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Characterization and Bioassay of a *Native beauveria* bassiana Isolate Against Key Insect Pests of Cotton

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DOI: https://doi.org/10.37229/fsa.fjb.2024.02.28

Abstract: Cotton (*Gossypium spp.*) production faces significant economic threats from diverse pests, leading to an over-reliance on synthetic insecticides and subsequent environmental and resistance issues. This study focuses on the isolation, molecular characterization, and biocontrol potential of a native entomopathogenic fungal isolate, *Beauveria bassiana*, obtained from cotton agro-ecosystems. The isolate was identified through morphological observation and phylogenetic analysis of the Internal Transcribed Spacer (ITS) region, showing 99.63–100% identity with reference strains and was accessioned in GenBank (PQ550640). Laboratory bioassays were conducted to evaluate the concentration-dependent virulence of the isolate against second-instar larvae/nymphs of three key pests: the cotton leafworm (*Spodoptera littoralis*), the pink bollworm (*Pectinophora gossypiella*), and the cotton aphid (*Aphis gossypii*). Results demonstrated significant dose-dependent mortality across all species ($P \leq 0.0052$). At the highest concentration (1×10^9 spores/mL), *A. gossypii* exhibited the highest susceptibility (90.3% mortality), followed by *P. gossypiella* (86.6%) and *S. littoralis* (85.3%). Even at lower concentrations (1×10^7 spores/mL), substantial mortality was recorded, particularly for *A. gossypii* (61.3%). These findings underscore the potent virulence and broad-spectrum activity of this indigenous strain, suggesting it is a promising candidate for integration into sustainable Integrated Pest Management (IPM) programs to reduce chemical dependency in cotton cultivation.

Key words: *Beauveria bassiana*, Cotton pests, Biological control, ITS sequencing, Virulence, Integrated Pest Management.

Future Science Association

Available online free at www.futurejournals.org

Print ISSN: 2572-3006

Online ISSN: 2572-3111

Received: 15 January 2024
Accepted: 10 February 2024
Published: 28 February 2024

Publisher's Note: FA stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Cotton (*Gossypium* spp.) is an essential global agricultural product, serving as the main source of natural fiber for the textile industry and as a notable oilseed crop. The cultivation of this crop is consistently threatened by a variety of arthropod pests, resulting in significant economic losses and an extensive reliance on synthetic

insecticides for protection ***(*Anwar *et al.*, 2022)**. Notable pests include the cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), a significant foliar feeder; the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), which bores into fruiting structures; and the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), a sap-sucking vector for plant viruses **(Rajendran *et al.*, 2018)**. The traditional approach to managing these pests has primarily relied on broad-spectrum chemical insecticides **(Ismail *et al.*, 2020)**. This approach, although effective in the short term, has resulted in significant adverse consequences, such as the emergence of insecticide resistance in pest populations, the loss of beneficial arthropods and natural enemies, environmental contamination, and increasing public health concerns **(Nadeem *et al*., 2022 and Shankarganesh *et al.*, 2022)**.

In addressing these challenges, Integrated Pest Management (IPM) strategies promote the integration of environmentally friendly and sustainable control methods ***(*Nadeem *et al.*, 2022; Zhou *et al.*, 2024).** Biological control, which employs natural enemies to reduce pest populations, is fundamental to integrated pest management (IPM) **(Galli *et al.*, 2024; Schaffner *et al.*, 2024)**. Entomopathogenic fungi (EPF) infect and eliminate insects via direct cuticular penetration, presenting a promising tool for pest management. These organisms function as specific biocontrol agents, exerting minimal effects on non-target organisms and the broader ecosystem **(Gielen *et al.*, 2024).** *Beauveria bassiana* (Bals.-Criv.) Vuill., a hypocrealean fungus, is extensively researched and utilized as an entomopathogenic fungus (EPF) due to its wide host range across various insect orders **(Iida *et al.*, 2023; Pedrini *et al.*, 2024; Sybilska *et al.*, 2024).**

