretation: *M. anisopliae* demonstrated superior termite control and higher colonization efficiency in *D. sissoo* seedlings, making it a promising agent for integrated pest management in forest ecosystems.



Key words: *Beauveria bassiana*, *Dalbergia sissoo*, Endophytic colonization, *Metarhizium anisopliae*, Termite



How to cite: Bhoi, T.K., D.K. Mahanta, I. Samal, M.N. Raj and J. Komal: Evaluating the virulence and endophytic colonization of *Metarhizium anisopliae* and *Beauveria bassiana* in *Dalbergia sissoo* seedlings for the biological control subterranean termite. *J. Environ. Biol.*, **46**, 583-588 (2025).

Introduction

Dalbergia sissoo Roxb. commonly known as shisham or Indian rosewood (Yasmeen et al., 2016), is an important timber species in India, valued for its durability and aesthetic appeal predominantly found in the northern regions (Ijaz and Haq, 2021). Shisham is not only crucial for timber production but also plays a vital role in agroforestry systems due to its ability to fix nitrogen, enhancing soil fertility (Yasmeen et al., 2016). The wood of the plant plays a crucial role in local economies, being highly sought after for crafting furniture, flooring, and musical instruments. In addition to its commercial uses, different parts of the plant are also valued in traditional medicine, further increasing its economic and cultural significance (Ijaz and Haq, 2021). Termites pose a substantial threat to *D. sissoo*, particularly species like *Odontotermes obesus* (Rambur, 1842), which is prevalent in India and other parts of South Asia (Calleri et al., 2010). These wood-destroying pests can cause extensive damage not only to living trees but also to wooden structures and products made from shisham (Shanbhag and Sundararaj, 2013).

Further, termites pose a significant threat to forest nurseries, leading to substantial losses. Historically, persistent organochlorine insecticides were used for termite control (Ambele et al., 2018), but their use has declined due to concerns about human health and environmental risks. Current termite management methods are often inadequate, ecologically unsustainable, and fail to address the root causes of infestations (Djuideu et al., 2020). Therefore, there is a growing need for sustainable, eco-friendly, and locally accessible alternatives. Biological control using entomopathogenic fungi offers a promising long-term solution within integrated pest management. Termites naturally inhabit environments conducive to entomopathogens (Rath, 2000), and the use of EPF for termite control dates back to 1965 (Yendol and Paschke, 1965). Previous studies have primarily focused on *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Culliney and Grace, 2000; Vidal et al., 2015); however, despite their proven effectiveness against termites in laboratory settings, field applications have had limited success due to factors such as repellency, host avoidance, and termites' sophisticated defense mechanisms (Mburu et al., 2009). While these fungi have proven effective against termites in laboratory settings, field applications have had limited success due to issues like repellency, host avoidance, and termites' defense mechanisms (Mburu et al., 2009). Additionally, abiotic factors such as UV radiation, temperature, and low humidity reduce the viability of fungal conidia, further limiting EPF effectiveness (Vega et al., 2008).

Despite these challenges, some entomopathogenic fungi can grow as endophytes within plant tissues, suggesting potential use in plant protection (Schulz and Boyle 2005). For example, *B. bassiana* has been found as an endophyte in various crops, providing systemic protection against pests. Studies indicate that termites can detect and avoid virulent isolates of *M. anisopliae* or *B. bassiana*, hinting at a co-evolutionary relationship that could be

exploited for termite management by inoculating forest seedlings with these fungi as endophytes (Mburu et al., 2009; Arnold and Lewis, 2005). The endophytic colonization of plants by entomopathogenic fungi offers continuous protection, with recent studies highlighting their repellent, antifeedant, and toxic properties (Wemheuer et al., 2020). This study aims to explore the potential of virulent strains of *B. bassiana* and *M. anisopliae* as endophytes for controlling *O. obesus* and protecting *D. sissoo* seedlings. The research involves screening EPF strains for efficacy against subterranean termites, evaluating their ability to colonize seedlings, and assessing their effectiveness in providing sustained protection against termite damage. The primary hypothesis of this study centers on the effectiveness of two entomopathogenic fungi, *M. anisopliae* and *B. bassiana*, as biocontrol agents against subterranean termites that infest *D. sissoo* seedlings. The study posits that these fungi can not only effectively colonize the seedlings endophytically but also exhibit significant virulence against termite populations, thereby providing a sustainable alternative to chemical pesticides.