The pathogenicity of *B. bassiana* is facilitated by the production of conidia (asexual spores) that adhere to the insect cuticle, germinate, and penetrate the host through enzymatic degradation and mechanical pressure **(Dannon *et al.*, 2020; Gabarty *et al.*, 2014).** Upon entering the hemocoel, the fungus multiplies, leading to the host's demise and subsequent sporulation on the carcass, thereby promoting further transmission **(Ma *et al.*, 2024)**. The efficacy of *B. bassiana* is well-documented; however, its virulence is notably strain-specific and exhibits considerable variation across different insect species and developmental stages. The identification and characterization of locally adapted or novel isolates are essential for the development of effective, region-specific biocontrol formulations **(Islam *et al.*, 2023).**

Comparative studies examining the dose-dependent virulence of a single *B. bassiana* isolate against *S. littoralis*, *P. gossypiella*, and *A. gossypii* under standardized laboratory conditions are scarce, despite the economic significance of these pests. These data are crucial for comprehending the host range spectrum of the pathogen and for establishing effective application concentrations for future field evaluations. This study aimed to: (1) isolate and characterize an indigenous entomopathogenic fungus from a cotton agro-ecosystem using morphological and molecular techniques; (2) identify the isolate through phylogenetic analysis of the Internal Transcribed Spacer (ITS) region of ribosomal DNA; and (3) assess the concentration-dependent pathogenicity of the identified *B. bassiana* isolate against second-instar larvae/nymphs of *S. littoralis, P. gossypiella, and A. gossypii* in a controlled laboratory bioassay. This study presents essential data that underpins the potential incorporation of this fungal isolate into integrated pest management programs for cotton.

2. Materials and methods

Tested insects

The study evaluated the efficacy of *Beauveria bassiana* against three economically significant insect pests:

- **The cotton leafworm (*Spodoptera littoralis*):** Second-instar larvae were used as representative foliar chewing pests.
- **The pink bollworm (*Pectinophora gossypiella*):** Second-instar larvae were selected as a key internal feeding pest of cotton bolls.

- **The cotton aphid (*Aphis gossypii*):** Second-instar nymphs were used as a model for sap-sucking hemipteran pests.

All insect populations were maintained under standard laboratory conditions (25 ± 2°C, 65 ± 5% RH, and a 16:8 h light: dark photoperiod) on their preferred host plants or artificial diet to ensure healthy, synchronized test cohorts for the bioassay.

Collection and isolation of entomopathogenic fungi

Insect cadavers suspected of fungal infection were collected from cotton fields infested with *S. littoralis*. Collected samples were surface-sterilized with 1% sodium hypochlorite (NaOCl) for 30 sec., followed by three washes with sterile distilled water to prevent external saprophytic contaminations. The samples were placed in sterilized polyethylene bags and stored at 4° C until fungal analysis **(Quesada-Moraga *et al.*, 2006).**

Selective medium for isolation of entomopathogenic fungi

Collected samples were placed on Sabouraud Dextrose Yeast Agar (SDYA) composed of peptone (10 gm), glucose (40 gm), yeast extract (2 gm), and Agar (15 gm). All ingredients were settled in 1000 ml of distilled water. The pH was set at 5.6±0.3 by diluted HCl at 25° C **(Sabouraud, 1892)**. Cultivated fungi were transferred onto a potato dextrose agar (PDA) petri dish to identify isolated fungi morphologically. All fungal colonies were incubated at 25±1° C and 95±5% R. H.

Fungal characteristics and morphological identification

Macroscopic morphological characteristics were evaluated based on color, texture, and reverse color. Suspensions of the investigated fungus were generated by placing a culture disc from the center of a 14-day fungal colony into a 50 mL flask containing 20 mL sterile 0.05% Tween 80 and stirring the mixture for 10 minutes with a magnetic stirrer. A hemocytometer was used to determine the conidial suspension's concentration. Isolated fungi were morphologically identified according to the description provided by Barnett and Hunter **(Barnett and Hunter, 1972)**. For microscopic examination, the mycelium of the fungus was placed on a coverslip after sporulation. After a drop of the mounting medium was applied, the growth was gingerly examined. The second coverslip was placed on top for light microscopy analysis, followed by high-definition digital photographs taken at 40X magnification **(Senthilkumar *et al.*, 2021)**.

Fungal DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from lyophilized mycelium using the DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified via polymerase chain reaction (PCR) using the universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR reactions were performed in a total volume of 100 µL containing 50 µL of Taq PCR Master Mix (Qiagen), 50-100 pmol of each primer, and 20-200 ng of template DNA. The thermal cycling program consisted of an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 5 min. PCR products were visualized on a 1% agarose gel stained with ethidium bromide and purified using a gel extraction method involving electro-elution and ethanol precipitation. Purified amplicons were sequenced in both directions using the Cy5/Cy5.5 Dye Primer Sequencing kit on an Open Gene automated DNA sequencing system (Visible Genetics, Canada) at the Regional Center for Mycology and Biotechnology, Al-Azhar University.

Sequence Analysis and Phylogenetic Identification

The obtained forward and reverse sequence reads were assembled to generate a consensus sequence. This consensus ITS sequence was used as a query in a nucleotide BLAST (Basic Local Alignment Search Tool) search against the NCBI GenBank non-redundant nucleotide database to identify homologous sequences. The top BLAST hits with the highest Max Scores and sequence identity

percentages were selected for further analysis. A multiple sequence alignment was generated using these reference sequences. A phylogenetic tree was constructed using the Maximum Likelihood method to visualize the evolutionary relationship between the query sequence and closely related fungal strains. All sequence analyses were conducted using standard bioinformatics tools.

Bioassay test

The bioassay was conducted to evaluate the concentration-dependent pathogenicity of the entomopathogenic fungus *Beauveria bassiana* against the three target insect species. A commercial isolate of *B. bassiana* was used in the experiment. Spore suspensions were prepared from a 10-day-old culture grown on Sabouraud Dextrose Agar (SDA) at 25 ± 1°C. Conidial concentration was determined using a hemocytometer and adjusted with a 0.05% aqueous solution of Tween® 80 to create five serial dilutions: 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 spores/mL.

For each insect species, synchronized cohorts of second-instar larvae (for *S. littoralis* and *P. gossypiella*) or nymphs (for *A. gossypii*) were used. The treatment was applied using a standard topical application method. Insects were individually treated with 1 µL of the appropriate spore suspension applied dorsally using a micro-applicator. Control groups were treated with 1 µL of the 0.05% Tween® 80 solution only.

The experimental design was completely randomized. Each treatment (concentration for each insect species) and the control was replicated four times, with each replicate consisting of 10 insects (n=40 insects per treatment). After treatment, insects were transferred to sterile plastic containers with a fresh supply of their respective diet or host plant leaves and maintained under controlled conditions (25 ± 2°C, 70 ± 5% RH, 16:8 L:D).

Mortality was assessed daily. Insects were considered dead if they showed no movement upon gentle prodding and exhibited visible mycosis (fungal outgrowth). The final cumulative mortality was recorded 7 days post-treatment (dpt). Mortality data from the control groups were used to correct treatment mortality using Abbott's formula when necessary. The corrected mortality percentages were subjected to a two-way Analysis of Variance (ANOVA) using a statistical software package (SPSS) to determine the effects of insect species, spore concentration, and their interaction. Treatment means were separated using Fisher's Least Significant Difference (LSD) test at the 0.05 probability level.

Data analysis

Data analysis encompassed morphological, molecular, and statistical methods to characterize the fungal isolate and evaluate its efficacy. Morphological identification based on colony and conidial features was confirmed molecularly through sequencing of the ITS region; BLASTn analysis revealed 99.63–100% identity with reference *Beauveria bassiana* strains, and phylogenetic placement firmly clustered the isolate within the *B. bassiana* clade. For the bioassay, corrected mortality data were analyzed using two-way ANOVA, which showed highly significant treatment effects ($P \leq 0.0052$) across all concentrations, and Fisher's LSD test ($\alpha = 0.05$) was applied for post-hoc mean separation, with superscript letters in the results table denoting significant differences between insect species at each dose.