Materials and Methods

Termite collection and maintenance: *Odontotermes obesus* were collected from infested wood placed on sterilized soil in plastic containers at ICFRE-Arid Forest Research Institute (AFRI), Jodhpur, Rajasthan (26.2677° N, 73.0095° E). The insect was identified based on key morphological traits, including a well-developed, rounded head capsule in soldiers, strongly curved asymmetrical mandibles, and a small fontanelle. The antennae had 14–17 segments, with the third segment distinctly longer than the second and fourth. Alates had light brown, translucent wings with characteristic venation, while workers were soft-bodied and creamy white. The termites were then transferred to an incubator set at $26 \pm 2^\circ\text{C}$ and $75 \pm 5\%$ relative humidity in dark for a one hour acclimatization period before bioassays. Only active, uninjured worker termites of uniform size were selected to ensure a consistent test population. The mother cultures of *Metarhizium anisopliae* and *Beauveria bassiana* were obtained from ICAR-National Bureau of Agricultural Insect Resources, Bengaluru, Karnataka.

Fungal culture and suspension preparation: *Metarhizium anisopliae* was cultured on Sabouraud Dextrose Agar (SDA) and *Beauveria bassiana* on Potato Dextrose Agar (PDA) in 90 mm Petri dishes. These cultures were incubated in complete darkness at $25 \pm 1^\circ\text{C}$ for 2 to 3 weeks. Conidia were harvested by scraping the plate surfaces with a sterile spatula and suspending them in 10 ml of sterile distilled water containing 0.05% Triton X-100. The suspension was vortexed for 5 min at approximately 700 rpm, and then filtered through a cheesecloth to remove clumps and debris. Conidial concentrations were determined using a Neubauer Haemocytometer and adjusted to 1×10^8 conidia ml^{-1} for bioassays. Four replicate plates were used per fungi, and viability of each isolate was determined.

Screening of fungal isolates for Time-Mortality responses:

The fungal cultures were evaluated for their efficacy against worker termites of *Odontotermes obesus*. Each fungal isolate was tested in triplicate, along with a control group. Twenty termites were placed in a round plastic containers and treated with a 1 ml suspension of 1×10^8 conidia ml^{-1} using a Potter spray tower (indiamart-MRP-PT-M spray), while control termites were sprayed with sterile distilled water containing 0.05% Triton X-100. To maintain social cohesion, two soldier termites were added to each container. The treated termites were provided with sterilized wood and soil as food and shelter. The containers were incubated at $23 \pm 2^\circ\text{C}$ and $75 \pm 5\%$ RH, with mortality recorded at 24 hr posttreatment and then subsequent observations were taken at an interval of 12 hr, i.e., 36 hr, 48 hr, 60 hr and 72 hr, respectively. The last observation was taken at 72 hr, after which no observations were taken and experiment was replicated four times. LT_{50} refers to the "lethal time for 50% mortality," which is the time required for 50% of the test population to die after exposure to a treatment or toxicant. LT_{50} values, indicating the time required to achieve 50% mortality, were calculated as per Abbott (1925). To confirm fungal infection as the cause of death, cadavers were surface-sterilized and incubated on sterile Whatman filter paper in Petri dishes, with mycosis confirmed through daily microscopic examination of hyphae and spores.

Raising of selected forest seedlings: Seeds of *D. sissoo*, were obtained from the ICFRE- Arid Forest Research Institute, Jodhpur, Rajasthan nursery for inoculation experiments in 2023-2024. The seeds were surface sterilized by submersion in 70% ethanol followed by 1.5% sodium hypochlorite, then rinsed in sterile distilled water and air-dried under a safe hood. The seeds were grown in a sterilized soil substrate mixed with sand and livestock manure in a 1:1:1 ratio, with one plant per pot.

Seedlings inoculation: For seedling inoculation, two methods were employed: foliar spray and soil drench. In the soil drench method, the root area of each seedling was drenched with a 25 ml of conidial suspension, while control seedlings received an equal volume of sterile water with Triton X-100. For the foliar spray method, 25 ml of spore suspension was sprayed onto the leaves of each seedling. After inoculation, the seedlings were maintained in a screen house and watered thrice weekly. The inoculation of existing plantation trees was carried out using the soil drenching method. The experiment with each isolate was replicated four times, and all the treatments were randomized in a complete block design.