3. Results

Identification and morphological characterization of isolated entomopathogenic fungus

Initial identification of the isolated entomopathogenic fungus was conducted through morphological analysis, which revealed features consistent with *Beauveria bassiana* (Bala.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) was identified as the initial fungus. The *B. bassiana* colony was discovered to be white and granular smooth. On a microscopic scale, it was observed that the hyphae had branched and produced conidiogenetic cells and a network of branching hyphae. The conidium of solitary cells of *B. bassiana* was both round and oval (Fig. 1).

a b

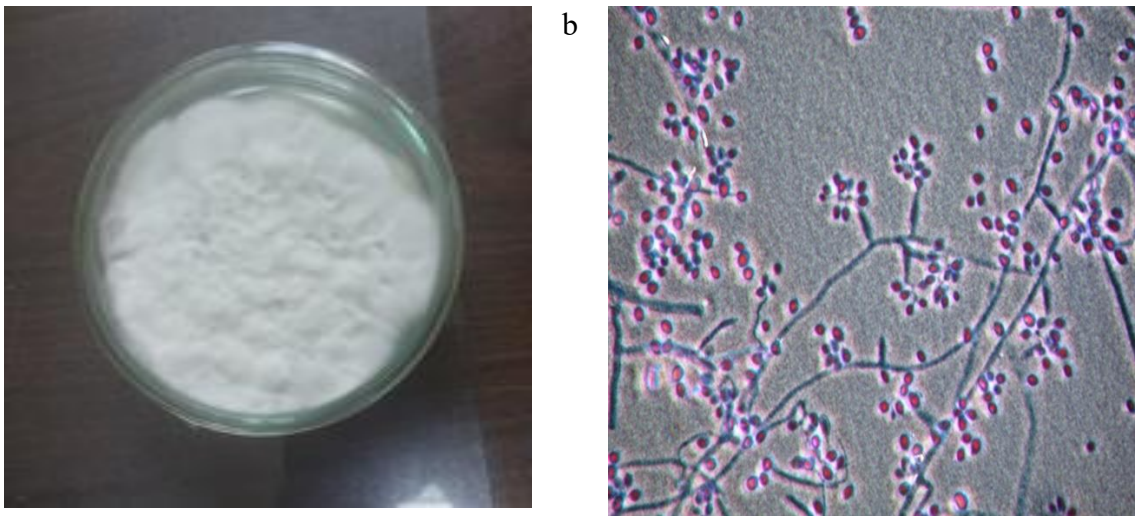

Fig. (1). Culture characteristics of the 14-day colony (a) and microscopic examination showing the conidiophore and conidia (b) for *Beauveria bassiana*

Sequence Analysis and BLAST Identification

The assembled ITS region sequence from the isolate was 458 base pairs in length. A BLASTn analysis revealed that the sequence showed extremely high similarity to multiple strains of *Beauveria bassiana* in the GenBank database. The top ten alignment results are summarized in Table 1. The query sequence exhibited 98-99% query coverage and 99.63% to 100% identity with the reference sequences of *B. bassiana*. The E-values for all top hits were 0.0, confirming a statistically significant match.

Table (1). Top BLASTn hits for the fungal ITS sequence against the NCBI GenBank database

Description	Scientific Name	Max Score	Total Score	Query Cover (%)	E value	Percent Identity	Accession
Beauveria bassiana voucher AUMC3873	*Beauveria bassiana*	981	981	98	0.0	100	MN710408.1
Beauveria bassiana strain B-Bug	*Beauveria bassiana*	981	981	99	0.0	99.63	MK862359.1
Beauveria bassiana isolate A2	*Beauveria bassiana*	981	981	98	0.0	100	MN428792.1
Beauveria bassiana strain HZBB160701	*Beauveria bassiana*	981	981	98	0.0	100	MH521027.1
Beauveria bassiana strain Ha5	*Beauveria bassiana*	981	981	98	0.0	100	MK418853.1
Beauveria bassiana strain Ha3	*Beauveria bassiana*	981	981	98	0.0	100	MK418851.1
Beauveria bassiana strain Ya5	*Beauveria bassiana*	981	981	98	0.0	100	MK418849.1
Beauveria bassiana strain Ya1	*Beauveria bassiana*	981	981	98	0.0	100	MK418845.1
Beauveria bassiana strain SSR5	*Beauveria bassiana*	981	981	98	0.0	100	MG763749.1
Beauveria bassiana strain VKBb03	*Beauveria bassiana*	981	981	99	0.0	99.63	MG548313.1