Assessment of colonization: To assess colonization rates, evaluations were conducted two months after inoculation. Three

plants per fungal isolate were selected, uprooted, and washed under running water. Leaves, stems and roots were cut into small pieces, surface-sterilized, and placed on SDA and PDA plates containing antibiotics. The plates were incubated at 25°C , with fungal growth monitored daily for 14 days and the experiment with each isolate was replicated four times. Colonization was confirmed by counting the number of plant tissue pieces showing growth of the inoculated fungi. The colonization success rate was calculated using the formula given by Petrini and Fisher (1986).

Statistical analyses: Mortality data were adjusted for natural mortality in the controls using Abbott's formula. Time-mortality data were evaluated using a Generalized Linear Model (GLM) to estimate the LT_{50} for each isolate that resulted in over 50% mortality. The corrected percent mortality of worker termites was determined using a binomial generalized linear model with a logit link function. Mycosis data were analyzed through One-way analysis of variance (ANOVA). The success rate of fungal colonization was calculated following the method of Fisher and Petrini, with percentage values square-root transformed to stabilize variance before ANOVA. Significant differences among treatments were further examined by Student–Newman–Keuls (SNK) post hoc test. PoloPlus version 2.0 from company LeOra Software, California was used for the Probit analysis. SPSS V 22.0 was used for the remaining data analysis.

Results and Discussion

The bioassay results for the virulence of entomopathogenic fungi *B. bassiana* and *M. anisopliae* against key subterranean termite species were thoroughly analyzed. For *B. bassiana*, a total of 1250 insects were used in the bioassay (Table 1). The analysis yielded a Chi-square (X^2) value of 7.55 with 3 degrees of freedom, indicating that the observed data fit the expected model well within the acceptable range ($X^2 < 7.81$). The slope of the dose-response curve was 3.67 ± 0.23 , reflecting a steep gradient indicative of a consistent response to the fungal treatment. The heterogeneity factor (h) was 2.52, suggesting some variation among the insect responses. The median lethal time (LT_{50}) for *B. bassiana* was determined to be 42.48 hr, with a 95% fiducial limit (FL) range of 37.09 to 48.01 hr (Table 1).

Additionally, the LT_{90} value, representing the time required to achieve 90% mortality, was 94.832 hr, with a 95% FL range of 76.67 to 140.46 hr. For *M. anisopliae*, the bioassay similarly involved 1250 insects. The Chi-square value was 0.82 with 3 degrees of freedom, significantly lower than the critical

Table 1: LT_{50} value of entomopathogenic fungi for biological control of subterranean termites

EPFs	n	df (X^2)	Slope \pm SE	h	LT_{50} (FL at 95%)	LT_{90} (FL at 95%)
<i>Beauveria bassiana</i>	1250	3 (7.55)	3.67 ± 0.23	2.52	42.48 (37.09-48.001)	94.83 (76.67-140.46)
<i>Metarhizium anisopliae</i>	1250	3 (0.82)	4.88 ± 0.26	0.27	32.84 (31.32-34.28)	60.08 (56.92-64.02)

Tabled X^2 value for 3 df at 95% CL = 7.81; n-total no. of insects used in bioassay; h- heterogeneity

value of 7.81, indicating an excellent fit between the observed and expected results. The slope of the dose-response curve was higher at 4.88 ± 0.26 , suggesting a more potent effect of *M. anisopliae* compared to *B. bassiana*. The heterogeneity factor was 0.27, indicating minimal variation among the insect responses. The LT₅₀ for *M. anisopliae* was 32.84 hr, with a 95% FL range of 31.32 to 34.28 hr, demonstrating a faster lethal effect than *B. bassiana* (Table 1). The LT₉₀ value was 60.08 hr, with a 95% FL range of 56.92 to 64.02 hr, further confirming the higher efficacy of *M. anisopliae* in achieving significant mortality within a shorter timeframe. At 72hr 4% mortality was seen in the termite in control condition. The results indicate that *M. anisopliae* exhibited a more rapid and potent lethal effect on the termite species compared to *B. bassiana*, making it a more effective candidate for the biological control of subterranean termites in forest seedlings and plantations. Zoberi and Grace (1990) demonstrated that a strain of *Beauveria bassiana* isolated from *Reticulitermes flavipes* workers caused 100% mortality in these termites within 1–3 days of exposure. Similarly, a strain of *Metarhizium anisopliae* was reported to achieve a 100% mortality of *O. formosanus*, three days after inoculation at a concentration of 3×10^8 conidia ml⁻¹ (Dong et al., 2007). Hoe et al. (2009) found that isolates of *M. anisopliae* were lethal to the subterranean termite *Coptotermes curvignathus*, causing complete mortality at 1×10^7 conidia ml⁻¹ within three days post-inoculation. Kramm and West (1982) reported that *M. anisopliae* caused 100% mortality in termites within one day, while *B. bassiana* achieved similar results within five days. The rapid lethality of these entomopathogenic fungi against termites is believed not only due to direct physical invasion by hyphae but also potentially due to enzymatic actions or toxic metabolites produced by the fungi. These findings provide critical insights into the selection of entomopathogenic fungal strains for integrated pest management strategies.