Phylogenetic Placement

A phylogenetic tree was constructed to confirm the taxonomic position of the isolate within the genus *Beauveria*. The tree (Fig. 2) clearly demonstrates that the query sequence clusters robustly with reference sequences of *Beauveria bassiana* strains, forming a distinct clade with strong bootstrap support. This class is separate from others including ascomycete fungi. The branch lengths within the *B. bassiana* clade are very short, indicating a high degree of genetic similarity and a close evolutionary relationship between the isolate and the referenced strains.

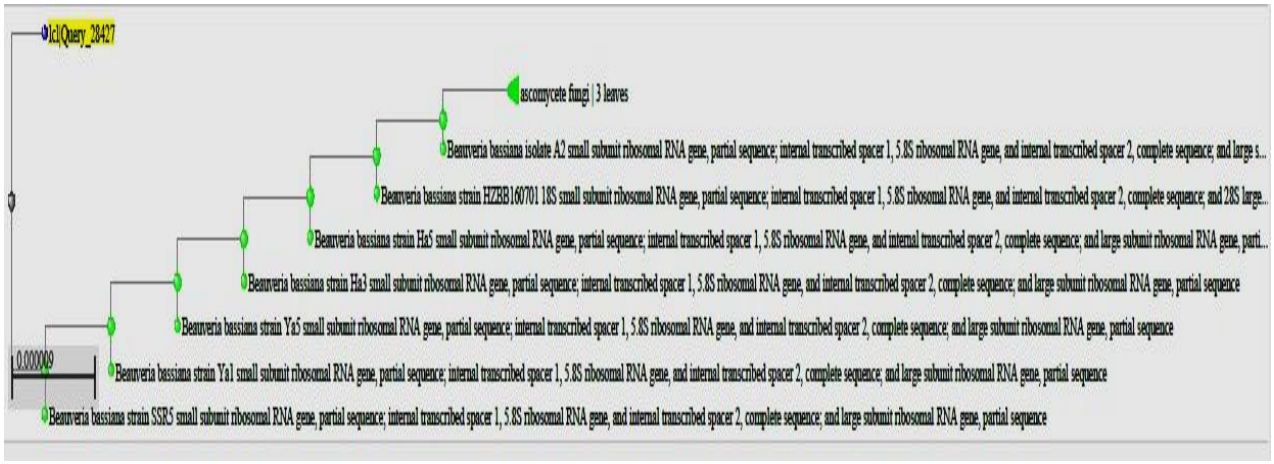

Fig. (2): Phylogenetic tree based on ITS sequences of rDNA of the fungal sample isolated in the present study (*Beauveria bassiana*)

Interpretation

Based on the molecular analysis, the fungal isolate was conclusively identified as *Beauveria bassiana*. The BLAST results provided definitive evidence, with sequence identity values at or near 100%. The phylogenetic analysis further supported this identification, placing the isolate firmly within the *B. bassiana* species complex. The minimal genetic distance observed in the tree suggests that it is a strain very closely related to other globally reported *B. bassiana* strains, such as SSR5, Ya1, and Ha3. The fungal isolate has been formally accessed in the gene bank under the identification number PQ550640".

Dose-Dependent Virulence of *Beauveria bassiana* Against Three Key Insect Pests

The bioassay demonstrated a clear dose-dependent virulence of *Beauveria bassiana* against the three insect species. Mortality rates increased significantly with higher spore concentrations, as evidenced in Table 2. At the highest concentration (1×10^9 spores/ml), *Aphis gossypii* exhibited the greatest susceptibility with 90.3% mortality, followed by *Pectinophora gossypiella* (86.6%) and *Spodoptera littoralis* (85.3%). The table further revealed that even at lower concentrations, such as 1×10^7 spores/ml, mortality was substantial, reaching 61.3% for *A. gossypii*, 51.0% for *P. gossypiella*, and 43.3% for *S. littoralis*. Statistical analysis supported these findings, with significant F-values and P-values ≤ 0.0052 across all concentrations, confirming that the observed mortality was a direct treatment effect. The results highlight the strong pathogenic potential of *B. bassiana* in a concentration-dependent manner.

Table (2). Mean percentage mortality (± standard error) of *Spodoptera littoralis*, *Pectinophora gossypiella*, and *Aphis gossypii* after treatment with different spore concentrations of the entomopathogenic fungus *Beauveria bassiana*

Tested insects	Mean mortality ± S.E. (%) at Different Spore Concentrations (spores/ml)				
	1×10^5	1×10^6	1×10^7	1×10^8	1×10^9
S. littoralis (2^{nd} instar larvae)	30.3 ± 0.3a	30.3 ± 0.3c	43.3 ± 0.3c	70.6 ± 0.9b	85.3 ± 0.9b
P. gossypiella (2^{nd} instar larvae)	34.0 ± 0.9b	41.6 ± 0.3b	51.0 ± 0.3b	72.3 ± 0.3b	86.6 ± 0.9b
A. gossypii (2^{nd} instar nymphs)	35.6 ± 0.6b	45.6 ± 0.3a	61.3 ± 0.6a	76.6 ± 0.6a	90.3 ± 0.6a
Control	-	-	-	-	-
F-value	14.36	27.75	200.27	17.27	30.17
P-value	0.0052 **	0.0009 ***	0.0000 ***	0.0032 **	0.0007 ***
$LSD_{0.05}$	1.15	2.31	2.21	2.58	1.63

Different superscript letters (a, b, c) within a column indicate statistically significant differences between insect species according to the Least Significant Difference (LSD) test at the 5% level.

P-value significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

4. Discussion

The present study successfully isolated and molecularly identified an indigenous *Beauveria bassiana* strain from a cotton agro-ecosystem, and demonstrated its significant, dose-dependent pathogenicity against three economically critical cotton pests: the cotton leafworm (*Spodoptera littoralis*), the pink bollworm (*Pectinophora gossypiella*), and the cotton aphid (*Aphis gossypii*). The findings contribute to the growing body of evidence supporting the use of this entomopathogenic fungus as a core component of integrated pest management (IPM) strategies in cotton.

The identification of the isolate as *B. bassiana* was confirmed by a combination of morphological traits and molecular analysis. The observed colony morphology and conidial characteristics align with classical descriptions of the species **(Norjma *et al.*, 2019)**. Definitive confirmation was achieved through sequencing of the Internal Transcribed Spacer (ITS) region of ribosomal DNA, a standard molecular marker for fungal taxonomy. BLASTn analysis revealed 99.63–100% identity with globally reported *B. bassiana* strains, and phylogenetic placement firmly clustered the isolate within the *B. bassiana* clade. This dual approach (morpho-molecular) is widely recommended for accurate identification of entomopathogenic fungi, as morphological traits alone can be variable and misleading **(Bich *et al.*, 2021; Gebremariam *et al.*, 2021).**

The bioassay revealed a clear concentration-dependent mortality for all three insect species, a hallmark of microbial insecticide activity. Notably, the cotton aphid (*A. gossypii*) exhibited the highest susceptibility, reaching 90.3% mortality at the highest concentration (1×10^9 spores/mL). This aligns with numerous reports highlighting the high vulnerability of aphids to *B. bassiana* infection, attributed to their soft cuticle and rapid conidial germination on their body surface **(Francis *et al.*, 2022; Im *et al.*, 2022; Xu & Xu, 2024)**. The observed mortality at lower concentrations (61.3% at 1×10^7 spores/mL) underscores the potent virulence of this isolate against sap-sucking pests, which are often difficult to control with conventional insecticides due to rapid resistance development **(Javar *et al.*, 2023; Oo *et al.*, 2017).**