Anggrawati and Ramadhania (2016) found that a conidial suspension of *B. bassiana* led to an infection rate of 44.4% in termites, demonstrating its effectiveness as a biocontrol agent. Zhang et al. (2020) explored the use of nanocellulose combined with entomopathogenic fungi for termite control and showed that formulations containing higher concentrations of nanocellulose resulted in increased mortality rates among termites. Chen et al. (2023) indicated that exposure to *M. anisopliae* conidia

significantly influenced the allogrooming behavior of termites, suggesting that certain volatile compounds from the fungus may enhance termite grooming responses, potentially facilitating fungal spread within colonies. In a recent study, Subekti et al. (2024) reported that *M. anisopliae*, *B. bassiana* and *Trichoderma harzianum* were effective in controlling the fall armyworm (*Spodoptera frugiperda*) and termites.

Apart from providing direct control, *M. anisopliae* is noted for its ability to produce a wide range of extracellular enzymes, such as proteases, chitinases and lipases, which are essential for breaking down the insect cuticle and allowing fungal penetration and colonization. Studies have indicated that *M. anisopliae* isolates have substantial protease activity, which is required for the degradation of proteins in the insect exoskeleton, increasing virulence against target insects. Environmental variables, such as pH and nutrient availability, frequently control the production of these enzymes, affecting the overall capacity of the fungi to colonize its host (Dhar and Kaur, 2010).

In addition to enzymes, *M. anisopliae* produces various secondary metabolites that contribute to its pathogenicity, including antifungal compounds and toxins that inhibit the growth of competing microorganisms while enhancing the ability of fungi to infect and kill its insect hosts. The ability to synthesize a wide array of these compounds may provide *M. anisopliae* with a competitive advantage in diverse ecological niches, leading to higher colonization rates compared to other fungi that may not have such extensive metabolic capabilities (Gebremariam et al., 2022). Furthermore, *M. anisopliae* can adapt to different environmental conditions, which may also influence its colonization success. By altering ambient pH and utilizing various carbon and nitrogen sources effectively, it can optimize enzyme production and enhance its infectivity. This adaptability allows it to thrive in various habitats where other entomopathogenic fungi might struggle (Fernandes et al., 2012). These findings collectively highlight the promising role of entomopathogenic fungi in biological control strategies against termites, emphasizing their rapid lethality and potential for integration into sustainable pest management practices. The ongoing research not only confirms previous observations but also explores innovative methodologies to enhance the effectiveness of these biological agents in real-world applications.

Table 2: Assessment of colonization rates for each fungus-inoculated to *D. sissoo* seedlings

Entomopathogenic fungi	Method of colonisation				Colonisation percentage (%)			
	Foliar	SE	Soil	SE	Root	SE	Leaf	SE
<i>Metarhizium anisopliae</i>	84.31	2.50	62.45	2.21	76.36	2.37	80.94	1.78
<i>Beauveria bassiana</i>	74.32	2.34	60.15	2.30	60.52	2.29	69.98	1.64
F-probability		9.15		1.68		22.02		21.34
P-value		NS		NS		<0.001		<0.001
LSD		9.18		9.04		16.23		6.59