The pink bollworm (*P. gossypiella*), an internal borer, also showed high susceptibility (86.6% mortality at 1×10^9 spores/mL). This result is consistent with recent work by **Omar *et al.* (2021)**, who reported that *B. bassiana* isolates were highly toxic to *P. gossypiella* larvae, with LC_{50} values in the range of 10^8–10^9 spores/mL. The ability of *B. bassiana* conidia to infect internally feeding larvae likely involves conidial adhesion to the insect during entry into bolls or through direct contact with treated surfaces, followed by cuticle penetration. The significant mortality achieved here confirms the potential of this fungus to target this cryptic and damaging pest stage **(Preisegger *et al.*, 2024).**

The cotton leafworm (*S. littoralis*), a foliar chewer with a thicker cuticle and more robust immune response, was the least susceptible among the three pests, though still showing substantial mortality (85.3% at 1×10^9 spores/mL). This relative tolerance has been noted in earlier studies. **Saad *et al.* (2019)** observed that *B. bassiana* isolates caused dose-dependent mortality in *S. littoralis* larvae, but the required concentrations were generally higher than for softer-bodied insects **(Athanassiou and Steenberg, 2007).** The lower susceptibility of lepidopteran larvae is often linked to behavioral and physiological defenses, including melanization and encapsulation responses in the hemocoel ***(Zhang et al.*, 2024).** Nevertheless, the mortality rates exceeding 70% at 1×10^8 spores/mL indicate that the tested isolate possesses considerable virulence even against this resilient pest.

The differential susceptibility observed among the three species can be explained by fundamental differences in their biology. Aphids possess a thin, permeable cuticle and a high surface-area-to-volume ratio, facilitating rapid fungal penetration. In contrast, lepidopteran larvae have a thicker, multi-layered cuticle and more active cellular immune responses. Furthermore, the feeding niche of *P. gossypiella* (inside bolls) may limit exposure compared to the externally feeding *A. gossypii*, yet our results show that even brief contact with conidia during larval movement can lead to fatal infection.

From a practical IPM perspective, the broad-spectrum activity of this *B. bassiana* isolate is highly advantageous. A single microbial agent that can suppress multiple pests—a chewing foliar pest, an internal borer, and a sap-sucking vector—simplifies application logistics and reduces the need for chemical insecticide mixtures. The dose-response data provide a critical baseline for determining effective field concentrations. For example, concentrations of 1×10^7 to 1×10^8 spores/mL induced mortality rates between 43.3% and 76.6%, which may be sufficient for population suppression when integrated with other control tactics. However, it must be emphasized that laboratory bioassays represent an optimal scenario with direct topical application. Field efficacy can be influenced by environmental factors (UV radiation, temperature, humidity), formulation quality, and insect behavior, which typically necessitate higher application rates to achieve comparable control.

Limitations and Future Research Directions

This study was conducted under controlled laboratory conditions; therefore, the next essential step is to evaluate the isolate's performance in field trials. Future work should focus on:

1. **Formulation development:** Creating wettable powders or oil-based formulations to enhance conidial stability, persistence, and rainfastness under field conditions.
2. **Integration with other IPM tools:** Investigating possible synergistic interactions with botanical extracts, reduced-risk insecticides, or other biological control agents (e.g., parasitoids) to enhance overall efficacy and delay resistance.
3. **Host-stage specificity:** Expanding bioassays to include other life stages (eggs, pupae, adults) to identify the most vulnerable window for intervention.
4. **Environmental safety:** Assessing the non-target effects of the isolate on beneficial arthropods, such as pollinators and natural enemies, to ensure its compatibility with sustainable agriculture.

5. Conclusion

In summary, the indigenous *B. bassiana* isolate characterized in this study exhibits strong virulence against three key cotton pests in a concentration-dependent manner. Its molecular confirmation and promising laboratory efficacy provide a solid foundation for its development as a biocontrol agent. Incorporating this fungus into cotton IPM programs could significantly reduce reliance on synthetic insecticides, mitigate resistance development, and contribute to more environmentally sustainable pest management.

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