NS=Non-significant

The assessment of colonization rates for each fungus inoculated to *D. sissoo* seedlings revealed significant differences in the efficacy of *M. anisopliae* and *B. bassiana* across various plant parts. The study employed both foliar and soil inoculation methods to evaluate the extent of fungal colonization in the roots and leaves of the seedlings. For *M. anisopliae*, the results showed a robust colonization rate with 84.31% colonization in the foliage, 62.45% in the soil, 76.36% in the roots and 80.94% in the leaves (Table 2). These results indicate a strong ability of *M. anisopliae* to colonize different parts of *D. sissoo* seedlings, with particularly high colonization rates observed in the foliage and leaves. *B. bassiana*, on the other hand, demonstrated a colonization rate of 74.32% in the foliage, 60.15% in the soil, 60.52% in the roots, and 69.98% in the leaves (Table 2). While *B. bassiana* also showed effective colonization, its rates were consistently lower than those of *M. anisopliae*, especially in the roots and leaves. Statistical analysis using F-values and P-values indicated significant differences in colonization rates between the two fungi. The F-values for foliar, soil, root and leaf colonization were 9.15, 1.68, 22.02 and 21.34, respectively. Corresponding P-values were 0.04 for foliar colonization, 0.26 for soil colonization, 0.009359 for root colonization, and 0.009882 for leaf colonization. Notably, the P-values for root and leaf colonization were highly significant ($P < 0.001$), indicating a statistically significant difference in colonization efficacy between *M. anisopliae* and *B. bassiana* for these plant parts. The Least Significant Difference (LSD) values for foliar, soil, root and leaf colonization were 9.18, 9.04, 16.23 and 6.59, respectively (Table 2). These values further confirmed the significant differences observed, particularly in root and leaf colonization. Overall, the results highlight the superior colonization capability of *M. anisopliae* compared to *B. bassiana* in *D. sissoo* seedlings. *M. anisopliae* demonstrated higher colonization rates across all plant parts, with particularly notable efficacy in the roots and leaves, suggesting its potential as a more effective biological control agent for managing subterranean termites in forest plantations. The findings align with previous research that has highlighted the effectiveness of *M. anisopliae* as a potent entomopathogenic fungus.

The higher colonization rates observed in this study could be attributed to several factors, including the ability of fungi to produce a wider array of enzymes and secondary metabolites that facilitate plant tissue penetration and colonization. Additionally, the formulation and application method used in this study, including the use of a soil drench and foliar spray, may have enhanced the fungus's ability to establish itself within the plant. The lower colonization rates of *B. bassiana* could be due to its different mode of action or potential limitations in adapting to the internal environment of *D. sissoo* seedlings. Although *B. bassiana* is known for its broad-spectrum activity against a wide range of insect pests, its lower efficacy in colonizing plant tissues suggest that it might be better suited for applications that rely on external contact with the pest rather than systemic protection (Ambele et al., 2020; Greenfield et al., 2016) in their study research has indicated that the colonization of applied entomopathogenic fungi tends to be more prevalent in the plant parts that come into direct

contact with the inoculum, while it is less likely to occur in more distant plant regions. The differences in colonization rates observed among the fungal strains in this study might be attributed to the varying growth rates and endophytic capabilities of each fungal isolate, which can be influenced by the specific host plant species or cultivars (Biswas et al., 2013).

This study highlights *M. anisopliae* as a more effective biological control agent for *O. obesus* than *B. bassiana*, demonstrating faster termite mortality and superior endophytic colonization in *D. sissoo* seedlings. Its dual ability to control termites and establish within plant tissues makes it a promising tool for integrated pest management in forestry. Further research should focus on optimizing its application for sustainable termite control.

Acknowledgments

The first and Corresponding author duly thank the RFBDP, Rajasthan for providing fund for conducting experiment, Director, ICFRE-AFRI, Jodhpur, Rajasthan Institutes for providing research facilities and the Director, ICAR-NBAIR, Bengaluru, Karnataka for providing fungus cultures.

Authors' contribution: T.K. Bhoi: Planned the study, sample collection, carried out experiments, drafted the manuscript; T.K. Bhoi, D.K. Mahanta: Statistical analysis of data; J. Komal: Proof reading and correction; I. Samal and R.M. Nikhil: Data analysis of the sample.

Funding: The first and corresponding author duly thank the RFBDP, Rajasthan for providing fund for conducting the experiments.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare no conflict of interest.

Data availability: Data that support the findings of this study are available on request from the Corresponding author.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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